Copper toxicity and cyanobacteria ecology in the Sargasso Sea

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Abstract

The closely related cyanobacteria *Synechococcus* and *Prochlorococcus* have different distributions in stratified water columns in the northern Sargasso Sea. The abundance of *Synechococcus* is relatively uniform with depth, but *Prochlorococcus* cell numbers are low within shallow mixed layers and high in and below the thermocline. Because free cupric ion (free Cu^{2+}) concentrations are high (up to 6 pM) in shallow mixed layers and lower in deeper water, there is an inverse relationship between *Prochlorococcus* densities and the free Cu^{2+} concentration. We explored the possibility of a causal underpinning for this relationship by examining the relative copper sensitivities of *Prochlorococcus* and *Synechococcus* in cultures and field populations. *Prochlorococcus* isolates from both the high- and low–light adapted ecotypes were inhibited at free Cu^{2+} concentrations that had no effect on *Synechococcus.* However, the high–light adapted strains were more copper resistant than their low–light adapted counterparts. When copper was added to *Prochlorococcus* from environments where the in situ free Cu^{2+} was low (in deeply mixed water columns and below the mixed layer in stratified conditions), net growth rates were substantially reduced and cells arrested in the G_1 and early S phases of the cell cycle. *Prochlorococcus* in shallow mixed layers were less sensitive to copper and were probably members of the copper-resistant high–light adapted ecotype. *Synechococcus* were relatively copper resistant across a range of environments in the Sargasso Sea. These observations are consistent with our hypothesis that copper plays a role in cyanobacteria ecology in the Sargasso Sea.

Human activities have produced a measurable increase in the concentrations of trace metals in even remote environments. For instance, the atmospheric flux of copper to Greenland ice has increased by over an order of magnitude from the pre-Roman period to the present (Hong et al. 1996).

These anthropogenic inputs are a cause for concern because even metals that are essential in trace amounts are toxic at higher concentrations. Copper is one example. It is a component of respiratory proteins and oxidases (Baron et al. 1995), yet free Cu^{2+} concentrations found in natural environments (in the picomolar to nanomolar range) have been shown to reduce cell division rates of phytoplankton in culture (Brand et al. 1986). Elevated free Cu^{2+} can decrease photosynthetic rates (Baron et al. 1995), interfere with the uptake of other essential trace metals such as manganese (Sunda 1989; Sunda and Huntsman 1998, and references therein), and disrupt enzyme function by both producing hydroxyl radicals and binding to –SH groups (Stauber and Florence 1987; Brown et al. 1994).

In most cases, copper is efficiently complexed in seawater (van den Berg et al. 1987; Coale and Bruland 1990; Moffett et al. 1990; Donat and van den Berg 1992; Moffett 1995; Kozelka and Bruland 1998; Tang et al. 2001). This is important because copper toxicity is a function of free Cu^{2+}, not of total copper (Sunda and Guillard 1976; Anderson and Morel 1978). Because of organic complexation, free Cu^{2+} concentrations are generally below 1 pM in both open ocean

and unpolluted coastal waters, even though total copper concentrations can be an order of magnitude higher close to the coast (Moffett et al. 1997; Kozelka and Bruland 1998; Tang et al. 2001). This helps explain the absence of differential copper sensitivity between phytoplankton clones isolated from oceanic and neritic waters (Brand et al. 1986).

Free Cu^{2+} can increase to potentially toxic levels only when the high-affinity organic chelators in seawater are saturated (Moffett et al. 1997). When this occurs, copper toxicity can influence the composition of the phytoplankton community. Cyanobacteria are one of the most sensitive species to copper toxicity in the laboratory, perhaps because their ancestors evolved in an anoxic environment where the concentration of bioavailable copper was low (Brand et al. 1986). The small size and high surface to volume ratio of these cells, which is an advantage in acquiring a limiting trace element, can also be a disadvantage if metals are present in potentially toxic concentrations. Larger eukaryotic cells tend to be more resistant to copper toxicity. For instance, cell division rates of the diatom *Skeleonema costatum* were reduced only when the free Cu^{2+} concentration was over 200 pM, whereas several isolates of the cyanobacteria *Synechococcus* could not survive at a free Cu^{2+} of 11 pM (Brand et al. 1986). It is therefore not surprising that polluted harbors with free Cu^{2+} concentrations over 100 pM do not contain abundant *Synechococcus* and are dominated by *S. costatum* (Moffett et al. 1997).

The objective of this study is to determine whether free Cu^{2+} concentrations of less than 10 pM in the relatively unpolluted open ocean can influence the structure of the cyanobacteria community by selectively inhibiting the cell division of specific species. The Sargasso Sea is an ideal place for such an investigation because cyanobacteria account for a large fraction of the phytoplankton biomass and productivity (Goericke and Welschmeyer 1993), and free Cu^{2+} gradients are predictable with season and with depth (Moffett et al. 1990; Moffett 1995). Total copper concentrations increase linearly from the surface to deeper water as a result of biological uptake coupled with regeneration and particle scavenging at depth (Bruland and Franks 1983). Free Cu^{2+} does not necessarily increase in parallel with total copper because copper speciation is dominated by very strong ligands, collectively referred to as L_1, whose concentrations are not uniform throughout the water column. In the mideuphotic zone, near the chlorophyll fluorescence maximum, L_1 concentrations are equal to or higher than those of total copper. As a result, the fraction of total copper that is organically bound is very high (>99%) (Moffett and Zika 1987; Moffett et al. 1990; Moffett 1995). Free Cu^{2+} concentrations are higher toward the surface in certain conditions (*see below*) and below the euphotic zone where the concentration of L_1 decreases (Moffett et al. 1990). Based on the low L_1 concentrations in deep water, these strong copper chelators are of recent biological origin and are not derived from refractory compounds (Moffett et al. 1990).

In the euphotic zone of the Sargasso Sea small relative changes in total copper and L_1, which appear to be driven by seasonal forcing of physical and biological processes, lead to significant gradients in free Cu^{2+}. The water column is well stratified during the summer in the northern Sargasso
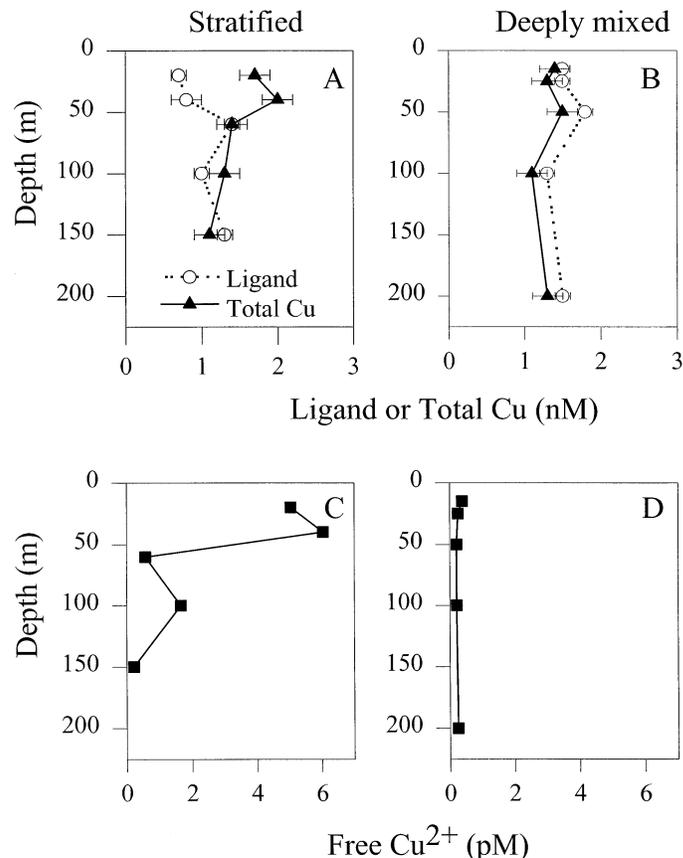

