Copper sensitivity of *Gonyaulax tamarensis*[1]

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Abstract

The copper sensitivity of the dinoflagellate *Gonyaulax tamarensis* was examined in artificial seawater medium. Two short term responses of the organism to copper toxicity are rapid loss of motility and reduced photosynthetic carbon fixation. The chelators tris(hydroxymethylamino)methane (Tris) and ethylenedinitrilotetraacetic acid (EDTA) were used to demonstrate that copper toxicity is a unique function of cupric ion activity. Copper additions to medium containing EDTA equilibrated with the chelator relatively slowly, resulting in misleading short term data. This kinetic effect was not seen when the major copper chelator was Tris or when the copper was added in a chelated form with EDTA. Variations in manganese concentrations over two orders of magnitude did not alter the results.

Cells of *G. tamarensis* are 100% nonmotile at a calculated cupric ion activity of $10^{-9.7}$ M with 50% of the cells nonmotile at $10^{-10.4}$ M. Nonmotile cells do not divide or grow larger. *Gonyaulax tamarensis* growth is totally inhibited at cupric ion activities that only partially inhibit the growth of four other species that have been studied extensively. Furthermore, this toxicity occurs at the calculated copper activity of natural waters, assuming only inorganic copper complexation. Thus organic chelation may be necessary before *G. tamarensis* can successfully compete with other algal species in coastal waters.

Concentrations of copper as low as 1 ppb can be toxic to various phytoplankton species (e.g. Steemann Nielsen et al. 1969; Steemann Nielsen and Kamp-Nielsen 1970; Mandelli 1969; Erickson 1972; Martin and Olander 1971). Complexing agents are thus important for reducing copper toxicity in culture media (Manahan and Smith 1973; Fitzgerald and Faust 1963). Steemann Nielsen and Wium-Andersen (1970) suggested that the effect of organic chelators in stimulating the growth of algae in seawater, as observed by Johnston (1963, 1964) and Barber and Ryther (1969), was due to their ability to decrease the activity of the free cupric ion. This interpretation has been supported by the computations of Jackson and Morgan (1978) using the results of Davey et al. (1973), Barber (1973), and Huntsman (unpublished), and by the laboratory results of Sunda and Guillard (1976) who found that copper uptake and toxicity were uniquely determined by the cupric ion activity for two species of marine algae.

We have found the response of the dinoflagellate *Gonyaulax tamarensis* to copper toxicity is a rapid loss of motility—a reaction to stress that provides a unique opportunity to study the organism's short term sensitivity to copper, relatively free from the potential complications of cellular exudates and other changes in the trace metal composition of the culture medium with time. Other types of chemical and physical stresses also cause this organism to become nonmotile. Temperature has been cited as a major factor (Prakash 1967; Yentsch et al. 1975), although Prakash also noted that nonoptimal conditions of nutrients, salinity, and light can induce a limited loss of motility. Nonmotile cells do not divide or increase in size if the stress is maintained, yet in almost all cases they can be "revived" if the stress has not been too severe and normal culture conditions are restored. This has led many researchers to refer to stress-induced nonmotile cells as cysts, but recent comparisons between laboratory cultures and cells of *G. tamarensis* taken from marine sediments indicate that this terminology may be incorrect (Anderson and Wall 1978). We will not call the stationary cell observed in laboratory cultures after exposure to toxic concentrations of copper a cyst, but

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Table 1. Modified Aquil seawater medium.

	Analyt. concn	Computed -log (free ion)	Computed major species
Bromide	6.2×10^{-4}M	3.2	Br^- 100%
Borate	3.53×10^{-4}M	4.0	H_3BO_3 74.3%, $B(OH)_4^-$ 25.7%
Calcium	7.4×10^{-3}M	2.18	Ca^{2+} 88.7%, $CaSO_4$ 10.0%
Carbonate	1.5×10^{-3}M	4.3	HCO_3^- 64.5%, $MgHCO_3^+$ 23.4%, $CaHCO_3^+$ 3.7%, $NaHCO_3$ 5%
Chloride	4.1×10^{-1}M	0.4	Cl^- 100%
Cobalt	9×10^{-10}M	12.5	CoY^{2-} 99.9%
Copper	1×10^{-7}M	13.0	CuY^{2-} 99.7%
EDTA (=Y)	5×10^{-5}M	12.1	CaY^{2-} 89.6%, MgY^{2-} 4.7%, MnY^{2-} 3.8%
Iron	4.5×10^{-7}M	21.0	$Fe(OH)_3(s)$ 81.1%, FeY^- 18.8%
Magnesium	4×10^{-2}M	1.46	Mg^{2+} 87%, $MgSO_4$ 12.3%
Manganese	2.3×10^{-6}	6.85	Mn^{2+} 6.2%, $MnCl^+$ 8.9%, MnY^{2-} 84.9%
Molybdate	1.5×10^{-7}M	6.82	MoO_4^{2-} 100%
Nitrate	1×10^{-4}M	4.0	NO_3^- 100%
Phosphate	1×10^{-5}M	8.5	HPO_4^{2-} 57.3%, $CaHPO_4$ 2.4%, $MgHPO_4$ 40.2%
Potassium	7.2×10^{-3}M	2.2	K^+ 97%, KSO_4^- 3%
Silicate	1.3×10^{-5}M	10.04	SiO_2 100%
Sodium	3.5×10^{-1}M	0.46	Na^+ 98.8%, $NaSO_4^-$ 1.2%
Strontium	3.5×10^{-5}M	4.5	Sr^{2+} 100%
Sulfate	2.1×10^{-2}M	2.0	SO_4^{2-} 51.4%, $CaSO_4$ 3.7%, $MgSO_4$ 23.4%, $NaSO_4^-$ 20.7%
Zinc	4×10^{-7}M	10.0	ZnY^{2-} 100%

Ionic strength = 0.5M, pH = 8.4. Contains vitamins as in f/2 medium (Guillard and Ryther 1962). Calcium and magnesium phosphate, calcium carbonate and manganese oxide solids not allowed to precipitate in computations.

simply a nonmotile cell—a temporary resting stage in the life cycle of *G. tamarensis*.

