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

4

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16

17 **ABSTRACT:**

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

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

38

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

43

44 **1. Introduction**

45

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

53 insignificant water sample volumes and considerable information on its chemical structure [6-
54 9]. Measurement of fluorescence is used to examine DOM spatial and temporal distribution in
55 the ocean [10-11], to study allochthonous and autochthonous sources of organic matter [12],
56 to trace riverine input of organic carbon to the ocean [13] and to calibrate satellite data in
57 remote sensing [14]. Fluorescence spectroscopy can be used as well for estimation of organic
58 pollutions in natural water [15], in recycled water systems [16] or wastewaters [17].

59 As it is presently accepted, the fluorescence of DOM is due to two basic groups of
60 fluorophores [8, 12, 18-21]. The first group shows emission within the wavelength range 380-
61 470 nm (so called humic-like fluorescence) under excitation within the wavelength range 220-
62 380 nm. The other group emits protein-like fluorescence with emission wavelength shorter
63 than 380 nm and has maximum of excitation at wavelengths shorter 305 nm; it is affiliated to
64 aromatic amino acids demonstrating tyrosine-like (emission wavelength of about 300 nm) or
65 tryptophan-like (about 340 nm) types of fluorescence bands [18]. Correlations between
66 tryptophan-like or tyrosine-like fluorescence and biochemical oxygen demand indicated the
67 presence of labile organic matter due to sewage in the riverine water and/or eutrophic impact
68 [12, 15]. Despite its importance, the true source of DOM protein-like fluorescence remains
69 unknown, because this type of fluorescence may derive from proteins, peptides or free amino
70 acids.

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

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

79 Because fluorescence properties of chromophores are generally tied to their molecular
80 structures and respond to changes on the molecular level, different MS components obtained
81 by SEC-HPLC may also reveal considerable information about the chemical structures
82 responsible for their fluorescence properties. To improve information provided by SEC-
83 HPLC, researchers frequently employed several techniques including UV-visible absorption,
84 2D- and 3D-fluorescence, NMR, DOC-analysis, PAGE etc. coupled in a single analytical
85 chromatographic run [8, 24, 27, 29-31].

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

92

93 **2. Materials and methods**

94 *2.1. Materials and water sampling sites*

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

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

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

111 The Onego Lake, the second largest lake in Europe, belongs to the basin of the Baltic Sea,
112 Atlantic Ocean, and is situated between Lake Ladoga (the largest in Europe) and the White
113 Sea. It covers an area of 9720 square km with the average depth 30 m. Onego Lake receives
114 1152 rivers; water balance is also provided by precipitation and water discharge from human
115 activities: industrial and household water, melioration-related drainage etc. The total flow of
116 terrigenous organic matter into the Onego lake ecosystem is determined by the spatial
117 structure of the biogeocenoses in the catchment area. The shores to the north and northwest
118 are high and rocky, built of layered granite and covered with spruce and pine forest. The
119 DOM in the Onego Lake is ~90% due to river runoff, and the rest is equally divided between
120 atmospheric precipitation and waste waters from three main industrial centers, Petrozavodsk,
121 Kondopoga, and Medvezhegorsk [34]. Surface water sampling was carried out away from
122 industrial facilities in the middle part of the Kondopoga Bay from a rowing-boat. Since the
123 study and comparison of polluted waters was not the aim of our work, we have sampled water
124 in non-high polluted area, far from settlements. On this reason we did not expect to find out
125 essential anthropogenic and/or eutrophic material in the Onego Lake water sample. The
126 phytoplankton and bacterial development in surface water may be increased due to
127 eutrophication of water in the Kondopoga Bay [35].

