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1 **Photoinduced production of substances with humic-like fluorescence,**
2 **upon irradiation of water samples from Alpine lakes**

3

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12

13 ***Abstract***

14

15 Evidence is here provided that irradiation of some lake water samples can trigger the formation of
16 fluorophores with humic-like properties, at the same time increasing water absorbance. This
17 phenomenon is the opposite of photobleaching, which is often observed when natural waters are
18 irradiated. Photogeneration of humic-like compounds can be highlighted in water samples where
19 the initial fluorescence signal of humic substances is low, so that photobleaching is minimised.
20 Samples that are most likely to show photoinduced formation of humic-like fluorophores are in fact
21 characterised by high values of protein-like vs. humic-like contribution ratios to fluorescence, as
22 evidenced by parallel factor (PARAFAC) analysis. Mountain lakes in late summer appear to be
23 suitable candidates to highlight the described phenomenon. In some cases, lake-water irradiation
24 caused a decrease in the spectral slope of the absorbance that, together with increasing absorbance
25 values, is consistent with an increase in molecular mass and aromaticity of organic matter. The

26 absorbance increase triggered by irradiation might play a role in screening biologically harmful UV
27 radiation, in mountain environments that would otherwise be characterised by very clear water,
28 where UV light can be easily transmitted along the water column.

29

30 **Keywords:** Aquagenic organic matter; Photochemistry; Fluorescence; PARAFAC analysis;
31 Spectral slope.

32

33 **Introduction**

34

35 Humic substances (HS) are important components of both chromophoric and fluorescent dissolved
36 organic matter (respectively, CDOM and FDOM) in natural waters, and play important roles as
37 metal complexing agents, radiation absorbers, and photosensitisers (Krachler and Krachler, 2021;
38 Nelson and Siegel, 2013; Zhou et al., 2019). Interaction between HS and metals or hydrophobic
39 pollutants contributes to keep these species dissolved, and to change their toxicity or bioavailability
40 (De Paolis and Kukkonen, 1997; Koukal et al., 2003; Suzuki and Shoji, 2020; Worms et al., 2015).
41 Furthermore, HS screen sunlight and they are a key factor in, among others, light penetration in the
42 water column, thermocline depth in stratified lakes, protection of living organisms against harmful
43 UV radiation, and contaminant degradation *via* photochemically produced reactive intermediates
44 (Berg et al., 2019; Remucal, 2014; Zhang et al., 2014; Shank et al., 2010; Sommaruga, 2001;
45 Sommaruga et al., 1999; Vione et al., 2014). Spectroscopic techniques (both absorption and
46 fluorescence) are widely used for HS detection and characterisation (Coble, 1996; Galgani et al.,
47 2011; Loiselle et al., 2009).

48 A major route of HS to surface waters is leaching from soil, in which case HS make up an important
49 fraction of allochthonous organic matter (Osburn and Stedmon, 2011). Precipitation water plays key
50 role in transferring soil HS to water basins (Nguyen et al., 2013; Tipping et al., 1999), where HS
51 can for instance undergo photobleaching upon exposure to sunlight (Brinkmann et al., 2003; Del

52 Vecchio and Blough, 2002; Helms et al., 2014). Photobleaching is the loss of chromophores (and
53 often also the fluorophores) induced by sunlight radiation, and it can be a very important
54 transformation route for biorecalcitrant compounds like HS (Clark et al., 2019; Gu et al., 2017; Niu
55 et al., 2014; Vähätalo and Wetzel, 2004).

56 The scenario is actually more complex, because HS are partially aquagenic. This means they can be
57 formed autochthonously in natural surface waters, from a range of precursors. Recent evidence
58 suggests that a complex network of biological and photoinduced processes may be responsible for
59 the production and processing of fluorescent organic matter, starting from extracellular polymeric
60 substances released by microorganisms (Fox et al., 2019; Yang et al., 2021). Aquagenic HS take
61 part in these processes, where they are both produced and consumed (He et al., 2016; Yang et al.,
62 2021), and where the exact roles of radiation and biological reactions are still unclear.

63 On the one side, photobleaching is a well-known, radiation-induced phenomenon involving HS
64 (Dainard et al., 2015; Del Vecchio and Blough, 2002; Helms et al., 2008; Yamashita et al., 2013).

65 On the other side, laboratory evidence suggests that compounds with HS-like properties, including
66 HS-like fluorescence, are produced by irradiation of precursors such as amino acids (tryptophan and
67 tyrosine) and phenols, including lignin breakdown compounds (Berto et al., 2018, 2016; Bianco et
68 al., 2014). Photoinduced formation of humic-like substances has opposite features compared with
69 photobleaching, because it causes an increase in long-wavelength (> 300 nm) absorbance. From a
70 mechanistic point of view, this photochemical process could involve dimerisation/oligomerisation,
71 as well as hydroxylation of the precursor molecules (see **Scheme S1** in the Supplementary Material,
72 hereinafter SM, for a possible example of such processes) (De Laurentiis et al., 2013; Hoffer et al.,
73 2004; Mabato et al., 2022). Indeed, evidence suggests that phenolic oligomers and poly-
74 hydroxylated aromatic compounds are reasonable humic fluorophores (Vione et al., 2021).

75 Unfortunately, very little is currently known about the possibility that formation of HS (or
76 otherwise, of compounds with HS-like fluorescence) may occur photochemically in real surface-
77 water samples. The main obstacle to highlight this process is the fact that existing HS in the sample

78 would undergo photobleaching, thereby potentially masking possible photoinduced formation of
79 additional HS-like compounds from non-humic precursors. Because of this problem, the ideal
80 sample to be irradiated should initially contain little to no HS, which is not easy to be attained.
81 However, past studies have found out that water from mountain lakes at the end of the boreal
82 summer season (mid September) exhibits fluorescence signals that can be assigned to proteins, or
83 even to sunlight-absorbing plankton pigments, but little to no HS fluorescence (De Laurentiis et al.,
84 2012). In contrast, HS signals are detected in samples taken from the same lakes, soon after ice
85 melting (mid June) (Bianco, 2013). Possible reasons for this finding are summer (June-August)
86 photobleaching of initially-occurring HS, plus high turnover of algal cells in late summer, including
87 important cell lysis.

88 Excitation-emission matrix (EEM) fluorescence spectroscopy is one of the most straightforward
89 ways to characterise organic matter in natural water samples (Minor et al., 2014; Stedmon et al.,
90 2003). The signals that are most often detected in EEM spectra are those of proteins and HS (Vione
91 et al., 2021). Protein fluorescence is detected based on the main fluorophores, *i.e.* tyrosine (Ex ~
92 230 nm; Em ~ 300 nm, where Ex means excitation wavelength and Em means emission
93 wavelength), and tryptophan (Ex ~ 230 nm; Em ~ 350 nm) (Coble, 1996; Stedmon et al., 2003;
94 Trubetskaya et al., 2016). In contrast, HS typically have two fluorescence peaks at Ex/Em =
95 ~250/ ~450 nm, and ~320/ ~450 nm (Bridgeman et al., 2011; Coble, 1996; Stedmon et al., 2003).

