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**Author(s):** Ignatev, Alexey; Tuhkanen, Tuula

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Monitoring WWTP performance using size-exclusion chromatography with simultaneous UV and fluorescence detection to track recalcitrant wastewater fractions

Alexey Ignatev, Tuula Tuhkanen



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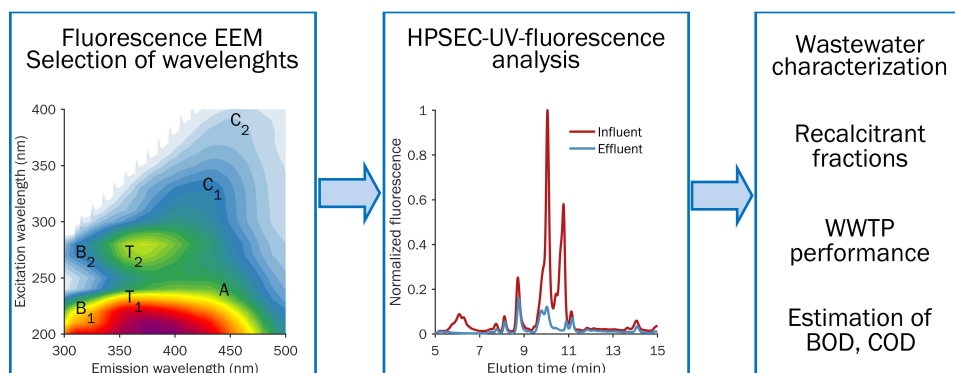
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1 **Monitoring WWTP performance using size-exclusion chromatography**  
2 **with simultaneous UV and fluorescence detection to track recalcitrant**  
3 **wastewater fractions**

4 Alexey Ignatev\*, Tuula Tuhkanen

5 Department of Biological and Environmental Science, University of Jyväskylä, PO Box 35, FI-

6 40014, Jyväskylä, Finland

7 \* Corresponding author

8 E-mail addresses:

9 [alexey.n.ignatev@ju.fi](mailto:alexey.n.ignatev@ju.fi), [alexignat@gmail.com](mailto:alexignat@gmail.com) (A. Ignatev),

10 [tuula.a.tuhkanen@ju.fi](mailto:tuula.a.tuhkanen@ju.fi) (T. Tuhkanen).

11

## Abstract

12 A trial monitoring of a typical full-scale municipal WWTP in Central Finland was aimed to  
13 explore applicability of high performance liquid chromatography – size exclusion chromatography  
14 (HPSEC) with simultaneous UV and fluorescence detection as a tool for advanced routine  
15 monitoring of wastewater treatment. High, intermediate, and low molecular weight (MW) fractions  
16 of wastewater influent and secondary effluent were characterized in terms of UV absorbance at  
17 254 nm (UVA<sub>254</sub>) and specific fluorescence represented tyrosine-like, tryptophan-like, and  
18 humic/fulvic-like compounds.

19 The activated sludge treatment removed  $97 \pm 1\%$  of BOD,  $93 \pm 2\%$  of COD,  $71 \pm 7\%$  of  
20 DOC, and  $24 \pm 7\%$  of TN, while reduction of total UVA<sub>254</sub> was  $50 \pm 6\%$ . Intensity of total  
21 fluorescence signal declined by ~80% for tyrosine-like, by 60-70% for tryptophan-like, and by 7-  
22 36% for humic/fulvic-like compounds. Low and intermediate MW humic/fulvic-like compounds  
23 fluorescing at  $\lambda_{ex}/\lambda_{em} = 390/500$  nm demonstrated recalcitrant behavior. Protein-like and  
24 humic/fulvic-like fractions of low MW < 1 kDa accounted for 60-65% of total UVA<sub>254</sub> and 50-70%  
25 of total fluorescence of whole influent and effluent samples. Strong linear correlations were  
26 observed between BOD, COD, DOC, UVA<sub>254</sub> and tyrosine-like, tryptophan-like fluorescence.

27 The analytical approach based on HPSEC with simultaneous UV and fluorescence detection  
28 allows rapid and advanced characterization of natural and anthropogenic organic matter in water  
29 treatment and distribution systems. Fairly good resolution archived in the HPSEC separation  
30 offers new opportunities for fingerprinting and tracking specific wastewater fractions.

31

## Keywords

32 Wastewater treatment;

33 Wastewater characterization;

34 Size-exclusion chromatography;

35 UV absorption;

36 Fluorescence;

37 Molecular weight distribution.

38

## 1. Introduction

39 In recent years, diverse contamination of the aquatic environment has been recognized by  
40 governmental regulators as a challenging and pervasive issue. The European Commission  
41 demands a strategic approach to control water pollution and calls for development of advanced  
42 but affordable analytical tools for routine monitoring (Directive, 2013). Of rising concern is  
43 current situation with emerging pollutants, which are not included in ongoing monitoring  
44 programs, but could pose significant risk (Directive, 2013).

45 In Europe, 95% of urban wastewater is collected and over 85% is treated according to the  
46 official requirements (Directive, 1991; European Commission, 2017). However, conventional  
47 wastewater treatment plants (WWTPs) incompletely remove emerging pollutants present at low  
48 concentrations, moreover, some soluble microbial products (SMPs) formed in activated sludge  
49 are more toxic and/or mutagenic than their parent compounds (Shon et al., 2006b). Thus,  
50 WWTPs become point sources of complex contamination of receiving water bodies. Importance of  
51 this issue rises in the light of water reuse. For example, Cyprus currently reuses 97% of treated  
52 wastewater (European Commission, 2017).

53 Performance of WWTPs and quality of wastewater effluent discharges are routinely  
54 assessed in terms of chemical and biochemical oxygen demand (COD and BOD), dissolved  
55 organic carbon (DOC), total nitrogen (TN), UV absorbance at 254 nm (UVA<sub>254</sub>), etc. However,  
56 these parameters are indicative and do not provide detailed information on properties of specific  
57 components of dissolved organic matter (DOM). At the same time, additional information is  
58 needed for contamination source tracking, designing and optimizing post-treatment, and risk  
59 assessment, particularly, due to impact of DOM on bioavailability of pollutants and xenobiotics  
60 (Choudhry, 1983).

61 While comprehensive characterization of DOM requires multi-method analytical approach  
62 (Abbt-Braun et al., 2004), many advanced techniques have limited applicability for routine  
63 monitoring of wastewater. For example, nuclear magnetic resonance (NMR) and pyrolysis-gas  
64 chromatography-mass spectrometry (py-GC/MS), being sensitive and powerful for structural  
65 analysis, are laborious, expensive, and require considerable operator expertise (Her et al., 2003).

66 Moreover, multicomponent polyfunctional nature of wastewater results in tangled NMR spectra  
67 and pyrochromatograms that are hard to interpret (Sillanpää et al., 2015).

68 Over the last decade, fluorescence spectroscopy has gained significant attention as a  
69 sensitive and inexpensive technique to control and optimize wastewater treatment (Carstea et  
70 al., 2016; Henderson et al., 2009; Mesquita et al., 2017). Rapid development is happening in  
71 the field of fluorescence sensors for online monitoring (Li et al., 2016). Due to high selectivity  
72 and sensitivity fluorescence detection can complement robust but limited UV-Vis spectroscopy.

