Microbial Ecology

Effects of Dissolved and Complexed Copper on Heterotrophic Bacterial Production in San Diego Bay

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Abstract

Bacterial abundance and production, free (uncomplexed) copper ion concentration, total dissolved copper concentration, dissolved organic carbon (DOC), total suspended solids (TSS), and chlorophyll *a* were measured over the course of 1 year in a series of 27 sample "Boxes" established within San Diego Bay. Water was collected through a trace metal-clean system so that each Box's sample was a composite of all the surface water in that Box. Bacterial production, chlorophyll *a*, TSS, DOC, and dissolved copper all generally increased from Box 1 at the mouth of the Bay to Box 27 in the South or back Bay. Free copper ion concentration generally decreased from Box 1 to Box 27 presumably due to increasing complexation capacity within natural waters. Based on correlations between TSS, chlorophyll *a*, bacterial production or DOC and the ratio of dissolved to free Cu ion, both DOC and particulate (bacteria and algae) fractions were potentially responsible for copper complexation, each at different times of the year. $CuCl_2$ was added to bacterial production assays from 0 to 10 μg L^{-1} to assess acute copper toxicity to the natural microbial assemblage. Interestingly, copper toxicity appeared to increase with decreases in free copper from the mouth of the Bay to the back Bay. This contrasts the free-ion activity model in which higher complexation capacity should afford greater copper protection. When cell-specific growth rates were calculated, faster growing bacteria (i.e. toward the back Bay) appeared to be more susceptible to free copper toxicity. The protecting effect of natural dissolved organic material (DOM) concentrated by tangential flow ultrafiltration (>1 kDa), illite and kaolinite minerals, and glutathione (a metal chelator excreted by algae under copper stress) was assessed in bacterial production assays. Only DOM concentrate offered any significant protection to bacterial production under increased copper concentrations. Although the potential copper protecting agents were allowed to interact with added copper before natural bacteria were added to production assays, there may be a temporal dose–response relationship that accounts for higher toxicity in short production assays. Regardless, it appears that effective natural complexation of copper in the back portions of San Diego Bay limits exposure of native bacterial assemblages to free copper ion, resulting in higher bacterial production.

Introduction

Copper has been shown to have deleterious effects on a number of marine organisms. It impacts marine phytoplankton and bacterial productivity [5, 52]. This toxicity is the foundation for the use of copper as an algicide and for copper-based paints used on ship hulls to prevent settling by marine organisms. Currently, water quality criteria for total dissolved copper is 2.9 μg L^{-1} [58], a level often difficult to achieve in effluents and industrial waterways with drydock facilities and ship maintenance activities. Stormwater runoff and industrial discharges also contribute to copper loading in estuarine and marine environments.

In natural aquatic systems, there is very little free copper. It is complexed by a number of different chelating agents including clay minerals [2, 12, 18], particulate matter [14, 57], colloidal dissolved organic material (DOM) [62, 63], and high affinity organic ligands [7, 22]. Organic ligands have also been shown to be present in estuarine sediments and their associated pore waters [48–

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50] which may be a major source of binding ligands in coastal waters. When natural waters are titrated with copper ion, a complexation capacity can be calculated from the dose–response curve of free copper. The strength of the copper–organic complex has been used to classify ligands in marine and estuarine waters. Inflections in titration curves indicate separate stability constants and binding ligands are identified by their stability as L_1, L_2, etc. [16, 65]. The exact chemical nature and origin of these ligands still remain largely unknown.

Some complexing ligands are believed to be produced by actively growing algae in response to copper stress [13, 19, 20, 36]. Currently, not enough is known about ligands found in the water column to state definitively if they are there solely as a response to copper stress or if their activity is a by-product of chemical moieties found in natural DOM, particulate matter (organic or mineral), and colloidal material. What has been well established is that copper toxicity to marine organisms can be mitigated by complexation with dissolved organic ligands [10, 11, 37, 55]. Based on a number of experiments using synthetic copper ligands and natural DOM to assess toxicity, a free-ion activity model has been developed in which toxicity may be assigned to the free-ion metal concentration (see recent review [8]). The phenomenon has also been taken into account in the biotic ligand model [15].

Only a few studies have exposed natural bacterial communities [32, 53] or cultures [23, 54] to increased concentrations of copper to determine impacts. In studies with natural microbial consortia, a synthetic chelating agent was used in an attempt to ameliorate copper toxicity. Results from these studies have largely confirmed the free-ion activity model where free copper increases were correlated with increased toxicity.

San Diego Bay is a large industrial and Naval port located in the Southwest border of the United States. Discharges and leaching from antifouling paints contribute approximately 31,000 kg year^{-1} copper loading to Bay waters [31]. Recent work within San Diego Bay has shown dissolved free copper concentrations are very low (ca. 10^{-11}–10^{-13} M), while most (99%) of the total copper is complexed with organic ligands, colloidal material, or particulates [67]. These studies in San Diego Bay have shown a correlation between copper toxicity and free copper ion concentration. These findings conform to the free-ion activity model. The present study was undertaken to assess the use of microbial production as a rapid (~1 h) assay for copper toxicity and potential complexation capacity in San Diego Bay [45]. The goal was to determine the impact of copper release and the immediate response of the microbial community. A number of different chelating agents were tested for their ability to ameliorate copper toxicity in San Diego Bay waters.

Methods

Study Area and Sampling. Composite water samples were collected aboard the *R/V ECOS* during a series of transects through San Diego Bay (−117°18′W, 32°66′N) from August 2000 through September 2001. The Bay was divided into 27 "Boxes." A ship track was run through each Box (from 1 to 27) with a Teflon flow-through system tapped and running into a acid-rinsed polyethylene collection carboy (20 L) such that each sample consisted of a composite of water through each Box (Fig. 1). Additionally, in July 2001, a sampling in the South part of the Bay was performed as part of another project. We were able to take samples for copper addition toxicity and the effects of glutathione addition. Measurement of pCu and dissolved copper was not made during this sampling.

Bacterial Production. Heterotrophic bacterial secondary production was determined using a modification of the method of Smith and Azam [51]. Briefly, 1 mL water samples were added to 2 mL centrifuge tubes containing ^{3}H-leucine (Amersham, 155–157 Ci mmol^{-1}) to create a final concentration of 20 nM. At t0, three replicates and one control killed with 57 μL 100% (w/v) trichloroactic acid (TCA; Fisher Scientific) were prepared. Samples were incubated at *in situ* temperature for 1–2 h, then killed with 100% TCA. Subsequent processing was identical to that reported by Smith and Azam [51]. Leucine incorporated into cell protein was converted to moles of carbon utilized by the conversion factor of Simon and Azam [47]. Various copper, mineral, dissolved organic carbon (DOC) and glutathione additions to production assays were performed as described below.

Dissolved Organic Carbon. Water samples from each Box were processed by filtering through 0.7-μm nominal pore size pre-combusted glass fiber filters (Whatman GF/F). Filtered water was transferred to 5-mL amber ampoules and acidified with 8 μL H_3PO_4. The ampoules were then heat-sealed and the samples frozen until DOC analysis. MilliQ blanks and method blanks (rinsed through syringe and filter unit) were processed before sampling, in the middle of the sampling transect and at the end of each sampling event. All blanks were <35 μM, but generally below 25 μM. An MQ1001 high temperature combustion TOC analyzer was used for the DOC analysis [43]. Standards, made from a 1–10 mg L^{-1} sodium phthalate solution were run with every 30 samples. Goodness of fit (r^2) for each set of standards was greater than 0.98.