96 The presence of multiple signals in the EEM spectrum of a natural water sample may require the
97 use of decomposition techniques to separate the different components, among which PARAllel
98 FACtor analysis (PARAFAC), introduced by Bro (1997) and popularised by Stedmon et al. (2003)
99 plays a major role. Indeed, spectral fluorescence data are multi-way (three-way) signals that depend
100 on the wavelength of light absorbed (excitation) and the wavelength at which fluorescence is
101 observed (emission). These data enter in a data cube that the PARAFAC algorithm fits by
102 minimising difference between the three-way model parameters (emission, excitation, and

103 contribution) and the observed signals that constitute the Excitation-Emission Matrices of
104 fluorescence (EEMs) (Andersen and Bro, 2003; Cory and McKnight, 2005; Murphy et al., 2013).
105 In this context, this work has the goal of figuring out if compounds with humic-like fluorescence
106 can actually be formed upon irradiation of samples of natural surface water. To minimise the
107 occurrence of humic fluorescence signals in the initial samples, the latter were taken from mountain
108 lakes in late summer. EEM spectroscopy, coupled with PARAFAC analysis, was mainly used to
109 monitor changes in fluorescent organic matter in the investigated samples.

110

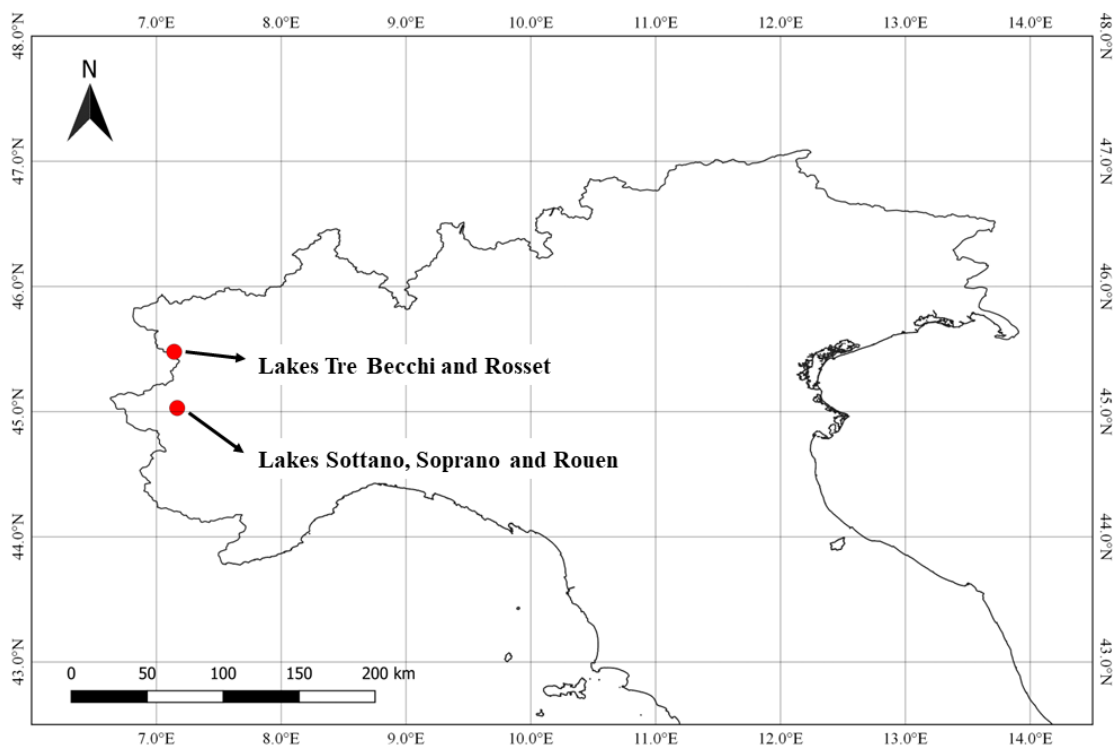
111 **Materials and methods**

112

113 *Alpine lakes under study*

114 The study lakes are located in the north-western part of the Italian Alps, in the Piedmont region (see
115 map in **Figure 1**). In particular, Lakes Rouen, Soprano, and Sottano are located in the Orsiera-
116 Rocciavrè Natural Park, at 2391, 2213, and 2102 m a.s.l., respectively, while Lake Rosset (2701 m
117 a.s.l.) and the three Lakes Tre Becchi (2727 m a.s.l., hereinafter called Tre Becchi A, B, and C
118 [A=Left; B=Central; C=Right]) are in the Gran Paradiso National Park. The lakes are small and
119 shallow, all located above the tree line, and mainly surrounded by rocks and grasses. During
120 sampling (mid-September 2018, *i.e.*, late boreal summer), the surface of the lakes was completely
121 ice-free.

122 Water samples (1 L from each lake) were kept refrigerated and in the dark during transport to the
123 laboratory, where they were vacuum-filtered with polyamide filters (0.45 mm pore size, Sartorius).
124 In order to prevent modifications caused by residual biological activity, the filtered samples were
125 kept in the dark at ~5 °C till the irradiation experiments.



126

127 **Figure 1.** Map showing the location of the lakes under study (Piedmont region, NW Italy).

128

129 The lake water samples were preliminary characterised for dissolved organic carbon (DOC),
 130 inorganic carbon (IC), and pH (see **Table S1** in the Supplementary Material, hereinafter SM). The
 131 DOC was obtained as the difference between total carbon and IC, using a Shimadzu TOC-VCSH
 132 instrument, based on the catalytic combustion method, and equipped with an ASI-V autosampler.
 133 The pH values were measured with a Metrohm combined glass electrode (code number
 134 6.0233.100).

135

136 ***Irradiation experiments***

137 Irradiation runs of lake water (80 mL) were carried out in 100 mL Duran®-glass flasks under a
 138 Philips TL K05 lamp, and irradiation was mainly from the top. This lamp emits most radiation in
 139 the wavelength range of 300-500 nm, with a broad band emission in the UVA region (maximum at
 140 365 nm). The lamp choice is motivated by the fact that UVA is the main component of sunlight UV,

141 and that a very important fraction of sunlight absorption (as absorbed photon flux density) by
142 chromophoric organic material is observed in the UVA range (Wolf et al., 2018).

143 At different irradiation times (between 7 and 72 h), 5- or 10-mL aliquots of lake water were
144 withdrawn for immediate EEM or UV-Vis characterisation, respectively. Each flask (80 mL of lake
145 water) was sampled for a maximum of two times, in order not to affect too much the solution
146 optical path (3 cm). Therefore, each series of EEM or UV-Vis spectra referred to a given lake was
147 obtained by independent irradiation of at least two separate aliquots of the same sample.

148 The spectral photon flux density of the lamp ($p^o(\lambda)$, see **Figure S1** in the SM) was obtained by
149 combination of two techniques: (i) wavelength-resolved lamp emission measurements, with a
150 calibrated Ocean Optics USB 2000 CCD spectrophotometer, and (ii) chemical actinometry, based
151 on 2-nitrobenzaldehyde (2NBA) (Galbavy et al., 2010; Willett and Hites, 2000).

