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Christina L. Dryden
Old Dominion University

Andrew S. Gordon
Old Dominion University

John R. Donat
Old Dominion University, jdonat@odu.edu

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Interactive regulation of dissolved copper toxicity by an estuarine microbial community

Christina L. Dryden
Department of Ocean, Earth, and Atmospheric Sciences, Old Dominion University, Norfolk, Virginia 23529

Andrew S. Gordon[1]
Department of Biological Sciences, Old Dominion University, Norfolk, Virginia 23529

John R. Donat
Department of Chemistry and Biochemistry, Old Dominion University, Norfolk, Virginia 23529

Abstract

Cultured marine microorganisms under copper stress produce extracellular compounds having a high affinity for copper (copper-complexing ligands). These ligands are similar in binding strength to those found in natural waters, but few studies have examined the relationship between copper, copper-complexing ligand concentrations, and natural microbial populations. A series of in situ experiments in the Elizabeth River, Virginia, revealed that an intact estuarine microbial community responded to copper stress by production of extracellular, high-affinity copper-complexing ligands. The rate of ligand production was dependent on copper concentration and resulted in a reduction of the concentration of free cupric ions, Cu^{2+}, by more than three orders of magnitude during a 2-week period in one experiment. We believe that this interactive response to copper stress represents a feedback system through which microbial communities can potentially buffer dissolved Cu^{2+} ion concentrations, thereby regulating copper bioavailability and toxicity.

Copper is widely used in industrial applications, notably as the active agent in antifouling coatings on ship hulls, and meeting regulatory criteria is costly to industry. The current acute water quality criterion for copper in Virginia waters is 5.9 $\mu g\ L^{-1}$ (92.8 nmol L^{-1}), based upon the National Ambient Water Quality Criterion for copper (U.S. Environmental Protection Agency [U.S. EPA] 1999). Controlling the release of dissolved copper from commercial and military shipping activity is a major concern, particularly in industrialized estuaries, because copper can be toxic to marine organisms (such as phytoplankton) at free cupric ion concentrations of 0.01 nmol L^{-1} or above (Sanders et al. 1983; Sunda and Ferguson 1983; Brand et al. 1986; Sunda et al. 1987). Therefore, understanding the dynamic ecological factors and feedbacks that affect the toxicity and bioavailability of dissolved copper is crucial for development of cost-effective management strategies for estuaries.

A major factor governing the toxicity and bioavailability of dissolved copper to marine organisms is its chemical speciation. Dissolved copper may exist in various forms (species): as free cupric ions (Cu^{2+}), inorganic complexes (e.g., with Cl^-, OH^-, CO_3^{2-}, and SO_4^{2-}), and as complexes with various organic ligands (e.g., humic substances, phytoplankton metabolites, proteins, etc.). The toxicity and nutrient availability of copper to marine organisms decrease as a result of complexation by natural organic ligands, indicating that the toxicity and availability of copper is controlled by the free cupric ion Cu^{2+} (Sunda and Guillard 1976; Anderson and Morel 1978; Brand et al. 1986). In marine and estuarine waters, many indigenous marine organisms are sufficiently sensitive to copper that they would be severely affected by copper toxicity at ambient copper concentrations in the absence of organic complexation (Hering et al. 1987; Coale and Bruland 1990). In most natural waters, the majority of dissolved copper (usually 95% or more) is complexed by strong organic ligands (usually termed L_1-class ligands) having conditional stability constants (K'_{CuL}) between $10^{11}-10^{13}$ (Coale and Bruland 1990; Moffett et al. 1990).

Although it has been known for some time that organic ligands control copper speciation in most natural waters, the sources of these ligands are not fully understood. In estuarine environments, ligands may derive from terrestrial sources, sediment, and, hypothetically, from water column processes (Sunda and Guillard 1976; Brand et al. 1986; Moffett et al. 1990; Bruland et al. 1991; Sunda and Huntsman 1995; Skrabal et al. 1997). Laboratory studies indicate that autotrophic picoplankton (the <2-μm component of the phototrophic planktonic microflora) (Moffett et al. 1990; Bruland et al. 1991; Gordon et al. 1996, 2000; Moffett and Brand 1996) and heterotrophic bacteria (Gordon et al. 2000) produce ligands having binding strengths similar to the L_1-class ligands observed in natural waters in response to elevated copper concentrations in culture. These culture observations have led to the hypothesis that a biological feedback system may regulate dissolved copper speciation in marine and estuarine waters (Bruland et al. 1991; Donat et al. 1994). While such a feedback system has been demonstrated in cultures of marine and estuarine microorganisms (Bruland et al.

[1] Corresponding author (agordon@odu.edu).