Fig. 1. The distribution of the high-affinity copper ligand L_1 and total Cu at the Bermuda Atlantic Time-series Station (BATS) in the Sargasso Sea in (A) a stratified and (B) a deeply mixed water column in July and March, respectively. (C, D) Free Cu^{2+} concentrations in each water column. Data from Moffett (1995).

and throughout the year in the southern Sargasso. Atmospheric deposition of trace metals is higher in the summer and copper accumulates in the shallow mixed layer (Fig. 1A; Moffett 1995; Jickells 1999). The concentration of L_1 is also lower within the mixed layer than just below it, probably because of photodegradation at the surface (Fig. 1A). This allows free Cu^{2+} to reach up to 6 pM close to the surface. Free Cu^{2+} concentrations are much lower deeper in the euphotic zone, where there is an excess of L_1 compared to total copper (Fig. 1C; Moffett et al. 1990; Moffett 1995). In the northern Sargasso Sea, the water column is deeply mixed during the winter, the atmospheric deposition of copper is low (Jickells 1999), and the concentration of L_1 is greater than that of total copper (Fig. 1B). As a result, free Cu^{2+} is less than 0.5 pM throughout the water column (Fig. 1D).

High concentrations of free Cu^{2+} associated with the saturation of the strongest ligands is generally thought of as more common in contaminated environments, such as polluted harbors (Moffett et al. 1997), than in the relatively pristine open ocean. However, because high free Cu^{2+} concentrations are a ubiquitous feature of shallow mixed layers in the tropical and subtropical Atlantic and Gulf of Mexico, our study site provides an opportunity to investigate a biological response at the species level to a widespread phe-

nomenon in the Atlantic Ocean. The extent of the increase in free Cu^{2+} toward the surface can vary from fourfold to over an order of magnitude, but all detailed free Cu^{2+} profiles from open ocean stratified water columns in these areas have shown some evidence for an increase in Cu^{2+} within the mixed layer, compared to below the thermocline (Buckley and van den Berg 1986; Moffett et al. 1990; Moffett 1995; Moffett and Zika 1987; this study). Variability in the extent of this increase might be due in part to the episodic nature of the atmospheric deposition of metals (Jickells 1999) and to changes in the depth of the mixed layer. High free Cu^{2+} concentrations may also be an inherently nonsteady state situation because scavenging removal of Cu is likely to be accelerated when it is less tightly complexed, and strong ligand production might be stimulated by Cu-sensitive organisms (van den Berg et al. 1987; Moffett et al. 1997).

We hypothesized that free Cu^{2+} might play a role in regulating cyanobacterial abundance in the Sargasso Sea because the dynamics of *Prochlorococcus* and *Synechococcus* could not be easily explained by other variables. The vertical distribution of *Synechococcus* is relatively constant with depth, regardless of the in situ free Cu^{2+} concentration (Olson et al. 1990). In contrast, there is a rough inverse correlation between free Cu^{2+} and *Prochlorococcus* abundance in stratified water columns in the northern Sargasso Sea (Moffett 1995). In shallow mixed layers, where free Cu^{2+} is high (Moffett 1995; this study), *Prochlorococcus* abundance is very low, and the bulk of the population is present below the mixed layer where the concentration of free Cu^{2+} is less than 1 pM (Olson et al. 1990; Moffett 1995). In environments where free Cu^{2+} is low in the mixed layer, the distribution of *Prochlorococcus* is more uniform with depth. This is the case in the subtropical Pacific, where both the fluvial dissolved flux and atmospheric deposition of metals are lower than in the Atlantic (Chester and Murphy 1990). The concentration of L_1 is greater than that of total copper, and as a result, free Cu^{2+} reaches only 0.01–0.2 pM (Coale and Bruland 1990). Under these conditions, *Prochlorococcus* are two orders of magnitude more abundant than *Synechococcus* at the surface throughout the year (Campbell and Vaulot 1993). These observations led us to hypothesize that *Prochlorococcus* might be more sensitive to Cu^{2+} toxicity than *Synechococcus,* and this may be one of the factors regulating their relative dominance.

Two approaches were used to test this hypothesis. First, the sensitivity of cultured isolates to environmentally relevant free Cu^{2+} concentrations was determined in the laboratory using *Prochlorococcus* isolates representing both the high- and low–light-adapted ecotypes (determined by chlorophyll [Chl] b/a_2 ratios) (Moore et al. 1998; Moore and Chisholm 1999) and *Synechococcus* clones isolated from the Sargasso Sea. Second, the copper sensitivity of in situ populations of both genera were examined by incubating water from different seasons and from different depths—representing environments with a range of ambient free Cu^{2+}—with added copper. Flow cytometry was used to follow changes in *Prochlorococcus* and *Synechococcus* cell numbers (the net growth rate) and *Prochlorococcus* DNA content per cell.

Table 1. Isolation coordinates and characteristics of *Synechococcus* and *Prochlorococcus* used in the culture experiments. See Waterbury et al. (1986) for more information on the *Synechococcus* isolates and Moore et al. (1988) and Moore and Chisholm (1999) for more information on the *Prochlorococcus* isolates.

Isolate name	Isolation coordinates	Location
Synechococcus		
WH 7805	33°44.8′N, 67°30′W	Sargasso Sea
WH 8103	28°30′N, 67°23.5′W	Sargasso Sea
Prochlorococcus		
Low–light adapted		
9313	37°30.8′N, 68°14.4′W	Gulf Stream
SS120	28°59′N, 64°21′W	Sargasso Sea
High–light adapted		
MED 4	43°12′N, 6°52′W	Mediterranean Sea
9311	37°30.8′N, 68°14.4′W	Gulf Stream
9401	Sargasso Sea	Sargasso Sea

Methods

Culture experiments—The *Synechococcus* isolates tested (WH 7805 and WH 8103) were supplied by John Waterbury of the Woods Hole Oceanographic Insititution (Waterbury et al. 1986). Both were isolated from the Sargasso Sea and have pigment compositions characteristic of open ocean strains (Table 1). The *Prochlorococcus* isolates examined were from a variety of locations (Table 1) and represented both the high- (MED 4, 9311, and 9401) and low–light adapted (SS120, 9313) ecotypes (Moore et al. 1998; Moore and Chisholm 1999). All experimental manipulations were done in a laminar flow hood. Sterile technique was used, although none of the *Synechococcus* and *Prochlorococcus* isolates used were axenic (Table 1). Marine bacteria can produce copper-binding compounds (Schreiber et al. 1990) and can also degrade metal chelator complexes (Thomas et al. 1998), so this was some cause for concern. By the end of the study, we had finally obtained an axenic culture of *Prochlorococcus* (Saito 2000), so we were able to do a control experiment to examine the effects of bacteria on our results. The presence of bacteria in *Prochlorococcus* cultures did not influence the degree of copper toxicity observed (Mann 2000*b*).

For all culture experiments, seawater for media was collected from the Sargasso Sea and stored in acid-cleaned polyethylene carboys. The water was sterilized by filtration through a 0.2-μm filter using an acid-washed borosilicate glass filter flask followed by microwave treatment (Keller et al. 1988). Nutrient and trace metal stocks were sterilized separately by filtration through acid-cleaned 0.02- or 0.2-μm syringe filters and were added at the final concentrations listed in Table 2. Population growth rates were determined by measuring in vivo fluorescence with a Turner fluorometer. Cultures were followed until the growth rate was constant for at least two transfers after the addition of copper. Cell division rates were determined using fluorescence as a proxy for cell number (Brand et al. 1986) and were set at zero if increases in fluorescence could not be maintained for two to three transfers.

Two experiments with cultured isolates were done: one to measure cell division rate as a function of free Cu^{2+} and a

Table 2. Media composition for culture experiments.