152 CCD measurement yields the raw lamp spectrum $i^o(\lambda)$ on a moles-of-photons basis, which is
153 proportional to the spectral photon flux density ($p^o(\lambda) = \mathfrak{G} i^o(\lambda)$, where \mathfrak{G} is the proportionality
154 factor, and $p^o(\lambda)$ has units of Einstein $L^{-1} s^{-1} nm^{-1}$). Actinometry yields the moles of photons
155 (Einstein units) that pass through the solution per unit volume and time, taking into account the
156 possible reflection/scattering processes at the different interfaces (for instance, air-glass, air-water,
157 or glass-water) (Galbavy et al., 2010). The degradation rate of 2NBA (R_{2NBA} , units of $mol L^{-1} s^{-1}$)
158 can be described as follows:

$$159 \quad R_{2NBA} = \Phi_{2NBA} P_{a,2NBA} = \Phi_{2NBA} \int_{\lambda} p^o(\lambda) [1 - 10^{-A(\lambda)}] d\lambda \quad (1)$$

160 where $\Phi_{2NBA} = 0.4 mol Einstein^{-1}$ is the photolysis quantum yield of 2NBA (Galbavy et al., 2010),
161 $P_{a,2NBA}$ [Einstein $L^{-1} s^{-1}$] the photon flux absorbed by 2NBA, and $A(\lambda)$ [unitless] the absorbance of
162 the irradiated solution. On this basis, the factor \mathfrak{G} can be obtained from known values, as follows
163 (Carena et al., 2019):

$$164 \quad \mathfrak{G} = \frac{R_{2NBA}}{\Phi_{2NBA} \int_{\lambda} i^o(\lambda) [1 - 10^{-A(\lambda)}] d\lambda} \quad (2)$$

165 To measure $R_{2\text{NBA}}$, aqueous solutions of 2NBA (80 mL, 80 $\mu\text{mol L}^{-1}$ initial concentration) were
166 irradiated under the lamp, and sampled (1.2 mL) after 0.5, 1, 2, 3, and 4 min. The 2NBA
167 concentration was quantified by means of liquid chromatography (HPLC-DAD instrument; Carena
168 et al., 2019). Then, $R_{2\text{NBA}}$ was obtained from the 2NBA time trend, by means of the initial slope
169 method.

170

171 *EEM and UV-Vis measurements*

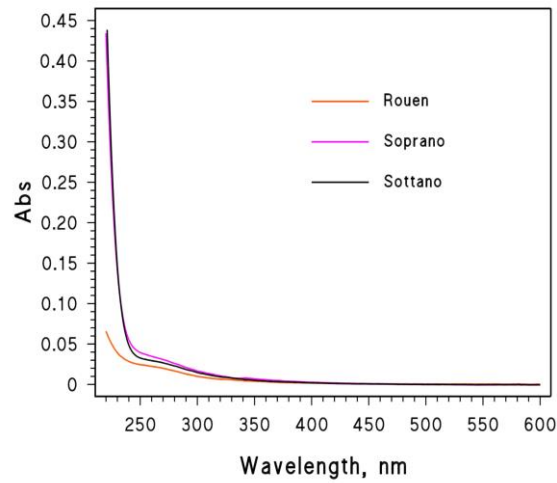
172 The fluorescence excitation–emission matrix (EEM) spectra were measured with a Cary Eclipse
173 fluorescence spectrofluorimeter, using 1-cm fluorescence quartz cuvettes. Excitation wavelengths
174 scan was from 220 till 500 nm, with 5 nm increment steps, while emission signals were taken from
175 220 till 600 nm, with 1 nm steps. Slit width was 10 nm on both excitation and emission. EEM
176 signals were normalised for the quinine sulphate unit (QSU; Fox et al., 2017). The QSU was
177 measured from the fluorescence signal emitted at 451 nm by an acidic quinine sulphate (QS)
178 aqueous solution (4 $\mu\text{g L}^{-1}$ QS + 0.1 N H_2SO_4), after excitation at 350 nm (Fox et al., 2019).

179 The UV-Vis absorption spectra of the alpine lake water samples were measured with a V-550 Jasco
180 spectrophotometer, using 5.0 cm optical path quartz cuvettes (Hellma). The absorption spectra thus
181 obtained are reported in **Figure 2**. In some cases, absorption spectra were also measured of
182 irradiated lake water samples, and the spectral slope S was calculated as a function of the irradiation
183 time. To do so, the 300-345 nm spectral interval was considered for the absorption spectra, because
184 the relevant data were well described by an exponential function (absorbance above 345 nm was
185 often negligible, and absorbance below 300 nm was often non-exponential, see **Figure 2**):

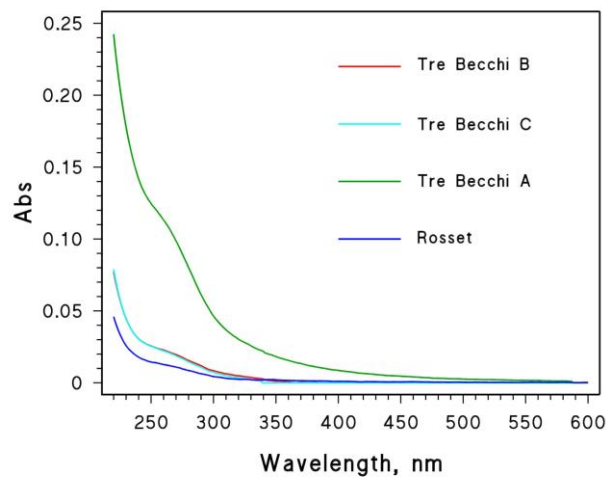
$$186 \quad A(\lambda) = A_0 e^{-S\lambda} \quad (3)$$

187 In **Eq. (3)**, $A(\lambda)$ is the absorbance at the wavelength λ , A_0 is the pre-exponential term, and S is the
188 spectral slope (Helms et al., 2008; Loiselle et al., 2009; Sharpless and Blough, 2014). The value of
189 S was obtained by a linear least-square fit procedure of $\ln A(\lambda)$ vs. λ data, with A_0 and S as free-
190 floating variables.

191



192



193

194

195 **Figure 2.** Absorption spectra (optical path length $b = 5$ cm) of the lake water samples under study.

196

197 ***PARAFAC Analysis***

198 The PARAFAC analysis of the EEM spectra was carried out with the Progmeef software
199 (<http://protee.univ-tln.fr/PROGMEEF.html>), with preliminary elimination of the Rayleigh and
200 Raman scattering signals, using Zepp's method (Zepp et al., 2004). The low absorbance values of
201 the samples (see **Figure 2**) made inner-filter correction unnecessary. PARAFAC was applied both
202 to all the initial EEM spectra of the lake water samples and, for every lake taken separately, to each
203 series of irradiation times. A range of 2 to 4 components was initially set, and the maximum number

204 of components ensuring a CONCORDIA score > 70% was chosen. In all the cases, a three-
205 component model proved suitable to reproduce sample fluorescence.

206

207 *Irradiation experiments coupled with FT-ICR MS measurements*

208 Separate solutions of tyrosine or tryptophan (each at 0.1 mM initial concentration) were irradiated
209 in a Pyrex glass photoreactor (130 mL volume), equipped with a 500 W Xenon lamp. The mass
210 spectrometric measurements (FT-ICR MS: Fourier Transform-Ion Cyclotron Resonance Mass
211 Spectrometry) of the irradiated samples, taken every 2 hours, were carried out with a Bruker 9.4 T
212 solariX instrument (100 - 800 Da mass range; average mass resolution about 350,000 was estimated
213 at m/z 319), equipped with an electro-spray ionisation (ESI) interface that was operated in negative
214 ion mode. Further experimental details are reported as Supplementary Material, **Text S1**.

