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Toxicity of a Mixture of Ten Metals to Phytoplankton*

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ABSTRACT: We studied effects of a mixture of ten heavy metals (Cu, Zn, Ni, Cr, Pb, Cd, Hg, As, Sb and Se) on phytoplankton growth in a natural assemblage of diatoms and dinoflagellates from Saanich Inlet, B. C. (Canada) and in laboratory cultures of the diatom *Thalassiosira aestivalis*. Growth was not inhibited at metal concentrations expected to occur in a moderately polluted estuary. However, at 5- or 10-fold higher metal concentrations, phytoplankton growth was inhibited both in natural populations and in laboratory cultures. Inhibition of natural assemblages could be attributed to Cu and Hg; deletion of these elements resulted in growth equivalent to that of control cultures containing no metals. Similarly, inhibition of *T. aestivalis* cultures was due to Cu in the metal mixture. With natural assemblages certain diatoms such as *Chaetoceros* sp., *Rhizosolenia* sp. or *Nitzschia delicatissima* were no longer dominant in cultures containing a metal mixture 5 times that in controls.

INTRODUCTION

There are many studies on single heavy metal ions affecting cultures or natural assemblages of phytoplankton. However, few workers have examined toxic effects of interactions of heavy metal ions. Effects of physiologically important cations on the toxicity of a heavy metal ion have been determined in several studies. Steemann-Nielsen et al. (1969) found that the toxicity of Cu to *Chlorella pyrenoidosa* was inversely related to pH and K^+ concentration; Harding and Whitton (1976, 1977) investigated the effects of pH, Mg and Ca ions on the toxicity of Zn to *Stigeoclonium tenue*; and Braek, et al. (1976) reported that Zn toxicity to *Phaeodactylum tricornutum* was increased at low Mg ion concentrations. Studies of the effects of two or more heavy metals on phytoplankton populations are rare. However, in the discharge of industrial wastes, metals are usually added to the sea as mixtures. Krock and Mason (1971) found no consistent interaction between Cu and Hg added to San Francisco Bay phytoplankton. Jensen and co-workers have examined the effects of Cu and Zn on marine phytoplankton when the metals were added singly (Jensen et al., 1974, 1976) or in combination (Braek et al., 1976). The two metals acted synergistically on *Amphidinium carterae*, two clones of *Skeletonema costatum* and a clone of *Thalassiosira pseudonana*, but antagonistically on *P. tricornutum*.

Recently it has been demonstrated that the toxicity of copper is a function of the copper-free ion activity in the culture medium (Sunda and Guillard, 1976; Anderson and Morel, 1978; Jackson and Morgan, 1978; Morel et al., 1978; Sunda and Lewis, 1978). Most of these studies have been conducted in artificial seawater medium of known composition, pH and ionic strength. Free copper concentrations were controlled using either ethylenediaminetetraacetic acid (EDTA) or trishydroxymethylaminomethane (Tris) as metal-ion buffers; therefore, the response (growth, duration of log phase, motility) of phytoplankton could be studied relative to calculated copper activities. While this approach is useful in the laboratory, extrapolation to natural systems is currently impossible since the activity of copper in natural seawater cannot be determined directly; moreover, copper activity cannot be calculated, since the activities and conditional stability constants of organic ligands capable of interacting with copper are not known. The importance of the latter difficulty is emphasized by studies suggesting that copper binding organic ligands are present in sufficient quantities to effect the speciation of copper in seawater (Batley and Florence, 1976; Florence and Batley, 1976; Williams and Baldwin, 1976; Sugai and Healy, 1978). More specifically, the results of Sugai and Healy (1978) and Gillespie and Vaccaro (1978)

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indicate that dissolved organic matter in Saanich Inlet waters exerts a significant influence on the speciation of copper.

