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HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHIC CHARACTERIZATION OF HUMIC SUBSTANCES AND DISSOLVED ORGANIC MATTER FROM BALTIC AQUATIC ENVIRONMENT

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In the present study the number-average molecular weights $\overline{M}_n$ *and weight-average molecular weights* $\overline{M}_w$ *of dissolved organic matter and humic substances from different aquatic environment were determined by high-performance size exclusion chromatography. Seawater samples from the low-salinity Baltic Sea, Kattegatt and Skagerrak were analyzed by HPSEC with fluorescence detection at excitation/emission wavelengths 350/450 and 310/450 nm. UV detection at 254 nm has been used for characterization of other aquatic HS samples from Estonia. The weight- and number-average molecular weights were calculated using polystyrene sulfonates and proteins as standard substances. Due to the enhanced separation efficiency obtained by experimental conditions used (0.02M phosphate buffer, pH 6.8), defined molecular weight fractions were separated in seawater DOM. The weight-average molecular weight for seawater DOM was in the range 600–1300.*

Multivariate classification and comparison by principal component analysis has been carried out on the data including 24 physical-and-chemical parameters of seawater samples. The plot of scores on the first two principal components revealed the clear grouping of samples in dependence on the sea region as well as on the depth of sampling. The plot of loadings allowed estimating the major variables, which are responsible for this grouping and to interpret the results basing on the knowledge of the biochemical processes occurring in the marine ecosystem.

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Introduction

The study of dissolved organic matter (DOM) in aquatic systems is very important because of its role in the carbon cycle. The majority of DOM in waters consists of humic substances (HS). HS are formed at chemical and biological degradation of plant and animal residues and at synthetic activities of microorganisms [1, 2]. HS are to be considered as precursors of oil shale. The organic part in oil shale, kerogen, is known to impact on humus formation. Kerogen is typically enriched in aliphatic carbon and hence HS originating from kerogen have a greater carbon content and aromaticity than those found in surface waters [3].

HS are operationally divided into fulvic and humic acids. Fulvic acid is soluble both in acidic and basic solution, whereas humic acid is nonsoluble in acid solution but soluble at higher pH values. Fulvic acid has, in general, a lower molecular weight and a higher percentage of carboxyl and hydroxyl groups than the humic one. Humic acid is usually more aromatic and hydrophobic than fulvic one. Both acids are complex mixtures of organic macromolecules without a distinct chemical structure. Several studies have indicated the presence of substructures and different functional groups in HS molecules. HS contain carbohydrates, aliphatic chains, polyphenols, carboxy benzene and other simple organic acids. The major functional groups in HS are carboxyl, hydroxyl and carbonyl groups [4].

The majority of research on aquatic HS has been carried out with river- and lakewater HS. Only few studies on seawater HS have been reported. The main reason is the difficulty of isolation of sufficient amounts of seawater HS to allow experimentation and chemical characterization [5]. It has been found that marine HS are less aromatic than fresh-water HS and predominantly unsaturated [6, 7].

Molecular weight (MW) is one of the fundamental properties of aquatic DOM. It is important in order to understand the physical and chemical characteristics and chemical reactivity of HS (and DOM). Molecular weight affects the behavior of HS with respect to adsorption, metal binding, bioreactivity, water treatment, electrostatic effects, etc. Molecular weight is a key factor controlling DOM reactivity. Lower molecular weight molecules have smaller radii, they are more hydrophilic, which results in greater bioavailability and rapid adsorption to mineral substrates. Higher molecular weight molecules are more aromatic and have greater metal-binding capacity [8]. The molecular weights of HS range from few hundred up to several thousand [9, 10]. MW of aquatic HS are considered to be generally in the range 500–10,000, with the majority of determinations in the range 500–2,000 [9, 11].

For determination of HS molecular weight, methods like ultrafiltration, size exclusion chromatography (SEC), viscosity measurements, X-ray scattering have been used [9, 12–15]. SEC is widely used for separation and

characterization of natural and synthetic polymers. In SEC, samples are fractionated on the basis of the molecular size and shapes of their components. Elution times enable to calculate the relative molecular weights. The calculations are based on relationships between molecular weights of appropriate standards and their hydrodynamic volume.

The application of high-performance SEC (HPSEC) has advantages in comparison with other molecular weight determination methods since it is relatively fast, non-destructive and requires no sample pretreatment. SEC curves express the average molecular weight (number-average $\overline{M}_n$ and weight-average $\overline{M}_w$) and also molecular weight distributions and the copolymer composition in each molecular weight fraction. HPSEC has been applied for characterization of marine sediments and waters [16–18].

UV detection is mostly used with SEC, since HS contain chromophores [16, 19–21]. Another important property of HS is exhibition of blue fluorescence. Several fluorophores exist in HS molecules, among them benzene rings, conjugated structures and carbonyl groups. SEC has been coupled with fluorescence detection for fulvic and humic acid characterization with organic mobile phase [22] and HPLC [7]. The fluorescence detection is preferred because of enhanced sensitivity and properties of DOM [23].

The objective of the present study was to characterize the aquatic DOM from the Baltic environment, namely from the Baltic Sea, Kattegatt and Skagerrak and from some Estonian waters using HPSEC. Previously, the same technique has been applied for characterization of hydrophobic and hydrophilic fractions isolated and fractionated from Baltic Sea DOM by the XAD method [24]. Some fundamental properties like molecular weights (weight- and number-average) and polydispersities were calculated for HS from different water-types.

Experimental

Equipment

One part of HPSEC experiments was carried out using the Jasco HPLC system, consisting of Jasco 880-PU solvent pump, Jasco 851-AS autosampler and Jasco 821-FP fluorescence detector coupled with the biocompatible BIOSEP-SEC-S2000 column (7.5 × 300 mm, Phenomenex, USA) with a quardcolumn (7.5 × 75 mm). The column packing consisted of hydrophilically bonded silica with particle size 5 μ and pore size 145 Å. Column efficiency using a protein mixture with 0.1M phosphate buffer, pH 6.8, was 75,900 plates/m. The molecular weight exclusion range for native proteins was 1000–300,000. The total permeation volume of the column (V_t, 11.58 mL) was determined with glucose, the void volume V_0 was 6.43 mL. The other part of HPSEC experiments was carried out using the HPLC system

including a pump (Inkrom, Estonia), injector (Rheodyne) and a UV detector (Knauer) coupled with the biocompatible BIOSEP-SEC-S2000 column as in the first part. The total permeation volume of the column (V_t, 14.35 mL) was determined with sodium azide (M = 65), the void volume V_0 was 7.42 mL as determined with blue dextran ($\overline{M}_w$ = 2,000,000).

Samples

Water samples from the Baltic Sea, Kattegatt and Skagerrak were collected in March 1995 and April 1996 during the expeditions with RV *Argos* (Table 1).

