Environ. Sci. Technol. **2003**, *37,* 2477−2485

Molecular Weight Characteristics of Humic Substances from Different Environments As Determined by Size Exclusion Chromatography and Their Statistical Evaluation

IRINA V. PERMINOVA,*,†
FRITZ H. FRIMMEL,‡
ALEXEY V. KUDRYAVTSEV,†
NATALIA A. KULIKOVA,§
GUDRUN ABBT-BRAUN,‡
SEBASTIAN HESSE,‡ AND
VALERY S. PETROSYAN†

Department of Chemistry, Lomonosov Moscow State University, 119992 Moscow, Russia, Divison of Water Chemistry, Engler-Bunte Institute, University of Karlsruhe, Engler-Bunte-Ring 1, 76131 Karlsruhe, Germany, and Department of Soil Science, Lomonosov Moscow State University, 119992 Moscow, Russia

Recorded molecular weights (MWs) for humic substances (HS) range from a few hundred to millions of daltons. For purposes of defining HS as a specific class of chemical compounds, it is of particular importance to ascertain if this broad range of MWs can be attributed to actual variability in molecular properties or is simply an artifact of the analytical techniques used to characterize HS. The main objectives of this study were (1) to establish if a preferential range of MWs exists for HS and (2) to determine any consistent MW properties of HS. To reach the goal, we have undertaken an approach to measure under standardized conditions the MW characteristics of a large set of HS from different natural environments. Seventy-seven humic materials were isolated from freshwater, soil, peat, and coal, such that each possessed a different fractional composition: humic acid (HA), fulvic acid (FA), and a nonfractionated mixture of HA and FA (HF). Size exclusion chromatography (SEC) was used as the analytical technique to determine molecular weight characteristics. The MW distributions were characterized by number (M_n) and weight (M_w) average MW, and by polydispersity. The complete range of M_w values varied within 4.7−30.4 kDa. The maximum M_w values were observed for peat HF and soil HA, whereas the smallest weights were measured for river water HF. Maximum values of polydispersity (3.5−4.4) were seen for peat HF and soil HA, while much lower values (1.6−3.1) were found for all preparations isolated with XAD-resins. Statistical evaluation showed consistent M_w and M_n variations with the HS source, while polydispersity was mostly a function of the isolation procedure used. A conclusion was made that HS have a preferential range of MW values that could characterize them as a specific class of chemical compounds.

Introduction

Humic substances (HS) are natural organic compounds comprising 50−90% of the organic matter of peat, lignites, and sapropels as well as of the nonliving organic matter of soil and water ecosystems (*1, 2*). Being the products of stochastic synthesis, HS are natural supramolecular polymers having elemental compositions that are nonstoichiometric and structures which are irregular and heterogeneous (*3*). Despite that there seem to be typical ranges for the kind and relative abundance of structural features. As a result, the conceptual molecular model of HS has evolved from a molecule to a molecular assembly. This new molecular model is characterized by properties that are not single valued but manifest a measurable distribution. Clearly, a single structural formula cannot be ascribed to any sample of HS; consequently, current definitions and classifications of HS are based on isolation procedures rather than on specific molecular features (*2*, *4*). To develop a definition of HS as a class of chemical compounds, the molecular properties of HS from a wide variety of natural environments are to be quantified and statistically evaluated. The latter is to reveal (1) whether the molecular parameters of HS are characterized with a preferential range of values that would be indicative of a class of chemical compounds and (2) whether the molecular properties of HS change regularly in accordance with the particular features of their formation that would facilitate development of classification rules for HS.

Until recently, quantitative information on the natural variability of molecular properties of HS has been scarce and somewhat contradictory. This is particularly true for molecular weight (MW). Recorded MWs for HS range from a few hundred to millions of daltons (*4*). To define HS as a specific class of chemical compounds, it is necessary to determine if the observed MW range can be attributed to variability in molecular properties, or whether it is simply an artifact of the analytical techniques used to characterize HS. The latter is of particular importance for size exclusion chromatography (SEC), the most frequently applied technique for analyzing HS (*5*, *6*). Reported MW values of HS from different sources as determined by SEC are summarized in Table 1. They vary from 0.3 to 700 kDa. Due to the supramolecular structure and polyelectrolytic properties of HS, application of SEC is likely to produce artifacts (*5*, *6*, *18*); hence, particular care should be exercised when interpreting SEC results. To highlight this problem for HS, a term apparent molecular weight (AMW) was introduced in the literature (*19*, *20*) as an indication of the strong dependence of the determined MW values on the experimental conditions used.

Nonsize exclusion effects (electrostatic repulsion and specific adsorption) contribute the greatest variability among measured MW values (*19*); however, such effects are suppressed when a proper mobile phase is used [e.g. 0.1 M NaCl, 0.002 M KH_2PO_4, and 0.002 M Na_2HPO_4 buffered to pH 6.8 (*21*) or 0.028 M phosphate buffer (*22*)]. Another source of MW variability is associated with a lack of proper calibration standards (*9*, *22*). This problem was the subject of particular consideration in our previous publication (*23*). We showed then, that out of the four polymer series used—polyacrylates, poly(methyl methacrylate)s, polysterenesulfonates, and polydextranes, the least charged polysterenesulfonates and

---

* Corresponding author phone/fax: +7(095)9395546; e-mail: iperm@org.chem.msu.ru.

† Department of Chemistry, Lomonosov Moscow State University.

‡ University of Karlsruhe.

§ Department of Soil Science, Lomonosov Moscow State University.

10.1021/es0258069 CCC: $25.00

TABLE 1. SEC-Determined Molecular Weight Characteristics of Humic Substances from Different Sources, As Reported in the Literature