In view of the problems discussed above, we explored the toxicities of the mixture of metals and selected pairs of these metals in an empirical manner (a) to define the approximate concentration of the metal mixture inhibiting growth, and (b) to determine which metals in the mixture were primarily responsible for the inhibition. The results reported in this paper are an extension of our previous studies on the relative toxicities of metals added singly to phytoplankton from Saanich Inlet, B. C., Canada (Hollibaugh et al., 1980).

MATERIALS AND METHODS

The materials and methods employed in this study are essentially the same as those described in our earlier paper (Hollibaugh et al., 1980). The metals in the mixture were Cu, Zn, Ni, Cr, Pb, Cd, Hg, As, Se and Sb. The concentrations of these metals reflected the higher concentrations found in an estuary moderately influenced by anthropogenic activity such as Narragansett Bay, Rhode Island, USA (Goldberg et al., 1977). Where data can be compared, the concentrations of some of the metals in Narragansett Bay often exceed those observed in Saanich Inlet, B. C. (Canada) by an order of magnitude (Table 1). Where data were not

Table 1. Comparison of trace metal concentrations in Narragansett Bay, RI, USA and Saanich Inlet, B. C., Canada

Metal	Narragansett Bay (nM)	Saanich Inlet (nM)
Cu	9.4– 52[1,2]	1.7– 8.2[5,6]
Zn	28 –133[1]	18[6]
Ni	43 –272[2,3]	16[6]
Pb	4 – 18[2,3]	0.5[6]
As	22[4]	16[6]

[1] Piotrowicz (1977).
[2] Duce et al. (1972).
[3] E. W. Davey and P. E. Soper, unpublished.
[4] Ray and Johnson (1972).
[5] H. L. Windom, unpublished.
[6] G. T Wallace and R. G. Smith, unpublished.

available (Cd, Cr, Hg, Sb and Se), the concentrations selected for use in the metal mixture were approximately an order of magnitude above those reported to be representative of average seawater (Brewer, 1975). The concentrations of the multielement metal mixture (ME) given in Table 2 reflect these considerations.

The metals in the ME mix were made up as four solutions in the first experiment: As, Pb and Sb were added separately, and all others were added from a combined stock solution 30 μM in each metal. In all other cases the metals were added separately from 30 μM stock solutions. Phytoplankton populations were exposed to ME mixtures ranging from 0.1 to 10.0 times the concentrations given in Table 2 (0.1 × ME to 10.0 × ME). Culture vessels were either 4-l polyethylene Cubitainers (Cole Palmer Instrument Co., Chicago, Illinois, USA) or 0.25-l glass bottles. Prior work at Saanich Inlet indicated that such containers initially washed with hydrochloric acid and thorougly rinsed with deionized water did not leach enough metal to affect the present experiments, except possibly at the 0.1 and 0.5 x concentrations. Adsorption of trace elements from solution onto container surfaces is a well-documented phenomenon and may have influenced the results at the lower metal concentrations tested

Table 2. Composition of Multi-Element (ME) mix. Salinity was 29 to 30 ‰. N as $NaNO_3$, Si as $Na_2SiO_3 \cdot 9H_2O$, and P as NaH_2PO_4 were added from 10, 10, 1 mM stock solutions as required

Metal	Compound	Amount added to filtered Saanich Inlet seawater $\mu g l^{-1}$	nM
As V	Na_2HAsO_4	5.0	67
Cd	$CdCl_2$	0.75	6.7
Cr	$K_2Cr_2O_7$	3.0	58
Cu	$CuSO_4 \cdot 5H_2O$	3.0	47
Hg	$HgCl_2$	0.15	0.75
Ni	$NiCl_2$	5.0	85
Pb	$PbCl_2$	3.0	14
Sb	$K(SbO)C_4H_4O_6 \cdot {}^1/_2H_2O$	1.5	12.3
Se	SeO_2	1.5	19
Zn	$ZnCl_2$	5.0	76

(Robertson, 1968; Subramanian et al., 1978). Daily measurement of the concentration of each metal in each container was logistically impossible.

