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Photochemical generation of photoactive compounds with fulvic-like and humic-like fluorescence in aqueous solution

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Abstract

The irradiation of L-tryptophan, L-tyrosine and 4-phenoxyphenol in aqueous solution produced compounds with similar fluorescence properties as humic substances, and with absorption spectra that were significantly extended into the UVA and visible regions compared to the starting compounds. The irradiated systems had photosensitizing properties, as proven by the photodegradation of 2,4,6-trimethylphenol and furfuryl alcohol (probes of excited triplet states and $^1O_2$, respectively). The described photochemical processes could constitute an additional pathway for the formation of humic substances in clear and shallow water bodies, which would be added to the complex network of reactions involving dissolved organic matter.

Keywords: Excitation-Emission Matrix; Photoinduced transformation; Triplet sensitizers; Singlet oxygen; Environmental photochemistry; Autochthonous and allochthonous organic matter; Photohumification.

Introduction

Humic substances are important light-absorbing components in surface waters and soil, and their water-soluble fractions (humic and fulvic acids, respectively HA and FA) account for a considerable proportion of the chromophoric dissolved organic carbon in aqueous environments (Loiselle et al., 2009; Del Vecchio and Blough, 2004). HA and FA partially originate from the microbial decomposition of animal and plant spoils and often reach surface-water environments by means of soil leaching and runoff (Vinebrooke and Leavitt, 1998). Surface-water humic substances affect, among others, the availability and transport of poorly soluble organic pollutants, toxic metals and nanoparticles (Town et al., 2012; Adegboyega et al., 2013). Sunlight absorption by HA and FA has an important impact on the spectrum of surface waters and sometimes strongly determines their color (Kutser et al., 2009; Banas, 2013), while the absorption of UV radiation protects aquatic organisms from UV-associated harmful effects (Laurion et al., 2000; Sommaruga, 2010).

HA and FA play an important role in surface-water photochemistry, because the absorption of radiation yields excited singlet states that by inter-system crossing can produce excited triplet states (T*) (Canonica et al., 1995; Golanoski et al., 2012). The T* transients of HA and FA can directly react with water-dissolved molecules by energy, electron or hydrogen transfer. Such processes can induce the transformation of organic pollutants, including phenylurea herbicides and sulfonamide antibiotics (Canonica et al., 2006; Canonica, 2007). T* also react with molecular oxygen to produce $^1O_2$ (Grandbois et al., 2008; Mostafa et al., 2013), which is for instance involved in the photochemical degradation of the pharmaceutical cimetidine and of several other heterocyclic compounds (Latch et al., 2003; Iesce et al., 2005).

Compounds with similar properties as humic acids or atmospheric humic-like substances (HULIS) can be produced by photochemical reactions in the presence of several components, such as phenolic molecules, fatty acids, surface-water CDOM and dead algae (Kieber et al., 1997; Nieto-Gligorovski et al., 2010; Ortega-Retuerta et al., 2010; Mostofa et al., 2011). The photogenerated material could also produce reactive transients (T*, $^1O_2$) under irradiation (De Laurentiis et al., 2013a).

To get further insight into the photohumification process, including the ability of the photogenerated compounds to produce reactive species, in this work we have irradiated low molecular weight compounds that are representative of the organic matter occurring in surface waters. The studied substrates include aromatic amino acids (L-tryptophan and L-tyrosine, contained in proteins) and 4-phenoxyphenol, which is a model compound for the 4-O-5 bonds in lignin (Park et al., 2013). The possible formation of substances with similar properties as HA and FA was assessed with excitation-emission matrix (EEM) fluorescence spectra and by the ability to sensitize photochemical reactions. The latter issue was studied by making use of 2,4,6-trimethylphenol and furfuryl alcohol as probe molecules (Canonica and Freiburghaus, 2001; Halladja et al., 2007). The EEM technique is a powerful tool to characterize organic matter in environmental water samples. Full fluorescence spectra are registered for excitation wavelengths that are sequentially varied, and fluorophores produce easily identified peaks in the resulting contour plots. One can thus differentiate protein material, humic substances, plankton pigments and fluorescent whitening agents (Coble, 1996; Mostofa et al., 2011).

Materials and methods

Reagents and materials. L-Tryptophan (TRP, purity grade 99%), L-tyrosine (TYR, 99%), 4-phenoxyphenol (4PP, 98%), 3,4-dihydroxyphenylalanine (DOPA, 99%), 2,4,6-trimethylphenol (TMP, 97%), furfuryl alcohol (FFA, 98%) and humic acid, sodium salt were purchased from Aldrich, methanol (gradient grade), acetic acid (99%), sodium acetate (98%) and phosphoric acid (85%) from Carlo Erba (Rodano, Italy). All reagents were used as received, without further purification. Water used was of Milli-Q quality.

