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Evaluation of aliphatic/aromatic compounds and fluorophores in dissolved organic matter of contrasting natural waters by SEC-HPLC with multi-wavelength absorbance and fluorescence detections

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ABSTRACT:

The analytical high performance size exclusion liquid chromatography (SEC-HPLC) with multi-wavelength absorbance and fluorescence detections and fluorescence 3D-excitation/emission matrix (EEM) were used for the analysis of average molecular size (MS), molecular size distribution of aliphatic/aromatic compounds and fluorophores in dissolved organic matter (DOM) from Suwannee River (SRDOM) and two Karelian lakes (Vodoprovodnoe Lake - L1DOM and Onego Lake - L2DOM). The average MS of DOM samples varied in the order SRDOM > L1DOM > L2DOM. The absorbance ratios A250/A365 and A210/A254 have been used for the DOM samples characterization. The absorbance ratio A250/A365 provided significant information about average MS of the bulk

aquatic DOMs but was not exactly correlated with the results of SEC-HPLC data. The absorbance ratio A210/A254 in combination with SEC was well correlated with the content of DOM aliphatic/aromatic compounds and could be used as an important water quality index to estimate the level of protein-like material in the aquatic DOM. Regardless of the origin (river or lakes), different geographical locations and different average MS of DOM, several similar types of humic-like SEC-separated fluorophores were found within the samples. In all DOM samples the decrease of the fluorophores emission maxima paralleled the decrease of their relative MS. The combination of SEC-HPLC with multi-wavelength fluorescence and absorbance detection and EEM analyses appears very useful for DOM characterization and for tracking of microbial activity resulting from anthropogenic and/or eutrophic impact in aquatic environments.

Key words: High performance SEC with multi-wavelength absorbance and fluorescence detections; EEM fluorescence spectroscopy; River and lakes dissolved organic matter (DOM); Fluorophores and aliphatic/aromatic compounds distribution; Anthropogenic and eutrophic impact in aquatic environments

1. Introduction

Dissolved organic matter (DOM), omnipresent in all aquatic ecosystems, is naturally occurring organic matter which consists of chemically complex compounds with different molecular size (MS) and photochemical properties. DOM affects a lot of processes in water photochemistry, including the fate of anthropogenic contaminants and the oxidation or transformation of organic material [1-5]. DOM is the primary absorber of sunlight and determines the optical properties of natural waters. One of the basic properties of aquatic DOM is fluorescence, which provides rapid and sensitive fluorescent analysis using

insignificant water sample volumes and considerable information on its chemical structure [6-9]. Measurement of fluorescence is used to examine DOM spatial and temporal distribution in the ocean [10-11], to study allochthonous and autochthonous sources of organic matter [12], to trace riverine input of organic carbon to the ocean [13] and to calibrate satellite data in remote sensing [14]. Fluorescence spectroscopy can be used as well for estimation of organic pollutions in natural water [15], in recycled water systems [16] or wastewaters [17].

As it is presently accepted, the fluorescence of DOM is due to two basic groups of fluorophores [8, 12, 18-21]. The first group shows emission within the wavelength range 380-470 nm (so called humic-like fluorescence) under excitation within the wavelength range 220-380 nm. The other group emits protein-like fluorescence with emission wavelength shorter than 380 nm and has maximum of excitation at wavelengths shorter 305 nm; it is affiliated to aromatic amino acids demonstrating tyrosine-like (emission wavelength of about 300 nm) or tryptophan-like (about 340 nm) types of fluorescence bands [18]. Correlations between tryptophan-like or tyrosine-like fluorescence and biochemical oxygen demand indicated the presence of labile organic matter due to sewage in the riverine water and/or eutrophic impact [12, 15]. Despite its importance, the true source of DOM protein-like fluorescence remains unknown, because this type of fluorescence may derive from proteins, peptides or free amino acids.

Very important factor influencing DOM fluorescence is its MS [19, 22]. After separation by size exclusion chromatography (SEC) the protein-like fluorescence was determined in the highest and medium MS fractions, while humic-like fluorescence was detected mainly in the low MS fractions [3, 4, 23]. The latter fraction also showed the highest capacity to sensitize the formation of singlet oxygen [24]. However, up to now the exact localization and distribution of fluorophores among the DOM differing in MS remained a challenge.

Size exclusion high-performance liquid chromatography (SEC-HPLC) is often used to characterize aquatic DOM due to its rapidity, availability and reproducibility [20, 25-28].

Because fluorescence properties of chromophores are generally tied to their molecular structures and respond to changes on the molecular level, different MS components obtained by SEC-HPLC may also reveal considerable information about the chemical structures responsible for their fluorescence properties. To improve information provided by SEC-HPLC, researchers frequently employed several techniques including UV-visible absorption, 2D- and 3D-fluorescence, NMR, DOC-analysis, PAGE etc. coupled in a single analytical chromatographic run [8, 24, 27, 29-31].

In this article we examined the localization of fluorescent and non-fluorescent species in several contrasting natural aquatic DOM. Our conclusions were based on the analysis of absorbance and fluorescence spectra obtained by SEC-HPLC with online information-rich multi-wavelength absorbance and fluorescence detections in combination with 3D-excitation/emission matrix (EEM). This approach was applied to genetically different DOM varied in MS and isolated from surface water of one river and two lakes.

2. Materials and methods

2.1. Materials and water sampling sites

The dry SRDOM sample (ref. number 1R101N, standard material) was obtained by using pre-filtration and concentration of allochtonous Suwannee River natural water by freeze drying following reverse osmosis [32-33] and purchased from IHSS. The samples of natural surface water were taken during expedition (August 2013) in two freshwater allochtonous lakes located in Karelia, the North of European part of Russia, Onego Lake and Vodoprovodnoe Lake. The lakes DOM samples were named L1DOM and L2DOM for Vodoprovodnoe Lake and Onego Lake, respectively.

