

# Photodegradation of fluorene in aqueous solution: Identification and biological activity testing of degradation products

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1	1Photodegradation of fluorene in aqueous solution: identification and
2	biological activity testing of degradation products
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9	HIGHLIGHTS
10	Degradation of fluorene under UV-Vis irradiation in water was investigated.
11	Twenty six photoproducts were characterized using GC-MS coupling.
12	Competing photodegradation pathways of fluorene under UV-Vis irradiation have been
13	suggested.
14	Some photoproducts of fluorene are biologically much more active than the parent compound.
15	
16	
17	ABSTRACT
18	Degradation of fluorene under UV-Vis irradiation in water was investigated and structural
19	elucidation of the main photoproducts was achieved using gas chromatography coupled with
20	mass spectrometry. Twenty-six photoproducts were structurally identified, mainly on the basis

21 of electron ionization mass spectra interpretation. The main generated transformation products

22 are hydroxy derivatives. Some secondary photoproducts including fluorenone, hydroxy

23 fluorenone, 2-biphenyl carboxylic acid, biphenylene, methanol fluorene congeners and hydroxy

24 fluorene dimers were also observed. A photodegradation pathway was suggested on the basis

25 of the chemical structures of photoproducts. Fluorene as well as its main photoproducts for

which chemical standards were commercially available were tested for their ability to elicit 26 27 cytotoxic, estrogenic and dioxin-like activity by using in vitro cell-based bioassays. None of 28 the tested compounds was cytotoxic at concentrations up to 100 µM. However, 2-29 hydroxyfluorene and 3-hydroxyfluorene exerted significant estrogenic and dioxin-like activity 30 on a concentration range of 3-30 µM, while fluorene and 9-hydroxyfluorene were weakly or 31 not active, respectively, in our assays. This supports the view that photodegradation processes 32 can generate by-products of higher toxicological concern than the parent compound and 33 strengthens the need to further identify transformation products in the aquatic environment.

Keywords: photolysis, photodegradation products, polycyclic hydrocarbons, fluorene, *in vitro* tests

36

#### 37 **1. Introduction**

The industrial and urban development which occurred in the second half of the 20<sup>th</sup> century 38 39 allowed the emergence of thousands of organic chemicals into aquatic environment without 40 prior study of their toxicity. Several decades later, a large number of these compounds have 41 shown negative impacts on human health and ecosystems even at very low concentrations. 42 Polycyclic Aromatic Hydrocarbons (PAHs) are among the most widespread pollutants in the environment. They are of special concern due to their negative characteristics, including 43 44 persistence, mobility in the environment and toxicity. The most environmental PAHs result from incomplete combustion of organic matter: forest fires, automobile exhaust, coal, oil 45 46 refining processes, etc.  $\begin{bmatrix} 1 & 2 & 3 \end{bmatrix}$  Many PAHs are toxic; their mainly adverse health effects are cancers in various tissues such as prostate, breast, pancreatic and cervical  $\begin{bmatrix} 4, 5, 6, 7 \end{bmatrix}$ , 47 cardiovascular diseases [<sup>8</sup>] and immunosuppression [<sup>9</sup>]. PAHs are also known to have endocrine 48 disrupting capabilities with consequent alteration of fertility in terrestrial and aquatic organisms 49 50 [<sup>10</sup>,<sup>11</sup>,<sup>12</sup>]. A number of PAHs are also found to bind the estrogen, androgen and aryl hydrocarbon receptors and either induce or inhibit the estrogen, antiandrogen and dioxin-like responses 51  $\begin{bmatrix}13, 14, 15\end{bmatrix}$ . For these reasons, PAHs have been listed as priority pollutants by both the US 52 53 Environmental Protection Agency (US EPA) and the European Union (EU). Despite a reduction 54 in PAHs emissions from fuel combustion, resulting from the substitution of coal by fossil fuels 55 since the 1960s and from the development of clean fuels and catalytic converters for diesel and gasoline engines in the recent years, the amounts of PAHs in aquatic environment remains 56 high.<sup>[16]</sup> PAHs can be introduced into the aquatic environment through different routes 57 including industrial and municipal wastewater, rainwater runoff, atmospheric deposition and 58 sediment- and air-water exchange [17, 18,16]. The concentration of PAHs in aquatic 59 environments varies widely depending on the nature of water (i.e. ground water versus surface 60 61 water) and the sampling location. In European surface water, the concentrations of PAHs were reported to range from 0.6 to 171.3 ng.L<sup>-1</sup> in rainwater [<sup>19</sup>,<sup>20</sup>], from 2 to 587 ng.L<sup>-1</sup> in river water 62  $[^{21}, ^{22}, 20]$ , from 5 to 1930 ng.L<sup>-1</sup> in seawater  $[^{23}, ^{24}]$ , from 1.4 to 5 ng.L<sup>-1</sup> in lake and reservoir 63 water [22,19] and from 4 to 1473 ng.L<sup>-1</sup> in wastewater [22,20]. In natural water environment, 64 65 the PAHs are eliminated mainly through processes of sorption to sediments, biodegradation and 66 photodegradation. Photodegradation remains the main route of PAHs degradation, especially 67 for the most recalcitrant ones. It has been reported that photolysis processes induced under

