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Measurement of the metal complexing ability of exudates of marine macroalgae

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Abstract

The metal complexing capacity of organic material released by three macroalgae was measured by the MnO_2 exchange method. After 30 days of growth the concentration of metal complexing ligands released into the media, and their conditional stability constants (log K') for complexes with copper, were: *Ectocarpus siliculosus*, 1.01×10^{-6} M, 10.15; *Audouinella purpurea*, 0.27×10^{-6} M, 9.95; *Antithamnion spirographidis*, 0.75×10^{-6} M, 9.77. Bioassay experiments with two planktonic microalgae in the presence of the released compounds were inconclusive and failed to confirm a free copper ion related response. The ecological effects of organic material released by macroalgae are probably not restricted to its chelating ability, but also arise from the toxicity of the material itself.

Following the initial studies by Fogg and Westlake (1955) many workers have investigated the release of metal complexing organic material by algae (*see* e.g. Johnston 1964; Steemann Nielsen and Wium-Anderson 1971; Lange 1974). Fresh impetus has been given recently to this area of research by the development of more sensitive techniques. Swallow et al. (1978), who studied eight algal species using a copper(II) ion-selective electrode with a detection limit of 1×10^{-6} M of ligands, found only one which produced copper complexing material. McKnight and Morel (1979) reported that nine out of 14 species of eucaryotic algae which they investigated produced more than 1×10^{-6} M of ligands, and van den Berg et al. (1979) found that three freshwater algae produced complexing ligands at $<10^{-6}$ M.

Both brown and red algae are common inhabitants of shallow water and the intertidal community. Their ability to concentrate and store heavy metals over long periods makes them admirably suitable for monitoring marine pollution (Bryan 1969; Haug et al. 1974; Myklestad et al. 1978). Many studies have been devoted to the characterization of the organic material excreted by these algae into the surrounding seawater, its contribution to the formation of humic substances, and its antibiotic and algicidal properties (e.g. Craigie and McLachlan 1964; Sieburth and Jensen 1969; Ragan and Craigie 1976; McEnroe et al. 1977; Sims et al. 1977). Several investigators have demonstrated that brown algae excrete polyphenols which are able to form complexes with a wide range of divalent cations (Khailov 1964; Johnston 1964; Barber 1973; Ragan et al. 1979), but no attempt has been made to measure their complexing ability quantitatively. Ragan et al. (1980) found some detoxification of zinc by brown algal polyphenols, and Fletcher (1975) explained the inhibition of two red algal species by similar material as antibiotic effects.

Our aim was to investigate the exudates of three marine macroalgae representative of the major taxonomic groups in coastal waters, *Ectocarpus siliculosus* (Phaeophyceae), *Antithamnion spirographidis*, and *Audouinella purpurea* (Rhodophyceae), and to determine the stability constants of their copper complexes by using the MnO_2 method (van den Berg and Kramer 1979*a*). Algal cultures used for this study were supplied by G. Russell.

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Experimental section

Reagents and materials—All the working storage containers used were cleaned according to the recommendations of Erickson (1978). Redistilled water, free of organic matter, was prepared daily and all chemicals used were of analytical grade.

A 4.1×10^{-3} M suspension of manganese dioxide was prepared as described by van den Berg and Kramer (1979*a*) and aged for several months. A 3×10^{-2} M stock solution of copper was made by dissolving copper sulfate in redistilled water and used to prepare working solutions.

Physical measurements—pH was measured with a Radiometer PM 52 pH meter and a glass electrode/solution/KCl (saturated solution)/calomel electrode system. The electrode was standardized at pH 2 (25°C) with a solution having an ionic strength similar to that of seawater (NaCl 0.69 M, HCl 0.01 M) and the pH of the experimental solutions was read in millivolts.

Concentrations of copper were measured by differential pulse anodic stripping voltammetry (DPASV) using a Princeton Applied Research model 174 polarographic analyzer equipped with a Metrohm model E410 hanging mercury drop electrode. The instrument was used in its differential pulse mode (scan rate 5 $mV \cdot s^{-1}$, modulation amplitude 25 mV, and pulse rate 0.5 s). Analyses were performed at 25°C in a cell fitted with a thermostatic jacket using 15-ml aliquots of filtered samples acidified with two drops of 6 M HNO_3. Before analysis by DPASV the solutions were purged for 5 min with "oxygen-free" N_2. Copper was plated at −0.5 V vs. a saturated calomel electrode for 3 min (150 s stirring, 30 s quiescent). Concentrations of copper were evaluated by reference to a calibration curve prepared by examination of filtered UV-irradiated seawater to which known amounts of copper had been added.