215 Aromaticity equivalent (X_c) and double-bond equivalent (DBE) values were used to get insight into
216 photodegradation product distribution. The DBE values were calculated based on **Eq. (4)**, for the
217 elemental composition $C_cH_hO_oN_n$ obtained by FT-ICR MS (Kourtchev et al., 2016):

$$218 \quad DBE = c - \frac{h}{2} + \frac{n}{2} + 1 \quad (4)$$

219 where c , h , o , and n correspond to the number of carbon, hydrogen, oxygen, and nitrogen atoms in
220 the neutral formula, respectively.

221 The value of X_c , which is used to identify aromatic and polyaromatic structures in a complex
222 mixture of compounds, was calculated as follows (Yassine et al., 2014):

$$223 \quad X_c = \frac{2C+N-H-2mO}{DBE-mO} + 1 \quad (5)$$

224 If $DBE \leq m O$, or $X_c \leq 0$, it was taken $X_c = 0$, where m is the fraction of O atoms in π -bond
225 structures of a compound. Because of the interest in amino acid, and because ESI is sensitive to
226 functional groups such as, for instance, carboxylic acid (R-COOH) and ester (R-COOR₁), we used
227 $m = 0.5$ for the calculation of the X_c value in this study (Wang et al., 2021; Yassine et al., 2014).

228 Reconstructed mass spectra were normalised to the intensity of the tyrosine ($m/z=180.0667$) and
229 tryptophan ($m/z=203.0826$) peaks, respectively. All the formulae of the compounds were written in
230 their neutral form to avoid confusion.

231

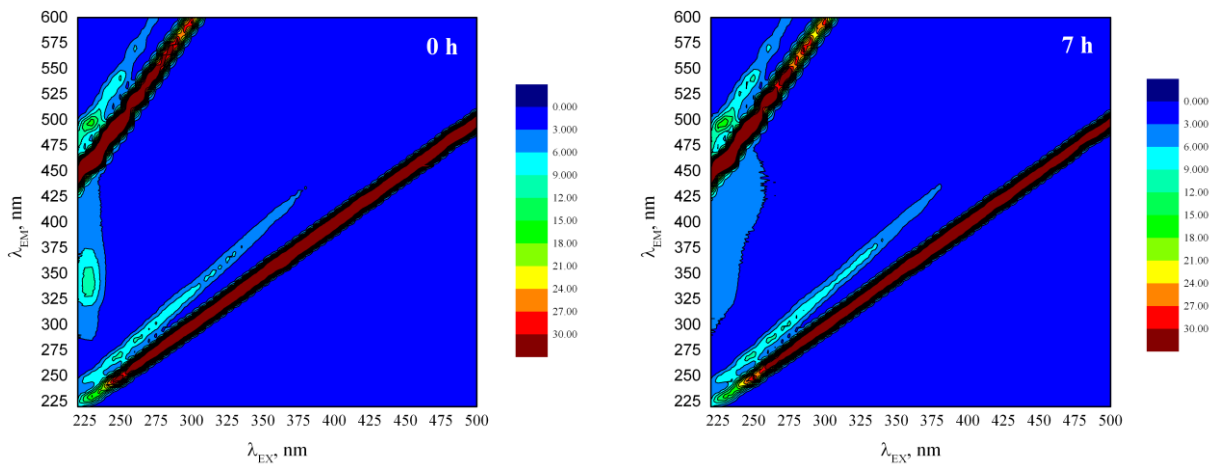
232 **Results and Discussion**

233

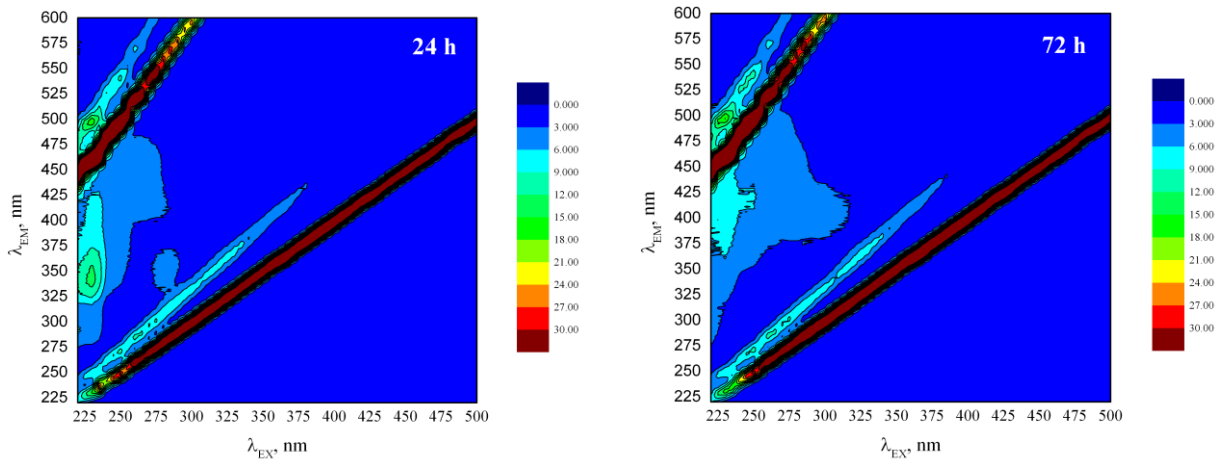
234 *Time evolution of lake-water samples upon irradiation: EEM spectra*

235 EEM spectra of the lake water samples were measured at different irradiation times. The
236 fluorescence signals of the lake water evolved upon irradiation, coherently with the fact that the
237 studied samples all showed non-negligible absorption in the UVA region (see **Figure 2**). In
238 particular, in the case of the lakes Tre Becchi B (**Figure 3**) and Soprano (**Figure 4**), some evidence
239 could be seen at first sight of a gradual shift in fluorescence from the protein/phenolic region
240 (emission wavelength λ_{EM} around 325-350 nm; Stedmon et al., 2003) to the humic one (λ_{EM} around
241 400-450 nm; Stedmon et al., 2003), which points to photoinduced formation of HS-like
242 fluorophores. In the other cases (Tre Becchi A, Tre Becchi C, Rosset, Rouen, Sottano, **Figures S2-**
243 **S6** in SM) there was often an increase in humic-like fluorescence, without a clear trend of the
244 signals in the protein region.

245 Control experiments carried out in the dark did not show significant changes in the fluorescence
246 spectra (data not shown), which suggests that any residual biological activity in the filtered lake
247 water samples was not able to affect fluorophores to a significant extent.