The locations of the sampling stations are shown in Fig. 1 and marked according to the Swedish Meteorological and Hydrological Institute (SMHI). From each station, water below the surface and from near the bottom were

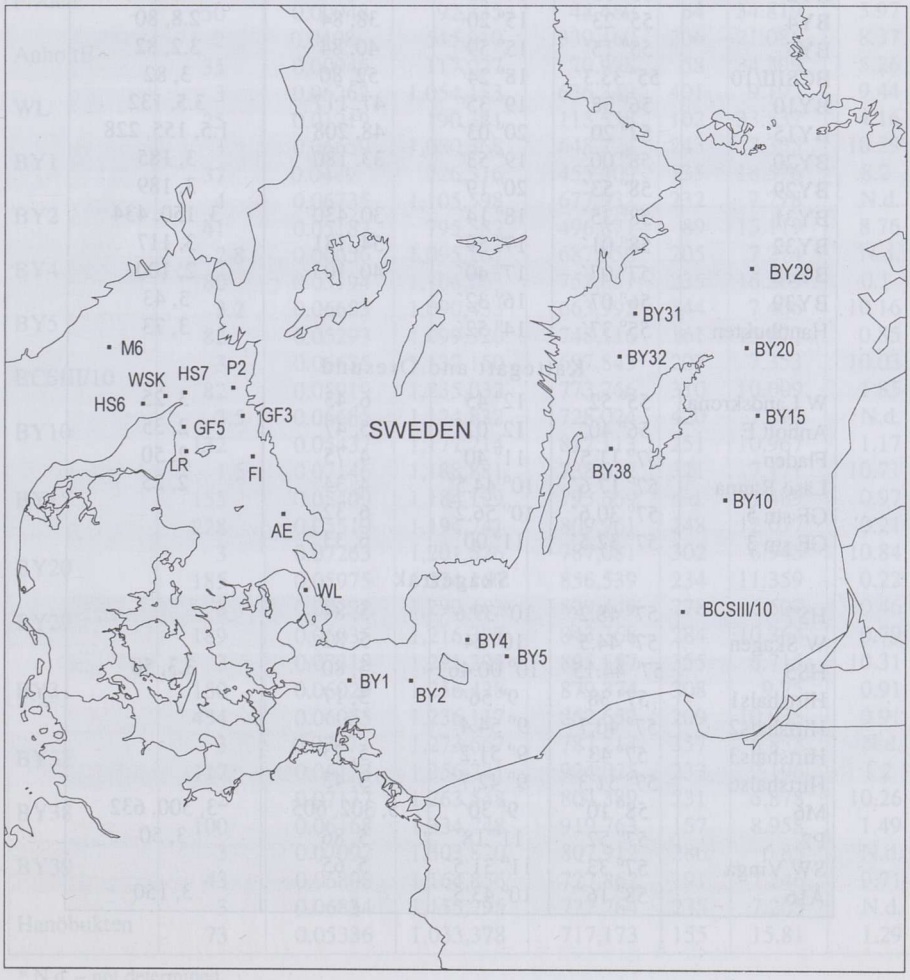

Fig. 1. Location of the sampling points in the Baltic Sea, Kattegatt and Skagerrak

taken. Samples were taken from Niskin bottles connected to CTD and stored in polyethylene bottles in the dark at 4 °C. Plastic gloves were used in all operations with the samples. Some physical-and-chemical characteristics of seawater samples are shown in Table 2.

Table 1. **Water Samples from the Baltic Sea, Kattegatt and Öresund, and Skagerrak Collected for the Molecular Weight Determinations by HPSEC**

Sample	Latitude	Longitude	Sampling depth, m	
			1995, March	1996, April
Baltic Sea				
BY1	55° 00′	13° 18′	31, 46	3, 37
BY2	55° 00′	14° 05′	21, 44	4, 41
BY4	55° 23′	15° 20′	38, 84	2.8, 80
BY5	55° 15′	15° 59′	40, 84	3.2, 82
BCSIII/10	55° 33.3′	18° 24′	52, 80	3, 82
BY10	56° 38′	19° 35′	47, 117	3.5, 132
BY15	57° 20′	20° 03′	48, 208	1.5, 155, 228
BY20	58° 00′	19° 53′	33, 180	3, 185
BY29	58° 53′	20° 19′		3, 189
BY31	58° 35′	18° 14′	30, 430	3, 150, 434
BY32	58° 01′	17° 59′	54, 181	3, 117
BY38	57° 07′	17° 40′	40, 103	2, 100
BY39	56° 07′	16° 32′		3, 43
Hanöbukten	55° 37′	14° 52′		3, 73
Kattegatt and Öresund				
W Landskrona	55° 52′	12° 45′	6, 43	3, 25
Anholt E	56° 40′	12° 07′	5, 47	2, 35
Fladen	57° 11.5′	11° 40′	4, 75	2, 50
Läsö Ränna	57° 17.6′	10° 44.5′	4, 34	2, 25
GF stn 5	57° 30.6′	10° 56.2′	6, 32	
GF stn 3	57° 32.5′	11° 00′	6, 33	
Skagerrak				
HS7	57° 48.2′	10° 39.6′	5, 84	
W Skagen	57° 44.5′	10° 24′	5, 82	
HS5	57° 44.15′	10° 00.46′	5, 80	3.3, 50
Hirtshals1	57° 38′	9° 56′	3, 17	
Hirtshals2	57° 40.7′	9° 54.4′	1	
Hirtshals3	57° 43′	9° 51.2′	5, 73	
Hirtshals6	57° 51.3′	9° 42.7′	5, 43	
M6	58° 10′	9° 30′	5, 302, 605	3, 300, 632
P2	57° 52′	11° 18′	5, 86	3, 50
SW Vinga	57° 33′	11° 31.5′	5, 65	
Å16	58° 16′	10° 43.5′		3, 150

Table 2. **Some Physical-and-Chemical Characteristics of Analyzed Seawater Samples**