HS sample	M_n, kDa	M_w, kDa	M_w/M_n	ref
Aquatic FA				
Missouri River, U.S.A.[a]	0.84	1.46	1.74	(*7*)
Yakima River, U.S.A.[a]	0.8	1.56	1.95	(*7*)
Suwannee River, U.S.A.[a]	1.36	2.31	1.7	(*7*)
Ohio River, U.S.A.[a]	0.71	1.33	1.9	(*7*)
Coal Creek, U.S.A.[a]	1.18	2.23	1.9	(*7*)
Minnesota groundwater[a]	0.64	1.00	1.6	(*7*)
Ogeechee River, U.S.A.[a]	0.81	1.64	2.04	(*8*)
McDonald's Branch, U.S.A.[a]	1.28	2.15	1.67	(*8*)
Lake Mekkojaervi, Finland, four samples[a]	5.24, 3.45, 3.63, 5.4	8.92, 9.93, 8.74, 16.73	1.7, 2.87, 2.4, 3.1	(*9*)
Lake Savojaeri, two samples[a]	4.31, 1.79	6.59, 4.75	1.53, 2.65	(*9*)
Nordic Ref FA[a]	3.87	6.10	1.58	(*9*)
surface waters of Sweden, 16 samples[a]	1.15−1.50	1.25−1.95	n.a.	(*10*)
Goeta River, Sweden, three samples[b]	1.21, 0.98, 1.06	2.67, 2.74, 2.79	2.21, 2.80, 2.64	(*11*)
Moscow River, Russia[c]	n.a.	0.3	n.a.	(*12*)
Lake Celyn, UK[d]	n.a	5	n.a	(*13*)
Aquatic HA and HF				
Nordic Ref HA[a]	5.06	19.44	3.84	(*9*)
Lake Mekkojaervi HA, Finland, 4 samples[a]	7.82, 4.27, 5.28, 6.64	20.28, 23.81, 28.63, 28.91	2.59, 5.57, 5.43, 4.35	(*9*)
Lake Savojaeri HA, Finland[a]	4.96, 3.58	20.97, 18.5	4.23, 5.17	(*9*)
Goeta River HA, Sweden[b]	1.2, 1.14	3.16, 3.15	2.63, 2.76	(*11*)
Everglades F1 HF, U.S.A.[a]	1.05	1.83	1.74	(*8*)
Everglades WCA HF, U.S.A.[a]	0.92	1.45	1.58	(*8*)
Aquatic DOM				
Suwannee River, U.S.A.[a]	1.33	2.19	1.6	(*7*)
Lake Fryxell, U.S.A.[a]	7.13	1.08	1.5	(*7*)
Lake Michigan porewater[a]	0.55, 0.77, 0.74	0.85, 1.12, 1.1	1.6, 1.5, 1.6	(*7*)
Utsjoki River, Finland[a]	0.63	4.48	7.1	(*9*)
Lake Mekkojaervi, Finland[a]	3.17, 3.78, 2.99, 2.93	8.60, 9.35, 12.57, 17.77	2.7, 2.5, 4.2, 6.1	(*9*)
Lake Savojaeri, Finland[a]	2.25, 1.04	11.63, 9.5	5.2, 9.1	(*9*)
Sediments FA, Goeta River[b]	1.20, 1.88	3.07, 3.99	2.56, 2.13	(*11*)
Aldrich HA[a]	1.63	4.1	2.5	(*7*)
soil FA and HA	range of molecular weights, kDa			
peat soil HA[c]	4−22			(*14*)
sod podzolic FA[d]	1−5			(*15*)
sod-podzolic HA[d]	100−700			(*16*)
soil (n.d.)[e]	100−200			(*17*)

[a] Polystyrenesulfonates are used as calibration standards. [b] Dimethylformamide (DMFA) is used as a mobile phase. [c] Proteins are used as calibration standards. [d] Calibration standards are not known. [e] Polydextrans are used as calibration standards.

nonionogenic polydextrans tend to be the calibration standards most adequate for HS under conditions that compensate for the effects of nonsize exclusion. Additional source of variability in MW values can be a difference in UV molar absorptivity and in a carbon content per molecule causing distortion of UV- or DOC-profiles of elution curves of HS (*24*). Finally, the aggregation of HS in the concentrated or acidified solutions as well as specific adsorption onto the gel can influence greatly the measured MW values. Problems of SEC application for the analysis of HS were in details addressed in a special issue of Soil Science (1999, V. 164, No 11).

To account for the above limitations of SEC we have undertaken an approach to measure MW distribution (MWD) of HS under standardized conditions specifically designed to compensate for nonsize exclusion effects. This implies the following: (1) use of electrolyte (0.028 M phosphate buffer) as a mobile phase—to eliminate electrostatic repulsion; (2) use of a buffer at pH 6.8 as a mobile phase—to prevent specific adsorption of HS onto the gel by keeping high dissociation degree of HS macromolecules; (3) use of diluted solutions of HS (2−4 mg of C/L) at pH 6.8 for analysis—to prevent aggregation of HS in solution and specific adsorption onto the gel, (4) use of a mobile phase for equilibration of the HS sample prior to analysis—to avoid an appearance of the "salt peaks" on the elution curve. Tandem UV-DOC detection was used to ascertain possible distortions of elution curves of HS. Two different calibration standards—nonionogenic polydextrans (PDX) and acidic polystyrenesulfonates (PSS), shown previously to best characterize SEC-behavior of HS under above conditions (*23*), were used to enumerate MW of HS. Given the difference in partial charge, the obtained estimates define for HS the upper and the lower bounds of molecular weight; thus, $MW_{PSS} < MW_{HS} < MW_{PDX}$.

To establish a range of preferential MW values of HS the above approach was applied to a large set of HS representing different natural environments. The number (M_n) and weight (M_w) average MW and the M_w/M_n ratio (polydispersity) (*25*) were calculated for each of the 77 samples studied. Following the approach developed by Rice and MacCarthy (*26*) and by Visser (*27*), a statistical evaluation of the data was conducted.

The main objectives of this study were (1) to determine whether a preferential range of MWs exists for HS and (2) to establish the MW limits intrinsic to HS and any consistent MW changes with respect to source and fractional composition.

Materials and Methods

Humic materials were isolated from different natural and anthropogenically influenced sources. They were either fractionated into humic acids (HA) and fulvic acids (FA) or used as a whole (nonfractionated mixture of HA and FA (HF)). The isolation technique and designation of the samples are given below.

Aquatic HA, FA, and HF (AHA, AFA, and AHF) were isolated from different locations in Russia and Germany. The river, marsh, and swamp waters are further identified by the use of an additional letter R (river), M (marsh), or S (swamp) and by the initial of the water body name. River water AHF were isolated from the rivers Moscow, Kad′, North Dvina (RMX, RKX, RND3) according to established methods (*28*). In brief, filtered water samples were acidified to pH 2 and passed through the XAD-2 resin. The sorbed fraction was recovered by back elution with 0.1 M NaOH, desalted on cation-exchange resin, and freeze-dried. The same procedure was applied for the isolation of AHF from the marsh (MMu8) and swamp waters (SMu4, SMu7 and SMu8) on the Island Mud'yug in the White Sea, nearby Arkhangelsk (SND11) and nearby Moscow (SSh2). All above locations are in Russia. Another river water sample AHF-RUL was provided by E. Gjessing.

Swamp water AFA and AHA were isolated from the brown water lake Hohlohsee (SHO6, SHO10, SHO13) in Schwarzwald (Germany) and from the Brunnenseemoor in the South of Germany (SBM7, SBM8, SBM9, SBM10, SBM11, SBM12). The isolation technique involved the use of XAD-8 resin for SHO samples (*29*) and of XAD-2 resin for SBM samples (*30*). Filtered water samples were acidified to pH 2 and passed through the XAD resin. The sorbed fraction is recovered by back elution with 0.1 M NaOH. Acidification of the extract to pH 2 led to the precipitation of HA. After the HA fraction was filtered off, the acidified solution was passed again through the XAD-resin. The adsorbed FA was eluted with 0.2 M NaOH and desalted on a cation-exchange resin. The same procedure was applied to the isolation of AHA and AFA from the groundwater (GFG1) sampled from the aquifer of Fuhrberg (Hannover, Germany). Wastewater HA and FA (WHA and WFA) were isolated as described in ref *29* from the reservoir Schwelvollert, near Halle-Leipzig (Germany). The water body is a basin, formed after surface mining, which had been filled with the concentrated wastewater from a brown coal (SV1). HA and FA isolated from the secondary effluent (ABV2) were obtained from the wastewater treatment plant Neureut (Karlsruhe, Germany).