Dissolved Copper Concentration. Trace metal-clean techniques were used for sampling and analysis of dissolved copper. Samples for dissolved copper were

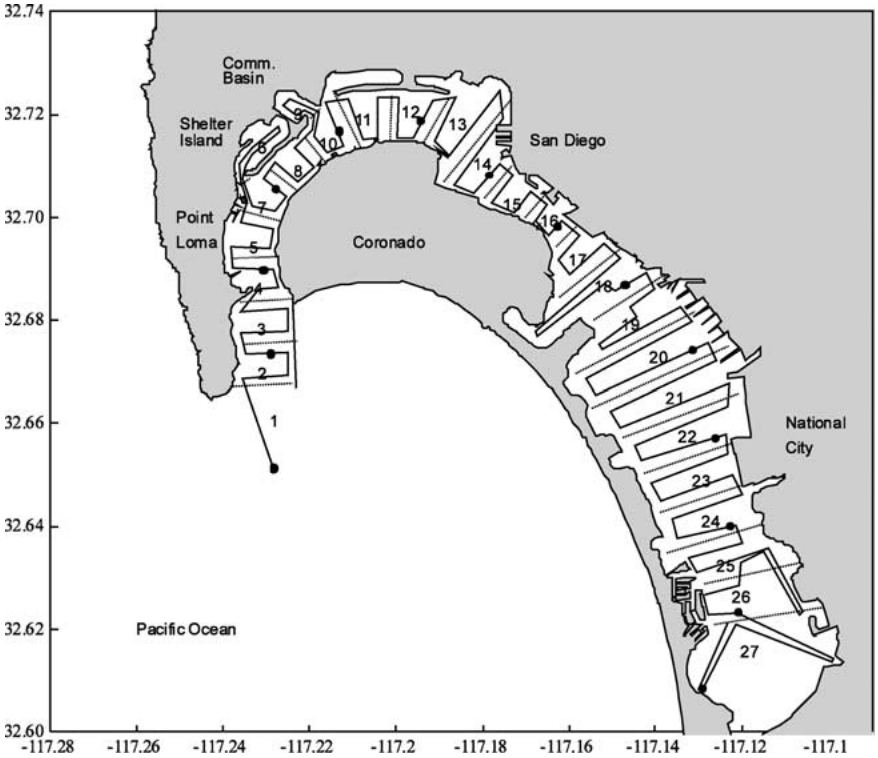

Figure 1. Sampling Box locations. Ship track shown in *heavier line*. *Dashed lines* indicate Box dividers.

collected through a separate Teflon system located on the bow of the research vessel, and were filtered *in situ* with 0.45-μm polypropylene Calix cartridge filters. The samples were acidified to pH ≤ 2 with Ultrex grade nitric acid in a class 100 polypropylene work station. The copper in the samples was preconcentrated by liquid–liquid extraction with APDC and DDDC [6], and measured by graphite furnace atomic spectrometry, using stabilized temperature platforms, and the method of standard additions.

Free Copper Ion Concentration and Copper Complexation Capacity. The electrode potential (mV) between an Orion 94-29 copper ion selective electrode (Cu-ISE) and an Orion Ag/AgCl double-junction reference electrode was used for the determination of free copper ion concentration [67] in a flowthrough system [66]. The Cu-ISE was calibrated with seawater Cu-activity buffers, made of 2×10^{-4} M Cu and either 1×10^{-3} M ethylenediamine (EN) or 1×10^{-3} M glycine in filtered (0.45 μm) seawater in a dark, class-100 work station, with constant stirring at 25 ± 0.1°C [3]. The measured activity of the free aqueous Cu(II) ion [or $Cu(II)_{aq}$] in the sample was reported as pCu, which is defined as $-\log$ a $Cu(II)_{aq}$, and it its equal to the concentrations of $Cu(II)_{aq}$ as the buffer system was also in terms of $Cu(II)_{aq}$.

The copper complexation capacity was measured by titration with copper. After calibration, the Cu-ISE and the reference electrode were allowed to equilibrate overnight in an aliquot of the seawater sample. Then, a fresh seawater sample (~250 mL) in a Teflon beaker was allowed to equilibrate with the electrodes for several minutes before starting the titration. The titrations were performed with a TTT 85 Titrator and an ABU 80 Autoburette (Radiometer Copenhagen), which were connected to a personal computer for continuous data recording. The titrant was 326–427 μg L^{-1} copper in 1 L of >18 MΩ water containing 32 g of NaCl. Titration was completed after 99 mL of the titrant were added, which was equivalent to an average increase in concentration of 98.5 μg L^{-1} ($n = 11$). Copper complexation capacity was determined from the change in slope of the titration curve.

Total Suspended Solids. Samples collected for TSS were analyzed by filtering approximately 900 mL through pre-dried and pre-weighed glass-fiber filters (1.2 μm nominal pore size). The filters were rinsed with deionized water to remove dissolved salts, dried, and weighed to determine the mass of the filtered solids.

Chlorophyll a Concentration. Chlorophyll *a* concentrations, as an estimator of total phytoplankton biomass, were measured in all 27 boxes. Seawater samples (100 mL) were filtered through 25-mm glass fiber filters (Whatman GF/F) at a differential pressure

of <25 mm Hg. The filters were placed in 15-ml polypropylene screw cap centrifuge tubes and kept frozen at <−20°C until processing. Photosynthetic pigments were extracted by adding 10 ml of absolute methanol to each filter. After storage in the dark for 2–3 h, the sample was shaken, centrifuged for 1–2 min, and chlorophyll fluorescence measured in a Turner Designs fluorometer (Model 10-005R). Fluorescence was measured before and after acidification with HCl to determine both chlorophyll *a* and phaeophytin concentrations [27].

Microbial Abundance. A second set of water samples (100 ml) from all 27 Boxes was preserved in 1% glutaraldehyde for determination of bacterial and cyanobacteria cell numbers. Bacterial numbers were determined from aliquots of the glutaraldehyde-preserved samples by staining the cells with DAPI and direct fluorescence microscopic counting. Cell numbers of photosynthetic cyanobacteria were determined from aliquots of the glutaraldehyde-preserved samples by direct microscopic counting of the cells as evidenced by fluorescence of cellular phycobiliproteins.

Copper Effects on Bacterial Production. Experiments were conducted throughout the sampling effort to determine the deleterious effects of copper ion on overall bacterial population productivity. Stock 200 μg L^{-1} dissolved copper solutions were prepared with $CuCl_2$ and MilliQ water (>18 MΩ conductivity). The solutions were filtered through Nalgene 0.2 μm CN filter units. The $CuCl_2$ solution was added to bacterial production assay tubes to give a final concentration between 5 and 50 μg L^{-1}. Filtered MilliQ was added to control and experimental tubes to make the volume consistent among all treatments.

Tangential Flow Ultrafiltration and HMW DOM Experiments. During the January 2001 sampling event, HMW (<1000 kDa) natural organic matter (NOM) was concentrated from Box 27 water using a Millipore PLAC 0.54 m^2 TFF cartridge. A peristaltic pump (Millipore) was used to maintain ~170 kPa pressure over the membrane system. All wetted parts were Teflon, polycarbonate, or Pharmed elastomer (tubing). The system was cleaned as per the manufacturer's instructions with 0.1 N NaOH followed by copious amounts (~40 L) of MilliQ water, until background DOC concentration (<25 μM) was established. Blanks for the system ranged from ~5 to 25 μM DOC. In the laboratory, post-cleaning rinses routinely reached this background level after about 20 L of MilliQ. The retention coefficient:

$$\left(\left(1 - \left[\frac{\text{permeate DOC}}{\text{ambient DOC}}\right]\right) \times 100\%\right) \qquad (1)$$

for TFF was 72%. The TFF concentrate was mixed with TFF permeate and added to production assay tubes to achieve final concentrations of 170, 175, 190 and 210 μM DOC. Mixtures were made so that the same volume was added to each production tube. Experiments were conducted with HMW DOC additions alone and in conjunction with $CuCl_2$ additions to determine added DOC stimulatory effects and DOC-related reduction in copper toxicity. Dilutions were made so that the total volume added to each tube was consistent.

Mineral Addition Experiments. Experiments were conducted in January 2001 to determine the effects of mineral additions on copper toxicity to natural bacterioplankton. Mineral solutions were made from powdered illite and kaolinite clays (Imani Natural Products, Park Rapids, MN, USA). Clay powders were extracted with dichloromethane: acetone (1: 1) using a Dionex ASE 200 accelerated solvent extractor for 10 min at 100°C at 10 MPa pressure. The extracted powders were dried at 50°C for 2 days prior to use. The clays were added to sterile TFF permeate from Box 27 (see TFF procedures above) in order to achieve final concentrations of 7.5 and 10 mg L^{-1} in production tubes (ambient TSS was 5.0 mg L^{-1}). Experiments were conducted with mineral additions alone and in conjunction with $CuCl_2$ additions to determine mitigating effects of copper toxicity. Dilutions were made so that the total volume added to each tube was consistent. Any production greater than the ambient amount from addition of clay minerals alone was subtracted from results obtained with copper plus mineral addition.

Glutathione Addition Experiments. During the May 2001 sampling event, dissolved glutathione, a potential copper-chelating biomolecule [36, 60], was added to bacterial production assays with and without the addition of dissolved copper. A stock glutathione solution was made using MilliQ water. Experiments were conducted with glutathione additions at final concentrations of 5 and 10 μg L^{-1} alone and in conjunction with $CuCl_2$ additions to determine any protecting effects of glutathione on copper toxicity. Dilutions were made so that the total volume added to each tube was consistent. Any glutathione-only additions that were greater than ambient production were subtracted from glutathione-plus copper addition experiments.