	Cell division rate	Cell cycle
Nitrilotriacetic acid (NTA)	10^{-4} M	None
EDTA	None	11.7 μM
NH$_4$Cl	50 μM	50 μM
Urea	100 μM	100 μM
NaH$_2$PO$_4$·H$_2$O	10 μM	10 μM
Na$_2$CO$_3$	40 μM	None
FeCl$_3$·6H$_2$O	7.3 μM	1.2 μM
Na$_2$MoO$_4$·2H$_2$O	3 nM	3 nM
MnCl$_2$·4H$_2$O	450 nM	5 nM
CoCl$_2$·6H$_2$O	160 nM	5 nM
NiCl$_2$·6H$_2$O	130 nM	10 nM
Na$_2$SeO$_3$	10 nM	10 nM
ZnSO$_4$·7H$_2$O	320 nM	8 nM

second to determine the effect of copper toxicity on the cell cycle. In the first experiment, isolates were grown in acid-washed borosilicate glass tubes in a 14 : 10 light : dark (LD) cycle at an irradiance of 30 μE m^{-2} s^{-1}. They were maintained in continuous batch culture for three or more transfers at constant cell division rates before copper was added as CuCl$_2$ at 0, 32, 65, 320, and 3,200 nM. After metals were buffered by 10^{-4} M nitrilotriacetic acid (NTA, Table 2), these copper additions increased the free Cu^{2+} concentrations from 0.07 to 1.2, 2.3, 11.2, and 112 pM, respectively. Free Cu^{2+} concentrations were calculated using the MINEQL program (Westall et al. 1976) with equilibrium constants from Morel and Hering (1993) corrected for ionic strength.

In the cell cycle experiment, the *Prochlorococcus* isolate MED 4 was grown in continuous light in acid-washed polycarbonate centrifuge tubes at an irradiance of 30 μE m^{-2} s^{-1}. After seven transfers, free Cu^{2+} in duplicate tubes was increased to 790 pM by the addition of 1×10^{-5} M CuCl$_2$. In this case, metals were buffered with 11.7×10^{-6} M ethylenediaminetetraacetic acid (EDTA) (Table 2). Samples were taken from the +Cu and control tubes 24 h after copper was added. Flow cytometry was used to determine cell abundance and DNA content per cell (*see below*).

Field experiments—Three stations with different vertical distributions of *Prochlorococcus* and *Synechococcus* and in situ free Cu^{2+} concentrations were investigated. Locations of the permanently stratified, seasonally stratified, and well or deeply mixed water columns sampled are shown in Fig. 2. Depth profiles of temperature and samples for enumerating cyanobacteria (Fig. 3) were obtained down to 200 m using a rosette equipped with Nisken bottles and a CTD.

Bottle incubations: Seawater was obtained for bottle incubations using acid-cleaned, Teflon-coated Go-Flo bottles suspended on Kevlar wire. Water was collected from 45 m in the deeply mixed station and from both within and below the mixed layer in the stratified water columns (Table 3). Further experimental manipulations were done under a laminar flow hood where 0, 2, and 5 nM total copper was added to duplicate or triplicate polycarbonate bottles. Bottles were placed in on-deck incubators that were screened as follows to mimic the in situ illumination and cooled with surface
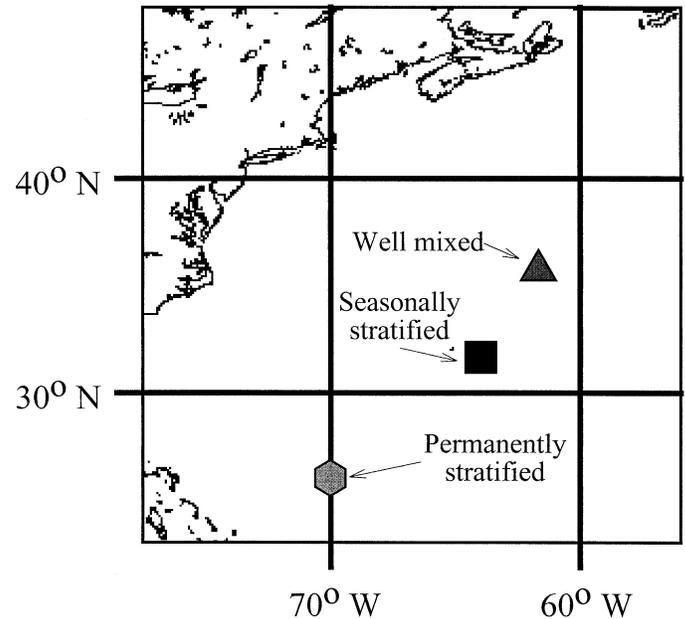

Fig. 2. Map of stations sampled in the Sargasso Sea. Different symbols represent different cruises. The water columns were deeply mixed, seasonally stratified, and permanently stratified in January 1997, June 1998, and February 1998, respectively.

seawater. All bottles were screened with one layer of dark blue Rosco 69 (18% transmission) optical gel (High Output) and were placed in clear plastic bags. Incubation bottles from the mixed layer or deeper in the euphotic zone of the stratified water columns were screened with an additional layer of light grey (50% transmission) or dark grey (12% transmission) optical gels (High Output), respectively. Samples were taken daily using acid-cleaned Teflon transfer caps and tubing via a positive pressure system. Additional aliquots for analysis of *Prochlorococcus* DNA content were taken at night from the stratified water column incubations.

Copper analysis: In all incubation bottles, total and free Cu^{2+} concentrations were determined from aliquots removed
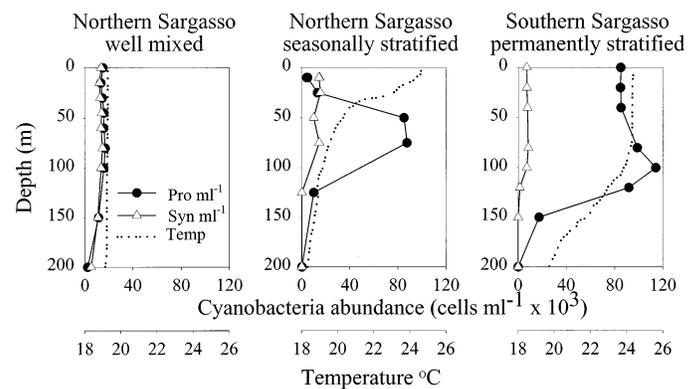

Fig. 3. Depth profiles of *Prochlorococcus* and *Synechococcus* abundance and temperature from (A) the deeply mixed and (B) the seasonally stratified northern Sargasso and from (C) the permanently stratified southern Sargasso.

Table 3. Characteristics of the on-deck bottle incubations, including the location and depth sampled, the amount of copper added, and the resulting total and free Cu^{2+} measured in the bottles. Standard error propagated in free Cu^{2+} calculations was estimated at $\pm 30\%$ (*see Methods*). *Prochlorococcus* and *Synechococcus* net growth rates ($k\ d^{-1}$) in the bottles are reported plus or minus the standard error. Net growth rates in the $+Cu$ bottles that are significantly different from the controls are in bold (99% confidence levels using an *F*-test to compare slopes, Sokal and Rohlf [1995]).