Primary production of the phytoplankton was determined by the ^{14}C method using Nuclear Enterprises equipment (low background beta counter type 6016 fitted with an anticoincidence unit, type 2079, in conjunction with a scaler-timer, ST 6).

Cultures of macrophytes and microplankton—Unialgal cultures of *Ectocarpus siliculosus* (Dillw.) Lyngb., *Antithamnion spirographidis* Schiffner, and *Audouinella purpurea* (Lightf.) Woelkerling were used in the study of the complexing ability of material released by macroalgae. *Chlorella salina* Kufferath, Plymouth culture collection 309, and *Phaeodactylum tricornutum* Bohlin, Plymouth culture collection 100, were used in bioassay experiments to test the toxicity of copper(II) ions in the presence of chelating material released by the macrophyta. The copper tolerance of the two microplankton species had not been examined before, and they were not selected on the basis of their insensitivity to copper which became apparent later.

The macroalgae were grown for 30 days at 16°C under constant irradiation in autoclaved, 2-liter Erlenmeyer flasks containing 1 liter of filtered, UV-irradiated seawater, which had been enriched to 5×10^{-4} M with nitrate and 0.75×10^{-4} M with phosphate. No trace elements were added because sufficient concentrations were present in the seawater. Irradiation was provided by two horizontal, 40-W Thorn Northlight fluorescent tubes, suspended 30 cm above the flasks containing the macroalgae.

To avoid loss of ligands by adsorption onto the container walls we immediately examined the filtrates by the MnO_2 method, and then deep-froze them in the dark until required.

Calibration of the MnO_2 method—As the procedure has been described in detail for freshwater (van den Berg and Kramer 1979*a*) and will be for seawater (van den Berg in prep.) only a brief description is given below.

The experiments were performed (in triplicate) at 25°C in a 500-ml flask contained in a thermostatic bath. For calibration 270 ml of UV-irradiated membrane-filtered seawater was brought to pH 4.5 with 5 M HCl and bubbled (vigorously) with N_2 for 30 min to remove the carbon-

Table 1. Species and stability constants used to calculate free copper concentrations in equilibrium with complexing ligands.

Species	log $K(\beta_2)$	Source
H_2O	13.93	Smith and Martell 1976
CuOH	5.81	
$Cu(OH)_2$	12.7	
CuCl	−0.09	
$CuCO_3$	5.75	
$Cu(CO_3)_2$	8.92	
Cu(gly)	8.14	Martell and Smith 1974
$Cu(gly)_2$	14.96	

Charges have been omitted for simplicity
μ = 0.7 (seawater)
Cl = 0.549

ate. MnO_2 suspension (2 ml) and 0.2 ml of a 4×10^{-2} M glycine solution were added and the pH was adjusted to 8.15 (±0.005). The dispersion was stirred slowly with a PTFE-coated magnetic stirring bar and blanketed with N_2. Increments (50 μl) of a suitable working solution of copper were added to give a final concentration of copper in the range 0.3–5.5×10^{-6} M (after correction for the initial copper concentration of the medium). During the additions the pH of the solution was kept constant by dropwise addition of dilute sodium hydroxide or hydrochloric acid as appropriate. After each addition of copper, 20 ml of the dispersion was removed and placed in a 30-ml polyethylene bottle which had previously been purged with nitrogen. The solutions were bubbled with nitrogen for 5 min, after which the bottles were tightly closed and transferred to a shaking water bath at 25°C. Next day the solutions were filtered through a 0.45-μm membrane filter which had been precleaned with 0.1 M HNO_3, and the first 3–4 ml of the filtrate were rejected. The remaining filtrate (≈17 ml) was transferred to a polyethylene bottle and acidified with one drop of concentrated nitric acid before storage. Total copper was determined in this solution using DPASV.

Measurements of the complexing capacity of the exudates were carried out (in duplicate) at pH 8.15 in the same fashion with 270 ml of the exudate in place of the seawater but omitting the glycine solution. Only 1.5 ml of the MnO_2 suspension was introduced into the medium.