Aquatic dissolved organic matter (ADOM) samples were collected from waters of the brown water lake on the Island Mud'yug (SMu4 and SMu8) and of Lake Brunnenseemoor (BM12). SKJA and SKJJB samples were provided by E. Gjessing. They were isolated by reverse osmosis in combination with a cation-exchange resin from a brown water lake in Norway (*31*).

Peat HF (PHF) were isolated from seven different samples of highland and lowland peat located in Tver region (Russia). The highland peat types were Sphagnum-Fuscum (T1), Sphagnum (T4, T5), sedge (T6), and woody-herbaceous (TH); the lowland peat types were woody (T7) and woody-herbaceous (TL). The isolation procedure was described elsewhere (*32*) and included a preliminary treatment with an ethanol−benzene (1:1) mixture followed up by alkaline (0.1 M NaOH) extraction. The extract was passed through a cation-exchange resin and concentrated on rotor-evaporator to HS content of 0.5−1 g C/L. The HS solution was kept at 4 °C in the dark.

Bottom sediments HF (BHF) were isolated from different locations in Northern Russia: North Dvina (RND13) and Luh (RLuh) rivers, and the brown water lake on the Island Mud'yug (SMu2). The isolation procedure was identical to the described above for peat HF.

Soil HA and FA (SHA and SFA) were isolated from different locations in Russia. The soil samples included the following: sod-podzolic soils under mixed wood land (PW94 and PW96), arable land (PP94 and PP96), and garden sites (PG94 and PG96) nearby Moscow and under mixed wood land nearby Novgorod (PWN); gray-wooded soils under deciduous wood land (GWW) and arable land (GWP) nearby Tula; typical (CTV) and meadow (CMV) chernozems (mollisols) nearby Voronezh. The SHA and SFA were obtained by alkali extraction with 0.1 M NaOH according to Orlov and Grishina (*33*). For calcareous soils (chernozems), the sample was pretreated with 10% HCl. The alkali extract was treated with 0.3 M KCl and centrifuged to remove the organomineral colloidal particles. The SHA and SFA were obtained by acidification of the supernatant to pH 1−2. The precipitated HA were desalted by dialysis. To isolate FA, the acidic supernatant was passed through XAD-2 resin. The sorbed fraction of SFA was recovered as described above for aquatic HF.

Soil Solution HF, HA, and FA (SSHF, SSHA, and SSFA) were isolated from water extracts of soils. SSHF-PP96 was isolated from sod-podzolic soil nearby Moscow (soil:water 1:5 v/v) using XAD-2. SSHA-BS1 and SSFA-BS1 were extracted from a podzol soil located near Bayreuth (Germany). The water extract was treated according to XAD-8 procedure (*30*).

Coal HA (CHA) were obtained from three commercial preparations: ALD (Aldrich humic acid), AGK (Biotechnology Ltd., Moscow), and RO (Carl Roth Ltd., Germany). They were desalted on a cation-exchange resin and freeze-dried.

Stock solutions of humic materials (100−500 mg of organic carbon (C) per liter) were prepared by concentration of the desalted extracts or by dissolving a weight of dried material. C content was measured using a Shimadzu 5000 TOC analyzer. For the SEC-analysis, portions of the stock solutions were diluted with a buffer identical in composition to the SEC mobile phase (0.028 M phosphate buffer) to a final concentration of 1−2 mg C/L.

SEC. All the SEC analyses were conducted according to Perminova et al. (*23*). For this purpose, a liquid chromatography system consisted of a solvent pump (Shimadzu LC 9A), a packed column, a UV−vis detector with variable wavelength, and a high-sensitive on-line DOC detector (Graentzel, Germany) was used. The UV-absorbance was measured at 254 nm. The SEC column was 25 × 200 mm packed with Toyopearl HW-50S. Phosphate buffer (0.028 M, pH 6.8) was used as a mobile phase at a flow rate of 1 mL/min. The SEC column was calibrated using polydextrans (PDX), kDa: 0.83, 4.4, 9.9, 21.4, 43.5, and sodium polystyrenesulfonates (PSS), kDa: 1.37, 3.8, 6.71, 8.0, 8.6, 13.4, 16.9. The standard kits were purchased from the Polymer Standard Service (Mainz). The calibration curve for PDX was expanded into the low MW region by means of mono- and oligosaccharides (180, 342, 504 Da) and glycerol. Blue dextran (2000 kDa) served as a void volume probe (V_0), methanol—as a permeation volume probe (V_p).

The distribution coefficient (K_d) of the analyte was calculated according to the known expression (*34*)

$$K_d = (V_e - V_0)/(V_p - V_0) \qquad (1)$$

where V_0, V_p, and V_e are void, permeation, and elution volumes, respectively.

SEC Data Treatment. The acquired data file was read by a self-designed "GelTreat"-program and treated as described in our previous publication (*35*). In brief, the program performs a pretreatment of the initial chromatogram (e.g. baseline correction, cutoff of peaks, smoothing) and converts it into K_d scale. It then calculates, on the basis of the calibration curve, an MWD profile and the corresponding numerical descriptors (averaged MWs, statistical moments, and partial integrals).

The calibration curves were obtained using the sets of ionogenic (PSS) and nonionogenic (PDX) polymers. The PSS calibration curve had a larger slope than the PDX one. It indicates that under the chosen experimental conditions the negatively charged PSS still experience electrostatic repulsion. The density of sulfonic groups in PSS polymers of 5.4 mmol/g is higher than that of the carboxylic groups in HS which varies on average between 2 and 5 mmol/g (*4*). In addition, the dissociation constants of sulfonic groups are higher than those of carboxylic groups. Hence, if the standards adequate to HS were available, e.g. polymers of aromatic carboxylic acids, then the corresponding calibration curve would have laid between PSS and PDX curves. If full compensation of the repulsive effects had been reached it should be close to the slope of PDX curve. Given these considerations, a use of the above calibration curves gives minimum (PSS) and maximum (PDX) estimates for the MW value of HS. We used the both calibration curves for calculation of the HS MW values in this publication.

Every new series of HS samples was preceded by the measurements of standards. To unify the calibration curves obtained for the different series, V_0 and V_p values were determined from the corresponding calibration curve as V_e at MW values of 70 000 and 32 Da, respectively, that represent the limits of the linear fractionation range of the gel. According to SEC theory, K_d values at the corresponding V_0 and V_p were put equal to 0 and 1, respectively. All the chromatograms were then converted into the K_d scale, and a unified calibration curve was calculated for all the series:

$$\log M = 4.845 - 3.43 \times K_d \tag{4}$$

Statistical Analysis. The mean, the median, the minimum (min), and maximum (max) values, the low and upper quartiles (Q25 and Q75, respectively), and the standard deviation (SD) for a particular parameter in the data set were determined using the common computational approach (*36*). The quartiles return the 25th and 75th percentile of values in a range. Min, median, and max return the same value as quartile when quart is equal to 0, 2, and 4.

Shapiro-Wilk (*W*) statistics were used to assess the normality of the distribution of values in the data set for a particular parameter. Contrary to chi-squared (χ^2) statistics, the Shapiro-Wilk statistics can be applied to small numbers of samples ($3 < n < 50$) (*36*). The information on the normality of the distribution is necessary to apply either the parametric *t*-test that operates on the assumption that the data exhibit normal distribution or the nonparametric Wilcoxon matched pairs test (*36*).