Water and phytoplankton were collected from CEE 77-2 (CEE: Controlled Experimental Ecosystem, Menzel and Case, 1977) on 8 June 1977. The CEE was initiated five weeks earlier as part of another experiment. Nutrients (N, Si and P) had been added on 7 June and at this time the water column had been thoroughly mixed by bubbling with air. An integrated 0–16 m sample was collected by pumping and was taken to the laboratory. The experiment was performed in 4-l plastic Cubitainers and five varying concentrations of the multielement metal mixture (ME) were tested.

The 4-l samples were incubated outdoors in a water bath kept at a constant temperature (11 °C). Natural sunlight was attenuated by covering the entire water

table with a translucent plastic tarp. The 0.25-l samples were incubated in a 12 °C cold room on a 17/7 L/D cycle at 120 $\mu Em^{-2}s^{-1}$ illumination from cool white fluorescent lights. When the water was collected, larger zooplankton were removed by filtration of the seawater through a 200 μm mesh net.

Growth was generally measured by *in vivo* chorophyll fluorescence at daily intervals. We recognize that fluorescence inhibition may be due to metal stress rather than an inhibition of growth, but fluorescence growth measurements gave the same results as growth measured by microscopic cell counts (compare Figs 1 and 2).

Cell counts were made using a Zeiss inverted microscope. Diversity indices, similarity indices, and cell carbon were calculated from cell counts as described in Thomas et al. (1977). *Thalassiosira aestivalis* (Gran and Angst, 1931) was isolated by D.L.R.S. from an enrichment culture of Saanich Inlet phytoplankton.

RESULTS

Natural Phytoplankton Populations

Phytoplankton species and nutrient concentrations of the initial water sample for the first experiment are given in Table 3. A pronounced decline in phytoplankton standing crop was observed during the first 2 d of this experiment. After recovery, a toxic effect was not observed in samples with less than a concentration five times (5 × ME) that of the original mixture (Fig. 1).

Table 3. Initial species composition of phytoplankton sample for Experiment I from CEE 77-2

Species	% by Carbon
Chaetoceros spp.	14
Corethron criophilum	1
Coscinodiscus spp.	2
Leptocylindrus danicus	4
Thalassiosira spp.	2
Licmophora abbreviata	1
Amphidinium spp.	11
Exuviella spp.	6
Gymnodinium spp.	6
Gyrodinium spp.	34
Peridinium spp.	3
Unidentified microflagellates	8
Other species	6
Total	100
Nutrients (μg-at l^{-1})	
NO_3-N	5.7
NH_3-N	1.2
SiO_4-Si	17
PO_4-P	0.9

Some stimulation of growth took place at the 0.5 × ME, and 1.0 × ME concentrations, and there was no growth at 10.0 × ME. At the end of the experiment all samples were examined for phytoplankton species composition.

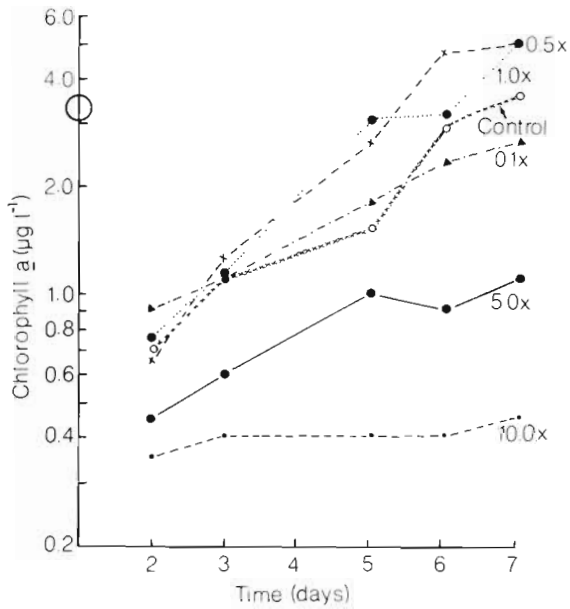

Fig. 1 Growth of natural phytoplankton assemblages from CEE 77-2 in varying concentrations of multielement (ME) mixture. Circle on ordinate: Day 0 values