The objectives of the work presented here are to use artificial seawater medium, two different chelators, and a wide range of total copper concentrations to demonstrate that the short term copper sensitivity of *G. tamarensis* can be quantified as a function of the cupric ion activity and to compare the results with available data on the toxicity of copper to other phytoplankton species.

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Methods

Cultures and medium—Our stock cultures of *G. tamarensis* (designated Isolate 429) were obtained from A. R. Loeblich at the Harvard University Biological Laboratories (Loeblich and Loeblich 1975 suggest the name *Gonyaulax excavata* for this species). The organism was originally isolated by C. Martin (Gloucester Marine Station, Massachusetts). Cells were grown at 16°C on a 14:10 h light-dark cycle with incident radiation of 60 μEin m^{-2} s^{-1} as measured by a quantum sensor. All medium was autoclaved for 15 min and equilibrated for 48 h before inoculation. We used sterile techniques throughout the experiments but did not test for bacterial contamination. To min-

Table 2. Computed trace metal speciation in two modifications of Aquil medium.

Species	-log (species concentration ( ) or activity { }), M								
	EDTA medium								
$(EDTA_T)$	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3
(Cu_T)	4.22	4.28	4.3	4.32	4.52	5.0	6.0	6.7	7.0
$\{Cu^*\}$	9.0	9.4	9.6	9.8	10.7	11.5	12.6	13.2	13.5
(Zn_T)	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4
$\{Zn^{2+}\}$	8.8	9.1	9.3	9.4	10.2	10.5	10.6	10.6	10.6
(Mn_T)	5.64	5.64	5.64	5.64	5.64	5.64	5.64	5.64	5.64
$\{Mn^{2+}\}$	6.7	6.7	6.8	6.8	7.1	7.4	7.4	7.5	7.5
(Fe_T)	6.35	6.35	6.35	6.35	6.35	6.35	6.35	6.35	6.35
$\{Fe^{3+}\}$	21.0	21.0	21.0	21.0	21.0	21.0	21.0	21.0	21.0
	Tris medium								
(Tris)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
(Cu_T)	4.5	4.7	5.3	5.5	5.7	6.0	7.0	8.0	9.0
$\{Cu^*\}$	9.1	9.4	9.7	10.0	10.2	10.5	11.7	12.8	13.6
(Zn_T)	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4
$\{Zn^{2+}\}$	9.2	9.3	9.6	9.8	10.0	10.2	10.6	10.7	10.7
(Mn_T)	7.64	7.64	7.64	7.64	7.64	7.64	7.64	7.64	7.64
$\{Mn^{2+}\}$	8.7	8.7	8.7	8.7	8.7	8.7	8.7	8.7	8.7
(Fe_T)	6.35	6.35	6.35	6.35	6.35	6.35	6.35	6.35	6.35
$\{Fe^{3+}\}$	21.0	21.0	21.0	21.0	21.0	21.0	21.0	21.0	21.0
$(EDTA_T)$	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3

Concentrations of ionic species corrected to activities using the Davies approximation for activity coefficients. Other chemical constituents and assumptions same as in Table 1.

imize adsorption effects, all glass flasks and beakers were coated with silicone (Davey et al. 1970). Glassware was routinely soaked for 12 h in 2 N HCl and rinsed with distilled, deionized, distilled water (DDD). All culture experiments were begun 3 h after the onset of the light cycle.

Experiments were carried out in batch culture in variations of Aquil (Table 1), an artificial seawater medium designed for studies of trace metals (Morel et al. 1975). Major salts were diluted with DDD water to a salinity of about 26‰. The equilibrium chemical speciation was computed with the program REDEQL2 (McDuff and Morel 1973), with several solids allowed to exceed saturation because of their slow precipitation kinetics. For each of the chelators used [EDTA, ethylenedinitrilotetraacetic acid, and Tris, tris(hydroxymethylamino)methane], adjustments were made to keep the activities of trace metals other than copper as constant as possible throughout different experiments (Table 2). Equilibrium con-

Table 3. Selected equilibrium constants used in REDEQL2 computations, ionic strength = 0.5 M.

Complex	log(K)
$H(EDTA)^{3-}$	10.00
$Ca(EDTA)^{2-}$	9.94
$Mg(EDTA)^{2-}$	7.94
$Mn(EDTA)^{2-}$	13.24
$Zn(EDTA)^{2-}$	15.74
$Cu(EDTA)^{2-}$	18.04
$Fe(EDTA)^{-}$	23.96
H(Tris)	8.17
$Cu(Tris)^{+}$	4.00
$Cu(Tris)_2$	7.60
$Cu(Tris)_3$	11.10
$Cu(B(OH)_4)+$	6.46
$CuCO_3(aq.)$	5.42
$Fe(OH)_3$ (solid)	37.81

Tris constants from Bai and Martell (1969). All others from Sillén and Martell (1964, 1971) or Ringbom (1963). Corrected for ionic strength using the Davies Approximation.

stants used for the major reactions that determine cupric ion activity in seawater are shown in Table 3. The Tris medium includes 5×10^{-7} M EDTA, added to chelate the metals ineffectively complexed by Tris.

To emphasize that the cupric ion activity has been calculated and not measured, the symbol pCu* will be used here instead of $-\log\{Cu^{2+}\}$. REDEQL2 calculations using the data of Sunda and Guillard (1976) agree with their results within 0.1 pCu* unit in all cases where there were no predicted copper precipitates.