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

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

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

139

140 *2.2. Preparation of stock DOM solutions for spectroscopic and chromatographic analyses*

141

142 The 100 ml of original freshwater samples were filtered using cellulose filters (Millipore)
143 with a pore size of $0.45 \mu\text{m}$ and freeze dried. Before the analysis 1 mg of each dry DOM
144 sample was completely dissolved in 2 ml of 30 mM phosphate buffer, pH 6.5 (PB). The 0.3
145 ml of each sample solution was diluted ten times using 30 mM PB and absorbance at 270 nm
146 was measured for diluted solutions with $C=50 \text{ mgL}^{-1}$. Using this data the A_{270} for the
147 concentrated DOM solutions ($C=500 \text{ mg L}^{-1}$) was calculated. Then all concentrated DOM
148 solutions were diluted using 30 mM PB to $A_{270} = 5 \text{ a.u.}$ As a result, the stock DOM
149 solutions had identical $A_{270}=5 \text{ a.u.}$ and different concentration ($C_{\text{SRDOM}}=362 \text{ mgL}^{-1}$,
150 $C_{\text{L1DOM}}=371 \text{ mgL}^{-1}$, $C_{\text{L2DOM}}=423 \text{ mgL}^{-1}$). Before further spectroscopic and chromatographic
151 analyses stock DOM solutions were stored in sealed glass bottles at $+4^\circ\text{C}$.

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

157

158 *2.3. UV-visible absorbance and 3D-excitation/emission matrix (EEM) fluorescence*
159 *spectroscopy*

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

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

167

168 *2.4. Analytical size exclusion high-performance liquid chromatography (SEC-HPLC) with*
169 *multi-wavelength absorbance and fluorescence detection*

170

171 SEC-HPLC of three DOM samples was conducted on a high-performance liquid
172 chromatographic system (Waters Corp., MA, USA). The multi-wavelength detector 2998,
173 working in the range 210-400 nm, was used for detection of absorbance. An Acquity
174 Fluorescence detector, connected directly to the waste line of the absorbance detector, was
175 used for fluorescence emission measurements and set to an excitation wavelength of 270 nm
176 and emission range of 300-600 nm. The column TSKgel G2000SW_{XL} (7.8 mm ID, 300 mm
177 L, particle size 5.0 µm) equipped with a guard column TSK G2000SW_{XL} (both - TOSOH
178 Bioscience, Japan) showed a fractionation range from 150 to 5 kDa for globular proteins.
179 Within experiments the 30 mM phosphate buffer was used as a sample buffer and as an eluent
180 with a flow rate of 1.0 mL min⁻¹. The use of the same buffer for sample dissolution and as an
181 eluent during SEC-procedure is the main requirement to avoid any additional peaks-artifacts,
182 especially at the end of the fractionation column's range. The void column volume (V_o),

183 determined with blue dextran 2000, was 5.6 mL. The permeation volume (V_p), determined
184 using low MW fluorescent amino acid tryptophan, was 13.1 mL. The injection volume was
185 0.02 mL. The stock DOM solutions applied on the column were eluted between about 5.6 and
186 13.1 mL and thus diluted to about 350 times. For this reason, the absorbance detector with
187 the absorbance limit of 2 a.u. could be used. The 30 mM phosphate buffer (pH-6.5) as the
188 mobile phase did not show any absorbance or fluorescence peaks. The entire SEC-HPLC
189 procedure was repeated three times (the deviations were no more than 3%). The recovery of
190 DOM samples on TSK-gel column was determined by comparing total peak areas of
191 chromatograms, detected at 210, 250, 270, and 365 nm for the same samples passing through
192 and bypassing the column. The recovery of all samples was about 94-98%. It means that
193 analytes did not adsorb onto the TSK-gel.

194 The full SEC-profiles of the A250/A365 and A210/A254 ratios were obtained by dividing
195 the chromatogram intensities detected at 250 or 210 nm by the intensities detected at 365 or
196 254 nm. The absorbance and fluorescent spectra at chromatographic points 1-9, 6a, 7a, and
197 8a were extracted from the data of multi-wavelength absorbance and fluorescent detectors.

198

199 **3. Results and discussion**

200

201 *3.1. Absorption spectra and fluorescence excitation-emission matrix of DOM from river and*
202 *lakes*

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

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

212 The EEM of all DOM samples exhibited two humic-like fluorescent maxima at excitation
213 wavelengths (λ_{ex}) 230 and 330 nm with emission wavelengths (λ_{em}) between 420 and 460
214 nm (Table 1, Fig. 2). The humic-like fluorescence intensity was more or less similar in the all
215 investigated DOM samples, and the highest emission intensity was found at $\lambda_{ex} = 230$ nm. In
216 the L1DOM and L2DOM samples the emission maxima at $\lambda_{ex}=330$ nm were red-shifted by
217 20-25 nm compared to the emission maxima at $\lambda_{ex}=230$ nm (Table 1), while in the SRDOM
218 at both λ_{ex} the emission maxima were identical (450 nm). Besides, L2DOM was the only
219 sample of the series to contain the protein-like fluorescence maxima centered at $\lambda_{ex} / \lambda_{em} -$
220 230/340 nm and 270/340 nm (Table 1, Fig. 2).