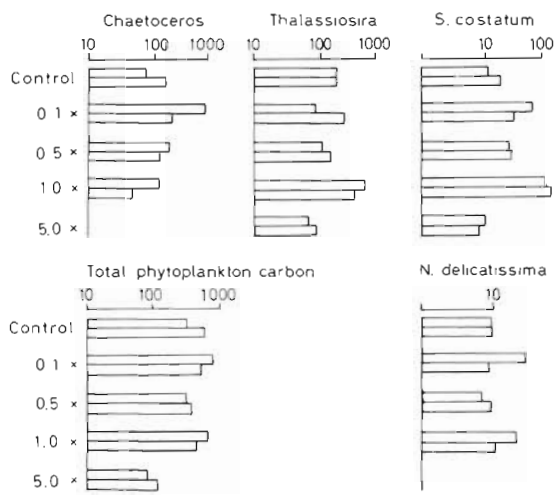

Fig. 2. Species composition expressed as carbon (μg Cl^{-1}), of phytoplankton assemblages from CEE 77-2 after growth for 8 d in the presence of varying concentrations of the ME mix. *Chaetoceros* spp. and *Nitzschia delicatissima* were not present at 5.0 × ME. Paired bars indicate abundances in duplicated experiments

Although the initial water sample in this first experiment was dominated by a dinoflagellate population (Table 3), four diatom taxa dominated on the eighth day (Fig. 2). The stimulation of growth at the 1.0 × ME

concentration resulted in increased standing crops of *Thalassiosira* sp. and *Skeletonema costatum*. The increase of growth in the 0.5 × ME treatment was not evident from the phytoplankton carbon or cell number data. *Nitzschia delicatissima* and *Chaetoceros* sp. were not present in the 5.0 × ME cultures; other taxa, not included in Figure 2, were also inhibited at this concentration and *Thalassiosira* sp. and *S. costatum* together represented nearly 100 % of the standing stock on Day 8.

The next experiment tested the hypothesis that the effects of the ME mix were due primarily to the Cu and Hg that it contained, rather than to the total metal load. A comparison of the relative toxicities of the metals in the ME mix to their relative concentrations in the ME mix suggested that Cu and Hg would reach a toxic concentration before the rest of the metals (Hollibaugh et al., 1980). Cu should become toxic at about 5 × ME (Cu = 235 nM) if all of the metals in the ME mix were assumed to act independently. In addition, distinctive morphological aberrations (clumps of daughter cells failing to separate after division and elongated, out-of-column frustules) were observed previously in *Thalassiosira aestivalis* grown at 5 × ME (Thomas et al., 1980). The same type of aberration had been observed consistently when *T. aestivalis* was grown in medium to which 100 to 500 nM of Cu had been added. All of this evidence suggested that the toxicity of the ME mix might be directly related to its Cu and Hg content.

Accordingly, the toxicities of Cu, the ME mix, the ME mix with Cu deleted (ME minus Cu), ME minus Hg, and ME minus both Cu and Hg were compared in a second experiment. The phytoplankton assemblage was collected at Station 77-1 in Saanich Inlet (48° 39.6'N, 123° 29.6'W) on 14 September, 1977. Nutrients were added to the phytoplankton assemblage to give initial nutrient concentrations of 10.1, 20.5, and 1.7 μg-at l^{-1} for N, Si, and P., respectively. Growth curves (Fig. 3) suggested that toxicity was determined primarily by the Cu and Hg in the 5 × ME mix. Growth of the phytoplankton was slightly improved when Hg was deleted from the mixture, but a much greater improvement was seen when Cu was deleted. The best growth was obtained when both Cu and Hg were deleted; 5.0 × and 10.0 × ME minus both Cu and Hg improved phytoplankton yield at the end of the log growth phase by 4.4 and 4.3 times over the control growth yield and greatly decreased the lag time. At these concentrations of the complete ME mix with Cu and Hg, growth yield was only 0.2 to 0.4 of the control, and the populations did not recover from their initial decline. The 5.0 × and 10.0 × ME minus Cu showed improved growth yields and decreased lag times compared to the ME mix, while 10.0 × ME minus Hg did