Statistical Analysis. Comparisons between mean values were performed using two-way ANOVA in InStat software (GraphPad.com, San Diego, CA, USA). Princi-

Table 1. pCu, dissolved copper, and heterotrophic bacterial production for San Diego Bay

	August 2000			*January 2001*			*May 2001*			*September 2001*		
Box	*pCu*	*Diss. Cu* ($\mu g\ L^{-1}$)	*Production* ($\mu g\ C\ L^{-1}\ d^{-1}$)	*pCu*	*Diss. Cu* ($\mu g\ L^{-1}$)	*Production* ($\mu g\ C\ L^{-1}\ d^{-1}$)	*pCu*	*Diss. Cu* ($\mu g\ L^{-1}$)	*Production* ($\mu g\ C\ L^{-1}\ d^{-1}$)	*pCu*	*Diss. Cu* ($\mu g\ L^{-1}$)	*Production* ($\mu g\ C\ L^{-1}\ d^{-1}$)
1	12.9	0.30	8.4	11.5	0.42	2.18	13.2	0.60	17.57	12.7	0.27	16.4
2	12.9	0.35	9.6	11.3	0.48	4.35	13.2	0.64	14.65	12.7	0.20	24.5
3	12.9	0.20	11.0	11.6	0.62	4.12	13.3	0.87	13.34	12.8	0.19	33.0
4	13.1	0.36	10.9	12.3	0.72	4.22	13.7	0.91	15.33	12.8	0.19	31.5
5	13.0	0.31	12.7	12.9	0.71	3.66	14.0	1.00	13.95	12.8	0.33	28.3
6	12.5	2.43	14.4	11.1	4.51	6.11	12.0	4.47	13.70	12.4	2.10	35.0
7	12.7	0.35	12.5	11.6	1.40	4.83	12.8	1.26	12.73	12.7	0.28	34.8
8	12.8	0.37	11.8	11.8	1.20	3.87	12.9	1.25	11.65	12.7	0.35	30.8
9	12.8	2.37	15.7	11.6	3.55	6.88	12.6	2.67	10.94	12.4	2.29	24.3
10	12.8	0.67	11.9	11.8	1.75	3.86	12.8	1.33	12.43	12.6	0.66	20.1
11	12.9	1.53	13.2	11.9	2.00	3.77	12.9	1.35	9.26	12.7	0.69	23.9
12	12.9	1.10	11.1	11.9	1.93	4.12	12.9	1.69	5.55	12.7	1.46	29.2
13	12.9	1.33	10.7	11.9	2.35	4.70	12.9	1.73	10.27	12.7	1.66	26.8
14	12.9	1.44	10.4	11.9	2.52	3.55	12.9	1.80	10.56	12.6	1.69	25.6
15	12.9	1.88	11.7	12.0	2.47	4.03	12.9	1.77	9.50	12.7	1.77	30.3
16	12.9	1.49	11.5	12.0	2.50	4.54	12.9	1.79	9.53	12.6	1.83	26.4
17	12.9	1.94	10.1	12.0	2.47	5.20	12.9	1.79	10.60	12.6	1.84	15.4
18	12.9	1.77	11.0	12.0	3.09	7.33	13.0	1.86	13.81	12.6	2.19	31.2
19	12.9	1.82	10.5	12.0	2.75	5.70	13.0	2.13	6.33	12.5	2.17	28.5
20	13.0	2.13	12.0	11.9	2.82	7.09	12.9	2.10	10.03	12.5	2.34	29.5
21	13.0	2.42	8.3	12.0	3.02	7.41	12.8	2.23	9.43	12.5	2.57	30.5
22	12.9	2.58	9.0	11.9	3.21	8.40	12.9	2.30	10.16	12.5	2.65	32.6
23	13.0	2.26	15.1	12.0	3.14	7.77	12.9	2.29	9.16	12.7	2.73	36.2
24	13.0	2.14	18.6	12.1	3.13	10.27	12.9	2.19	12.58	12.8	2.58	39.8
25	13.0	2.16	18.7	12.2	3.01	10.53	13.0	2.29	14.97	12.8	2.40	36.1
26	13.0	2.14	19.2	12.2	2.83	10.50	13.0	2.33	17.29	12.8	2.73	35.4
27	13.1	2.19	17.9	12.4	2.34	11.85	13.1	2.19	16.02	12.9	2.18	37.3

Average standard deviation for production was ±7%.

pal components analyses (PCA) and regression analyses were performed with Matlab software. For presentation in tables, standard deviations were averaged. Error bars indicate sample standard deviations.

Results

Ambient Conditions. Dissolved copper showed a consistent pattern for each of the seasonal samplings. In general, dissolved copper was lowest in Box 1 with incrementally increasing amounts found in subsequent boxes (Table 1). The most notable exceptions were in Box 6 and in Boxes 21–23. Box 6 is located in the Shelter Island basin, which hosts a marina harboring numerous pleasure craft. Corollary analyses often showed elevated concentrations of DOC, NH_4^+ and NO_3^- at this station indicating potential point sources of elevated anthropogenic impacts (data not shown). Boxes 21–23 span the vicinity of the San Diego Navy Base where large-scale use of copper-based antifouling paints occurs. Free copper ion concentration (which is expressed as pCu, or the negative logarithm of the free copper ion concentration) generally showed slight decreases moving from Box 1 to Box 27. Free copper ion concentration was elevated in Box 6; however, it did not show significant elevation in Boxes 21–23 compared to adjacent Boxes 20 and 24 ($P > 0.05$).

At equilibrium and for well-defined systems, the copper complexation stability constant is defined by the equation:

$$K'\left[\frac{[\mathrm{Cu_d}]}{[\mathrm{Cu_f}][L]}\right] \qquad (2)$$

where K' is the stability constant, L is the complexation capacity (or complexing ligand concentration), Cu_d is the dissolved copper concentration and Cu_f is the uncomplexed or free Cu ion concentration ($Cu(II)_{aq}$). The ratio of Cu_d to Cu_f is commonly referred to as the alpha coefficient (α). A series of titrations was performed in the laboratory to resolve the relationship between the stability constant and the complexating ligand(s) concentration. If K' were relatively constant, one would expect a linear relationship between the measured complexation capacity and the ratio of dissolved to free Cu. However, there was no statistically significant correlation observed for any sampling events in this study ($r^2 < 0.40$, $P > 0.05$). This indicates that either the stability constants for Cu complexation in the Bay had considerable variability, or that there were appreciable differences between the ISE-derived measurements of free copper and the subsequent laboratory determinations of complexation capacity. In order to assess the variability in the α coefficient (subsequently just termed α), we performed a PCA. Data were normalized by dividing each α by its entire season's standard deviation. Results of this analysis indicated that over 80% of the variability in α over the course of the four samplings could be explained in the first principal component, and 9% could be explained in the second. Due to the proportionality of Eq. (2), it follows that K' or the complexation capacity ($[L]$) could potentially be assigned to the first principal component. In plotting the data, there was an almost linear relationship between the first principal component and box number, potentially indicating an incremental increase in the stability constant from the mouth to the back Bay. The lack of clustering when the first and second principal component are plotted against one another indicates another factor is responsible for the variability in the Bay, perhaps the complexation capacity itself (Fig. 2). Due to the variability in a prominent factor controlling α and the lack of correlation of α with measured ligand concentrations, a proxy for natural water complexation capacity was represented by using only the coefficient α. In addition, the complexation capacities measured by titration were always 5–10 times higher than the dissolved copper concentrations (data not shown) indicating residual complexation capacity above the total dissolved copper concentration.

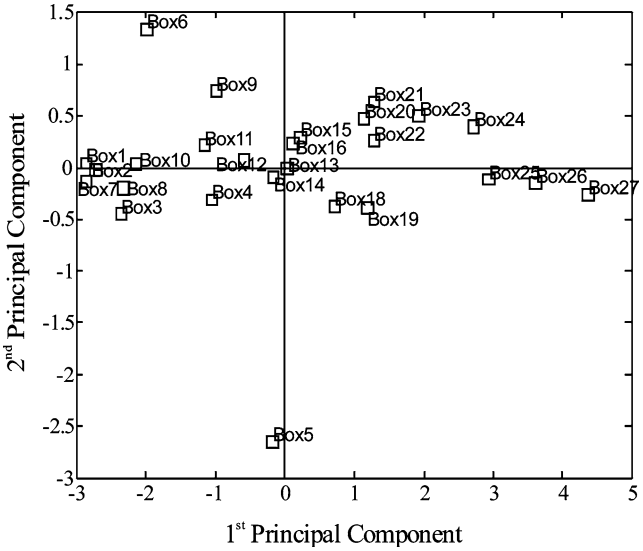

Figure 2. Results of principal components analysis of variability in α over four samplings.