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

225

226 3.2. SEC-HPLC analysis of DOM from river and lakes with multi-wavelength UV detection

227

228 SRDOM, L1DOM and L2DOM samples were characterized by SEC-HPLC on
229 TSKgelG2000SW_{XL} - column in 30 mM phosphate buffer, pH 6.5. All chromatograms
230 showed an intense hump with rather well-resolved peaks at the tailing edge (Fig. 3a) and two
231 small peaks close to void column volume (inset to Figure 3a). It should be noted that all
232 samples exhibited rather similar shapes and the same absorbance peaks and shoulders at any
233 wavelength detection in the range 210-400 nm, when multi-wavelength UV detector was
234 used. As an example the chromatograms detected at 365 nm were presented (Figure 3b).

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

242 The differences in the average relative MS of the investigated aquatic DOM samples are
243 well consistent with the ratio A250/A365 (Table 1): the higher is the average MS of DOM,
244 the lower is the ratio value. To get deeper insight into the nature of DOM of different origin,
245 the behavior of this ratio as a function of V_e has been studied. Fig. 3c presents the variation of
246 A250/A365 values vs. V_e for SRDOM, L1DOM and L2DOM samples. SRDOM and
247 L1DOM exhibited similar tendency: in the first part of the column fractionation range (from
248 6.2 to 9.8 ml) the A250/A365 values gradually increased, while in the second part (from 9.8
249 ml to $V_p=13.1$ ml) several local maxima and minima of A250/A365 values were observed.
250 The maximal A250/A365 values exactly corresponded to the tops of the absorbance peaks 6,
251 7, 8 and 9, and the minimal values belong to the chromatographic points 6a, 7a, 8a between
252 the local peaks (Table 2, Fig. 3c). The shape of A250/A365 values in the first part of the
253 L2DOM chromatogram showed an intense broad hump with a maximum of about 11 ± 2.0 ,
254 located at $V_e=6.5-7.5$ ml, that is likely different from SRDOM and L1DOM. However, it is
255 clear seen from Figure 3b that L2DOM absorbance at 365 nm is very low between $V_e=6-8$ ml
256 leading to low signal-to-noise ratio (Figure 3c). On this reason we cannot do exact conclusion
257 about the behavior of A250/A365 ratio in this V_e -region for L2DOM. Nevertheless the
258 second part of the L2DOM chromatogram showed the same 6, 7, 8 and 9 peaks, but local
259 maxima and minima had approximately 1.5 times higher values.

260 The A250/A365 ratio was used by several researchers to monitor DOM molecular size
261 [27, 37, 38, 40], a negative relationship between average MS of DOM and the A250/A365
262 ratios was observed. This trend was found as well for the bulk SRDOM, L1DOM and
263 L2DOM (Table 1), but after SEC-HPLC fractionation this correlation was no longer observed
264 (Tables 2, Fig. 3c). We assume therefore that absorbance ratio A250/A365 may be used only
265 for a rough MS estimation of the bulk aquatic DOM.

266 It is well known that anthropogenic organic matter from municipal wastewater and/or
267 eutrophic process in aquatic environments exhibit strong protein-like fluorescence [12, 15-
268 17]. All proteins composed of 20 amino acids and only three of them (phenylalanine, tyrosine
269 and tryptophan) contain aromatic rings. The weight content of aromatic amino acids in
270 proteins is about 10% [41]. On this reason the aromaticity of proteins is generally low and
271 proteins are mostly aliphatic. The presence of protein-like matter could be detected as well by
272 A210/A254 ratio, because protein-like compounds (proteins, short and long peptides or free
273 amino acids) have specific absorption spectra with in order or more higher A210/A254 ratio,
274 than typical aquatic DOM samples [39, 27]. Her et al., 2008 [39] first used the absorbance
275 ratio A210/A254 for analysis of protein DOM compounds after SEC fractionation. The
276 wavelength 210 nm was chosen to detect DOM functional groups (carboxy and amide groups)
277 absorbing light at shorter wavelengths. It should note that carboxy and amide groups are not
278 aliphatic, but they are an essential part of proteins with possessed mostly aliphatic properties.
279 The wavelength 254 nm was chosen, because it is widely used for detection of aromatic
280 compounds .