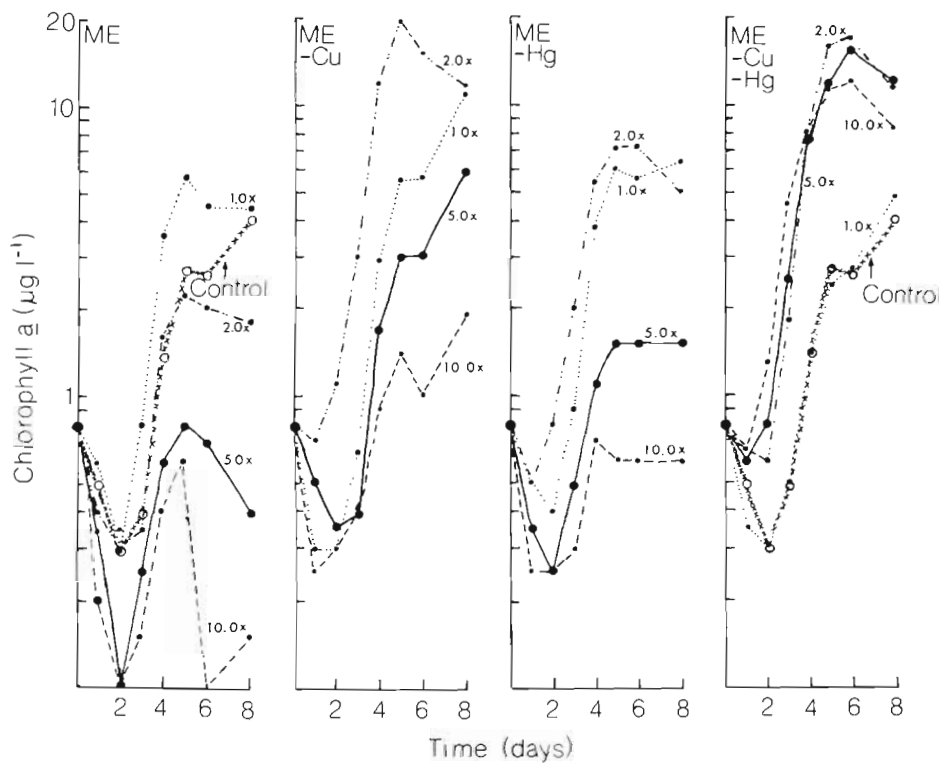

Fig. 3. Growth of natural phytoplankton assemblages exposed to varying concentrations of ME mix, ME mix with Cu deleted (ME minus Cu), ME minus Hg, and ME minus both Hg and Cu. Growth of controls shown in right- and left-hand panels only