248



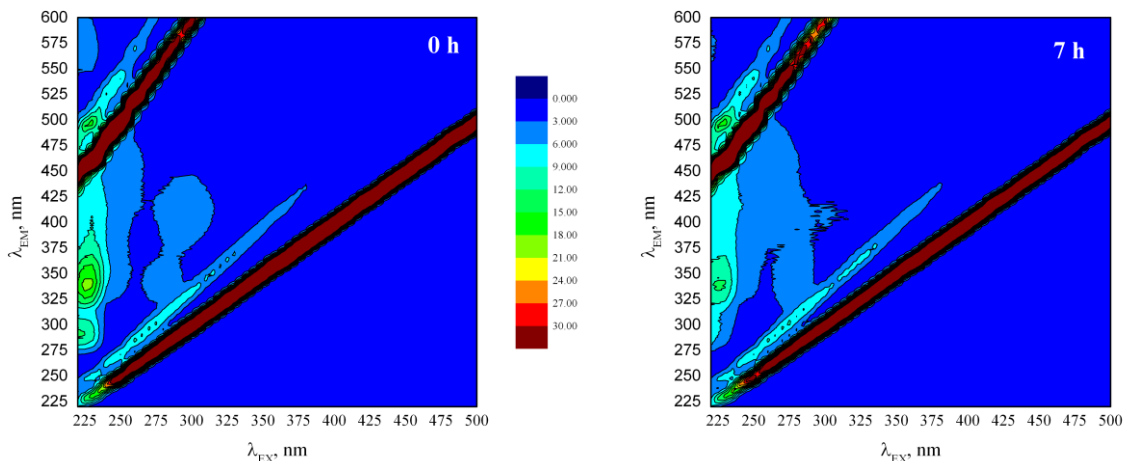
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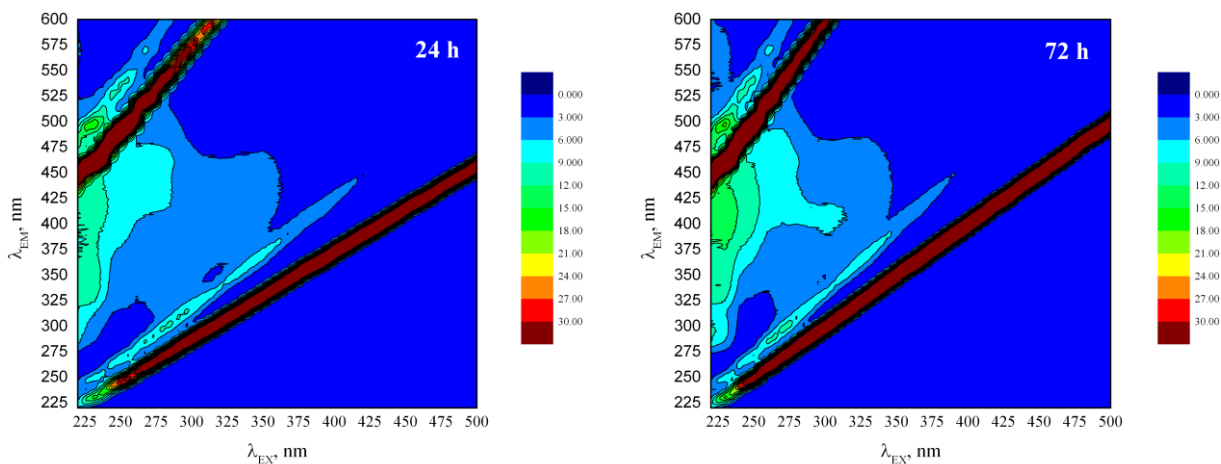
251 **Figure 3.** Trends of the EEM fluorescence spectra of water sampled from Lake Tre Becchi B, as a
 252 function of the irradiation time. EEM signals were normalised for the quinine sulphate unit.

253

254



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258 **Figure 4.** Trends of the EEM fluorescence spectra of water sampled from Lake Soprano, as a
259 function of the irradiation time. EEM signals were normalised for the quinine sulphate unit.

260

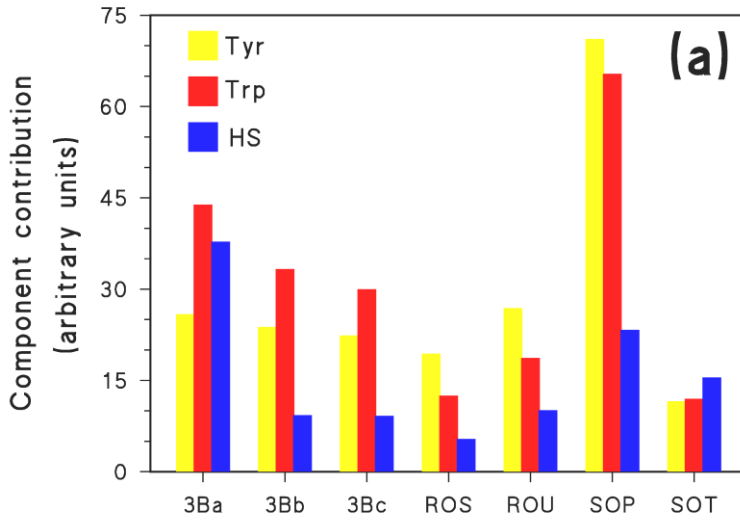
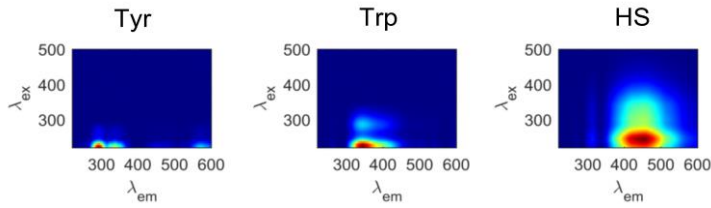
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262 **PARAFAC analysis**

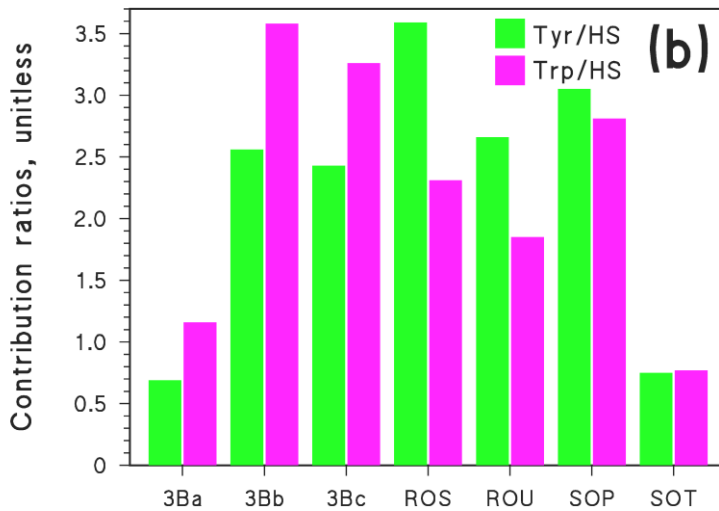
263 PARAFAC analysis of the EEM spectra of lake water samples before irradiation showed the
264 presence of three components (see **Figure 5**). The first had Ex/Em ~ 230/300 nm, which is usually
265 assigned to a protein component produced by fluorescence emission of tyrosine.