Another possible explanation for the lack of correlation between α and complexation capacity calculated from copper titrations is a possible temporal change in the complexation capacity during sample storage. ISE measurements were performed "real-time" through a shipboard flowthrough system while complexation capacity measurements (copper titrations) were performed on refrigerated, stored samples. There is very likely a systematic error associated with each type of measurement. It is possible that some changes in complexation capacity occur

Table 2. Total dissolved solids, dissolved organic carbon, and calculated complexation capacity for San Diego Bay

	August 2000			*January 2001*			*May 2001*			*September 2001*		
Box	*TSS* ($mg\ L^{-1}$)	*DOC* ($mg\ L^{-1}$)	α	*TSS* ($mg\ L^{-1}$)	*DOC* ($mg\ L^{-1}$)	α	*TSS* ($mg\ L^{-1}$)	*DOC* ($mg\ L^{-1}$)	α	*TSS* ($mg\ L^{-1}$)	*DOC* ($mg\ L^{-1}$)	α
1	0.36	2.18	8.22×10^{4}	0.59	1.36	4.58×10^{3}	3.17	1.50	3.28×10^{5}	1.93	2.99	4.67×10^{4}
2	1.06	ND	9.59×10^{4}	0.56	ND	3.30×10^{3}	2.58	1.59	3.50×10^{5}	ND	2.55	3.46×10^{4}
3	1.00	1.91	5.48×10^{4}	0.48	1.20	8.51×10^{3}	2.40	1.76	5.99×10^{5}	5.72	2.66	4.13×10^{4}
4	1.16	1.80	1.56×10^{5}	0.43	1.19	4.95×10^{4}	3.12	1.64	1.57×10^{6}	3.11	2.85	4.13×10^{4}
5	1.28	1.92	1.07×10^{5}	0.45	1.26	1.94×10^{5}	2.56	1.56	3.45×10^{6}	3.10	2.49	7.18×10^{4}
6	2.28	1.81	2.65×10^{5}	1.28	1.39	1.96×10^{4}	1.52	1.65	1.54×10^{5}	1.76	1.55	1.82×10^{5}
7	1.48	1.77	6.05×10^{4}	0.52	1.31	1.92×10^{4}	3.19	1.64	2.74×10^{5}	4.39	2.39	4.84×10^{4}
8	1.62	1.78	8.05×10^{4}	0.54	1.26	2.61×10^{4}	5.13	1.48	3.42×10^{5}	3.88	2.64	6.05×10^{4}
9	2.74	2.07	5.16×10^{5}	1.03	1.39	4.87×10^{4}	3.34	1.65	3.67×10^{5}	5.00	2.31	1.98×10^{5}
10	2.40	1.75	1.46×10^{5}	0.59	1.37	3.81×10^{4}	3.79	1.75	2.89×10^{5}	2.95	2.62	9.06×10^{4}
11	3.20	1.77	4.19×10^{5}	0.66	1.46	5.48×10^{4}	4.32	1.69	3.70×10^{5}	2.91	1.96	1.19×10^{5}
12	2.68	2.09	3.01×10^{5}	0.71	1.42	5.29×10^{4}	3.26	1.74	4.63×10^{5}	2.50	3.16	2.52×10^{5}
13	2.44	2.11	3.64×10^{5}	0.73	1.51	6.44×10^{4}	4.28	1.70	4.74×10^{5}	2.60	2.68	2.87×10^{5}
14	1.52	2.02	3.94×10^{5}	0.83	1.53	6.90×10^{4}	3.04	1.76	4.93×10^{5}	3.42	2.44	2.32×10^{5}
15	3.60	2.15	5.15×10^{5}	0.98	1.52	8.52×10^{4}	2.63	1.65	4.85×10^{5}	4.41	2.29	3.06×10^{5}
16	2.24	2.26	4.08×10^{5}	0.96	1.78	8.62×10^{4}	3.02	1.65	4.90×10^{5}	2.16	2.70	2.51×10^{5}
17	1.60	ND	5.31×10^{5}	0.69	1.64	8.52×10^{4}	6.83	1.63	4.90×10^{5}	2.16	2.63	2.53×10^{5}
18	1.46	ND	4.85×10^{5}	0.64	1.67	1.07×10^{5}	2.72	1.66	6.41×10^{5}	1.75	2.95	3.01×10^{5}
19	1.96	2.60	4.99×10^{5}	0.68	1.65	9.48×10^{4}	2.51	1.79	7.34×10^{5}	2.55	3.04	2.37×10^{5}
20	1.62	0.88	7.34×10^{5}	0.61	1.70	7.72×10^{4}	2.78	1.81	5.75×10^{5}	1.93	3.39	2.55×10^{5}
21	1.59	2.54	8.34×10^{5}	0.69	1.73	1.04×10^{5}	2.91	1.86	4.85×10^{5}	2.68	3.80	2.80×10^{5}
22	1.22	2.69	7.07×10^{5}	0.85	1.68	8.79×10^{4}	2.85	1.74	6.30×10^{5}	3.30	2.92	2.89×10^{5}
23	2.02	2.73	7.79×10^{5}	1.04	1.72	1.08×10^{5}	3.11	1.81	6.27×10^{5}	3.91	3.35	4.72×10^{5}
24	2.08	2.33	7.38×10^{5}	1.35	1.84	1.36×10^{5}	3.79	1.84	6.00×10^{5}	5.00	3.59	5.61×10^{5}
25	2.68	3.29	7.45×10^{5}	1.35	1.94	1.65×10^{5}	5.28	1.88	7.90×10^{5}	9.12	3.39	5.22×10^{5}
26	3.31	2.93	7.38×10^{5}	2.35	2.20	1.55×10^{5}	3.76	1.93	8.03×10^{5}	6.19	3.38	5.94×10^{5}
27	ND	3.12	9.51×10^{5}	4.98	1.95	2.03×10^{5}	7.48	2.00	9.51×10^{5}	6.56	3.79	5.97×10^{5}

Table 3. Goodness of fit from regression analysis of α against TSS, DOC concentration, chlorophyll *a* or bacterial production

Date	*TSS* (r^2; *P*) *n* = 27	*DOC* (r^2; *P*) *n* = 27[b]	*Chl a* (r^2; *P*) *n* = 27	*Production* (r^2; *P*) *n* = 27
August 2000	0.16; 0.046	0.34; <0.0030	0.62; <0.0001	0.21; 0.016
January 2001	0.27; 0.0066	0.22; 0.0154	0.23; 0.0124	0.46; 0.0001
May 2001	0.14; 0.062	0.60; <0.0001	0.40; 0.0007	0.01; 0.65
September 2001	0.22; 0.0172	0.42; 0.0002	0.49[a]; <0.0001	0.27; 0.0057

[a]Negative slope.
[b]Three DOC samples from August 2000 were contaminated and not included in calculation.

during sample collection, storage, and processing in the laboratory (copper titrations). For instance, it has been shown that introduced copper ions may initially sorb to "weak" ligands, but over time exchange to stronger binding ligands [1]. The sampling location (San Diego Bay) is a dynamic system with temporal input and output fluxes of copper whereas the sample storage containers are static systems perhaps allowing copper exchange between weak and strong ligands during storage time. Even though both α, calculated by ISE, and complexation capacity, from copper titrations, show a general trend of increasing with increasing Box number from the mouth to the back Bay, differences in systematic measurement error (although perhaps consistent within any particular measurement protocol) might not allow for a direct correlation between the two types of measurements.

Bacterial production was lowest in Box 1 for all samplings except May 2001 (Table 1). In general, production increased as Box number increased—i.e. as a function of increasing distance from the mouth of the Bay (Table 1). During August 2000 and January 2001, there was a significant increase in production in Box 6. These two sampling events had elevated NH_4^+ concentrations in Box 6 that could explain the higher production values.

