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Phototransformation of l-tryptophan and formation of humic substances in water

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13 **Photo-transformation pathways of L-tryptophan and the related formation of**
14 **humic-like substances**

15

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29

30

31 ***Abstract***

32 The photo-transformation pathways of aqueous L-tryptophan were studied under simulated sunlight. It
33 is known that humic substances can be formed from proteins by photo-chemical processes in surface
34 waters, but the role of the single amino acids and their transformation pathways are not yet known.

35 The irradiated L-tryptophan solutions were analyzed by absorption, fluorescence, Nuclear Magnetic
36 Resonance (NMR) and Electronic Magnetic Resonance (EPR) spectroscopies, chromatography,

37 potentiometry and mass spectrometry (MS). The solutions appeared turbid after irradiation, thus
38 nephelometry and Dynamic Laser Light Scattering were used to characterize the suspended particles.
39 About 95% of L-tryptophan was degraded in 8h irradiation, undergoing deamination and
40 decarboxylation of the amino acid moieties to release ammonium and formate. The MS signal at m/z
41 146 suggested the formation of 3-ethylindole, while pH-metric and NMR data revealed the presence of
42 hydroxylated compounds. The phototransformation intermediates of L-tryptophan had fluorescence
43 and absorption spectra similar to those of humic substances, were able to produce $\bullet\text{OH}$ upon
44 irradiation, and tended to aggregate by both ionic and hydrophobic interactions.
45 For the first time we got insight into the nature of the compounds derived by the photo-transformation
46 of L-tryptophan, the behavior of which was quite different from that of the previously studied L-
47 tyrosine, although both compounds produced humic-like materials.

48

49 **Keywords**

50 Environmental Photochemistry; Aromatic amino acids; Tryptophan; Surface waters; Humic
51 substances; Dissolved Organic Matter.

52

53

54 **1. Introduction**

55

56 The chromophoric dissolved organic matter plays a key role in water ecosystems. It absorbs sunlight,
57 reducing the penetration of ultraviolet-B radiation in the water columns and thereby protecting the
58 aquatic organisms (Sommaruga and Augustin 2006; Piccini 2009). Moreover, when irradiated it yields
59 reactive transient species that have an important role in the photo-transformation of water-dissolved
60 organic molecules. The composition of chromophoric dissolved organic matter affects water
61 photochemistry, and one of its main photoactive components is the humic substances (Ma 2010). The
62 latter mainly derive from the microbial degradation of plants residues. However, recent research
63 suggests that humic-like compounds can be formed upon irradiation of phenolic molecules and
64 aromatic amino-acids in aqueous solution (De Laurentiis 2013a/b/c; Bianco 2014; Berto 2016). The
65 humic-like character of the reaction intermediates, not shared by the original substrate, involves
66 peculiar spectroscopic and chemical properties as well as the photochemical production of transient
67 species such as singlet oxygen and reactive triplet states (Bianco 2014). Although it is well known that
68 humic substances are partly formed from proteins, the effect of sunlight irradiation on amino acids
69 dissolved in surface waters is still unknown. This work wants to clarify the photo-degradation
70 pathways of L-tryptophan (Bianco 2014) and to assess the acid-base and aggregation properties of the
71 phototransformation by-products.

72

73 2. Experimental

74

75 2.1. Chemicals

76 Tetraethylammonium chloride, tetrabutylammonium chloride, tetrabutylammonium bromide,
77 potassium chloride, potassium carbonate, sodium acetate, formic acid (~98%), trifluoroacetic acid
78 (99%), methanesulfonic acid (>99.5%), and methanol (>99.8%) were from Sigma Aldrich (St. Louis,
79 Missouri, United States). 5,5-Dimethyl-1-pyrroline N-oxide (DMPO, >98%) was from Cayman
80 Chemicals (Ann Arbor, United States). Phosphoric acid (85%) and acetic acid (100%) were provided
81 by Carlo Erba Reagents (Cornaredo, Italy). L-tryptophan and benzene were from Merck (Darmstadt,
82 Germany). Deuterated dimethyl sulfoxide was from Eurisotop (Saint-Aubin, France). Standard KOH
83 and HCl solutions were from Merck and they were standardized against, respectively, potassium
84 hydrogen phthalate and sodium carbonate (both from Sigma Aldrich). Water used was of Milli-Q
85 quality.