73 Whole wastewater samples are often analyzed using excitation-emission matrix (EEM)  
74 fluorescence spectroscopy, which covers a wide range of excitation and emission wavelengths  
75 ( $\lambda_{ex}/\lambda_{em}$ ), usually, from ~200 to ~500 nm. Interpretation of EEM spectra via peak-picking, regional  
76 integration, parallel factor analysis (PARAFAC), in principle, allows discriminating different types  
77 of DOM from natural and anthropogenic sources (Chen et al., 2003; Yang et al., 2015). However,  
78 mitigation of the inner-filtering in samples with high DOC content requires additional steps:  
79 controlled dilution (Henderson et al., 2009) and/or algebraic correction (Kothawala et al., 2013).  
80 Shifting, overlapping, and broadening of peaks complicate EEM analysis (Yang et al., 2015).  
81 PARAFAC mathematically decomposes EEM spectra into simulated fluorescing components that  
82 are independent spectrally, but not necessarily physicochemically (Li et al., 2014). It leads to  
83 inconsistencies across EEM-PARAFAC studies (Ishii and Boyer, 2012).

84 Many of the aforementioned issues are avoided in high performance liquid  
85 chromatography – size-exclusion chromatography (HPSEC), which is widely used to characterize  
86 DOM in raw and drinking water (Sillanpää et al., 2015) and also in wastewater (Chon et al.,  
87 2017; Guo et al., 2011; Her et al., 2003; Imai et al., 2002; Jarusutthirak and Amy, 2007; Nam  
88 and Amy, 2008; Shon et al., 2006a, 2004; Szabo et al., 2016; Yan et al., 2012). HPSEC  
89 separates DOM components according to their molecular weight (MW) into fractions. In absence  
90 of secondary interactions, longer elution time corresponds to smaller MW. Secondary  
91 interactions, such as adsorption on the column material, hydrophobic and electrostatic effects,  
92 lead to advanced or delayed elution that violates the mechanism of purely steric exclusion (Le  
93 Coupanec et al., 2000). While hydrophobic effects are important at high ionic strength of eluent,

94 the role of electrostatic effects (particularly, ion exclusion) increases with decrease of eluent ionic  
95 strength (Specht and Frimmel, 2000). Eluents of low ionic strength (2-20 mM) provide high  
96 chromatographic resolution for anionic compounds, however, elution of cationic compounds is  
97 delayed due to the ion exclusion (Le Coupanec et al., 2000). Eluents with high ionic strength  
98 may be undesirable because “macromolecule aggregation in wastewater will be destroyed with  
99 the addition of Na<sup>+</sup>, which can substitute divalent metal ions (such as Ca<sup>2+</sup>) involved in  
100 extracellular polymeric substances bridging” (Guo et al., 2011).

101 Small sample volume, minimal pre-treatment, ease and speed of the analysis are among  
102 practical advantages of HPSEC over other characterization techniques (Michael-Kordatou et al.,  
103 2015). In many studies, HPSEC fractionation of wastewater is monitored by UVA<sub>254</sub> and online  
104 DOC, while use of fluorescence detection is less common.

105 The current work aims to examine applicability of HPSEC with simultaneous UV and  
106 fluorescence detection for routine monitoring of WWTP performance and advanced  
107 characterization of DOM in wastewater influent and effluent. The object of this study is municipal  
108 WWTP Nenäinniemi (Jyväskylä, Finland), operated by Jyväskylän Seudun Puhdistamo Oy. The  
109 WWTP serves 155 000 residents, uses a conventional activated sludge treatment and has an  
110 average wastewater flow of around 40 000 m<sup>3</sup> day<sup>-1</sup>, out of which about 13% is produced by local  
111 industry. The treated wastewater effluent is discharged into lake Päijänne. No effluent post-  
112 treatment was deployed at the time of this study.

## 113 **2. Materials and methods**

### 114 **2.1. Sampling and sample preparation**

115 All samples of wastewater influent (referred herein as “influent”) and the secondary  
116 effluent (referred herein as “effluent”) were 24 h composite. Preliminary EEM fluorescence  
117 analyses were done for one influent and one effluent samples collected on March 19, 2017.  
118 During the main sampling campaign samples were collected from the WWTP daily for two weeks,  
119 March 26-31 and April 3-6, 2017 (in total, 10 sampling days). To obtain results presented in



120 Section 3.2.3 additional influent and effluent samples were collected and analyzed in July-  
121 September 2017.

122 Upon delivery to the university laboratory, influent and effluent samples were ultra-  
123 centrifuged at 6 000 rpm for 30 min to facilitate further filtration. Additional experiments  
124 confirmed that the centrifugation did not affect HPSEC chromatograms. The supernatant was  
125 immediately filtered through disposable 0.45  $\mu\text{m}$  cellulose acetate syringe filters (VWR, USA) that  
126 were pre-washed with 20 mL of ultrapure water and then air purged. The first few mL of filtrate  
127 were discarded.

128 HPSEC and EEM fluorescence measurements were done within 24 h after sampling.  
129 Filtered 20 mL samples for DOC analysis were stored at  $-20\text{ }^{\circ}\text{C}$  in screw cap polypropylene tubes  
130 (Sarstedt, Germany) and analyzed within one month.

## 131 **2.2. EEM fluorescence spectroscopy**

132 EEM fluorescence spectra of influent and effluent samples were obtained using a Perkin-  
133 Elmer SL 55 spectrofluorimeter. The samples were diluted fivefold with ultrapure water. The  
134 number of replicates was one.

135 The excitation interval was 200-500 nm (step 10 nm) and the emission interval was 300-  
136 600 nm (step 0.5 nm). The emission cut-off filter was 290 nm, the scan rate was  $300\text{ nm min}^{-1}$ ,  
137 and the slit widths were 10 nm for both excitation and emission. Voltage of the photomultiplier  
138 (type R928) was set to "Auto".

139 Single emission spectra were combined into EEMs in MATLAB (R2016b, MathWorks, USA).  
140 Inner-filtering was corrected algebraically, according to (Kothawala et al., 2013), using UV/Vis  
141 spectra obtained with a Shimadzu UV-1800 spectrophotometer. The corrected EEM was  
142 normalized by Raman scatter peak of water, as suggested by (Lawaetz and Stedmon, 2009).  
143 During data processing, first order Rayleigh light scattering peak, caused by fine colloidal  
144 particles, was removed from the EEMs for visual clarity.

## 145 **2.3. DOC, BOD, and COD**

146 DOC measurements were done using a total organic carbon analyzer Shimadzu TOC-L  
147 equipped with an autosampler ASI-L. The non-purgeable organic carbon method was selected.

148 Neither influent nor effluent samples were diluted. Calibration was done in the DOC range 0-100  
149 mgC L<sup>-1</sup> using freshly prepared solutions of potassium phthalate. Prior analyses, vials were  
150 calcined at 400 °C for 4 h in air. All samples were acidified with HCl and purged with N<sub>2</sub> to strip  
151 dissolved inorganic carbon. A single DOC measurement included three 100 µL injections. Two  
152 duplicate analyses were done on two different days using two independent calibration curves,  
153 and the results were averaged.

154 BOD (7 days) and COD analyses were done by NabLabs Oy (Jyväskylä, Finland) according to  
155 Finnish standards specified in Supplementary material Table S1.