Motility counts—The loss of motility induced by copper toxicity was quantified by pipetting 0.15 ml of culture onto a depression slide and counting the relative numbers of motile and nonmotile cells (under a low power objective with the diaphragms closed so that a maximum of 10 cells was visible at any one instant). The procedure was repeated at four nonoverlapping locations per slide until a minimum of 200 individual cells had been counted for each culture. A cell was considered motile if it showed any visible movement at all during the few seconds of observation. This does not distinguish cells that are actively swimming from those that are moving sporadically, as they often do before the complete cessation of movement. An indication of the reproducibility of this technique is given in Table 4 for two different cultures.

Table 4. Percentage of motile cells in two sample cultures.

No. of motility determinations†	Range	Mean motile cell percentage	SD
15	74.1 - 86.0	80.2	4.4
15	40.0 - 48.0	44.5	2.8

† Minimum of 200 cells counted for each determination.

Carbon fixation—Photosynthetic carbon fixation was measured with $[^{14}C]NaHCO_3$ (New England Nuclear) by the methods of Strickland and Parsons (1972), except that the 5% NaCl solution was cleaned of trace metal contamination by passage through a column of Chelex 100 (Biorad).

Separate addition of copper and chelator—For experiments designed to test the response of *G. tamarensis* to different cupric ion activities when unchelated copper was added to cultures grown with a chelator already present, 16 μCi of ^{14}C were added to 1,200 ml of exponentially growing cells at a density of about 3,000 cells ml^{-1}, and 50-ml portions were poured into triplicate flasks that had previously been spiked with varying copper solutions. In one case, dark bottle and formaldehyde-killed controls were also used. After 1 and 23 h, 10-ml samples were removed and filtered through 2.4-cm Whatman GF/C filters which were then rinsed with Aquil and placed in a container with fumes from concentrated HCl for 24 h to drive off labeled bicarbonate. ^{14}C activity was measured in Aquasol (New England Nuclear) on a Beckman LS-133 liquid scintillation system. The percentage of motile cells was determined 2 and 24 h after the initial copper exposure. The pH was monitored periodically.

Simultaneous addition of copper and

chelator—For experiments where copper was added in solution with the chelator, cells were grown in a medium (Table 1) containing a low initial concentration of chelator ($10^{-6.3}$ M EDTA). Concentrations of trace metals were correspondingly reduced to $10^{-7.65}$ M manganese, $10^{-8.4}$ M zinc, $10^{-6.35}$ M iron, and 10^{-9} M copper. ^{14}C was added to the cultures as described previously. In one experiment, concentrated solutions of copper and Tris were placed in empty flasks and culture added to 50 ml to obtain the desired pCu* values with 10^{-3} M Tris; in another, concentrated solutions of manganese, zinc, EDTA, and copper were used to give the desired pCu* levels and the final trace metal concentrations shown in Table 2 for $10^{-4.3}$ M EDTA medium. (A control experiment indicated that omission of the manganese and zinc additions did not alter the results significantly.) After adding the chelator and copper, we obtained motility and ^{14}C data as described previously.

Manganese variations—Two experiments were conducted to determine the effects of different concentrations of manganese on the copper sensitivity of *G. tamarensis:* one where the concentration of manganese was decreased two orders of magnitude to $10^{-7.64}$ M in medium chelated with EDTA and another where it was increased to $10^{-5.64}$ M in medium with Tris as the major chelator. The percentage of motile cells was determined in each experiment 2 and 24 h after the copper additions.

Pre-equilibration of the medium—To test the effect of a longer equilibration time between the addition of copper to chelated medium and the initial exposure of the cells to the toxicity, we added copper to media chelated with $10^{-4.3}$ M EDTA and 10^{-3} M Tris to give six flasks at each of two pCu* levels for each chelator. The pH was adjusted to 8.4 with NaOH and three control flasks from each category were inoculated with 3 ml of exponentially growing cells to a final volume of 50 ml. After 24 h, the remaining pre-equilibrated flasks were inoculated with cells, as was a second set of control