86

87 2.2 Apparatuses and procedures

88 Summarized experimental info is provided below, see [Berto \(2016\)](#) for further details. Solutions of L-
89 tryptophan 1.0 mmol L⁻¹ were irradiated in a solar simulator (Solarbox, CO.FO.ME.GRA) for 0–24 h,
90 where the longest time corresponds to a fluence of 2.4·10⁶ J m⁻². Dark control runs were carried out.
91 The solutions were analyzed immediately after irradiation or lyophilized. The radiation of the Xe lamp
92 equipping the Solarbox was filtered with a 320 nm cut-off filter, producing negligible emission below
93 300 nm.

94 Before and after 8h irradiation, L-tryptophan solutions underwent characterization by pH-metric
95 titrations, UV-vis spectrophotometry (V-550, Jasco), fluorescence Excitation-Emission Matrix
96 spectroscopy (Cary Eclipse, Varian), nephelometry (DRT 1000, HF Instrument), Dynamic Laser Light
97 Scattering (ALV–NIBS instrument) and Nuclear Magnetic Resonance (AVANCE200, Bruker). Before

98 spectrophotometric measurements, solutions were filtered with Millipore syringe filters (0.45 μm pore
99 size) to eliminate suspended particles.

100 A mass profile of the irradiated solutions was obtained with a Thermo Finnigan Advantage Max ion
101 trap spectrometer, equipped with electrospray ionization source used in the positive ion mode. The
102 flow rates of sheath and auxiliary gas (N_2) were set at 25 and 5 (arbitrary unit), respectively. The
103 tuning parameters were: capillary voltage at 25 V, tube lens offset at 5.00 V. The source voltage was
104 4.5 kV, the capillary temperature 250 $^\circ\text{C}$. The lyophilized product was dissolved in 50/50
105 water/methanol – formic acid 0.1% and injected into the mass analyzer.

106 The production of $\bullet\text{OH}$ in the irradiated systems was assessed by irradiating 1.0 mmol L^{-1} L-
107 tryptophan in the presence of 3.0 mmol L^{-1} benzene, and by monitoring the formation of phenol from
108 the reaction between benzene and $\bullet\text{OH}$ (Chiwa, 2015; note that neither benzene nor $\bullet\text{OH}$ absorb lamp
109 radiation). The generation of $\bullet\text{OH}$ by L-tryptophan -photodegradation products was also monitored by
110 Electron Paramagnetic Resonance (EPR) spectroscopy with a Miniscope MS 100 (Magnettech, Berlin,
111 Germany) spectrometer, employing 5,5-dimethyl-1-pyrroline N-oxide as the spin trapping agent. The
112 instrument settings were: microwave power 10 mW; modulation 1000 mG; scan range 120 G; center
113 of field approximately 3345 G. In details, 2 mL of 1 mmol L^{-1} L-tryptophan aqueous solution,
114 continuously stirred in a quartz vial, were irradiated above 280 nm with a Hg-Xe lamp (Oriel
115 Instruments, Stratford, United States) in order to induce L-tryptophan photodegradation. The
116 irradiation was stopped after 1h, and 0.20 mL of the solution were added to 0.20 mL of an aqueous
117 solution of the spin-trap (0.4 mol L^{-1}). The solution was then irradiated applying a filter (cut off 400
118 nm) to avoid direct spin-trap photoexcitation. Solution aliquots of 50 μL were withdrawn after
119 different times of irradiation (namely 5, 10 and 20 min) in a glass capillary and the EPR spectrum
120 recorded. EPR spectra were also recorded with L-tryptophan solutions (0.5 mmol L^{-1}) in the presence
121 of the spin trap (0.2 mol L^{-1}) and on a spin-trap solution alone (0.2 mol L^{-1}) after 5, 10 and 20 minutes
122 of irradiation above 400 nm. The experiments were repeated twice with similar results.

123 L-tryptophan was quantified at different irradiation times by a High Performance Liquid
124 Chromatograph – Diode Array Detector (Elite LaChrom, VWR-Hitachi) equipped with column Merck
125 LiChroCART RP-18 (125 mm × 4 mm × 5 μm), using 60 μL injection volume and 1.0 mL min⁻¹
126 eluent flow rate. An isocratic method was applied with a 10/90 ratio of methanol/acetate buffer (5
127 mmol L⁻¹, pH 4). The signal of L-tryptophan was detected at 278 nm and the retention time was 5.1
128 min. The same instrument and column were used to quantify phenol formation from benzene, with an
129 isocratic mixture of 7/93 methanol/phosphoric acid (5 mmol L⁻¹, pH 3). The detection wavelength was
130 270 nm, phenol retention time 14.8 min. The diode-array signal (whole absorption spectrum) was used
131 to check that the 14.8 min peak was actually phenol.