#### 156 2.4. HPSEC method

157 Influent and effluent samples were analyzed using an HPLC Shimadzu LC-30AD equipped  
158 with online degassing units Shimadzu DGU-20A5R and DGU-20A3R, a column oven Shimadzu  
159 CTO-20AC, an autosampler Shimadzu SIL-30AC, a photodiode array (PDA) detector Shimadzu  
160 SPD-M20A, and a fluorescence detector Shimadzu RF-20A XS. The column was a silica-based  
161 Yarra SEC-3000 (300×7.6 mm, Phenomenex, USA).

162 An HPSEC method developed for wastewater analysis by (Szabo et al., 2016; Szabo and  
163 Tuhkanen, 2010) was adopted after minor modifications. Particularly, sodium acetate eluent was  
164 replaced with phosphate buffer to avoid quenching of tyrosine-like fluorescence by acetate ions  
165 (Feitelson, 1964). The both eluents demonstrated identical separation in preliminary  
166 experiments.

167 5 mM phosphate buffer with  $\beta(\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}) = 0.45 \text{ g L}^{-1}$  and  $\beta(\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}) = 0.39$   
168  $\text{g L}^{-1}$  (pH 6.8, ionic strength 10 mM) was used as the eluent at a flow rate of 1 mL min<sup>-1</sup>.  
169 Analytical grade Na<sub>2</sub>HPO<sub>4</sub> · 2H<sub>2</sub>O and NaH<sub>2</sub>PO<sub>4</sub> · 2H<sub>2</sub>O were purchased from VWR, Belgium, and  
170 Merck, Germany, respectively. Ultrapure water was generated using an “Ultra Clear UV plus TM”  
171 system (SG Water, Germany). Eluent was pre-filtered through GF/F grade (0.45 µm) cellulose  
172 acetate filters (Whatman, Germany).

173 Neither influent nor effluent samples were diluted for HPSEC analyses. Ionic strength of  
174 samples was not adjusted. The injection volume was 15 µL for influent and 30 µL for effluent.  
175 The autosampler temperature was 4 °C, and the column oven temperature was 25 °C. Elution

176 was stopped after 35 min for all the samples. After every 8-10 runs a blank injection of ultrapure  
177 water was done to test the column and lines for possible contamination. The HPLC system was  
178 regularly flushed with aqueous solutions of HPLC grade methanol or acetonitrile (Merck,  
179 Germany) to prevent contamination build-up.

180 The cell temperature of the PDA detector was 40 °C and the slit width was 1.2 nm.  
181 Sensitivity of the fluorescence detector was set to “high”, the fluorescence cell temperature was  
182 25 °C, and the response time was 1.5 s. The data acquisition rates were 12.5 Hz and 10.0 Hz for  
183 the PDA and the fluorescence detectors, respectively.

184 The PDA and fluorescence detectors worked in tandem, and a 3D UV spectrum ( $\lambda = 200$ -  
185 400 nm vs. elution time) and two fluorescence signals for two different  $\lambda_{ex}/\lambda_{em}$  were recorded  
186 simultaneously during each chromatographic run. To obtain HPSEC-fluorescence chromatograms  
187 for the eight selected  $\lambda_{ex}/\lambda_{em}$  each sample was injected four times. Thus, four replicate HPSEC-UV  
188 were obtained for each sample, they were averaged during data processing.

189 Void volume, determined with blue dextran (Sigma-Aldrich, Sweden), was 5.5 mL (elution  
190 time 5.5 min), and permeation volume, determined with acetone, was 12.5 mL (elution time 12.5  
191 min). The size-exclusion column was calibrated with polystyrene sulfonate standards of 210,  
192 1 600, 3 200, 4 800, 6 400, 17 000, and 32 000 Da (Sigma-Aldrich, Germany), which are  
193 commonly used for HPSEC calibration (Shon et al., 2006a).

## 194 2.5. Processing of HPSEC data

195 Raw chromatographic data were exported from the proprietary software (Shimadzu  
196 LabSolutions LC/GC Version 5.51) to ASCII text files and processed in MATLAB using in-house  
197 scripts. Each chromatogram was integrated numerically using MATLAB function trapz. Horizontal  
198 axis was taken as the baseline, and individual fractions were separated with perpendicular drops.

199 Total fluorescence and total UVA<sub>254</sub> were calculated by integration of corresponding  
200 chromatograms in the elution time range 4.5–30.0 min. Thus, total fluorescence and total UVA<sub>254</sub>  
201 account for all HPSEC fractions combined and represent properties of whole influent and effluent  
202 samples.

203 In this study, fluorescence and UVA<sub>254</sub> are expressed in terms of [mV] and [mAU], elution  
 204 time is given in [min], thus, fractional (and total) fluorescence and UVA<sub>254</sub> have dimensions of  
 205 [mV min] and [mAU min], respectively.

206 Removal efficiencies were calculated according to the following equation

$$207 \quad \text{Removal efficiency} = (1 - \text{Area}_{\text{eff}} / \text{Area}_{\text{inf}}) 100\% \quad (1)$$

208 where  $\text{Area}_{\text{eff}}$  and  $\text{Area}_{\text{inf}}$  are the corresponding areas (total or fractional) of HPSEC  
 209 chromatograms of influent and effluent sampled on the same day. Removal efficiencies were  
 210 calculated for each day of the main monitoring period (in total, 10 days) and then were averaged.

211 Number-averaged MW ( $\bar{M}_n$ ), weight-averaged MW ( $\bar{M}_w$ ), and dispersity ( $\mathcal{D}_M$ ) were  
 212 calculated with equations (2)-(4) according to (Her et al., 2002).

$$213 \quad \bar{M}_n = \frac{\sum_{i=1}^n h_i}{\sum_{i=1}^n (h_i / MW_i)} \quad (2)$$

$$214 \quad \bar{M}_w = \frac{\sum_{i=1}^n (h_i \cdot MW_i)}{\sum_{i=1}^n h_i} \quad (3)$$

$$215 \quad \mathcal{D}_M = \bar{M}_w / \bar{M}_n \quad (4)$$

216 where  $h_i$  and  $MW_i$  are the height of HPSEC chromatogram and the estimated MW corresponding  
 217 to the eluted volume  $i$ , and  $n$  is the number of data points. The elution time range 4.5-12.5 min  
 218 was taken to calculate  $\bar{M}_w$ ,  $\bar{M}_n$ , and  $\mathcal{D}_M$ .

219 UV absorbance ratio index (URI) was calculated as ratio of UVA<sub>210</sub> to UVA<sub>254</sub> (Her et al.,  
 220 2008).

### 221 3. Results and discussion

#### 222 3.1. Analysis of EEM fluorescence spectra

223 Prior the main sampling campaign, EEM fluorescence spectra of one influent and one  
 224 effluent samples were analyzed to select optimal  $\lambda_{\text{ex}}/\lambda_{\text{em}}$  for the further HPSEC monitoring. The  
 225 EEM fluorescence spectra (Fig. 1) demonstrate intense tyrosine-like peaks B<sub>1</sub> and B<sub>2</sub> and  
 226 tryptophan-like peaks T<sub>1</sub> and T<sub>2</sub>, which can be related to the dissolved amino acids, free and  
 227 bound to proteins, and other organic compounds with similar fluorescence properties (Fellman et

228 al., 2010). Tyrosine residues in proteins and peptides do not emit fluorescence in the presence  
229 of tryptophan, because the emission energy of tyrosine residues is transferred to the excitation  
230 energy of the neighboring tryptophan residues (Yamashita and Tanoue, 2003). Thus, tyrosine-like  
231 fluorescence may indicate more degraded peptide material, while tryptophan-like fluorescence  
232 may represent intact proteins and less degraded peptide material (Fellman et al., 2010).

