

**Selected properties of
different molecular
size fractions of humic
substances isolated
from surface Baltic
water in the Gdańsk
Deep area**

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Humic substances
Baltic water
Heavy metals
Complexation

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Abstract

Humic substances were isolated from surface Baltic water in the Gdańsk Deep area and fractionated by ultrafiltration on membrane filters of pore sizes 1000, 5000 and 10 000 Daltons. 97% of the material obtained was smaller than 10 000 D. The fractions were characterised by UV-VIS and IR spectroscopy, and analysed for elemental composition and functional group concentration. The nitrogen and phenolic hydroxyl group concentrations confirmed the autochthonous origin of the fractions. The aromaticity of the molecules was found to increase with molecular size, and the greatest saturation of aromatic rings with carboxyl groups was found in the fraction smaller than 1000 D. Complexation capacity was not directly connected with molecular size. All the fractions possessed a larger capacity for lead than cadmium, the complexation of the latter being detected only in the < 1000 D fraction.

1. Introduction

Marine humic substances are a class of biogenic, refractory, yellow organic compounds constituting the largest fraction of dissolved organic matter in seawater. Their importance to the environment is due to their ubiquity and the functions they perform within it (Jackson, 1975). They are a mixture of molecules, the properties of which are spread across a broad continuum. In order to acquire more homogenous fractions of these highly complex and heterogeneous substances, they are usually separated into fulvic and humic acid fractions. Derived from soil chemistry, the criterion differentiating these

fractions is based solely on their solubility in organic acids (Kononowa, 1966; Schnitzer and Khan, 1972). Even though there are no distinct differences between these two fractions and their decomposition products (Thurman, 1985), such fractionation has often been used in studies on freshwater humic substances.

In this paper the traditional criterion has been replaced by fractionation based on molecular size. This approach is underpinned by the assumption that any interaction between the marine environment and humic substances, such as flocculation, or uptake by organisms, is influenced by the size of the latter rather than their solubility in acids.

Among the most common methods of measuring molecular size are gel permeation chromatography and ultrafiltration. Applications of the former are limited, owing to gel-humic substance interactions which could influence the molecular size distribution (Gjessing, 1970). The latter technique is a relatively simple process in which molecules are retained by or pass through a membrane of a definite pore size. Since flow rates are higher and the adsorbent area is smaller, the results achieved using ultrafiltration are less affected by adsorption. Nevertheless, problems do arise in practice from nonuniform pore size, concentration-dependent retention (Carlson *et al.*, 1985) and aggregation of humic substances (Buffle *et al.*, 1978), but despite its disadvantages, ultrafiltration is commonly used to study organic matter in seawater, and it is this technique that has been applied in the present work.

One important feature of humic substances is their ability to complex metals in seawater and thus modify their geochemical behaviour and toxicity towards marine organisms. Of great ecological importance, this process has given rise to a number of extensive studies into the interactions of humic substances with heavy metals (Turner *et al.*, 1987).

Organic complexation is usually quantified by amperometric titration with the selected metal (Srna *et al.*, 1980; Raspor *et al.*, 1984). The application of the very sensitive Anodic Stripping Voltammetry technique (ASV) using a mercury-film electrode permits measurements at quite realistic overall metal levels, close to their actual total concentration in seawater. The titration is based on the assumption that where a metal occurs in the presence of excess ligand, only the free metal in equilibrium with the complex is detected by ASV. The parameter determined in such studies – the complexation capacity – is the ability of the ligand to ‘mask’ a metal with respect to the sensing technique (Nürnberg and Raspor, 1982). There are reports on the altered bioavailability of metals bound in forms inaccessible to ASV (Florence *et al.*, 1983; Srna *et al.*, 1980).

The objectives of the present investigations were:

- to isolate dissolved humic substances from Baltic seawater, and to determine their molecular size distribution;
- to characterise the different molecular size fractions obtained;
- to quantify their interactions with lead and cadmium under conditions closely reflecting the natural seawater system;
- to obtain a preliminary assessment of the correlation between molecular size, selected physical and chemical properties, and the ability of the fractions to complex lead and cadmium.