In general, TSS and DOC concentrations were highest in the back Bay (Box 27) and lowest at the mouth of the Bay (Table 2). TSS varied almost an order of magnitude in August 2000 and January 2001, but only varied by a factor of ~2–3 in May 2001 and September 2001. DOC range did not vary substantially with season; however, absolute values were highest in late summer and fall samplings (August 2000 and September 2001). To investigate possible causative relationships between α and other parameters, a series of linear regression analyses was performed. There were significant linear relationships between the ratio of dissolved to free Cu (α) and DOC, Chl *a* and bacterial production during at least one of the seasons (Table 3). Goodness of fit was low ($r^2 < 0.61$) for all parameters during the May 2001 and September 2001 samplings, but was highest for DOC in both samplings (Table 3).

Heterotrophic Bacterial Production with Copper Additions. There was a general trend toward decreasing production with increasing concentrations of added $CuCl_2$. Initial experiments were done with water from Box 27 since it generally had the highest production and greatest calculated copper complexation capacity. There was an exponential decrease in bacterial production with increasing concentrations of added copper in Box 27 water during January 2001 (Fig. 3), even at levels below the measured complexation capacity of the adjacent Box 26 (14.9 μg L^{-1}). During May 2001 (Fig. 4A) and September 2001 (Fig. 4B), four samples (Box 1, 4, 21 and 27) were assayed for production response to added copper. Box 1 appeared to show the least impact from increasing concentrations of copper while Box 27 showed the highest.

Effects of DOC Additions. Additions of ambient ultrafiltered DOM (UDOM) showed significant amelioration of copper impacts on bacterial production in Box

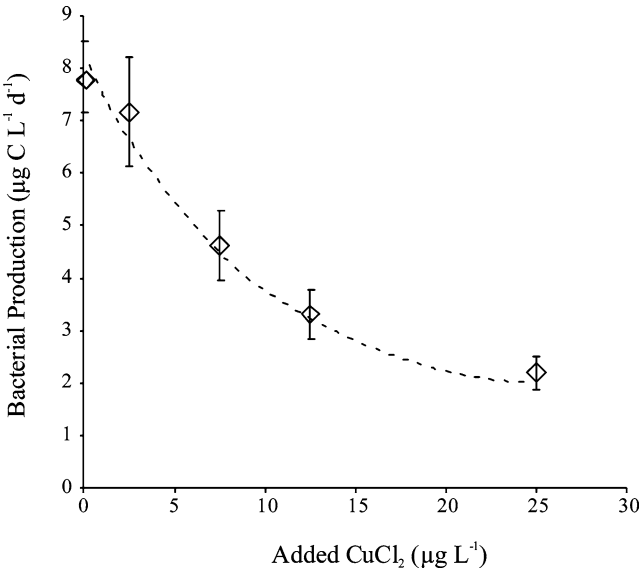

Figure 3. Bacterial production in response to added $CuCl_2$, January 2001, Box 27. *Dashed line* exponential equation: $y = 1.94 + 6.23e^{-0.11x}$ $r^2 = 0.99$, $P < 0.05$.

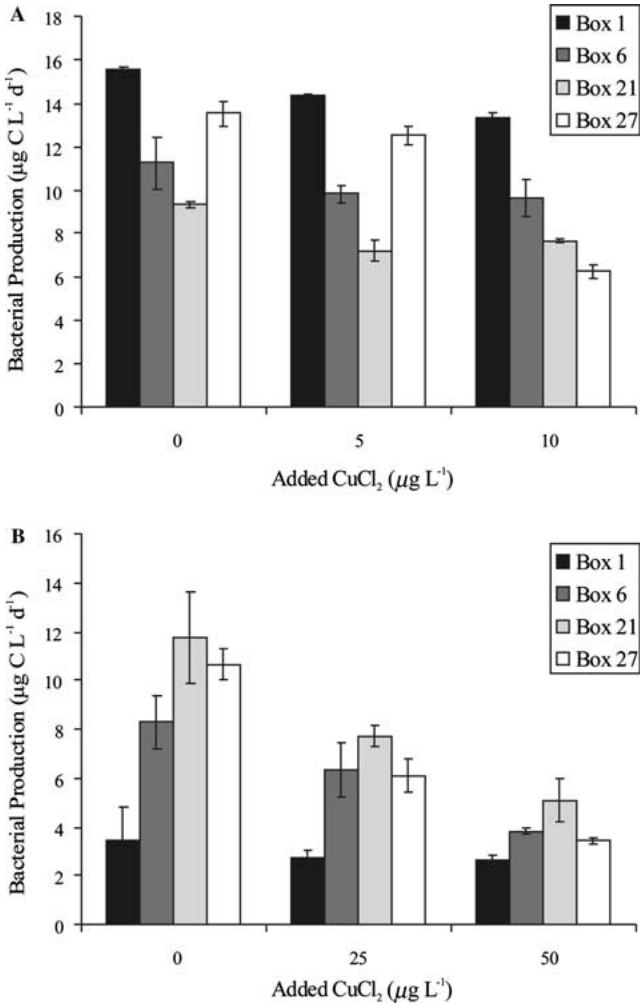

Figure 4. Bacterial production in response to added $CuCl_2$, (A) May 2001; (B) September 2001.

27 during the January 2001 sampling. Control incubations in which UDOM was added alone (without copper additions) showed no statistically significant production increase with increasing UDOM concentrations (data not shown). From 12.5 to 25 μg L^{-1} added $CuCl_2$, there was significant increase in production with UDOM additions from 25 to 65 μM above ambient concentration ($P < 0.05$). The mitigating effect was greater at lower copper concentrations and appeared, at least at the highest copper concentrations, to increase with increasing UDOM (Fig. 5). However, even in the highest added UDOM experiment, mean values for production were lower than ambient conditions with no added copper.

Effects of Mineral Additions. Neither illite nor kaolinite addition reduced the toxicity of copper to natural bacterioplankton (data not shown; $P > 0.05$). Controls with added minerals but no added copper showed no statistically significant increases in bacterial

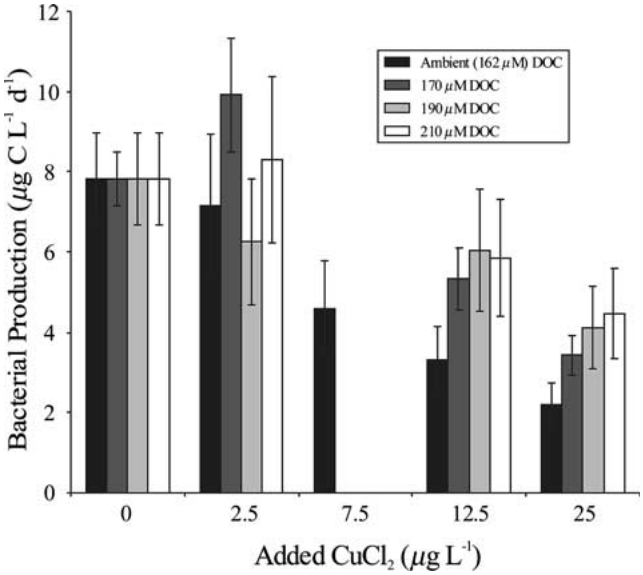

Figure 5. Bacterial production with different $CuCl_2$ and natural DOC concentrate additions. Values represent final $CuCl_2$ and DOC concentrations. Due to sample size limitations, no DOC concentrate was added to the 7.5 μg L^{-1} $CuCl_2$ addition experiment.

production ($P > 0.05$), indicating no metabolic stimulation from the assay itself. In fact, the highest kaolinite additions appeared to have an inhibitory effect on bacterial production which has been shown to occur in freshwater environments with *Ceriodaphnia* [38].

Effects of Glutathione Additions. In May 2001, there was no significant protection effect offered by glutathione in any of the studies (data not shown; $P > 0.05$). In fact, there were several instances when glutathione addition appeared to be toxic to microorganisms. During July 2001, there was also no statistically significant protecting effect of adding glutathione ($P > 0.05$).