233 Peaks A, C<sub>1</sub>, C<sub>2</sub>, and M represent fluorescence of natural humic/fulvic matter and also  
234 anthropogenic humic/fulvic-like fluorescing compounds (Carstea et al., 2016). Peaks A and C are  
235 always observed together in EEM fluorescence spectra of dissolved humic and fulvic standards  
236 isolated by International Humic Substances Society (IHSS) (Alberts and Takács, 2004). Some  
237 studies link peak A with fulvic-like compounds, whereas peaks C and M – with humic-like  
238 compounds (Chen et al., 2003). However, such interpretation seems to be rather simplified since  
239 the existing classification into humic and fulvic compounds is operational and based solubility,  
240 not on substantial differences in chemistry. In fact, the same fluorophores are found in both  
241 humic and fulvic compounds, and a clear distinction between them based uniquely on  
242 fluorescence properties is not always possible (Senesi et al., 1991).

243 “Red-shifted” peaks A and C<sub>2</sub>, with emission at longer wavelengths, can be related to highly  
244 conjugated aromatic compounds of high MW, whereas “blue shifted” peaks C<sub>1</sub> and M, with  
245 emission at shorter wavelengths, are thought to contain compounds of lower aromaticity and  
246 lower MW (Fellman et al., 2010). Recently, peak M was suggested as indicative of mixing old and  
247 new humic/fulvic material as the result of recent microbial activity (Coble et al., 2014), since  
248 bacterial degradation is considered to be more often a source rather than a sink of humic/fulvic-  
249 like fluorescence (Coble et al., 2014; Stedmon and Markager, 2005)

250 Eight  $\lambda_{ex}/\lambda_{em}$  were selected near maxima of EEM peaks B<sub>1</sub>, B<sub>2</sub>, T<sub>1</sub>, T<sub>2</sub>, A, C<sub>1</sub>, and C<sub>2</sub> to  
251 represent variety of fluorophores and to provide high fluorescence intensity for the further HPSEC  
252 monitoring (Table 1).

## 253 3.2. Characterization of influent and effluent

254 Conventional surrogate parameters (BOD, COD, DOC, etc.) of whole influent and effluent  
255 samples are summarized in Supplementary material Table S1. These parameters are routinely  
256 measured 2-3 times per week to control the WWTP.

### 257 3.2.1. HPSEC fractionation

258 A comparison of typical HPSEC-UV-fluorescence chromatograms of influent and effluent is  
259 given in Fig. 2 and Supplementary material Fig. S1. The chromatograms exhibit fairly good  
260 resolution and provide descriptive information on the nature of wastewater DOM. A quick visual  
261 inspection of such chromatograms can directly give a rough determination of problematic  
262 fractions that are not efficiently removed during treatment (for example, fractions III and IV). Both  
263 influent and effluent demonstrated individual elution profiles, so-called fingerprints, which can  
264 assist defining type and origin of wastewater (Peuravuori and Pihlaja, 1997). These elution  
265 profiles were reproducible, although significant day-to-day variations in UVA<sub>254</sub> and fluorescence  
266 intensities were observed.

267 For each sample, resolved peaks were combined into eight fractions, denoted I-VIII. MW of  
268 influent and effluent fractions were estimated using the calibration curve (Supplementary  
269 material Fig. S2), which is consistent with the data previously obtained by (Szabo et al., 2016) for  
270 similar conditions.

271 Use of calibration standards for MW estimation assumes that the standards and  
272 wastewater components behave similarly in a size-exclusion column used. Considering  
273 multicomponent nature of wastewater and diverse secondary interactions, any choice of size-  
274 exclusion standards is ambiguous. Thus, HPSEC calibration provides apparent rather than true  
275 MW. Apparent MW can be used to compare samples in a particular experiment, but should not  
276 be interpreted literally (Yan et al., 2012).

277 In a first approximation, compounds of high MW > 10 kDa eluted in fraction I, fraction II  
278 represented compounds of intermediate MW 3-10 kDa, fraction III consisted of compounds of  
279 MW 1-3 kDa, and fractions IV-VII combined compounds of low MW < 1 kDa. All peaks appeared

280 after acetone elution time 12.5 min, i.e. outside the calibration range, were deliberately  
281 combined into fraction VIII, for which estimation of MW is not possible.

282 High resolution archived in the range of low MW < 1 kDa can be useful for contamination  
283 tracking in groundwater recharge and water recycling, since fractions of low MW account for  
284  $67 \pm 24\%$  of the dissolved organic nitrogen in wastewater effluents, contain many precursors of  
285 disinfection by-products (Pehlivanoglu-Mantas and Sedlak, 2008), and account for most  
286 genotoxicity found in secondary effluents (Wu et al., 2010).

### 287 3.2.2. Influence of $\lambda_{\text{ex}}/\lambda_{\text{em}}$ on elution profiles

288 Monitoring different  $\lambda_{\text{ex}}/\lambda_{\text{em}}$  belonging to the same type of fluorescence (for example,  
289 tryptophan-like T<sub>1</sub>: 230/355 nm and T<sub>2</sub>: 270/355 nm) resulted in noticeable variations in elution  
290 profiles (Supplementary material Fig. S3-S6). Averaged total and fractional fluorescence  
291 intensities of influent and effluent samples measured at different  $\lambda_{\text{ex}}/\lambda_{\text{em}}$  are provided in  
292 Supplementary material Tables S2-S4.

293 Total intensity of tyrosine-like fluorescence ( $\lambda_{\text{em}} = 310$  nm) at the shorter  $\lambda_{\text{ex}} = 220$  nm was,  
294 on average, ~30% higher for effluent and ~10% higher for influent, compared to the longer  $\lambda_{\text{ex}} =$   
295 270 nm.

296 Total intensity of tryptophan-like fluorescence ( $\lambda_{\text{em}} = 355$  nm) measured at the shorter  $\lambda_{\text{ex}} =$   
297 230 nm was ~10% higher for effluent, but ~25% lower for influent, compared to the longer  $\lambda_{\text{ex}} =$   
298 270 nm. These variations were mostly due to low MW fractions VI and VII. A two-fold increase in  
299 tryptophan-like fluorescence of effluent fraction VII observed at the shorter  $\lambda_{\text{ex}}$  is particularly  
300 interesting.

301 For humic/fulvic-like fluorescence shorter  $\lambda_{\text{ex}}$  and  $\lambda_{\text{em}}$ , in general, resulted in higher  
302 intensity across all influent and effluent fractions.

303 Effluent fraction VII, eluted around 11 min, had a very strong UV absorbance at  $\lambda < 230$  nm  
304 (Supplementary material Fig. S7) that was probably caused by low MW carboxylic and amino  
305 acids formed during biological processes (Jarusutthirak and Amy, 2007). Such high UV  
306 absorbance could negatively affect accuracy of fluorescence detection for effluent fractions VI  
307 and VII due to inner filter effect at short  $\lambda_{\text{ex}} < 230$  nm.