natural sunlight can be significantly accelerated by dissolved organic matters. In advanced 68 oxidation processes involving UV light for water and wastewater purification, the ultimate 69 objective is to achieve complete mineralization of organic pollutants to carbon dioxide, water, 70 71 or at least to produce small organic intermediates more soluble, readily biodegradable and thus less toxic than their parent compounds  $[2^{5}, 2^{6}, 2^{7}]$ . Numerous studies have been conducted on the 72 73 photodegradation of PAHs. In most of these studies, much interest has been focused on the 74 optimization of degradation conditions. The evaluation of the degradation efficiency only relies 75 on the disappearance of the initial pollutants. In this way, the kinetics of disappearance are 76 evaluated, as well as the monitoring of the mineralization rate achieved along the process. 77 Unfortunately, in the course of the degradation of these pollutants, some by-products potentially more toxic and sometimes even more stable than the parent compounds may be formed  $[^{28}, ^{29}]$ . 78 79 For example, studies carried out by Gala et al., Bertilsson et al., Shemer et al. and Woo et al. 80 have shown that exposure to UV irradiation may increase the toxicity of many PAHs to a variety of aquatic organisms [<sup>30</sup>,25,<sup>31</sup>,27]. However, very little is known about the nature of the 81 82 substances responsible for this increase in toxicity. In this regard, we were interested in the 83 identification of the phototransformation by-products of PAHs. In the present study, fluorene 84 (Flu) has been chosen as a model compound for several reasons. First, according to our knowledge, identification of photoproducts of this PAH in water has never been reported. 85 86 Second, fluorene is one of the US EPA and EU priority PAHs because it has water solubility 87 significantly higher than those of PAHs with larger molecular weights. Third, a previous study 88 has shown that 2-hydroxyfluorene, which could be a possible photodegradation product of fluorene in water, exhibits estrogenic activity [<sup>32</sup>]. The primary objectives of the present 89 90 research were the elucidation of the photoproducts chemical structures and that of the reaction 91 pathways leading to their formation during UV-Vis irradiation of fluorene in aqueous solution. 92 The kinetics of fluorene disappearance and those of appearance of some photoproducts were 93 examined. In vitro bioassays based on human MELN and fish PLHC-1 cell lines were carried 94 out to evaluate the estrogenic and dioxin-like potency of photoproducts for which chemical 95 standards were commercially available.

#### 97 **2. Materials and methods**

#### 98 2.1 Chemicals, reagents and sample preparation

99 Standards of fluorene (Flu,  $\geq$  99% purity), 2-hydroxyfluorene (2OH-Flu,  $\geq$  98% purity), 3-100 hydroxyfluorene (3OH-Flu,  $\geq$  98% purity), 9-hydroxyfluorene (9OH-Flu,  $\geq$  96% purity), were 101 purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). HPLC-grade methylene 102 chloride (DCM), acetonitrile (ACN), methanol (MeOH), pyridine, dimethylsulfoxide (DMSO) 103 and the silvlation reagent: N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% of 104 trimethylchlorosilane (TMCS) were also purchased from Sigma-Aldrich (France). Ultrapure 105 water used for photolysis experiments and SPE extraction was obtained by purification of 106 drinking water using a Millipore Milli-Q device (resistivity < 18 M $\Omega$ .cm; DOC < 0.1 mg.L<sup>-1</sup>; 107 Millipore, CA, USA). A stock solution of individual compounds: Flu, 2OH-Flu, 3OH-Flu and 9OH-Flu, each one at 10 µg.mL<sup>-1</sup>, was prepared in amber vials by dissolving the appropriate 108 109 amount of each chemical in anhydrous methylene chloride. It was used for the development of 110 the GC-MS analytical method. All the standard chemicals were prepared in DMSO for bioassay 111 testing.