132 The Total Inorganic Nitrogen (sum of NO₂⁻, NO₃⁻ and NH₄⁺) and the formate anion were determined
133 by ion chromatography with electrochemical suppression (DX500, Dionex). An injector with a 20 μL
134 loop was used. For the analysis of ammonium the eluent was methanesulfonic acid 16 mmol L⁻¹, for
135 the anions it was 9 mmol L⁻¹ K₂CO₃. The Total Organic Nitrogen was calculated as the difference
136 between Total Nitrogen and Total Inorganic Nitrogen. Total Nitrogen was measured with a Shimadzu
137 Total Organic Carbon (TOC-V CSH) analyzer equipped with ASI-V autosampler. The instrument was
138 fed with zero-grade air (free of volatile organic compounds and CO₂) and calibrated with KNO₃
139 standard solutions.

140

141 3. Results and Discussion

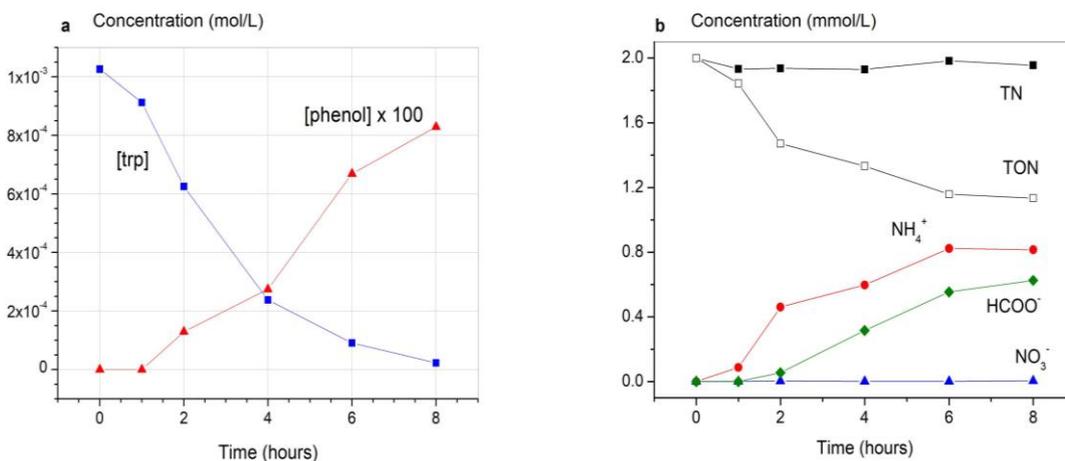
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143 3.1. Phototransformation of L-tryptophan

144 The irradiation of 1 mmol L⁻¹ L-tryptophan (pH 6.0) caused its transformation with 95% degradation
145 in 8h (Figure 1a). No transformation was observed in the dark at the same time scale. The time trends
146 of nitrite, nitrate and ammonium during irradiation were compared with that of the Total Organic
147 Nitrogen and they are reported in Figure 1b, together with the time trend of formate (likely formed by
148 decarboxylation).

149 The Total Organic Nitrogen (initial concentration 2 mmol_N L⁻¹) progressively decreased with
150 increasing irradiation time, down to 1.2 mmol_N L⁻¹ after 8h, and its residual was only partially
151 accounted for by the L-tryptophan not yet degraded. The main fraction consists of nitrogen-containing
152 transformation intermediates. The observed decrease by about 0.8 mmol_N L⁻¹ in 8h irradiation
153 corresponds almost exactly to the observed increase of ammonium. The constant value of Total
154 Nitrogen during irradiation excludes loss of nitrogen as volatile species (e.g., N₂). Moreover, nitrite
155 was below detection limit (<0.001 mmol L⁻¹) and nitrate reached a concentration of only 0.004 mmol
156 L⁻¹ after 8h. This behavior suggests that only one of the nitrogen-containing groups of L-tryptophan
157 underwent (partial) transformation into inorganic nitrogen. This is most likely the amino group, which
158 is released as NH₃ (deamination) by the first excited singlet state of the zwitterionic L-tryptophan
159 (Sobolewski 2009).