Because extrapolations from high metal and ligand concentrations are unreliable (Saar and Weber, 1979; Gamble *et al.*, 1980), the experiments were done at concentrations of humic substances and metals close to those present in Baltic water. In order to include the seawater 'matrix' effect on the complexation process, organic-free seawater served as supporting solution in the ASV titration.

2. Experimental

The source of the humic substances was surface Baltic water in the Gdańsk Deep area, sampled from on board r/v 'Kopernik', anchored at 54°50'N, 19°20'E. They were isolated by sorption on Amberlite XAD-8 resin. Collected in polyethylene buckets in the integrating mode from 1 to 4 June 1992, the 5 dm³ subsamples of water were subsequently filtered on board (Whatman GF/C), acidified to pH 2 (HCl, Merck) and pumped by peristaltic pump through three columns containing Amberlite XAD-8. The adsorbed humic substances were then eluted with 0.1 M NaOH. The details of the procedures for resin cleansing, column preparation, and elution and desalting of the adsorbed humic substances are given in Grzybowski and Pempkowiak (1991). Some 300 dm³ of seawater were processed.

The humic substances obtained were separated into three fractions of different molecular weight by ultrafiltration carried out in a stirred, pressurised Amicon 202 ultrafiltration cell. Diaflo membrane filters of pore sizes 1000, 5000 and 10 000 D (Daltons) were used. Prior to filtration, the filters had been conditioned by being soaked for 24 h in redistilled water, and then flushed with redistilled water pumped through the ultrafiltration set until no UV-VIS absorbance (100 mm quartz cell) could be detected in the ultrafiltrate. The ultrafiltration was performed with an ultrafiltrate-to-concentrate volume ratio not exceeding 5, in order to minimise concentration polarisation and filter clogging. The resultant fractions were concentrated in a vacuum evaporator. Their concentration and ash content were determined gravimetrically.

IR spectra were measured using the KBr technique with a Perkin–Elmer 257 FT–IR spectrophotometer. The UV–VIS absorbance at 250, 365, 465 and 665 nm was measured with a Zeiss Specord spectrophotometer in a 1 cm quartz cell; doubly distilled water was used as reference.

The total acidity was determined by the barium hydroxide method and the carboxyl groups by the calcium acetate exchange reaction (Schnitzer and Khan, 1972; Perdue *et al.*, 1980).

Voltammetric measurements were done with a PP–04 Polarograph (Telpod, Poland) in a 50 cm³ Teflon cell with a saturated calomel electrode, a platinum wire electrode and rotating glassy carbon electrode coated with a mercury film. 4×10^{-5} M mercury (II) nitrate solution in UV-irradiated seawater was used to pre-deposit the film. The experimental conditions used for the voltammetry were the following: mode – D.P.A.S.V., deposition time – up to 30 min., pulse height – 50 mV, pulse repetition – 50 ms, deposition potential – -0.800 V, scan rate – 20 mV s^{-1} , resting period – 20 s. The voltammograms were recorded with an X–Y recorder. Cleaning of the ultra-filtration cell, the Teflon cell and the glassware was performed according to the usual trace-metal-analysis procedures (Mart *et al.*, 1980). The solution was purged with high-purity nitrogen for 10 minutes.

Organic-free seawater was acquired by 12 h UV irradiation of the unacidified sample in quartz tubes by means of a medium-pressure mercury lamp.

3. Results and discussion

3.1. Physico-chemical characteristics of the fractions

Fig. 1 shows the molecular size distribution of the humic substances isolated. As a result of irreversible sorption on the filters, the sum of the fractions is 98%.

Molecules larger than 5000 molecular weight units ($D = \text{Daltons}$) share in the distribution to a very small degree. This phenomena is probably caused by the flocculation of high-molecular, terrestrial material owing to the increase in salinity (Sholkowitz, 1976). A similar distribution has been obtained by Makinen *et al.* (1994) in humic substances isolated from Baltic water. However, in some samples extracted from Polish (Pempkowiak *et al.*, 1979) and Swedish coastal waters (Plechanow *et al.*, 1983) no fraction larger than 5000 D was detected at all. The observed difference is probably due to the different separation technique, or the input of terrigenous material.