Irradiation experiments. Solutions to be irradiated (10 mL) were placed in cylindrical Pyrex glass cells (4.0 cm diameter, 2.3 cm height), tightly closed with a lateral screw cap and irradiated from the top under magnetic stirring. The irradiation device was a Philips TL40W/05 lamp, emitting radiation above 300 nm with emission maximum at 365 nm. The UV irradiance (290-400 nm) on top of the solutions was 21.0 ± 1.0 W m^{-2}, measured with a CO.FO.ME.GRA. (Milan, Italy) power meter. The volumetric photon flux in solution was $(1.19\pm0.08)\cdot10^{-5}$ Einstein L^{-1} s^{-1}, actinometrically determined with the ferrioxalate method (Kuhn et al., 2004). After irradiation (typical irradiation times were 2h, 4h, 8h, 16h and 24h), the cells were withdrawn from the lamp and the irradiated solutions underwent characterization with different techniques.

Analytical determinations. The time trends of TRP, TYR, 4PP, TMP (T* probe; Canonica and Freiburghaus, 2001) and FFA ($^1O_2$ probe; Halladja et al., 2007) were monitored by High-Performance Liquid Chromatography coupled with Diode Array Detection (HPLC-DAD). The VWR-Hitachi Elite LaChrom instrument was equipped with L-2130 quaternary pump for low-pressure gradients (incorporating a degassing unit), L-2200 autosampler, L-2300 column oven (kept at 40 °C) and L-2455 DAD detector. The column used in most cases was a Merck LiChroCART RP-18 (125 mm × 4 mm × 5 μm), with 60 μL injection volume and flow rate of 1.0 mL min^{-1}. Eluent solutions were CH_3OH (A), 5 mM acetate buffer at pH 4 (B) and H_3PO_4 at pH 3 (C). TRP was isocratically eluted with 10% A + 90% B, with retention time t_R = 5.3 min and detection wavelength λ = 278 nm. TYR was eluted with 1% A + 99% B, with t_R = 2.9 min and λ = 274 nm. Under the same elution conditions, DOPA had t_R = 2.0 min and was detected at 280 nm. 4PP was eluted with 55% A + 45% C (t_R = 6.8 min, λ = 262 nm), TMP with 60% A + 40% C (t_R = 4.5 min, λ = 210 nm), FFA with 15% A + 85% C (t_R = 4.0 min, λ = 230 nm). Problems were encountered with FFA in the presence of TRP, because of co-elution with TRP degradation intermediates. To increase resolution and avoid peak overlap, elution was carried out with a Merck Chromolith Performance RP18e column (100 mm length, 3 mm diameter) at 0.8 mL min^{-1} flow rate (injection volume 10 μL), with 1% A + 99% B.

Fluorescence EEM (Excitation-Emission Matrix) spectra were taken with a Varian Cary Eclipse spectrofluorimeter, using a fluorescence quartz cuvette with 1.000 cm optical path length.

EEM spectra were obtained with 600 nm $\min^{-1}$ scan rate. The slit width on both excitation and emission (5 or 10 nm) was a compromise between spectral resolution and signal intensity. Before and after each EEM measurement, the intensity of the Raman signal of water was used as a test of stability. Absorption spectra were taken with a Varian Cary 100 Scan UV-Vis double-beam spectrophotometer, using quartz cuvettes with 1.000 cm path length. Nephelometric measures were carried out with a DRT 1000 nephelometer by HF Instrument, calibrated in standard formazine units (NTU) as prescribed by the 7025 ISO norm (ISO, 1999). Particle diameters were determined by Dynamic Laser Light Scattering using an ALV-NIBS instrument, measuring scattered light for at least 20 s at 298 K. The pH values of the solutions were measured with a combined glass electrode connected to a Metrohm 713 pH meter.

Results and Discussion

Direct phototransformation of L-tryptophan (TRP)

The irradiation of 1 mM TRP (pH 6.6) caused transformation with half-life time of ~8 h and 90% degradation in 24 h (see Figure SM1 in the Supplementary Material, hereafter SM). No transformation was observed in the dark at the same time scale. The EEM of TRP before irradiation showed the typical peaks at excitation/emission wavelengths (Ex/Em) around 250/350 and 300/350 nm (Figure 1a). Peak intensities decreased with irradiation time, consistently with the observed decrease of TRP concentration, and a new peak at Ex/Em ~ 350/460 nm appeared for irradiation time >8 h. Such a peak overlaps with peak C of humic substances (Coble, 1996). The EEM of the sample irradiated for 24 h is reported in Figure 1b.

