Copper chlorins in the water and sediments of the Baltic Sea

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Abstract

Copper chlorins, complexes of copper with the most abundant derivatives of chlorophyll a in this environment, were investigated in the water and sediments of the Baltic Sea using HPLC and LC–MS. Extracts of the organic substance adsorbed from water on XAD–2 resin revealed the presence of allo-phaeophytins a, oxygenation products of Cu-phaeophytin a. The sediments contained small amounts of Cu-phaeophytin a and Cu-pyrophaeophytin a. The results indicate that in the investigated area copper is incorporated into the chlorin complexes of phytoplankton origin in the surface water and released from the complexes above the bottom or within the sediments.

1. Introduction

The speciation of copper in seawater has been a subject of continuous research over the last twenty years. In the beginning, most scientists assumed, mainly on the basis of calculations, that copper occurred in seawater predominantly in inorganic carbonate and hydroxide complexes. On the other hand, experimental studies have shown (Buckley and van den Berg, 1986; Hanson *et al.*, 1988) that the majority (40–99%) of copper ions in seawater are complexed by organic ligands. Although at present this seems to be the prevailing view, the extent of complexation and the nature of the ligands still remain vague.

Voltammetric and sorption methods indicate that the ligands complexing copper belong to at least two classes – one with a high conditional stability constant ($\log K_{L1} = 11$–13) and low concentration (2–5 nM), the other with a lower stability constant ($\log K_{L2} = 8$–10) and higher concentration (6–200 nM) (Coale and Bruland, 1990; Moffet et al., 1990).

Copper is included among the 'nutrient' trace metals (Li, 1991), as the free copper ion concentration is often observed to decrease with depth in the ocean. One of the causes of such a distribution may be photodecomposition of the organic copper complexes. Moffet and Zika (1987) have shown that sunlight causes photolysis of the complexes, although the process is slow (Moffet et al., 1990). They also observed a maximum of copper binding in the region of the chlorophyll maximum, which points to the biological origin of the L_1 ligands. It is several years since phytoplankton was confirmed as a significant factor controlling copper speciation in seawater (Wangersky, 1986), but the mechanism of this control is as yet unresolved. The strong belief in humic substances as the only ligands complexing copper in seawater has now subdued, and the large-scale chelation of copper by certain phytoplankton and bacteria exudates (Swallow et al., 1978; McKnight and Morel, 1979, 1980) is also in doubt.

It is worth mentioning here that humic substances in seawater are defined operationally, as substances adsorbing on Amberlite XAD–2 resin or other neutral sorbents. Similarly, 'dissolved' complexes are determined optionally, depending on the filtration conditions. Thus, such a 'solution' contains more or less particulate matter. Chester et al. (1988) studied the solid-state speciation of copper in surface water particulates and organic sediments. Their work showed that around 50% of copper in the surface water particulates is bound up in organic associates. When the material is deposited on the bottom, some of the organic forms are destroyed to an extent dependent on the diagenetic environment of deposition. These workers are of the opinion that the preservation of the organic copper fraction in sediments reflects primary production in the overlying waters. A correlation was found between copper and silica in material collected in sediment traps in Antarctic regions (Westerlund and Ohman, 1991), which suggests the removal of copper from the water column by diatoms or other skeletal algae.

The results of our recent studies (Kowalewska, 1990; Kowalewska et al., in press) indicate that these strong copper chelators might be porphyrin-type ligands. Numerous results confirming this supposition may be found in the literature. Slowey et al. (1967) were the first to point to porphyrins as the organic ligands complexing copper in seawater. Smith and Windom (1980) found that 90% of organic copper complexes have a molecular mass

of the order of magnitude 10^3. Osterroht *et al.*   (1983, 1988) observed a correlation between the amount of organically bonded copper and absorption at 400 nm of the organic extract isolated from Baltic seawater; this is the wavelength at which the Soret band, characteristic of the porphyrin system, occurs. EPR studies of estuarine materials have revealed the presence of copper-porphyrin spectra in the humic acids isolated from living and dead *Spartina alterniflora,* a plant growing in the highly productive warm-temperate salt marshes (Filip *et al.*, 1988; Alberts *et al.*, 1989).

In this study, an investigation of copper chlorins, derivatives of chlorophyll *a*, was carried out in the water and recent sediments of the Baltic Sea.

2. Experimental

Sample collection

Figure 1 shows the location of sampling stations during the cruises of r/v ' Oceania ' in June 1989 and May 1991. Seawater samples were collected in polyethylene containers for on-board extraction of organic complexes. The sediment samples were collected with a core sampler. The top 0–5 cm of the core was transferred to a polyethylene container and stored in a freezer for later analysis.

Extraction of the organic matter from water

After collection, the seawater sample (50 dm^3) was left to stand overnight, then filtered through a Whatman GF/C glass fibre filter and passed through a column with Amberlite XAD–2 resin (30 × 1 cm bed) at a rate of 25 cm^3 min^{-1}. The bed was then washed with 100 cm^3 of redistilled water and the sorbed organic substance eluted with 250 cm^3 of acetone. The acetone solution was evaporated under vacuum to reduce the final volume of the solution to 25 cm^3. Chlorins were transferred to hexane by extraction in an acetone:hexane:water system (25:5:5 v/v/v). The hexane solution was transferred to a vial, evaporated to dryness in a stream of argon and stored in a freezer prior to HPLC analysis.