Discussion

Copper Toxicity to Bacterial Production. Our most significant finding was that short-term copper toxicity appeared to increase as pCu values increased from Box 1 to Box 27 (an increase in pCu indicates a decrease in free-ion concentration). The ratio alpha generally increased from the mouth to the back Bay (c.f. Table 2). One would expect there to be greater complexation of added Cu^{2+} at Box 27 than at Box 1. The free-ion activity or biotic ligand model would then predict less impacted production with higher pCu. We observed the opposite trend. We noted a decrease in production with added Cu^{2+} for Boxes 1, 6, 21 and 27 during May 2001 and September 2001 (Figs. 4A, 4B). The decrease in production with 10 μg L^{-1} (final concentration) added copper were then related to α (Fig. 6). In this representation,

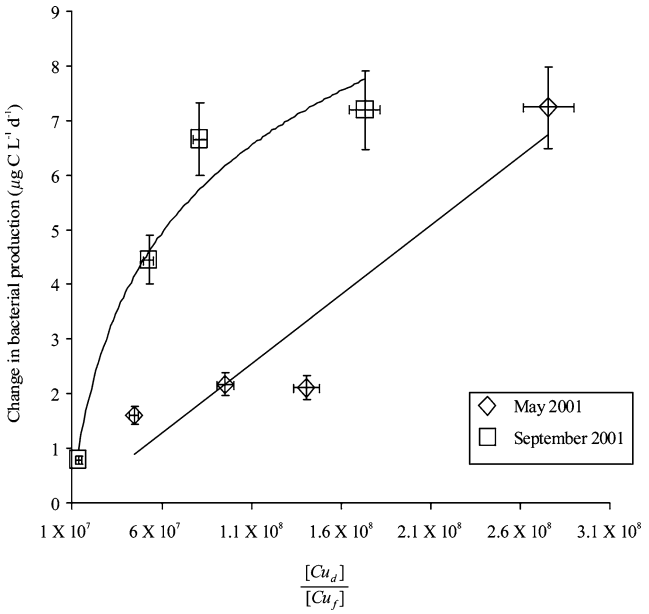

Figure 6. Change in production from addition of 10 μg L^{-1} (final concentration) $CuCl_2$ in relation to a. May, 2001, linear regression gave the best goodness of fit: $r^2 = 0.89$, $P < 0.001$; September 2001, logarithmic gave best goodness of fit: $r^2 = 0.95$, $P < 0.03$.

higher α values were correlated with greater decreases in production. The expectation would be that impact on bacterial activity would decrease with increasing copper complexation capacity, or the coefficient α. The results from this study are at odds with (1) the idea that toxicity in bioassays are inversely related to complexation capacity in natural waters and (2) and with toxicity studies conducted as part of this overall effort using *Dendraster excentricus*, *Mytilus galloprovincialis*, and *Strongylocentrotus purpuratus* embryo EC50 assays [45]. In these studies, it was found that copper toxicity decreased from the mouth to the back Bay (with higher measured complexation capacity and higher α) even though aqueous copper concentrations (free Cu^{2+}) of about 1×10^{-11} M were equally toxic regardless of the source water (i.e. mouth or back Bay) [45]. In earlier experiments, a proxy for complexation capacity was determined by copper toxicity to test organisms [25, 54, 55].

There may be several factors that could explain our results in light of previous work. We wanted to have as short an incubation time as possible in order to determine the immediate effects of copper addition. The time of interaction between the bacteria, natural seawater milieu and copper ion may be a factor in the observed effects. Copper addition studies by Sunda and Ferguson [53] showed no change in reaction kinetics between 3 and 5 h of incubation for bacterial amino acid utilization. Recently, it has been shown that complexation between free copper ion and NOM occurs rapidly, usually less than 1–2 h [59]. However, in freshwaters, TSS-free copper interactions took much longer, on the order of 5–6 h [1]. Incubation times for this study were on the order of 1–2 h which is a standard time for minimizing secondary utilization of added isotope.

Throughout our sampling, pCu values were generally higher in the South Bay than at the mouth (Table 1). This means that organisms in the South Bay have less chronic exposure to free copper than organisms at the mouth. There may be a temporal dose–response relationship between free copper that has not fully complexed with *in situ* ligands and exposure to the bacterial assemblage. If this is the case, higher complexation capacity would provide more protection, but less free copper exposure to bacterial cells, making them more susceptible to rapid shock with added copper. Future studies might take advantage of this potential time-dependent dose response. Assays could be conducted with various lag times between addition of copper and copper complexing agents (NOM, minerals, glutathione, etc). In this manner, acute copper impacts versus chronic copper exposures could be assessed.

Results from this study differed from the findings of Sunda and Ferguson [53] wherein bacterial incubations with the lowest productivity (based on amino acid utilization) were most sensitive to copper ion additions. In this work, we found that copper toxicity was negatively correlated with bacterial production (Fig. 6). In other words, the fastest growing assemblages were the most sensitive to copper ion additions. In the laboratory, microbial cells have been shown to be more susceptible to toxicants during log phase than during stationary phase growth [33, 41, 42]. If we make the assumption that higher production values were coincident with a higher proportion of the microbial assemblage growing at log phase, perhaps growth state might explain the increased toxicity at higher growth rates. Calculating cell-specific bacterial production (g C $cell^{-1}$ day^{-1}) using DAPI cell counts and heterotrophic production data and plotting by Box number for the four boxes in which copper was added showed a logarithmic increase moving from the mouth to the back portions of San Diego Bay (Fig. 7). Extrapolating laboratory growth characteristics to assemblage-level microbiology in field samples is always problematic; however, if actively growing and reproducing bacterial cells are in fact more susceptible to copper toxicity, one would expect greater impacts where production and cell abundance were highest. DOC and nutrient concentrations, like production, increased from the mouth to the back Bay. If growth and reproduction were more limited at the mouth than in the back Bay, there may be a higher proportion of production allocated to cell maintenance as opposed to active growth and reproduction [34]. If maintenance metabolism and active growth in natural systems were analogous to stationary and log phases in laboratory growth studies, copper toxicity could be highest with the most active cells.

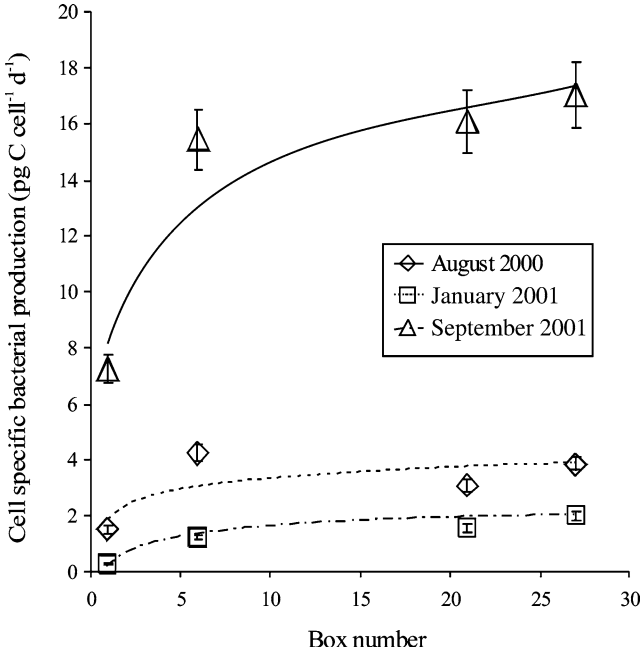

Figure 7. Cell-specific production related to sample box number for August 2000, January 2001, and September 2001 samplings. Logarithmic growth curve correlations: August 2000, $r^2 = 0.52$, $P > 0.05$; January 2001, $r^2 = 0.96$, $P < 0.05$; September 2001, $r^2 = 0.90$, $P < 0.05$.

Protection from Copper Toxicity. A number of natural and synthetic agents such as inorganic ions, organic chelating agents, clay minerals, dissolved humic materials and bulk dissolved organic matter have been used to ameliorate copper toxicity to freshwater, estuarine and marine bacteria (see [8] and [12] for reviews). Although higher particulate loading has been shown to impact copper toxicity [57], we did not observe any protecting effect from the addition of "local" clay minerals. San Diego receives very little rainfall during the year (~25 cm year^{-1}; www.sdcwa.org) and very little runoff, particularly if there have been no recent rain events. Therefore particulate matter in the water column might not be rich in clay minerals and may comprise other materials.

Glutathione was chosen as a putative protective agent because it is produced by algae under copper stress [9, 36]. Incubation studies using marine algae in defined media have shown that copper toxicity can be ameliorated by the addition of thiol compounds [36, 61]. Glutathione-copper binding kinetics are rapid and well within the incubation time used for this study [35]. There may be several explanations why no reduction of toxicity was seen in this study. The concentrations of both copper and copper binding ligands in the reported studies were much lower than added here. Also, this study was conducted with natural water already containing copper, binding ligands, and microbial communities. However, glutathione additions were performed at or above the added copper concentrations. Glutathione–copper binding may be partially controlled by natural substances in the water column, either through competitive binding or slowing association reactions. Additionally, glutathione may not confer copper ion protection to bacteria in the same way it does to algae.