The headwaters of the Suwannee River are in the Okefenokee Swamp, peat-filled wetland straddling the Georgia–Florida line in the United States. This river characterized by the high concentration of natural organic matter, the relatively low levels of inorganic salts, and the

minimal anthropogenic impact. Wetlands in the floodplain are mainly associated with the tributary creeks branching off the main channel, and consist of cypress and deciduous hardwoods (swamp tupelo, river birch, ogeeche tupelo, and thers). The Suwannee River further runs southward into Florida. The catchment area of the upper Suwannee River during its flow is characterized by the surrounding vegetation (loblolly pine, laurel oak, magnolia, swamp chestnut and others) [33].

The Onego Lake, the second largest lake in Europe, belongs to the basin of the Baltic Sea, Atlantic Ocean, and is situated between Lake Ladoga (the largest in Europe) and the White Sea. It covers an area of 9720 square km with the average depth 30 m. Onego Lake receives 1152 rivers; water balance is also provided by precipitation and water discharge from human activities: industrial and household water, melioration-related drainage etc. The total flow of terrigenous organic matter into the Onego lake ecosystem is determined by the spatial structure of the biogeocenoses in the catchment area. The shores to the north and northwest are high and rocky, built of layered granite and covered with spruce and pine forest. The DOM in the Onego Lake is ~90% due to river runoff, and the rest is equally divided between atmospheric precipitation and waste waters from three main industrial centers, Petrozavodsk, Kondopoga, and Medvezhegorsk [34]. Surface water sampling was carried out away from industrial facilities in the middle part of the Kondopoga Bay from a rowing-boat. Since the study and comparison of polluted waters was not the aim of our work, we have sampled water in non-high polluted area, far from settlements. On this reason we did not expect to find out essential anthropogenic and/or eutrophic material in the Onego Lake water sample. The phytoplankton and bacterial development in surface water may be increased due to eutrophication of water in the Kondopoga Bay [35].

The third sampling site, small ultrafresh Vodoprovodnoe Lake with depth not exceeding 2 m, is located close to Kandalaksha Bay in the area of the Pertsov White Sea Biological Station of the Lomonosov Moscow State University far from industrial facilities. The water in

Vodoprovodnoe Lake has a characteristic brown color, which comes from high concentration of humic substances [36]. Both Vodoprovodnoe Lake and Suwannee River can serve as a source of DOM in the absence of anthropogenic impact. Surface water sampling was carried out in the middle of the lake from a rowing-boat.

The content of dissolved organic carbon (DOC) in both Karelian lakes original freshwater was made using high temperature catalytic combustion (TOC-500, Shimadzu). The DOC content in Vodoprovodnoe Lake was 15.1±4.2 mgC L^{-1} and in Onego Lake – 6.9±1.6 mgC L^{-1}. Water samples were stored in sealed plastic bottles in the dark at +4°C.

2.2. Preparation of stock DOM solutions for spectroscopic and chromatographic analyses

The 100 ml of original freshwater samples were filtered using cellulose filters (Millipore) with a pore size of 0.45 μm and freeze dried. Before the analysis 1 mg of each dry DOM sample was completely dissolved in 2 ml of 30 mM phosphate buffer, pH 6.5 (PB). The 0.3 ml of each sample solution was diluted ten times using 30 mM PB and absorbance at 270 nm was measured for diluted solutions with C=50 mgL^{-1}. Using this data the A270 for the concentrated DOM solutions (C=500 mg L^{-1}) was calculated. Than all concentrated DOM solutions were diluted using 30 mM PB to A270 = 5 a.u. As a result, the stock DOM solutions had identical A270=5 a.u. and different concentration (C_{SRDOM}=362 mgL^{-1}, C_{L1DOM}=371 mgL^{-1}, C_{L2DOM} =423 mgL^{-1}). Before further spectroscopic and chromatographic analyses stock DOM solutions were stored in sealed glass bottles at +4°C.

Phosphate buffer (pH 6.5) was prepared in water at concentration of 30 mM by mixing three volumes of 30 mM Na_2HPO_4 solution (pH 9.8) and seven volumes of 30 mM NaH_2PO_4 solution (pH 4.5). Water was purified using a reverse osmosis RIOS 5 and Synergy (Millipore) device (resistivity 18 MΩ cm, DOC < 0.1 mg L^{-1}). All reagents were of the highest grade available.

2.3. UV–visible absorbance and 3D-excitation/emission matrix (EEM) fluorescence spectroscopy

The stock DOM solutions were diluted 10 times in 10 mM phosphate buffer (pH 6.5) and the UV-visible absorption spectra were recorded on a Cary 3 spectrophotometer (Varian, Cary, USA) in a quartz cuvette with optical path 1 cm using wavelengths from 200 to 700 nm. The absorbance ratios A250/A365 and A210/A254 were calculated (Table 1).

For the EEM analysis, recorded using a Cary Eclipse fluorescence spectrophotometer (Varian, Cary, USA), the stock DOM solutions were diluted 100 times by water to reach an absorbance of 0.05 at 270 nm in 0.03 mM phosphate buffer (pH 6.5).