Station	Sampling depth, m	Absorbance at 254 nm	SEC peak area, a.u.		TOC, μM	Salinity, mg/L	Oxygen, mg/L
			Ex. 310 nm	Ex. 350 nm			
P2	3	0.02345	267,930	168,015	140	29.302	7.77
	50	0.00986	81,930	50,314	86	34.887	6.2
Å16	3	0.02606	319,810	193,916	184	28.202	7.91
	150	0.00914	65,212	42,771	90	34.991	6.4
M6	3	0.02484	302,543	173,729	173	28.209	7.78
	300	0.00822	58,279	38,204	82	35.071	6.31
	632	0.00853	71,757	46,332	81	34.997	6.78
HS5	3.3	0.02525	292,396	178,517	186	29.734	7.96
	50	0.0098	78,301	36,021	73	34.94	6.78
LR	2	0.03424	452,783	253,059	131	24.37	8
	25	0.01065	124,054	68,571	68	34.634	5.79
Fladen	2	0.037	481,104	286,394	132	22.464	N.d.*
	50	0.00948	92,735	48,480	64	34.819	5.97
AnholtE	2	0.0408	545,910	339,460	206	21.085	8.37
	35	0.00945	117,027	70,998	58	34.399	5.26
WL	3	0.06262	1,054,153	636,349	401	9.19	9.44
	25	0.01219	190,381	113,538	102	33.837	4.16
BY1	3	0.06659	1,080,868	648,224	243	7.335	10.29
	37	0.0489	726,316	455,509	135	18.576	8.2
BY2	4	0.06735	1,105,598	677,932	232	7.278	N.d.
	41	0.05183	795,582	496,871	89	15.119	8.76
BY4	2.8	0.06656	1,095,267	687,650	205	7.274	N.d.
	80	0.05194	1,106,263	752,651	235	16.269	0.1
BY5	3.2	0.06683	1,090,433	663,992	344	7.406	10.16
	82	0.05293	1,099,320	748,116	161	15.877	0.85
BCSIII/10	3	0.06635	1,137,150	697,843	207	7.353	10.03
	82	0.05919	1,235,032	773,766	310	10.009	1.85
BY10	3.5	0.06685	1,124,832	728,024	420	7.297	N.d.
	132	0.05437	1,177,214	812,113	251	10.967	1.17
BY15	1.5	0.07146	1,188,881	738,032	321	7.171	10.71
	155	0.05409	1,184,199	793,669	n.d.	11.489	0.97
	228	0.05519	1,194,741	809,961	248	12.077	0.21
BY20	3	0.07263	1,201,626	787,081	302	6.945	10.84
	185	0.05975	1,224,160	856,539	234	11.359	0.22
BY29	3	0.07292	1,290,463	829,449	278	6.607	10.46
	189	0.06035	1,216,397	843,368	284	10.364	0.79
BY31	3	0.07318	1,271,205	805,557	355	6.715	10.31
	150	0.06029	1,256,428	879,374	308	9.75	0.91
	434	0.06055	1,236,117	852,652	209	10.035	0.91
BY32	3	0.0717	1,273,667	787,214	357	6.853	N.d.
	117	0.06123	1,356,731	924,025	233	9.196	1.2
BY38	2	0.07137	1,263,548	804,389	231	6.878	10.26
	100	0.06168	1,334,748	919,763	157	8.958	1.49
BY39	3	0.07092	1,303,820	807,912	286	6.85	N.d.
	43	0.06808	1,164,856	727,862	191	7.246	9.71
Hanöbukten	3	0.06834	1,155,295	722,764	235	7.205	N.d.
	73	0.05336	1,033,378	717,173	155	15.81	1.29

* N.d. – not determined.

The salinity and oxygen were measured by SMHI according to their standard methods. University of Göteborg, Sweden (Analytical and Marine Chemistry Department) supplied total organic carbon (TOC) data. UV absorbance was determined with a Hewlett Packard 8452A diode array spectrophotometer with quartz cuvette (10-mm path length) at 254 nm against MilliQ water.

Water samples from Estonia were collected from Pirita River, Tallinn, and from Tallinn Bay in 2000/2001. Other river-, lake- and seawater samples from Saaremaa Island were collected in August and October 2000. All samples were taken 0.2 m below the surface. Samples were filtered and stored at 4 °C in dark.

HPSEC Method

In principle, the accuracy of molecular weight determination is achieved only when appropriate standards of the same chemical nature and conformation (in solution), as those of the actual sample are available. Since HS and DOM are a heterogeneous mixture of polyelectrolytes, which change conformation according to the conditions at which they are analyzed and no HS molecular weight standard exist, the results obtained in the present study have to be regarded as nominal molecular weights.

The column was calibrated using polystyrene sulfonate sodium salts (American Polymer Standards Corp.). PSS were selected as calibration compounds because of their reported similarity to HS in respect to their elution properties [2, 19]. PSS have charge density most similar to DOM [8]. However, HS are considered to be more branched and cross-linked than PSS [25]. The PSS standards have narrow relative molecular mass distribution and are characterized by weight-average $\overline{M}_w$ and number-average $\overline{M}_n$ molecular weights and also by peak-average molecular weight $\overline{M}_p$, which corresponds to the molecular weight at the maximum peak height.

For calibration the semi-logarithmic relationship between the logarithm of the PSS molecular weight and the retention time was used. The $\overline{M}_w$ and $\overline{M}_n$ for PSS were 1,430/1,200; 4,800/4,400; 6,500/5,900 and 16,000/14,500. The $\overline{M}_p$ values for used PSS were 1,370; 4,600; 6,200 and 15,200. The following protein standards, obtained from Institute of Chemistry (Tallinn, Estonia) were also used for calibration: immunoglobulin (160,000), ovalbumin (45,000), lactalbumin (18,000) and insulin (5,700).

SEC chromatograms were obtained using the following procedure: a 100-μL (or 54-μL) sample was injected into the size exclusion column, the flow rate of 0.020M phosphate buffer with pH 6.8 was 0.5 (or 1.0) mL/min. The fluorescence intensity at 350/450 and 310/450 nm (excitation/emission) and the absorbance at 254 nm were used for detection. Chromatograms were

recorded and processed by HPLC software Borwin (JMBS Developments, France) or by LabVIEW software (National Instruments, USA). The average $\overline{M}_w$ and $\overline{M}_n$ were calculated using the formulas:

$$\overline{M}_w = \Sigma(h_i * M_i)/\Sigma h_i$$

and

$$\overline{M}_n = \Sigma(h_i)/\Sigma(h_i/M_i)$$

where h_i is the detector output and M_i the molecular weight, both on i-th retention volume [26].

Principal Component Analysis (PCA)

Principal component analysis (PCA) is a well-known chemometrical technique for multivariate data investigation [27]. PCA effects multivariate data reduction by transforming the data into orthogonal components, which are the linear combination of the original variables. As a result, a maximum possible amount of variance within in the initial data set is concentrated in as few principal components as possible. One of the main applications of PCA in natural science is the classification of samples (objects) on the basis of their physical-and-chemical parameters. The discriminating ability of PCA has been proven on numerous data sets combining very different features of samples [e.g. 28–34].

In this work, the data matrix under treatment consisted of 47 seawater samples for which 24 different physical-and-chemical parameters were measured. The following groups of parameters constituted the raw data set: absorbance at 254 and 280 nm; SEC peak area (with fluorescence detection); pH; salinity; results of chemical analysis of sea water, including content of different anions as well as amount of major elements of biological importance (N, P, O, etc.); the total amount of organic carbon; average molecular weights ($\overline{M}_w$ and $\overline{M}_n$) and molecular weight distribution expressed as polydispersity ($\overline{M}_w / \overline{M}_n$). The selection of the data measured is presented in Table 2. The entire data set is available from the authors on request.

Not all the parameters were obtained for each sample during the experimental work. For this reason the raw data table was incomplete. It was decided to fill these empty places by the average for the rest of the column in order to obtain the full data matrix ready for further mathematical analysis. Then, because of its nonhomogeneity (parameters with different orders of magnitude were collected into this data set), the raw data matrix needed for some kind of preprocessing [35]. The mean centering – subtracting of the column average from the each element of this column – and normalization of columns to the length equal to one were chosen for this aim. Then the preprocessed data was subjected to PCA.