Extraction of chlorins from sediment samples

A frozen sediment sample (60–130 g wet weight) was allowed to thaw, and then centrifuged to remove water. Next, the sediment was flushed with acetone (*ca* 2 cm^3 g^{-1} of sediment), sonicated for 15 mins, then centrifuged. The extraction procedure was usually repeated three times. The collected supernatant fractions were evaporated under vacuum. The residue was dissolved in a few millilitres of acetone, transferred to a vial and the solvent removed in a stream of nitrogen.
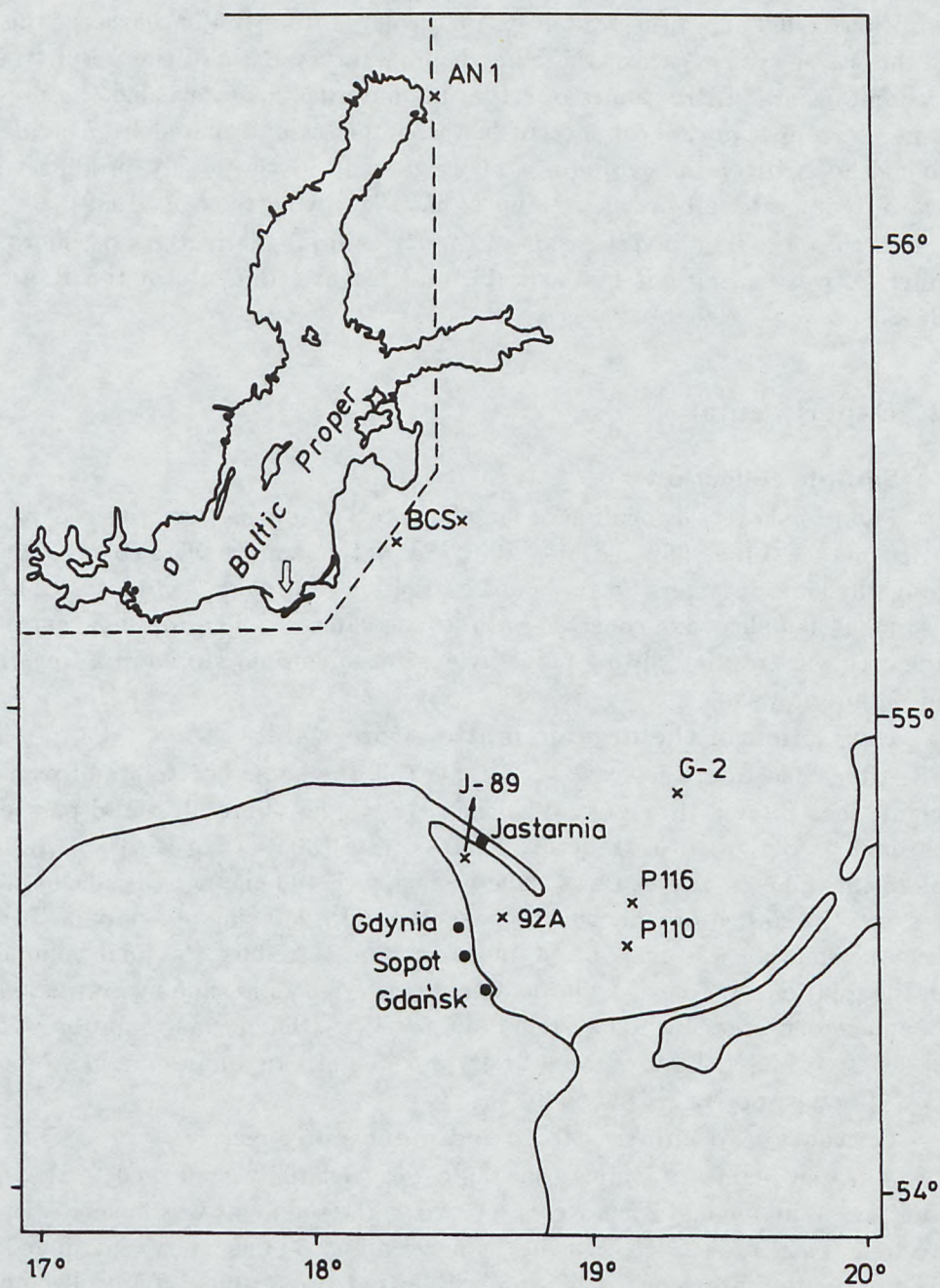
Fig. 1. Location of the sampling stations

High-performance liquid chromatography

The Amberlite XAD–2 resin extract dissolved in acetone, was injected into a Knauer Chrom-a-Scope liquid chromatograph (20 μl) equipped with a diode-array type detector. Separations were carried out at room temperature on a Merck Lichrospher 100 RP end-capped column (250 $\times$ 4 mm, 5 μm) with a Merck Lichrospher 100 RP18 end-capped precolumn (4 $\times$ 4 mm, 5 μm) in an isocratic system. The mobile phase was 90:10 (v/v) acetone:water at a flow rate of 2.0 ml min^{-1}. During the analysis, the spectrum was scanned in the 360–700 nm range every 0.1 sec and recorded by IBM computer.

The chlorophyll a, chlorophyll b and chlorophyllide a standards were extracted from leaves of higher plants and purified by TLC on Merck Kieselgel 60 plates in a 20:35 (v/v) acetone:hexane system. Phaeophytin a and phaeophorbide a were obtained in the same way after prior acidification of the acetone pigment extract. Pyrophaeophytin a was provided by Dr B. Keely of the University of Bristol. Cu-phaeophytin a and Cu-pyrophaeophytin a were obtained by the method described by Verne-Mismer (1988) from copper acetate and the relevant phaeopigments in a chloroform – methanol solution.

The cultures of *Monoraphidium contortum* isolated from the Baltic Sea, were obtained from the University of Gdańsk's Marine Plant Ecology Laboratory collection. The algae were cultivated on an f/2 growth medium prepared on the basis of natural seawater.

Combined liquid chromatography – mass spectrometry (LC–MS)

LC–MS analyses were performed using a HPLC set by Waters Associates (MS 600 Silk quaternary delivery system, Rheodyne 7125 injection valve, Waters 484 UV/visible variable wavelength detector). The liquid chromatography separations were carried out on a Novapak C_{18} RP column (100 $\times$ 5 mm, 5 μm) in an acetone:methanol:water gradient system at an effluent rate of 1.0 ml min^{-1}. The HPLC set was linked on line to a Finnigan MAT TSQ–70 quadrupole mass spectrometer via a Finnigan MAT TSP–2 thermospray interface (source conditions: discharge ionisation 1200 V, vaporizer temperature 65°C, source temperature 250°C, repeller 0 V (Eckardt *et al.*, 1990)). Negative-ion mass spectra were recorded by scanning the region from m/z 400 to 1200 in 2 sec.

3. Results and discussion

The HPLC chromatograms of the seawater samples are shown in Figures 2 and 3. The first was collected in Puck Bay, *i.e.* in the inner part
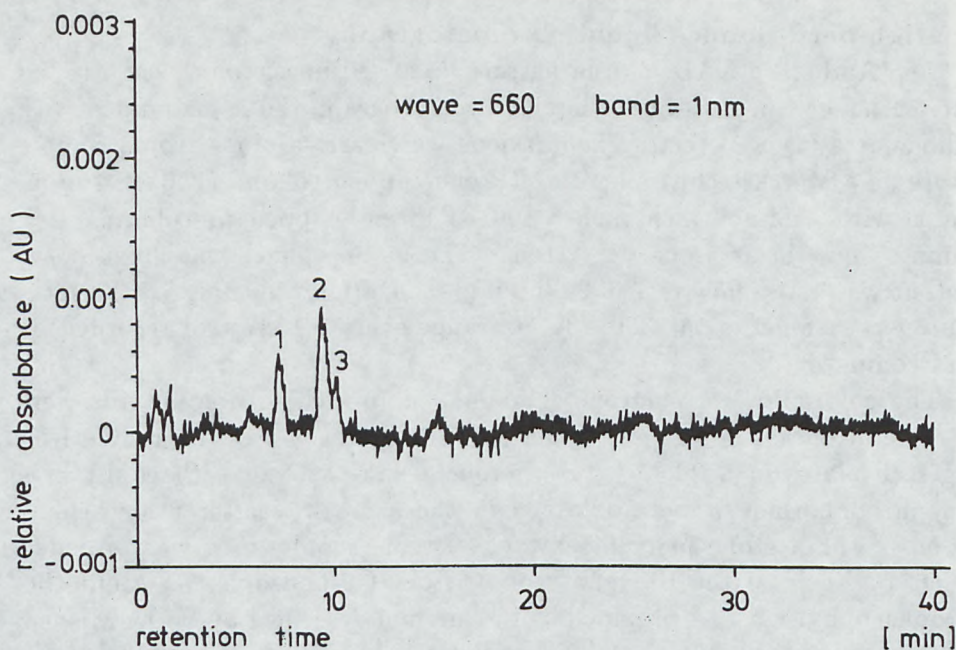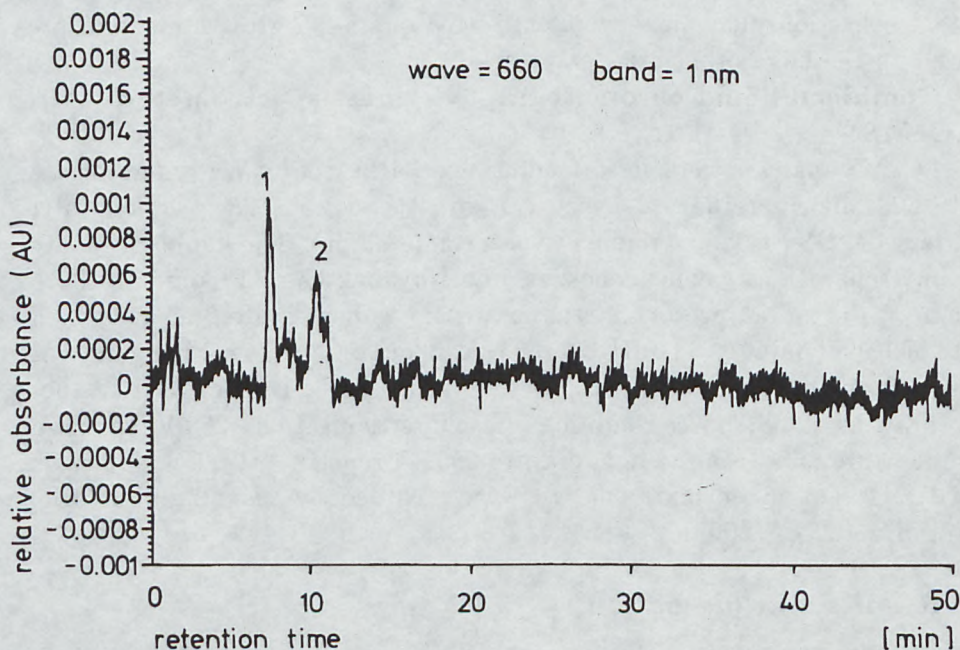
Fig. 2. Acetone/hexane extract of XAD–2 resin with sorbed organic complexes from seawater collected at station J–89: 1 — (λ_{max} – 402, 668 nm), 2 — (λ_{max} – 410, 644 nm), 3 — (λ_{max} – 412, 648 nm)