2.4. Analytical size exclusion high-performance liquid chromatography (SEC-HPLC) with multi-wavelength absorbance and fluorescence detection

SEC-HPLC of three DOM samples was conducted on a high-performance liquid chromatographic system (Waters Corp., MA, USA). The multi-wavelength detector 2998, working in the range 210-400 nm, was used for detection of absorbance. An Acquity Fluorescence detector, connected directly to the waste line of the absorbance detector, was used for fluorescence emission measurements and set to an excitation wavelength of 270 nm and emission range of 300-600 nm. The column TSKgel G2000SW$_{XL}$ (7.8 mm ID, 300 mm L, particle size 5.0 µm) equipped with a guard column TSK G2000SW$_{XL}$ (both - TOSOH Bioscience, Japan) showed a fractionation range from 150 to 5 kDa for globular proteins. Within experiments the 30 mM phosphate buffer was used as a sample buffer and as an eluent with a flow rate of 1.0 mL min^{-1}. The use of the same buffer for sample dissolution and as an eluent during SEC-procedure is the main requirement to avoid any additional peaks-artifacts, especially at the end of the fractionation column's range. The void column volume (Vo),

determined with blue dextran 2000, was 5.6 mL. The permeation volume (Vp), determined using low MW fluorescent amino acid tryptophan, was 13.1 mL. The injection volume was 0.02 mL. The stock DOM solutions applied on the column were eluted between about 5.6 and 13.1 mL and thus diluted to about 350 times. For this reason, the absorbance detector with the absorbance limit of 2 a.u. could be used. The 30 mM phosphate buffer (pH-6.5) as the mobile phase did not show any absorbance or fluorescence peaks. The entire SEC-HPLC procedure was repeated three times (the deviations were no more than 3%). The recovery of DOM samples on TSK-gel column was determined by comparing total peak areas of chromatograms, detected at 210, 250, 270, and 365 nm for the same samples passing through and bypassing the column. The recovery of all samples was about 94-98%. It means that analytes did not adsorb onto the TSK-gel.

The full SEC-profiles of the A250/A365 and A210/A254 ratios were obtained by dividing the chromatogram intensities detected at 250 or 210 nm by the intensities detected at 365 or 254 nm. The absorbance and fluorescent spectra at chromatographic points 1-9, 6a, 7a, and 8a were extracted from the data of multi-wavelength absorbance and fluorescent detectors.

3. Results and discussion

3.1. Absorption spectra and fluorescence excitation-emission matrix of DOM from river and lakes

The UV-visible absorption spectra of the Suwannee River DOM (SRDOM) and Karelian lakes DOM (L1DOM and L2DOM) appeared to be featureless without major maxima, and decreased gradually with increasing wavelength with some shoulder between 260–280 nm, the shoulder was more pronounced for the L2DOM sample (Fig. 1). In addition, essential differences in the slopes were observed between the samples, expressed by the absorbance ratios such as A210/A254 and A250/A365. These ratios were used by several researchers to

monitor changes in DOM chemical peculiarities [27, 37-39]. Both ratios varied in the order L2DOM > L1DOM > SRDOM (Table 1) and seemed to be connected with their structural properties.

The EEM of all DOM samples exhibited two humic-like fluorescent maxima at excitation wavelengths (λex) 230 and 330 nm with emission wavelengths (λem) between 420 and 460 nm (Table 1, Fig. 2). The humic-like fluorescence intensity was more or less similar in the all investigated DOM samples, and the highest emission intensity was found at λex = 230 nm. In the L1DOM and L2DOM samples the emission maxima at λex=330 nm were red-shifted by 20-25 nm compared to the emission maxima at λex=230 nm (Table 1), while in the SRDOM at both λex the emission maxima were identical (450 nm). Besides, L2DOM was the only sample of the series to contain the protein-like fluorescence maxima centered at λex / λem - 230/340 nm and 270/340 nm (Table 1, Fig. 2).

The analysis of absorption spectra and EEM revealed quite distinct differences between optical properties of DOM samples. To get additional information about their structural peculiarities we carried out their analysis by SEC-HPLC equipped with multi-wavelength absorbance and fluorescence detectors.

3.2. SEC-HPLC analysis of DOM from river and lakes with multi-wavelength UV detection

SRDOM, L1DOM and L2DOM samples were characterized by SEC-HPLC on TSKgelG2000SW$_{XL}$ - column in 30 mM phosphate buffer, pH 6.5. All chromatograms showed an intense hump with rather well-resolved peaks at the tailing edge (Fig. 3a) and two small peaks close to void column volume (inset to Figure 3a). It should be noted that all samples exhibited rather similar shapes and the same absorbance peaks and shoulders at any wavelength detection in the range 210-400 nm, when multi-wavelength UV detector was used. As an example the chromatograms detected at 365 nm were presented (Figure 3b).

The separation by SEC is commonly described by size exclusion effect: the higher is the elution volume (Ve), the lower is the molecular size (MS). Whereas all samples showed rather similar elution profiles, the chromatographic curves of L1DOM and especially L2DOM samples were shifted to higher Ve in comparison with SRDOM (Fig. 3a, b). The average MS of samples varied therefore in the order SRDOM > L1DOM > L2DOM. All DOM samples were obtained by using freeze drying procedure (see Materials and methods). On this reason the differences in MS of DOM samples investigated do not depend on freeze drying.

The differences in the average relative MS of the investigated aquatic DOM samples are well consistent with the ratio A250/A365 (Table 1): the higher is the average MS of DOM, the lower is the ratio value. To get deeper insight into the nature of DOM of different origin, the behavior of this ratio as a function of Ve has been studied. Fig. 3c presents the variation of A250/A365 values vs. Ve for SRDOM, L1DOM and L2DOM samples. SRDOM and L1DOM exhibited similar tendency: in the first part of the column fractionation range (from 6.2 to 9.8 ml) the A250/A365 values gradually increased, while in the second part (from 9.8 ml to Vp=13.1 ml) several local maxima and minima of A250/A365 values were observed. The maximal A250/A365 values exactly corresponded to the tops of the absorbance peaks 6, 7, 8 and 9, and the minimal values belong to the chromatographic points 6a, 7a, 8a between the local peaks (Table 2, Fig. 3c). The shape of A250/A365 values in the first part of the L2DOM chromatogram showed an intense broad hump with a maximum of about 11±2.0, located at Ve=6.5-7.5 ml, that is likely different from SRDOM and L1DOM. However, it is clear seen from Figure 3b that L2DOM absorbance at 365 nm is very low between Ve=6-8 ml leading to low signal-to-noise ratio (Figure 3c). On this reason we cannot do exact conclusion about the behavior of A250/A365 ratio in this Ve-region for L2DOM. Nevertheless the second part of the L2DOM chromatogram showed the same 6, 7, 8 and 9 peaks, but local maxima and minima had approximately 1.5 times higher values.