308           Complexation with transition metal ions, such as  $\text{Fe}^{3+}$ , should be also considered when  
309 analyzing wastewater samples, because these ions can inhibit fluorescence of humic and fulvic  
310 compounds of various MW (Cabaniss, 1992).

### 311           **3.2.3. Correlations between DOC, COD, BOD, total UVA<sub>254</sub>, and total fluorescence**

312           Tyrosine-like and tryptophan-like fluorescence is often considered as a surrogate indicator  
313 of biologic activity and DOM bioavailability (Fellman et al., 2010).

314           Strong linear correlations (Pearson coefficient  $\rho > 0.9$ ) were observed between DOC, COD,  
315 BOD and total UVA<sub>254</sub>, total tyrosine-like, total tryptophan-like fluorescence of influent and  
316 effluent (Fig. 3 and Supplementary material Fig. S8). These correlations complement previous  
317 findings by (Bridgeman et al., 2013; Christian et al., 2017; Hudson et al., 2008; Hur and Cho,  
318 2012) and suggest that protein-like compounds comprise a major share of fluorescing DOM in  
319 influent. The observed variations in DOC, COD, and BOD of influent reflects fluctuating patterns of  
320 diverse anthropogenic activity, including industrial discharge emissions.

321           At the same time, humic/fulvic-like fluorescence at  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 390/500$  nm did not  
322 demonstrate significant correlations with DOC, COD, and BOD neither for influent nor for effluent  
323 (Pearson coefficient  $\rho < 0.6$ ). It is known, that certain fractions of natural humic and fulvic  
324 compounds in wastewater derive from drinking water sources (Shon et al., 2006b). For example,  
325 (Guo et al., 2011) detected a humic/fulvic-like fraction of MW  $\sim 650$  Da in both wastewater and  
326 tap water from the same geographical area. In Jyväskylä, drinking water is produced mainly from  
327 lake water. During the monitoring period, the rate of raw water abstraction and weather  
328 conditions, which affect quality and quantity of surface runoff water, were stable. Thus, a steady  
329 concentration of natural humic/fulvic compounds could be expected in the urban water system  
330 and, at the end, in the influent. For this reason, fluorescence at  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 390/500$  nm may be  
331 indicative of recalcitrant humic and fulvic compounds of natural origin.

332           Even though conventional COD and BOD measurements are required by official  
333 regulations, HPSEC-UV-fluorescence analysis can provide additional interim data to be used, for  
334 example, in early warning systems. Further research, covering a wider timespan and a larger



335 number of samples, is needed to determine whether the observed correlations are statistically  
336 reliable and to identify possible seasonal variations.

#### 337 3.2.4. MW distributions

338 Percentage contributions of individual fractions to the total UVA<sub>254</sub> and the total  
339 fluorescence of influent and effluent were calculated for each sampling day and afterwards were  
340 averaged. Fig. 4 and Supplementary material Fig. S9 demonstrate that, among all fractions,  
341 influent and effluent fractions VI had the strongest UV and fluorescence response (at different  
342  $\lambda_{ex}/\lambda_{em}$ ) and contributed to 25-30% of total UVA<sub>254</sub> and 30-40% of total fluorescence. Compounds  
343 with MW < 1 kDa (fractions IV-VII combined) accounted for 60-65% of the total UVA<sub>254</sub> and 50-  
344 70% of total fluorescence (at different  $\lambda_{ex}/\lambda_{em}$ ) of influent and effluent. Among low MW fractions,  
345 fraction IV was predominantly humic/fulvic-like, fractions V and VI exhibited all types of  
346 fluorescence, whereas fraction VII was predominantly tryptophan-like with moderate  
347 humic/fulvic-like fluorescence and low tyrosine-like fluorescence.

348 Compounds with MW > 1 kDa (fractions I-III combined) contributed to 20-25% of the total  
349 UVA<sub>254</sub> and 10-15% of the total tyrosine-like, tryptophan-like fluorescence of influent. Among  
350 them, fraction I (MW > 10 kDa) demonstrated only protein-like fluorescence, while fractions II  
351 (MW 3-10 kDa) and III (MW 1-3 kDa) exhibited also noticeable humic/fulvic-like fluorescence.

352 Finally, compounds eluted outside the calibration range (fraction VIII, where MW estimation  
353 is not possible) contributed to 10-15% of the total UVA<sub>254</sub> and 15-20% of the total tryptophan-like  
354 and humic-fulvic-like fluorescence. Interestingly, ~40% of tyrosine-like fluorescing compounds in  
355 influent eluted outside the calibration range, while in effluent less than 20% of tyrosine-like  
356 fluorescing compounds demonstrated delayed elution. The late elution could be caused, for  
357 example, by specific interactions between wastewater components and the column material or by  
358 ion exclusion of cationic compounds (Le Coupannec et al., 2000).

359 Most fractions simultaneously exhibited tyrosine-like, tryptophan-like, and humic/fulvic-like  
360 fluorescence, suggesting that protein-like and humic/fulvic-like matter eluted together. Humic  
361 and fulvic compounds are known to interact with proteins and other biomolecules and form  
362 various associations including covalent bonded structures (Arenella et al., 2014; Sutton and

363 Sposito, 2005). Some humic-bound proteinaceous constituents may be more resistant to  
364 biological degradation (Saadi et al., 2006). However, HPSEC-UV-fluorescence analysis does not  
365 allow to discriminate whether the observed fractions consisted of individual compounds or  
366 aggregates.

367 Number-averaged MWs, calculated with eq. (2) from HPSEC-UV-fluorescence  
368 chromatograms, were in the range 154-238 Da for both influent and effluent (Supplementary  
369 material Table S5). Weight-averaged MWs and dispersities of tyrosine-like and tryptophan-like  
370 compounds were 2-4 times higher than those of humic/fulvic-like compounds.

371 The above findings support previously published data. For example, DOM in effluent from a  
372 Japanese WWTP was found to have weigh-averaged MW 380-830 Da (Imai et al., 2002). Highest  
373 UVA<sub>254</sub> was reported for effluent fraction with MW 0.3-5 kDa (Shon et al., 2004). A bimodal  
374 distribution of DOM was observed for a bench-scale activated sludge reactor fed with a synthetic  
375 wastewater: 30-50% of the DOM had low MW < 1 kDa, while another 25-45% had high MW > 10  
376 kDa (Jarusutthirak and Amy, 2007). Effluent from a pilot WWTP in China also exhibited a bimodal  
377 MW distribution: multiple peaks with high UVA<sub>254</sub> and high protein-like fluorescence at  $\lambda_{ex}/\lambda_{em} =$   
378 278/353 nm were observed in the range of MW 0.1-2 kDa, and a wide peak with low UVA<sub>254</sub> but  
379 intense protein-like fluorescence was detected in the range of MW 10-20 kDa (Guo et al., 2011).  
380 HPSEC-DOC-UVA<sub>254</sub> chromatograms of effluent from a WWTP in USA displayed three segments:  
381 20-60 kDa with high DOC and very low UVA<sub>254</sub>; 1-20 kDa with high DOC and high UVA<sub>254</sub> (with the  
382 maximum around 2 kDa); and < 1 kDa with high DOC and moderate UVA<sub>254</sub> (Nam and Amy,  
383 2008). Effluent from a WWTP in Korea had five presumably aromatic peaks in the range of MW  
384 700-2050 Da and three protein-like peaks at 480, 890, and 1690 Da, however, high MW  
385 compounds were not detected (Chon et al., 2017).