Table 3. **The Molecular Weights of Seawater DOM Expressed as Weight- and Number-Average Molecular Weights ($\overline{M}_w$ and $\overline{M}_n$) and Resolution of HPSEC Chromatograms (fluorescence detection at excitation/emission 350/450 nm)**

Station	Depth, m	$\overline{M}_w$	$\overline{M}_n$	Resolution M, in brackets percentage of the total peak area	$\overline{M}_w / \overline{M}_n$
P2	3	932	717	1135 (57); 522 (12); <400 (31)	1.2999
	50	746	627	884 (63); <400 (37)	1.1898
Å16	3	966	734	1292 (40); 687 (14); 552 (11); <400 (34)	1.3161
	150	798	646		1.2353
M6	3	958	745	1282 (40); 715 (13); 464 (47)	1.2859
	300	850	676		1.2574
	632	854	683	1000 (67); <400 (33)	1.2504
HS5	3.3	985	754	1217 (54); 454 (46)	1.3064
	50	810	657		1.2329
LR	2	699	525	897 (47); <400 (53)	1.3314
	25	602	459		1.3115
Fl	2	718	532	936 (44); <400 (56)	1.3496
	50	692	501		1.3812
AE	2	729	528	951 (44); 473 (9); <400 (47, 2 peaks)	1.3807
	35	551	467	638 (67); <400 (33)	1.1799
WL	3	770	560	990 (45); <400 (55)	1.3750
	25	686	500		1.3720
BY1	3	979	734	1242 (49); 468 (51)	1.3338
	37	968	735	1242 (47); 625 (13); 408 (39)	1.3170
BY2	4	976	741	1269 (45); 680 (8); 454 (47)	1.3171
	41	958	737	1138 (62); 405 (38)	1.2999
BY4	2.8	971	749	1265 (45); 641 (20); 401 (36)	1.2964
	80	1018	767		1.3272
BY5	3.2	967	748	1143 (64); 407 (36)	1.2928
	82	963	751		1.2823
BCSIII/10	3	957	741		1.2915
	82	978	749	1157 (63); 406 (37)	1.3057
BY10	3.5	986	754	1330 (39); 553 (61)	1.3077
	132	983	747	1260 (48); 642 (12); 423 (41)	1.3159
BY15	1.5	974	759	1145 (65); 410 (35)	1.2833
	155	953	741	1151 (58); 430 (42)	1.2861
	228	979	748		1.3088
BY20	3	898	617		1.4554
	185	909	621		1.4638
BY29	3	914	625		1.4624
	189	903	619		1.4588
BY31	3	930	631	1236 (41); 516 (23); <400 (36)	1.4739
	150	927	624	1162 (50); 458 (14); <400 (36)	1.4856
	434	928	625	1193 (46); <400 (54)	1.4848
BY32	3	934	638		1.4639
	117	919	619		1.4847
BY38	2	949	634	1112 (63); <400 (37)	1.4968
	100	965	642	1135 (62); <400 (38)	1.5031
BY39	3	960	638	1179 (53); <400 (47)	1.5047
	43	997	649		1.5362
Hanöbukten	3	956	637		1.5008
	73	996	636		1.5660

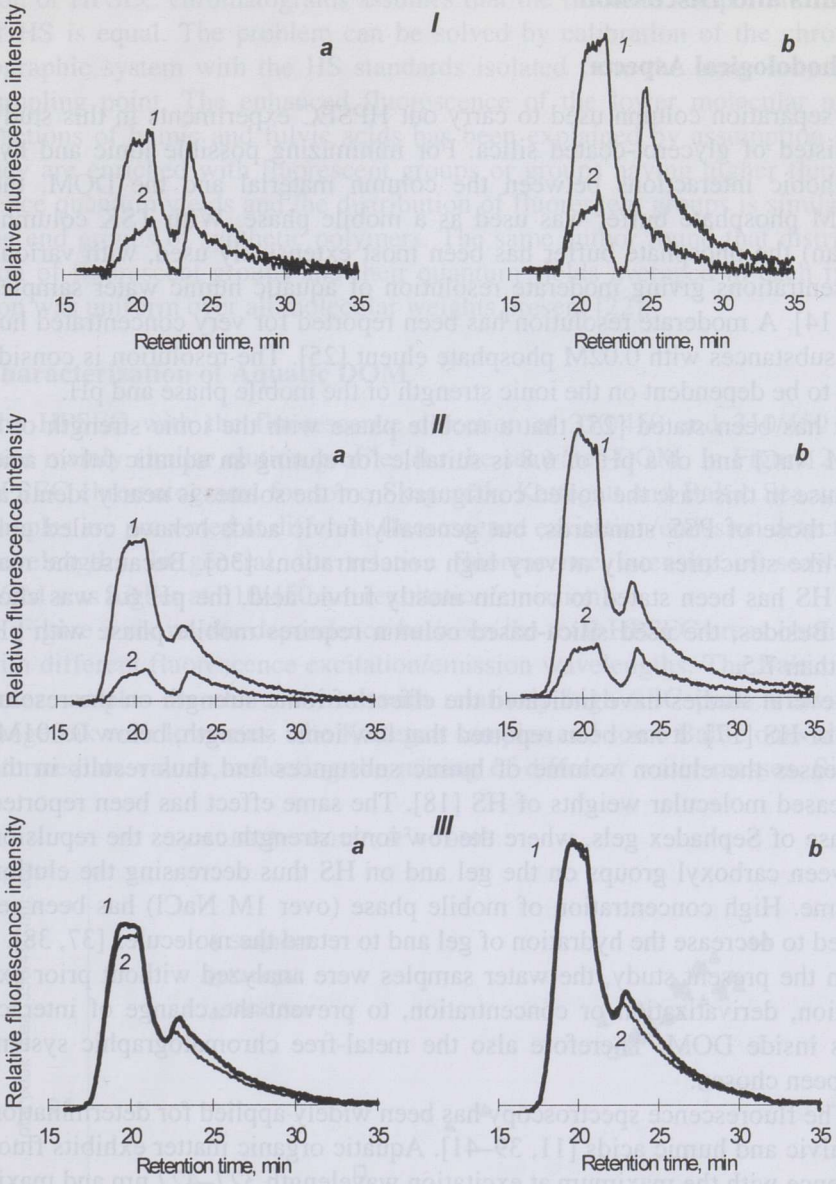

Fig. 2. HPSEC chromatograms of seawater DOM from (*I*) Skagerrak (station M6), (*II*) Kattegatt (station WL), and (*III*) the Baltic Sea (station BY15).
Column: BIOSEP-SEC-S 2000 (300 × 7.5 mm); eluent: 0.02M phosphate buffer, pH 6.8; flow rate 0.5 mL/min; fluorescence detection/excitation emission (*a*) 350/450 and (*b*) 310/450 nm.
I: *1* – salinity 28 mg/L, depth 3 m; *2* – salinity 35 mg/L, depth 632 m
II: *1* – salinity 9 mg/L, depth 3 m; *2* – salinity 34 mg/L, depth 25 m
III: *1* – salinity 7 mg/L, depth 1.5 m; *2* – salinity 12 mg/L, depth 228 m

Results and Discussion

Methodological Aspects

The separation column used to carry out HPSEC experiments in this study consisted of glycerol-coated silica. For minimizing possible ionic and hydrophobic interactions between the column material and the DOM, the 0.02M phosphate buffer was used as a mobile phase. With TSK columns (Japan) the phosphate buffer has been most extensively used, with various concentrations giving moderate resolution of aquatic humic water samples [10, 14]. A moderate resolution has been reported for very concentrated humic substances with 0.02M phosphate eluent [25]. The resolution is considered to be dependent on the ionic strength of the mobile phase and pH.