The UV–VIS spectra of the fractions did not differ qualitatively, increasing monotonically towards high frequency. This effect is common to humic

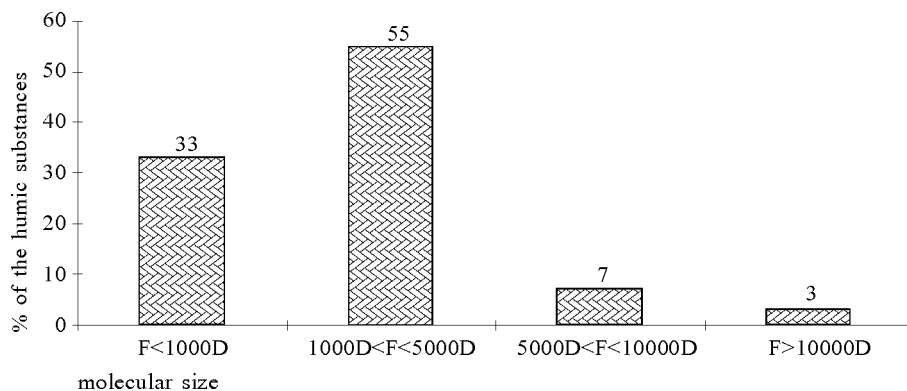


Fig. 1. Molecular size distribution of humic substances isolated from Baltic surface water

substances isolated from different environments. The slope of the spectrum is usually quantified by the ratio of absorbance at 465 and 665 nm (E_4/E_6), and 250 and 365 nm (E_2/E_3). These factors are accepted as indicators of age and humification (Schnitzer and Khan, 1972). The data presented in Tab. 1 show that E_4/E_6 and E_2/E_3 decrease with increasing molecular size.

Table 1. UV-VIS spectral parameters of different molecular size fractions of humic substances isolated from Baltic surface water

Molecular size	Parameter:	
	E_4/E_6	E_2/E_3
fraction < 1000 D	8.0	3.7
1000 D < fraction < 5000 D	6.1	3.4
5000 D < fraction < 10000 D	5.9	2.7

The observed decrease in E_4/E_6 and E_2/E_3 parameters with increasing molecular size has also been observed in humic substances isolated from freshwater (De Haan, 1983) and soil (Chen *et al.*, 1977). According to the latter, the magnitude of the E_4/E_6 ratio is inversely proportional to the degree of condensation. The measurements of visible light absorbance in analysed solutions of the same concentration showed that the high-molecular fraction absorbs more strongly. The same phenomenon was observed by Stewart and Wetzel (1980) and has been ascribed to the greater aromatic component of the molecules.

Furthermore, the results of the elemental composition analysis (Tab. 2) confirm the assumption that the ‘aromaticity’ of the fractions increases with their molecular size. A direct correlation between E_4/E_6 and $[H]/[C]$ ratios has also been observed by Ertel and Hedges (1983) in terrestrial humic substances. It should be stressed, however, that an $[H]/[C]$ ratio decreasing with increasing molecular size could also have resulted from a higher unsaturated bond content (Flaig *et al.*, 1975).

Table 2. Elemental composition of different molecular size fractions of humic substances isolated from Baltic surface water

Molecular size	Elemental composition [%]			Molecular quotient	
	C	H	N	$[H]/[C]$	$[N]/[C]$
non-fractionated humic substances	44.8	5.8	3.6	1.56	0.08
fraction < 1000 D	44.1	6.1	4.1	1.65	0.08
5000 D > fraction > 1000 D	46.5	6.0	3.5	1.55	0.07
5000 D < fraction < 10000 D	48.1	5.0	3.4	1.25	0.06
fraction > 10000 D	48.9	5.3	2.8	1.30	0.05

The results for the ‘raw’ humic substances are in general agreement with the data obtained by Pempkowiak *et al.* (1976) for material sampled from Baltic water. The only exception is the lower nitrogen concentration, which probably resulted from using NaOH as eluant instead of $NH_3 \times H_2O$. This phenomenon has been also reported for humic substances isolated from marine sediments (Rashid and King, 1971).