Description	Depth (m)	Added Cu (nM)	Total Cu (nM)	Free Cu^{2+} (pM)	*Prochlorococcus* ($k\ d^{-1}$)	*Synechococcus* ($k\ d^{-1}$)
Deeply mixed	45	0	1.2 ± 0.2	0.6	0.4 ± 0.02	0.5 ± 0.10
Mixed layer		2	3.0 ± 0.5	4.7	**0.1 ± 0.05**	0.5 ± 0.04
		5	6.4 ± 0.5	9.0	**0.0 ± 0.01**	0.6 ± 0.03
Seasonally stratified						
Mixed layer	16	0	1.2	3.6	-0.1 ± 0.03	0.0 ± 0.02
		5	4.4 ± 0.4	9.0	-0.2 ± 0.11	**-0.1 ± 0.02**
Below mixed layer	85	0	1.0 ± 0.1	0.1	0.1 ± 0.05	-0.0 ± 0.05
		2	2.8	3.0	**-0.1 ± 0.04**	-0.2 ± 0.16
		5	5.0	12.0	**-0.5 ± 0.16**	-0.2 ± 0.11
Permanently stratified						
Mixed layer	50	0	2.4 ± 0.3	2.0	0.1 ± 0.02	0.1 ± 0.02
		2	2.0 ± 0.3	2.0	**-0.0 ± 0.04**	0.1 ± 0.03
		5	4.2 ± 0.3	8.0	**-0.1 ± 0.03**	0.1 ± 0.04
Below mixed layer	125	0	0.9	0.2	0.1 ± 0.03	0.3 ± 0.04
		5	5.2 ± 0.8	8.0	**-1.2 ± 0.23**	0.2 ± 0.06

at the end of the experiment. Samples were filtered through 0.2 μm Poretics filters and frozen until analyzed. Thawed samples were ultraviolet (UV)-irradiated and analyzed for both total copper and free Cu^{2+} by cathodic stripping voltammetry as described in Moffett (1995). Free Cu^{2+} was calculated in most samples from titration data obtained using cathodic stripping voltammetry with benzoylacetone as the competing ligand. The standard error propagated by these calculations was estimated to be $\pm 30\%$. The protocol was similar to that described in Moffett (1995) and Moffett et al. (1997), except in this study, we used an Eco Chemie Microautolab voltammetric analyzer coupled to a Metrohm VA 663 static mercury drop electrode.

Sample preservation and DNA staining with Sybr Green 1: All field samples, as well as selected aliquots from the culture experiments, were preserved in a final concentration of 0.1% glutaraldehyde for 10 min at room temperature in the dark. The samples were then immediately placed in liquid nitrogen (Vaulot et al. 1989). *Prochlorococcus* DNA content per cell was determined by adding potassium citrate at a final concentration of 30 μM and the DNA stain Sybr Green 1 at a $1:10^{-4}$ final dilution (Molecular Probes). All working stocks of Sybr Green I were diluted in DMSO (Marie et al. 1997). Samples were incubated at room temperature in the dark for 15 min before being analyzed by flow cytometry. DNA fluorescence histograms were modeled and divided into G_1, S, and $G_2 + M$ using ModFit software (Verity Software House, Inc.).

Flow cytometry: Cell number and DNA content per cell were quantified using a modified EPICS 753 flow cytometer. The instrument was configured as in Binder et al. (1996), except that it was used in single-beam mode and the optical filters were arranged to detect green fluorescence from Sybr Green I, which is excited at 488 nm. After passing through

630- and 560-nm short-pass dichroic filters, green signals were selected using a 515-nm long-pass filter. Samples were delivered at a constant rate using a syringe pump (Harvard Apparatus, model 22). Unstained samples were run at a flow rate of 10 μl min^{-1}. Sybr Green stained samples were run at lower flow rates (5 to 7 μl min^{-1}) after pre-equilibrating sample lines with the stain for at least 1 h.

Determination of net growth rates: Net growth rates ($k\ d^{-1}$) were determined from linear regressions of the natural log of cell number versus time. Regression coefficients are reported plus or minus the standard error in Table 3 (Sokal and Rohlf 1995).

Results and discussion

Sensitivity of Prochlorococcus *and* Synechococcus *cultures to free cupric ion concentration*—Based on the distribution of *Prochlorococcus* and *Synechococcus* in the Sargasso Sea, we hypothesized that *Synechococcus* would be more resistant to high free Cu^{2+} than *Prochlorococcus*. In order to test this hypothesis, the cell division rates of cultured isolates were measured at various free Cu^{2+} concentrations. Two *Synechococcus* isolates were tested, WH 7805 and WH 8103. The former was one of the most copper-sensitive clones out of seven cyanobacteria investigated in an earlier study (Brand et al. 1986). The latter was used because it is one of the few isolates with the high phycourobilin pigment content characteristic of the dominant type of *Synechococcus* in the Sargasso Sea (Olson et al. 1988). We found that the cell division rates of both isolates were not affected by free Cu^{2+} up to 112 pM (log free Cu^{2+} = -9.9), which is at least an order of magnitude higher than the observed in situ concentrations in the Sargasso Sea (solid bar, Fig. 4).

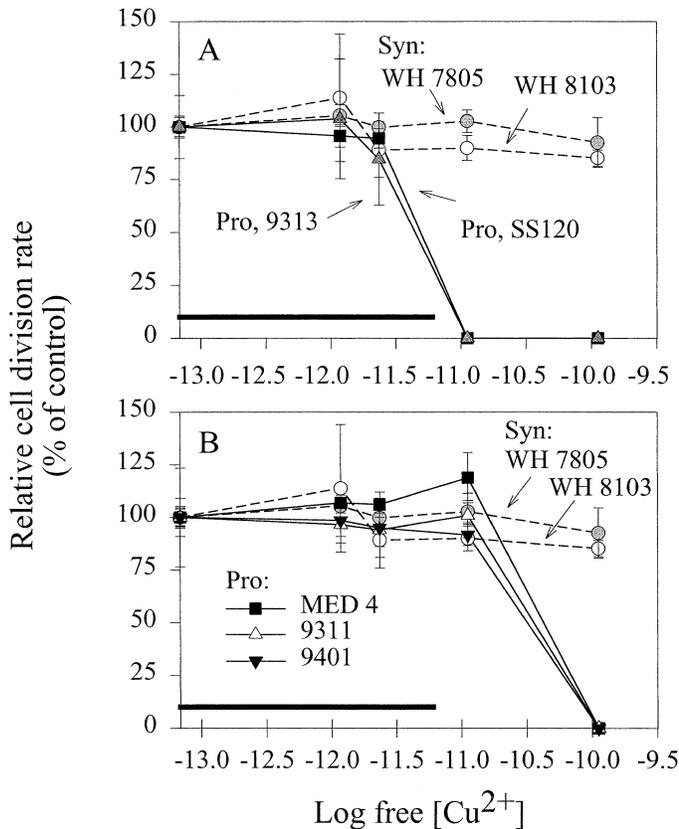*Prochlorococcus* were more sensitive to free Cu^{2+} than

Fig. 4. Cell division rates of *Prochlorococcus* and *Synechococcus* isolates as a function of free Cu^{2+}. Rates are expressed relative to the uninhibited control culture. (A) *Synechococcus* and low–light adapted *Prochlorococcus*. (B) *Synechococcus* and high–light adapted *Prochlorococcus* ecotypes. The average cell division rate of the controls was 0.6 d^{-1}, with a range of 0.35 to 0.86 d^{-1}. The dark bar represents the range of free Cu^{2+} observed in the Sargasso Sea (Moffett 1995). For more detail on the isolates used, see Table 1.

Synechococcus, but there were differences in sensitivity between the two ecotypes. The low–light adapted isolates SS120 and 9313 (Moore et al. 1998; Moore and Chisholm 1999) could not grow at free Cu^{2+} above 2.3 pM (log free $Cu^{2+} = -11.6$, Fig. 4). This is less than half the highest in situ concentration observed in the Sargasso Sea (Moffett 1995). However, growth rates of the high–light adapted ecotypes (MED 4, 9311, and 9401) were only significantly reduced at 112 pM (log free $Cu^{2+} = -9.9$, Fig. 4). Thus the cell division rates of these cells, although clearly more sensitive to free Cu^{2+} than *Synechococcus,* were not significantly reduced at free Cu^{2+} concentrations that are typical of Sargasso Sea waters (solid bar, Fig. 4).