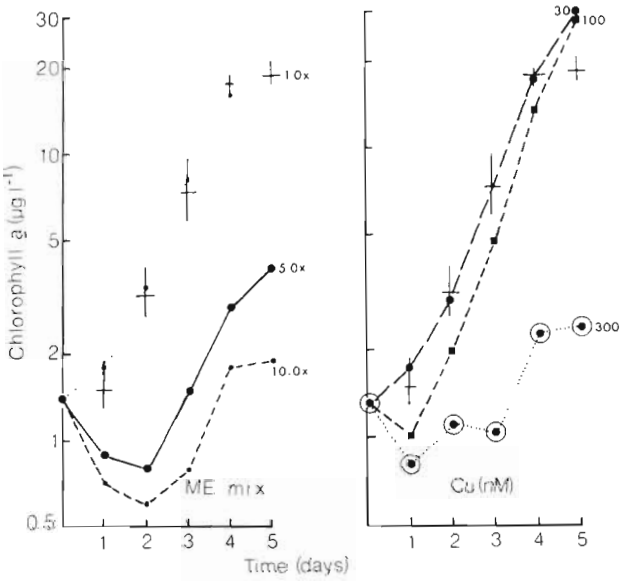

Fig. 4. Growth of a natural phytoplankton assemblage contained in glass bottles and exposed to varying concentrations of ME mix and Cu. Cu concentration expressed in nM. Crosses: range and mean of 3 controls

not recover from the initial decline and growth yields at both 5.0 × and 10.0 × ME minus Hg were only slightly greater than those of the complete ME mix at these concentrations.

The cell carbon of the initial assemblage in this second experiment was dominated by the centric diatoms *Chaetoceros* sp., *Melosira* sp., and *Skeletonema* sp. with some small percentages of the dinoflagellates *Gymnodinium* sp. and *Peridinium* sp. At the end of the experiment some of the treatments were examined microscopically to determine if any species shifts had occurred. The control culture (no metal additions) contained the same centric diatoms as those found initially and also *Rhizosolenia* sp. In the 5.0 × ME culture *Chaetoceros* sp. and *Rhizosolenia* sp. were no longer dominant and most of the assemblage was made up of the centric diatoms *Skeletonema* sp. and *Thalassiosira* sp. When Cu and/or Hg were deleted from the metal mix, the assemblage was similar to that of the control; *Chaetoceros* sp. and *Rhizosolenia* sp. were again dominant along with *Skeletonema* sp. and *Thalassiosira* sp. Species diversity indices (Shannon and Weaver, 1963) in the

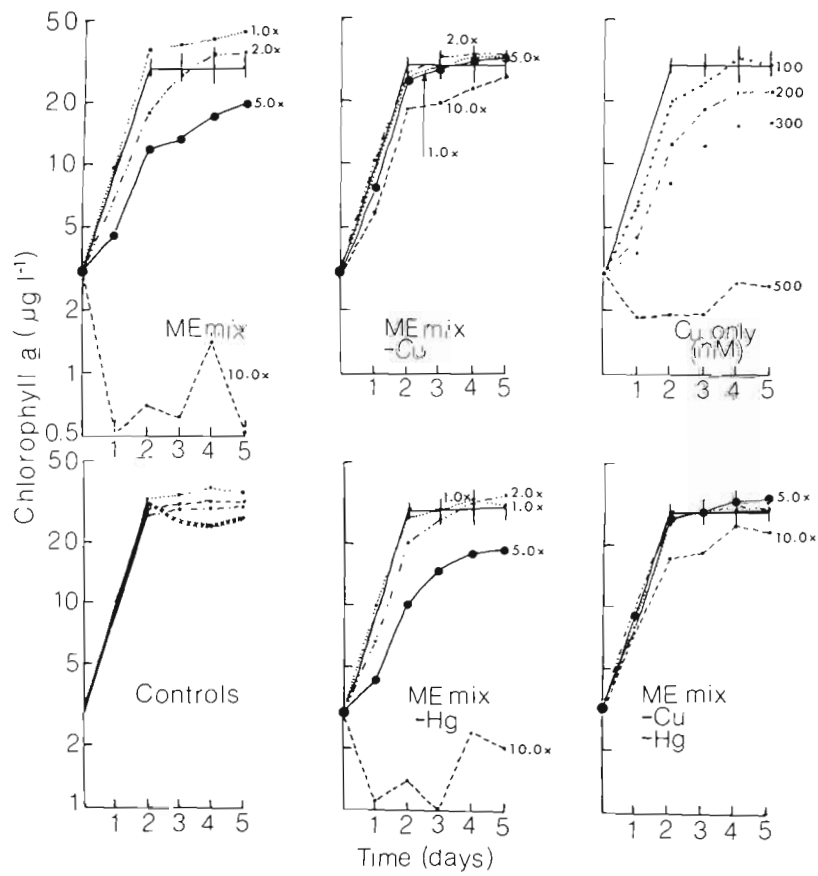

Fig. 5. *Thalassiosira aestivalis*. Growth in varying concentrations of ME mix (ME minus Cu, ME minus Hg, ME minus both Hg and Cu) and Cu. Cu concentration in nM