The nitrogen concentration in the fractions is typical of marine humics (Stuermer, 1975). The question whether the measured nitrogen is a component of the humic substances or originates from compounds connected by low-energy bonds is still unresolved. The isolation method applied allows some weakly attached nitrogen compounds to be removed (Thurman and Malcolm, 1981; MacCarthy and O’Cinneide, 1974). Assuming that the nitrogen detected is a constituent of the structure, one may conclude that its content decreases with increasing molecular size.

Fig. 2 shows IR spectra of different molecular size fractions of the humic substances.

The identification of the transmission bands has been based on the published data on IR investigations of soil, fresh- and seawater humus (Schnitzer, 1971; MacCarthy and Rice, 1985). The spectra obtained comprise bands typical of marine humic substances (Stuermer, 1975); the differences are quantitative. A noticeable feature of the spectra obtained is the

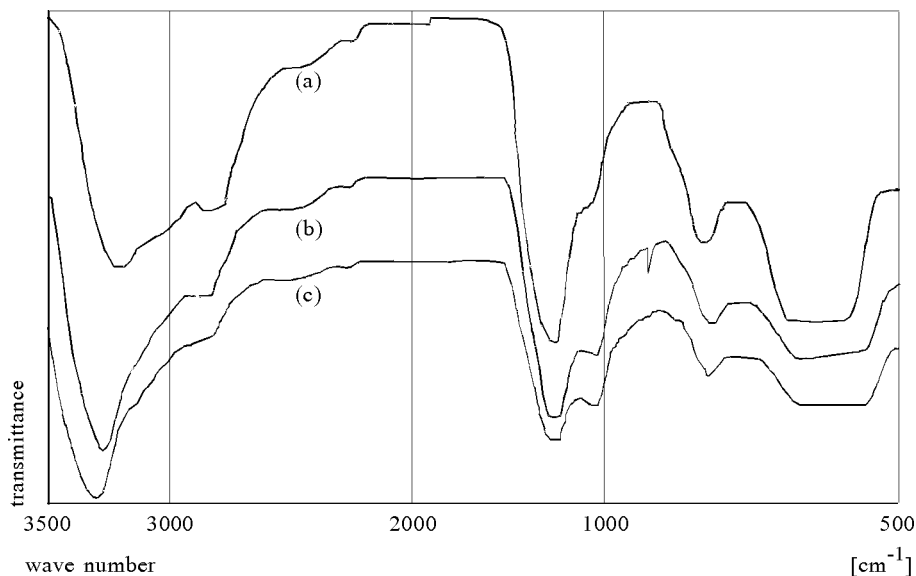


Fig. 2. IR spectra of different molecular size fractions of humic substances isolated from Baltic surface water: fraction < 1000 D (a), 1000 D > fractions > 5000 D (b), 5000 D < fractions < 10000 D (c)

1640 cm^{-1} band, diminishing with decreasing molecular size, attributed to C=C vibrations in aromatic structures. The absorption at about 2950 cm^{-1} , which can be assigned to the stretching vibrations of aliphatic moieties, is more intensive for the < 1000 D fraction. The absorption bands at 1550 and 1420 cm^{-1} corresponding to COO^- are not strong, indicating that the samples contained very few mineral constituents. However, one has to be conscious of the fact that quantitative analysis of IR spectra based solely on transmission-band size may be erratic owing to the non-reproducibility of the measurements. In order to quantify data from the IR spectra, the areas of the bands selected have been measured, logarithmised and 'normalised' with reference to the 1650 cm^{-1} band, to which a value of 1 has been attributed (Fig. 3). It should be emphasised that the different fraction values obtained are not comparable with one another and describe solely the internal characteristics of the fractions.