One factor that must be taken into account when interpreting copper toxicity data is the influence of other metals on the results. Competitive interactions in which a high concentration of one metal alleviates the toxic effects of another are important in determining copper toxicity and have been documented for Cu and Fe (Murphy et al. 1984; Stauber and Florence 1985), Cu and Mn (Sunda 1989; Sunda and Huntsman 1998), and Cu and Zn (Sunda and Huntsman 1998). In other words, the free Cu^{2+} concentration that is toxic to cy-

anobacteria will change in response to the free ion concentrations of other metals. In spite of this, the relative sensitivity to copper of *Synechococcus* and the two *Prochlorococcus* ecotypes has been consistent in laboratory experiments using several media with different free trace metal ion concentrations (data not shown, Mann 2000*b*). *Synechococcus,* followed by the high–light adapted *Prochlorococcus,* were always more copper resistant than *Prochlorococcus* from the low–light adapted ecotype. As a result, we feel confident that these differences in sensitivity to copper are ecologically relevant. Moreover, the free Cu^{2+} concentrations that produced toxic effects in the laboratory are consistent with the results of the field experiments described below.

Copper sensitivity of Prochlorococcus *and* Synechococcus *in the Sargasso Sea*—Based on the results from cultured isolates, we expected that *Prochlorococcus* in the field would be more affected by free Cu^{2+} than *Synechococcus* because the cell division rates of at least some of the former strains were reduced at environmentally relevant free Cu^{2+}. In addition, since *Prochlorococcus* in the high–light adapted ecotype are more copper resistant, it would make sense if these cells dominated the *Prochlorococcus* population in mixed layers, where both irradiance and free Cu^{2+} are high. To investigate these ideas, the sensitivity of cyanobacteria from environments with different in situ free Cu^{2+} concentrations to added copper was analyzed using bottle incubations from several depths and locations.

Three stations were investigated, including both a seasonally stratified and a deeply mixed water column in the northern Sargasso Sea and a permanently stratified water column in the southern Sargasso. These water columns were divided into low and high free Cu^{2+} environments based on in situ measurements of copper. Two low free Cu^{2+} environments were sampled: the entire water column in the deeply mixed northern Sargasso and the region below the mixed layer in the stratified water columns. In the former case, free Cu^{2+} was 0.6 pM at 45 m (Table 3) in a mixed layer of approximately 200 m (Fig. 3A). This is in good agreement with the 0.5 pM free Cu^{2+} found throughout the upper 200 m in an earlier analysis of a similar water column (Moffett 1995). Free Cu^{2+} below the mixed layer in the stratified water columns was even lower and ranged from 0.1 to 0.2 pM (Table 3). However, free Cu^{2+} concentrations in stratified water columns varied substantially with depth (Moffett 1995; this study) and were 10- to 36-fold higher at the surface than below the mixed layer (Table 3). The mixed layers of both the seasonally and permanently stratified water columns, which contained 2.0 to 3.6 pM of free Cu^{2+} (Table 3), were considered high Cu^{2+} environments. For comparison, the highest in situ free Cu^{2+} reported for the Sargasso Sea is 6 pM (measured in a shallow mixed layer in the seasonally stratified northern Sargasso Sea [Moffett 1995]).

In the deeply mixed water column where free Cu^{2+} concentrations were low, the abundance of *Prochlorococcus* and *Synechococcus* was roughly equal (1.5×10^4 ml^{-1}) and constant with depth until cell numbers declined between 100 and 200 m (Fig. 3A). In the stratified water columns, the distribution of *Synechococcus* was relatively uniform with

depth. In contrast, *Prochlorococcus* abundance varied inversely with free Cu^{2+} and cell numbers were higher in deeper water where free Cu^{2+} was lower. In the northern Sargasso Sea, *Prochlorococcus* numbers within the shallow mixed layer decreased by 20-fold compared to those below the thermocline. This occurred to a much lesser extent in the deeper mixed layer of the southern Sargasso. *Prochlorococcus* numbers declined slightly at the surface but remained significantly higher than those of *Synechococcus* (Fig. 3B,C).

Phytoplankton assemblages from each of these environments were incubated in bottles on deck and either 0, 2, or 5 nM of copper was added. These high amounts were necessary to saturate the copper ligand L_1 and thus elevate the free Cu^{2+} concentration (the toxic species) to 2 pM or higher. Both total copper and free Cu^{2+} were measured in each bottle at the end of the incubation period (*see Methods*). These analyses indicated that none of the incubations performed in this study were inadvertently contaminated by copper (Table 3). Some samples had significantly lower concentrations than predicted based on the copper added. This could reflect wall loss (Coale 1991) or uptake by suspended particles that were subsequently removed during filtration. In spite of this loss, free Cu^{2+} in the Cu-spiked samples was higher than in the controls, in some cases substantially so (Table 3). The large increase in free Cu^{2+} reflects saturation of the L_1-type ligands that dominate copper speciation at ambient levels. As a result, increases in free Cu^{2+} were statistically significant, in spite of the large standard error propagated in the calculations. The addition of 2 nM copper generally resulted in free Cu^{2+} concentrations that are environmentally realistic, whereas the 5 nM copper addition increased free Cu^{2+} to 1.3 to 2 times the highest concentration seen in situ (Table 3).

Low Cu^{2+} environments—deeply mixed water column and below the mixed layer in stratified water columns: Added copper drastically reduced *Prochlorococcus* net growth rates in both low Cu^{2+} environments, without affecting those of *Synechococcus*. In incubations from the deeply mixed water column, this differential effect is easily illustrated by a comparison of the flow cytometric scatterplots from the control and +5 nM Cu bottles (Fig. 5). Net growth rates of *Prochlorococcus* in this experiment were significantly depressed by the addition of copper, decreasing from 0.4 d^{-1} to 0.1 and 0.0 d^{-1} with the addition of 0, 2, and 5 nM Cu, respectively (Fig. 6A; Table 3). The net growth rate of *Synechococcus,* on the other hand, was not significantly inhibited and ranged from 0.5 to 0.6 d^{-1} in both the control and +Cu bottles (Fig. 6B; Table 3).

Prochlorococcus net growth rates also decreased significantly when copper was added to water from below the mixed layer (where the in situ free Cu^{2+} was low) in both the seasonally and permanently stratified water columns. At the seasonally stratified station, *Prochlorococcus* net growth rates decreased from 0.1 $\pm$ 0.05, to -0.1 ± 0.04, and -0.5 ± 0.16 d^{-1} for the control and 2 and 5 nM copper bottles, respectively (Fig. 7A; Table 3). *Prochlorococcus* net growth rates decreased even further to -1.2 ± 0.23 d^{-1} (Fig. 7A; Table 3) when 5 nM of copper was added to deep water from the permanently stratified station. In contrast, the ad-
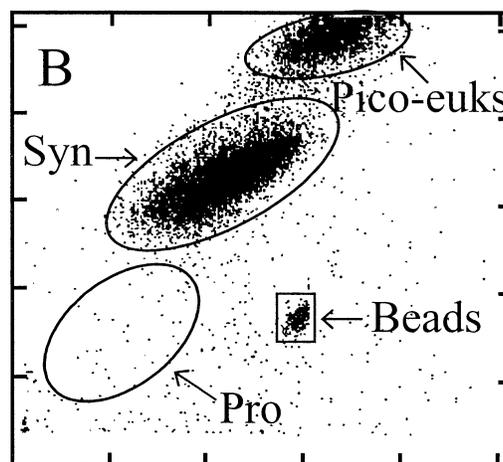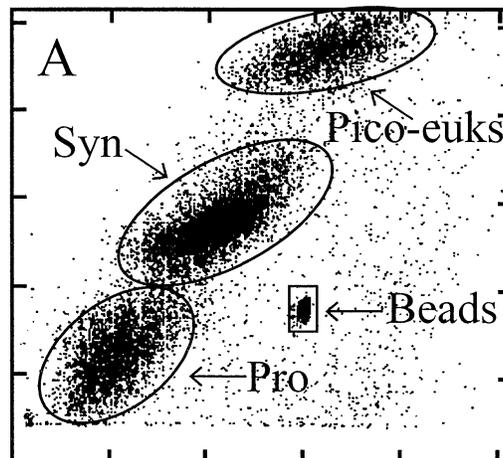
Fig. 5.   Flow cytometric scattergrams of side scatter (a proxy for cell size) versus red fluorescence per cell relative to standard beads in (A) the control and (B) a +5 nM copper bottle 4 d after copper was added. Water for these on-deck incubations was taken from the deeply mixed water column at 45 m in the northern Sargasso Sea. Populations of picoeukaryotes, *Synechococcus, Prochlorococcus,* and 0.474 μm standard beads are identified. Note that recognizable *Prochlorococcus* were virtually eliminated by the addition of copper.

dition of copper to water from below the mixed layer in both the seasonally and permanently stratified water columns had no significant effect on *Synechococcus* net growth rates (Fig. 7B; Table 3).