Recent investigations into the kinetics of copper–NOM and copper–TSS interactions have shown that as many as 1–2 h (for NOM) to 5–6 h (for TSS) are needed for maximum copper sorption [1, 38]. While these factors may have played a part in copper-only additions in this study, there is less reason to believe they were critical to the copper protection studies. For all production assays with added copper and putative protecting agents (NOM, minerals, glutathione), the copper and agent were added to the assay at least 8 h prior to the introduction of the natural seawater sample to give the copper and agent time to interact before exposure to bacterioplankton. However, we are unsure to what degree the seawater milieu affected the copper–agent interaction upon its introduction into the incubation vessel. If the introduction of seawater caused an immediate dissociation of the copper–agent complex, the subsequent recomplexation may have needed time to occur, thus potentially allowing bacterioplankton exposure to free copper ion. Future studies should include a time-dependent response element so that these factors can be determined.

Copper Complexation. There are three main proposed origins of copper binding ligands in natural environments: (1) production in response to copper stress by algae or other organisms [19, 21, 36]; (2) components of natural dissolved or colloidal organic matter [4, 24, 29, 46, 63, 64]; or (3) components of clays and other suspended particulate matter [2, 12, 17, 26, 30]. Natural samples probably include material from all of these sources. Based on correlations between Chl *a*, DOC, TSS and α alone, it would appear that biological processes impact copper complexation of water in San Diego Bay. DOC and POC, in the form of algae (Chl *a*) and bacteria (production) may facilitate "colloidal pumping" in which temporal aggregation of colloids into larger sinking particles moves water column metals to the sediments [28, 39]. While we did not concentrate DOM from size ranges other than >1 kDa, it has been shown in Narragansett Bay, Rhode Island (USA) that the strongest copper binding ligand are in the 1–8 kDa range [62] while in Galveston Bay, TX (USA), ligands within the ultrafiltered DOM had stability constants up to 60 times greater than in the LMW (permate) fraction [56]. This also appears to be the case in the Chesapeake Bay where copper complexing ligands were found to be <10,000 kDa [22]. In this Chesapeake Bay study, XAD resin

extraction removed ~50% of the copper complexation capacity indicating a humic acid nature of the ligands. It was also found that most of the L_1, or strong class of ligands, was retained on a 1 kDa nominal molecular weight cutoff (NMWCO) ultrafilter, and that the fraction <1 kDa retained the weaker, or L_2 ligands. Our results are intriguing in this light because we found some copper protection from the addition of DOC concentrate (>1 kDa), but not enough to bring production back in line with no copper controls (Fig. 5).

Although there was no observed protection offered by glutathione additions there was significant correlation between Chl *a* and α during the August 2000 sampling. However, data from this study fail to support a direct relationship between phytoplankton ligand production and phytoplankton abundance. Abundance and activity are generally not well correlated in natural systems, and there may be a lag between growth of algae and the leaching of cellular components such that high levels of complexing ligands exist for weeks after bloom conditions. There was a significant correlation between bacterial production and α for the January 2001 samplings (Table 3). Correlation between production or Chl *a* and pCu yielded regression coefficients <0.20 for all samplings. This would indicate that free copper concentrations were in and of themselves not toxic to the natural phytoplankton or bacterial assemblages. Lack of correlation between Chl *a* and bacterial production and either of these with α during some seasons indicates decoupling between algal abundance and bacterial activity and copper complexation within San Diego Bay.

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References

1. Achterberg, EP, Van Elteren, JT, Kolar, ZI (2002) Species kinetics and heterogeneous reactivity of dissolved cu in natural freshwaters. Environ Sci Technol 36: 914–920
2. Babich, H, Stotzky, G (1977) Reductions in toxicity of cadmium to microorganisms by clay-minerals. Appl Environ Microbiol 33: 696–705
3. Belli, SL, Zirino, A (1993) Behavior and calibration of the copper(Ii) ion-selective electrode in high chloride media and marine waters. Anal Chem 65: 2583–2589
4. Bianchi, TS, Lambert, CD, Santschi, PH, Guo, LD (1997) Sources and transport of land-derived particulate and dissolved organic matter in the Gulf of Mexico (Texas shelf/slope): the use of lignin-phenols and loliolides as biomarkers. Org Geochem 27: 65–78
5. Brand, LE, Sunda, WG, Guillard, RRL (1986) Reduction of marine-phytoplankton reproduction rates by copper and cadmium. J Exp Mar Biol Ecol 96: 225–250
6. Bruland, KW, Coale, KH, Mart, L (1985) Analysis of seawater for dissolved cadmium, copper and lead—an intercomparison of voltammetric and atomic-absorption methods. Mar Chem 17: 285–300
7. Bruland, KW, Donat, JR, Hutchins, DA (1991) Interactive influences of bioactive trace-metals on biological production in oceanic waters. Limnol Oceanogr 36: 1555–1577
8. Campbell, PGC (1995) Interactions between trace metals and aquatic organisms: a critique of the free-ion activity model. In: Tessier, A, Turner, DR (Eds.) Metal Speciation and Bioavailability in Aquatic Systems. Wiley, New York, pp 45–102
9. Chapman, JS, Diehl, MA, Lyman, RC (1993) Biocide susceptibility and intracellular glutathione in *escherichia-coli*. J Indust Microbiol 12: 403–407
10. Coale, KH, Bruland, KW (1988) Copper complexation in the Northeast Pacific. Limnol Oceanogr 33: 1084–1101
11. Coale, KH, Bruland, KW (1990) Spatial and temporal variability in copper complexation in the North Pacific. Deep Sea Res 37: 317–336
12. Collins, YE, Stotzky, G (1989) Factors affecting the toxicity of heavy metals to microbes. In: Beveridge, TJ, Doyle, RJ (Eds.) Metal Ions and Bacteria. Wiley, New York, pp 31–90
13. Croot, PL, Moffett, JW, Brand, LE (2000) Production of extracellular Cu complexing ligands by eucaryotic phytoplankton in response to Cu stress. Limnol Oceanogr 45: 619–627
14. Davis, JA (1984) Complexation of trace metals by adsorbed natural organic matter. Geochim Cosmochim Acta 48: 679–691
15. Di Toro, DM, Allen, HE, Bergman, HL, Meyer, JS, Paquin, PR, Santore, RC (2001) Biotic ligand model of the acute toxicity of metals. 1. Technical basis. Environ Toxicol Chem 20: 2383–2396
16. Donat, JR, Bruland, KW (1995) Trace elements in the oceans. In: Salbu, B, Steinnes, E (Eds.) Trace Elements in Natural Waters. CRC Press, Boca Raton
17. Du, Q, Sun, ZX, Forsling, W, Tang, HX (1997) Adsorption of copper at aqueous illite surfaces. J Colloid Interface Sci 187: 232–242
18. Farrah, H, Hatton, D, Pickering, WF (1980) Affinity of metal-ions for clay surfaces. Chem Geol 28: 55–68
19. González, JM, Santana-Casiano, JM, Perez-Peña, J, Millero, FJ (1995) Binding of Cu(II) to the surface and exudates of the alga *Dunaliella tyertiolecta* in seawater. Environ Sci Technol 29: 289–301
20. González-Dávila, M, Santana-Casiano, JM, Laglera, LM (2000) Copper adsorption in diatom cultures. Mar Chem 70: 161–170
21. Gordon, AS, Donat, JR, Kango, RA, Dyer, BJ, Stuart, LM (2000) Dissolved copper-complexing ligands in cultures of marine bacteria and estuarine water. Mar Chem 70: 149–160
22. Gordon, AS, Dyer, BJ, Kango, RA, Donat, JR (1996) Copper ligands isolated from estuarine water by immobilized metal affinity chromatography: temporal variability and partial characterization. Mar Chem 53: 163–172
23. Gordon, AS, Howell, LD, Harwood, V (1994) Responses of diverse heterotrophic bacteria to elevated copper concentrations. Can J Microbiol 40: 408–411
24. Guo, LD, Santschi, PH, Warnken, KW (2000) Trace metal composition of colloidal organic material in marine environments. Mar Chem 70: 257–275