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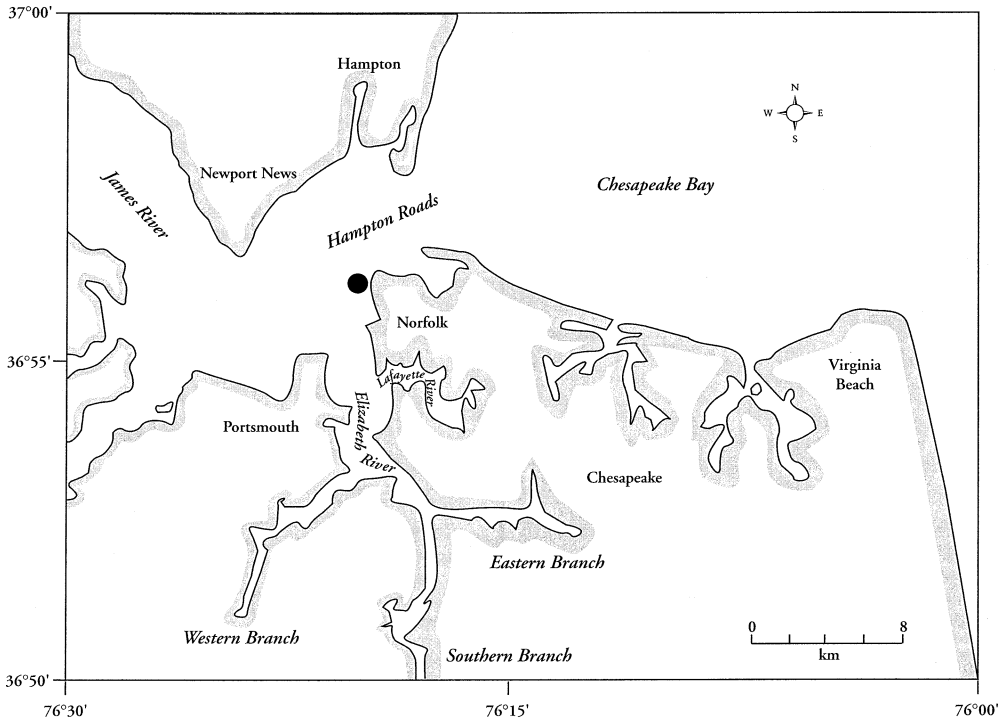

Fig. 1. Map of Hampton Roads and the Elizabeth River. The Elizabeth River consists of a main stem and four branches (Lafayette River, Eastern Branch, Western Branch, and Southern Branch). The study site is indicated on map with a black circle.

1991; Moffett and Brand 1996), it has not previously been demonstrated in natural microbial communities.

The objective of our study was to examine the dynamics of copper-complexing ligand production by natural microbial assemblages under realistic environmental conditions in situ, thus avoiding the potential pitfalls inherent in extrapolation of laboratory studies to field conditions. This response would have important implications for our understanding of the fate and effects of copper in estuarine systems, since it would demonstrate that natural microbial communities could participate in a negative feedback loop that influences the bioavailability and toxicity of copper to themselves and to other estuarine biota.

Materials and methods

Study location—The study site was located at the mouth of the Elizabeth River, Virginia, adjacent to Norfolk Naval Base, which is home to the North Atlantic Fleet and is the largest naval base in the world. The Elizabeth River is an urban, industrialized subestuary of the Chesapeake Bay. The experimental station is well mixed and flushed because of its close proximity to the mouth of the Chesapeake Bay (Fig. 1).

Sample collection, pre-equilibration, and incubation—In May and November 2000, June 2001, and July 2002, estuarine seawater was collected from a depth of 1 m at the mouth of the Elizabeth River (Fig. 1). During sample collection, the temperature, salinity, and pH were measured. Using trace metal clean techniques, study-site surface water was collected into acid-cleaned, polycarbonate carboys using acid-cleaned Teflon tubing and a peristaltic pump (Bruland et al. 1979; Flegal et al. 1991; Donat et al. 1994). To minimize copper and ligand loss to bottle walls by adsorption, each acid-cleaned polycarbonate incubation bottle was pre-equilibrated for 1 week with 0.22-μm filtered site water at 4°C prior to each experiment. Water used for pre-equilibration had the same copper amendment that was used in the experimental bottles.

Under a laminar flow hood, pre-equilibrated incubation bottles were filled with unfiltered site water and either received one of several experimental treatments or were left unaltered. These treatments included addition of copper (100 and 200 nmol L^{-1}) with and without addition of the metabolic poison sodium azide (15 mmol L^{-1}). Dark incubation bottles were used with selected treatments, including the unaltered and addition of copper without azide. Within 10 h after water collection, the incubation bottles were placed in a moored array at the study site approximately 1 m below the water's surface. After 1 or 2 weeks of incubation, the bottles were retrieved for analysis. Each incubated bottle was immediately subsampled for total dissolved copper, copper speciation/complexation, and microbial enumeration under a laminar flow hood following trace metal clean techniques (Bruland et al. 1979; Flegal et al. 1991). Total dissolved copper and copper speciation subsamples were filtered, using a peristaltic pumping system, through 0.22-μm MSI polycarbonate cartridge filters (Fisher Scientific). Total dissolved copper subsamples were acidified to pH 2 with HCl (Optima

Table 1. Summary of initial in situ data during experiments performed in the Elizabeth River, Virginia.