386 Though detailed structural analysis, such as NMR, is rarely done for fractionated  
387 wastewater, there is consensus that high MW fractions (> 10 kDa) consist of aliphatic  
388 polysaccharide-like compounds and amino sugars with low aromaticity, intermediate MW  
389 fractions (1-10 kDa) include various protein-like and aromatic humic/fulvic-like compounds, and  
390 low MW fractions (< 1 kDa) contain organic acids, amino acids and peptides, simple sugars, and

391 persistent anthropogenic chemicals (Chon et al., 2017; Guo et al., 2011; Huber, 1998;  
392 Jarusutthirak and Amy, 2007; Nam and Amy, 2008).

### 393 **3.2.5. UV absorbance ration index (URI)**

394 URI (ratio of  $UVA_{210}$  to  $UVA_{254}$ ) characterizes relative density of non-aromatic moieties (Her  
395 et al., 2008). Conjugated double bonds and aromatic structural elements have high  $UVA_{210}$  and  
396 high  $UVA_{254}$ , while non-aromatic moieties (amino, carbonyl, carboxyl, etc.) have high  $UVA_{210}$  and  
397 relatively low  $UVA_{254}$ . Thus, higher URI corresponds to higher relative density of non-aromatic  
398 moieties. URI helps distinguishing aromatic humic/fulvic compounds from less aromatic protein-  
399 like compounds (Shon et al., 2006a). For example, Suwannee River standard humic and fulvic  
400 acids correspondingly have URI 1.59 and 1.88 (Her et al., 2008), while for proteins of bovine  
401 serum albumin with low aromaticity URI 13.50 (Shon et al., 2006a).

402 Averaged URI of influent and effluent fractions and whole samples are presented in Table  
403 2. The lowest URI (corresponding to the highest density of conjugated double bonds) was  
404 observed for influent and effluent fractions II-V and VIII indicating humic/fulvic-like nature of  
405 these fractions. High URI of influent and effluent fraction I (MW > 10 kDa) can be indicative of  
406 polysaccharides and amino sugars (Shon et al., 2006a). In contrast to humic/fulvic-like  
407 compounds and proteins, polysaccharide-like material has low  $UVA_{254}$  and low fluorescence per  
408 unit of DOC. Therefore, MW distributions obtained from HPSEC-UV<sub>254</sub>-fluorescence  
409 chromatograms may underestimate high MW fractions and overestimate intermediate and low  
410 MW fractions (Her et al., 2002).

411 The highest URI was observed for effluent fraction VI. Considering intense tyrosine-like and  
412 tryptophan-like fluorescence, it is likely that fraction VI included considerable amounts of low MW  
413 amino acids formed during biological processes (Jarusutthirak and Amy, 2007). At the same  
414 time, co-elution of inorganic ions with strong  $UVA_{210}$ , such as nitrate, could also increase  
415 calculated URI of low MW fractions VI, VII (Szabo and Tuhkanen, 2010).

### 416 **3.3. Assessment of the WWTP performance**

417 Overall performance of the WWTP is summarized in Table 3. The values of BOD, COD, DOC,  
418 and TN removals are typical for a conventional biological treatment.

419 The WWTP removed half of organic carbon with conjugated double bonds, monitored by  
420 UVA<sub>254</sub>. A high removal was observed for the total tyrosine-like fluorescence, followed by a  
421 satisfactory decline of the total tryptophan-like fluorescence. Reduction of the total humic/fulvic-  
422 like fluorescence did not exceed 36%, and a particularly low decline was observed at  $\lambda_{ex}/\lambda_{em} =$   
423 390/500 nm. These results correlate well with a recent EEM-PARAFAC study by (Riopel et al.,  
424 2014), where “a large decrease (~60%) in the protein-like components was observed, followed  
425 by a smaller decrease (~28%) in the signal of the humic/fulvic-like components, and little to no  
426 change in the fulvic-like signal” before and after biological treatment of a municipal wastewater.

427 The observed reduction of fluorescence and UVA<sub>254</sub> was lower than the reduction of BOD  
428 and COD, which suggests presence of organic compounds “invisible” for fluorescence and UV  
429 detection (for example, sugars). Apart from that, “fresh” fluorescing material formed during  
430 microbial metabolism (de novo synthesis) could additionally contribute to fluorescence signal of  
431 effluent. Moreover, certain organic compounds present in influent and capable of fluorescence  
432 quenching could be degraded during WWTP treatment (Saadi et al., 2006).

433 URI of whole wastewater samples increased almost three-fold during the treatment (Table  
434 2) pointing out that, overall, humification did not occur (Maizel and Remucal, 2017). At the same  
435 time, the treatment noticeably increased dispersity of protein-like compounds (Supplementary  
436 material Table S5), which can be explained in terms of high MW of extracellular polymeric  
437 substances secreted by microorganisms in activated sludge.

438 Analysis of HPSEC-UV-fluorescence chromatograms allows tracking individual tyrosine-like,  
439 tryptophan-like, and humic/fulvic-like fractions. Observed removal efficiencies considerably  
440 varied for fractions of different MW (Supplementary material Table S6). Fractions IV and V (MW <  
441 1 kDa) demonstrated recalcitrant behavior with removal efficiencies significantly below 50% for  
442 UVA<sub>254</sub> and fluorescence at different  $\lambda_{ex}/\lambda_{em}$  and especially for humic/fulvic-like fluorescence. The  
443 highest removal efficiencies (above 80%) were observed for fraction I (MW > 10 kDa). Fractions  
444 III-VIII, in general, had significantly higher removal efficiencies for tyrosine-like and tryptophan-like  
445 fluorescence than for humic/fulvic-like fluorescence. Slight increase of humic/fulvic-like

446 fluorescence of fractions V-VII observed at  $\lambda_{ex}/\lambda_{em} = 390/500$  nm may indicate formation of new  
447 fluorescing material, for example, as result of sludge digestion.

448 According to the calculated URI values (Table 3), aromatic character of wastewater  
449 fractions II-V, VII, and VIII slightly increased during the biological treatment due to less efficient  
450 removal of humic/fulvic-like compounds compared to protein-like compounds. Formation of non-  
451 aromatic microbial products of high and low MW, probably, caused the noticeable increase of URI  
452 of fractions I and VI.

#### 453 4. Conclusions

454 Comparative interpretation of HPSEC-UV-fluorescence chromatograms of wastewater  
455 influents and effluents can shed light on the behavior of protein-like and humic/fulvic-like  
456 fractions in WWTPs and provide a deeper understanding of anthropogenic organic matter in  
457 urban hydrological cycle.

458 HPSEC-UV-fluorescence analysis of municipal wastewater using the proposed method  
459 revealed seven fractions within the calibration range. Compounds of low MW  $< 1$  kDa were  
460 resolved into four fractions and accounted for 60-70% of total UVA<sub>254</sub> and 70-80% of total  
461 fluorescence.

462 Most fractions were mixtures of protein-like and humic/fulvic-like matter. The fraction of  
463 high MW  $> 10$  kDa had low fluorescence and low UVA<sub>254</sub>, exhibiting non-aromatic character.