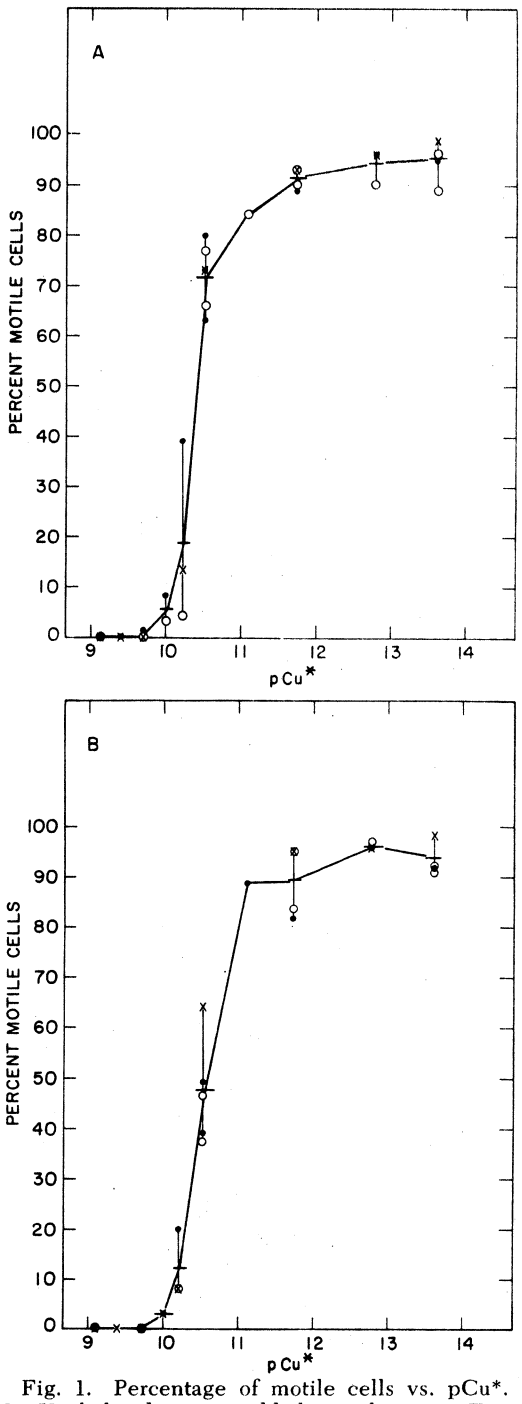

Fig. 1. Percentage of motile cells vs. pCu*. ●—Unchelated copper added to cultures in Tris medium; ○—copper plus Tris added to cultures in medium containing $10^{-6.3}$ M EDTA; ×—unchelated copper added to cultures in Tris medium containing increased manganese. Curves connect mean at each level of pCu*. A—2 h after copper additions; B—24 h after copper additions.

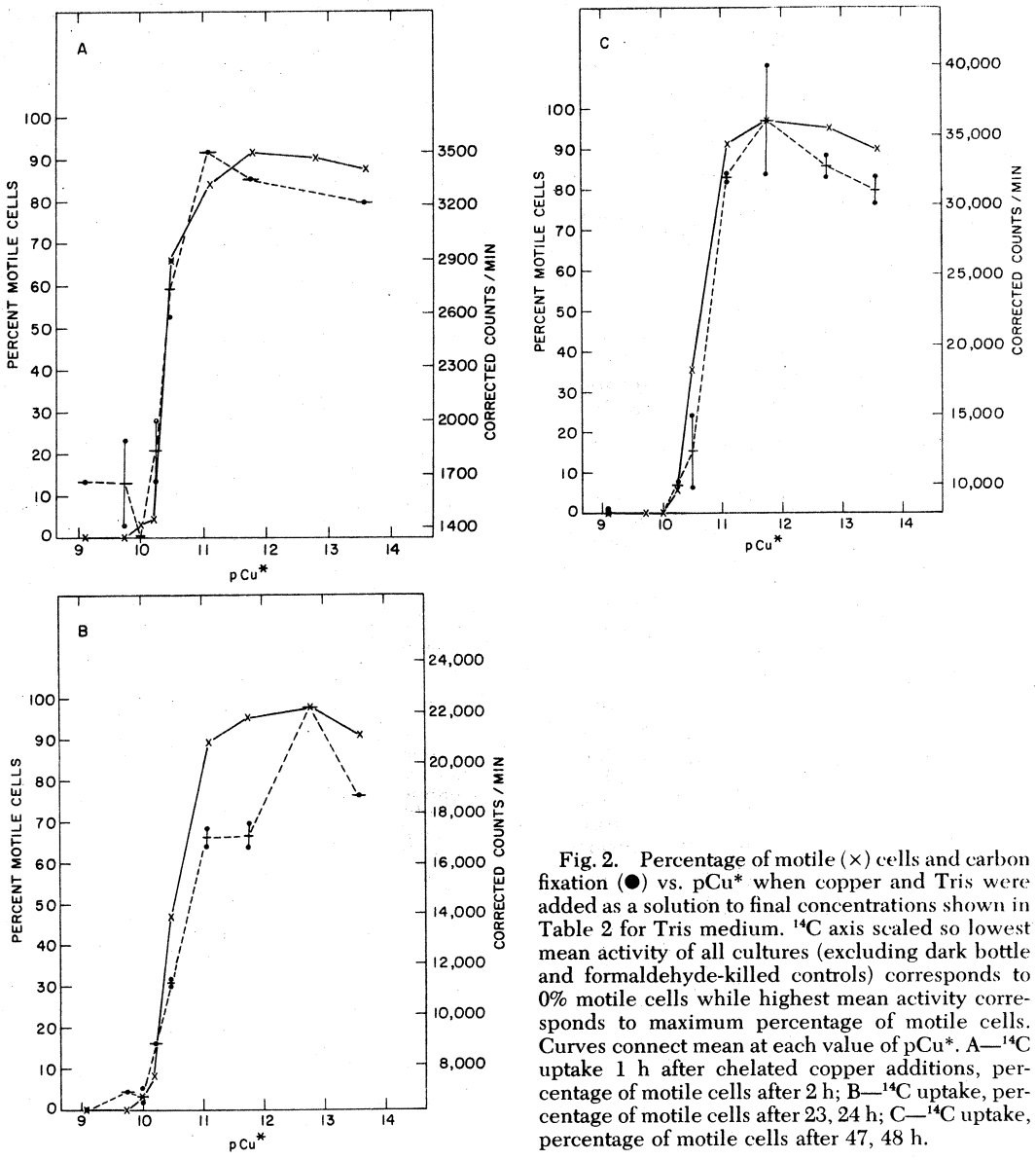

Fig. 2. Percentage of motile (×) cells and carbon fixation (●) vs. pCu* when copper and Tris were added as a solution to final concentrations shown in Table 2 for Tris medium. ^{14}C axis scaled so lowest mean activity of all cultures (excluding dark bottle and formaldehyde-killed controls) corresponds to 0% motile cells while highest mean activity corresponds to maximum percentage of motile cells. Curves connect mean at each value of pCu*. A—^{14}C uptake 1 h after chelated copper additions, percentage of motile cells after 2 h; B—^{14}C uptake, percentage of motile cells after 23, 24 h; C—^{14}C uptake, percentage of motile cells after 47, 48 h.

flasks that were first spiked with copper to indicate whether the inoculum sensitivity had changed. Motility percentages were determined 2 and 24 h after each inoculation.