The absorbance ratios at 2950 and 1640 cm^{-1} support the assumption that the degree of aromatisation increases with molecular size. The < 1000 D fraction shows a higher COOH group content (1725 cm^{-1} band) with reference to the 1650 cm^{-1} band, which may be attributed to the stronger saturation of aromatic rings with carboxyl groups. The increasing ratio of the 3200 cm^{-1} band (polymeric hydrogen-bonded OH) *vs.* the other bands

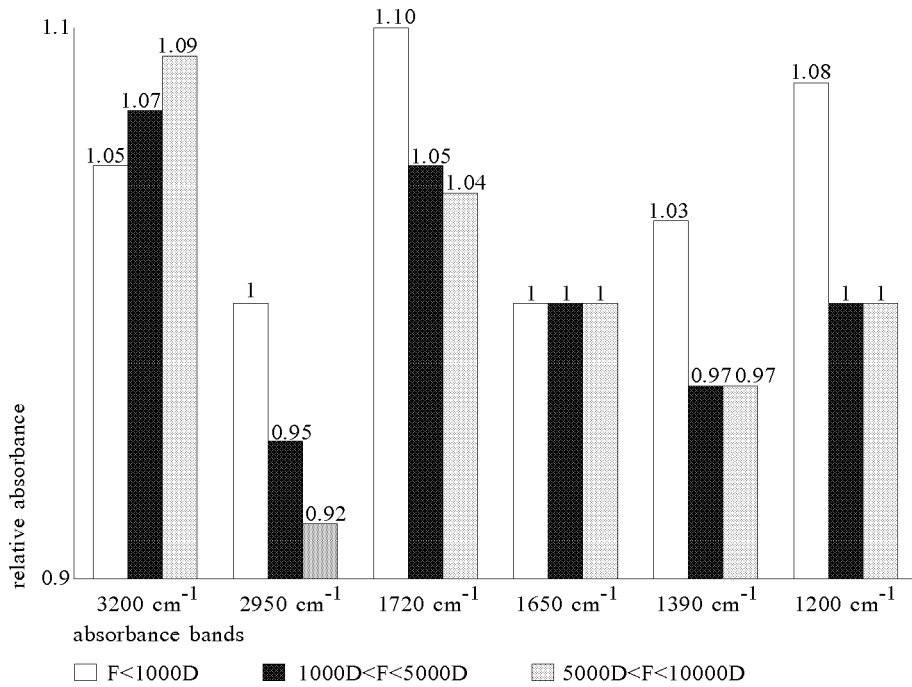


Fig. 3. Relative IR absorbance of different molecular size fractions of humic substances isolated from Baltic surface water

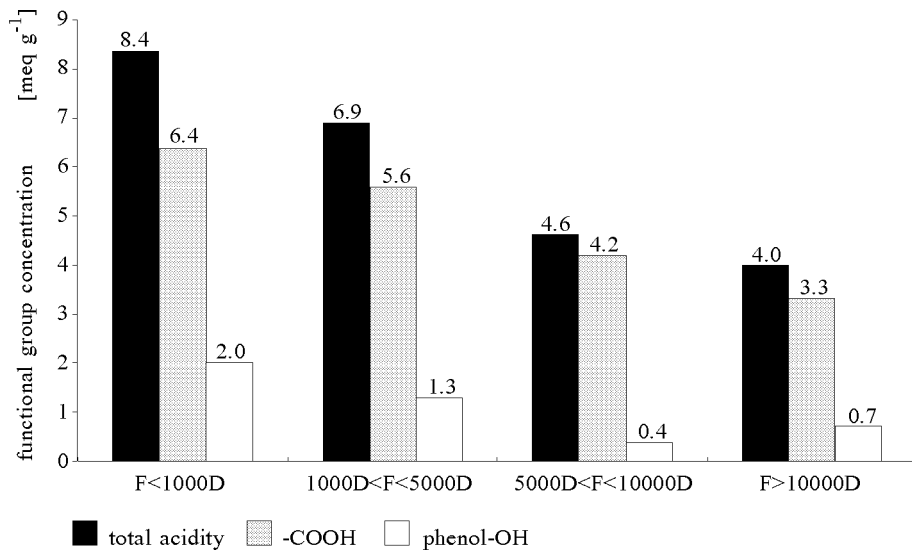


Fig. 4. Concentration of carboxyl and phenolic hydroxyl groups in different molecular size fractions of humic substances isolated from Baltic surface water

may be evidence for the presence of inter- or intra-molecular hydrogen bonds in the larger molecules.