Calculation of the ligand concentration and the stability constants

The adsorption of copper on the exchanger at constant pH, temperature, and ionic strength is described by the Langmuir equation

$$\Gamma_{ads} = \Gamma_{max} \cdot [Cu^{2+}] / \left(\frac{1}{B} + [Cu^{2+}] \right) \quad (1)$$

in which Γ_{ads} is the number of moles of Cu adsorbed per mole of MnO_2, Γ_{max} is the maximum value of Γ_{ads}, and B is the binding constant. Mass balance equations for copper and glycine species present in seawater at pH 8.15 are

$$[Cu_{diss}] = [Cu^{2+}] + [CuCl^+] + [Cu(OH)^+] + [Cu(OH)_2^0] + [Cu(gly)] + [Cu(gly)_2], \quad (2)$$

and

$$[gly_{total}] = [gly_{free}] + [Cu(gly)] + 2[Cu(gly)_2]. \quad (3)$$

Equation 2 can be further simplified to

$$[Cu_{diss}] = a \cdot [Cu^{2+}] + [Cu(gly)] + [Cu(gly)_2] \quad (4)$$

in which $a = 1 + [Cl^-] \cdot K^{CuCl} + [OH^-] \cdot K^{CuOH} + [OH^-]^2 \cdot \beta^{Cu(OH)_2}$ = constant, because the concentrations of the inorganic ligands are not significantly affected by variations in the copper concentration.

Calculations were performed using stability constants taken from Martell and Smith (1974) and Smith and Martell (1976) (Table 1) which had been corrected for seawater ionic strength by extrapolation or by use of the equation of Davies. In the presence of MnO_2

$$[Cu_{ads}] = [Cu_{total}] - [Cu_{diss}]. \quad (5)$$

$[Cu_{ads}]$ was calculated from Eq. 5 and $[Cu^{2+}]$ from Eq. 2 and 4 by the method of successive approximations. From these values we then calculated Γ_{max} and B from Eq. 1.

The mass balance equations for the

copper and ligand (L) species present in the exudates of the macroalgae at the same pH as the calibration experiment are

$$[Cu_{diss}] = a \cdot [Cu^{2+}] + [CuL], \quad (6)$$

and

$$[L_T] = [L] + [CuL] \quad (7)$$

with

$$K' = [CuL]/[Cu^{2+}][L]. \quad (8)$$

$[Cu^{2+}]$ is then calculated by use of Eq. 1 and 5 and [CuL] is obtained from Eq. 6. Substitution of Eq. 8 into 7 gives

$$[Cu^{2+}]/[CuL] = (K' \cdot [L_T])^{-1} + [Cu^{2+}]/[L_T]. \quad (9)$$

The plot of Eq. 9 as $[Cu^{2+}]/[CuL]$ vs. $[Cu^{2+}]$ is a straight line because K' and L_T are constant. $[L_T]$ is calculated from $(\text{slope})^{-1}$ and K' from slope/Y-intercept. The confidence limits of K' are calculated from a least-squares fit to the data.

Results and discussion

The results of the calibration of the MnO_2 method with glycine (Fig. 1a) show that there was a linear relationship over the entire range of copper additions and it is therefore not necessary to extrapolate or approximate the data as in some other methods (e.g. Shuman and Woodward 1977). The difference between the Γ_{max} and log B values reported here (0.229 and 8.85) and those of 0.78 and 7.6 reported previously for a medium having an ionic strength of 0.01 (van den Berg and Kramer 1979*a*) may be attributable both to the effects of seawater on the surface of the exchanger and to the fact that different preparations of stock suspensions may give different "qualities" of exchanger. The calibration was performed in the absence of carbonate ions, after purging with nitrogen. However the pH varied in some of the samples during the period of equilibration. The final pH values of all samples from the third calibration were pH 8.15 ± 0.02. For this reason only the data for this calibration are presented in Fig. 1a. Although the other two calibrations provided values for B and Γ_{max} in close agreement with those from the third run, the 2σ variance calculated from a least-squares fit to the data was greater.

When the results of the titration of the filtrates from the three macroalgae with Cu^{2+} are plotted in accordance with Eq. 9 (Fig. 1b,c,d), straight lines are obtained. This suggests that only one ligand or complexing site is operative, or, at least, is predominant over this range of free copper(II) ion concentrations. The free copper(II) ion concentration varied from 10^{-10} M to 10^{-9} M during these determinations, because most dissolved copper was complexed by released organic material. Formation of CuL_2 complexes, if it occurs with these ligands, would tend to take place at relatively high ligand and metal ion concentrations, and would produce curvature in the plots of Eq. 9. Cu_2L complexation would result in two straight-line sections of decreasing slope, unless two sites with exactly the same conditional stability constants for copper were present on the ligand molecules. The assumption that only 1:1 (CuL) complexes were formed over the range of copper concentrations tested is therefore warranted. As a consequence it is possible to calculate molar ligand concentrations (rather than ligand equivalents or complexing sites) and conditional stability constants for complexes with copper by linear regression of the data in Fig. 1b,c,d (Table 2). Various amounts of complexing ligands were released by the three macroalgae tested: 1.01×10^{-6} M by *Ectocarpus*, 0.75×10^{-6} M by *Antithamnion*, and 0.27×10^{-6} M by *Audouinella*. On a dry weight basis the release of complexing ligands by *Antithamnion* was actually greatest, the amounts being about a tenth (on a dry weight basis) of those reported by van den Berg et al. (1979) for freshwater microplanktonic species.