Irradiation considerably modified the absorption spectrum of the solution, with decrease of the TRP band around 280 nm and a progressive increase of absorbance above 330-350 nm (Figure 1c). For irradiation ≥8 h, the spectrum above 350 nm showed the typical absorption behavior of humic substances, namely an exponential decrease of absorbance with increasing wavelength that is presumably caused by charge-transfer interactions of compounds in supra-molecular assemblies (Del Vecchio and Blough, 2004; Sulzberger and Durish-Kaiser, 2009). However, such a spectrum could be due as well to radiation scattering by particles (Salaices et al., 2002; Minero and Vione, 2006). Nephelometric measures showed an increase of turbidity upon irradiation (Figure 1d), suggesting photochemical formation of particles from TRP. Further characterization by laser light scattering indicated the occurrence of particles with hydrodynamic diameters in the range of 200-350 nm, for irradiation times ≥8 h. Turbidity of samples irradiated for 24 h was decreased by ~10% upon filtration at 5 μm pore diameter and it was practically eliminated by filtration at 0.45 or 0.10 μm. Absorbance also decreased upon filtration at 0.45 or 0.10 μm, by ~25% at 300 nm and by ~60% at 400 nm (see Figure SM2 in SM). However, the genuine absorption spectra above 350 nm still showed an exponential decrease of the absorbance with increasing wavelength (Figure SM2), thereby confirming similarity with the spectra of FA and HA. Considering that the solution pH did not undergo significant changes upon irradiation, which excludes precipitation of TRP, the formation of particles suggests that irradiated TRP may be transformed into compounds of higher molecular weight.

Direct phototransformation of L-tyrosine (TYR)

TYR (1 mM initial concentration, pH 6.5) was transformed upon irradiation and no transformation was observed in the dark. The photoreaction kinetics was considerably slower compared to TRP: about 60% of initial TYR was still present in solution after 24 h irradiation (see Figure SM1 in SM). Before irradiation, the EEM spectrum showed the typical TYR peaks at Ex/Em ~ 200/300 and 240-290/300-340 nm (Figure 2a). Irradiation caused a decrease of peak intensity, while new peaks appeared at Ex/Em ~ 250/390-430 and 280-300/390-430 nm (Figure 2b,c). The former overlaps with peak A of humic substances, the latter with peak M of FA (Mostofa et al., 2011; Coble, 1996). The material produced by TYR irradiation was considerably more fluorescent than that derived from TRP: indeed, that shown in Figure 1b is the irradiated TRP sample with the highest fluorescence intensity.

The TYR absorption band near 275 nm did not change significantly in intensity for irradiation ≤8 h (Figure 2d), mostly because of limited TYR transformation (≤15%) and probably also for the formation of compounds with similar absorption spectrum. At longer irradiation times (16 h, 24 h) the spectra underwent considerable change, with a substantial absorbance increase that was extended into the visible (irradiated solutions appeared pale yellow). The exponential-like decrease of absorbance with increasing wavelength can be exclusively attributed to radiation-absorbing species, because the presence of particles was excluded by measures of nephelometry and laser light scattering.

TYR has been found to produce compounds with similar absorption properties as HA or FA, in the presence of clay minerals in the dark (Bosetto et al., 1997). Focusing on similarities between humic substances and melanins, it has been suggested that the transformation of TYR into 3,4-dihydroxyphenylalanine (DOPA) was a key step in the process (Bosetto et al., 1997). In our experiments DOPA was not detected upon TYR irradiation, which might be assigned to negligible formation or very fast transformation. Irradiation of 1 mM DOPA did not yield fluorescence signals in the region of humic substances, and the percentage of DOPA transformation after 24-h irradiation was <5%. Despite this, an increase of the absorbance above 300 nm was observed in the irradiated solutions and the absorption was extended into the visible, causing the development of a yellow color. The slow direct phototransformation of DOPA suggests that it might not be an important TYR intermediate under our experimental conditions (otherwise DOPA should accumulate to reach detectable concentration), unless degradation processes sensitized by other intermediates play an important role (*vide infra*). Moreover, TYR is a phenolic compound and the direct photolysis of phenols under UVA irradiation proceeds *via* homolytic breaking of the O-H bond to yield the corresponding phenoxy radicals (De Laurentiis et al., 2013b). The latter evolve into dimeric species, rather than into hydroxylated compounds such as DOPA (Rayne et al., 2009).