#### 112 2.2 Irradiation device

113 Photolysis experiments were conducted in a cylindrical pyrex glass reactor (200 mm long x 25 114 mm internal diameter, effective reaction volume: 50 mL). This reactor is surmounted by two 115 tubes closed by valves allowing constant pressure (1 atm), constant supply of oxygen and 116 removal of the sample to be analysed. A UV lamp immersed in a cylindrical quartz water jacket 117 tube was placed in the middle of the reactor. The lamp used in our experiments was a high-118 pressure mercury vapour lamp (HPL-N 125W/542 E27 SG) manufactured from Philips-France 119 (Ivry sur Seine, France). It emits a polychromatic radiation with wavelengths ranging from 350 120 nm to 750 nm. Approximately 15% of the emitted light power is in the range 350-400 nm with 121 a further 85% between 400 and 750 nm. The luminous flux emitted from this lamp was reported 122 by the manufacturer to be 6200 lm. Prior to photolysis experiments, the lamp was allowed 123 warming up for 10 min to achieve equilibrium intensity. Homogeneity of the reaction medium 124 was carried out using a magnetic stirrer. During irradiation period, the reaction temperature was 125 maintained between 23 and 25 °C by adjusting the flow rate of cooling water. The reactor was 126 wrapped in an aluminium foil to optimize UV-visible irradiation of the solution and to avoid 127 emission outside the reactor. The control samples were continuously stirred and kept in the dark

- 128 under similar experimental conditions as the irradiated solution. For photolysis experiments, a
- 129 fluorene solution at 2 mg.L<sup>-1</sup> was prepared by dissolving 1 mg of fluorene standard in 500 mL
- 130 of Milli-Q water. It was prepared just prior to use to reduce any possible biological, physical
- 131 and/or chemical transformation due to prolonged storage of fluorene.

#### 132 2.3 Extraction of residual fluorene and its photoproducts

133 Solid phase extraction (SPE) has been used to extract and enrich fluorene and its generated 134 photoproducts from water solutions. The protocol employed derives from methods described in our previous studies.<sup>[33]</sup> Briefly, Oasis<sup>®</sup> HLB cartridges (200 mg x 6 mL, Waters, France) 135 were placed into a SPE vacuum manifold (Supelco, Bellefonte, PA, USA), conditioned with 3 136 137 mL of ACN, then 3 mL of distilled water, and equilibrated with 3 mL of a mixture of 138 methanol/water (5:95, v/v). Sample volumes of 50 mL were loaded on the cartridges, and the 139 aqueous solution was eluted as waste at a rate of approximately 2.5 mL.min<sup>-1</sup>. The retained 140 compounds (fluorene and its photodegradation products) were eluted by loading four times 2.5 141 mL of methanol. The organic extracts were evaporated to complete dryness under a gentle 142 nitrogen stream, and dry residues were resuspended in 0.5 mL of DCM, sonicated and then 143 divided into two equal parts. The first one was completely dried under a gently stream of 144 nitrogen and reconstituted into 50 µL of DMSO for bioassay analysis. The second aliquot was 145 subdivided again into two equal parts; the first one was directly analysed by GC/MS, while the 146 second was derivatized with BSTFA according to the method already described by Kinani et 147 al. prior to GC/MS analysis [33].

#### 148 2.4 GC-MS analysis

149 Residual fluorene and generated photoproducts (derivatized and non-derivatized extracts) were 150 analysed using a "450-GC" gas chromatograph equipped with a "CP-8400" autosampler and 151 coupled to a "240-MS" ion trap mass spectrometer (Varian, Les Ulis - France). The chromatographic separation was performed on a 60 m "Factor four VF-10-MS" (10% phenyl, 152 153 90% methylpolysiloxane) capillary column (internal diameter: 0.25 mm, film thickness: 0.25 154 μm) from Varian. All experiments were performed by automatically injecting 1.0 μL of sample in the splitless mode at a rate of 50  $\mu$ L.s<sup>-1</sup>. High purity (99.999%) helium was used as the carrier 155 gas at a constant flow of 1.4 mL.min<sup>-1</sup> hold by electronic pressure control. The injector 156 157 temperature was set at 280 °C. The split valve opened after 2.0 min, with a split ratio of 40/100. The capillary column was ramped from an initial temperature of 50 °C, held for 0.5 min, at 10 158 °C.min<sup>-1</sup> up to 320 °C where it was held for 7.5 min. The total duration of GC analysis was 35.0 159

