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

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

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Abstract

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

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

potentiometry and mass spectrometry (MS). The solutions appeared turbid after irradiation, thus nephelometry and Dynamic Laser Light Scattering were used to characterize the suspended particles.

About 95% of L-tryptophan was degraded in 8h irradiation, undergoing deamination and decarboxylation of the amino acid moieties to release ammonium and formate. The MS signal at *m*/*z* 146 suggested the formation of 3-ethylindole, while pH-metric and NMR data revealed the presence of hydroxylated compounds. The phototransformation intermediates of L-tryptophan had fluorescence and absorption spectra similar to those of humic substances, were able to produce $^{\bullet}OH$ upon irradiation, and tended to aggregate by both ionic and hydrophobic interactions.

For the first time we got insight into the nature of the compounds derived by the photo-transformation of L-tryptophan, the behavior of which was quite different from that of the previously studied L-tyrosine, although both compounds produced humic-like materials.

Keywords

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

1. Introduction

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

2. Experimental

2.1. Chemicals

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

2.2 Apparatuses and procedures

Summarized experimental info is provided below, see Berto (2016) for further details. Solutions of L-tryptophan 1.0 mmol L^{-1} were irradiated in a solar simulator (Solarbox, CO.FO.ME.GRA) for 0–24 h, where the longest time corresponds to a fluence of $2.4 \cdot 10^6$ J m^{-2}. Dark control runs were carried out. The solutions were analyzed immediately after irradiation or lyophilized. The radiation of the Xe lamp equipping the Solarbox was filtered with a 320 nm cut-off filter, producing negligible emission below 300 nm.

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

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

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

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

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

The Total Inorganic Nitrogen (sum of NO_2^-, NO_3^- and NH_4^+) and the formate anion were determined by ion chromatography with electrochemical suppression (DX500, Dionex). An injector with a 20 µL loop was used. For the analysis of ammonium the eluent was methanesulfonic acid 16 mmol L^{-1}, for the anions it was 9 mmol L^{-1} K_2CO_3. The Total Organic Nitrogen was calculated as the difference between Total Nitrogen and Total Inorganic Nitrogen. Total Nitrogen was measured with a Shimadzu Total Organic Carbon (TOC-V CSH) analyzer equipped with ASI-V autosampler. The instrument was fed with zero-grade air (free of volatile organic compounds and CO_2) and calibrated with KNO_3 standard solutions.

3. Results and Discussion

3.1. Phototransformation of L-tryptophan

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

The Total Organic Nitrogen (initial concentration 2 $mmol_N$ L^{-1}) progressively decreased with increasing irradiation time, down to 1.2 $mmol_N$ L^{-1} after 8h, and its residual was only partially accounted for by the L-tryptophan not yet degraded. The main fraction consists of nitrogen-containing transformation intermediates. The observed decrease by about 0.8 $mmol_N$ L^{-1} in 8h irradiation corresponds almost exactly to the observed increase of ammonium. The constant value of Total Nitrogen during irradiation excludes loss of nitrogen as volatile species (e.g., N_2). Moreover, nitrite was below detection limit (<0.001 mmol L^{-1}) and nitrate reached a concentration of only 0.004 mmol L^{-1} after 8h. This behavior suggests that only one of the nitrogen-containing groups of L-tryptophan underwent (partial) transformation into inorganic nitrogen. This is most likely the amino group, which is released as NH_3 (deamination) by the first excited singlet state of the zwitterionic L-tryptophan (Sobolewski 2009).

Alkalimetric titrations were performed on the irradiated solutions, and the titration curves were elaborated by the BSTAC software (De Stefano 1993) with the approach reported in Berto (2016). The concentrations and pK^H values of the dissolved species thus obtained were very well-matched with those of ammonium and formate detected in the solutions at the same irradiation time. We could thus assume that the protogenic capacity of the irradiated solutions was mainly due to ammonium and formate. Moreover, a third protogenic group with pK^H=10.4 is consistent with a phenolic moiety, and it suggests hydroxylation of the C_6 aromatic ring of L-tryptophan.