The *W* test was applied to the data sets of MW characteristics grouped by source and fractional composition of a humic material. For almost all the data sets studied, the corresponding values of parameters *W* exceeded the critical *W* values at $P = 95\%$, indicating that the data exhibited the normal distribution. The Bartletts's test was used to examine the heterogeneity of the variances of the data sets. Its application to the data sets did not reveal heterogeneity of the variances. This allowed us to use the parametric *t*-test for evaluating the probability that the mean of a particular parameter exhibited by two data sets could be observed within the same population.

Results and Discussion

Molecular Weight Distribution of HS of Different Source and Fractional Composition: General Trends. The typical UV/DOC-chromatograms of humic materials from peat, soil, and aquatic environments are given in Figure 1. All SEC-chromatograms of HS show a unimodal distribution, some of them contain subpeaks and shoulders. Those positioned at $K_d = 0$ are characteristic to almost all the samples of PHF and SHA used in this study (Figure 1a,b). Their presence is related to somewhat insufficient fractionation range of the gel. At the same time, the lower MW samples (SFA, AHF) (Figure 1c,d) have no peaks at $K_d = 0$ and are rather symmetrical. Despite the described MWD distortions of the higher MW samples, the same gel-matrix was used for the complete set of HS samples. This was done to facilitate a direct comparison of the SEC-characteristics of HS samples of different source and fractional composition.

A comparison between the DOC- and UV-profiles of the humic materials from the various sources (Figure 1) shows quite different UV-absorptivity of the HS used. So, SHA and PHF exhibit the largest UV-absorptivity, whereas SFA and AHF—much less. To eliminate the influence of the different UV-absorptivities on the MW characteristics of HS, only DOC-profiles were used for calculation of MWD. The corresponding MWDs are given in Figure 1 (right-hand side). On the basis of MWD obtained, M_n, M_w, and M_w/M_n were calculated (Table 2). The average relative *SD*s for all MW characteristics studied varied from 3 to 7%. This shows a good reproducibility of the obtained results.

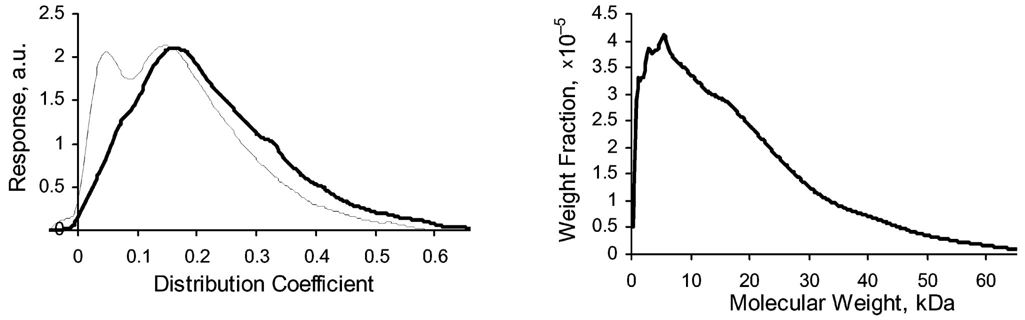
The complete range of M_w variability on PDX-scale accounted for 4.7–30.4 kDa, or 1.6–14.4 kDa on the PSS-scale. This range is much narrower than that traditionally reported for HS: from hundreds to millions of Daltons (*4*). The M_w^{PSS} of 2–5 kDa and M_n^{PSS} of 1–2 kDa obtained in this study for aquatic FA, HA, and HF corroborate well the values cited in Table 1. Based on the obtained M_w values, humic materials can be arranged in the following order: peat HF ≅ bottom sediments HF ≅ sod-podzolic and gray wooded soil HA > chernozem HA ≅ coal HA > soil FA ≅ swamp FA ≅ swamp HF ≅ peat DOM > river HF. The obtained trends are in agreement with the data in Table 1.

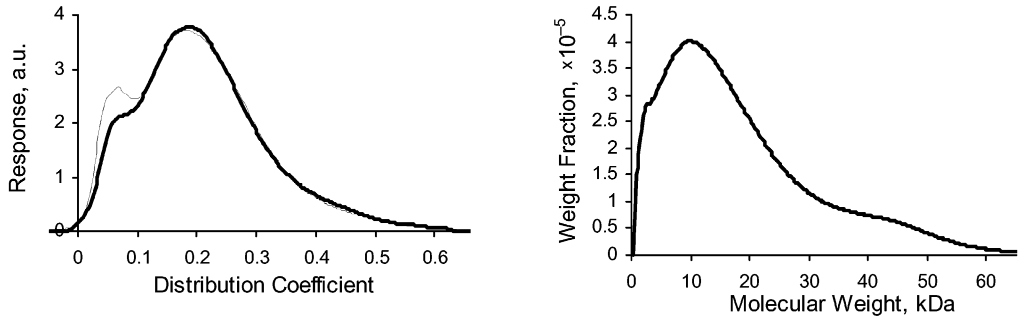
Considering that the HS samples studied represent very different environments and stages of humification: brown coal–peat–soil; groundwater–freshwater–wastewater; it can be concluded that HS have a preferential range of MW values. The above range can therefore be considered as an estimate of the M_w limits for HS as a class of chemical compounds. To verify an obtained estimate, further MW measurements on HS samples from the sources missing in the data set studied (e.g. marine environments) are to be conducted.

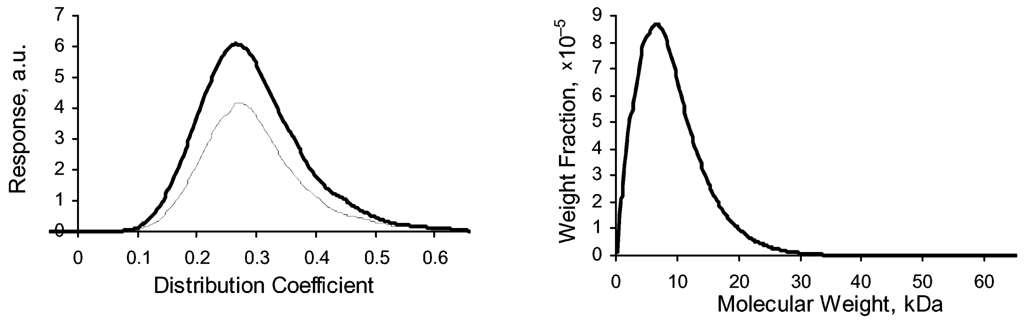
The M_w/M_n values varied within the range of 1.64–4.40 and were consistent with the isolation approach used. Maximum values of polydispersity (3.5–4.4) were observed for nonfractionated peat HF and soil HA, while much lower values (1.6–3.1) were found for all preparations isolated with XAD resins (SFA, AHF, AFA). The value of 4.40 recorded for SHA-PG96 can be considered as an outlier from the data set analyzed. The second largest M_w/M_n value is 3.74 and registered for another soil HA (SHA-PP96). Both are much higher than those of the other nine soil HA (2.13–3.17) and are also beyond the range of peat HF (2.66–3.47). The M_w/M_n values for aquatic HS vary between 1.66 and 3.06. The latter are in close agreement with those reported for aquatic HS in the literature: 1.5–2.04 (*7*, *8*, *11*).