160 Alkalimetric titrations were performed on the irradiated solutions, and the titration curves were
161 elaborated by the BSTAC software (De Stefano 1993) with the approach reported in Berto (2016). The
162 concentrations and pK^H values of the dissolved species thus obtained were very well-matched with
163 those of ammonium and formate detected in the solutions at the same irradiation time. We could thus
164 assume that the protogenic capacity of the irradiated solutions was mainly due to ammonium and
165 formate. Moreover, a third protogenic group with pK^H=10.4 is consistent with a phenolic moiety, and
166 it suggests hydroxylation of the C₆ aromatic ring of L-tryptophan.



167

168

169 **Figure 1 a)** Phototransformation of 1.0 mmol L⁻¹ L-tryptophan alone ([trp]). Time trend of phenol
 170 ([phenol], concentration multiplied by 100) upon irradiation of 1.0 mmol L⁻¹ L-tryptophan and 3.0
 171 mmol L⁻¹ benzene. **b)** Concentration of Total Nitrogen (TN), Total Organic Nitrogen (TON), formate,
 172 ammonium and nitrate ions as a function of irradiation time (the irradiated substrate was here 1.0
 173 mmol L⁻¹ L-tryptophan). Phenol is formed by the reaction between •OH and benzene. The TON
 174 decreases, whereas ammonium and formate increase, suggesting the loss of amino-acidic moieties
 175 under irradiation.

176

177

178 The MS direct infusion analysis of the degradation products of 1.0 mmol L⁻¹ L-tryptophan after 8h
 179 irradiation showed a peak at *m/z* 146 that could correspond to the [M+H⁺] ion of 3-ethylindole. It
 180 would be formed upon deamination and decarboxylation processes, which would also produce formic
 181 acid and ammonium as already discussed (Figure 1b).

182 We used benzene as probe molecule to assess the possible generation of hydroxyl radicals during
 183 photo-transformation. Benzene does not undergo direct photolysis upon irradiation above 300 nm, and
 184 it reacts quickly with •OH to produce phenol (Albinet 2010). L-tryptophan solutions (1 mmol L⁻¹)
 185 containing 3 mmol L⁻¹ benzene were thus irradiated for up to 8h to monitor the time trend of phenol
 186 (Figure 1a). Note that phenol was not detectable in the first hour of irradiation, and it started to be
 187 detected only when L-tryptophan degradation reached ~40%. This finding suggests that •OH was not
 188 directly produced by irradiated L-tryptophan, but rather by excitation of its transformation