Copper uptake—To determine whether significant quantities of copper could be lost to the *G. tamarensis* cells during the toxicity experiments, we grew 1-liter cultures in both EDTA and Tris medium to densities of 4,000 cells ml^{-1} and kept duplicate flasks containing medium but no cells under the same culture conditions. Copper was added in duplicate to flasks containing 100-ml volumes withdrawn from the cultures and the media. The flasks were swirled immediately and 10-ml subsamples filtered through separate 1.0-μm pore-size membrane filters. Cells were exposed to copper for <3 min

before this filtration. The filtrate was analyzed for total copper by atomic absorption spectrophotometry, with atomization by flame for the EDTA medium and the HGA-2100 graphite furnace for the Tris medium. Twenty-four hours after the copper was added, additional samples were filtered and analyzed. Motility was determined 2 and 24 h after the copper addition.

Results

Copper toxicity in Tris medium—The response of *G. tamarensis* to the cupric ion activity was the same whether the copper was added to medium already chelated with Tris or added in solution with Tris (Figs. 1 and 2). All cells became nonmotile below pCu* 9.7 while above pCu* 11.0 there was no evident inhibition of motility. An increase in the concentration of manganese by two orders of magnitude gave similar results (Fig. 1). A 48-h experiment with Tris demonstrated very little change in motility from the 2- or 24-h results (Fig. 2).

Photosynthetic fixation of ^{14}C decreased with increasing cupric ion activity in Tris medium (Fig. 2). The ^{14}C data show good agreement with the motility counts in demonstrating a rapid decrease in carbon uptake at cupric ion activities below 10^{-11} M. To emphasize this effect, the extreme values of the uptake data have been plotted so as to span the same range as the corresponding motility data. Microscopic examination of certain low pCu* flasks revealed that all cells were nonmotile, yet the amount of ^{14}C taken up by these cultures increased over 48 h. Formaldehyde-killed controls evidenced no significant ^{14}C uptake, while dark bottle controls had <5% of the activity found in the cultures with no motile cells.

Copper toxicity in EDTA medium—Dramatic differences in the toxicity response of *G. tamarensis* were observed in EDTA medium at the same pCu*, depending on the precise mode of copper addition to the cultures. If the copper was added as a copper sulfate solution (with no EDTA), all cells became nonmotile

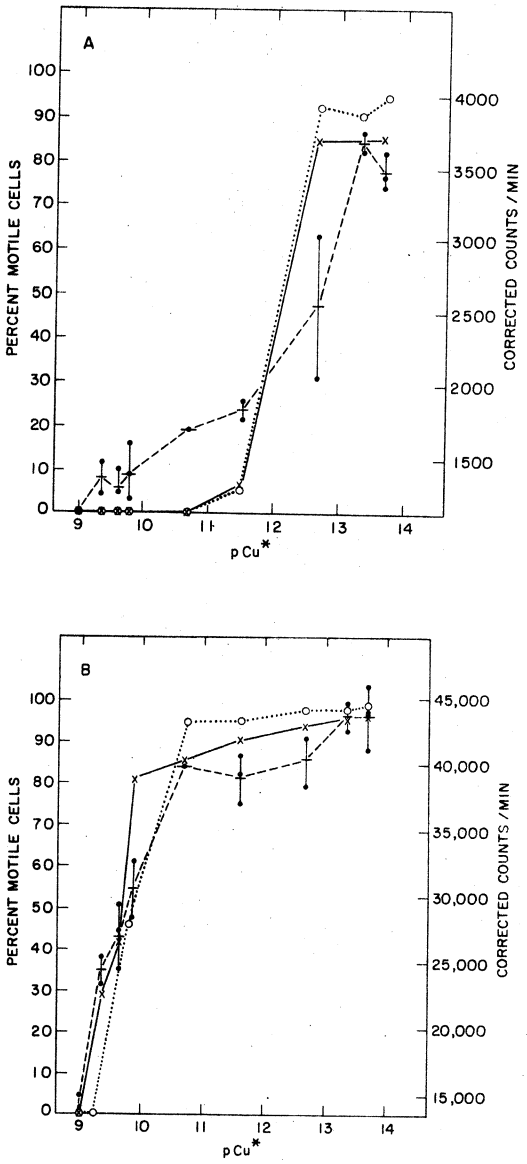

Fig. 3. Percentage of motile cells and carbon fixation vs. pCu* in EDTA medium. ^{14}C axis scaled so lowest mean activity of all cultures corresponds to 0% motile cells while highest mean activity corresponds to maximum percentage of motile cells obtained in same experiment. Curves connect mean at each value of pCu*. A—^{14}C uptake (●) 1 h after unchelated copper additions to cultures in EDTA medium; percentage of motile cells (×) 2 h after unchelated copper addition to cultures in EDTA medium; percentage of motile cells (○) 2 h after copper additions to cultures in EDTA medium with Mn_T reduced to $10^{-7.64}$ M. B—^{14}C uptake after 23 h, percentage of motile cells after 24 h.