The A250/A365 ratio was used by several researchers to monitor DOM molecular size [27, 37, 38, 40], a negative relationship between average MS of DOM and the A250/A365 ratios was observed. This trend was found as well for the bulk SRDOM, L1DOM and L2DOM (Table 1), but after SEC-HPLC fractionation this correlation was no longer observed (Tables 2, Fig. 3c). We assume therefore that absorbance ratio A250/A365 may be used only for a rough MS estimation of the bulk aquatic DOM.

It is well known that anthropogenic organic matter from municipal wastewater and/or eutrophic process in aquatic environments exhibit strong protein-like fluorescence [12, 15-17]. All proteins composed of 20 amino acids and only three of them (phenylalanine, tyrosine and tryptophan) contain aromatic rings. The weight content of aromatic amino acids in proteins is about 10% [41]. On this reason the aromaticity of proteins is generally low and proteins are mostly aliphatic. The presence of protein-like matter could be detected as well by A210/A254 ratio, because protein-like compounds (proteins, short and long peptides or free amino acids) have specific absorption spectra with in order or more higher A210/A254 ratio, than typical aquatic DOM samples [39, 27]. Her et al., 2008 [39] first used the absorbance ratio A210/A254 for analysis of protein DOM compounds after SEC fractionation. The wavelength 210 nm was chosen to detect DOM functional groups (carboxy and amide groups) absorbing light at shorter wavelengths. It should note that carboxy and amide groups are not aliphatic, but they are an essential part of proteins with possessed mostly aliphatic properties. The wavelength 254 nm was chosen, because it is widely used for detection of aromatic compounds .

On Fig. 3d the variation of A210/A254 values vs. Ve for the three DOM samples are presented. During all column fractionation range the SRDOM and L1DOM exhibited more or less constant low A210/A254 values (2.3-1.6) (Table 2). These results generally coincide with the ones presented by Her et al. [39] and Yan et al. [27] for SRHA and SRFA standards.

However, the L2DOM sample had high A210/A254 values at the fractionation start that gradually decreased from 8 to 2 at the second part of the column fractionation range.

The analysis of A210/A254 behavior vs. Ve demonstrated that L2DOM from Karelian Onego Lake was enriched in high MS aliphatic compounds in comparison with Suwannee River DOM and L1DOM from Karelian Vodoprovodnoe Lake. These data correlate well with the EEM analysis of DOM samples (see Fig. 2).

The compounds eluted in the second part of column fractionation range for all investigated DOM samples demonstrated low A210/A254 values and therefore had mainly aromatic nature. On the other hand, the ratio A250/A365 demonstrated that aromatic compounds located in the second part of column fractionation range differ in the slopes of absorbance spectra (Table 2) and chemical structure.

3.3. SEC-HPLC analysis of humic-like and protein-like fluorescence distribution with multi-wavelength fluorescence detection

On Fig.4 the UV chromatograms of the SRDOM, L1DOM and L2DOM were compared with the two fluorescence chromatograms set at Ex/Em - 270/450 nm for detection of humic-like fluorescence and 270/345 nm for visualization of protein-like fluorescence.

Regardless of the DOM sources and their different average MS, the humic-like fluorescence (dashed black lines) showed similar profiles with several well resolved peaks 5, 6, 7, 8 and 9 corresponding to the absorbance peaks (solid black lines). The emission spectra at the Ve corresponding to the top of humic-like fluorescent peaks 5-9 and the chromatographic points 1-4, 6a, 7a and 8a, were extracted from the data of multi-wavelength fluorescence detector. The emission maxima presented in Table 2. It was found, that in all DOM samples the gradual decrease of emission maxima paralleled the decrease of DOM molecular size. The emission spectra of several humic-like peaks presented on Figure 5

(d,e,f). It should be noted, that Wu et al. [29] and Her et al. [42], who conducted chromatographic experiments with offline EEM-detection, also noted some small blue shift of emission maxima in parallel with decreasing MS.

The fluorescence chromatograms of all DOM samples set at Ex/Em 270/345 nm for visualization of protein-like fluorophores are shown on Fig. 4 (red lines). All chromatograms had similar shape, but in L2DOM the intensities of peaks 5-9 were about twice higher than in SRDOM or L1DOM. Moreover, only the L2DOM protein-like chromatogram was located above the baseline in the fractionation region between 6.2 and 9.0 ml (Fig. 4). These data and those of EEM (see Fig. 2) allowed us to suggest that protein-like matter of L2DOM were in higher amounts than in the other samples and distributed along the all column fractionation range. The protein-like fluorescence was found in the first part of the column fractionation range as an individual emission peak with maximum at 345 nm (Fig. 5f, chromatographic points 2 and 3, Table 2) and as a shoulder at 345 nm in the second part of the column fractionation range (Fig. 5f, humic-like peaks 4,6,8, Table 2,). Additionally, the presence of protein-like matter in the L2DOM was confirmed by UV spectra of chromatogtaphic points 2 and 3 with high absorbance at 210 nm and maximum at 280 nm (Fig. 5c) as characteristic absorbance spectra of proteins [43]. In contrast, in SRDOM and L1DOM the protein-like fluorescence was not found in the all column fractionation range (Fig. 5 a,b,d,e, Table 2,). In L2DOM a blue shift (5-20 nm) in the emission maxima of the individual humic-like fluorophores with identical Ve is the result of overlapping of humic-like fluorophores with protein-like ones distributed throughout the all column fractionation range.