The absence of humic fluorescence upon DOPA irradiation, despite the absorbance increase, suggests that humic-like absorption and fluorescence might not be closely interconnected. Because fluorescence is a more selective index of the occurrence of HA and FA compared to the absorbance (Nebbioso and Piccolo, 2013), the formation of humic substances *via* the DOPA pathway under direct photochemistry conditions can be strongly questioned.

Direct phototransformation of 4-phenoxyphenol (4PP)

The irradiation of 0.1 mM 4-phenoxyphenol (4PP) at pH 6.5 caused its transformation, with an half-life time of ~7 h and almost complete degradation after 24 h (see Figure SM1 in SM). No transformation of 4PP took place in the dark. Irradiation caused an overall increase of the solution absorbance, which after 8 h was significantly extended into the visible range (see Figure 3).

Fluorescence had a considerable initial increase upon irradiation, followed by a slow decrease. The new fluorescence signals partially overlapped with the emission of 4PP, but separated peaks appeared at Ex/Em = 200-250/400-450 nm and 300/400-450 nm, in the respective regions of peaks A and M of humic substances (Mostofa et al., 20111; Coble, 1996). The fluorescence spectrum of Figure 3c (24 h irradiation) shows that the peak at Ex/Em = 200-250/400-450 nm actually consists of two very near peaks with Ex ~210 and 230 nm.

A fluorescence peak with Ex/Em = 320/400-450 nm, similar to peak M, has been observed upon interaction between 4PP and non-fluorescent 4-carboxybenzophenone (De Laurentiis, 2013c). Therefore, it is possible that part of the fluorescence emission of irradiated 4PP is caused by inter-molecular interactions between photogenerated compounds.

Photochemical activity of irradiated solutions

Irradiation of TRP, TYR and 4PP caused the appearance of fluorescence peaks overlapping with peaks A, C and/or M of humic substances. HA and FA are well-known photosensitizers in surface waters. The ability of the fluorescent solutions (1 mM TRP, 1 mM TYR or 0.1 mM 4PP, pre-irradiated for 16 h) to produce reactive species under irradiation was tested by use of furfuryl alcohol (FFA) and trimethylphenol (TMP) as probe molecules for $^1O_2$ and T*, respectively. The probes were added (to obtain 0.1 mM initial concentration) after the 16 h pre-irradiation time. Blank experiments were carried out with the spiked solutions in the dark, or by irradiating TMP or FFA alone in Milli-Q water, yielding negligible or very low transformation of the probes.

Pre-irradiated TRP and TYR induced significant degradation of TMP, which disappeared within 2-4 h. The TYR system was more photoactive compared to the TRP one (see Figure 4a,b). The irradiation of 0.1 mM TMP + 1 mM TRP and of 0.1 mM TMP + 1 mM TYR (without pre-irradiation) was also carried out, to check for the possibility that TRP or TYR could directly photosensitize the degradation of TMP. In the case of TRP the degradation of the probe molecule was delayed by 3-4 h compared to the pre-irradiated system, suggesting that time was required for the photochemical formation of photoactive compounds. TYR was much less able than TRP to sensitize the probe transformation without pre-irradiation (Figure 4b), indicating that the photochemical production of photoactive intermediates from TYR would require more than 4 h. Important sensitization of TMP degradation by either TRP or TYR would thus be excluded, and photoactivity should be attributed to their phototransformation intermediates. The irradiation test with 4PP and TMP could not be carried out because of chromatographic interferences.

The transformation of FFA by the pre-irradiated systems was considerably slower compared to TMP: the half-life time was ~4 h for pre-irradiated TRP, ~8 h for TYR and ~50h for 4PP (Figure 4c-e). When FFA was added to TRP, TYR and 4PP without pre-irradiation, it was observed a delay in phototransformation that ranged from 1-2 h for TRP to ~10 h for TYR, and to over 50 h for 4PP. The described experiments suggest that the compounds formed upon phototransformation of TRP, TYR and 4PP can act as photosensitizers.

The photosensitization ability of the pre-irradiated solutions (16 h pre-irradiation time) was compared to that of Aldrich HA, which are more photoactive than the humic substances usually found in natural waters (Zeng et al., 2002). To choose the HA concentrations at which to carry out the tests, it was decided to simulate the absorbance of the irradiated solutions (16 h pre-irradiation time) at the maximum emission wavelength of the lamp (365 nm). No spectral interference by TRP, TYR or 4PP is expected at this wavelength value. Therefore, pre-irradiated TYR and 4PP were compared with 10 mg L^{-1} HA, and pre-irradiated TRP with 20 mg L^{-1} HA (see Figure SM3 in SM).