Statistical Evaluation of the Molecular Weight Characteristics of HS. To determine consistent MW changes with respect to source and fractional composition, the corresponding statistical evaluation of the data set was conducted. The humic materials studied were grouped by source (soil, peat, river water, swamp water) and composition (HA, FA, HF). Five subsets—PHF ($n = 8$), SFA ($n = 9$), SHA ($n = 11$), AFA-*S* ($n = 8$), AHF-R ($n = 8$)—were formed. The descriptive statistics were calculated as described in the experimental part which included mean, median, min, max, Q25, Q75, SD, and *W*-statistics (Table 3). According to the results of the Shapiro-Wilk statistic, the data for all parameters tested are normally distributed at $P = 95\%$ for all the HS classes used. This is consistent with a close agreement between mean and median observed for all data sets. The box and whisker plots are used to compare the descriptive statistics calculated for M_w (Figure 2) and M_w/M_n (Figure 3).

Trends in Class-Averaged M_w Values. As seen from Figure 2, the HS classes formed can be easily segregated into two groups by a magnitude of M_w value. PHF and SHA comprise


a) **Peat HF (PHF)**


b) **Soil HA (SHA)**


c) **Soil FA (SFA)**

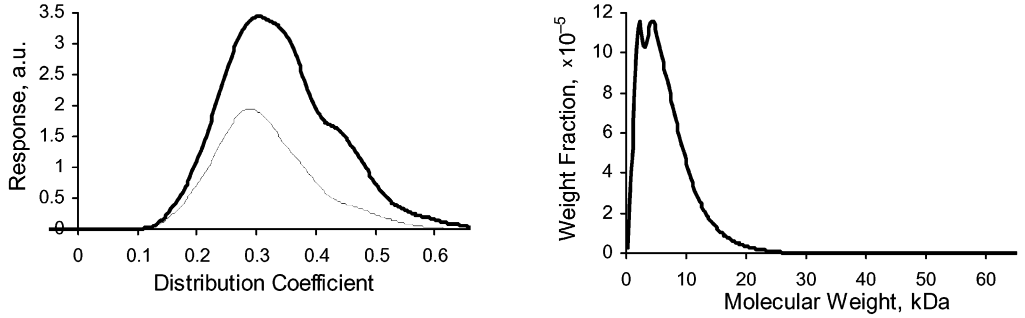

d) **Aquatic HF from River Water (AHF-R)**

FIGURE 1. Typical SEC-chromatograms of HS from different environments (column-packing—Toyopearl HW-50S, eluent—0.028 M phosphate buffer, pH 6.8) (left-hand side) and the corresponding differential MWD (right-hand side). The thick line shows DOC-profile, the thin line—UV profile. DOC- and UV-response is given in arbitrary units. (a) peat HF (PHF-T7), (b) soil FA (SFA-PW94), (c) soil HA (SHA-PW94), (d) aquatic HF (AHF-RMX2).

a group of high MW HS, whereas SFA and three classes of aquatic HS comprise a group of low MW HS. There is a significant difference in M_w between those two groups. At the same time, inside of the groups, the ranges for M_w of different classes overlap substantially. The performed *t*-test ($P > 95\%$) showed that the data sets which did not exhibit the statistically significant differences of the means for M_w were the following pairs: PHF and SHA; SFA and AFA-S. This could be the result of similarities in the average M_w of the HS from the given source, but it cannot be excluded that it could be due to the small number of samples from which the sets are constructed.

According to the *t*-test, the mean M_w of SHA is statistically distinct from that of SFA (higher). The mean M_w of aquatic FA and HF are distinct from that of PHF and SHA (lower). The mean M_w of SFA differs statistically from that of AHF-R

TABLE 2. Molecular Weight Characteristics of Humic Materials Used in This Study[a]