After the development of the EEM fluorescence analysis in DOM chemistry [44], it was accepted that DOM of different origin were characterized by different EEM spectra [6, 7, 18]. Very recently in contrast to this assumption Wünsch et al. [8] after analysis of 90 peer-reviewed PARAFAC models revealed that some fluorescence components reoccur irrespective of aquatic DOM sources. Our results are well consistent with this suggestion,

because regardless of DOM origin (river or lakes) and different geographical locations our samples possessed a number of fluorophores with similar emission maxima, chromatographic behaviors and relative MS.

As known from the literature, strong correlations have been found between protein-like fluorescence and biochemical oxygen demand in natural water, which indicates water impacted by microbial activity [15-16]. The significant protein-like fluorescence, which has been detected only in L2DOM could be resulting from microbial activity stimulated by biogenic and mineral substances flowed with wastewater to the Kondopoga Bay of the Onego Lake.

4. Conclusions

- The SEC-HPLC with information-reach multi-wavelength fluorescence and absorbance detections and 3D-fluorescence EEM analysis were used for the characterization of river and lakes DOM of different genesis and geographical locations. It was found that independently on UV-detection in the range 200-400 nm the average MS of DOM samples varied in the order SRDOM > L1DOM > L2DOM.
- Regardless of the DOM sources and their different average MS, several similar types of humic-like SEC-separated fluorophores were found. In all DOM the decrease of the fluorophores emission maxima paralleled the decrease of their MS. The blue-shifted emission maxima by 5-20 nm in L2DOM are primarily related to the admixture of some compounds providing protein-like fluorescence distributed through out all fractionation range of the SEC-column.
- The absorbance ratios A250/A365 and A210/A254 have been applied for characterization the DOM fractionated by SEC-HPLC. The A250/A365 ratio may be used only for a rough estimation of the bulk aquatic DOM molecular size, but after SEC-HPLC the negative

correlation between MS and this ratio was no longer observed. The absorbance ratio A210/A254 was well correlated with the content of DOM aliphatic compounds (proteins or peptides). The using of A250/A365 and A210/A254 ratios in tandem provides successful information about SEC-distribution of different structural aromatic and aliphatic compounds of DOM.

- The SEC-HPLC with multi-wavelength fluorescence and absorbance detection is a very useful technique to more accurate DOM analysis and tracking of microbial activity resulting from anthropogenic impact in water reservoir.

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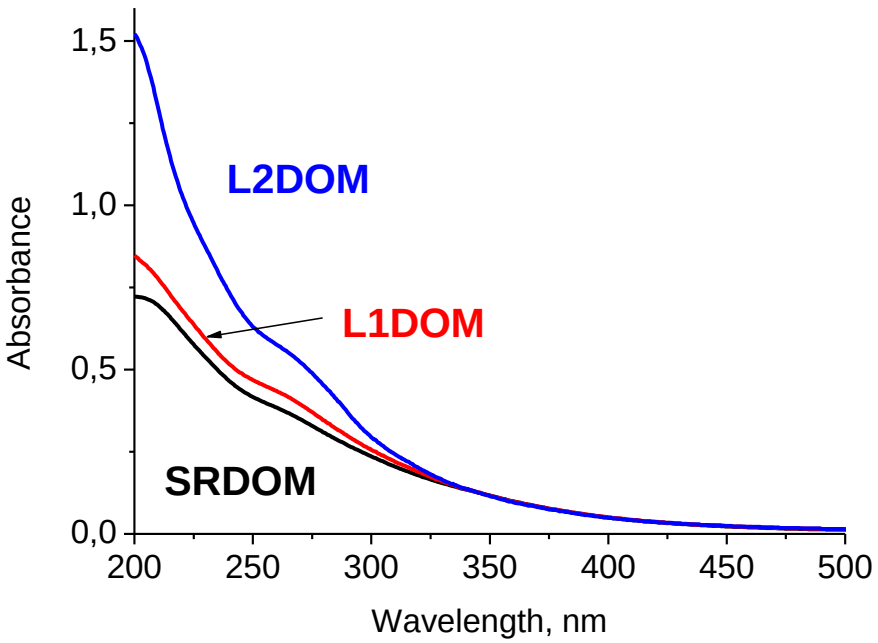

Figure 1. Absorbance spectra of DOM from Suwannee River (SRDOM) and Karelian Lakes Vodoprovodnoe (L1DOM) and Onego (L2DOM) in 10 mM phosphate buffer (pH-6.5) normalized at a wavelength 400 nm (0.05 a.u.) to see differences in slopes.