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

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

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

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

296

297 *3.3. SEC-HPLC analysis of humic-like and protein-like fluorescence distribution with multi-*
298 *wavelength fluorescence detection*

299

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

303 Regardless of the DOM sources and their different average MS, the humic-like
304 fluorescence (dashed black lines) showed similar profiles with several well resolved peaks 5,
305 6, 7, 8 and 9 corresponding to the absorbance peaks (solid black lines). The emission spectra
306 at the V_e corresponding to the top of humic-like fluorescent peaks 5-9 and the
307 chromatographic points 1-4, 6a, 7a and 8a, were extracted from the data of multi-wavelength
308 fluorescence detector. The emission maxima presented in Table 2. It was found, that in all
309 DOM samples the gradual decrease of emission maxima paralleled the decrease of DOM
310 molecular size. The emission spectra of several humic-like peaks presented on Figure 5

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

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

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

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

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

346

347 **4. Conclusions**

348

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

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

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

371

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376

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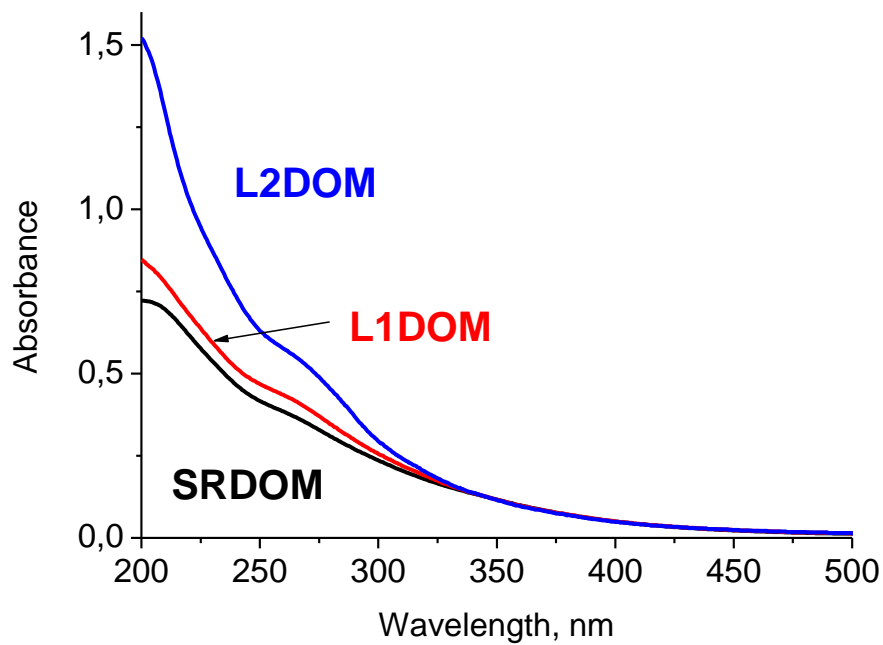
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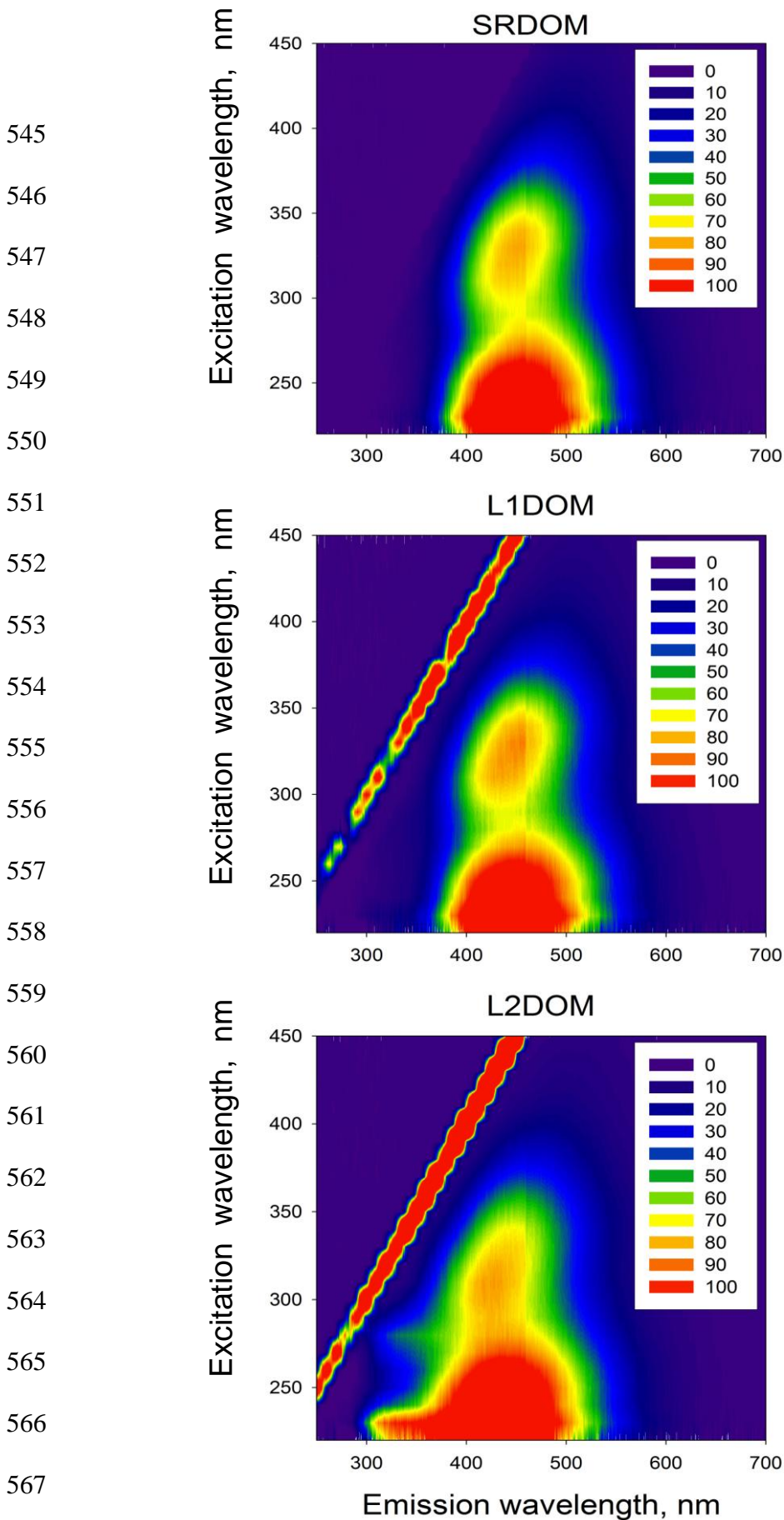
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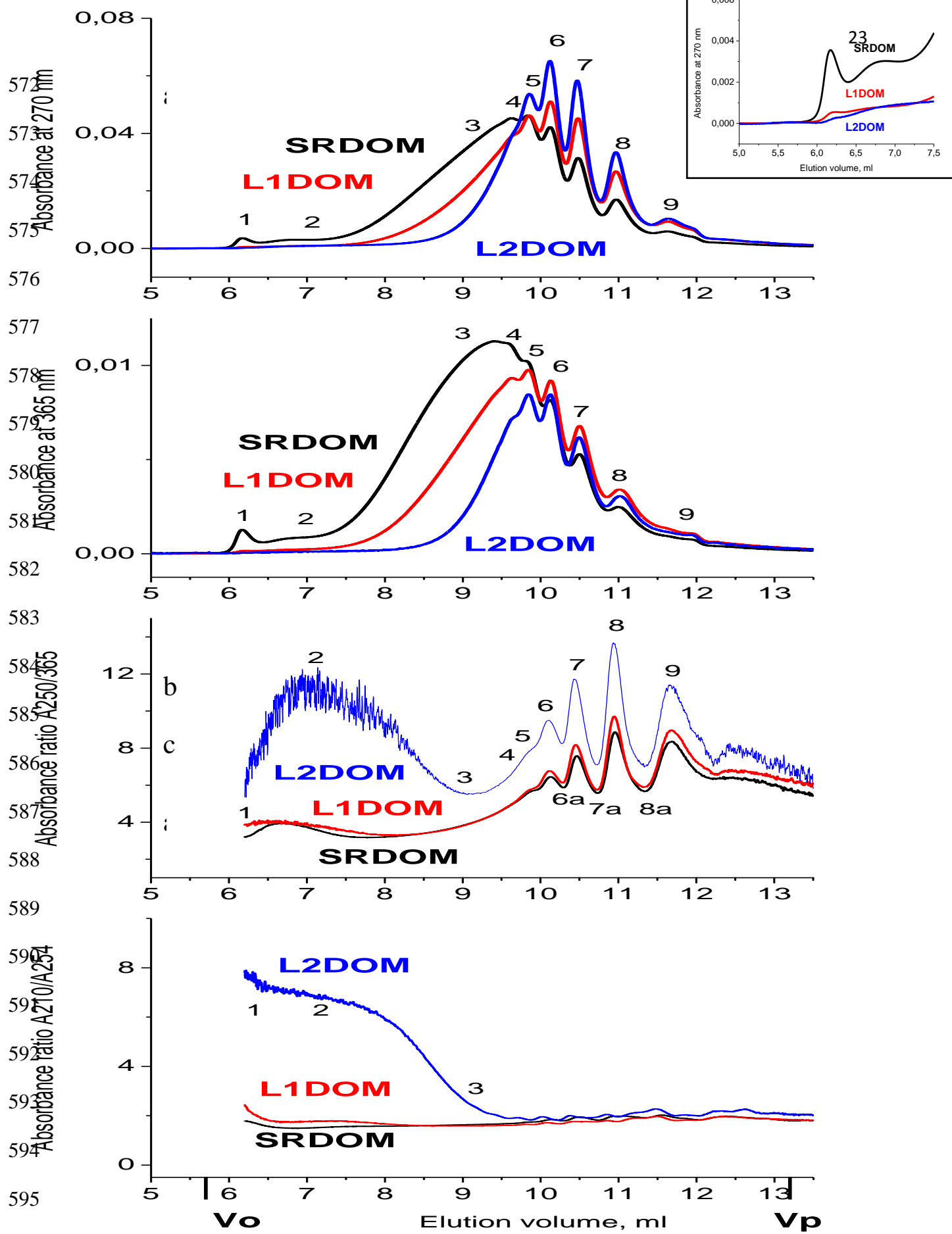
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Figure 1. Absorbance spectra of DOM from Suwannee River (SRDOM) and Karelian Lakes Vodoprovodnoe (L1DOM) and Onego (L2DOM) in 10 mM phosphate buffer (pH-6.5) normalized at a wavelength 400 nm (0.05 a.u.) to see differences in slopes.