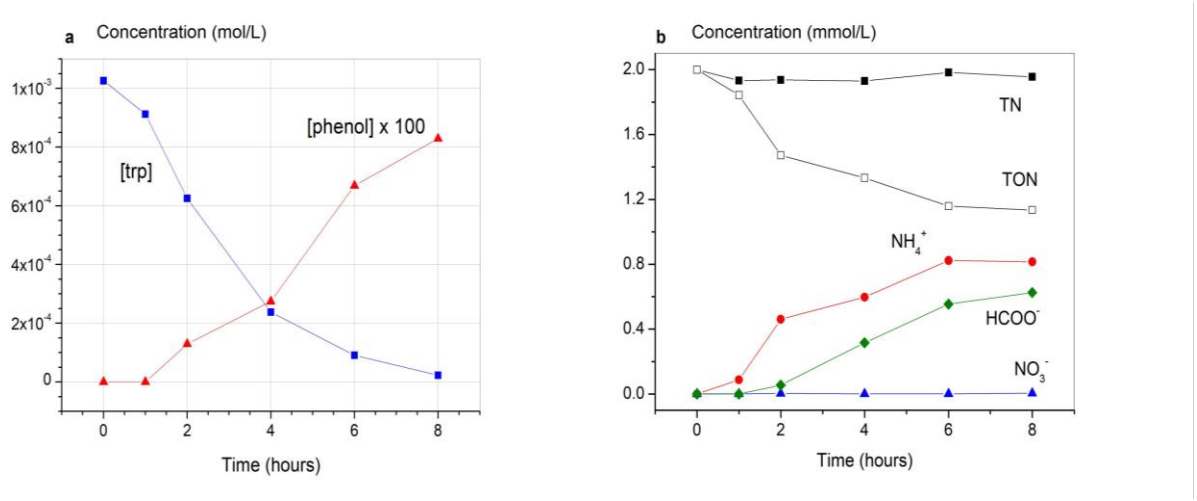

Figure 1 a) Phototransformation of 1.0 mmol L^{-1} L-tryptophan alone ([trp]). Time trend of phenol ([phenol], concentration multiplied by 100) upon irradiation of 1.0 mmol L^{-1} L-tryptophan and 3.0 mmol L^{-1} benzene. **b)** Concentration of Total Nitrogen (TN), Total Organic Nitrogen (TON), formate,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

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

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

intermediate(s). This outcome is consistent with the previous observation that they were the L-tryptophan transformation intermediates to be responsible for the photoinduced production of $^1O_2$ and reactive triplet states (Bianco 2014). The production of •OH by irradiated humic substances is still a subject of debate in the literature but it has been shown that hydroxylated compounds, like those tentatively detected here by titration, may be involved in •OH photogeneration (Tafer 2016).