160 min. The manifold, ion trap electrodes and transfer line temperatures were set at 120 °C, 220 161 °C and 300 °C, respectively. The mass spectrometer was operated in internal ionization source and the acquisition was performed in full scan mode. Both chemical ionization (CI) and electron 162 163 ionization (EI) modes were used for a reliable structural elucidation of photodegradation 164 products of fluorene. The instrument was automatically tuned using the ions resulting from 165 electron ionization of perfluorotributylamine. In EI, the ionization energy was 70 eV and the 166 filament current was 10 µA. Spectra were recorded using the automatic gain control (AGC) 167 function with a target value of 20.000. CI was performed using methanol as reagent gas. 168 Automatic reaction control (ARC) parameters were 100 µs for the maximum ionization time, 169 and 2000 for the target value. In all experiments, the multiplier voltage was set to 1850 V ( $10^5$ 170 gain) by automatic tuning. Ions were scanned over a 50-600 m/z range, with a scan rate of 1 171 scan.s<sup>-1</sup>. For the experiments carried out with fluorene at 0.1 ppm in raw lake water (see section 172 3.3), GC-MS analyses were performed with the same chromatographic conditions, using 173 selected ion storage in the EI mode. The two major ions (see Table 1) were selected for each 174 compound. The sensitivity was also enhanced by increasing the filament current and multiplier 175 voltage values up to 50  $\mu$ A and 2000 V, respectively.

176

#### 177 2.5 In vitro bioassays

#### 178 2.5.1 Estrogenic activity

179 The MELN reporter cell line has been used in order to assess the estrogenic activity of 180 commercially available photoproducts of fluorene. This cell line was obtained by stable 181 transfection of MCF-7 human breast cancer cells by an ERE-bGlob-Luc-SVNeo plasmid [<sup>34</sup>]. 182 The cells were routinely cultured in phenol red containing Dulbecco's Modified Eagle's 183 Medium (DMEM), supplemented with 5% foetal calf serum (FCS), 1% nonessential amino 184 acids and penicillin/streptomycin (50 U/mL each) in a 5% CO<sub>2</sub> humidified atmosphere at 37 185 °C. For experiments, cells were left to incubate for 2 days in phenol red free DMEM 186 supplemented with 3% dextran charcoal coated-FCS (DCC medium) before seeded in white 187 opaque 96-wells culture plates at a density of 50,000 cells per well. Serial dilutions of reference 188 or test chemicals were added in triplicates 24 h later and then left to incubate for 16 h. In all 189 assays, DMSO in the culture medium was always at 0.1% v/v, including in cell controls. At this 190 concentration, no effect on cell viability or luciferase activity was observed. After cell exposure,

the medium was removed and replaced by 50  $\mu$ L of DCC medium containing 0.3 mM of Dluciferin and the luminescence signal was measured in living cells for 2 s per well with a microtiter plate luminometer (μBeta, Wallac). Relative luminescence units (RLU) were converted to relative response units expressed as percent of maximal luciferase activity induced by the positive control (17β-Estradiol (E2) at 10 nM).

196 2.5.2 Dioxin-like activity

197 The dioxin-like activity was monitored by using the fish hepatic PLHC-1 cell line (ATCC, 198 #CRL-2406) as described by Louiz et al. [15]. The cells were routinely grown at 30 °C in E-199 MEM culture media supplemented with 10% FCS and 1% antibiotics in a 5% CO<sub>2</sub> humidified 200 atmosphere. For experiments, cells were seeded in 96-well plates at a density of approximately 201 50,000 cells per well. After 24 h of incubation, cells were exposed to test chemicals or sample 202 extracts for 4 h incubation period. Then, plates were processed for 7-ethoxyresorufin-Odeethylase (EROD) activity in intact cells, as previously described.<sup>[15]</sup> Results were expressed 203 204 as percent of EROD activity induced by the positive control (tetrachlorodibenzodioxin (TCDD) 205 at 0.3 nM).

#### 206 2.5.3 Cytotoxicity

The ability of test chemicals to alter cell viability after a 24 h-exposure was assessed in the PLHC-1 cell line by using the MTT assay [<sup>35</sup>], as previously described [<sup>36</sup>]. The results were expressed as relative to DMSO treated control cells.