treated cultures did not differ greatly from the control. In all the treated cultures, except 5.0 × ME, the similarity index (Whittaker, 1960) was > 80 % of that of the control. In the 5.0 × ME culture the similarity was 72 % of the control. This reflects the decrease in dominance of *Chaetoceros* sp. and *Rhizosolenia* sp. Total phytoplankton carbon concentrations in the 5.0 × ME and 5.0 × ME minus Cu treatments (419 and 243 μgC l^{-1} respectively) were lower than those of the control (991 μgC l^{-1}) at the end of the experiment. In all other treatments final phytoplankton carbon concentrations were greater than that in the control. This compares favorably with the results for *in vivo* fluorescence given in Figure 3.

As part of this experiment, we incubated the natural assemblage at 1.0 ×, 5.0 ×, and 10.0 × ME in 0.25-l glass bottles at 12 °C to see if changes in container material and size (surface to volume ratio) might affect the results. In glass bottles we also found inhibition at 5.0 × and 10.0 × ME as compared with the control and 1.0 ×. Thus the container material and surface to volume ratio did not seem to have an effect on metal mixture toxicity. These results are shown in Figure 4.

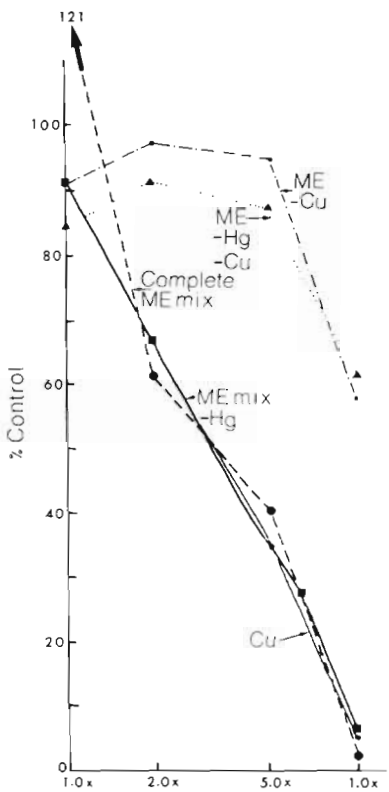

Fig. 6. *Thalassiosira aestivalis*. Growth in varying concentrations of ME mix (ME minus Cu, ME minus Hg, ME minus both Hg and Cu) and Cu, expressed as percent of growth attained by controls at end of logarithmic growth phase (Day 2). Cu plotted as multiples of its concentration in complete ME mix

Thalassiosira aestivalis

Although the two experiments with natural assemblages gave comparable results in that centric diatoms were the dominant algae at the end of the experiments, natural assemblages can differ in their species composition. Therefore we isolated a representative diatom, *Thalassiosira aestivalis*, and repeated the experiments. The cultures were incubated in glass bottles in the cold room. Figure 5 shows growth curves of *T. aestivalis* with metal mixtures (ME), ME minus Cu, ME minus Hg, and ME minus both Cu and Hg. A set of treatments with Cu alone at several concentrations was also included.

Most of the toxicity of the ME mixture was due to the Cu it contained. The removal of Hg still resulted in some toxicity at 5.0 × ME and 10.0 × ME. Growth of the samples is expressed as a percentage of the growth of the controls at the end of the exponential growth phase (Fig. 6). This demonstrates that the inhibition of the ME mixture was definitely due to Cu although 10.0 × ME minus Cu or 10.0 × ME minus both Cu and Hg were somewhat toxic. These results are comparable to those found with ME mixtures and natural assemblages (Fig. 3).