In the case of pre-irradiated TRP, the photosensitized degradation of both TMP and FFA was comparable to that observed with 20 mg L^{-1} HA. Compared to 10 mg L^{-1} HA, pre-irradiated TYR induced similar photodegradation of FFA and faster photodegradation of TMP. In contrast, FFA transformation with pre-irradiated 4PP was slower than with 10 mg L^{-1} HA.

The ability of the phototransformation intermediates of TRP and TYR to act as photosensitizers suggests that they could also play a role in the photodegradation of TRP and TYR at relatively high irradiation times. Actually, it is well known that the two amino-acids undergo photosensitized transformation in the natural environment (Boreen et al., 2008; Remucal and McNeill, 2011).

Conclusions

Irradiation of TRP, TYR and 4PP yielded compounds with fluorescence properties similar to those of humic substances. Furthermore, the irradiated material developed considerable absorbance above 300 nm, which was also extended into the visible region. The irradiated solutions contained compounds that could act as photosensitizers, as shown by their ability to photoinduce the degradation of TMP (T* probe) and FFA ($^1O_2$ probe). Formation of particles upon irradiation of

TRP suggests that some extent of photochemical polymerization may be operational in that system. The photogenerated compounds had a number of properties (absorption, fluorescence, photoactivity) similar to humic substances.

The described photoprocesses could play a role in the presence of concentrated organic material undergoing degradation (including depolymerization/hydrolysis of biopolymers), such as plant or algal spoils (Mostofa et al., 2011). The ability of TRP, TYR and 4PP to absorb sunlight, which is confined to the UVB region (see Figures 1c, 2d and 3d) limits the environmental importance of their direct photolysis to clear and shallow water bodies. These conditions are met for instance in mountain lakes located above the treeline, which are also rich in aquagenic (protein-like) material and relatively poor in soil-derived humic substances (Sommaruga and Augustin, 2006; De Laurentiis et al., 2012). Under these conditions, photochemistry could be an additional and potentially important pathway for the formation of substances with humic properties.

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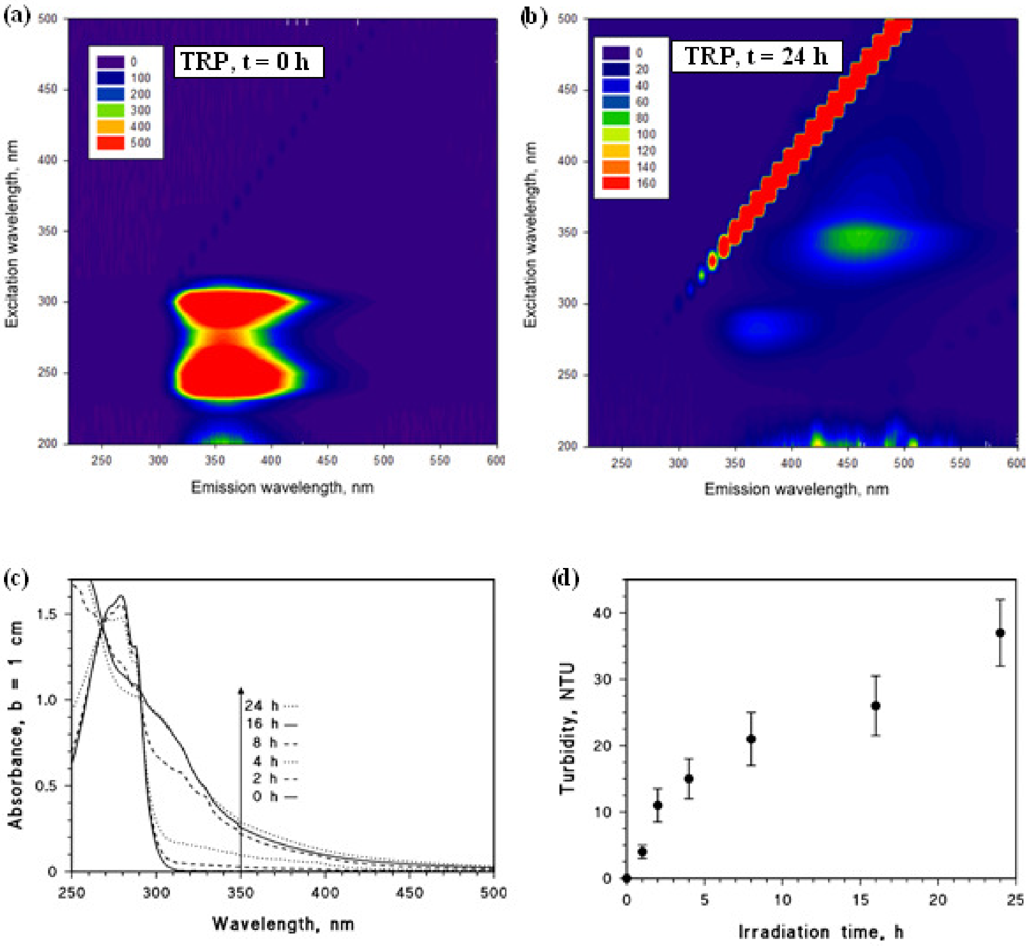