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

3.2. Spectroscopic characterization of the irradiated systems

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

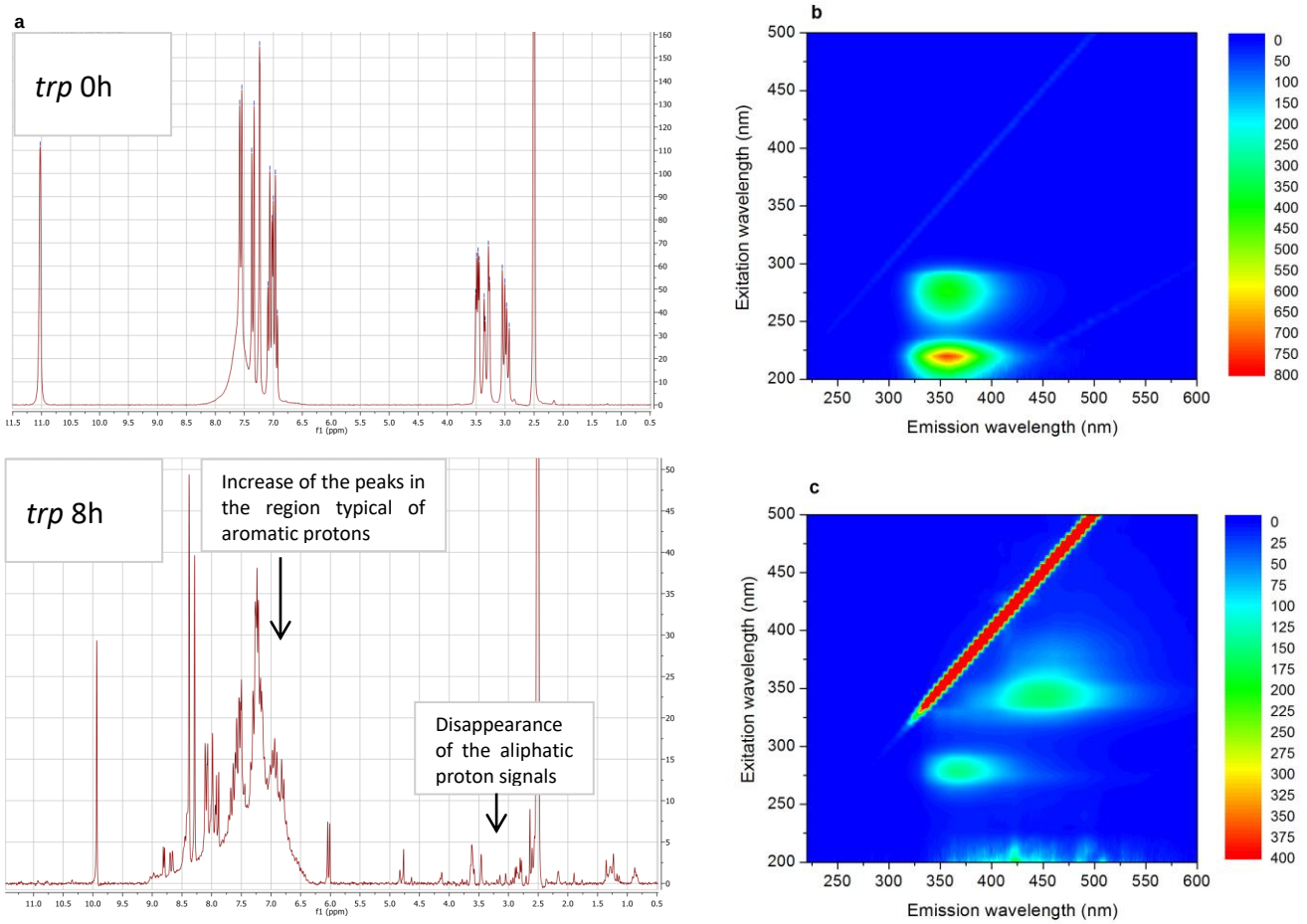

Figure 2 a) ^{1}H-NMR spectra of L-tryptophan before and after 8h irradiation (solvent: deuterated dimethyl sulfoxide); b) Excitation-Emission Matrix (EEM) of L-tryptophan 10 µmol L^{-1} (1 mmol L^{-1}, diluted 1:100). c) EEM of 1 mmol L^{-1} L-tryptophan subjected to 8h irradiation and filtration before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

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

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

3.3. Suspended particles characterization

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

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

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

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

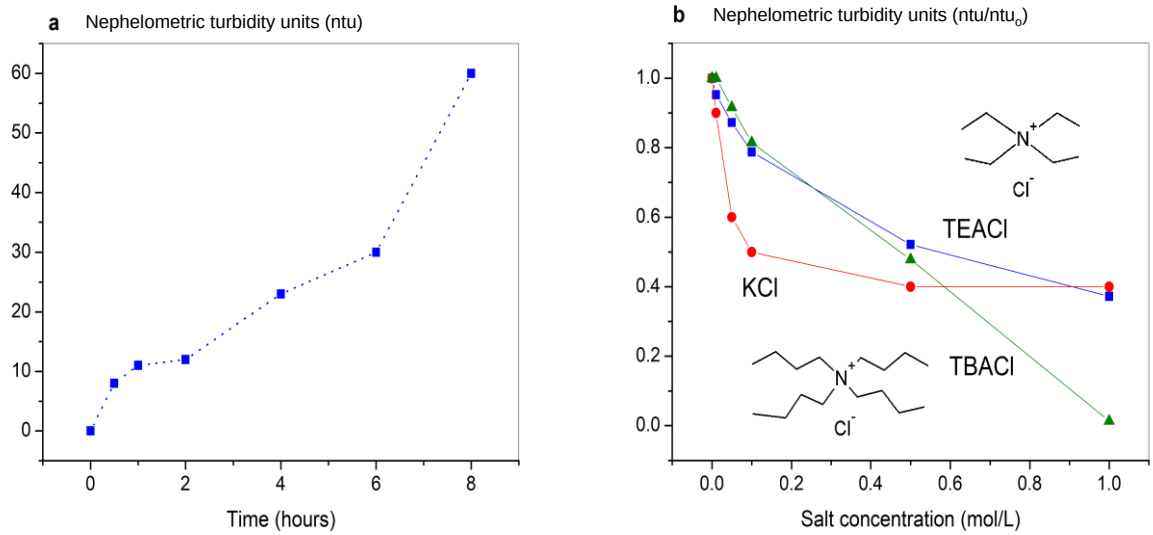

Figure 3 a) Turbidity of 1 mmol L^{-1} L-tryptophan, as a function of the irradiation time. **b)** Turbidity of 1 mmol L^{-1} L-tryptophan irradiated for 8h, after addition of different amounts of background salts. The turbidity of the solutions increased upon irradiation, but it was reduced by salt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

4. Conclusions

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

The pH-metric data suggest the formation of by-products with phenolic functionalities, and NMR spectra suggest the occurrence of substitution processes on the aromatic ring. A hydroxylation of the L-tryptophan C_6 aromatic ring is consistent with the occurrence of $^{\bullet}OH$ radicals, photogenerated by L-tryptophan transformation intermediates but not by L-tryptophan itself.

The photochemical production of $^{\bullet}OH$ (this work), of $^1O_2$ and of reactive triplet states (Bianco 2014) suggests that L-tryptophan phototransformation intermediates behave as photosensitizers, in a similar way as humic substances. The irradiated solutions of L-tryptophan show the presence of suspended particles, which are affected by addition of salts in a similar way as previously observed with humic substances. The detected particles could be formed upon by-product aggregation via both polar and hydrophobic interactions.

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

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