266

267



268



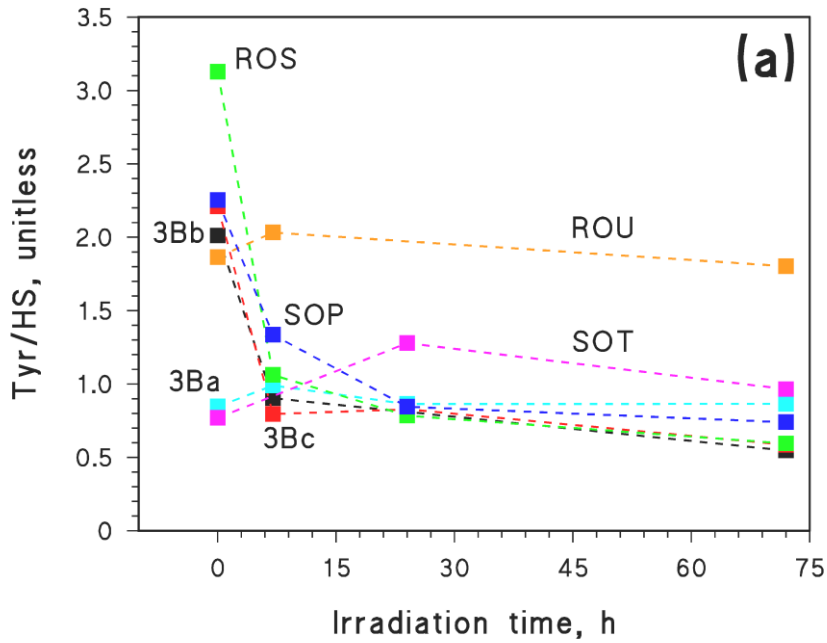
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271 **Figure 5. (a)** Contributions (arbitrary units) of the three fluorescence components, found by
 272 PARAFAC analysis of EEM spectra of the investigated lake-water samples before irradiation.
 273 Component acronyms: Tyr = tyrosine-like component; Trp = tryptophan-like; HS = humic-like. The
 274 EEM traces of the three components are shown in the upper figure panel. Lake acronyms: 3Ba =
 275 Tre Becchi A; 3Bb = Tre Becchi B; 3Bc = Tre Becchi C; ROS = Rosset; Rou = Rouen; SOP =
 276 Soprano; SOT = Sottano. **(b)** Component contribution ratios, Tyr/HS and Trp/HS, in the
 277 investigated lake-water samples.

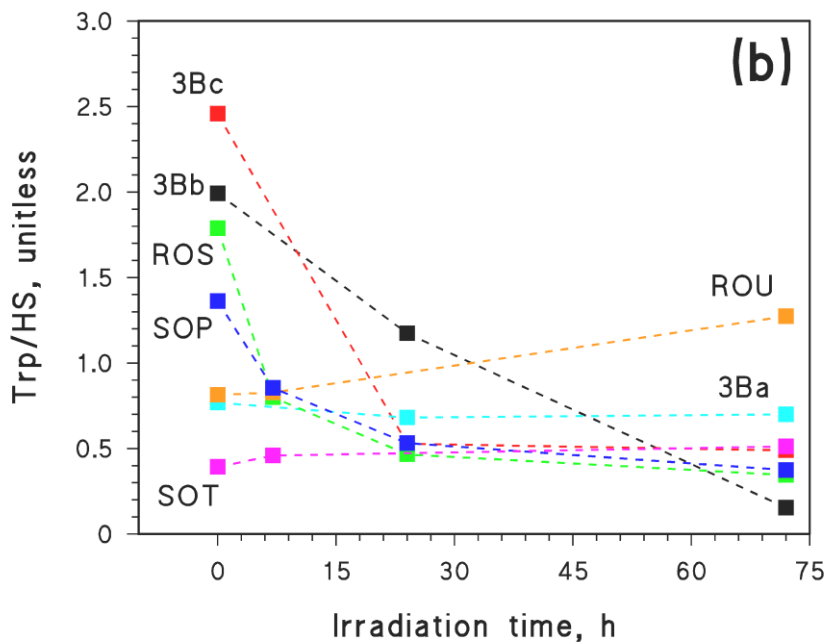
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279 The second component had Ex/Em \sim 230/350 nm, which is also a protein component produced by
280 fluorescence emission of tryptophan. The third component had Ex₁/Em₁ \sim 250/450 nm and Ex₂/Em₂
281 \sim 330/450 nm, which respectively overlap with peaks A and C of humic substances. Hereinafter,
282 these three components will be labelled as Tyr, Trp, and HS, respectively. The PARAFAC
283 contributions can be related to pseudo-concentrations (arbitrary units) and give insight into the
284 fluorescence intensity of each component among the lakes. As shown in **Figure 5a**, Lake Soprano
285 (SOP) had the highest Tyr and Trp contributions, which were minimum in Lake Sottano (SOT).
286 Concerning HS, Tre Becchi A (3Ba) was the most impacted lake, followed by SOP.
287 Using the Tyr/HS and Trp/HS contribution ratios, one can compare the relative fluorophore
288 contributions under the hypothesis that fluorescence quantum yields are constant between samples.
289 **Figure 5b** shows that 3Ba and SOT were the lakes with the lowest Tyr/HS and Trp/HS contribution
290 ratios.

291 PARAFAC analysis was also applied to time series of EEM spectra, derived from irradiation
292 experiments. In particular, each time series from each irradiated lake-water sample was processed
293 separately, always finding similar components as before (Tyr, Trp, and HS). The time evolution of
294 the component contribution ratios is shown in **Figure 6** (**6a**: Tyr/HS; **6b**: Trp/HS). A decrease of
295 contribution ratio over irradiation time was observed in the case of samples from lakes Rosset
296 (ROS), Soprano (SOP), Tre Becchi B (3Bb), and Tre Becchi C (3Bc), which were also the samples
297 with the highest values of contribution ratios before irradiation. The observed decrease in the
298 Tyr/HS and Trp/HS ratios was largely due to an increase in HS fluorescence contribution, which
299 was apparent in all the samples except for Tre Becchi A (see **Figure S7** in SM). The overall results
300 warrant two observations: (i) there is here experimental evidence that HS-like fluorescence can be
301 produced upon irradiation of lake water samples, which in several cases led to a reduction in the
302 Tyr/HS and Trp/HS contribution ratios, and (ii) the phenomenon was best observed in samples
303 where initial contribution ratios were high, most likely because low HS contribution to initial
304 fluorescence minimises the impact of photobleaching on HS-like fluorescence signals.



305



306

307

308 **Figure 6.** Time trend of the PARAFAC contribution ratios Tyr/HS (a) and Trp/HS (b), upon UVA
 309 irradiation of the lake water samples under study. Lake acronyms: 3Ba = Tre Becchi A; 3Bb = Tre
 310 Becchi B; 3Bc = Tre Becchi C; ROS = Rosset; Rou = Rouen; SOP = Soprano; SOT = Sottano.