Date	Temp (°C)	Salinity	Total dissolved Cu (nmol L^{-1})	Autotrophic picoplankton numbers (10^8 L^{-1})	Bacterioplankton numbers (10^6 ml^{-1})
May 2000	16.0	16.4	12.3	0.8	4.5
Nov 2000	14.5	16.5	16.2	0.5	2.3
Jun 2001	21.8	18.2	19.2	1.2	4.9
Jul 2002	25.6	13.2	15.5	1.4	5.3

Grade, Fisher Scientific). Copper speciation subsamples were not acidified but were immediately frozen after collection until analyzed. Microbial enumeration subsamples were unfiltered, fixed immediately with 2.5% glutaraldehyde, stored at 4°C, and analyzed within 1 week of collection (Porter and Feig 1980).

Total dissolved copper determination—Total dissolved copper concentrations were determined using cathodic stripping voltammetry (CSV), as described by Campos and van den Berg (1994). The only modification made to the Campos and van den Berg (1994) CSV method included the use of a 4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid (HEPPS) buffer solution (Sigma; final concentration 0.01 mol L^{-1}) rather than a borate buffer solution. The analytical system consisted of an EG&G PARC (Princeton) 264A polarographic analyzer interfaced with an EG&G PARC (Princeton) 303A hanging mercury drop electrode.

Copper complexation and speciation analysis—Copper speciation was determined at natural pH using competitive ligand equilibration–adsorptive cathodic stripping voltammetry (CLE/ACSV) with salicylaldoxime (SA) as the competitive ligand (Campos and van den Berg 1994). The theory, application, and limitations of CLE/ACSV methods for determining copper-complexing ligand concentrations are discussed in detail elsewhere (e.g., Donat and van den Berg 1992; van den Berg and Donat 1992; Campos and van den Berg 1994; Donat et al. 1994; Bruland et al. 2000). The final concentrations of the HEPPS buffer and SA in a sample to be analyzed were 0.01 mol L^{-1} (resulting pH 8.2) and 2 μmol L^{-1}, respectively. An analytical competition strength, log $\alpha_{Cu(SA)2}$, of 3.8 (*see* Bruland et al. 2000 for description and definition) was used for all titrations to detect the strongest class of Cu-binding ligands.

Copper-complexing ligand and conditional stability constant calculations—To obtain ligand concentrations (C_L) and conditional stability constants (K'_{CuL}), the data from the CLE/ACSV measurements and Ruzic/van den Berg linearization were used (Ruzic 1982; van den Berg 1982; Campos and van den Berg 1994). A detailed description of the theory behind the calculations used is presented in Campos and van den Berg (1994) and Rue and Bruland (1995). Using ligand concentrations and conditional stability constants obtained by CLE/ACSV, the overall copper speciation and free Cu^{2+} ion concentrations were calculated with the chemical equilibrium modeling program MINEQL+©.

Enumeration of bacterioplankton and autotrophic picoplankton—Epifluorescent direct counts of bacterioplankton and autotrophic picoplankton followed the method of Porter and Feig (1980) using the DNA stain 4′6-diamidino-2-phenylindole (DAPI). The DAPI-stained samples were observed under ultraviolet excitation for total counts and blue light excitation for the chlorophyll autofluorescence of the autotrophic picoplankton (Affronti 1990). The average counting error was 9% using the Porter and Feig (1980) method and counting 20 fields. Every field had a minimum of 30 individual cells of each type (i.e., bacterioplankton and autotrophic picoplankton).

Results and discussion

Initial incubation conditions and total dissolved copper recoveries—The initial conditions for all incubations are presented in Table 1. Study site salinities ranged from 13.2 to 18.2 on the sampling dates, and water temperature varied from 14.5°C in November to 25.6°C in July. Total dissolved copper concentrations ranged from 12.3 to 19.2 nmol L^{-1}, five to eight times lower than the current acute copper criterion in Virginia waters (93 nmol L^{-1}) (U.S. EPA 1999). Initial bacterial concentrations varied twofold, from 2.3 to 5.3×10^6 ml^{-1}, and autotrophic picoplankton numbers varied threefold, from 0.5 to 1.4×10^8 L^{-1}. Peak bacterial and autotrophic picoplankton numbers were observed during July 2002, the study month that also had the highest temperature (25.6°C) and the lowest salinity (13.2).

When the bottle contents were analyzed after incubation, 41% to 120% of the copper added to experimental bottles was measured in the dissolved form (Tables 2, 3). The balance was presumably partitioned between particulate matter, including cells, and the container walls (despite preconditioning of bottles with site water). Achterberg et al. (2003) observed similar total dissolved copper recoveries and removal by particles. Dissolved copper concentrations in these experiments ranged from 45 to 190 nmol L^{-1}, compared to ambient concentrations of 10 to 50 nmol L^{-1} throughout the Elizabeth River (Donat unpubl. data). The copper concentrations to which microbial communities were exposed in this study, then, were realistic relative to Elizabeth River ambient copper concentrations and to the acute copper criterion for Virginia waters (93 nmol L^{-1}) (U.S. EPA 1999).