The results of the functional group analysis, presented in Fig. 4, are indirect confirmation of the above conclusions.

The content of COOH and phenolic-OH groups decreases with growing molecular size. This tendency has also been observed in humic substances isolated from marine sediments (Rashid and King, 1970). The phenolic hydroxyl group concentration is confirmation of the autochthonous origin of the samples: values below 2 meq g^{-1} are one of the features distinguishing marine from terrestrial humic substances (Schnitzer and Khan, 1978).

3.2. Complexing properties

The complexing capacity measurements were preceded by the determination of metals of interest in the seawater (electrolyte) and in the humic substances analysed. The respective concentrations of Pb and Cd in the filtered subsamples of UV-irradiated Baltic water ($\text{pH} \approx 8.0$, salinity = 7.2 PSU) were $0.11 \pm 0.02 \mu\text{g dm}^{-3}$ and $0.06 \pm 0.02 \mu\text{g dm}^{-3}$ (ASV measurements). The results lie within the range of concentrations reported for these metals in Baltic water (Kremling and Petersen, 1984). The ASV detection limit determined in seawater depleted of organics was $0.02 \mu\text{g dm}^{-3}$ for lead and $0.01 \mu\text{g dm}^{-3}$ for cadmium. The AAS measurements did not detect lead or cadmium in the humic substances.

The complexing capacities of the humic-substance solutions were determined by the method proposed by Chau *et al.* (1974): the solution was titrated with standard addition of metal and ASV was applied to measure the electroactive (uncomplexed) metal concentration. The plot of the metal concentration *vs.* the added metal inflects at the point where the complexation capacity has been exhausted. The titration data are usually compared with the model proposed by Ruzic (1984), which assumes a 1:1 stoichiometry of complex formation. This model, however, has limited applicability in studies of the more extensively complexed ligands and in solutions of low ligand-to-metal ratio (the so-called surface saturation effect (Buffle *et al.*, 1987)).

Because in the present paper both of the above cases apply, the measurements of the complexation capacity obtained from the Ruzic plot are of low precision. In order to improve their precision, the kinetics of the complexations have been measured. In the presence of Ca and Mg in the solution, the time scale of trace-metal equilibration with organic ligands is longer than the deposition period (Hering and Morel, 1988). The principle of the method involves monitoring the change in the peak height corresponding to the non-complexed metal with the time elapsing after the addition of the

standard. If the metal is not undergoing complexation, the height remains constant. In order to determine the influence of metal sorption on the cell walls, ASV measurements were done in seawater deprived of organics by UV irradiation. Tests run in UV-irradiated seawater showed no diminution of peak heights after as long as 180 min following the addition of standard. Therefore, it was assumed that within the time scale of the experiment the sorption of metal ions remains negligible and the diminishing of peak height results solely from the evolution of a non-electroactive metal-humic complex.

The complexing capacities presented in Tab. 3 were obtained from the analysis of the ASV titration curves, which was supplemented at each titration point by a comparison of two peak heights: one directly following electrodeposition, the other after 45 min of equilibration. The ultimate complexation capacity was assumed to have been attained when no difference between them was observed.

Table 3. Lead and cadmium complexation capacities of different molecular size fractions of humic substances isolated from Baltic surface water

Molecular size	Cadmium	Lead
fraction < 1000 D	$0.20 \pm 0.05 \mu\text{g mg}^{-1}$	$0.40 \pm 0.10 \mu\text{g mg}^{-1}$
1000 D < fraction < 5000 D	*	$0.55 \pm 0.05 \mu\text{g mg}^{-1}$
5000 D < fraction < 10000 D	*	$0.25 \pm 0.05 \mu\text{g mg}^{-1}$

* below the detection limit [$0.01 \mu\text{g dm}^{-1}$].