DISCUSSION

Discharge of metals into the sea exerts complex effects on phytoplankton. Metals are usually discharged as mixtures, and each metal has a different relative toxicity. Hollibaugh et al. (1980) have shown that Hg and Cu are the most toxic of ten metals tested singly. Also, metals can affect different species of phytoplankton according to the relative susceptibility of each species. Finally, metals may have synergistic effects. Thus, the effect due to mixtures of metals may be greater than the effect of each metal alone. In the experiments described here we have investigated some of these complexities.

Mixtures of metals at concentrations that approximate conditions in a moderately polluted estuary (Narragansett Bay) were generally without demonstrable effect on natural assemblages or *Thalassiosira aestivalis*. However, when concentrations increased 5- or 10-fold, the mixture inhibited growth. In natural assemblages, the toxicity of these mixtures was due to Cu and Hg in the mixture. With *T. aestivalis*, toxicity was due to Cu. That is, when these metals were deleted from the mixture, the algae grew as well as those in control cultures to which no metals were added.

While overall growth of natural assemblages was reduced by 5.0 × ME, the most striking effect was the

elimination of the centric diatoms *Chaetoceros* sp. and *Rhizosolenia* sp., and the pennate diatom *Nitzschia delicatissima* as dominant organisms. Deletion of Cu and/or Hg from 5.0 × ME restored the dominance of *Chaetoceros* sp., and *Rhizosolenia* sp. *Skeletonema* sp. and *Thalassiosira* sp. retained dominance in all treatments. General species diversity did not change much upon treatment with 5.0 × ME as compared to the control, but species similarity to the control was reduced.

We made some preliminary experiments on the effects of Cu-Zn pairs, Cu-Ni pairs, and Cu-Cd pairs of metals on *Thalassiosira aestivalis* to test for synergistic or antagonistic effects (unpublished). In one experiment there was no additive toxicity between Cu and Zn, but in another there was an additive effect with these two metals. Cu-Ni pairs and Cu-Cd pairs showed an additive effect in one experiment. However, these results are not considered conclusive. The algae were only grown for 3 d in each experiment and such results deserve more extensive investigation, which was not possible during our stay at the Saanich Inlet laboratory. Braek et al. (1976) demonstrated synergistic effects between Cu and Zn with three species of phytoplankton in cultures, but found antagonism between the two metals with another species. Synergism could be due to preferential binding of a less toxic over a more toxic ion by protein constituents within the cells, thus potentially raising the intracellular activity of the more toxic metal. Synergism could also arise extracellularly when metals less toxic than Cu are able to displace Cu from inorganic or organic ligands occurring in seawater and thus increase the free ion activity of copper. It has recently been demonstrated that Cu^{2+} ion activities rather than concentrations, determine Cu toxicities (Sunda and Guillard, 1976; Anderson and Morel, 1978; Morel et al., 1978).

Lack of definition of the chemical nature and total concentration of the metal species in these experiments necessarily impose some degree of uncertainty on the interpretation of the results. The activities of the free metal ions reflect complex equilibria with the surface of the culture vessel and with organic and inorganic ligands present in the seawater medium. The data necessary to compute the speciation and the techniques necessary to verify the calculated free ion activities of the metals in these experiments are not yet available. However, we feel the empirical approach applied here is useful in identifying the relative toxicity of various trace metals in natural waters where the identity and concentration of potentially important organic ligands are still unknown. Such an approach can also show which metals are most likely to produce synergistic or antagonistic effects and can focus attention on the most critical elements in the matrix.

Despite these uncertainties, we conclude that concentrations less than 5 times those in the original metal mix (designed to simulate trace metal concentrations in a moderately polluted estuary) can significantly inhibit the growth of natural phytoplankton assemblages. Most of the inhibition is attributed to the presence of copper and mercury in the metal mixture.

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