210 **3. Results and discussion** 

#### 211 3.1 Identification of fluorene photoproducts by GC-MS

212 The comparison between chromatograms of irradiated and non-irradiated solutions of fluorene 213 revealed the presence of twenty six photoproducts. The identified compounds are summarized 214 in Table 1. Among the twenty six compounds, those which were commercially available, such 215 as 9-fluorenone, 2-hydroxyfluorene (2OH-Flu), 3-hydroxyfluorene (3OH-Flu) and 9-216 hydroxyfluorene (9OH-Flu), were quickly and unambiguously identified by comparing 217 retention times and mass spectra with those of reference compounds. The other compounds 218 were identified on the basis on their EI and CI mass spectra interpretation. BSTFA 219 derivatization was helpful since it permitted to establish the number of exchangeable hydrogen 220 atoms for each photolysis product.

221

#### Table 1

222 Six products with M = 182 were detected with close retention times (ranging from 25.7 to 29.3) 223 min.) and identical mass spectra. Compared to fluorene mass weight, those compounds are 224 shifted by + 16 amu, suggesting addition of an oxygen atom. In derivatized extracts, the 225 corresponding molecular and pseudo molecular ions are shifted by + 72 amu (one hydrogen 226 atom replaced by Si(CH<sub>3</sub>)<sub>3</sub>) in EI and CI, leading to the conclusion that these compounds are 227 hydroxylated derivatives of fluorene (OH-Flu). Owing to the analysis of standard solutions, the 228 chromatographic peaks at 26.4, 26.5 and 29.1 were unambiguously attributed to, 9OH-Flu, 229 20H-Flu and 30H-Flu, respectively, and both other peaks were logically assumed to 230 correspond to 1-hydroxyfluorene (1OH-Flu) and 4-hydroxyfluorene (4OH-Flu).

231 A product is detected at 26.5 min. Its main ions in EI are m/z 180 and m/z 152, corresponding 232 to the molecular ion M<sup>+</sup> and to [M-CO]<sup>+</sup>, respectively. The EI and CI mass spectra and the 233 retention time of this product were consistent with those observed for the authentic standard of 234 9-fluorenone. This result suggests that a CH<sub>2</sub> group of fluorene has been oxidized into CO, as 235 depicted in Figure 1. A similar oxidation was reported by Rivas et al. who proposed a detailed 236 mechanism of oxidation of fluorene by hydroxyl radicals. 9-fluorenone, 9-fluorenol and 237 dibenzofuran were formed during the first step of the reaction; further oxidation of these 238 products proceeded via hydroxylation and cleavage of the fluorene ring  $[^{37}]$ .

239

#### Figure 1

240 Nine products with M = 196 were detected. They were divided into two groups on the basis of 241 their EI mass spectra. The four first eluted compounds (27.8, 29.8, 31.8 and 31.9 minutes) correspond to isomers of hydroxyfluorenone (OH-Flu-one): EI mass spectra of some isomers 242 of OH-Flu-one are displayed in the NIST mass spectra database  $[^{38}]$ . These spectra are identical 243 244 so that the different isomers could not be determined owing to the database. It is to be noted 245 that 2-benzyl-1,4-benzoquinone provides the same EI mass spectrum [38]. Its formation has 246 been considered since 1.4-naphtoquinone was detected as a photolysis product of naphthalene in several previous studies  $[^{39}, ^{40}]$ . In the present work, BSTFA derivatization of the compounds 247 of interest led to the addition of one trimethylsilyl (TMS) group to each compound (M = 268), 248 249 showing the presence of one exchangeable hydrogen atom, in agreement with OH-Flu-one 250 isomers. The five other products with M = 196 (RT = 30.1, 30.2, 30.3, 30.4 and 30.5 minutes) 251 displayed EI mass spectra with ions at m/z 178, m/z 166 and m/z 165, resulting from