Figure 1. EEM fluorescence spectrum of 1 mM TRP: **(a)** before irradiation; **(b)** after 24 h irradiation (the linear feature is the Rayleigh scattering). **(c)** Absorption spectrum of TRP (initial concentration 1 mM) as a function of irradiation time (indicated as figure legend). **(d)** Time evolution of turbidity with irradiation (error bars represent ±σ of replicate runs). For EEMs, the intensity of the Raman signal of water at Ex/Em = 230/248 nm was 5.5±0.9 (arbitrary units) and the slit width was 5 nm on both excitation and emission.

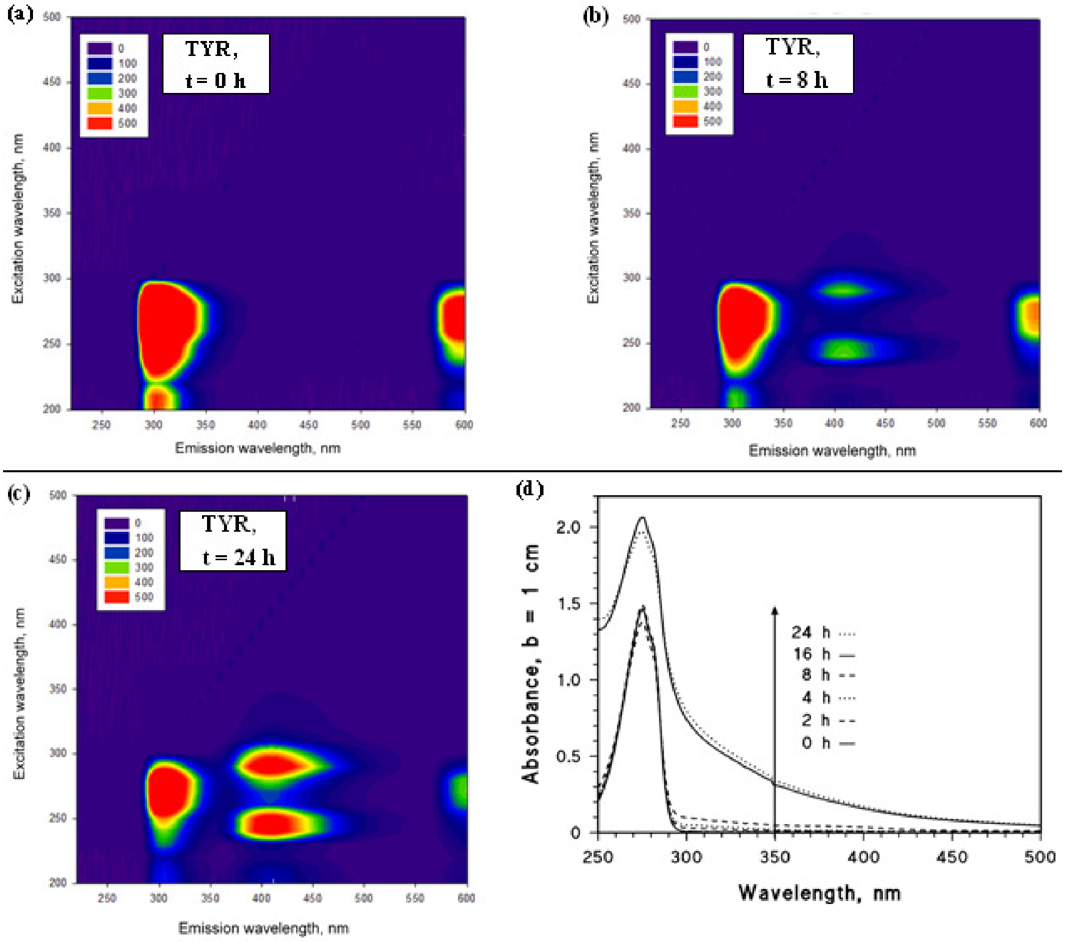