Fig. 3. Acetone/hexane extract of XAD–2 resin with sorbed organic complexes from seawater collected at station AN 1: 1 — (λ_{max} – 402, 668 nm), 2 — (λ_{max} – 406, 644)

of the Gulf of Gdańsk (J–89), and the second on the open sea (AN–1). The chromatograms registered at 660 nm show two distinct peaks – one at $t_R = 7.5$ min and the other at $t_R = 10$ mins. The absorption spectra of these peaks are of the chlorin type (Tab. 1).

Table 1. Elution order and spectral characteristics of the investigated chlorins (Lichrospher 100RP18e, 90:10 acetone-water, 2 ml min^{-1})

t_R [min]	Compound	Spectral data maxima [nm]
1.3	phaeophorbide a	410, 666
2.4	Cu-pyrophaeophorbide a	420, 648
4.0	chlorophyll b	458, 642
5.3	chlorophyll a	428, 662
6.7	phaeophytin b	434, 650
9.8	phaeophytin a	410, 664
8.8	Cu-phaeophytin a allo-forms	408, 648
9.2	—"—	408, 646
9.9	—"—	408, 644
10.9	—"—	402, 652
11.9	Cu-phaeophytin a	408, 642
8.6	Cu-pyrophaeophytin a allo-forms	418, 648
9.6	—"—	420, 650
11.0	—"—	420, 650
18.6	Cu-pyrophaeophytin a	422, 648

For comparison, the chromatograms of Cu-phaeophytin a (Fig. 4) and Cu-pyrophaeophytin a (Fig. 5) were obtained under the same conditions. The standards contain considerable amounts of derivatives, as copper chlorins very readily form oxygenated derivatives (so-called allo-forms), even more easily than chlorophylls do.

Figure 6a shows a chromatogram of the laboratory culture extract of the green alga *Monoraphidium contortum*, from the Gulf of Gdańsk. The chromatography was also performed after acidification of the extract (Fig. 6b).

Comparison of all the chromatograms presented and their spectral characteristics (Table 1) indicates that allomers of phaeophytin a ($t_R = 7.5$ min) and of Cu-phaeophytin a (t_R about 10 mins) were present in the seawater extracts.

The sediment extracts analysed by the LC–MS method revealed weak signals corresponding to the mass of Cu-phaeophytin a ($t_R = 41$ min) and Cu-pyrophaeophytin a ($t_R = 54$ min). Examples of the relevant RIC traces
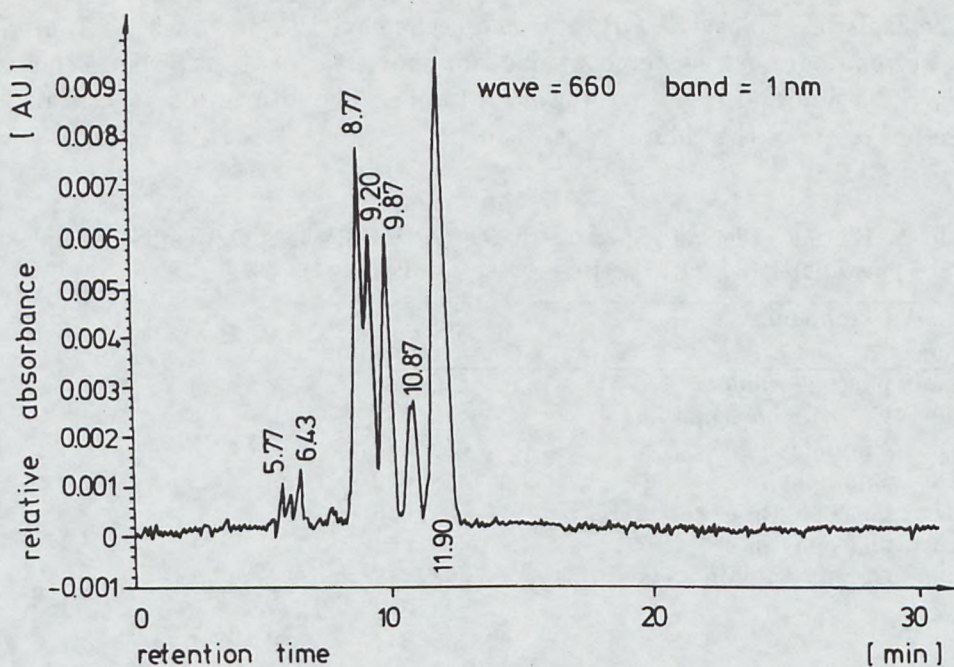
Fig. 4. UV-visible chromatogram of Cu-phaeophytin *a* standard. Merck Lichrospher 100RP18e, (250 × 4 nm, 5 µm), mobile phase: 90:10 acetone-water, 2 ml min⁻¹
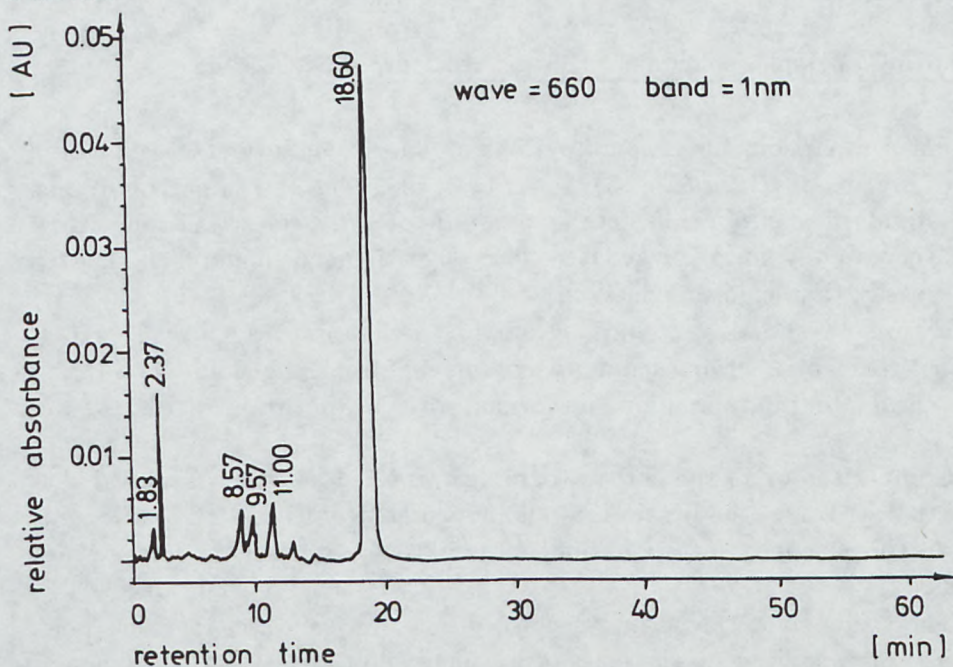