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



596 **Figure 3.** SEC-HPLC chromatograms with absorbance detection at 270 nm (a) and at 365
 597 nm (b), absorbance ratios A_{250}/A_{365} (c) and A_{210}/A_{254} (d) of DOM from Suwannee River
 598 (SRDOM - black lines) and Karelian Lakes Vodoprovodnoe (L1DOM - red lines) and Onego
 599 (L2DOM - blue lines). *Inset:* zoom on the 5 to 7.5 ml zone of the chromatograms

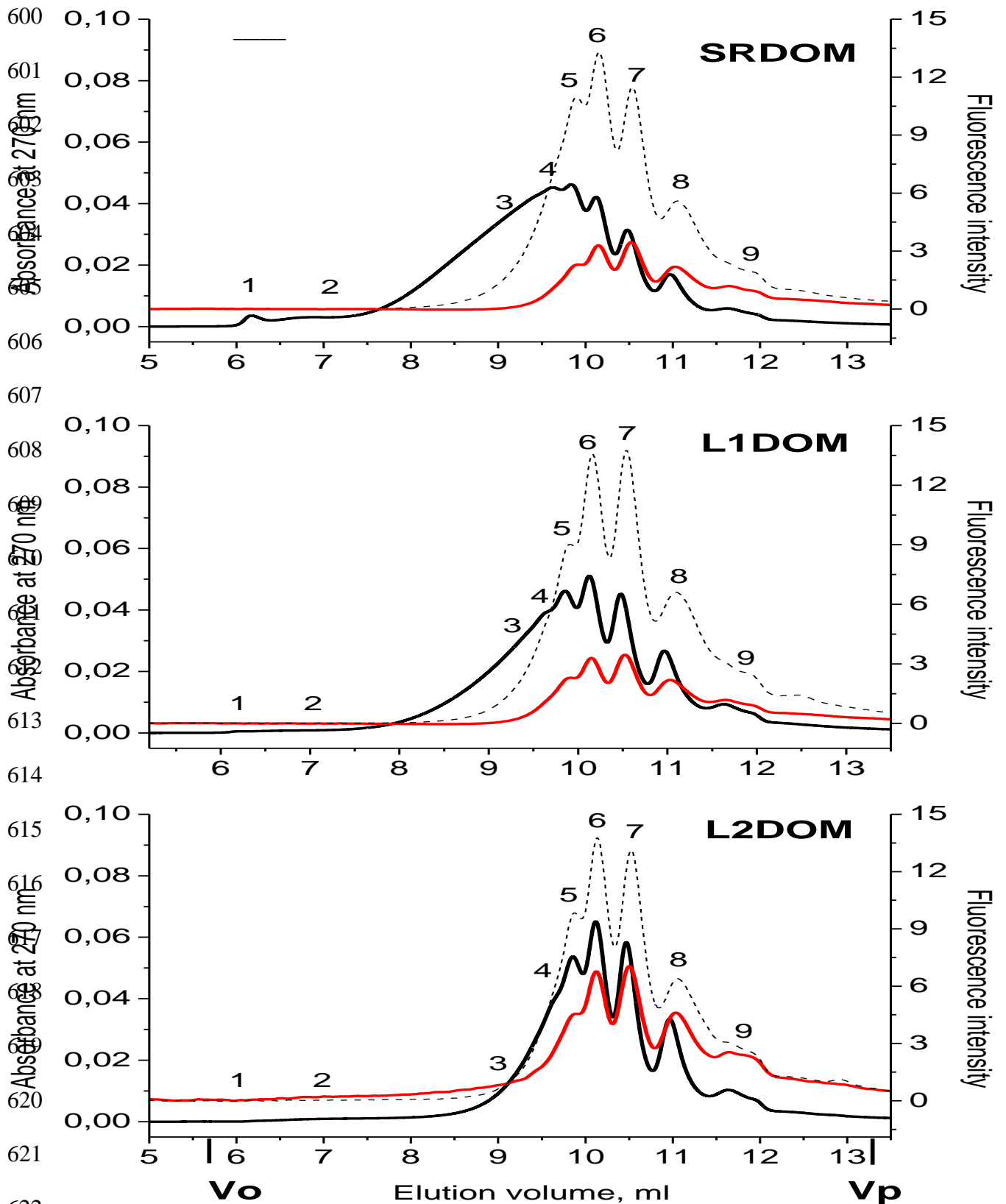


Figure 4. SEC-HPLC chromatograms with absorbance detection at 270 nm (solid black lines), and fluorescent chromatograms with detection at Ex/Em wavelength 270/450 nm (dashed black lines) and Ex/Em wavelength 270/345 nm (red lines) of DOM from Suwannee River (SRDOM) and Karelian Lakes Vodoprovodnoe (L1DOM)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