	M_n	M_w	M_w/M_n	M_n^{PSS}	M_w^{PSS}
Peat Humic Substances (PHF)					
PHF-T194	6.1 ± 0.3	18.2 ± 0.5	2.97 ± 0.16	1.87 ± 0.09	8.40 ± 0.12
PHF-T494	7.1 ± 0.4	22.4 ± 0.5	3.16 ± 0.22	2.18 ± 0.06	10.34 ± 0.01
PHF-T594	7.02 ± 0.06	19.3 ± 0.5	2.75 ± 0.07	2.11 ± 0.08	8.58 ± 0.16
PHF-T694	5.9 ± 0.5	17.0 ± 0.7	2.88 ± 0.20	1.70 ± 0.19	7.37 ± 0.3
PHF-T794	7.0 ± 0.4	18.6 ± 0.5	2.66 ± 0.22	2.09 ± 0.16	8.14 ± 0.28
PHF-T1094	5.8 ± 0.2	20.1 ± 0.3	3.47 ± 0.06	1.67 ± 0.05	9.23 ± 0.11
PHF-TH94	5.5 ± 0.6	17.1 ± 0.1	3.15 ± 0.30	1.56 ± 0.24	7.41 ± 0.06
PHF-TT94	7.0 ± 0.3	20.64 ± 0.05	2.94 ± 0.11	2.07 ± 0.07	9.23 ± 0.03
Soil Fulvic Acids (SFA)					
SFA-CM94	5.0 ± 0.4	10.2 ± 0.9	2.04 ± 0.04	1.58 ± 0.21	4.41 ± 0.3
SFA-CTV94	6.8 ± 0.5	12.6 ± 0.4	1.87 ± 0.10	2.21 ± 0.09	5.19 ± 0.12
SFA-GW94	6.3 ± 0.4	13.9 ± 0.3	2.21 ± 0.08	1.91 ± 0.14	5.80 ± 0.14
SFA-PG94	6.1 ± 0.3	12.3 ± 0.5	2.02 ± 0.07	1.83 ± 0.13	4.91 ± 0.16
SFA-PG96	5.58	11.3	2.02	1.76	4.17
SFA-PP94	5.1 ± 0.5	9.0 ± 0.3	1.76 ± 0.13	1.53 ± 0.21	3.39 ± 0.10
SFA-PP96	4.01	9.05	2.25	1.13	3.2
SFA-PW94	5.54 ± 0.4	9.01 ± 0.05	1.64 ± 0.10	1.76 ± 0.04	3.37 ± 0.02
SFA-PW96	4.8 ± 0.3	9.9 ± 0.3	2.05 ± 0.07	1.58 ± 0.15	4.31 ± 0.21
Soil Humic Acids (SHA)					
SHA-CM94	7.0	16.6	2.38	1.78	6.51
SHA-CTV94	6.9 ± 0.3	14.7 ± 0.2	2.13 ± 0.07	2.11 ± 0.13	6.11 ± 0.08
SHA-GP94	7.84	20.3	2.59	2.36	8.99
SHA-GW94	8.67	20.4	2.35	2.72	9.02
SHA-PG94	8.64	20.3	2.35	2.68	8.94
SHA-PG96	4.24	18.7	4.40	1.31	6.9
SHA-PP94	8.64	19.4	2.25	2.73	8.51
SHA-PP96	5.34	20.0	3.74	1.70	8.50
SHA-PW94	6.8	17.0	2.19	2.91	8.05
SHA-PW96	5.78	18.3	3.17	1.97	7.8
SHA-PWN	6.53	16.0	2.45	1.94	6.79
Aquatic Fulvic Acids from Swamp Water (AFA-S)					
AFA-SHO6	5.31	10.8	2.03	1.56	4.20
AFA-SHO10	6.04	13.1	2.17	1.80	5.32
AFA-SBM7	6.4	12.3	1.93	1.96	4.64
AFA-SBM8	6.1	11.8	1.95	1.84	4.51
AFA-SBM9	6.4	12.4	1.95	1.95	4.76
AFA-SBM10	6.5 ± 0.2	11.8 ± 0.3	1.82 ± 0.07	1.95 ± 0.1	4.35 ± 0.2
AFA-SBM11	6.37	11.0	1.73	1.97	4.28
AFA-SBM12	7.1 ± 0.1	12.4 ± 0.2	1.74 ± 0.07	2.24 ± 0.1	4.63 ± 0.2
Aquatic Humic Substances from River Water (AHF-R)					
AHF-RHPL	2.9 ± 0.3	5.2 ± 0.2	1.80 ± 0.08	0.79 ± 0.09	1.79 ± 0.08
AHF-RKX2	3.84	8.01	2.09	1.06	2.98
AHF-RMC	4.4 ± 0.3	8.6 ± 0.4	1.97 ± 0.14	1.25 ± 0.13	3.32 ± 0.15
AHF-RMX2	3.9 ± 0.2	6.7 ± 0.2	1.73 ± 0.08	1.11 ± 0.07	2.38 ± 0.09
AHF-RMX8	3.5 ± 0.1	6.3 ± 0.3	1.83 ± 0.04	0.96 ± 0.05	2.24 ± 0.13
AHF-RND3	4.2 ± 0.3	7.9 ± 0.3	1.86 ± 0.12	1.23 ± 0.12	2.96 ± 0.09
AHF-RND14	4.23	9.52	2.25	1.18	3.95
AHF-RUL	3.1 ± 0.4	5.8 ± 0.3	1.89 ± 0.14	0.84 ± 0.13	2.02 ± 0.12
Aquatic Humic Substances from Swamp Water (AHF-S)					
AHF-SMU4	4.27	10.2	2.38	1.21	4.05
AHF-SMU7	4.26	10.3	2.42	1.20	4.00
AHF-SMU8	3.48 ± 0.02	7.3 ± 0.3	2.11 ± 0.08	0.97 ± 0.01	2.75 ± 0.16
AHF-SND11	3.35	10.3	3.06	0.93	2.21
AHF-SSH2	5.60	11.3	2.01	1.23	3.11
Aquatic Humic Acids from Swamp Water (AHA-S)					
AHA-SHO10	7.57	16.1	2.13	2.29	6.72
AHA-SHO13	4.17	12.2	2.93	1.23 ± 0.12	3.96 ± 0.09
AHA- SBM10	5.30	8.79	1.66	1.62	3.28
Aquatic and Peat Dissolved Organic Matter (ADOM and PDOM)					
ADOM-SBM12	4.74	9.79	2.07	1.36	3.77
ADOM-SKJA	4.78	12.4	2.58	1.37	5.14
ADOM-SKJB	4.65	10.2	2.19	1.34	4.00
ADOM-SMU4	4.7	10.7	2.32	1.38	4.02
ADOM-SMU8	3.6 ± 0.1	6.7 ± 0.3	1.87 ± 0.03	1.01 ± 0.03	2.42 ± 0.13
PDOM-TH	3.8 ± 0.1	11.7 ± 0.6	3.10 ± 0.04	1.05 ± 0.02	5.00 ± 0.12
PDOM-TT	3.54 ± 0.05	10.5 ± 0.2	2.96 ± 0.04	0.97 ± 0.02	4.30 ± 0.09
Bottom Sediments HS from Rivers and Swamps (BHF-R and BHF-S)					
BHF-RLUH	5.46	13.4	2.45	1.73	5.7
BHF-RND13	6.7 ± 0.1	17.01 ± 0.05	2.56 ± 0.03	2.01 ± 0.02	7.42 ± 0.03
BHF-SMU2	5.8 ± 0.5	19.1 ± 0.2	3.30 ± 0.31	1.67 ± 0.19	8.54 ± 0.15

TABLE 2 (Continued)

	M_n	M_w	M_w/M_n	M_n^{PSS}	M_w^{PSS}
			Coal Humic Acids (CHA)		
CHA-AGK	6.7 ± 0.4	14.7 ± 0.3	2.23 ± 0.31	1.81 ± 0.14	5.90 ± 0.14
CHA-ALD	5.92	11.8	1.99	1.80	4.53
CHA-RO	3.99	10.9	2.74	1.21	3.4
			Soil Solution Fulvic and Humic Acids, and HF (SSFA, SSHA, and SSHF)		
SSFA-BS1	6.70	11.4	1.70	2.14	4.48
SSHA-BS1	6.1 ± 0.2	11.4 ± 0.5	1.86 ± 0.02	1.8 ± 0.1	4.50 ± 0.3
SSHF−PP96	4.39	7.06	1.61	1.30	2.53
			Wastewater Fulvic and Humic Acids (WFA and WHA)		
WFA-ABV2	2.57	4.72	1.83	0.69	1.60
WHA-ABV2	9.67	30.4	3.14	2.90	14.35
WFA-SV1	3.13	5.90	1.88	0.87	2.08
WHA-SV1	3.56	6.80	1.91	0.99	2.45
			Suwannee River (IHSS) and Groundwater (G) HA and FA, Soil HF		
AFA-IHSS	4.8 ± 0.3	8.37 ± 0.09	1.77 ± 0.11	1.41 ± 0.13	3.12 ± 0.05
AHA-IHSS	6.9 ± 0.2	12.8 ± 0.3	1.86 ± 0.02	2.13 ± 0.07	5.11 ± 0.14
AHA-IHSS	6.9 ± 0.2	12.8 ± 0.3	1.86 ± 0.02	2.13 ± 0.07	5.11 ± 0.14
AFA-GFG1	3.2 ± 0.2	5.2 ± 0.2	1.60 ± 0.03	0.91 ± 0.07	1.76 ± 0.09
AHA-GFG1	3.82	7.19	1.88	1.08	2.60
SHF-CO94	6.2 ± 0.7	19.5 ± 0.9	3.16 ± 0.26	1.89 ± 0.10	8.87 ± 0.38

[a] M_n and M_w—number and weight average MW, respectively, evaluated using PDX calibration curve; M_w/M_n—polydispersity; M_n^{PSS} and M_w^{PSS}—values of M_n and M_w evaluated using PSS calibration curve. All MW are given in kilodaltons (kDa); ± is a standard deviation and is given for the samples measured with replicates ($n = 3$).