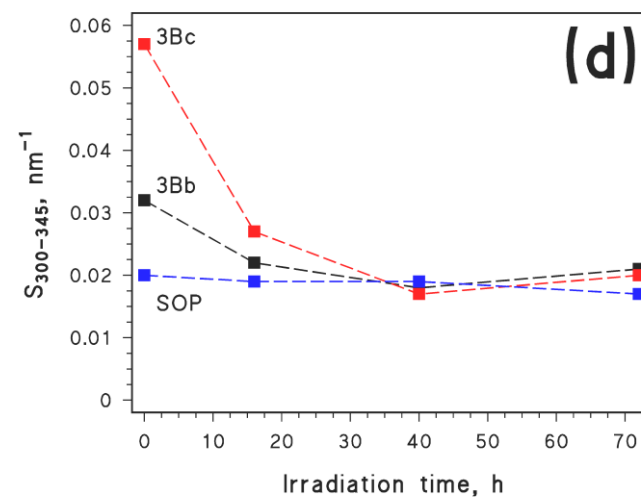
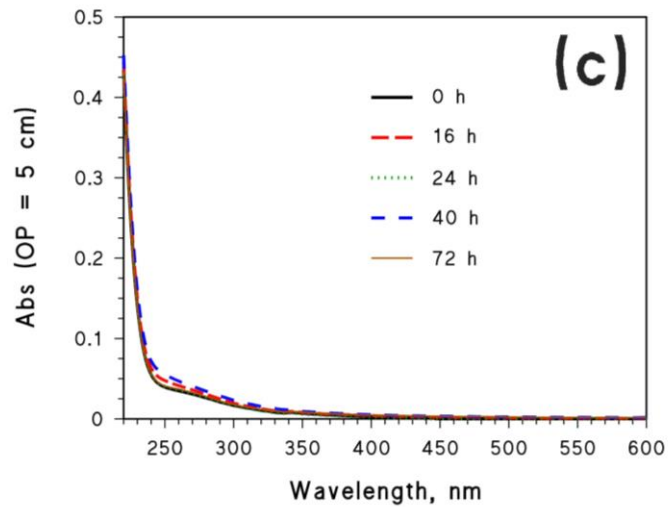
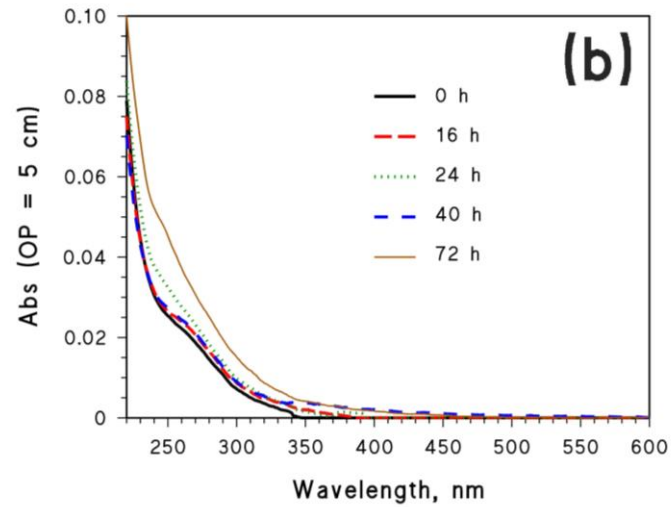
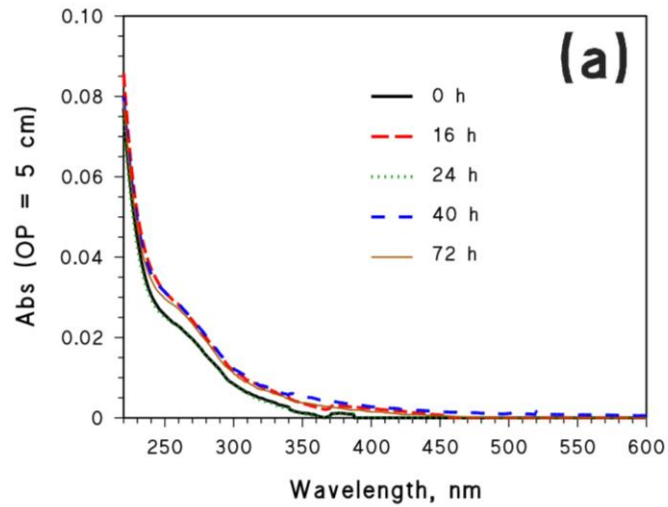
311

312 A decrease of Tyr/HS and Trp/HS ratio over time could be consistent with photoinduced
313 transformation of protein-like into HS-like fluorophores (Berto et al., 2018, 2016). However, the
314 same phenomenon could also be explained by different phenomena, such as for instance the
315 transformation of non-fluorescent precursors into HS-like fluorophores, or an increase in
316 fluorescence intensity of already existing fluorophores upon photoprocessing.

317 To corroborate evidence over the production of HS-like fluorophores, three samples (Tre Becchi B,
318 Tre Becchi C, Soprano) were chosen for additional irradiation experiments, where the absorbance
319 was monitored as a function of irradiation time. As shown in **Figure 7a-c**, lake-water absorbance
320 increased upon irradiation, which is the opposite of photobleaching and indicates that photoinduced
321 formation of chromophores took place. The same finding about increasing absorbance has already
322 been reported in irradiation experiments of synthetic solutions, where formation of compounds with
323 HS-like fluorescence has been observed starting from phenolic or amino-acidic precursors (Berto et
324 al., 2016; Bianco et al., 2014).

325 The time evolution of the spectral slope S was determined in the same samples. The results reported
326 in **Figure 7d** indicate that S decreased with irradiation in two out of three cases, *i.e.*, Tre Becchi B
327 and Tre Becchi C. The spectral slope S is inversely correlated with the molecular mass of CDOM
328 (Helms et al., 2008) and, in two cases, an increase in lake-water absorbance upon irradiation
329 occurred alongside with a decrease in S . No significant S decrease was observed in the sample from
330 Lake Soprano (**Figure 7d**), which was also the sample showing the slightest variation of absorbance
331 upon irradiation (**Figure 7c**). These findings suggest that, in some irradiated lake water samples,
332 increasing absorbance might be associated with an increase in the molecular mass of organic matter.