252 eliminations of H<sub>2</sub>O, H<sub>2</sub>C=O and CH<sub>3</sub>O, respectively. After many attempts to find likely 253 chemical structures corresponding to  $C_{13}H_8O_2$ , we finally concluded that the only possibilities 254 corresponding to these EI mass spectra were structures of fluorene methanol (MeOH-Flu), in 255 agreement with the solution suggested by the NIST database. Since photolysis had been 256 performed in pure water, the only explanation for the addition of a carbon atom to Flu was 257 through the formation of a dimer species, for which a mechanism of formation is proposed in 258 Figure 2. The first eluted isomer (RT = 30.1 min.) was determinated to be 9MeOH-Flu. As a 259 matter of fact, its EI mass spectrum displays a m/z 178 ion (loss of H<sub>2</sub>O from M<sup>+</sup>) in greater 260 abundance than for other isomers (82% vs 24%). The supporting information file SI-1 shows 261 the dissociation pathways proposed to interpret the EI mass spectrum of 9MeOH-Flu. The easier 262 loss of water from 9MeOH-Flu than for other MeOH-Flu isomers is easily rationalized on the 263 basis of the  $\alpha,\beta$  concerted elimination of H<sub>2</sub>O involving a hydrogen atom of the sp3 carbon, 264 which cannot occur for other isomers.

265

#### Figure 2

266 Four compounds with M = 348 were detected. The first one elutes at 37.1 min. while the three 267 other ones are coeluted between 38.4 and 38.6 minutes. All are assumed to be isomers of 268 hydroxylated dimers of fluorene. Their formation is discussed below. Five products with M =269 198 were detected, one eluted at 28.5 min. and three between 31.2 and 31.6 min. For the first 270 one, the EI mass spectra obtained from non-derivatized extracts showed major fragment ions at 271 m/z 181 ([M-OH]<sup>+</sup>) and m/z 153 ([M-COOH]<sup>+</sup>), issued from characteristic losses for 272 compounds containing a carboxylic group. In chemical ionization, this molecule exhibits a base 273 peak at m/z 181 consistent with water elimination. The EI mass spectrum obtained from 274 derivatized extracts exhibits a molecular ion at m/z 270 and a MH<sup>+</sup> pseudo molecular ion at m/z 275 271 issued from auto protonation, a phenomenon classically observed in ion trap mass 276 spectrometers. The major fragment ions are observed at m/z 181 [M-OSi(CH<sub>3</sub>)<sub>3</sub>]<sup>+</sup>, m/z 153 277  $[MH-HOSi(CH_3)_3-CO]^+$  and m/z 73  $[Si(CH_3)_3]^+$ . The main ions of the CI mass spectrum are 278 m/z 271 (MH<sup>+</sup>) and m/z 181 corresponding to [MH-HOSi(CH<sub>3</sub>)<sub>3</sub>]<sup>+</sup>. Based on these mass 279 fragmentation patterns and on the results of MS databases researches, this compound was 280 identified as 2-biphenylcarboxylic acid (BPCA). Two mechanisms can be advanced to explain 281 the formation of this compound. The first one involves direct oxidation of fluorene while the 282 second one involves oxidation of 9-fluorenone, which has been previously identified as a 283 photoproduct of fluorene. The four other compounds with M = 198 may correspond either to

dihydroxyfluorene congeners, either to structures such as those displayed in the supporting information file SI-2, which are dialdehydes analoguous to those reported by several authors in their studies devoted to photocatalysis of naphthalene  $[39,^{41},^{42}]$ . The molecular weight of these three compounds is shifted to M = 342 after BSTFA derivatization, thus indicating that two exchangeable hydrogen atoms were replaced by a TMS group and allowing to conclude in favor of dihydroxyfluorene congeners.