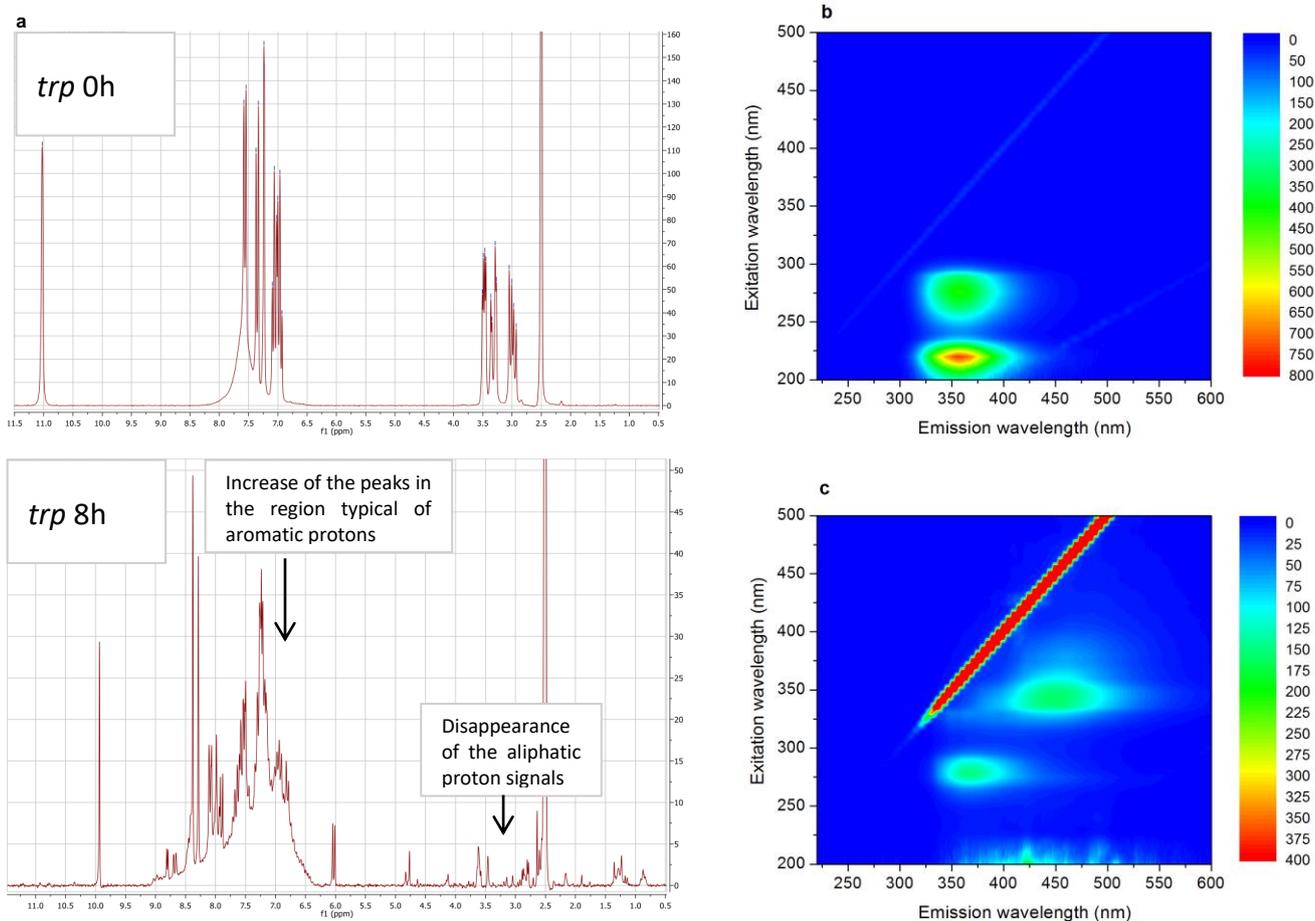
189 intermediate(s). This outcome is consistent with the previous observation that they were the L-
190 tryptophan transformation intermediates to be responsible for the photoinduced production of $^1\text{O}_2$ and
191 reactive triplet states (Bianco 2014). The production of $\bullet\text{OH}$ by irradiated humic substances is still a
192 subject of debate in the literature but it has been shown that hydroxylated compounds, like those
193 tentatively detected here by titration, may be involved in $\bullet\text{OH}$ photogeneration (Tafer 2016).

194 The photo-induced production of $\bullet\text{OH}$ by L-tryptophan photoproducts was also investigated by spin
195 trapping - EPR spectroscopy. We irradiated a 1 mmol L^{-1} L-tryptophan solution above 280 nm for 1h
196 to induce the formation of photoproducts, which absorb visible light. This solution was added to a
197 concentrated 5,5-dimethyl-1-pyrroline N-oxide solution (10^{-1} molar range). The EPR spectrum under
198 these experimental conditions did not evidence the presence of radical species (data not shown for
199 brevity). The solution containing the L-tryptophan photoproducts was then irradiated above 400 nm for
200 5, 10 and 20 minutes. After 20 minutes of irradiation the 5,5-dimethyl-1-pyrroline N-oxide radical
201 adduct [DMPO-OH] \bullet was detected, suggesting $\bullet\text{OH}$ photoproduction. No signal was detected with
202 either L-tryptophan (not previously irradiated)/5,5-dimethyl-1-pyrroline N-oxide or 5,5-dimethyl-1-
203 pyrroline N-oxide alone at any irradiation time. These results further confirm that the photogeneration
204 of $\bullet\text{OH}$ was induced by the excitation of L-tryptophan photoproducts, while the parent compound was
205 not able to generate free radicals under irradiation.

206 207 *3.2. Spectroscopic characterization of the irradiated systems*

208 The UV-visible and Excitation-Emission Matrix (EEM) spectra were recorded on L-tryptophan
209 solutions before and after 8h of irradiation. The irradiated solutions were filtered and diluted by 1:10
210 (UV-vis) and 1:100 (EEM). The EEM data are shown in Figure 2b and they confirm the occurrence of
211 humic-like signals (Galgani 2011; Coble 1996). The significance of this finding is enhanced by the fact
212 that ~50% of the protein-like fluorescence of natural organic matter is actually due to free tryptophan
213 and tyrosine loosely bound to organic matter by weak hydrophobic interactions (Trubetskaya 2016).