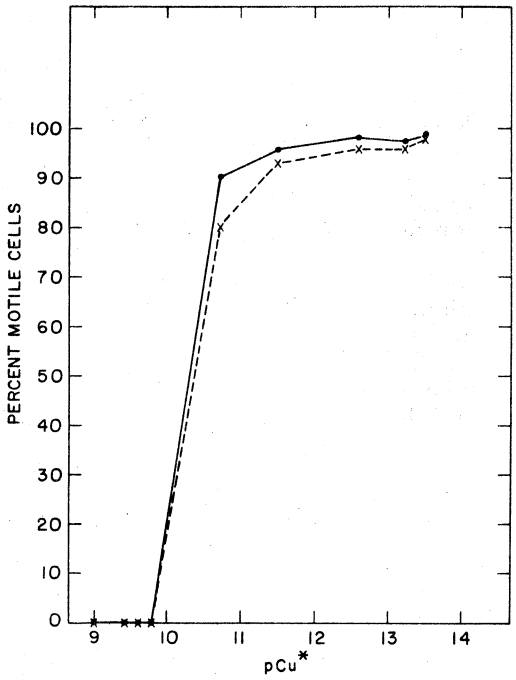

Fig. 4. Percentage of motile cells vs. pCu* 2 h (×) and 24 h (●) after copper, EDTA, zinc, and manganese were added as a solution to final concentrations shown in Table 2 for EDTA medium.

below pCu* 10.7 after 2 h (Fig. 3A). This corresponds to a much greater apparent sensitivity than that observed with Tris medium (Figs. 1A and 2A). After 24 h a significant revival was observed as the motility curve was shifted by over two orders of magnitude in the direction of decreased sensitivity (Fig. 3B). All cells were nonmotile only at pCu* 9.0, corresponding to a sizable apparent decrease in sensitivity in comparison with the Tris results. Decreasing the manganese concentration two orders of magnitude had no effect on the 2- or 24-h data (Fig. 3). If the copper was added as a solution containing an excess of EDTA (plus sufficient manganese and zinc to make the final metal activities the same as those in Table 2 for the EDTA medium), the cells became 100% nonmotile at pCu* 9.8 and below, with only a slight revival at low cupric ion activities after 24 h (Fig. 4). These results are similar to the Tris data in Fig. 1.

Pre-equilibration of the medium—To determine whether the shift between the 2- and 24-h data in the EDTA medium was due to a chemical change in the medium or to cell adaptation, we conducted an experiment in which EDTA and Tris media (including copper) were pre-equilibrated for 24 h before inoculation. Control cultures were inoculated into fresh medium minutes after copper addition (both at the time of initial medium preparation and at the time of inoculation into the pre-equilibrated medium).

In the Tris medium, there was no significant difference in motility between the controls and the pre-equilibrated medium (Table 5), and the motility remained essentially unchanged after 24 h. In the EDTA medium, the controls behaved as expected, with a large initial exaggeration of the copper sensitivity followed after 24 h by an apparent insensitivity in comparison to the Tris data. The pre-equilibrated EDTA medium dem-

Table 5. Effect of increased equilibration time of copper chelator on motility.

	EDTA medium				Tris medium			
	immediate cell inoculation†		cell inoculation after 24 h‡		immediate cell inoculation†		cell inoculation after 24 h ‡	
pCu*	10.7	11.5	10.7	11.5	10.1	11.0	10.1	11.0
Percent motile cells								
- 2 h	5	62	59	74	22	57	24	53
-24 h	91	94	59	74	20	61	18	68

† Motility results shown are averages from two sets of triplicate control flasks spiked with copper at zero and 24 h, followed immediately by cell inoculation

‡ Copper additions to medium followed 24 h later by cell inoculation. Values are averages from triplicate flasks.

Table 6. Concentrations of copper in culture filtrate, filtered medium, and unfiltered medium.

	Culture filtrate				Filtered medium				Unfiltered medium			
EDTA medium												
Copper added ($\times 10^{-5}$M)	6.0	5.0	3.0	1.0	6.0	5.0	3.0	1.0	6.0	5.0	3.0	1.0
pCu*	9.0	9.6	10.7	11.5	9.0	9.6	10.7	11.5	9.0	9.6	10.7	11.5
Measured total copper ($\times 10^{-5}$M)†												
- 3 min	4.6	3.9	2.8	1.1	6.0	5.1	3.0	1.0	6.1	5.1	3.1	1.0
-24 h	6.0	4.9	3.0	1.0	6.0	5.0	3.0	1.0	6.1	5.0	3.0	1.1
Tris medium												
Copper added ($\times 10^{-6}$M)	30	5.0	2.0	1.0	30	5.0	2.0	1.0	30	5.0	2.0	1.0
pCu*	9.1	9.7	10.2	10.5	9.1	9.7	10.2	10.5	9.1	9.7	10.2	10.5
Measured total copper ($\times 10^{-6}$M)†												
- 3 min	29	5.1	2.0	1.0	29	5.0	2.0	1.0	30	5.0	2.0	1.0
-24 h	29	5.0	2.0	1.0	29	5.1	2.1	1.0	30	5.0	2.2	1.2

Values are averages from replicate flasks.
† Filtering occurred within 3 min of, and 24 h after, copper additions to chelated medium.

onstrated remarkable constancy over time and reasonable agreement with the Tris data. (Allowances must be made for the differences in pCu* between the Tris and EDTA media in Table 5.) This demonstrates that the high toxicity at 2 h in EDTA medium spiked with copper sulfate is caused by a slow equilibration between copper and EDTA in the solution, leading to a dramatic overestimate of copper sensitivity in comparison with a preequilibrated solution. It also demonstrates that the subsequent underestimate of the sensitivity after 24 h (i.e. Fig. 3B compared to Fig. 4) is linked to the initial shock at the time copper sulfate was added.

Copper "uptake"—Within 3 min of adding copper sulfate solutions to cultures in the EDTA medium, up to 25% of the copper was removed from the medium along with the cells upon filtration (Table 6). This initial copper loss occurred at the lower pCu* values where the concentrations of copper and EDTA were nearly equal. Adding copper to EDTA medium without cells did not result in any measurable loss attributable to adsorption on the walls of the flasks. After 24 h the concentrations of copper measured in the culture filtrate were the same as in the filtered and unfiltered medium. Motility 2 and 24 h after the copper additions showed a shift in apparent sensitivity similar to that shown in Fig. 3. With Tris as the major chelator, there was no detectable loss of copper to the cells either in the first 3 min after the copper addition or 24 h later (Table 5).