It has been stated [25] that a mobile phase with the ionic strength of a 0.1M NaCl and of a pH of 6.8 is suitable for eluting an aquatic fulvic acid because in this case the coiled configuration of the solutes is nearly identical with those of PSS standards, but generally fulvic acids behave coiled colloid-like structures only at very high concentrations [36]. Because the marine HS has been stated to contain mostly fulvic acid, the pH 6.8 was chosen. Besides, the used silica-based column requires mobile phase with pH less than 7.5.

Several studies have indicated the effect of ionic strength on the resolution of HS [17]. It has been reported that low ionic strength, below 0.001M, decreases the elution volume of humic substances and thus results in the increased molecular weights of HS [18]. The same effect has been reported in case of Sephadex gels, where the low ionic strength causes the repulsion between carboxyl groups on the gel and on HS thus decreasing the elution volume. High concentration of mobile phase (over 1M NaCl) has been reported to decrease the hydration of gel and to retard the molecules [37, 38].

In the present study, the water samples were analyzed without prior extraction, derivatization or concentration, to prevent the change of interactions inside DOM. Therefore also the metal-free chromatographic system has been chosen.

The fluorescence spectroscopy has been widely applied for determination of fulvic and humic acids [11, 39–41]. Aquatic organic matter exhibits fluorescence with the maximum at excitation wavelength 327–427 nm and maximum emission at 450–520 nm [42]. The fluorescence detection at two excitation/emission wavelengths (310/450 and 350/450 nm) has been used in the present study. The 350/450 nm is commonly used to detect HS. The excitation at 350 nm and emission at 450 nm have been suitable for chromophores characteristic of Baltic Sea HS [43]. The 310/450 nm has been chosen because of the reported fluorescence maximum of HS for marine shallow transitional water samples [40].

The problem of using fluorescence detection is that the fluorescence per unit mass of HS can vary within several orders of magnitude. The integra-

tion of HPSEC chromatograms assumes that the fluorescence per unit mass of HS is equal. The problem can be solved by calibration of the chromatographic system with the HS standards isolated from the same source or sampling point. The enhanced fluorescence of the lower molecular mass fractions of humic and fulvic acids has been explained by assumption that they are enriched with fluorescent groups or groups having higher fluorescence quantum yields and the distribution of fluorescent groups is similar to the end groups of synthetic polymers. The same author found that distribution of fluorescent groups and their quantum yields averaged in each fraction was uniform over all molecular weights present [22].

Characterization of Aquatic DOM

The HPSEC with the fluorescence detection at 350/450 and 310/450 nm gave mainly similar elution profiles for the seawater DOM. In Figure 2 the HPSEC chromatograms for some Skagerrak, Kattegatt and Baltic Sea water samples are presented at different fluorescence excitation/emission detection wavelengths. In general, the relative fluorescence intensity of seawater DOM was higher at 310/450 nm (excitation/emission).

Figure 3 shows the dependence between the total HPSEC areas obtained with different fluorescence excitation/emission wavelengths. The Baltic Sea water samples are characterized with relatively high SEC peak areas and Skagerrak with low ones. The Kattegatt samples and some Baltic ones show intermediate values, reflecting the mixing of different water masses. Since

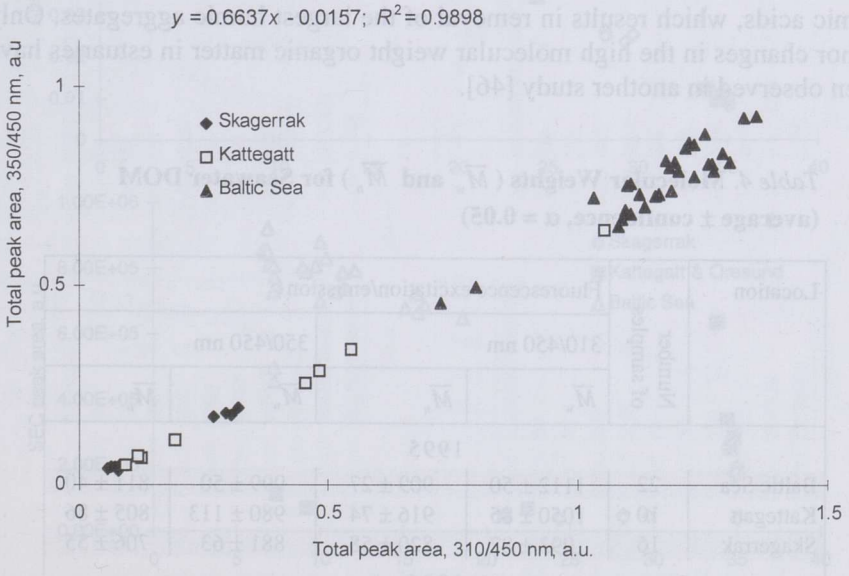

Fig. 3. The dependence between the total HPSEC peak areas of seawater DOM from the Baltic Sea, Kattegatt and Skagerrak obtained with fluorescence detection at excitation/emission wavelengths 350/450 and 310/450 nm

subpeaks were present in the HPSEC chromatograms, separation of SEC peak into several molecular weight fractions was possible.

Table 3 reports the average molecular weights ($\overline{M}_w$ and $\overline{M}_n$) and polydispersity for investigated seawater samples. The results where $\overline{M}_w$ of DOM is less than 400 are uncertain because of the lack of suitable PSS standards for this region. The presence of low-molecular mass acids such as succinic and malonic, with molecular mass 170, has been explained by degradation of fulvic acids. The aliphatic acids C_{15}–C_{17} with molecular mass 310 have been identified from HPSEC chromatograms of the natural waters with DOC detection [44]. The polydispersity ($\overline{M}_w / \overline{M}_n$) changed from 1.19–1.57, which is in accordance of values reported for different aquatic fulvic acids and dissolved organic matter (1.5–1.9) [45]. The polydispersity is close to 1 for the more homogeneous humic matter.

The chemical and microbial degradation of humic fraction has been reported to be the source for more homogeneous organic matter [14]. Average molecular weights (expressed as $\overline{M}_w$ and $\overline{M}_n$) for all samples are given in Table 4.

The $\overline{M}_w$ values exceed the $\overline{M}_n$ being close to 1000. The influence of salinity on the DOM molecular weight expressed as $\overline{M}_w$ is presented in Fig. 4. The average molecular weight was relatively constant despite of salinity changes. At the same time absorbance and SEC peak area clearly showed the dependence on salinity (Fig. 5). Both parameters decreased with increasing salinity. The increasing salinity (ionic strength) causes flocculation of humic acids, which results in removal of the largest humic aggregates. Only minor changes in the high molecular weight organic matter in estuaries have been observed in another study [46].