214



215

216

217 **Figure 2** a) ¹H-NMR spectra of L-tryptophan before and after 8h irradiation (solvent: deuterated
 218 dimethyl sulfoxide); b) Excitation-Emission Matrix (EEM) of L-tryptophan 10 μmol L⁻¹ (1 mmol L⁻¹,
 219 diluted 1:100). c) EEM of 1 mmol L⁻¹ L-tryptophan subjected to 8h irradiation and filtration before the
 220 measurements. The linear features are due to the Rayleigh–Tyndall scattering (Baker 2002). The
 221 irradiated L-tryptophan solutions show an increase of the NMR peaks attributed to aromatic protons,
 222 suggesting the occurrence of hydroxylation processes. Their EEM spectra show humic-like features.
 223 *trp*: L-tryptophan. ¹H-NMR: Hydrogen - Nuclear Magnetic Resonance.

224

225

226 Compared to the original amino acid, the Nuclear Magnetic Resonance analysis of the samples
 227 irradiated for 8h showed a noticeable increase of the peaks attributed to aromatic protons. Before
 228 irradiation, L-tryptophan had 11 resolved peaks between 7.7 and 6.9 ppm, while irradiation induced
 229 the appearance of signals in a wider region together with an almost complete disappearance of the
 230 aliphatic proton signals (δ 3 - 3.5 ppm) (Figure 2). These results suggest the occurrence of poly-

231 substituted aromatic rings. The disappearance of the aliphatic proton signals suggests that the photo-
232 transformation process affects also the amino-acidic portion of the molecule, as previously pointed out.

233

234 3.3. Suspended particles characterization

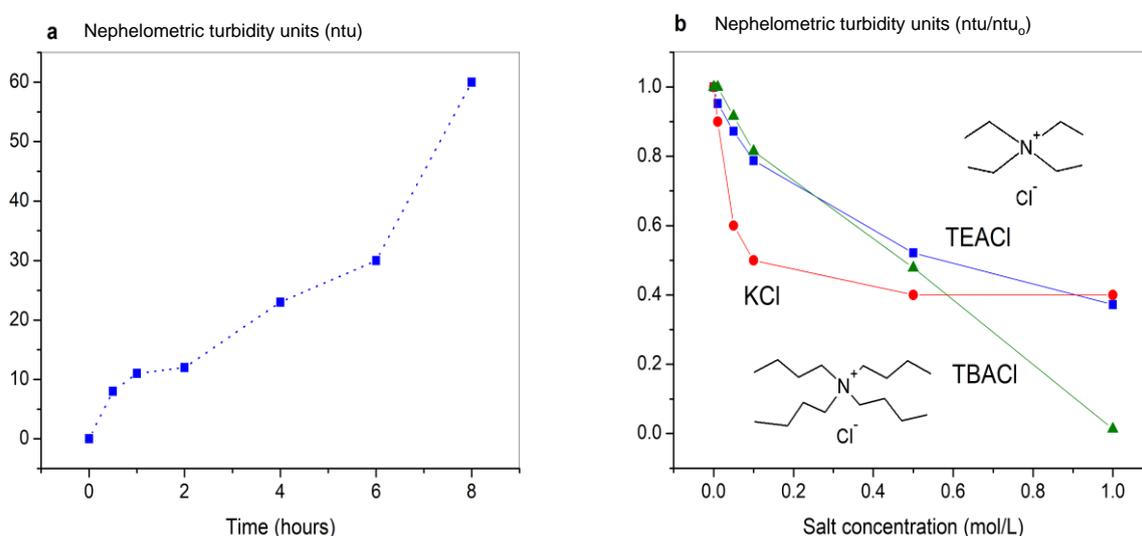
235 The solutions of L-tryptophan appeared turbid and pale-yellow after irradiation. The turbidity was
236 quantified nephelometrically, and the diameters of the formed particles were measured by Dynamic
237 Laser Light Scattering. The turbidity increase during irradiation was gradual (Figure 3a), while light
238 scattering measurements suggested that most of the particles had hydrodynamic diameters in the range
239 of 52 ± 12 nm after 4h irradiation, and 64 ± 22 nm after 8h. The occurrence of larger particles was also
240 shown, with hydrodynamic diameters peaking at 590 and 1210 nm for irradiation times of 4 and 8h,
241 respectively.