Copper-complexing ligand production—The ligand production rate (Fig. 2) increased with increasing copper additions in every experiment except the November 2000 ex-

Table 2. Total dissolved copper (Cu) concentration, ligand concentration (C_L), and conditional stability constants (K'_{CuL}) in incubation bottles with 100 nmol L^{-1} copper added (no azide added).

Date	Incubation time (weeks)	Total dissolved Cu (nmol L^{-1})	C_L (nmol L^{-1})	K'_{CuL}
May 2000	0	46.9±3.3	88.4±3.0	12.5±0.2
	1	51.2±4.3	106.2±3.8	12.7
Nov 2000	0	45.3±1.5	75.6±3.7	12.6±0.2
	1	54.8±4.6	72.1±7.7	12.3
	2	52.7±3.8	70.5±7.0	12.5
Jun 2001	0	93.1±13.2	65.4±6.9	12.2±0.5
	1	120.1±3.3	146.1±5.1	13.0
	2	80.9±3.8	124.8±9.3	13.0
Jul 2002	0	76.9±5.8	83.4±5.3	12.5±0.3
	1	104.4±7.5	153.9±7.7	12.7

periment, in which 100 nmol L^{-1} was added. These experimental data indicate that ligand production increased in response to above-ambient copper concentrations. A similar production of strong copper-complexing ligands in response to added copper has also been observed in cultures of the cyanobacterium *Synechococcus* (Moffett and Brand 1996) and heterotrophic bacteria (Schreiber et al. 1990; Gordon et al. 2000). A net loss of ligands was observed in control incubations with no added copper and in copper-treated samples containing the biological poison azide. Ligand loss could be due to photodegradation, biodegradation, or ligand adsorption onto container walls. Biologically mediated ligand production, and not leakage from cells or release due to cellular lysis, is the likely candidate, because ligand concentrations showed either no change or a net decrease in the azide-killed controls.

Light/dark incubations were used to determine whether ligand production is linked to photosynthesis (Fig. 3). Light and dark production was not significantly different for the control and the 200 nmol L^{-1} Cu treatment in June 2001. However, incubations carried out in light and dark bottles (June 2001 and July 2002) showed a reduction in the rate of L_1-class ligand production in the dark bottles to which 100 nmol L^{-1} copper had been added. These findings indi-

Table 3. Total dissolved copper (Cu) concentration, ligand concentration (C_L), and conditional stability constants (K'_{CuL}) in incubation bottles with 200 nmol L^{-1} copper added (no azide added).

Date	Incubation time (weeks)	Total dissolved Cu (nmol L^{-1})	C_L (nmol L^{-1})	K'_{CuL}
May 2000	0	81.9±7.2	84.1±1.1	12.7±0.3
	1	93.9±8.9	121.7±17.1	12.7
Nov 2000	0	99.4±6.3	66.6±2.5	12.7±0.3
	1	161.0±6.9	98.7±1.4	12.8
	2	190.1±12.5	156.2±4.0	12.9
Jun 2001	0	155.8±10.6	107.6±6.5	12.5±0.2
	1	156.9±2.8	160.7±13.9	12.5
	2	115.5±8.4	124.9±1.5	12.5

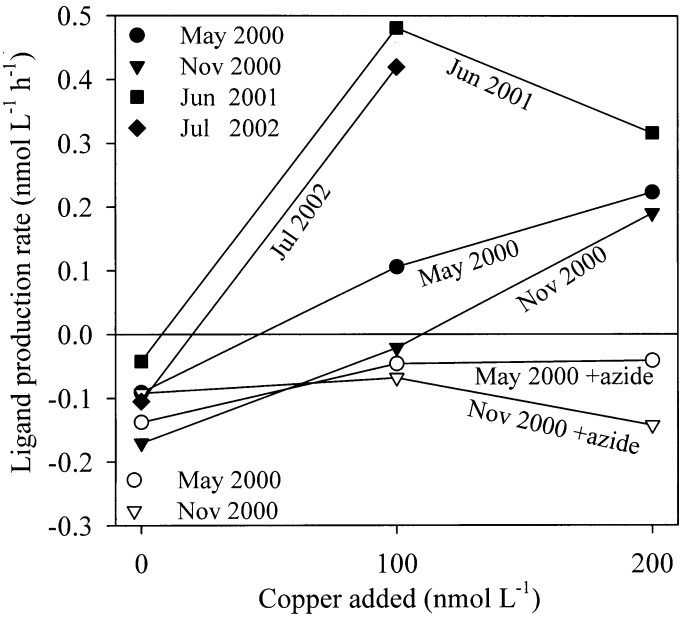

Fig. 2. Rates of ligand (L_1) production by intact microbial communities (solid symbols) and by azide-killed controls (open symbols) as a function of copper concentration. Ligand production rates were determined from the change in ligand concentration during the first week of incubation.