Figure 2. EEMs of TYR (initial concentration 1 mM) at different irradiation times: **(a)** 0 h, **(b)** 8 h, **(c)** 24 h. **(d)** Absorption spectrum of the TYR solution at different irradiation times. For EEMs, the intensity of the Raman signal of water at Ex/Em = 230/248 nm was 4.0±0.8 (arbitrary units) and the slit width was 5 nm on both excitation and emission.

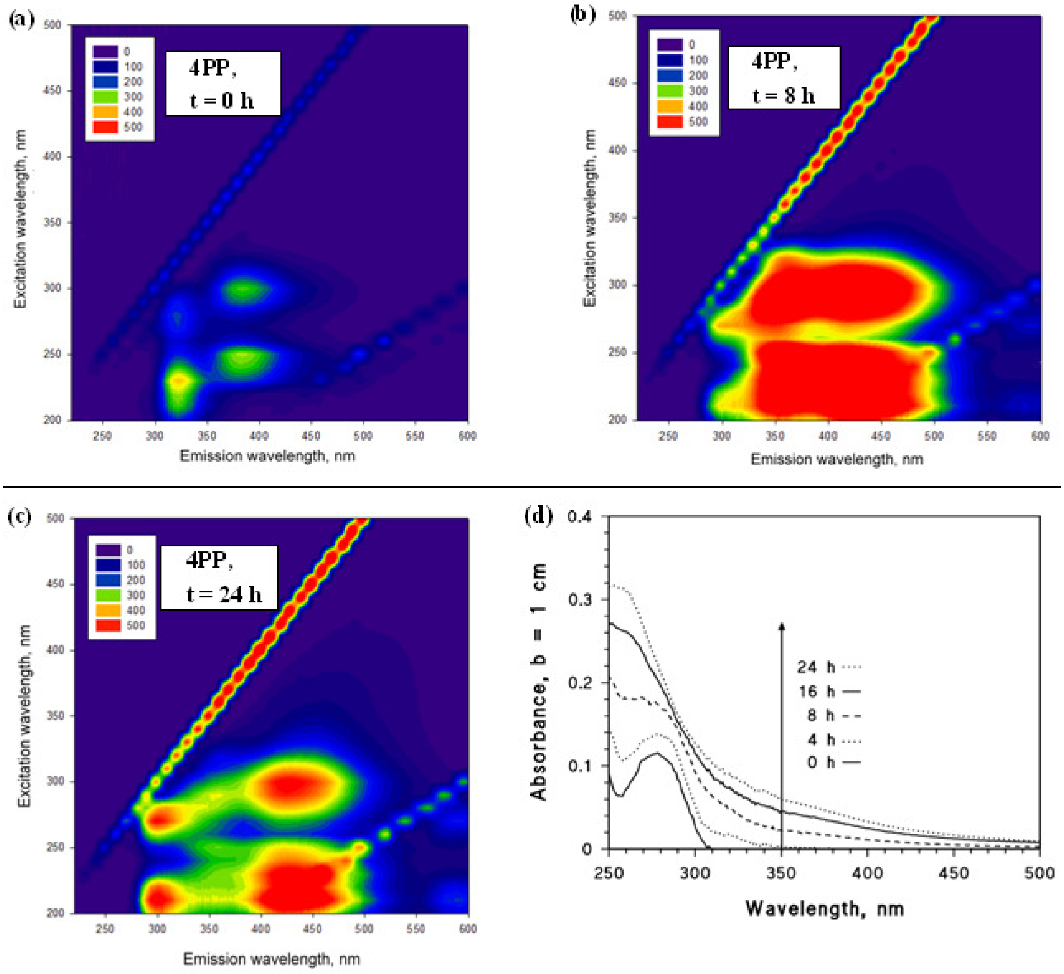

Figure 3. EEMs of 4PP (initial concentration 0.1 mM) at different irradiation times: **(a)** 0 h, **(b)** 8 h, **(c)** 24 h. **(d)** Absorption spectrum of the 4PP solution at different irradiation times. For EEMs, the intensity of the Raman signal of water at Ex/Em = 230/248 nm was 16.5±0.6 (arbitrary units) and the slit width was 10 nm on both excitation and emission.