This uptake of copper by the cells, followed by a release back into the medium is consistent with the results of Mandelli (1969) who found that the copper content of cells of various species of phytoplankton decreased with time from the initial spiking, reaching equilibrium within 15 to 30 min. Mandelli used a seawater medium chelated with a $10^{-5.33}$ M EDTA and total concentrations of copper ranging from $10^{-5.32}$ M to $10^{-6.1}$ M for the uptake experiments. A time-dependency of cellular metal content has also been re-

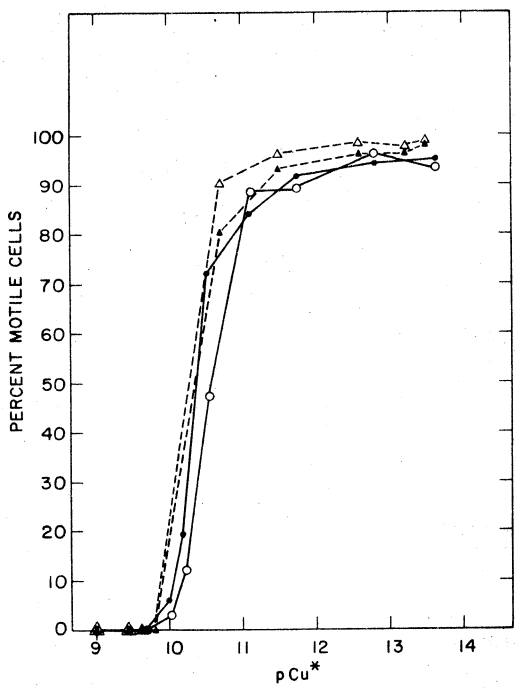

Fig. 5. Motility data vs. pCu* for two chelators at both 2 and 24 h. ●—Combined Tris data 2 h after Cu addition, chelated and unchelated; ○—combined Tris data 24 h after Cu addition, chelated and unchelated; △—combined EDTA data after 2 h, chelated copper added; ▲—combined EDTA data after 24 h, chelated copper added.

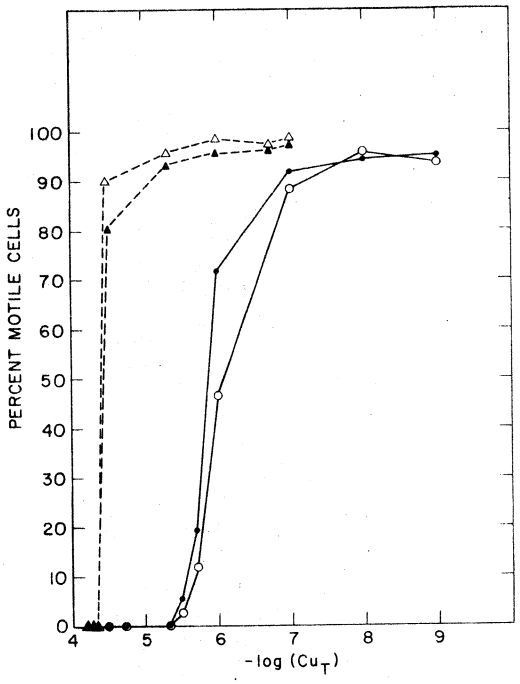

Fig. 6. Motility data vs. $-\log(Cu_T)$. Symbols as in Fig. 5.

ported for zinc and mercury (Davies 1973, 1976).

Discussion

Experiments with two different chelators and a wide range of copper concentrations demonstrate that copper toxicity in *G. tamarensis* is determined by the cupric ion activity and not the total concentration of copper. To arrive at this conclusion, we must first disregard certain experiments where kinetic phenomena introduce misleading results. Unchelated copper added to EDTA medium gave results that indicate an extreme 2-h sensitivity, followed by a shift to a significantly lower sensitivity after 24 h (Fig. 3). This shift in toxicity is not observed when copper is added in chelated form (Fig. 4) nor when copper added to EDTA medium is allowed to equilibrate 24 h before cells are added (Table 4). In contrast to the EDTA data, when Tris is the major chelator, toxicity results are relatively constant over time and do not depend on whether the copper is added to the cultures unchelated or chelated. It thus appears that the kinetics of EDTA-copper chelation in the seawater medium are slower than those of the Tris-copper system. Copper added to a culture containing a nearly equimolar amount of EDTA presumably does not reach equilibrium instantaneously, perhaps due to the time necessary for some of the chelator to dissociate from the abundant cations, calcium and magnesium. As can be seen in Table 1, immediately before the copper addition the EDTA is essentially totally bound to calcium, magnesium, and manganese. In the Tris medium, however, 63% of the Tris is in free ligand form while 37% is bound to hydrogen so that the Tris can chelate the copper without intermediate dissociations.

It seems reasonable to argue that when

copper is first added to a culture grown with EDTA, the cells may be exposed to a concentration of cupric ions significantly exceeding the available chelator at that time, resulting in potentially significant cellular adsorption or complexation. An initial uptake of the "excess" cupric ions (Table 6) accounts for the high short term sensitivity shown in Fig. 3A. The reduced sensitivity after 24 h (Fig. 3B) is harder to explain. The two obvious possibilities are medium conditioning and cell adaptation, but preliminary experiments to distinguish between these have been inconclusive. However, the presence of sizable quantities of discarded thecae and of various lysis products suggests that the medium after 24 h is quite different chemically from the well defined Aquil medium for which the pCu* values have been computed. With Tris medium, and with chelated copper additions to EDTA medium, the cells are apparently not exposed to the initial copper shock so that it is reasonable to expect the equilibrium cupric ion activities to be attained very rapidly and to change little with time.