It should be emphasised that the results presented here are basically conditional, *i.e.* dependent on the kind of humic substances, the experimental conditions, and the content of the supporting electrolyte. Therefore the absolute values obtained cannot be generalised to other types of humic substances without some restrictions.

The weighted sums of the results lie within the range of the complexing capacities obtained by Pempkowiak (1989) for the 'raw' (non-fractionated) humic substances isolated from Gdańsk Deep water. The observed low degree of organic cadmium complexation agrees well with the observations of Florence (1982), who reports that up to 85% of cadmium in seawater exists in electroactive complexes.

The high complexation ability of the low-molecular fraction has also been observed in humic substances isolated from lakes (Giesy, 1983) and estuaries (Smith, 1976; Haekel, 1984). In the study of cadmium complexation by humus isolated from marshes, John *et al.* (1988) reported that the highest proportion of cadmium was associated with the 1000–10000 D fraction.

This discrepancy might be due to the fact that that experiment was performed in freshwater solutions. The possible influence of NaCl was also observed by Haekel (1984), who found cadmium bound by humic substances (only in the low-molecular fraction) solely in samples to which NaCl had been added to obtain ocean salinity.

A strong affinity for lead seems to be an immanent feature of humic substances: it has been reported in material isolated from freshwater (Alberts *et al.*, 1984), seawater (Florence, 1982), marine sediments (Raspor *et al.*, 1984) and soil (Saar and Weber, 1979).

4. Conclusions

About 90% of humic substances isolated from a sample of surface Baltic water were smaller than 5000 D. The available data indicates that this distribution is typical for humic substances dissolved in Baltic water.

The nitrogen content and the presence of phenolic hydroxyl groups in all of the fractions are proof of the autochthonous origin of the material analysed. The results of physico-chemical analyses of the fractions show that their properties change monotonically with molecular size. In combination with elementary composition data, the UV-VIS spectra suggest that aromaticity increases with molecular size. In the IR spectra of the fractions, the band representing the aliphatic moieties is the most prominent in the low-molecular fraction. Semi-quantitative analysis of these spectra indicates that the ratio of aliphatic to aromatic components decreases with increasing molecular size – unlike the degree of COOH-group saturation of aromatic rings. The latter tendency finds indirect confirmation in the results of functional group analysis: their concentrations increase with decreasing molecular size.

The complexation properties are not directly connected with the size of the molecules. Apart from the salinity, the crucial feature determining whether humic substances are capable of complexation seems to be the concentration of carboxylic and phenolic groups. Detection of cadmium complexation solely in the smallest size fraction indicates that the metal-binding sites of the fractions are specific and discriminatory, as they display a greater affinity for lead (the titrations were done separately for lead and cadmium in order to avoid competition between them). This phenomenon can be explained by the concept of organic ligand interactions with ‘hard’ and ‘soft’ metals discussed by Buffle (1988). Lead, one of the ‘hard’ metals, forms stronger complexes than cadmium at sites containing oxygen and nitrogen. The organic ligands ‘preferred’ by cadmium contain sulphur – an element whose concentration in aquatic humic substances rarely exceeds 1% (Perdue, 1983). Taking into account ligand structure, Pinheiro *et al.* (1994) offered

another explanation: they proved that humic substances have a greater diversity of complexing sites (a higher degree of heterogeneity) available for lead than for cadmium.

The fact that cadmium is bound by the smallest molecules may have a significant influence on its uptake by organisms via a cell membrane – a process strictly controlled by the size of the metal-ligand complexes. Additionally, the most active fractions in the complexation process are, as a result of strong functional group substitution, highly water-soluble – hence bio-available. Their predominantly aliphatic character makes them less prone to colloidal coagulation and, subsequently, to sedimentation (the flocculation caused by an increase in salinity affects mainly the high-molecular fractions). Therefore, the participation of marine humic substances in immobilising heavy metals by removing these to the sediments, seems to be restricted to the waters containing suspended, sorptive particles.

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