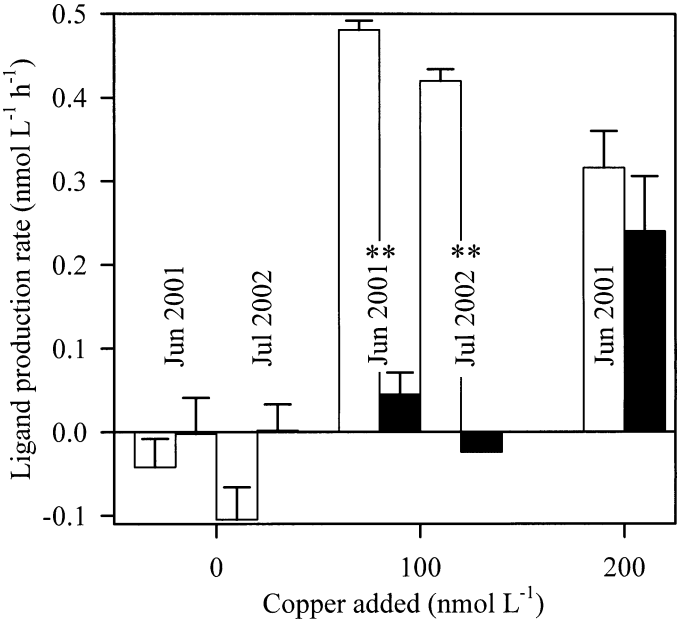

Fig. 3. Rates of ligand (L_1) production by an intact microbial community incubated under ambient light exposure and in the dark (white bars are light bottles and black bars are dark bottles). July 2002 data only collected for unaltered and 100 nmol L^{-1} copper treatment. ** indicates a significant difference ($P < 0.01$; *t*-test) after 100 nmol L^{-1} copper addition. Rates of ligand production in bottles with no copper addition and with a 200 nmol L^{-1} addition were not significantly different. Error bars are standard deviations.

cate that different populations within the microbial community may be responsible for L_1 class ligand production at different copper concentrations and that heterotrophic processes (probably bacterial) can significantly contribute to L_1-class ligand production under some conditions (i.e., copper stress). These results along with culture studies by Gordon et al. (2000) and Schreiber et al. (1990) support the suggestion by Croot et al. (2000) that heterotrophic bacteria could be a major biological source of copper chelators.

Copper-complexing ligands and Cu^{2+}—With an addition of 100 nmol L^{-1} copper, an increase in ligand concentrations was observed over the first incubation week for May 2000, June 2001, and July 2002 (Fig. 4A; Table 2). In November 2000, no increase in ligand concentration was detected over the 2-week incubation. The decrease in dissolved (i.e., filterable) ligand concentrations from week 1 to week 2 in the June 2001 incubation may be attributed to a loss of filterable bound copper species, either from adsorption onto container walls, particle surfaces, or coagulation of colloidal copper species (Wells et al. 1998).

A ligand concentration increase over the first week of incubation for May 2000, June 2001, and July 2002 resulted in a decrease in free Cu^{2+} ion concentration (Fig. 4B). In November 2000, the Cu^{2+} ion concentration did not significantly increase or decrease, which is expected since the ligand concentration remained constant. During June 2001, a decrease in the free Cu^{2+} ion was observed from week 1 to 2; however, as discussed previously, the ligand concentration decreased instead of increasing as expected. Therefore, the decrease in the free Cu^{2+} ion was due to that 33% loss of total dissolved copper and not to increased complexation by ligands or changes in conditional stability constant.

The culture study by Brand et al. (1986) provides a useful reference for which phytoplankton might be impacted by the free Cu^{2+} ion concentrations found in our study. The free Cu^{2+} ion concentrations that have been reported to reduce the relative reproductive rates of several phytoplankton classes in that study are indicated as horizontal lines on Fig. 4B. In the May 2000, November 2000, and July 2002 experiments, the free Cu^{2+} ion concentrations present at the beginning of the experiments after 100 nmol L^{-1} copper was added were high enough to reduce the relative reproductive rates of only the more copper-sensitive cyanobacterium *Synechococcus.* In June 2001, at time 0, all phytoplankton classes represented would have been affected. The free Cu^{2+} ion concentration decreased for May 2000, June 2001, and July 2002 during the entire incubation. With no change in the ligand concentration in November 2000, the free Cu^{2+} ion concentration showed little reduction during the 2-week incubation with only *Synechococcus* being affected.