The values of the conditional stability constants for copper complexation at pH 8.15 are quite similar for the three macroalgae: log K' is 10.15 for *Ectocarpus*, 9.77 for *Antithamnion*, and 9.95 for *Audouinella*, with a confidence limit (2σ) of 0.16 calculated from the least-squares fit

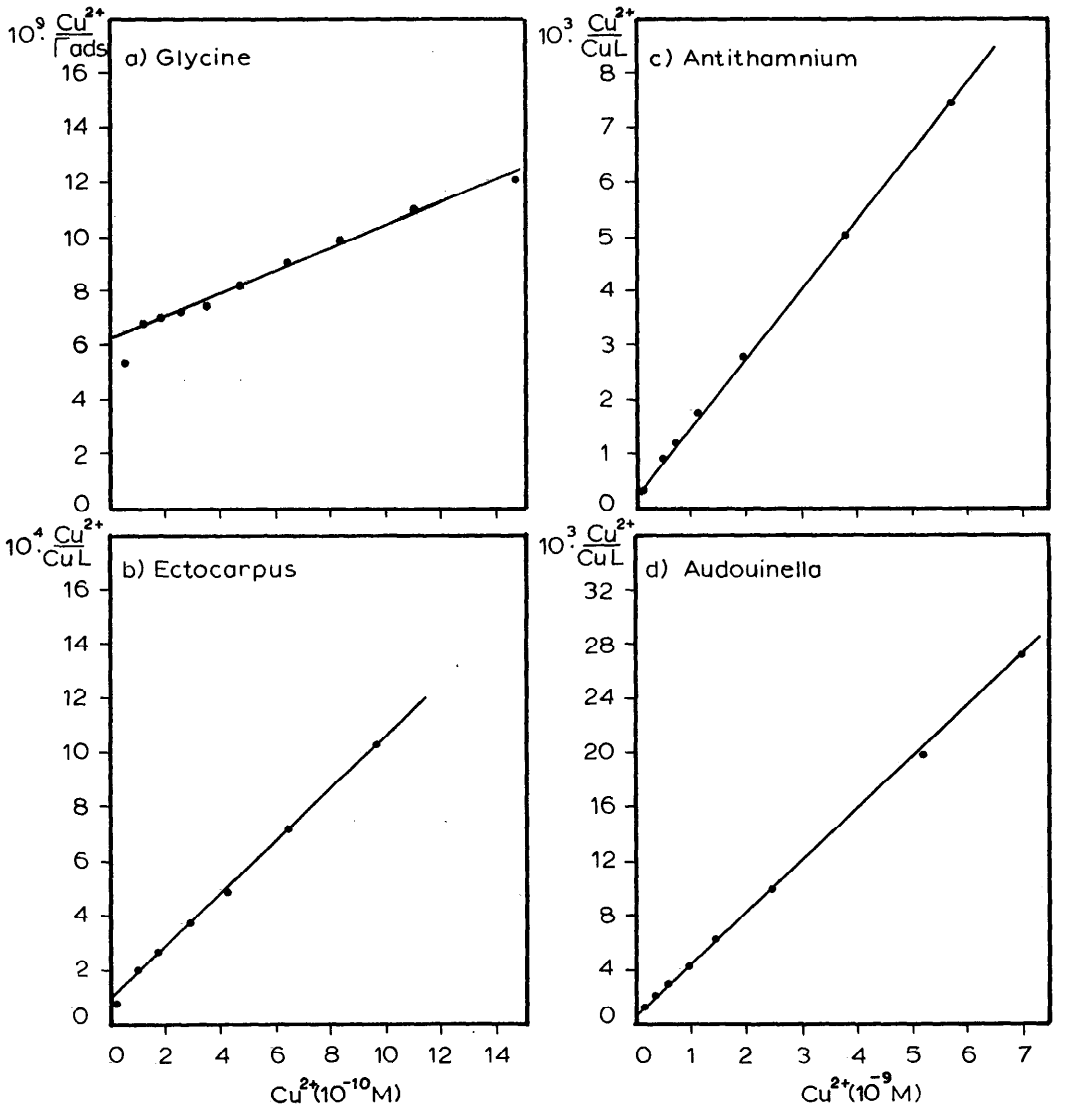

Fig. 1. a. Titration of MnO_2 with Cu in presence of glycine. $[Cu^{2+}]$ and Γ_{ads} have been calculated by Eq. 1, at 25°C, pH = 8.15, μ = 0.7 (seawater). b, c, d. Titration of exudates with Cu^{2+} in presence of MnO_2 showing monoligand binding. $[Cu^{2+}]$ and [CuL] have been calculated from Eq. 9, at 25°C, pH = 8.15, μ = 0.7 (seawater).

of the data. These values are very high, especially for seawater in which there is competition by calcium and magnesium ions. EDTA, for instance, in seawater has a value for K' slightly less than that of the ligand released by *Ectocarpus*. The values are significantly higher than those reported by Mantoura et al. (1978) for fulvic substances in seawater, and by van den Berg and Kramer (1979*b*), Buffle et al. (1980), and Tuschall and Brezonik (1980) for complexing material in freshwater. The complexing ligands released by the macroalgae investigated here are possibly similar to the hydroxamate siderophores from filamentous blue-green algae

which have values for log K' as high as 10.3 in freshwater medium (McKnight and Morel 1980), or similar to the copper metallothioneins released by *Neurospora crassa* (Lerch 1980) and present in eels (Noel-Lambot et al. 1978). Metallothioneins serve to store copper in the fungus *N. crassa* when it is grown in a medium enriched in copper. The release of complexing ligands by macroalgae may be a mechanism for the removal of copper from the cells. Seeliger and Edwards (1979) showed that two benthic red algae store copper in a copper-enriched medium, and release it in an organic form in a copper-poor medium. However, the copper concentrations in our media were quite low, between 0.1 × and 0.3 × 10^{-6} M Cu, much lower than the concentration of released ligands.

On the other hand, the release may not be related to the copper concentration but to the concentration of another element. The release of copper complexing hydroxamate siderophores by blue-green algae reported by McKnight and Morel (1980), for instance, was induced by lack of iron. In our investigation, we added only nitrate and phosphate to the cultures, so the iron concentration may have been low and this may have led to a similar effect.