Table 4. **Molecular Weights ($\overline{M}_w$ and $\overline{M}_n$) for Seawater DOM (average ± confidence, $\alpha = 0.05$)**

Location	Number of samples	Fluorescence excitation/emission			
		310/450 nm		350/450 nm	
		$\overline{M}_w$	$\overline{M}_n$	$\overline{M}_w$	$\overline{M}_n$
1995					
Baltic Sea	22	1112 ± 50	909 ± 27	999 ± 50	811 ± 40
Kattegatt	10	1050 ± 85	916 ± 74	980 ± 113	805 ± 86
Skagerrak	16	993 ± 87	820 ± 58	881 ± 63	706 ± 55
1996					
Baltic Sea	30	1216 ± 52	794 ± 57	1107 ± 41	759 ± 60
Skagerrak	9	1198 ± 193	886 ± 178	1042 ± 118	773 ± 114
Kattegatt	8	1145 ± 33	856 ± 46	810 ± 98	582 ± 99

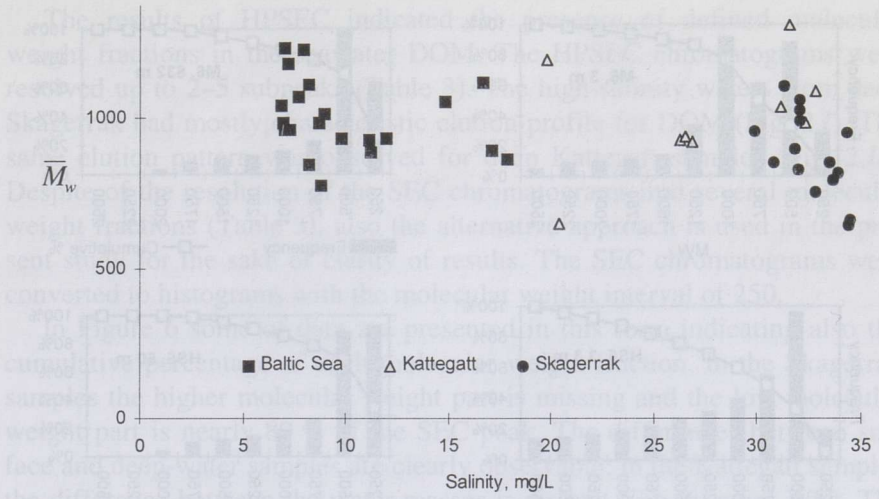

Fig. 4. DOM weight-average molecular weight ($\overline{M}_w$) changes with salinity in the Baltic Sea, Kattegatt and Skagerrak

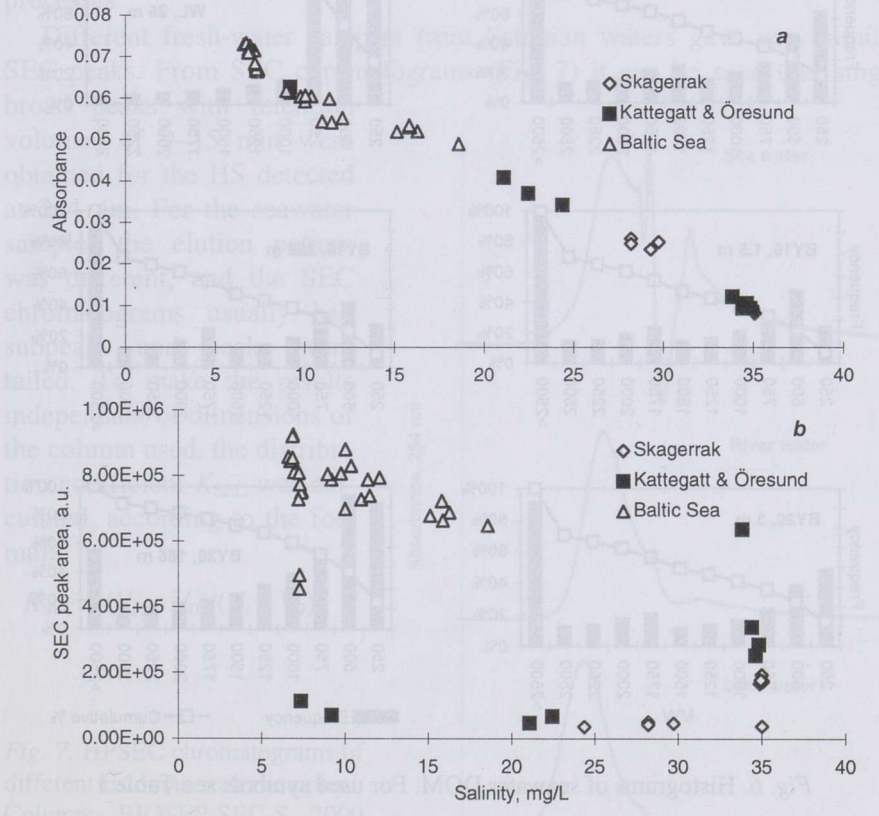

Fig. 5. Dependence of DOM characteristics on salinity: *a* – absorbance, *b* – HPSEC peak area