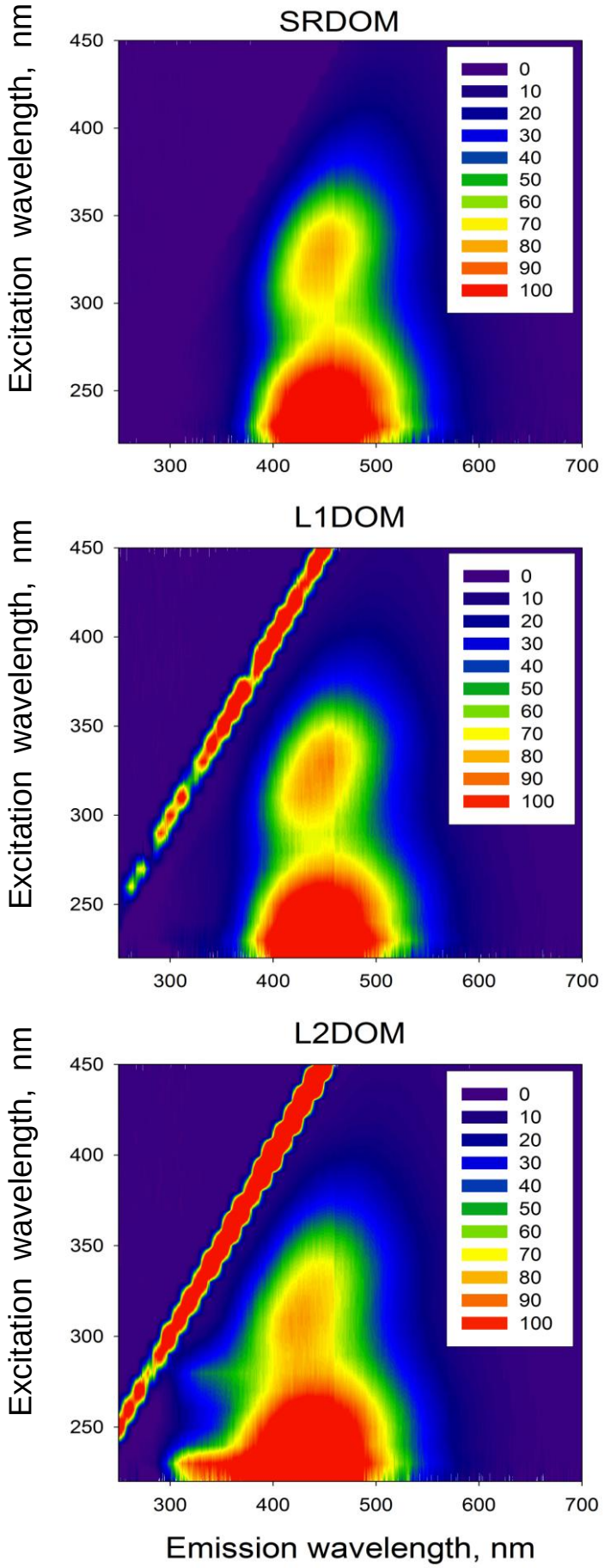

Figure 2. The EEM of DOM from Suwannee River (SRDOM) and Karelian Lakes Vodoprovodnoe (L1DOM) and Onego (L2DOM) at A270=0.05 a.u. in 0.3 mM phosphate buffer, pH 6.5. Fluorescence intensity expressed by different colors in the write upper corner of each graphic.

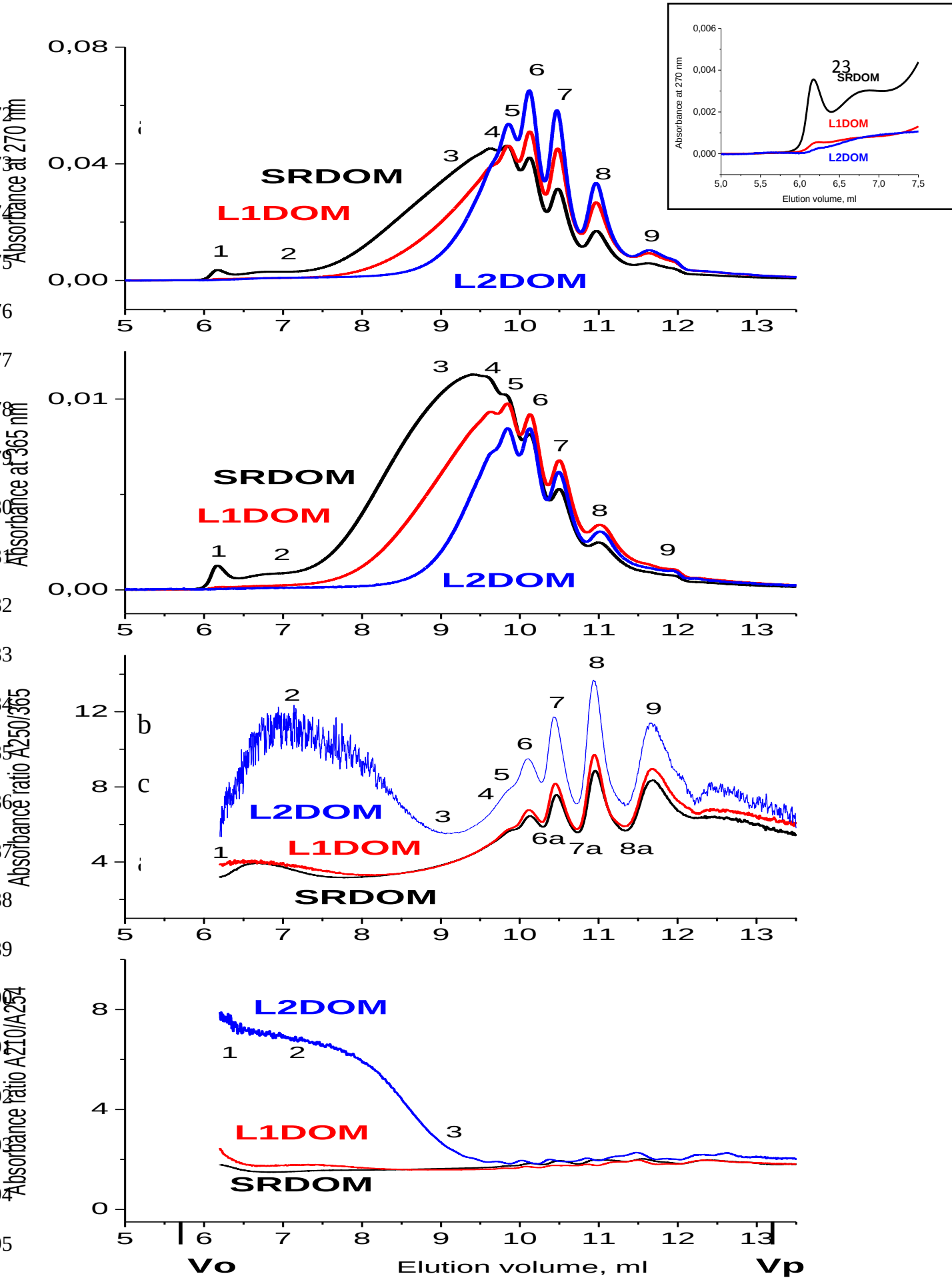

Figure 3. SEC-HPLC chromatograms with absorbance detection at 270 nm (a) and at 365 nm (b), absorbance ratios A250/A365 (c) and A210/A254 (d) of DOM from Suwannee River (SRDOM - black lines) and Karelian Lakes Vodoprovodnoe (L1DOM - red lines) and Onego (L2DOM - blue lines). *Inset*: zoom on the 5 to 7.5 ml zone of the chromatograms