If the experiments using unchelated copper additions to EDTA medium are disregarded because of the apparent equilibration problems shown in Fig. 3, Table 5, and Table 6, the remaining motility results are seen to be a direct function of pCu* for the two chelators (Fig. 5). When the same motility data are plotted against total concentrations of copper, a separate function is seen for each chelator (a result to be expected since only one concentration was used for each), but no general relationship is evident for the combined data (Fig. 6). These results are consistent with the laboratory results of Sunda and Guillard (1976) and the calculations of Jackson and Morgan (1978) who found that copper toxicity was uniquely determined by the cupric ion activity for various algal species.

The effect of copper-EDTA kinetics on the results of these short term toxicity experiments has far reaching implications for similar trace metal studies in the literature. This is especially true for experiments where copper or some other metal is added to healthy cultures and short term responses such as carbon fixation, growth rate, or nutrient uptake are measured. In these cases it seems that the results are indicative of each culture's affinity for the free metal ions at the moment of spiking as well as of its ability to release the metal back into the medium over time. An organism's sensitivity under these conditions is not necessarily the same as that determined under true equilibrium conditions.

The different types of experiments reported here make it possible to quantify the short term sensitivity of *G. tamarensis* to the activity of the cupric ion. ^{14}C fixation was found to be a relatively good measure of short term copper sensitivity, although there was a significant amount of photosynthesis in nonmotile cells that could not divide without some change in the level of copper toxicity. Total inhibition of motility was consistently demonstrated at pCu* 9.7 and below, with about 50% of the cells nonmotile at pCu* 10.4 after both 2 and 24 h. Above pCu* 11.0, there were no evident changes in the motility results with decreasing amounts of copper.

There are few published data that quantitatively evaluate the sensitivity of various species of algae to copper toxicity. Sunda and Guillard (1976) found total growth inhibitions below the pCu* levels of 8.3 and 8.4 for the estuarine diatom *Thalassiosira pseudonana* (clone 3H) and the green alga *Nannochloris atomus* (clone GSB Nanno), with 50% growth rate inhibition at levels of 8.6 and 9.3. They also calculated the cupric ion activity used in experiments by Davey et al. (1973) on an open ocean strain of *T. pseudonana* (clone 13-1) and reported total growth inhibition below pCu* 8.0 and 50% inhibition at 9.3. Complexation of copper by borate ions was not included in Sunda and Guillard's calculations but the pCu* results reported here for *G. tamarensis* do include the effects of this possible complex. Computations on their data with REDEQL2 indicate that borate complexation does not affect pCu*

results when the Tris concentration is 5×10^{-3} M or greater, while the maximum increase of 0.4 pCu* units occurs at a Tris concentration of 10^{-3} M. Fortunately, most of their experiments were conducted with Tris concentrations near 10^{-2} M, so valid comparison can be made with their results.

Exponentially growing cells of *Skeletonema costatum* (clone Skel) inoculated into Aquil medium showed no reduction in growth rate or cell yield at pCu* 8.5 or above (Morel et al. 1978).

Toxicity data for these species are difficult to compare with ours for *G. tamarensis* because the data are based on growth rates, which we have not determined. However, motility results do give some indication of growth response since our observations indicate that cells with copper-induced nonmotility will not divide or increase in size as long as the culture medium remains unchanged. Short term motility results cannot provide sufficient resolution to show conclusively that *G. tamarensis* needs a lower cupric ion activity than other algae to achieve optimal growth. However, it is clear that the toxicity effects do result in total growth inhibition at a pCu* of about 9.7, an activity at which *T. pseudonana* (clone 3H), *T. pseudonana* (clone 13-1), and *N. atomus* (clone GSB Nanno) would have growth rates inhibited by only 40, 20, and 20% (Sunda and Guillard 1976). Furthermore Sunda and Guillard first observed copper toxicity effects 2 days after inoculation, in marked contrast to the almost immediate loss of motility in *G. tamarensis*. At pCu* 9.7, *S. costatum* (clone Skel) divides at its maximum rate (Morel et al. 1978).

On the basis of a mean value of 1.4×10^{-8} M copper in coastal seawater samples (Chester and Stoner 1974) we can estimate the cupric ion activity in natural seawater, assuming no copper complexation other than with inorganic ligands. For pH 8.2, REDEQL2 calculations give a pCu* of 9.6, which agrees well with Sunda and Guillard's (1976) estimate of 9.7. Because this calculation does not include organic complexation, it represents maximum toxicity. At this pCu*, *G. tamarensis* would not be able to grow or divide while growth of other species would be inhibited partially or not at all. The role of organic complexation could thus be significant in the ecology of natural systems.

One important approach to recent research into the causes or triggering mechanisms of toxic dinoflagellate blooms has been to consider the stimulatory effects of organic compounds entering the ocean in overland runoff or coastal upwelling. In general, these studies have concentrated on the role of organic chelators in assisting in transport or availability of metal ions as nutrients (e.g. Prakash and Rashid 1968; Doig and Martin 1974; Kim and Martin 1974). The reduction in the toxicity of certain metal ions through chelation has been mentioned briefly in relation to red tide outbreaks, but no data are available at this time (Martin and Martin 1973). The data presented here suggest that the growth of *G. tamarensis* may be totally inhibited by copper toxicity in natural waters under conditions that leave other algae relatively unaffected and that organic chelation of this copper may be necessary before the cells can successfully multiply to bloom proportions.

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