Fig. 5. UV-visible chromatogram of Cu-pyrophaeophytin *a* standard. Merck Lichrospher 100RP18e (250 × 4 mm, 5 µm), mobile phase: 90:10 acetone-water, 2 ml min⁻¹
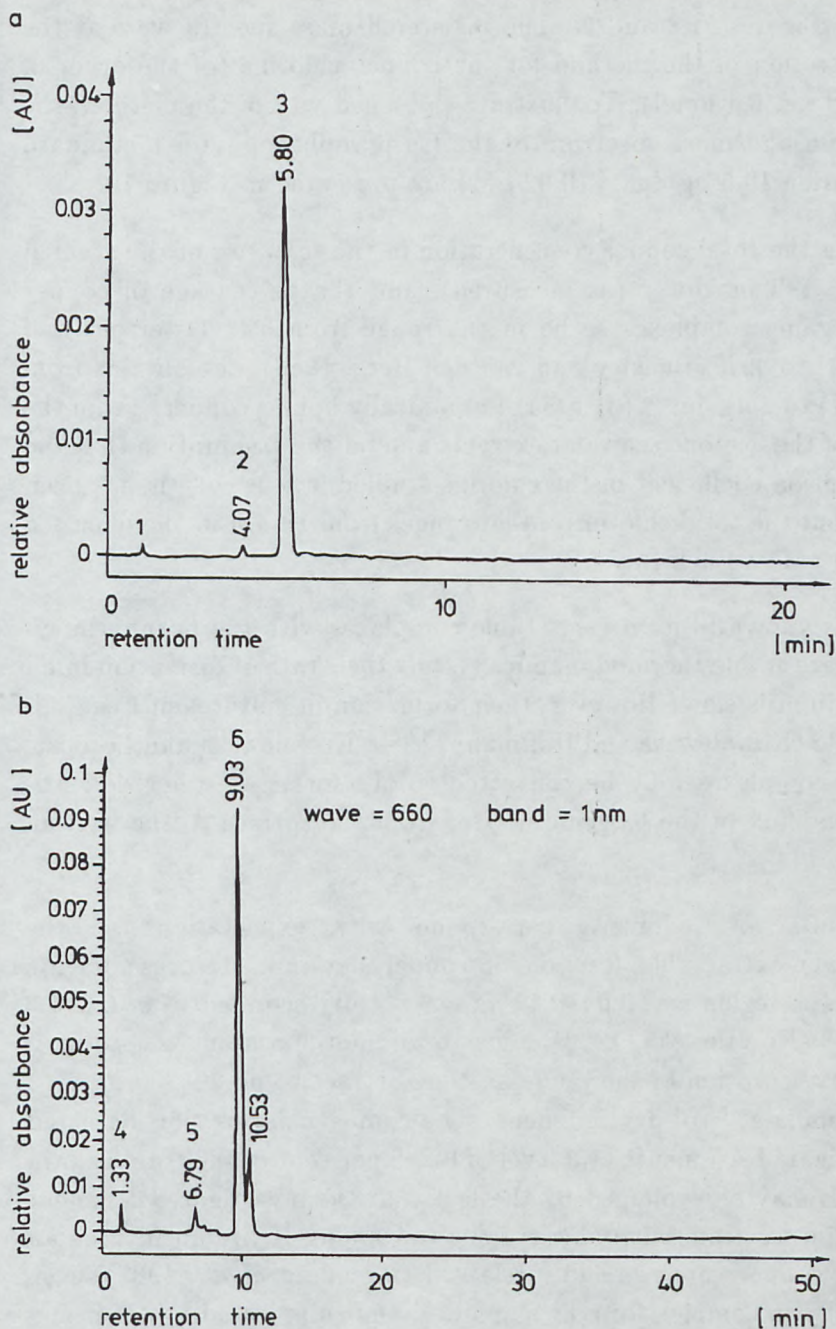
Fig. 6. UV-visible chromatograms of acetone chloropigment extract from green algae *Monoraphidium contortum* before (a) and after (b) acidification with 0.1 M HCl: 1 — chlorophyllide *a*, 2 — chlorophyll *b*, 3 — chlorophyll *a*, 4 — phaeophorbide *a*, 5 — phaeophytin *b*, 6 — phaeophytin *a*, spectra in Table 1. Merck Lichrospher 100 RP18e (250 × 4 mm, 5μm), mobile phase: 90:10 acetone-water, 2 ml min^{-1}, absorption at 660 nm

are given in Figures 7, 8 and 9. The registered mass spectra were at the limits of detection of the method for the copper chlorins (of the order of 0.1 μg cm^{-3} *i.e.* 0.1 nmol). To illustrate the sensitivity of the method, the chromatogram and mass spectrum of the Cu-pyrophaeophytin *a* standard of concentration 19.5 μg dm^{-3} (0.02 μM) are presented in Figure 10.

Assuming the total copper concentration in the seawater of the studied region to be 1–2 μg dm^{-3} (as measured), and the percentage of copper bound in organic complexes to lie in the range from 5% (Osterroht and Wenck, 1983) to 99% (Buckley and van den Berg, 1986), one obtains from 0.05 (0.8 nM) to 2 μg dm^{-3} (31 nM) of organically bound copper. From the absorption of the acetone seawater extracts and on the assumption that the molar absorption coefficient of the chlorins studied is 5×10^4, it has been calculated that the total chlorin content ranges from 10 nM in the open sea waters to 60 nM in Puck Bay.