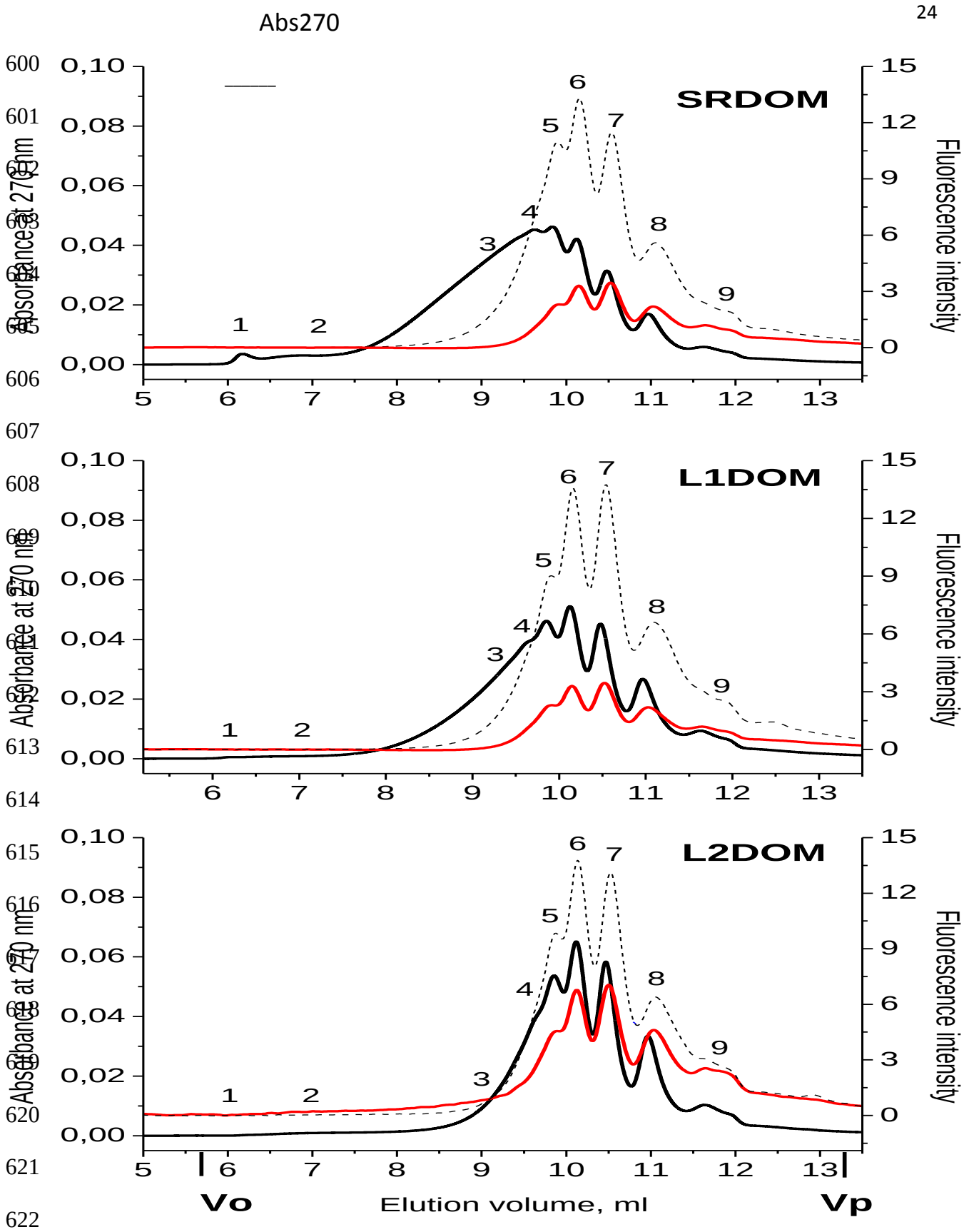

Figure 4. SEC-HPLC chromatograms with absorbance detection at 270 nm (solid black lines), and fluorescent chromatograms with detection at Ex/Em wavelength 270/450 nm (dashed black lines) and Ex/Em wavelength 270/345 nm (red lines) of DOM from Suwannee River (SRDOM) and Karelian Lakes Vodoprovodnoe (L1DOM) and Onego (L2DOM).