An increase in ligand concentrations was observed over a 1-week period for May 2000, June 2001, and November 2000 incubations with an addition of 200 nmol L^{-1} copper (Fig. 5A; Table 3). Ligand concentrations continued to increase in copper-amended containers up to 2 weeks in November 2000, but in June 2001, ligand concentrations decreased after 1 week (Fig. 5A). This ligand decrease was similar to that observed with the addition of 100 nmol L^{-1} Cu in June 2001. Again, the decrease may be attributed to

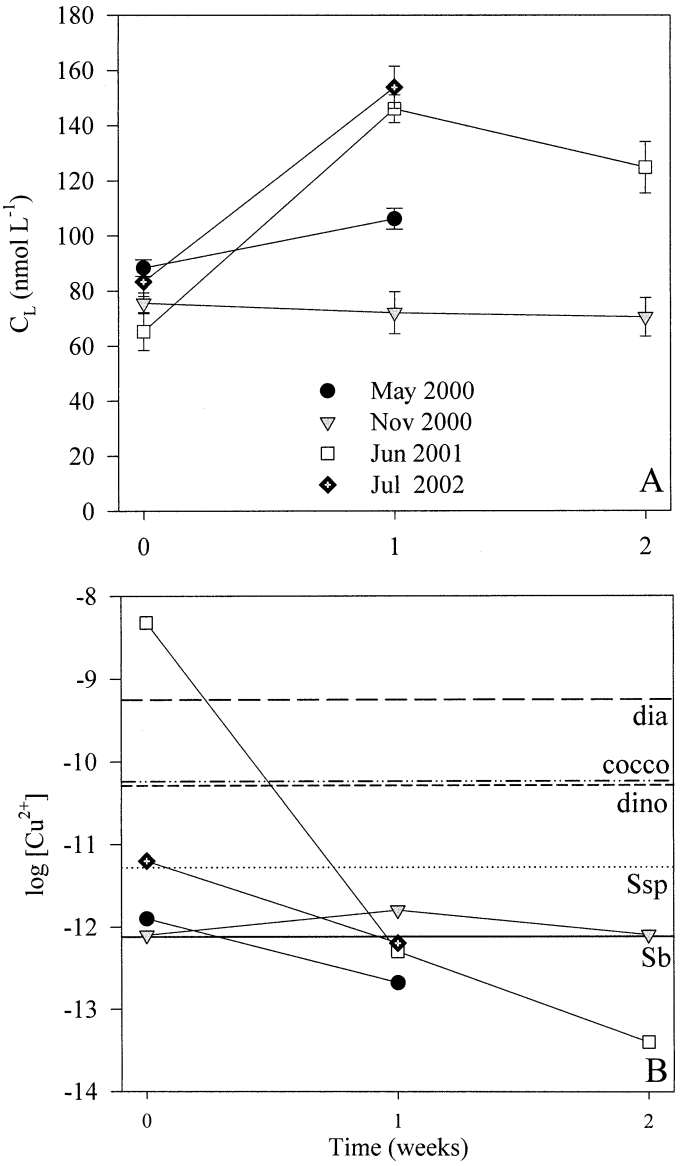

Fig. 4. Ligand (L_1) concentration as a function of time after A) 100 nmol L^{-1} copper addition in three seasonal in situ incubations and B) corresponding free Cu^{2+} ion concentrations as a result of increased ligand concentrations in response to 100 nmol L^{-1} copper addition. Error bars are standard deviations. The lines in panel B represent the free Cu^{2+} ion concentrations causing reduced reproductive rates for diatoms (dia), coccolithophores (cocco), dinoflagellates (dino), *Synechoccocus* sp. (Ssp), and *Synechoccocus bacillaris* (Sb) (Brand et al. 1986).

a loss either from adsorption onto container walls, particle surfaces, or coagulation of colloidal copper species (Wells et al. 1998).

As the ligand concentration increased during May 2000 and June 2001, the concentration of free Cu^{2+} ion decreased (Fig. 5B). In May 2000, the free Cu^{2+} ion concentration decreased by approximately an order of magnitude 1 week after 200 nmol L^{-1} copper was added to the incubation bottles, and in June 2001, the free Cu^{2+} ion concentration decreased more than three orders of magnitude (Fig. 5B). Al-