TABLE 3. Descriptive Statistics of Molecular Weight Characteristics of Humic Substances Grouped by Source and Fractional Composition (kDa)

	mean	median	Q25	Q75	min	max	SD	*W*
				Peat Humic Substances (PHF), *n* = 8				
M_n[a]	6.4	6.6	5.9	7.0	5.5	7.1	0.7	0.838
M_w	19.2	19.0	17.6	20.4	17.0	22.4	1.9	0.949
M_w/M_n	3.00	2.95	2.82	3.15	2.66	3.47	0.26	0.960
				Soil Fulvic Acids (SFA), *n* = 9				
M_n	5.5	5.5	5.0	6.1	4.0	6.8	0.8	0.983
M_w	10.8	10.3	9.0	12.3	9.0	13.9	1.8	0.895
M_w/M_n	1.99	2.02	1.87	2.05	1.64	2.25	0.20	0.938
				Soil Humic Acids (SHA), *n* = 11				
M_n	7.0	7.0	5.8	8.6	4.2	8.7	1.5	0.926
M_w	18.3	18.7	16.6	20.3	14.7	20.4	2.0	0.897
M_w/M_n	2.73	2.38	2.25	3.17	2.13	4.40	0.74	0.768[b]
				Aquatic Fulvic Acids from Swamp Water (AFA-S), *n* = 8				
M_n	6.3	6.4	6.0	6.5	5.3	7.1	0.5	0.925
M_w	11.9	12.1	11.4	12.4	10.8	13.1	0.7	0.937
M_w/M_n	1.91	1.94	1.78	1.99	1.73	2.17	0.15	0.948
				Aquatic Humic Substances from River Water (AHF-R), *n* = 9				
M_n	3.9	3.9	3.5	4.2	2.9	4.7	0.6	0.953
M_w	7.4	7.9	6.3	8.4	5.2	9.5	1.4	0.957
M_w/M_n	1.91	1.86	1.79	1.97	1.73	2.25	0.17	0.894
				Complete Set, *n* = 77				
M_n	5.5	5.5	4.2	6.5	2.6	9.8	1.5	
M_w	12.8	11.8	9.0	16.9	4.7	30.4	5.0	
M_w/M_n	2.31	2.13	1.88	2.59	1.60	4.40	0.56	

[a] M_n and M_w−number and weight average MW, respectively, evaluated using PDX calibration curve. [b] The *W* values labeled with an asterisk are lower than the critical value of *W*-statistic and indicate that the data set of the given parameter does not exhibit a normal distribution.

(higher) but could not be distinguished from that of AFA-S. It is of interest that the mean M_w of river HF is statistically different from swamp FA (lower). This observation suggests that MW is indicative of the specific features of HS formation, e.g. of the level of microbiological activity in the given environment: the higher the biological activity (river), the lower the MW of the HS that are formed. This is true not only for aquatic environments. For example, the trend in M_w of SHA (Table 2) shows that those of chernozems (mollisols) are 2−3 kDa lower than those of sod-podzolic or gray-wooded soil. Chernozems are characterized by much higher microbiological activity than the other two soil-types (*2*). To verify such subtle differences, much larger data sets are needed.

Trends in Class-Averaged M_w/M_n Values. The most striking observations from comparing the box and whisker plots in Figure 3 are extreme similarity of all the descriptive statistics calculated for polydispersity of SFA, AHF-R, and AFA-S. Considering that all these classes of HS were isolated with a use of the XAD-technique, a conclusion can be made that it is the isolation and/or fractionation technique that determines polydispersity of a humic material rather than the peculiar features of the environment where it was formed.

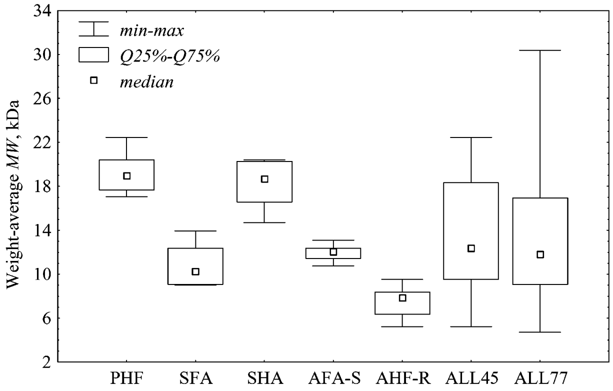


FIGURE 2. Box whisker plot of class-averaged medians, quartiles, and ranges of M_w (PDX-scale) for the HS grouped by source and fractional composition. "ALL45" designates a joint set of the subsets of PHF, SFA, SHA, AFA-S, and AHF-R. "ALL77" designates the complete set of all HS samples used in this study.

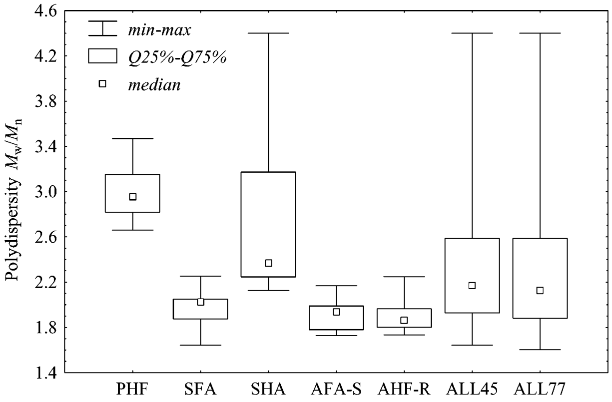


FIGURE 3. Box whisker plot of class-averaged medians, quartiles, and ranges of M_w/M_n for the HS grouped by source and fractional composition. "ALL45" designates a joint set of the subsets of PHF, SFA, SHA, AFA-S, and AHF-R. "ALL77" designates the complete set of all HS samples used in this study.

This conclusion supports similar findings reported in the literature on the minimum values of polydispersity measured for samples isolated by XAD resins (*9*).

To estimate the statistical significance of the observed trends in the mean values for M_w/M_n of HS grouped by source, the *t*-test was used. The obtained results confirm the trends elucidated by Figure 3 that the class-averaged polydispersity for SFA, AHF-R, and AFA-S are not statistically different from each other at the given P (>95%). In addition, there is no statistically significant distinction between PHF and SHA. At the same time, SHA is statistically distinct from SFA on the basis of mean M_w/M_n (higher); PHF differs significantly from SFA, AHF-R, and AFA-S (higher).

The performed *t*-tests point out that the groups of HS that differ significantly with respect to both mean M_w and M_w/M_n are the following pairs: PHF and AHF-R; PHF and SFA; PHF and AFA-S; SHA and AHF-R; SHA and SFA; and SHA and AFA-S. There was no significant difference between the pairs of PHF and SHA and AFA-S and SFA.

Considering that the above statistical evaluation involved 45 out of 77 samples used in this study, the similarity in statistical characteristics (range, median, upper and lower quartiles) for M_w and M_w/M_n values (Figures 2 and 3, respectively) obtained for the subgroups and the complete set is noteworthy. The subgroups represented only five environments, whereas the complete set was composed of more than 10 different sources and fractions of HS. This results from generalization of the trends elucidated by statistical analysis.

The observed trends show that HS appear to have a preferential range of MW values that characterizes them as a specific class of chemical compounds. The MW properties change regularly in accordance with the particular features of the formation of and isolation of HS: average MWs tended to change consistently with the source, and polydispersity was more dependent on the isolation and/or fractionation approach used. Hence, the MW characteristics possess a substantial discriminating power and can be used as descriptors of the molecular properties of HS. Extension of the approach used in this study on quantification and statistical evaluation of the other molecular parameters of HS (e.g. elemental composition, structural-group composition) can contribute greatly in developing definition and classification of HS as a class of chemical compounds.