Copper is known to form very stable complexes with the porphyrin system. They are stable thermodynamically, but their rate of formation in an aqueous medium is slow. However, their formation in cultures of living algae is possible (Kowalewska and Hoffmann, 1989; Kowalewska and Łotocka, 1991). The complexes may be converted to allo-forms or other derivatives during the flux of the biogenic matter from the surface to the bottom sediments.

The results for sediments correspond with expectations as the acetone/hexane-extractable fraction of copper determined for sediments from this Baltic region was 0.04–0.10 μg Cu g^{-1} dry sediment, *i.e.* 0.6–1.6 nmol Cu g^{-1} (Kowalewska, 1990). The total chlorin content was estimated from the absorption of the crude acetone extract to be 20–300 μg g^{-1} (0.02–0.33 μmole g^{-1}) of dry sediment. The copper chlorins thus occurred in the investigated sediments at a level of 0.2–8 per cent of the total chlorin content. This may be explained by the fact that the investigated sediment samples originated from eutrophic regions containing H_2S, and in such an environment copper can be readily released (Kremling *et al.*, 1987), even from a porphyrin complex forming sulphides, and substituted by other metal ions such as Ni^{2+} or VO^{2+} in the porphyrin-type ligand. Besides, as has already been mentioned, copper chlorins, like Cu-chlorophyll, can be transformed to a variety of different derivatives in the water column and sediments during diagenetic processes, which makes the study more difficult.