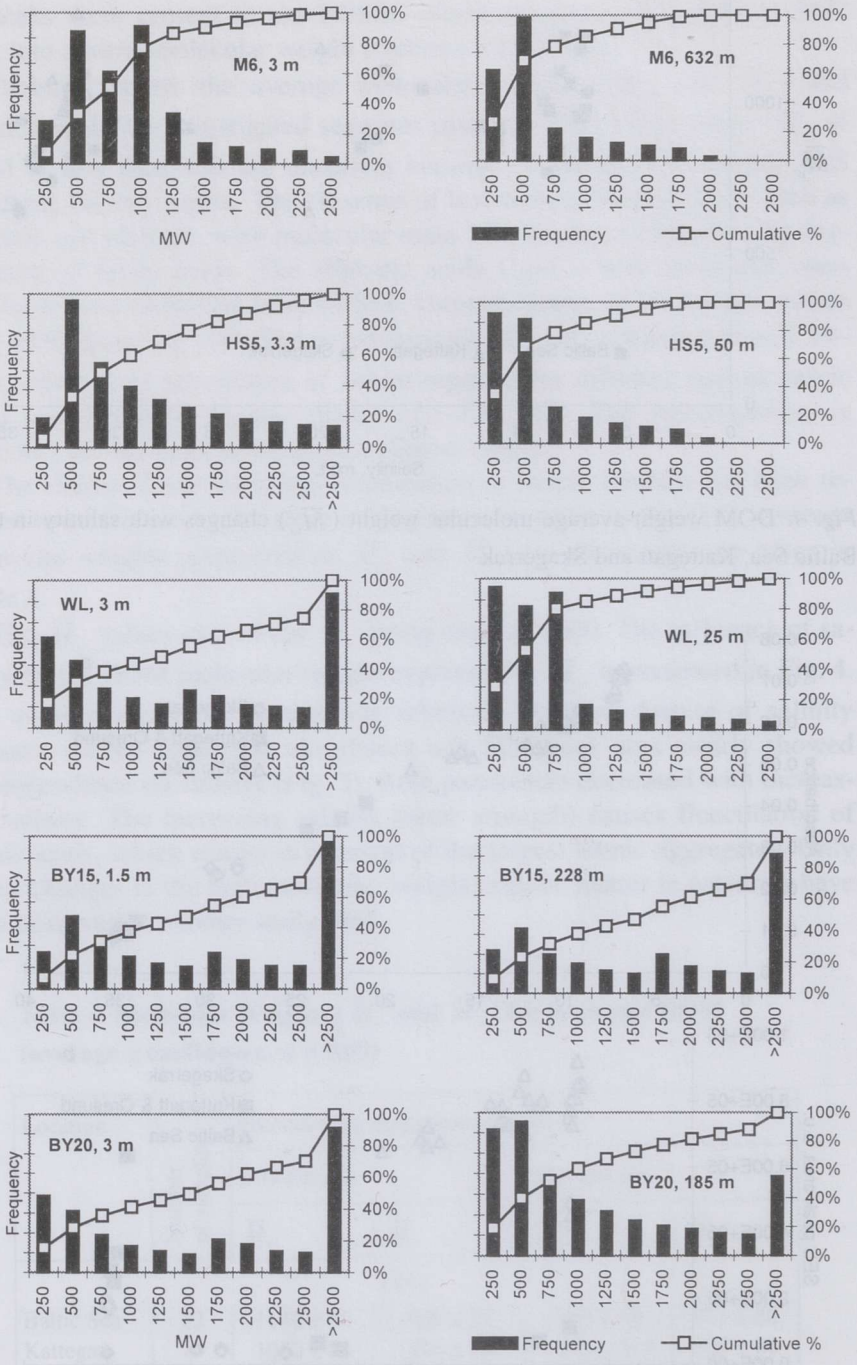

Fig. 6. Histograms of seawater DOM. For used symbols see Table 1

The results of HPSEC indicated the presence of defined molecular weight fractions in the seawater DOM. The HPSEC chromatograms were resolved up to 2–5 subpeaks (Table 3). The high-salinity waters from deep Skagerrak had mostly characteristic elution profile for DOM (Fig. 2,*I*). The same elution pattern was observed for deep Kattegatt samples (Fig. 2,*II*). Despite of the resolution of the SEC chromatograms into several molecular weight fractions (Table 3), also the alternative approach is used in the present study for the sake of clarity of results. The SEC chromatograms were converted to histograms with the molecular weight interval of 250.

In Figure 6 some of data are presented in this form indicating also the cumulative percentage of each molecular weight fraction. In the Skagerrak samples the higher molecular weight part is missing and the low molecular weight part is nearly 80 % of the SEC peak. The differences between surface and deep-water samples are clearly observable. In the Kattegatt samples the difference between the water masses is distinct (Fig. 6, point WL). The water with low organic matter concentration flows into the Baltic Sea and the organic-rich Baltic Sea water in the surface flows out. Baltic Sea water samples show relatively similar histograms thus reflecting efficient mixing processes.

Different fresh-water samples from Estonian waters gave very similar SEC peaks. From SEC chromatograms (Fig. 7) it can be seen that single broad peaks with retention volumes of 9–15 min were obtained for the HS detected at 254 nm. For the seawater samples the elution pattern was different, and the SEC chromatograms usually had subpeaks and peaks were tailed. To make the results independent of dimensions of the column used, the distribution coefficient K_{SEC} was calculated, according to the formula

$$K_{SEC} = (V_R - V_0)/(V_t - V_0)$$

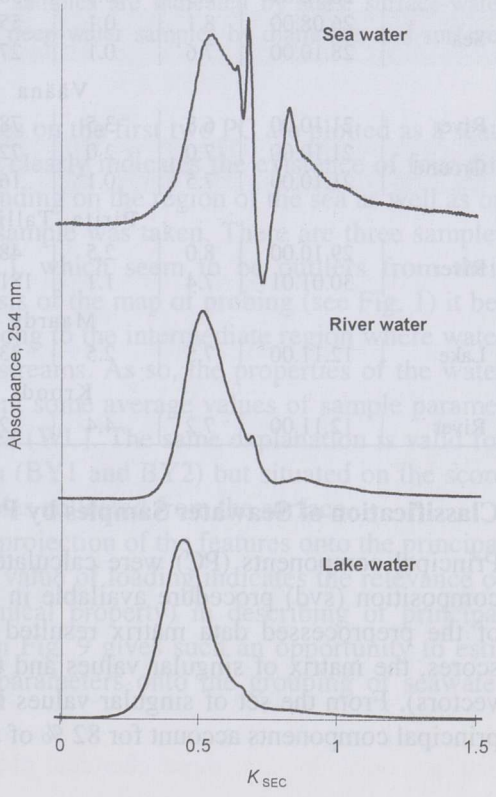

Fig. 7. HPSEC chromatograms of different Estonian water samples. Column: BIOSEP-SEC-S 2000 (300 × 7.5 mm); eluent: 0.02M phosphate buffer, pH 6.8

where V_R is the retention volume of the solute;
V_t is the total permeation volume of the column;
V_0 is the exclusion volume of the column.

The HS in all analyzed samples were eluted from SEC column within the separation range.

The obtained SEC chromatograms are clearly related to the origin of HS. Allochthonous HS derived from terrestrial organic matter (lake- and river-water samples in the present study) are characterized with one broad peak, lower K_{SEC} values and higher molecular weight. Autochthonous HS derived from *in situ* biological production (seawater samples) are characterized with substructured peaks, higher K_{SEC} values and lower molecular weight (Fig. 7; Table 5).

Table 5. **Average Molecular Weights ($\overline{M}_w$ and $\overline{M}_n$), Polydispersities ($\overline{M}_w / \overline{M}_n$) and KSEC Values of Aquatic DOM from Different Estonian Water Samples (SEC column calibration with proteins)**

Water type	Sampling date	pH	HS, mg/L	$\overline{M}_n$	$\overline{M}_w$	$\overline{M}_w / \overline{M}_n$	K_{SEC}
			Koigi, Saaremaa				
Lake	26.08.00	6.7	16.2	1646	6223	3.78	0.479
	28.10.00	6.3	16.2	1500	9423	6.28	0.443
Sea	26.08.00	8.1	0.1	551	4915	8.92	0.525
	28.10.00	7.6	0.1	271	3773	13.92	0.547
			Vääna				
River	21.10.00	6.8	3.5	786	6127	7.80	0.473
Ground	21.10.00	7.0	2.0	722	4993	6.92	0.476
	21.10.00	7.5	0.1	163	3610	22.15	0.451
			Pirita, Tallinn				
River	29.10.00	8.0	2.5	485	4537	9.36	0.515
	30.01.01	7.4	1.1	1815	5405	2.98	0.511
			Maardu				
Lake	12.11.00	7.5	2.5	331	2542	7.68	0.537
			Kroodi				
River	12.11.00	7.2	4.4	224	2083	9.30	0.569

Classification of Seawater Samples by PCA

Principal components (PC) were calculated through the singular value decomposition (svd) procedure available in MATLAB environment. The svd of the preprocessed data matrix resulted in three matrices: the matrix of scores, the matrix of singular values and the matrix of loadings (i.e. eigenvectors). From the set of singular values it was estimated that the first five principal components account for 82 % of a total data variance.