High Cu^{2+} environments—within the mixed layer in stratified water columns: The in situ concentration of free Cu^{2+} had a strong effect on the copper sensitivity of *Prochlorococcus*. Cells from the mixed layer, where the in situ free Cu^{2+} was already high, were much less sensitive to copper than those from deeper water. These data are consistent with
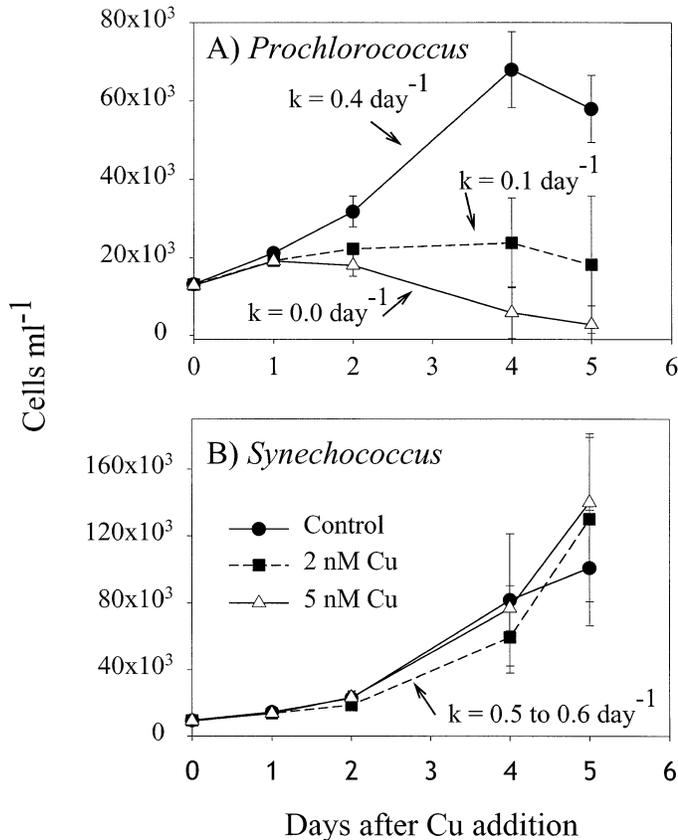
Fig. 6. (A) *Prochlorococcus* and (B) *Synechococcus* abundance and net growth rates in triplicate bottles incubated on deck with 0, 2, and 5 nM added copper. Free Cu^{2+} concentrations were 0.6, 4.7, and 9.0 pM, respectively (*see Table 3*). Water for these incubations was taken from 45 m in a deeply mixed water column (January 1997). The net growth rates were calculated over the first 4 d.

Fig. 7. *Prochlorococcus* and *Synechococcus* net growth rates in bottles incubated with 0, 2, and 5 nM added copper. Picomolar free Cu^{2+} concentrations are noted. (A) *Prochlorococcus* and (B) *Synechococcus* from the seasonally and permanently stratified water columns. ML and BML refer to incubations of water from the mixed layer and below the mixed layer, respectively. Error bars represent the standard errors of the net growth rate regressions, and significant differences between the net growth rates in the control and +Cu bottles are marked with an asterisk (*).

a mixed layer dominated by *Prochlorococcus* from the high–light adapted, copper-resistant ecotype (*see below*; Fig. 4). *Prochlorococcus* net growth rates were either not affected by the addition of copper or decreased slightly (Fig. 7A) in incubations from the mixed layer of the seasonally and permanently stratified water columns, respectively. In the latter, net growth rates did decrease from 0.1 ± 0.02 to -0.1 ± 0.03 d^{-1} when the addition of 5 nM copper increased the free Cu^{2+} to 8 pM. However, this change was small when compared to the net growth rate decrease from 0.1 ± 0.03 to -1.2 ± 0.23 d^{-1} that occurred when *Prochlorococcus* from below the mixed layer were exposed to the same free Cu^{2+} concentration (Fig. 7A; Table 3). The resistance to copper toxicity in *Synechococcus* followed the opposite pattern; the only significant decrease in *Synechococcus* net growth rates occurred in the mixed layer of the seasonally stratified water column, where the in situ free Cu^{2+} concentrations were already high (Fig. 7B; Table 3). As a result, the sensitivity of *Prochlorococcus* and *Synechococcus* to copper was similar in incubation bottles from the mixed layer (Fig. 7; Table 3).

As discussed above, the concentration of trace metals other than copper must be considered in interpreting these results. For example, higher concentrations of other metals in
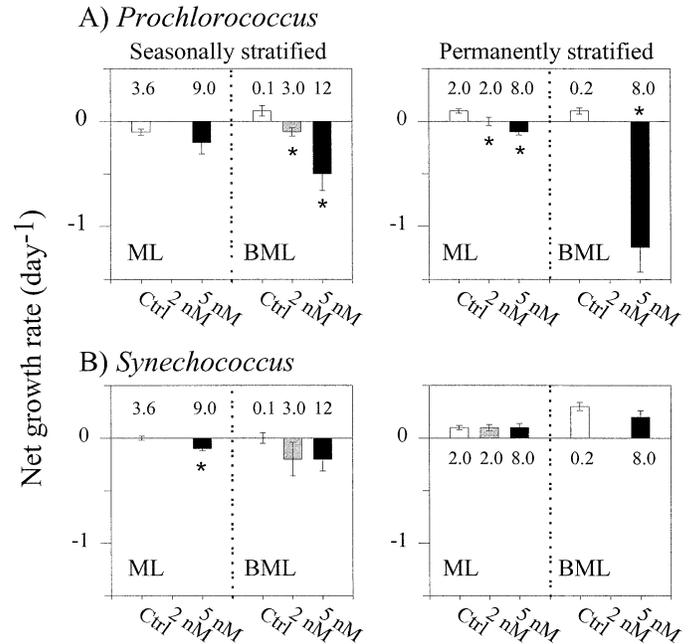
neritic, as compared to oceanic, waters is one possible explanation for the enhanced resistance to added copper sometimes found in neritic phytoplankton communities (Sunda 1987). If the concentrations of other metals are higher within the mixed layer than in the mideuphotic zone, then changes in the ratio of Cu to other metal ions could explain some of the increased resistance to copper toxicity seen in *Prochlorococcus* populations from the surface. The concentration of soluble Mn^{2+} does increase by roughly a factor of two from the mideuphotic zone to the mixed layer in the Sargasso Sea (Bruland and Franks 1983; Sunda and Huntsman 1988). However, because free Cu^{2+} concentrations increase by at least a factor of four to over an order of magnitude in the same conditions (Buckley and van den Berg 1986; Moffett et al. 1990; Moffett 1995; Moffett and Zika 1987; this study), we do not think any variation in the Cu:Mn ratio was substantial enough to affect the degree of resistance to added copper. In any case, changes in the Cu:Mn ratio within the euphotic zone were not great enough to influence the copper resistance of *Synechococcus,* which was relatively constant with depth.