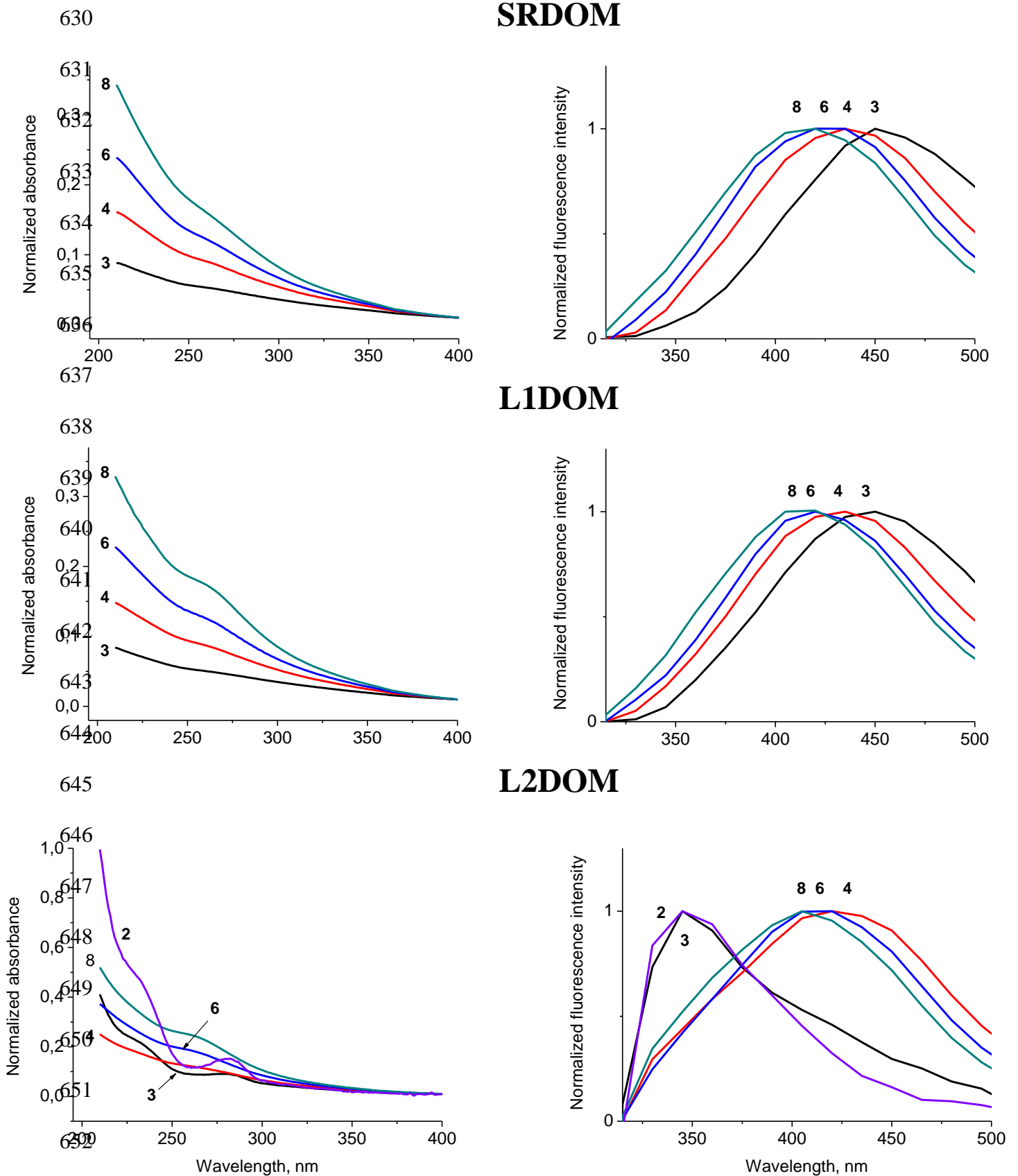


Figure 5. Absorbance spectra, normalized at 400 nm (a,b,c) and emission spectra, normalized at a maximum (d,e,f) of compounds, eluted at the chromatographic points 2, 3, 4, 6 and 8 after SEC-HPLC of DOM from Suwannee River (SRDOM) and Karelian Lakes Vodoprovodnoe (L1DOM) and Onego (L2DOM). Absorbance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

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Sample name	Absorbance ratios		Fluorescence maxima (nm)	
	A210/A254	A250/A365	Protein-like ($\lambda_{ex}/\lambda_{em}$)	Humic-like ($\lambda_{ex}/\lambda_{em}$)
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

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662 **Table 1.** Absorbance ratios A210/A254 and A250/A365, protein-like and humic-like
663 fluorescence maxima ($\lambda_{ex}/\lambda_{em}$) of DOM from Suwannee River (SRDOM) and Karelian
664 Lakes Vodoprovodnoe (L1DOM) and Onego (L2DOM).

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Number of chromatograp hicpoint	Elut ion volu me(ml)	SRDOM			L1DOM			L2DOM		
		A250/ 365	A210/ 254	Em max(nm)	A250/365	A210/2 54	Em max (nm)	A250/3 65	A210/2 54	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	10.1 2	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

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674 *sh, Shoulder

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

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