242 The presence of particles suggests the occurrence of aggregation phenomena among the
243 phototransformation intermediates (Net 2010). To gain insight into the related processes, a solution of
244 L-tryptophan irradiated for 8h was added with increasing concentrations of background salts having
245 different chaotropic properties (Marcus 2009) or with an organic solvent, with the aim of changing the
246 solution properties. The turbidity of the irradiated systems decreased upon addition of methanol,
247 compared to the addition of a comparable volume of water, suggesting that aggregation involved
248 hydrophobic interactions. In additional experiments, increasing concentrations of background salts
249 (KCl, tetraethylammonium chloride and tetrabutylammonium chloride) were added separately to
250 different aliquots of the irradiated solution before turbidity measurements (Figure 3b). The salting-in
251 effect is evident in all cases, likely caused by the association between the added ions and charged or
252 electronegative groups on the reaction intermediates.

253 At low salt concentration, the most marked effect was observed upon addition of KCl (K^+ and Cl^- ions
254 are water structure breakers, differently from the tetrabutylammonium and tetraethylammonium
255 cations (Marcus 2009)). The addition of 1 mol L^{-1} tetrabutylammonium chloride considerably
256 decreased the turbidity of the solution (Figure 3b), and the effect was already visible to the naked eye.
257 The same behavior was observed upon addition of tetrabutylammonium bromide at the same

258 concentration value (data not shown). This result suggests a specific effect of the tetrabutylammonium
 259 alkyl chains on the solubility of the L-tryptophan intermediates, because the occurrence of relatively
 260 long chains in tetrabutylammonium is the main difference between this and the other added cations.
 261 The tetrabutylammonium cation can probably interfere with the self-aggregation of solutes, which
 262 supports the idea that the formation of aggregates also involved hydrophobic interactions.
 263 Interestingly, [Piccolo \(1999\)](#) obtained similar results as ours when working with humic substances
 264 solutions.

265



266

267 **Figure 3 a)** Turbidity of 1 mmol L⁻¹ L-tryptophan, as a function of the irradiation time. **b)** Turbidity of
 268 1 mmol L⁻¹ L-tryptophan irradiated for 8h, after addition of different amounts of background salts. The
 269 turbidity of the solutions increased upon irradiation, but it was reduced by salt addition. TEACl:
 270 tetraethylammonium chloride. TBACl: tetrabutylammonium chloride. ntu: nephelometric turbidity
 271 units.

272

273 4. Conclusions

274 The data collected in this work highlighted, for the first time to our knowledge, that the photo-
275 transformation of L-tryptophan into humic-like compounds mainly proceeds via deamination and
276 decarboxylation with release of free ammonium and formate. Such a process would be consistent with
277 the detection of 3-ethylindole, formed from L-tryptophan by loss of the carboxylic and amino
278 functions. Interestingly, about 60% of the initial organic nitrogen was retained in the transformation
279 intermediates after almost complete disappearance of the primary compound.

280 The pH-metric data suggest the formation of by-products with phenolic functionalities, and NMR
281 spectra suggest the occurrence of substitution processes on the aromatic ring. A hydroxylation of the
282 L-tryptophan C₆ aromatic ring is consistent with the occurrence of •OH radicals, photogenerated by L-
283 tryptophan transformation intermediates but not by L-tryptophan itself.

284 The photochemical production of •OH (this work), of ¹O₂ and of reactive triplet states (Bianco 2014)
285 suggests that L-tryptophan phototransformation intermediates behave as photosensitizers, in a similar
286 way as humic substances. The irradiated solutions of L-tryptophan show the presence of suspended
287 particles, which are affected by addition of salts in a similar way as previously observed with humic
288 substances. The detected particles could be formed upon by-product aggregation via both polar and
289 hydrophobic interactions.

290 The formation of compounds with absorption, fluorescence and photosensitizing properties similar to
291 those of humic substances is a feature that the photochemistry of L-tryptophan shares with L-tyrosine
292 (Bianco 2014; Berto 2016). However, tyrosine phototransformation did not involve the amino-acidic
293 function but it rather proceeded by aromatic ring hydroxylation and dimerization, yielding water-
294 soluble intermediates with protogenic functions similar to those of the parent molecule (Berto 2016).

295

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