Net growth rates and grazing: We have interpreted the variations in net growth rates among the cyanobacteria populations in the incubation bottles as arising from differences in resistance to copper toxicity. However, other explanations are possible. For instance, because net growth rates are a function of both cell division rate (intrinsic growth rate) and

mortality, it is possible that the changes in cell number we measured with the addition of copper could be due to differences in grazing-induced mortality. We believe that this is not the case for several reasons. Grazing-induced mortality is probably lower in the +Cu bottles than in the controls because the cell division rates of ciliates in culture are reduced by free Cu^{2+} of less than 1 pM (Stoecker et al. 1986). Even if grazing pressure were higher in the +Cu bottles, *Prochlorococcus* and *Synechococcus* would have to have different and extremely selective grazers in order to account for the observed decreases in *Prochlorococcus* net growth rates while *Synechococcus* were unaffected (Fig. 6). It seems unlikely that cyanobacteria grazers would be so specific as to ignore stable and, in the deeply mixed water column, rapidly growing *Synechococcus* populations when *Prochlorococcus* were disappearing—particularly when marine protozoa in the laboratory can obtain higher cell division rates and cell yields from *Synechococcus* and bacteria than from *Prochlorococcus* (Caron et al. 1991; Christaki et al. 1999).

Prochlorococcus DNA content per cell: Our interpretation that metal toxicity was the underlying cause of net growth rate changes in the +Cu bottles is directly supported by data on *Prochlorococcus* DNA content per cell. (These analyses were not done for *Synechococcus* because of technical limitations.) As an intrinsic cell property, DNA concentration is not directly affected by grazing. Instead, DNA content reflects the physiological state of the cell and the progression through the cell cycle. Division in *Prochlorococcus* is tightly synchronized to the LD cycle, and there is a discrete DNA synthesis phase (S), even when the cells are growing faster than one division per day (Shalapyonok et al. 1998). As a result, the eukaryotic terms G_1, S, and $G_2 + M$ are applied for convenience. During the day, most cells are in G_1 and have one complement of DNA. DNA synthesis generally starts after dusk and reaches a peak around 1900 h, followed by the G_2 phase and mitosis (Vaulot et al. 1995; Liu et al. 1997; Shalapyonok et al. 1998; Mann 2000*a*). Samples for DNA analysis were taken at night when cells in the $G_2 +$ M stage of the cell cycle should be present if the populations are actively dividing (Vaulot et al. 1995; Liu et al. 1997; Shalapyonok et al. 1998; Mann 2000*a*).

DNA histograms in both control and +Cu bottles are similar within the mixed layer, but in deeper water, changes in DNA content per cell are consistent with the increased sensitivity of these populations to copper. In the permanently stratified southern Sargasso, samples for DNA analysis were taken 12 h after copper was added (day 0). Cells in the G_2 + M phase of the cell cycle were present in both the control and +5 nM Cu bottles from the mixed layer, as would be expected from dividing populations (Fig. 8A). Increasing the free Cu^{2+} in water from below the mixed layer had a more dramatic effect. No cells in the G_2 + M phase were present in the +5 nM Cu bottles (Fig. 8B). Either *Prochlorococcus* arrested in the G_1 and early S phases or the timing of the cell cycle was significantly disrupted by copper toxicity. There was also a significant left shoulder to the G_1 peak of the +Cu cells, indicating a decrease in DNA content or some change in the cells that would make the DNA less available for staining (Fig. 8B). Similar DNA histograms, with cells
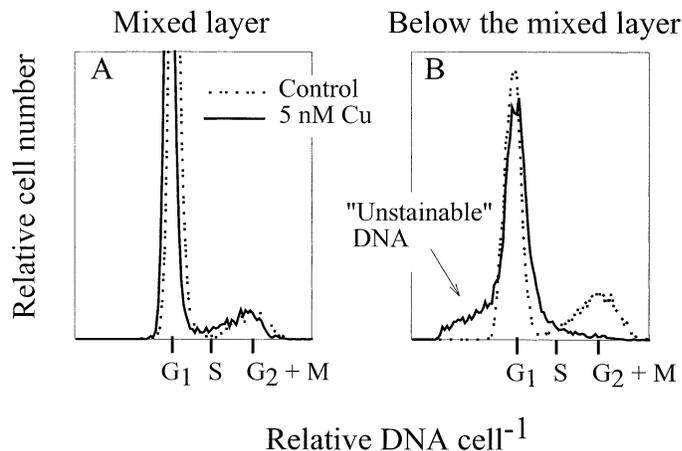

Fig. 8. Distribution of relative DNA per cell in *Prochlorococcus* 12 h after 5 nM copper was added to bottles from (A) within and (B) below the mixed layer of the permanently stratified southern Sargasso Sea.

present only in G_1 and early S and an apparent loss of DNA content, were also seen in the +Cu bottles from below the mixed layer in the seasonally stratified water column (Mann 2000*b*).

DNA histograms from a *Prochlorococcus* isolate were examined to verify that metal toxicity was directly responsible for the loss of stainable DNA and the arrest of cells in G_1 and early S described above. The high–light adapted isolate MED 4 was grown in constant light until the cell cycle was asynchronous. This allowed sampling at any time without complications from the synchronization of cell division to the LD cycle. In the experimental tubes, the free Cu^{2+} concentration was increased to 790 pM. This quickly decreased the cell division rate from 0.26 in the control to 0.06 d^{-1}. Twenty-four hours after copper was added, cell numbers were about 40% lower in the +Cu culture than in the control (data not shown). This decrease in cell number and division rate was accompanied by the arrest of cells in the G_1 and early S phases and an apparent loss of DNA content per cell (Fig. 9). Although this pattern of cell cycle arrest might not be associated specifically with copper toxicity, DNA molecules are one target of the reactive hydroxyl radicals generated when intracellular copper concentrations are high (Brown et al. 1994; Ueda et al. 1998).

Overview and additional considerations—Data from both the net growth rate and the DNA content per cell indicate that *Prochlorococcus* from environments with high in situ free Cu^{2+} were much more resistant to copper toxicity than cells acclimated to lower free Cu^{2+} concentrations. We hypothesize that these differences in copper sensitivity are the result of dominance of each environment by a different ecotype. In this view, the *Prochlorococcus* population in mixed layers (where both irradiance and free Cu^{2+} concentrations are high) is dominated by the high-light ecotype. Members of the low–light adapted ecotype, which is more copper sensitive, account for the bulk of the *Prochlorococcus* population below the mixed layer and in the deeply mixed water column where the in situ free Cu^{2+} concentrations are low.
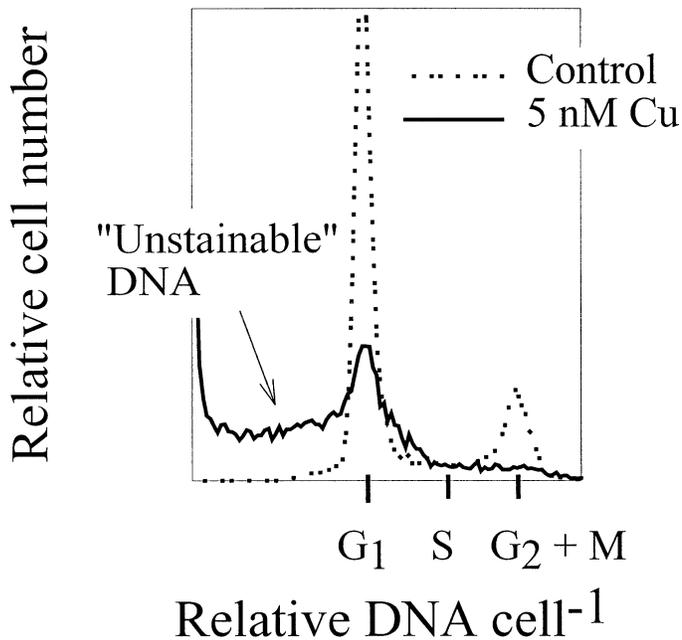
Fig. 9. Distribution of relative DNA per cell in a high–light adapted *Prochlorococcus* isolate, MED 4, grown in continuous light with or without added copper. Samples were taken 1 d after copper was added when the cell division rate in the +Cu tubes began to decrease. Cells exposed to high free Cu^{2+} are arrested in the G_1 and early S phases of the cell cycle. Note that the +Cu culture has some degraded or unstainable DNA to the left of the G_1 peak.