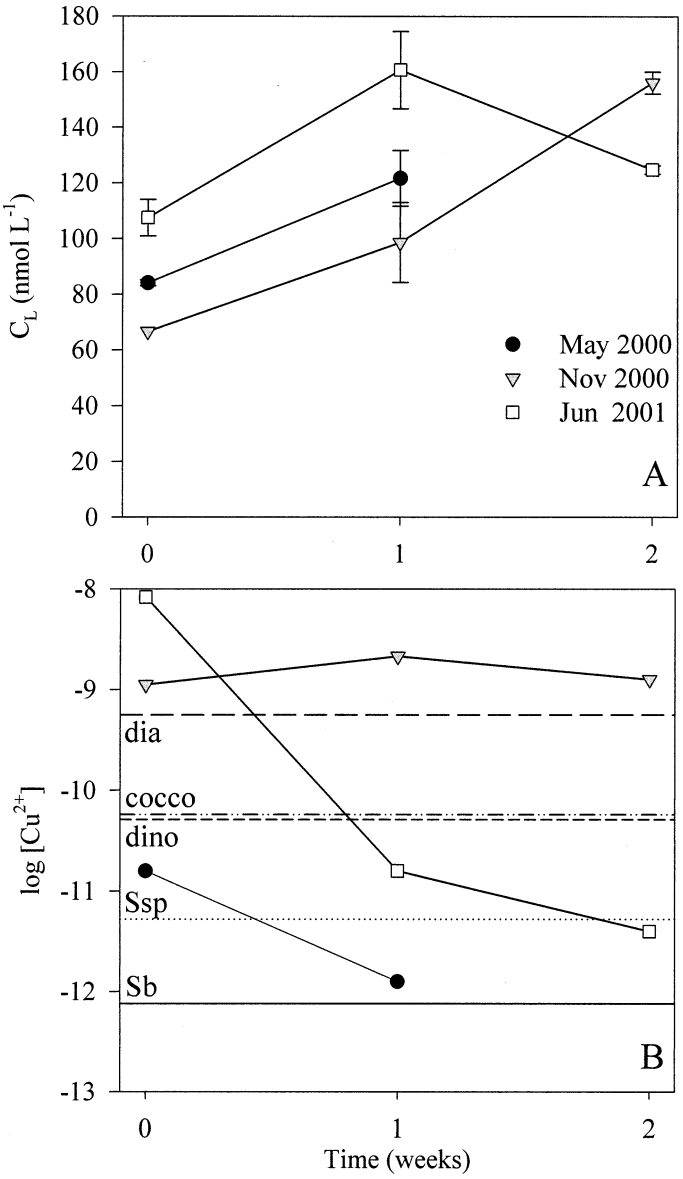

Fig. 5. Ligand (L_1) concentration as a function of time after A) 200 nmol L^{-1} copper addition in three seasonal in situ incubations and B) corresponding free Cu^{2+} ion concentrations as a result of increased ligand concentrations in response to 200 nmol L^{-1} copper addition. Error bars are standard deviations. The lines in panel B represent the free Cu^{2+} ion concentrations causing reduced reproductive rates for diatoms (dia), coccolithophores (cocco), dinoflagellates (dino), *Synechoccocus* sp. (Ssp), and *Synechoccocus bacillaris* (Sb) (Brand et al. 1986).

though the ligand concentration increased in November 2000, there was an equivalent increase in dissolved copper, resulting in no change in the free Cu^{2+} ion. Additional ligand production would have been necessary to reduce the free Cu^{2+} ion concentrations significantly in November 2000. The differences among initial free Cu^{2+} ion concentrations in May 2000, June 2001, and November 2000 trials was largely caused by variations in dissolved copper owing to variable loss of copper from solution. At every sampling time and in each trial, the free Cu^{2+} ion concentration was only a small fraction of the total copper (~2.5% to 0.005%), consistent with previous reports dissolved copper speciation in estuaries (Donat et al. 1994; Kozelka and Bruland 1998).

In a survey of algal species, Brand et al. (1986) found that cyanobacteria were the most sensitive to copper toxicity; dinoflagellates and coccolithophores had intermediate sensitivity; and diatoms were the least sensitive. In the November 2000 and June 2001 experiments, the free Cu^{2+} ion concentrations present at the beginning of the experiments after 200 nmol L^{-1} copper was added were high enough to reduce the relative reproductive rates of all the phytoplankton classes listed in Fig. 5B. However, in the May 2000 experiment, the lower Cu^{2+} ion concentration would cause a reduced reproductive rate in only the more copper-sensitive cyanobacterium *Synechococcus.* In May 2000, the free Cu^{2+} ion concentration decreased to a level that would affect the growth rate of only the most sensitive cyanobacterial species, *Synechococcus bacillaris.* Likewise, in June 2001, the decrease in the free Cu^{2+} ion concentration observed over weeks 1 and 2 would be expected to affect only *Synechococcus.* Since the November 2000 free Cu^{2+} ion concentration showed little reduction during the 2-week incubation, the free Cu^{2+} ion concentration would have continued to affect all the phytoplankton presented in Fig. 5B during the entire incubation.

Analogous to marine phytoplankton, Sunda and Ferguson (1983) found that marine bacteria show toxic responses to relatively low Cu^{2+} ion concentrations (15 to 30 pmol L^{-1}). Cultures of heterotrophic bacteria have also been reported to produce dissolved, high-affinity copper ligands in response to elevated Cu^{2+} concentrations (Schreiber et al. 1990; Gordon et al. 2000). Based on these culture studies, and since the intact microbial community contained marine bacteria as well as marine phytoplankton, the marine bacteria could be producing copper-complexing ligands in response to elevated Cu^{2+} ion concentrations.