#### 290 *3-2 Mechanistic approach of the photodegradation of fluorene*

291 As demonstrated in what follows, the sp3 carbon atom of fluorene leads to a particular behavior 292 under UV-Vis irradiation in comparison with other PAHs. As a matter of fact, one of the main 293 mechanisms generally proposed to rationalize oxidation of PAHs under UV photolysis begins 294 by the formation of a radical molecular cation from the irradiated molecule through electron removing  $[^{43}]$ . The solvated electron released by the molecule is assumed to be transferred to 295 296 dissolved oxygen yielding a superoxide anion which may, in turn, react with water to provide 297  $H_2O_2$  (see the upper left side of Figure 1). UV photolysis of  $H_2O_2$  yields hydroxy radicals [<sup>44</sup>]. 298 The mechanism of electron removing in the aqueous phase may be compared with that of 299 electron ionization in the gas phase, which is widely used in mass spectrometry.  $\Pi$  electrons are 300 known to be much more easier to abstract than  $\sigma$  electrons. That is why ionization is expected 301 to occur on one of the aromatic rings, as observed in mass spectrometry. In this study, ionization 302 on one of both aromatic rings can easily account for the formation of 1-, 2-, 3- and 4-303 hydroxyfluorene (Figure 1, left pathway) but cannot lead to the formation of 9-hydxoxyfluorene 304 (9OH-Flu) and fluorenone (Flu-one). The detection of large amounts of 9OH-Flu and Flu-one 305 suggests a mechanism, competing with electron removing, consisting in the direct cleavage of 306 one of the C-H bonds in which the sp3 carbon atom is involved, as suggested in the center 307 pathway of Figure 1. In the present case, the elimination of H<sup>·</sup> from this carbon atom leads to a 308 fully conjugated radical which is particularly stable. A direct reaction between this radical and 309 a HO<sup>.</sup> radical leads to 9OH-Flu but also to other hydroxyfluorene isomers because of mesomeric 310 effects. We suggest that fluorenone results from dehydrogenation of fluorenol as the "kinetic" 311 approach (see below) shows a good correlation between the increase in Flu-one concentration 312 and the decrease in 9OH-Flu concentration. Furthermore, we observed when analyzing standard 313 samples that 9OH-Flu is spontaneously converted into Flu-one in a few hours, at ambient 314 temperature, even sheltered from light. It is to be noted that in a previous study, Dabrowska et 315 al. established that the formation of fluorenone precedes that of fluorenol when irradiating

316 fluorene not in water but in methylene chloride and hexane [<sup>45</sup>]. Sabaté et al. reached the same 317 conclusion performing irradiation in an ethanol/water mixture  $[^{46}]$ . Both studies focused on the 318 stability of fluorene under photolysis and only Flu, OH-Flu and Flu-one were detected owing 319 to a UV-vis spectrophotometer; other by-products were not detected or not considered. The 320 formation of BPCA is assumed to result from oxidation of Flu-one through addition of a 321 hydroxy radical. Since dihydroxyfluorene (diOH-Flu) isomers could not be identified, their 322 formation has been assumed to result from a hydroxy radical addition onto 9OH-Flu and/or 323 onto another OH-Flu isomer. Finally, the formation of hydroxy-9-fluorenone may result either 324 from dehydrogenation of hydroxy-9-fluorenol, via a mechanism analogous to that involved in 325 the reduction of 9OH-Flu into Flu-one, either from attack of a hydroxy radical onto the later.

We proposed the mechanism displayed in Figure 2 to rationalize the formation of 9MeOH-Flu through a dimer intermediate. The formation of other MeOH-Flu isomers with the CH<sub>2</sub>OH group bound to an aromatic ring in 1, 2, 3 or 4 position can be explained with analogous mechanisms considering a dimer with a C-C bond between the sp3 carbon atom and a carbon atom of an aromatic ring (Reaction pathways are suggested in the supplementary information file SI-3). The detection of biphenylene (M = 152) at 18.0 min. is in agreement with the suggested mechanism.

333 With the aim to establish how photolysis products of Flu are related to each other and if the 334 evolutions of relative amounts are in agreement with the proposed reaction pathways, we 335 followed relative concentrations as a function of the irradiating time up to 120 minutes. A "real" 336 kinetic study was not the purpose of its work; it would have required possessing standard 337 compounds for all the photolysis products and would have been very complicated to achieve 338 since several photolysis pathways are undoubtedly in competition. Given the unexpected 339 evolution of the fluorene concentration as a function of the irradiation time, kinetics 340 experiments were carried out three times and provided results with a good repeatability (RSD 341 < 15%)". Figure 3 displays the evolution of the relative amounts of fluorene and its 342 photoproducts. Since concentrations of isomeric compounds undergo the same evolution, sums 343 of amounts were plotted for much clarity. It is to be noted that the amounts reported in figure 3 344 correspond to the areas of chromatographic peak integrated on the total ionic current (TIC) in 345 the EI mode and normalized to the initial TIC value which corresponds to the fluorene peak 346 area at t<sub>0</sub>. Considering the likely differences in response factors between compounds, the 347 relative amounts plotted allow the monitoring of a given photoproduct but they cannot be used 348 to compare concentrations of different photoproducts.