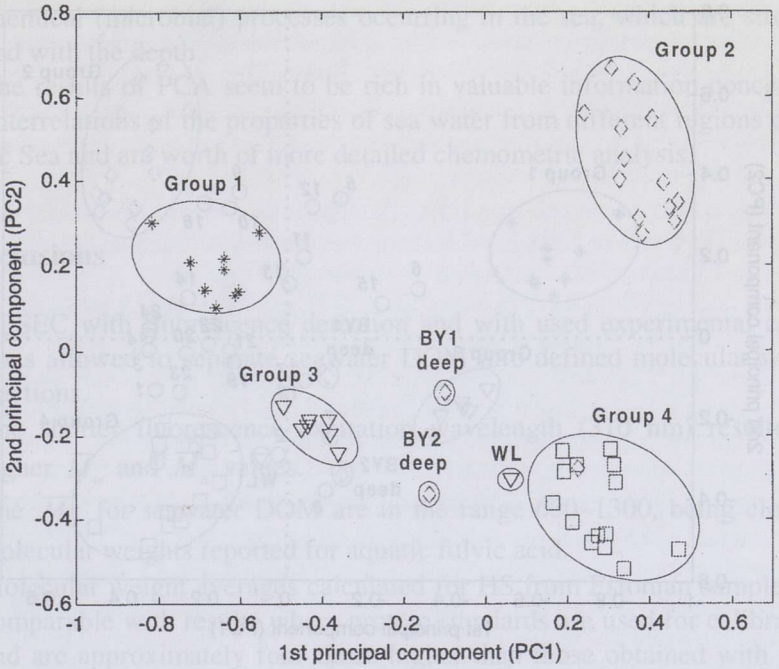

Fig. 8. Score plot derived from physical-and-chemical characteristics of seawater. Skagerrak and Kattegatt deep-water samples are indicated by stars, surface-water samples by triangles, the Baltic Sea deep-water samples by diamonds and surface-water samples by squares

The scores of the water samples on the first two PC are plotted as a scatter diagram in Fig. 8. This figure clearly indicates the existence of four different groups in the data set depending on the region of the sea as well as on the depth from which the water sample was taken. There are three samples (BY1 deep, BY2 deep and WL), which seem to be outliers from their groups. However, from the analysis of the map of probing (see Fig. 1) it became clear that these samples belong to the intermediate region where water masses are mixed due to strong streams. As so, the properties of the water samples taken from there represent some average values of sample parameters from different parts of the sea (WL). The same explanation is valid for the samples taken from the depth (BY1 and BY2) but situated on the score plot (see Fig. 8) close to the samples collected from the surface.

The loading plot provides the projection of the features onto the principal components. It is known that the value of loading indicates the relevance of each variable (physical-and-chemical property) in describing of principal components. The biplot shown in Fig. 9 gives such an opportunity to estimate the influence of different parameters onto the grouping of seawater samples.

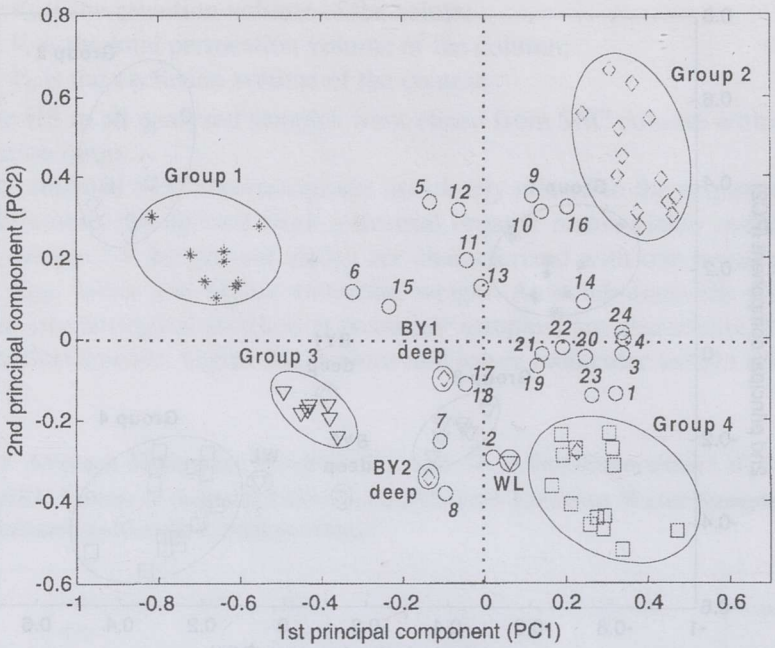

Fig. 9. Biplot for the first two principal components based on physical-and-chemical characteristics of sea water. The meaning of symbols is the same as in Fig. 8. The numbered circles are related to loadings

From this plot the discriminating ability of variables *6* (salinity), *1*, *3* and *4* (absorbance and SEC peak area) and *20–22* (average molecular weights) for separation of samples into different groups along the PC1 axes (46 % of the total variability) is evident. This "geographical" grouping is mainly based on different content of organic matter in seawater samples from the regions close to the Atlantic Ocean and the inner parts of the Baltic Sea.

It could be noticed that the variables *1*, *3* and *4* are highly correlated with the features *20–21* as well as with the total content of organic carbon (variable *23*). The possible reason is that absorbance and fluorescence are due to chromophores and fluorophores existing in HS molecules and the molecular weights are calculated using the respective detectors signal according to formulas presented under experimental. The salinity of seawater is higher in Skagerrak and Kattegatt regions in comparison with the Baltic Sea, which also influence this kind of clustering.

The distribution of samples onto the groups depending on the sampling depth (along the second PC axis) is caused by the variables with high loadings concerning the PC2 (43 % of the total variability). In our case such parameters as the content of nitrogen in different forms (variables *11–13*), phosphorus (variables *9* and *10*) and oxygen (variable *8*) collectively define a loading pattern characteristics of deep-water samples as opposed to the surface ones. In fact, these chemical elements are involved into different

biochemical (microbial) processes occurring in the sea, which are strongly related with the depth.

The results of PCA seem to be rich in valuable information concerning the interrelations of the properties of sea water from different regions of the Baltic Sea and are worth of more detailed chemometric analysis.

Conclusions

- HPSEC with fluorescence detection and with used experimental conditions allowed to separate seawater DOM into defined molecular weight fractions.
- The shorter fluorescence excitation wavelength (310 nm) resulted in higher $\overline{M}_w$ and $\overline{M}_n$ values.
- The $\overline{M}_w$ for seawater DOM are in the range 600–1300, being close to molecular weights reported for aquatic fulvic acid.
- Molecular weight averages calculated for HS from Estonian samples are comparable with results where protein standards are used for calibration, and are approximately four times higher than those obtained with polystyrene sulfonate standards.
- Principal component analysis on the data set containing the physical-and-chemical parameters of seawater samples has shown a very good clustering depending on the region of the Baltic Sea and the depth from which the samples originate. The analysis of the features causing this separation is possible.

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