Acknowledgments

I.V.P. thanks the University of Karlsruhe and DAAD for a support of her stay as a visiting scientist at the Division of Water Chemistry. A.V.K. appreciates financial support of the Robert Havemann Scholarship (Germany). N.A.K. thanks the Lomonosov Moscow State University for a 2002 scholarship award. We extend our deepest appreciation to Prof. J. Grebmeier (University of Tennessee, U.S.A.) and Dr. K. Hatfield (University of Florida, U.S.A.) for useful comments and editorial work on the manuscript. Anonymous referees are acknowledged for constructive critique on the data presentation. This research was supported by the grants of the BMBF (143/97-RUS) and of the Russian Foundation for Basic Research (RFBR-01-03-32664).

Literature Cited

(1) Thurman, E. M. *Organic Geochemistry of Natural Waters*; Martinus Nijhof/Dr. W. Junk Publishers: Dordrecht, The Netherlands, 1985.
(2) Orlov, D. S. *Soil Humic Acids and General Theory of Humification*; Moscow State University Publisher: Moscow, 1990.
(3) MacCarthy, P.; Rice, J. A. In Proceedings of Chapman Conference on the Gaia Hypothesis, San Diego, CA, March 7−11, 1988; Schneider, S. H., Boston, P. J., Eds.; MIT Press: Cambridge, 1990; pp 339−345.
(4) Stevenson, F. *Humus Chemistry. Genesis, Composition, Reaction*, 2nd ed.; Wiley & Sons: New York, 1994.
(5) De Nobili, M.; Gjessing, E.; Sequi, P. In *Humic Substances II;* Hayes, M. H. B., MacCarthy, P., Malcolm, R. L., Swift, R. S., Eds.; John Wiley & Sons: Chichester, 1989; pp 561−592.
(6) De Nobili, M.; Chen, Y. *Soil Sci.* **1999**, 164, 825−833.
(7) Chin, Yu-P.; Aiken, G.; O'Loughlin, E. *Environ. Sci. Technol.* **1994**, *28*, 1853−1858.
(8) Cabaniss, S. E.; Zhou, Q.; Maurice, P. A.; Chin, Y.-P.; Aiken, G. R. *Environ. Sci. Technol.* **2000**, *34*, 1103−1109.
(9) Peuravuori, J.; Pihlaya, K. *Anal. Chim. Acta.* **1997**, *337*, 133−149.
(10) Dahlen, J.; Backstrom, M.; Ephraim, J.; Boren, H., Allard, B. *Chemosphere* **1999**, *38*, 783−794.
(11) Plechanov, N. *Org. Geochem.* **1983**, *5*, 143−149.
(12) Varshal, G. M.; Velyukhanova, T. K.; Sirotkina, I. S. *Hydrochem. Mater.* **1973**, *59*, 143−151 (In Russian).
(13) Mantoura, R. F. C.; Riley, J. R. *Anal. Chim. Acta* **1975**, *78*, 193−200.
(14) Piccolo, A.; Mirabella, A. *Sci. Total Environ.* **1987**, *62*, 39−46.
(15) Karpukhin, A. I.; Fokin, A. D. In *The Particular Features of Soil Processes in Sod-podzolic Soils;* Moscow, 1977; pp 102−114 (In Russian).
(16) Ammosova, Ya. M.; Balaganskaya, E. D. *Eurasian J. Soil Sci.* **1991**, *7*, 29−39.
(17) Dell'Agnola, G.; Ferrari, G.; Maggioni, A. *Ric. Sci.* **1964**, *B4*, 347−352.
(18) Swift, R. S. *Soil Sci.* **1999**, *164*, 790−802.
(19) Shamet, K. A.; Thurman, E. M.; Randtke, S. J. In *Humic substances in the global environment and implications on human health*; Senesi, N., Miano, T. M., Eds.; Elsevier: Amsterdam, 1994; pp 769−774.
(20) Tadanier, C. J.; Berry, D. F.; Knocke, W. R. *Environ. Sci. Technol.* **2000**, *34*, 2348−2353.

(21) Perminova, I. V. *Soil Sci*. **1999**, *164*, 834−840.
(22) Zhou, Q.; Cabaniss, S. E.; Maurice, P. A. *Water Res.* **2000**, *34*, 3505−3514.
(23) Perminova, I. V.; Frimmel, F. H.; Kovalevskii, D. V.; Abbt-Braun, G.; Kudryavtsev, A. V.; Hesse, S. *Water Res.* **1998**, *32*, 872−881.
(24) O'Loughlin, E.; Chin, Yu-P. *Water Res.* **2001**, *35*, 333−338.
(25) Belenkii, B. G.; Vilenchik, L. Z. Modern liquid chromatography of macromolecules. *Journal of Chromatography Library*, *25*; Elsevier: Amsterdam, 1983.
(26) Rice, J. A.; MacCarthy, P. *Org. Geochem*. **1991**, *17*, 635−648.
(27) Visser, S. A. *Environ. Sci. Technol*. **1983**, *17*, 412−417.
(28) Aiken, G.; Thurman, E. M.; Malcolm, R. L.; Walton, H. F. *Anal. Chem*. **1979**, *51*, 1799−1803.
(29) Abbt-Braun, G.; Frimmel, F. H.; Lipp, P. *Z. Wasser-Abwasser Forsch*. **1991**, *24*, 285−292.
(30) Abbt-Braun, G.; Frimmel, F. H. In *Refractory Organic Substances in the Environment;* Frimmel, F. H., Abbt-Braun, G., Heumann, K. G., Hock, B., Luedemann, H.-D., Spiteller, M., Eds.; John Wiley & Sons: 2002; pp 1−38.
(31) Gjessing, E. T.; Alberts, J. J.; Bruchet, A.; Egeberg, P. K.; Lydersen, E.; McGown, L. B.; Mobed, J. J.; Muenster, U.; Pempkowiak, J.; Perdue, E. M.; Ratnawerra, H.; Rybacki, D.; Takacs, M.; Abbt-Braun, G. *Water Res.* **1998**, *32*, 3108−3124.
(32) Lowe, L. E. *Sci. Total Environ*. **1992**, *113*, 133−145.
(33) Orlov, D. S.; Grishina, L. A. *Handbook of Humus Chemistry;* Moscow State University Publisher: Moscow, 1981. (In Russian).
(34) Yau, W. W.; Kirkland, J. J.; Bly D. D. *Modern Size-Exclusion Liquid Chromatography;* John Wiley & Sons: New York, 1979.
(35) Kudryavtsev, A. V.; Perminova, I. V.; Petrosyan, V. S. *Anal. Chim. Acta* **2000**, *407*, 193−202.
(36) Dmitriev, E. A. *Mathematical Statistics in Soil Science;* Moscow State University Publisher: Moscow, 1995; 320 pp (In Russian).

Received for review May 20, 2002. Revised manuscript received March 12, 2003. Accepted March 21, 2003.

ES0258069