464 High removal efficiencies were observed for most tyrosine-like and tryptophan-like  
465 fractions. At the same time, low MW humic/fulvic-like fractions, especially those fluorescing at  
466  $\lambda_{ex}/\lambda_{em} = 390/500$  nm, demonstrated recalcitrant behavior in the WWTP.

467 Since composition of wastewater can significantly differ, preliminary selection of optimal  
468  $\lambda_{ex}/\lambda_{em}$  for fluorescence monitoring should be done individually for each WWTP considering  
469 possible seasonal variations. For this purpose, EEM fluorescence spectroscopy can be  
470 recommended. This study focused on fluorescing and UV absorbing wastewater fractions. Thus,  
471 certain categories of organic compounds (for example, polysaccharides) were not covered.

472 HPSEC analysis of complex water matrices, such as wastewater, requires minimal sample  
473 preparation and allows collecting an extensive dataset within short time (~30 min per analysis)

474 using multiple detectors connected in a series. Although unambiguous interpretation of UV and  
475 fluorescence spectra is challenging, the proposed HPSEC-UV-fluorescence approach is promising  
476 for fingerprinting and tracking specific wastewater fractions and advanced monitoring of WWTP  
477 performance.

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## 484 Appendix A. Supplementary material

485 Supplementary material related to this article can be found online at

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**Figure 1.**

EEM fluorescence spectra of (a) influent and (b) effluent after fivefold dilution. DOC before dilution: 58.0 mgC L<sup>-1</sup> (influent) and 19.6 mgC L<sup>-1</sup> (effluent). Sampling date: March 19, 2017. (2-column fitting image)

**Figure 2.**

Normalized HPSEC chromatograms of influent and effluent with (a)-(c) fluorescence and (d) UV detection. DOC 89.2 mgC L<sup>-1</sup> (influent) and 27.2 mgC L<sup>-1</sup> (effluent). Additional horizontal axis at the bottom provides estimation of MW. Bar diagrams on the right show averaged removal efficiencies for individual fractions and total signals, mean  $\pm$  SD ( $n = 10$ ). Sampling date: March 30, 2017. Additional HPSEC-fluorescence chromatograms are given in Supplementary material Fig. S1. (2-column fitting image)

**Figure 3.**

Correlations between DOC, COD, BOD and total UVA<sub>254</sub>, total tyrosine-like, and total tryptophan-like fluorescence of influent (triangles) and effluent (circles) samples.  $\rho$  is Pearson correlation coefficient. Linear equations were obtained using robust regression (MATLAB function robustfit). Number of influent/effluent pairs: 25 for DOC, 17 for COD and BOD. For additional fluorescence  $\lambda_{\text{ex}}/\lambda_{\text{em}}$  refer to Supplementary material Fig. S8. (2-column fitting image)

**Figure 4.**

Percentage contribution of individual fractions into (a)-(c) total fluorescence and (d) total UVA<sub>254</sub> of influent and effluent. Mean  $\pm$  SD ( $n = 10$ ). For additional fluorescence  $\lambda_{\text{ex}}/\lambda_{\text{em}}$  refer to Supplementary material Fig. S9. (2-column fitting image)

**Table 1.**  $\lambda_{\text{ex}}/\lambda_{\text{em}}$  selected for the HPSEC-fluorescence monitoring and corresponding EEM peaks.

Fluorescence type	EEM peak	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)
Tyrosine-like	B <sub>1</sub>	220/310
	B <sub>2</sub>	270/310
Tryptophan-like	T <sub>2</sub>	230/355
	T <sub>1</sub>	270/355
Humic/fulvic-like	A	240/440
	A	270/500
	C <sub>1</sub>	330/425
	C <sub>2</sub>	390/500

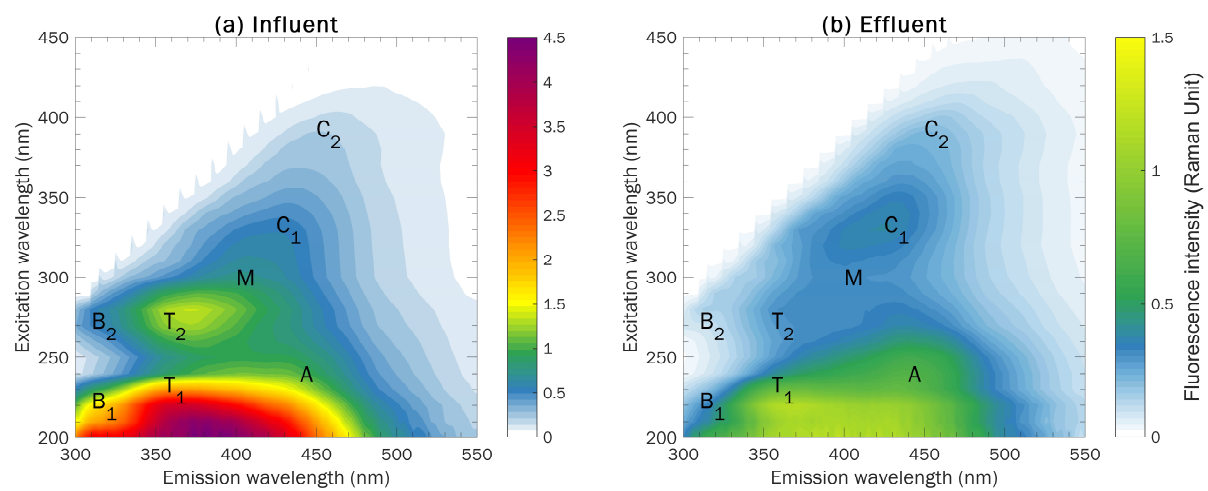
**Table 2.** URI of influent and effluent fractions and whole samples. Mean  $\pm$  SD ( $n = 10$ ).