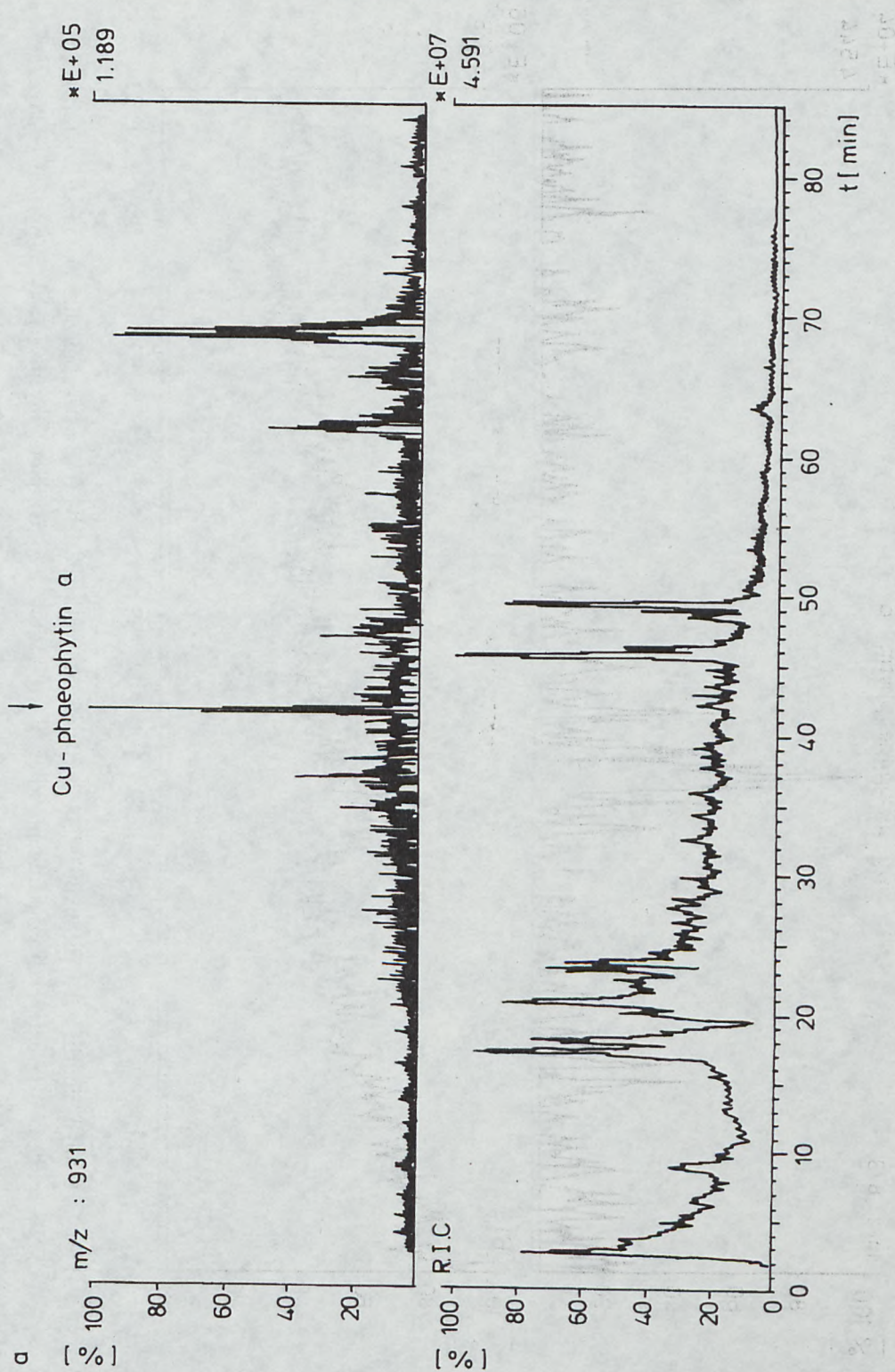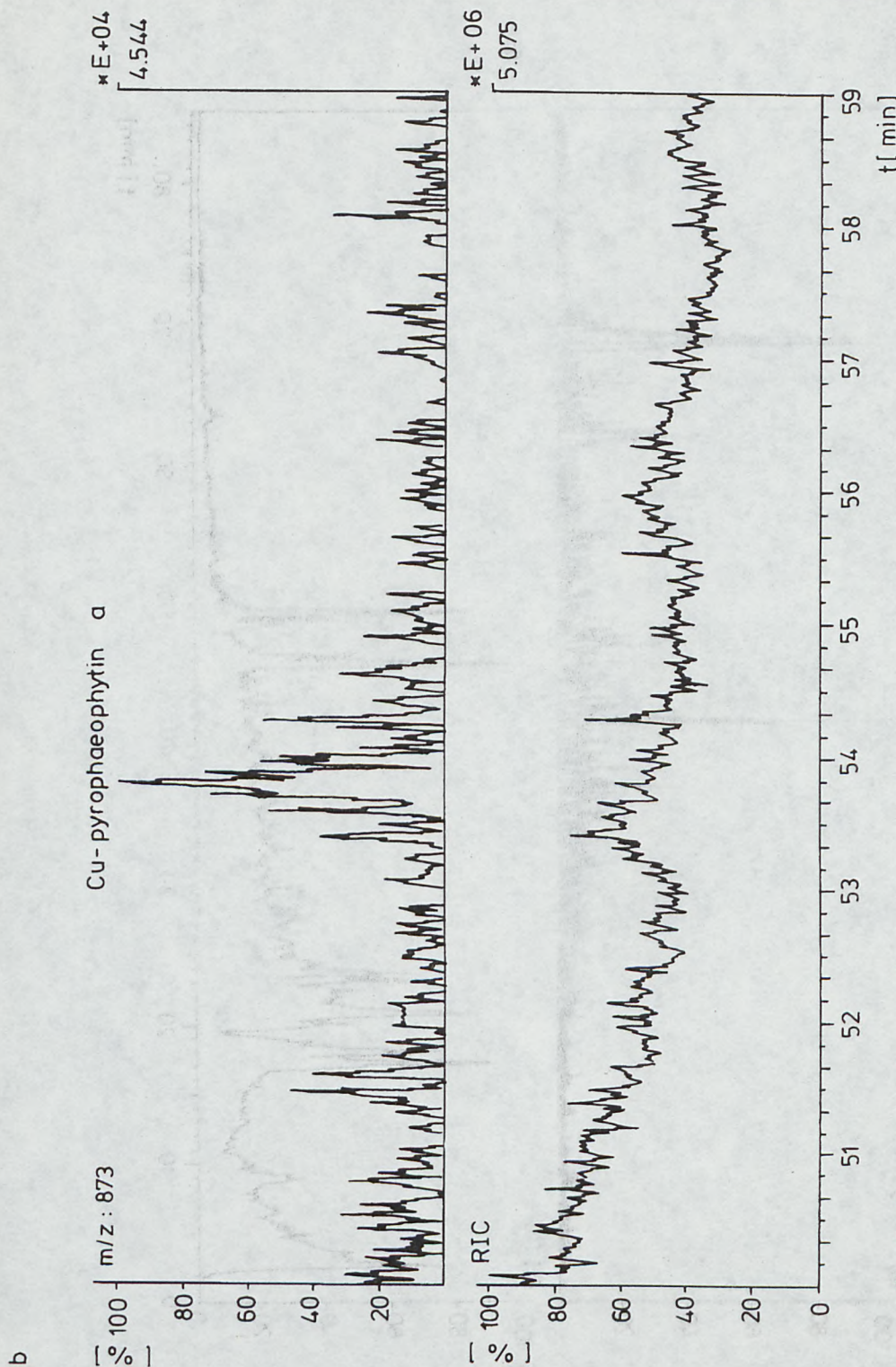
Fig. 7. Copper chlorin mass chromatograms in comparison with the RIC (reconstructed current chromatogram) of the total acetone extract from sediment collected at station G–2: Cu-phaeophytin *a* (a), Cu-pyrophaeophytin *a* (b)
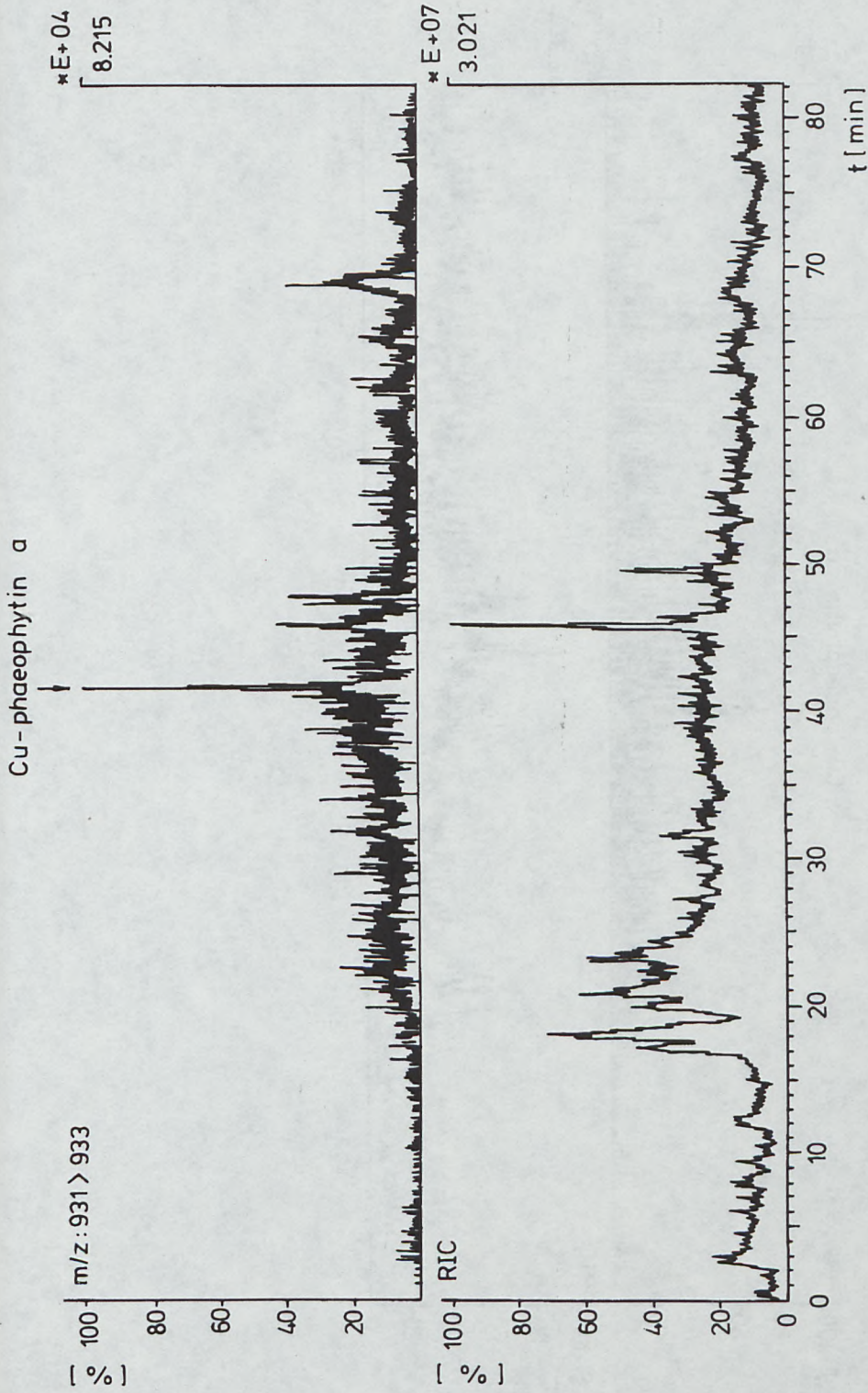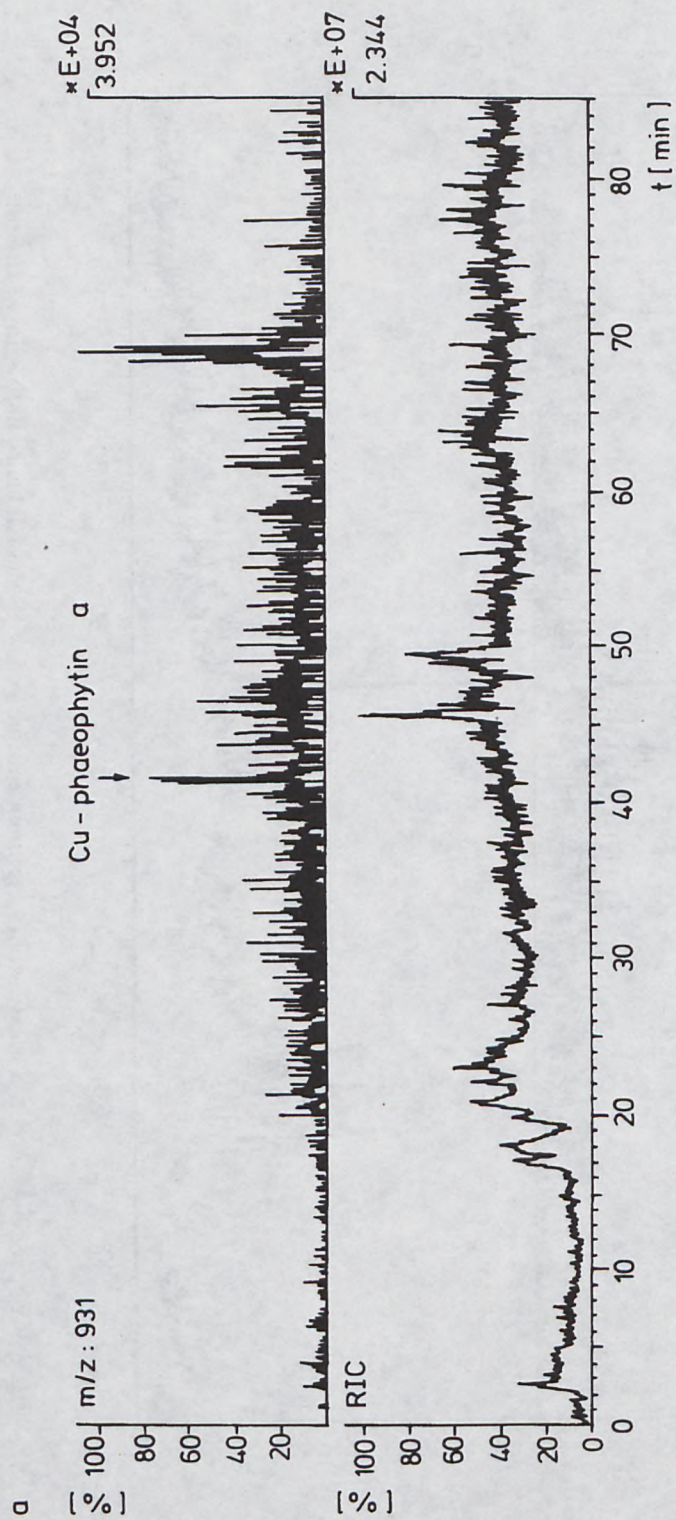
Fig. 8. Copper phaeophytin *a* mass chromatogram in comparison with the RIC chromatogram of the total acetone extract of sediment collected at station AN–1
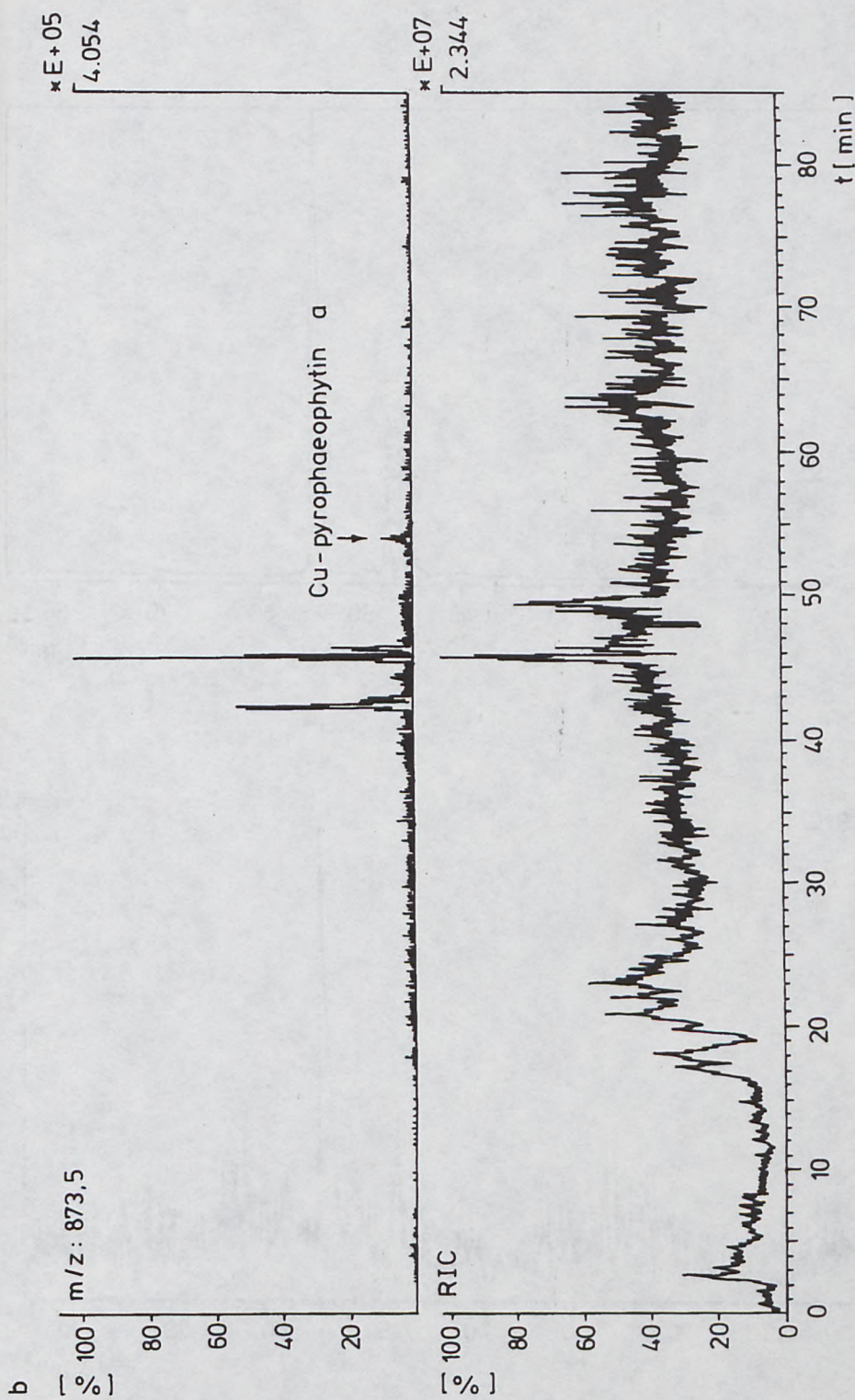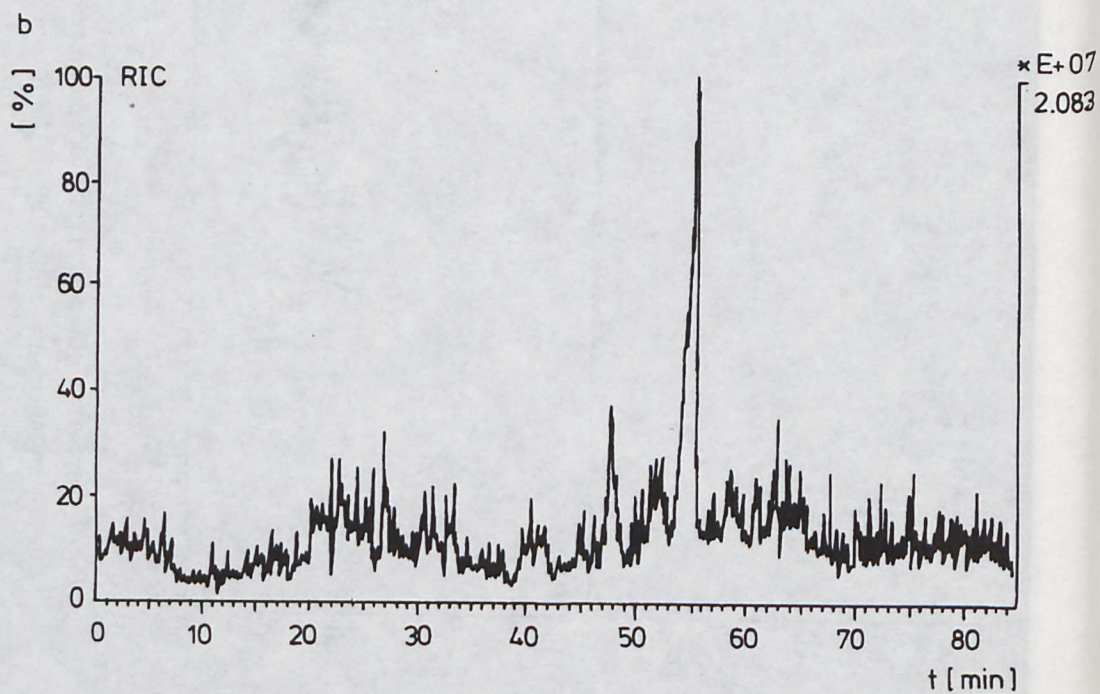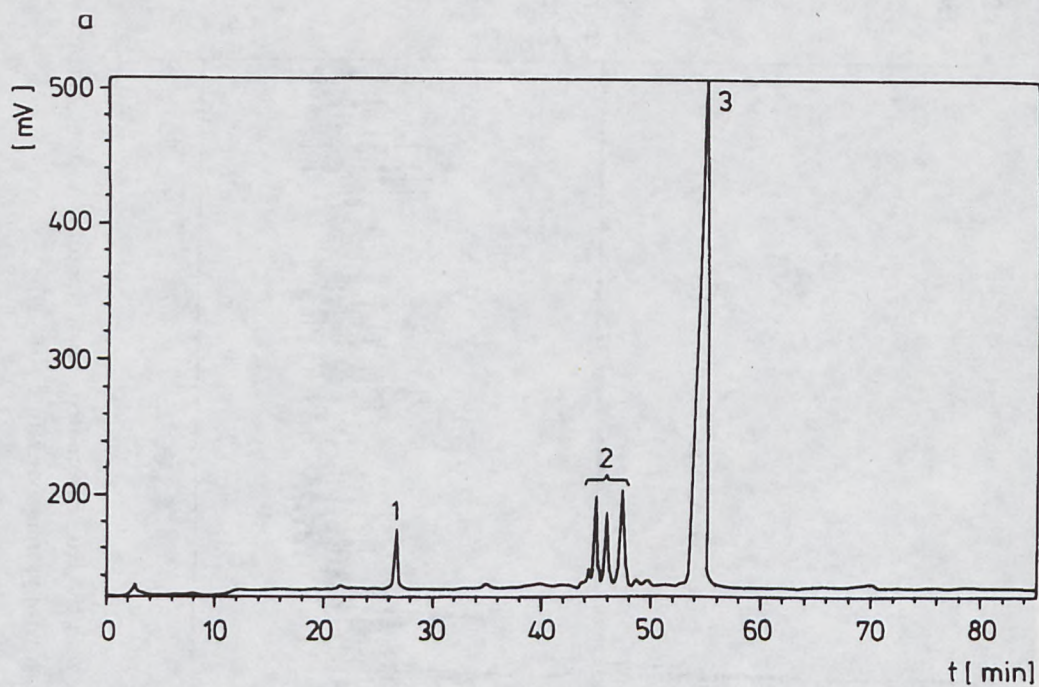
Cu-phaeophytin a