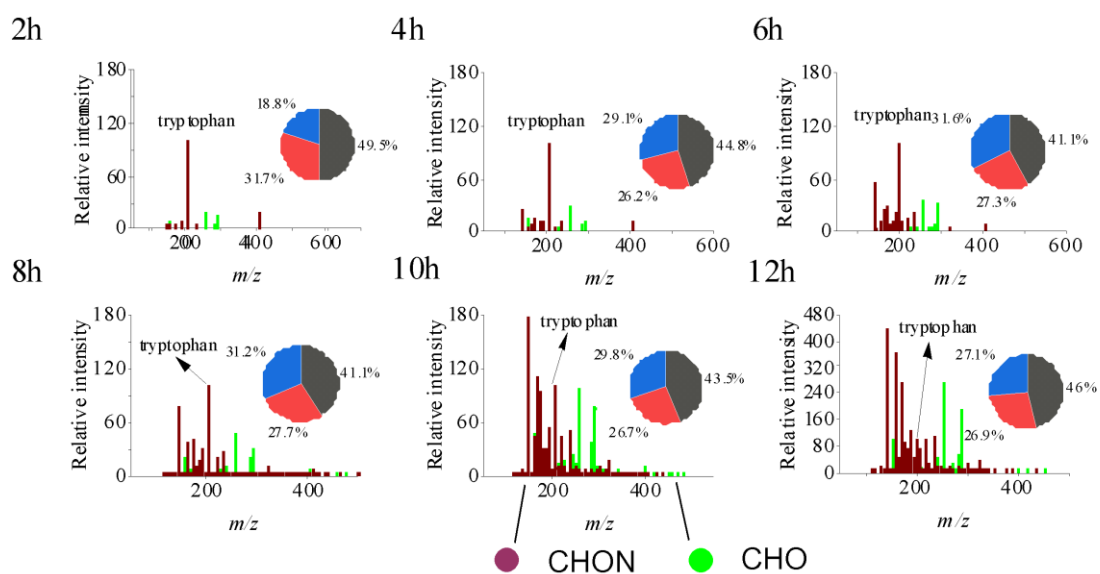
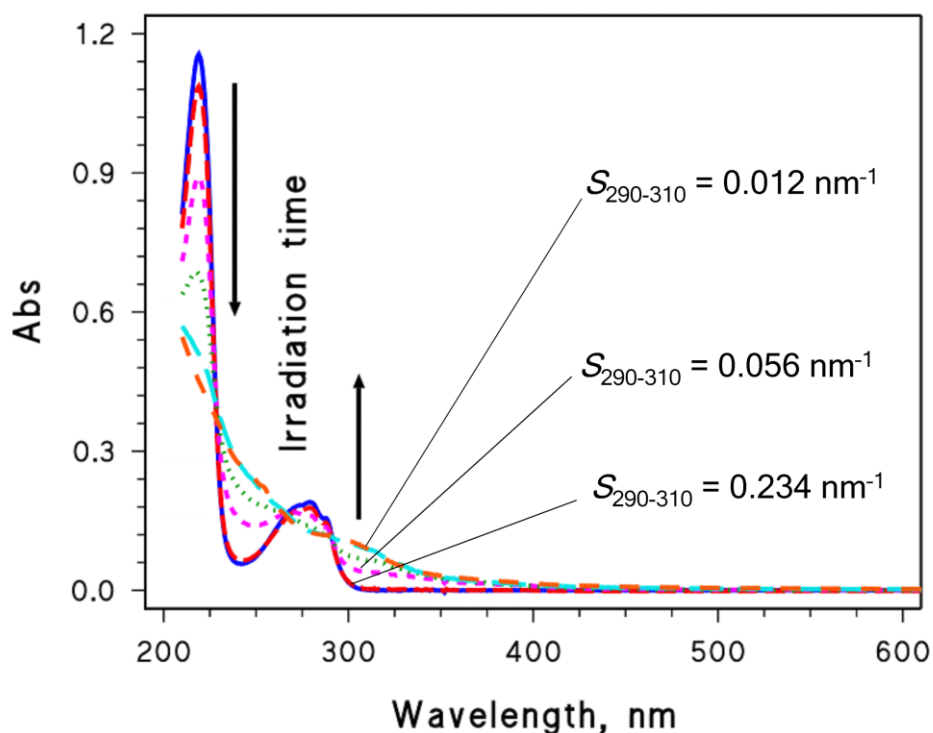
333 **Figure 8** reports the results of FT-ICR MS measurements of irradiated tryptophan solutions, which
334 shows the following: (i) The photodegradation of tryptophan produced compounds with both higher
335 and lower molecular mass, compared to the parent molecule. In particular, for irradiation times $>$
336 2h, ~30% of the detected signals could be attributed to polyaromatic compounds (*i.e.*, compounds
337 having more than one aromatic ring, $X_c > 2.7$).



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341 **Figure 7.** Trend of the absorbance (measured over an optical path length of 5 cm) as a function of the irradiation time, for the following lake
342 samples: (a) Tre Becchi B; (b) Tre Becchi C; (c) Soprano. (d) Time trend of the spectral slope S (calculated over the wavelength interval of 300-345
343 nm) for the three irradiated samples: 3Bb = Tre Becchi B; 3Bc = Tre Becchi C; SOP = Soprano.

(a)**(b)**

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Figure 8. (a) Reconstructed mass spectra of 0-12 hours photo-degradation of 0.1 mM tryptophan, obtained by FT-ICR MS (colour code: CHON vs. CHO compounds). Inset pie charts show the fractions of compounds (CHO and CHON), identified by X_c values (●: $X_c < 2.50$; ●: $2.50 \leq X_c < 2.71$; ●: $X_c \geq 2.71$). (b) Time evolution of the absorption spectrum of 0.1 mM tryptophan during the irradiation experiments (measured over a 1 cm optical path length, after 1:3 dilution with 10 mM phosphate buffer at pH 7). Values of the spectral slope S , calculated in the wavelength interval of 290-310 nm, are also highlighted.

352 Therefore, alongside with fragmentation, formation of larger structures was operational. (ii) The
353 absorbance of the irradiated solution at $\lambda > 300$ nm increased with irradiation. (iii) The value of S
354 decreased with irradiation in the same wavelength interval.

355 Based on literature reports, formation of compounds with higher molecular mass is the most likely
356 reason of the observed spectral changes (**Figure 8**), including the increase in long-wavelength (*i.e.*,
357 > 300 nm) absorbance, and the decrease in S (Berto et al., 2018; Bianco et al., 2014; Jiang et al.,
358 2021; Smith et al., 2016). Formation of compounds with higher molecular mass was also observed
359 in the case of tyrosine irradiation (see **Figure S8** in SM). Interestingly, parallel increases in both
360 absorbance and molecular mass upon irradiation have also been reported in the case of
361 environmentally significant phenolic compounds, which are also known photochemical sources of
362 HS-like substances (Smith et al., 2016; Vione et al., 2019; Tang et al., 2020). It is possible that such
363 processes occur in at least some of the irradiated lake water samples, and account for similar effects
364 on the absorption spectra.

365

366 **Conclusions**

367

368 Here we provide evidence that irradiation is able to trigger significant formation of HS-like
369 fluorescent compounds, in some water samples collected from mountain lakes in late summer.
370 Fluorophore formation could be best highlighted in samples showing initial high values of protein-
371 like vs. HS-like contribution ratios to fluorescence, as derived from PARAFAC analysis. In such
372 samples, possible photobleaching of initially occurring HS-like fluorophores would have limited
373 ability to interfere with photoinduced generation of further HS-like fluorescing compounds. Sample
374 choice was probably important, as mountain lakes in late summer often show low intensity of HS-
375 like fluorescence.

376 Fluorescence increase in the humic region took place alongside with an increase in sample
377 absorbance (which is the opposite to photobleaching) and, sometimes, a decrease in spectral slope.

378 Both phenomena increase radiation absorption of lake water at higher wavelengths. This event has
379 potential ecologic significance, because radiation-absorbing compounds screen biologically harmful
380 UV radiation. Therefore, photoinduced chromophore formation in otherwise clear water
381 environments might play a role in decreasing UV stress to living organisms, and the interplay
382 between this phenomenon and photobleaching (likely taking place at the same time) will require
383 further investigation.

384 The origin of the fluorescence increase will also require further investigation. Photochemical
385 precursors of HS-like substances (tyrosine, tryptophan, and several phenols) are all able to produce
386 compounds with higher molecular mass when irradiated, which also leads to an increase in long-
387 wavelength absorbance. Such a scenario, though reasonable, still has to be conclusively
388 demonstrated.

389

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391

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