25. Hering, JG, Sunda, WG, Ferguson, RL, Morel, FMM (1987) A field comparison of 2 methods for the determination of copper complexation—bacterial bioassay and fixed-potential amperometry. Mar Chem 20: 299–312
26. Holm, TR, Zhu, XF (1994) Sorption by kaolinite of Cd^{2+}, Pb^{2+} and Cu^{2+} from landfill leachate-contaminated groundwater. J Contam Hydrol 16: 271–287
27. Holm-Hansen, O, Riemann, B (1978) Chlorophyll *a* determination: improvements in methodology. Oikos 30: 438–447
28. Honeyman, BD, Santschi, PH (1989) A brownian-pumping model for trace metal scavenging: evidence from Th isotopes. J Mar Res 47: 950–995
29. Hongve, D, Skogheim, OK, Hindar, A, Abrahamsen, H (1980) Effects of heavy-metals in combination with NTA, humic-acid, and suspended sediment on natural phytoplankton photosynthesis. Bull Environ Contam Toxicol 25: 594–600
30. Ikhsan, J, Johnson, BB, Wells, JD (1999) A comparative study of the adsorption of transition metals on kaolinite. J Colloid Interface Sci 217: 403–410
31. Johnson, HD, Grovhoug, JG, Valkirs, AO (1998) Copper loading to US Navy harbors. Technical Document #3052 3052
32. Jonas, RB (1989) Acute copper and cupric ion toxicity in an estuarine microbial community. Appl Environ Microbiol 55: 43–49
33. Kachlany, SC, Levery, SB, Kim, JS, Reuhs, BL, Lion, LW, Ghiorse, WC (2001) Structure and carbohydrate analysis of the exopolysaccharide capsule of Pseudomonas putida G7. Environ Microbiol 3: 774–784
34. Kjelleberg, S, Hermansson, M, Marden, P, Jones, GW (1987) The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment. Ann Rev Microbiol 41: 25–49
35. Leal, MFC, van den Berg, CMG (1998) Evidence for strong copper(I) complexation by organic ligands in seawater. Aquat Geochem 4: 49–75
36. Leal, MFC, Vasconcelos, MTSD, van den Berg, CMG (1999) Copper-induced release of complexing ligands similar to thiols by *Emiliania huxleyi* in seawater cultures. Limnol Oceanogr 44: 1750–1762
37. Lewis, AG, Whitfield, PH, Ramnarine, A (1972) Some particulate and soluble agents affecting the relationship between metal toxicity and organisms survival in the calanoid copepod *Euchaeta japonica*. Mar Biol 17: 215–221
38. Ma, HZ, Kim, SD, Allen, HE, Cha, DK (2002) Effect of copper binding by suspended particulate matter on toxicity. Environ Toxicol Chem 21: 710–714
39. Martin, J-M, Dai, M-H, Cauwet, G (1995) Significance of colloids in the biogeochemical cycling of organic carbon and trace metals in the Venice Lagoon (Italy). Limnol Oceanogr 40: 119–131
40. Moffett, JW, Brand, LE (1996) Production of strong, extracellular Cu chelators by marine cyanobacteria in response to Cu stress. Limnol Oceanogr 41: 388–395
41. Mong, HB, Brown, LR, Kim, JK (1995) Effect of zinc and calcium on the intracellularly uptake of cadmium and growth of *Escherichia coli*. J Microbiol 33: 302–306
42. Perkins, J, Gadd, GM (1993) Cesium toxicity, accumulation and intracellular-localization in yeasts. Mycol Res 97: 717–724
43. Qian, JG, Mopper, K (1996) Automated high performance, high-temperature combustion total organic carbon analyzer. Anal Chem 68: 3090–3097
44. Riemann, B, Lindgaard-Joergensen, P (1990) Effects of toxic-substances on natural bacterial assemblages determined by means of [H-3] thymidine incorporation. Appl Environ Microbiol 56: 75–80
45. Rivera-Duarte, I, Rosen, G, Lapota, D, Chadwick, D, Kear-Padilla, L, Zirino, A (2005) Copper toxicity to larval stages of three marine invertebrates and copper complexation capacity in San Diego Bay, California. Environ Sci Technol, Article 10.1021/es040545j S0013-936X(04)00545-0 Web Release Date: January 21, 2005
46. Santschi, PH, Lenhart, JJ, Honeyman, BD (1997) Heterogeneous processes affecting trace contaminant distribution in estuaries: the role of natural organic matter. Mar Chem 58: 99–125
47. Simon, M, Azam, F (1989) Protein content and protein synthesis rates of planktonic marine bacteria. Mar Ecol Prog Ser 51: 201–213
48. Skrabal, SA, Donat, JR, Burdige, DJ (1997) Fluxes of copper-complexing ligands from estuarine sediments. Limnol Oceanogr 42: 992–996
49. Skrabal, SA, Donat, JR, Burdige, DJ (1997) Fluxes of strong copper-complexing ligands from estuarine sediments: a source to the water column? Abstr Pap Am Chem Soc 213: 161-GEOC
50. Skrabal, SA, Donat, JR, Burdige, DJ (2000) Pore water distributions of dissolved copper and copper-complexing ligands in estuarine and coastal marine sediments. Geochim Cosmochim Acta 64: 1843–1857
51. Smith, DC, Azam, F (1992) A simple, economical method for measuring bacterial protein synthesis rates in seawater using ^{3}H-leucine. Mar Microb Food Webs 6: 107–114
52. Starr, TJ, Jones, ME (1957) The effect of copper on the growth of bacteria isolated from marine environments. Limnol Oceanogr 2: 33–36
53. Sunda, WG, Ferguson, RL (1983) Sensitivity of natural bacterial communities to additions of copper and to cupric ion activity: a bioasssay of copper complexation in seawater. In: Wong, CS, Boyle, E, Bruland, KW, Burton, JD, Goldberg, ED (Eds.) Trace Metals in Sea Water. Plenum Press, New York, pp 871–891
54. Sunda, WG, Gillespie, PA (1979) Response of a marine bacterium to cupric ion and its use to estimate cupric ion activity in seawater. J Mar Res 37: 761–777
55. Sunda, WG, Lewis, JAM (1978) Effect of complexation by natural organic-ligands on toxicity of copper to a unicellular alga, monochrysis-lutheri. Limnol Oceanogr 23: 870–876
56. Tang, DG, Warnken, KW, Santschi, PH (2001) Organic complexation of copper in surface waters of Galveston Bay. Limnol Oceanogr 46: 321–330
57. Tubbing, DMJ, Santhagens, LR, Admiraal, W, Vanbeelen, P (1993) Biological and chemical aspects of differences in sensitivity of natural-populations of aquatic bacterial communities exposed to copper. Environ Toxicol Water Quality 8: 191–205
58. US EPA (1997) Water quality standards; establishment of numeric criteria for priority toxic pollutants for the state of California; proposed rule. 40 CFR Part 131. Federal Register 62: 150–150
59. Vandenberg, cMG, Donat, JR (1992) Determination and data evaluation of copper complexation by organic-ligands in sea-water using cathodic stripping voltammetry at varying detection windows. Anal Chim Acta 257: 281–291
60. Vasconcelos, MTSD, Leal, MFC (2001) Adsorption and uptake of Cu by *Emiliania huxleyi* in natural seawater. Environ Sci Technol 35: 508–515
61. Vasconcelos, MTSD, Leal, MFC, van den Berg, CMG (2002) Influence of the nature of the exudates released by different marine algae on the growth, trace metal uptake, and exudation of Emiliania huxleyi in natural seawater. Mar Chem 77: 187–210
62. Wells, ML, Kozelka, PB, Bruland, KW (1998) The complexation of 'dissolved' Cu, Zn, Cd and Pb by soluble and colloidal organic matter in Narragansett Bay, RI. Mar Chem 62: 203–217
63. Wen, L-S, Santschi, PH, Gill, G, Paternostro, C (1999) Estuarine trace metal distributions in Galveston Bay: importance of colloidal forms in the speciation of the dissolved phase. Mar Chem 63: 185–212

64. Wen, L-S, Santschi, PH, Tang, DG (1997) Interactions between radioactively labeled colloids and natural particles: evidence for colloidal pumping. Geochim Cosmochim Acta 61: 2867–2878
65. Zirino, A, Belli, SL, Van der Weele, DA (1998) Copper concentration and Cu(II) activity in San Diego Bay. Electroanalysis 10: 423–427
66. Zirino, A, Clavell, C, Seligman, PF, Barber, RT (1983) Cu and pH in the Peruvian Upwelling System. Mar Chem 12: 25–35
67. Zirino, A, Van der Weele, DA, Belli, SL, DeMarco, R, Mackey, DJ (1998) Direct measurement of $Cu(II)_{aq}$ in seawater at pH 8 with the jalpaite ion-selective electrode. Mar Chem 61: 173–184