Population density and copper additions—Copper additions of 100 and 200 nmol L^{-1} affected the population density of both autotrophic picoplankton and bacterioplankton (Fig. 6). Both the 100 and 200 nmol L^{-1} copper concentrations resulted in a reduction of the population density of autotrophic picoplankton and bacterioplankton with time. However, neither population was completely eliminated, even by the 200 nmol L^{-1} copper addition. Several factors may explain why copper additions did not totally eliminate all the autotrophic picoplankton and bacterioplankton. One set of factors could be the amelioration of any copper toxicity by production of strong copper-complexing ligands, which results in a reduction of free Cu^{2+} ion concentration. Also, since a significant portion of the added copper was lost to adsorption on container walls or particles, an additional reduction in copper toxicity could occur, resulting in the remaining autotrophic picoplankton and bacterioplankton cells. Another plausible rationale could be that some remaining cells may be resistant to copper toxicity (Gordon et al. 1993). During all four trials, autotrophic picoplankton were somewhat more affected by copper additions than were bacterioplankton. This observation is consistent with laboratory studies of copper sensitivity in various bacterioplank-

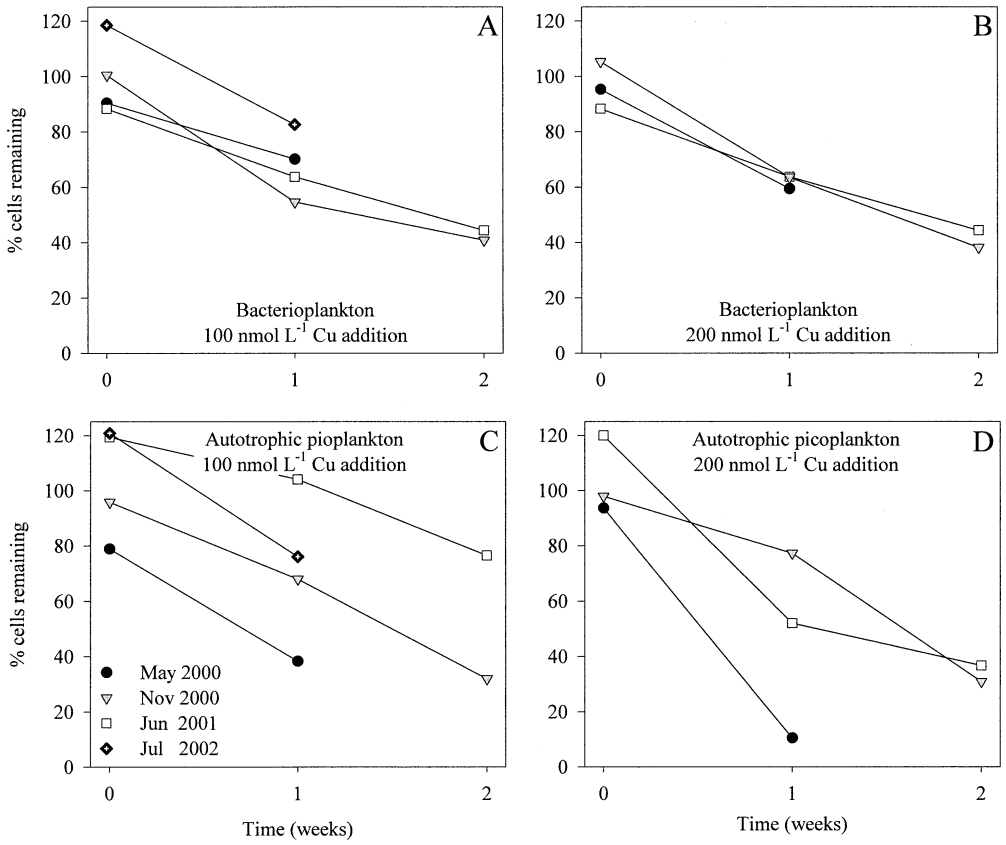

Fig. 6. A, B) Effect of copper addition on abundance of bacterioplankton, and C, D) autotrophic picoplankton during in situ incubation of the intact microbial community. Abundances shown are the percent cells remaining in comparison to control bottles to which no copper was added (average counting error was 9%).

ton and autotrophic picoplankton species that generally show that autotrophic picoplankton species, such as some *Synechococcus* sp., are among the most sensitive to copper (Brand et al. 1986).

Elevated copper concentrations, within realistic limits, induced the natural estuarine microbial communities of the Elizabeth River, Virginia, to produce L_1 class copper-complexing ligands, which dramatically reduced the free Cu^{2+} ion concentrations in the water column. Our in situ data indicate that estuarine microbial communities have the capacity to respond to copper stress and ameliorate copper toxicity by actively buffering the free Cu^{2+} ion through organic ligand production. Production is not due simply to cell death and lysis or it would have been observed in the azide-killed controls. Production of these ligands will potentially affect the interaction of copper with all organisms in the estuary as well as its biogeochemical cycling. This detoxification pathway is a negative feedback loop that needs to be taken into account when predicting the impact of copper discharge into estuarine systems. The results of this study indicate that both autotrophic picoplankton and bacterioplankton can be important contributors to production of L_1 class ligands.

To our knowledge, this is the first study to show that in situ exposure of an intact indigenous estuarine microbial community to copper stress led to production of L_1 class ligands, resulting in amelioration of copper toxicity. This information contributes to our understanding of the complex processes taking place in copper-polluted estuarine environments. Clearly, additional measurements in different environments will be required before generally applicable models of copper-responsive ligand production can be developed. In addition, other sources of copper-complexing ligands, such as sediment pore waters (Skrabal et al. 1997), and the contribution by heterotrophic bacteria need to be taken into account in any general models of copper biogeochemistry in estuaries.

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