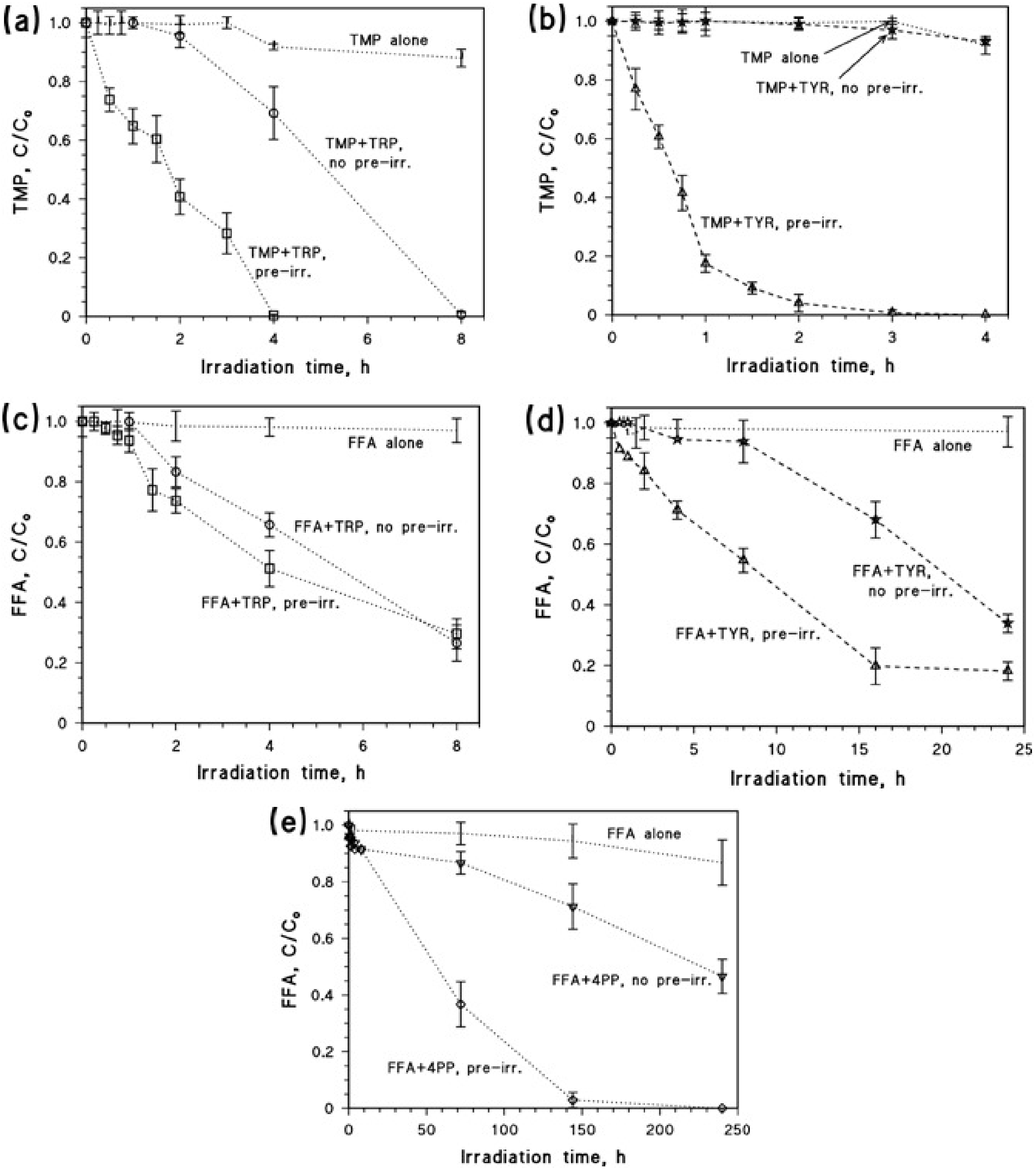

Figure 4. Phototransformation of TMP (0.1 mM initial concentration), sensitized by TRP (**a**) and TYR (**b**), with and without pre-irradiation for 16 h of 1 mM TRP and 1 mM TYR. Phototransformation of FFA (0.1 mM initial concentration), sensitized by TRP (**c**), TYR (**d**) and 4PP (**e**), with and without pre-irradiation for 16 h of 1 mM TRP, 1 mM TYR and 0.1 mM 4PP. The time evolution of TMP and FFA alone is also reported on the plots. Error bars represent ±σ of replicate experiments.