Fig. 9. Copper chlorin mass chromatograms in comparison with the RIC chromatogram of the total acetone extract of sediment collected at station 92 A: Cu-phaeophytin *a* (a), Cu-pyrophaeophytin *a* (b)
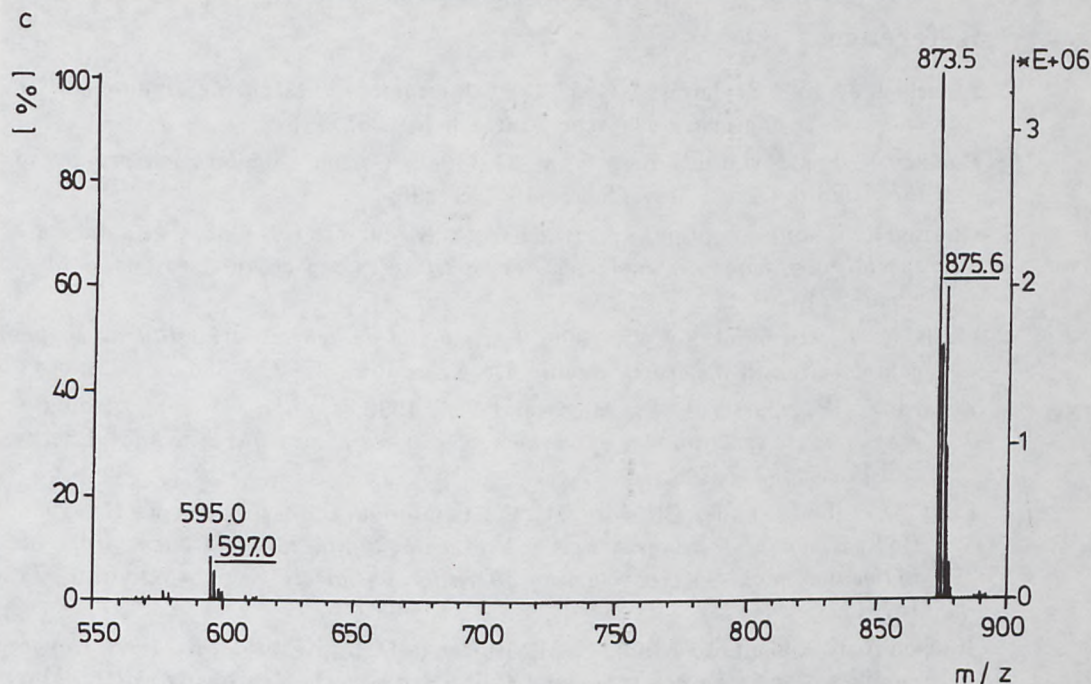
a

b

c

Fig. 10.   UV-visible chromatogram (a), RIC chromatogram (b) and MS spectrum (c) from LC–MS analysis of Cu-pyrophaeophytin *a* standard. HPLC and MS conditions as detailed in experimental section

4. Conclusions

- Extracts of seawater revealed the presence of compounds with the same retention time and the same spectrum as allo-Cu-phaeophytins *a*.

- Sediment extracts contained small amounts of copper chlorins (Cu-phaeophytin *a* and Cu-pyrophaeophytin *a*) at the detection level (*i.e.* 0.1 nmol).

- The results indicate that in the investigated basin copper is incorporated into chlorin complexes of phytoplankton origin in the surface water and released from these complexes just above the bottom or in the bottom sediments.

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