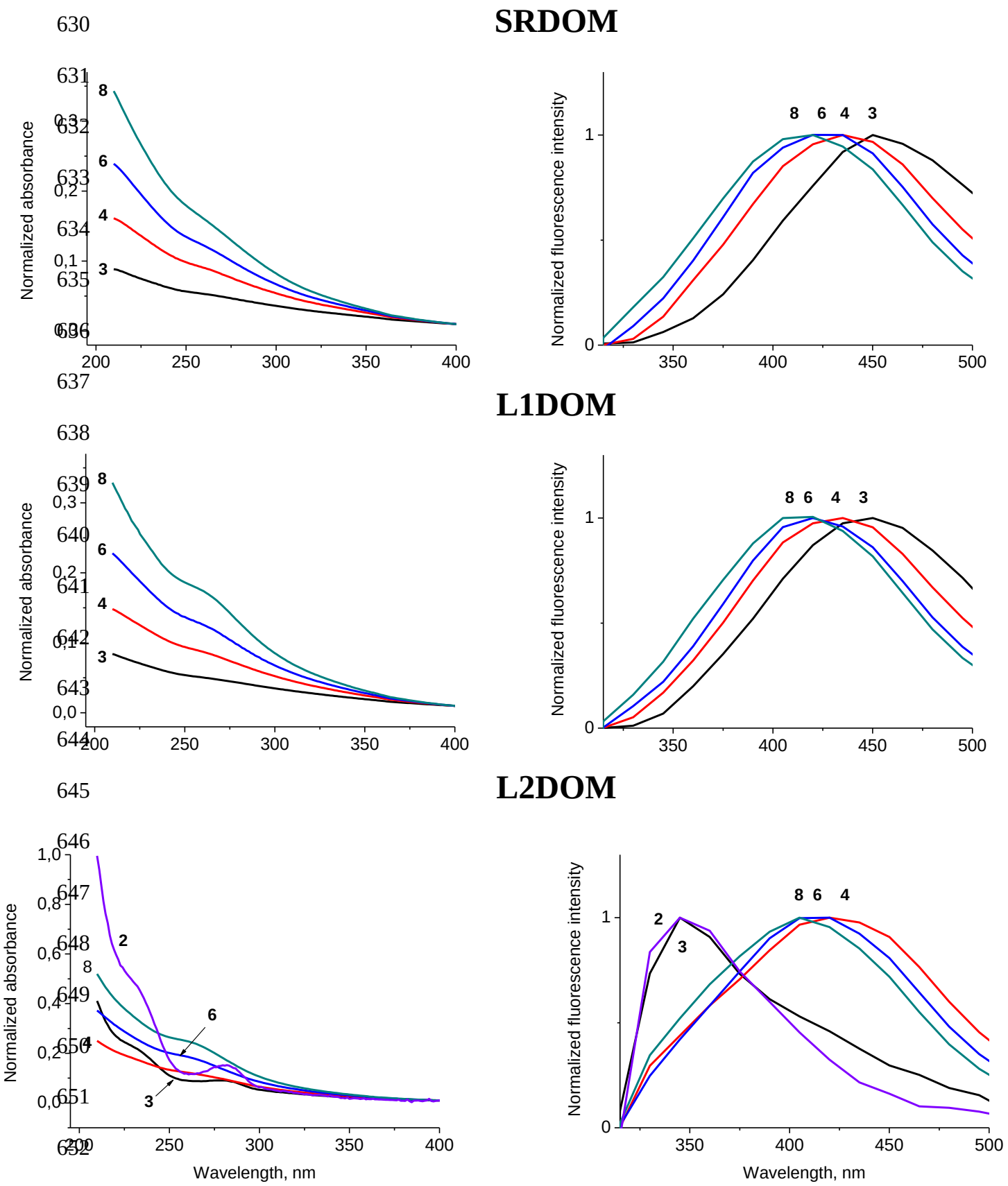

Figure 5. Absorbance spectra, normalized at 400 nm (a,b,c) and emission spectra, normalized at a maximum (d,e,f) of compounds, eluted at the chromatographic points 2, 3, 4, 6 and 8 after SEC-HPLC of DOM from Suwannee River (SRDOM) and Karelian Lakes Vodoprovodnoe (L1DOM) and Onego (L2DOM). Absorbance spectra were extracted from the data of multi-wavelength absorbance detector and fluorescence spectra – from the data of multi-wavelength fluorescence detector.

Sample name	Absorbance ratios		Fluorescence maxima (nm)	
	A210/A254	**A250/A365**	**Protein-like (λex/λem)**	**Humic-like (λex/λem)**
SRDOM	**1.71**	**4.58**	**-**	**230/450** **330/450**
L1DOM	**1.73**	**5.21**	**-**	**230/440** **330/460**
L2DOM	**2.14**	**7.05**	**230/340** **270/340**	**230/420** **330/445**

Table 1. Absorbance ratios A210/A254 and A250/A365, protein-like and humic-like fluorescence maxima (λex/λem) of DOM from Suwannee River (SRDOM) and Karelian Lakes Vodoprovodnoe (L1DOM) and Onego (L2DOM).

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Number of chromatographicpoint	Elution volume(ml)	SRDOM			L1DOM			L2DOM		
		A250/365	A210/254	Em max(nm)	A250/365	A210/254	Em max (nm)	A250/365	A210/254	Em max (nm)
1	6.20	3.18	1.76	-	3.83	2.34	-	6.46	7.84	-
2	7. 0	3.85	1.51	-	4.05	1.76	-	11.45	6.86	345
3	9.0	3.53	1.54	450	3.53	1.63	450	6.37	4.09	345
4	9.60	5.12	1.76	435	5.13	1.76	435	6.84	1.89	425sh* 345
5	9.80	5.43	1.70	435	5.64	1.72	430	7.45	1.89	415 sh 345
6	10.12	6.33	1.64	430	6.67	1.64	420	9.47	1.65	415 sh 345

6a	10.2	5.90	1.69	420	6.11	1.67	420	8.31	1.89	415sh 345
7	10.5	7.37	1.76	420	8.13	1.77	420	11.84	1.76	410sh 345
7a	10.7	5.59	1.83	420	5.72	1.83	420	7.06	1.95	410sh 350
8	11.0	8.35	1.89	410	9.69	1.90	410	13.69	2.02	405sh 345
8a	11.3	5.60	1.89	410	5.85	1.88	410	7.06	2.15	405sh 345
9	11.6	7.78	1.82	410	8.95	1.83	410	11.45	2.02	405sh 345

*sh, Shoulder

Table 2. The absorbance ratios A250/A365, A210/A254 and emission maxima (Em max) of chromatographic points 1-9, 6a, 7a and 8a after SEC-HPLC of DOM from Suwannee River (SRDOM) and Karelian Lakes Vodoprovodnoe (L1DOM) and Onego (L2DOM).

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