The presence of these strong chelating compounds may well explain the marked tolerance of *Ectocarpus* and the red seaweeds to metallic poisons, such as copper and mercury, as well as their well known ship fouling potentialities (Harris 1943; Russell and Morris 1973). The relationship between metal toxicity and the free, uncomplexed, metal concentration is now fairly well established for several metals (e.g. Sunda and Guillard 1976; Anderson and Morel 1978; Jackson and Morgan 1978; Canterford and Canterford 1980). We therefore tested the ability of these released chelating compounds to reduce copper toxicity, using two microplanktonic species as test organisms because of the comparative ease with which their photosynthetic activity can be measured; however, the results of these experiments were rather inconclusive. In the presence of the released organic material one organism, *Phaeodactylum*, was inhibited at all copper levels relative to its growth in UV-irradiated seawater, while the other organism, *Chlorella*, was stimulated at low, and inhibited at high, copper concentrations. We did not observe the "simple" Cu^{2+} related response as found for instance by Jackson and Morgan (1978) and Sunda and Guillard (1976).

Table 2. Production (μmol·liter^{-1}) of complexing ligands by three macroalgae and conditional stability constants, log K'_L, of copper complexes of these exudates (pH = 8.15).

	Dry wt (mg)	Ligands produced	Ligands per mg dry wt (μmol·mg^{-1})	log K'_L
Ectocarpus	367	1.01	2.76×10^{-3}	10.15±0.15
Antithamnion	241	0.75	3.09×10^{-3}	9.77±0.12
Audouinella	286	0.27	0.94×10^{-3}	9.95±0.16

Algal organic material has previously been shown to stimulate growth of *Phaeodactylum* (Prakash et al. 1973) and a similar observation has been made by Ragan et al. (1980) by the addition of zinc and algal polyphenols.

The ecological significance of the organic substances released by marine macroalgae may not be restricted to one particular effect. Their strong chelating capacities certainly play an important role by increasing or decreasing the availability of trace metals for other species and themselves, but excreted matter itself can also be responsible for toxic effects, the relative importance of which remains in question.

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