Another possible explanation for different degrees of copper sensitivity in *Prochlorococcus* populations is the induction of copper resistance mechanisms within a genetically homogeneous population only when in situ free Cu^{2+} concentrations are high. This may be the case to some extent, but a partitioning of ecotypes on the basis of both irradiance and free Cu^{2+} concentrations is probable since, to date, all high–light adapted *Prochlorococcus* have been more resistant to copper toxicity than cells in the low–light adapted ecotype (Fig. 4; Table 2). In situ populations of *Prochlorococcus* are also genetically diverse (Urbach and Chisholm 1998). Genetically distinct ecotypes have been shown to coexist in the field (Moore et al. 1998), and members of the high–light adapted ecotype predominate within the mixed layer (West et al. 2001). In addition, stepwise acclimation to increasing concentrations of copper, which would allow for the induction of copper resistance mechanisms, had no effect on the copper sensitivity of *Prochlorococcus* isolate SS120 (data not shown).

Overall, free Cu^{2+} levels in the Sargasso Sea are more likely to affect *Prochlorococcus* than *Synechococcus*. The latter were clearly more resistant to copper toxicity than *Prochlorococcus* in experiments with cultured isolates (Fig. 4) and in environments where the in situ free Cu^{2+} concentrations were low (Figs. 5–7; Table 3). The copper resistance of *Synechococcus* was relatively constant with depth and did not increase with the in situ free Cu^{2+} concentration (Fig. 7; Table 3). *Synechococcus* net growth rates decreased with the addition of copper only in the 5 nM Cu bottle from the seasonally stratified mixed layer, where free Cu^{2+} concentra-

tions were already high (Fig. 7; Table 3). This suggests that the free Cu^{2+} concentrations in the mixed layer are high enough to exert a selective pressure on *Prochlorococcus*, but not on *Synechococcus*.

The molecular basis for the differences in copper sensitivity between cyanobacteria species and *Prochlorococcus* ecotypes is unknown. Strategies bacteria use to gain copper tolerance include copper efflux mechanisms such as Cu ATPases, sequestration by periplasmic or outer membrane proteins, adsorption onto polysaccharides on the cell surface, and reduced intracellular uptake (Cooksey 1993; Brown et al. 1994; Cervantes and Gutierrez-Corona 1994), as well as production of extracellular copper chelators (Moffett and Brand 1996). For example, *Synechococcus* produces a strong copper binding ligand in concentrations that are regulated by the amount of metal present (Moffett and Brand 1996). This ligand has the same binding constant as the copper-binding L_1 found in situ, indicating that cyanobacteria could influence trace metal speciation in the oceans (Moffett and Brand 1996). Mechanisms in high–light adapted *Prochlorococcus* designed to deal with intense irradiance may also confer resistance to copper because both can cause damage through oxidative stress. In any case, acquiring resistance to copper for *Prochlorococcus* may involve some cost because cells in environments with low in situ free Cu^{2+} concentrations are clearly not copper resistant. Costs could either be direct, such as the ATP needed to run a Cu efflux pump, or indirect if copper resistance involves a reduced access to other metals such as iron.

Although it appears that copper toxicity must be considered to fully understand the regulation of cyanobacteria abundance in the Sargasso Sea, other variables such as irradiance, nutrient limitation, and grazing pressure are also undoubtedly important. For example, differences in free Cu^{2+} concentrations and irradiance might explain the low abundance of *Prochlorococcus* at the surface of the seasonally stratified northern Sargasso compared to the mixed layer of the southern Sargasso Sea (Fig. 3). Presumably, as we argued above, acquiring resistance to copper involves some cost, and free Cu^{2+} is about twice as high in the seasonally stratified mixed layer than in the southern Sargasso Sea (Table 3; Moffett 1995). This is a small increase in free Cu^{2+} concentration, but it might be significant—particularly if copper tolerance is achieved using an energy-intensive process. The mixed layer in the seasonally stratified water column is also about three times shallower than in the permanently stratified southern Sargasso Sea, so cells above the thermocline will also be trapped at a higher irradiance than those in the deeper mixed layer (Fig. 3). This is important because high irradiance, in addition to being a direct stress for phytoplankton, increases the toxicity of copper (Rai et al. 1995; Lupi et al. 1998). Synergistic relationships between free Cu^{2+} and irradiance were probably reduced in the incubations compared to the actual water column because the irradiance was lower and the polycarbonate bottles would have attenuated UV wavelengths of less than approximately 350 nm.

Nutrient concentrations must also be considered because soluble phosphorous concentrations are lower at the surface of the Atlantic than in the Pacific (Wu et al. 2000). This is

one possible explanation (in addition to the lower free Cu^{2+} concentrations) for the high abundance of *Prochlorococcus* in Pacific surface waters (Campbell and Vaulot 1993; Liu and Campbell 1997). Soluble reactive phosphorous and $NO_3 + NO_2$ concentrations are low in the mixed layer of the seasonally stratified Sargasso Sea. However, they are as low in the permanently stratified mixed layer further to the south (Cavender-Bares et al. 2001). Because *Prochlorococcus* are 20-fold more abundant at the surface of the latter (Fig. 3), nutrient concentrations might not play a large role in determining the distribution of these cyanobacteria unless the fluxes of nutrients are significantly different in the two stratified water columns.

Higher loss rates at the surface could also explain why *Prochlorococcus* concentrations in shallow mixed layers are lower than those at depth. However, evidence for a higher grazing rate in the mixed layer compared to deeper water is equivocal. The concentrations of potential *Prochlorococcus* predators are higher at the surface in some cases (Arenovski 1994; Caron et al. 1999), but not in others (Weisse and Scheffel-Moser 1991; Arenovski 1994; Caron et al. 1999). The disappearance of labeled prey in surface waters of the Sargasso Sea is also highly variable, ranging from 0 to 0.6 d^{-1} (Caron et al. 1999). Viral lysis could also be important, but it is unclear to what extent this can determine cyanobacteria population abundance (Fuhrman and Suttle 1993; Waterbury and Valois 1993). For *Prochlorococcus,* the titer of specific viruses is lower within shallow mixed layers than in deeper water (Sullivan et al. pers. comm.), but there is also the intriguing possibility that increasing free Cu^{2+} concentrations could induce lysogenic viruses to enter a lytic phase (Sode et al. 1997).

In this study, we have shown that cell division rates of *Prochlorococcus* are more likely to be affected by the free Cu^{2+} concentrations found in the Sargasso Sea than those of *Synechococcus*. Thus, picomolar quantities of free Cu^{2+} might be a significant factor in regulating the relative abundance of these cyanobacteria in the Sargasso Sea. Although all *Prochlorococcus* isolates were more sensitive to copper than *Synechococcus,* members of the high–light adapted clade were more copper resistant than the low–light adapted strains. Cell division rates of the latter are inhibited at free Cu^{2+} concentrations that are not uncommon in the surface waters of the Sargasso Sea. The results of field experiments, in which copper was added to natural communities of cyanobacteria collected from environments with different in situ free Cu^{2+} concentrations, were consistent with the differential sensitivities to copper seen in the isolated strains. We hypothesize that conditions in the surface waters of stratified water columns select for the more copper-resistant, high–light adapted *Prochlorococcus* ecotypes. Although cells of this ecotype are relatively resistant to copper toxicity, the abundance of *Prochlorococcus* cells in shallow mixed layers is still low. This may be because of the cost of maintaining cell integrity and cell division rates in the face of both high free Cu^{2+} concentrations and high irradiance.

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