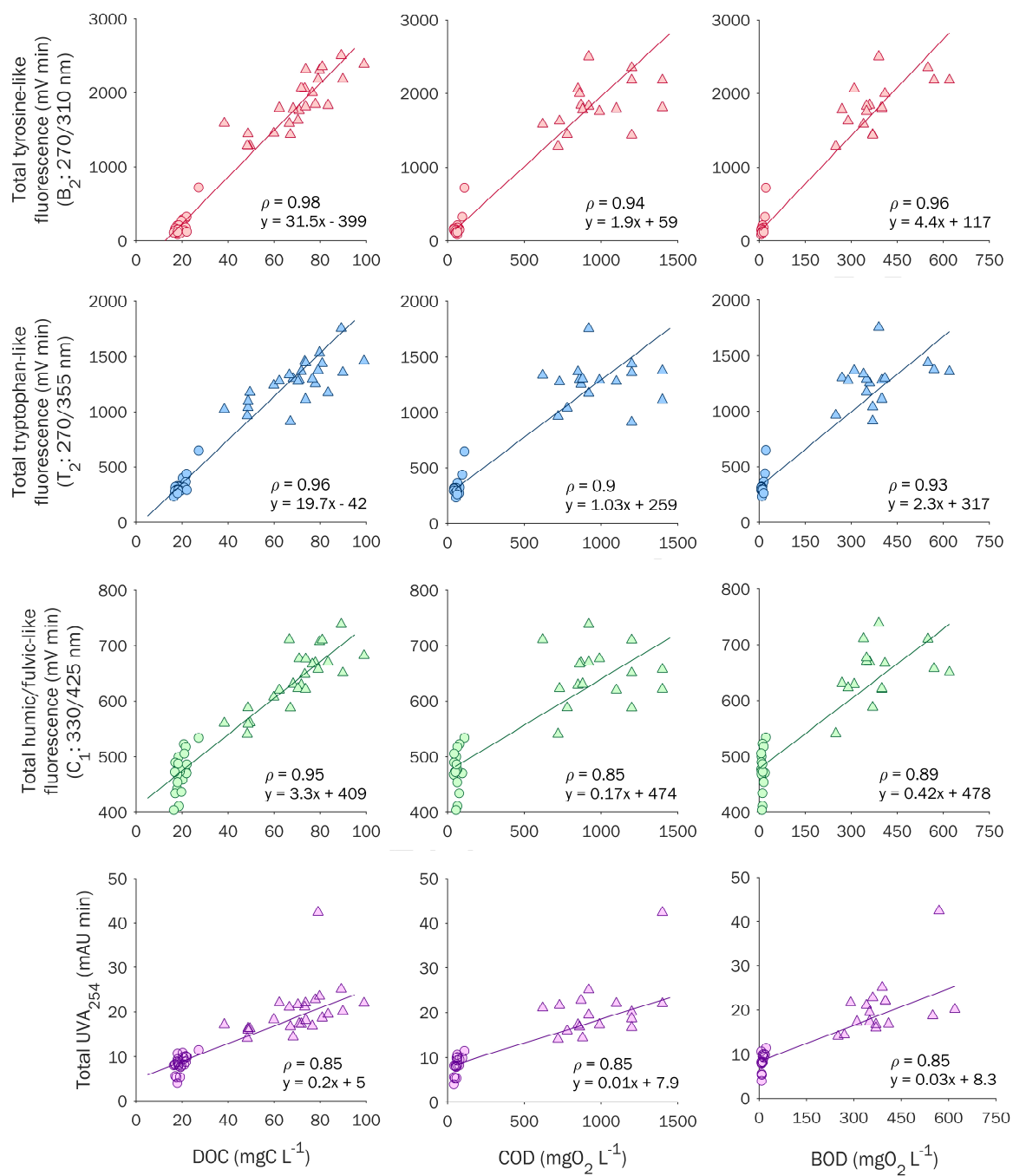
<b>Fraction</b>	<b>Influent</b>	<b>Effluent</b>
I	6.1 $\pm$ 0.8	15.1 $\pm$ 2.7
II	3.0 $\pm$ 0.4	2.7 $\pm$ 0.3
III	2.6 $\pm$ 0.4	2.3 $\pm$ 0.3
IV	2.9 $\pm$ 0.5	2.5 $\pm$ 0.3
V	3.2 $\pm$ 0.8	2.9 $\pm$ 0.6
VI	3.7 $\pm$ 0.4	28.6 $\pm$ 4.1
VII	3.9 $\pm$ 0.3	3.1 $\pm$ 0.4
VIII	2.9 $\pm$ 0.4	2.6 $\pm$ 0.5
Whole sample	3.4 $\pm$ 0.4	10.0 $\pm$ 1.1

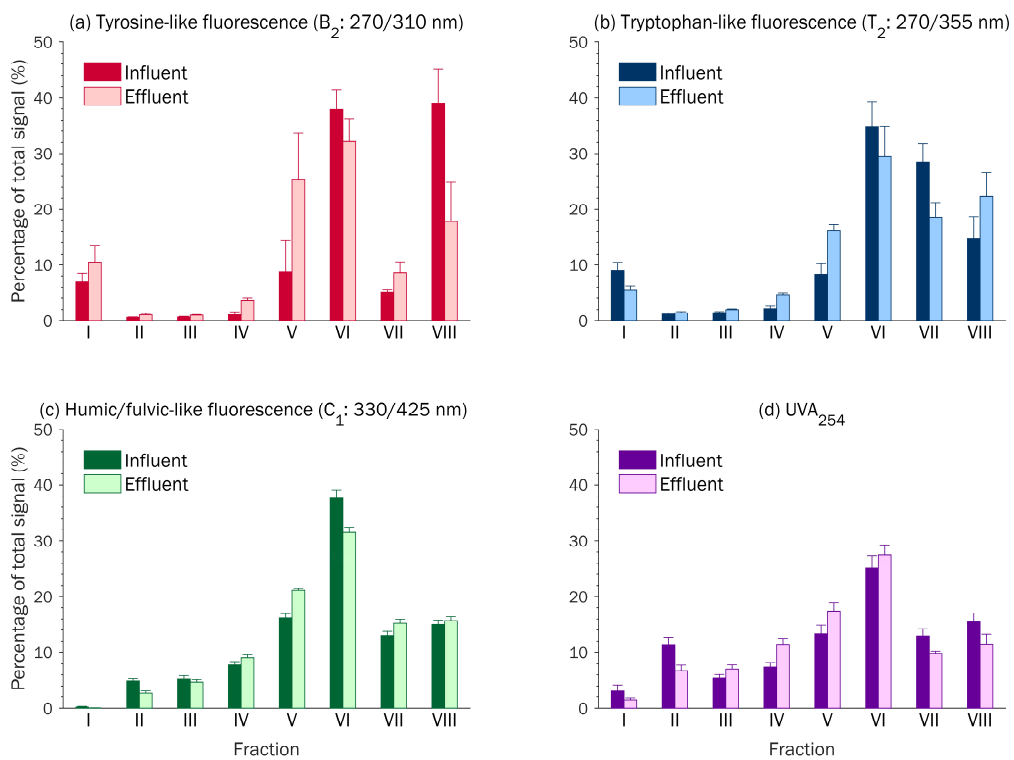
**Table 3.** Overall efficiency of the WWTP. Mean  $\pm$  SD.

Parameter		Removal (%)
BOD		97 $\pm$ 1
COD		93 $\pm$ 2
DOC		71 $\pm$ 7
TN		24 $\pm$ 7
Total UVA <sub>254</sub>		50 $\pm$ 6
Total fluorescence:		
Tyrosine-like	B <sub>1</sub> : 220/310 nm	80 $\pm$ 5
	B <sub>2</sub> : 270/310 nm	83 $\pm$ 5
Tryptophan-like	T <sub>1</sub> : 230/355 nm	55 $\pm$ 5
	T <sub>2</sub> : 270/355 nm	70 $\pm$ 3
Humic/fulvic-like	A: 240/440 nm	36 $\pm$ 4
	A: 270/500 nm	32 $\pm$ 2
	C <sub>1</sub> : 330/425 nm	25 $\pm$ 4
	C <sub>2</sub> : 390/500 nm	7 $\pm$ 3









Low MW compounds accounted for ~60% of UVA<sub>254</sub> and ~70% of wastewater fluorescence.

Conventional biological treatment reduced total UVA<sub>254</sub> by ~50%.

In total, 55-83% of protein-like and < 36% of humic-like fluorescence was removed.

Recalcitrant humic-like fractions were detected at  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 390/500$  nm.

Linear correlations between BOD, COD, DOC and fluorescence, UVA<sub>254</sub> were observed.