349 The concentration of Flu decreases by a factor four during the first fifteen minutes of irradiation, 350 then increases until one hour of irradiation and decreases again during the following hour. 351 Figure 3 shows evidence that the fast decrease in Flu concentration corresponds to dimers 352 formation. Dimers partially dissociate in turn to give biphenylene and MeOH-Flu isomers, 353 which are assumed to eliminate 'CH<sub>2</sub>OH radicals under irradiation to provide fluorene again 354 (Figure 2), thus explaining the increasing relative amount of Flu after the first fifteen minutes 355 of irradiation. In competition with this reaction pathway, the mechanisms suggested in Figure 356 1 appear to be slower. The kinetics features are in good agreement with the proposed 357 mechanisms since the concentrations in Flu-one and BCPA increase as that in 9OH-Flu 358 decreases. Amounts of diOH-Flu isomers increase then slowly decrease as those of OH-Flu 359 isomers increase.

360

#### Figure 3

#### 361 *3.3 Irradiation in more realistic conditions*

362 The above experiments were performed with a relatively high concentration of fluorene to allow 363 fullscan detection in GC-MS and thus characterization of photoproducts. To get closer of 364 natural conditions, additional experiments were carried out with organic matter containing water taken from Lake Palaiseau (France) spiked with fluorene at 100 µg.L<sup>-1</sup>. Water evaporation 365 366 under mild conditions led to a dry matter residue of 230 mg. L<sup>-1</sup>. Experiments were conducted 367 with irradiation times of 60 and 120 min. The sample preparation process was almost the same 368 as the one described in section 2.3; a filtration step on a PTFE membrane (diam. 13 mm, pore 369 size: 0.45 µm, Sigma-Aldrich, France) has only been added to remove suspended matter prior 370 to solid phase extraction. GC-MS analyses were performed with the chromatographic 371 conditions previously described but using selected ion storage in the EI mode. The two major 372 ions (see Table 1) were selected for each compound; the method sensitivity was enhanced by 373 increasing both filament current and electron multiplier voltage (see section 3.2). For both 374 irradiation times, the GC-MS chromatograms showed the photoproducts characterized earlier 375 but those at M = 348, which were assumed to result from dimerization and hydroxylation of 376 fluorene and those at M = 196 corresponding to methanol-fluorene congeners. The 377 disappearance of dimerized species using a solution at 0.1 ppm may be easily explained as the 378 probability of reaction between two molecules decreases when the concentration decreases. 379 Non-detection of methanol-fluorene congeners is logical as well, given that their formation 380 requires a first dimerization step (see the mechanisms depicted in figure 1 and supporting

information file 3). Additional experiments were performed in the fullscan mode, with the aim to possibly detect photoproducts issued from photo-induced reactions between fluorene and some components of dissolved organic matter. None was detected, either because such reactions do not occur or, more likely, because detection in the fullscan mode provides too high detection thresholds in comparison with the expected concentration ranges for such products.

386

#### 387 *3.4 In vitro activity of hydroxyfluorenes*

388 Amongst all the identified degradation products, 20H-Flu, 30H-Flu and 90H-Flu were 389 commercially available as chemical standards and could thus be tested for their biological 390 activity using *in vitro* cell-based assays. While none of the test compounds was found to alter 391 cell viability at concentrations up to 100 µM (data not shown), two of them, i.e. 2OH-Flu and 392 3OH-Flu, were able to activate the estrogen (ER) and aryl hydrocarbon (AhR) receptors. As 393 shown in Figure 4, 20H-Flu and 30H-Flu exerted a significant estrogenic activity in the MELN 394 cell line, yielding up 50% and 100% of luciferase induction, respectively. For both chemicals, 395 the EC50 was found to be 10 µM. Conversely, the parental compound fluorene was only weakly 396 estrogenic (weak effect at > 30  $\mu$ M) while 9OH-Flu was not active at the test concentrations. 397 The *in vitro* estrogenic activity of 2OH-Flu has been previously reported in yeast-based assays 398  $[^{47}, ^{48}]$ , we confirm here its activity in a mammalian cell-based assay and report for the first time 399 the estrogenic potency of 3OH-Flu. In addition, the position of the hydroxyl group on the 400 molecule has a strong influence on the biological activity. Both 3OH-Flu and 2OH-Flu have a 401 phenol ring in their structure which confers structural similarity with the endogenous hormone 402 17ß-estradiol and thus contributes to their ability to bind to and activate the ER [48]. 403 Conversely, 90H-Flu, which has no phenolic group in its structure, was unable to activate the 404 ER in our assay.

405

#### Figure 4

406 Interestingly, 20H-Flu and 30H-Flu were also potent inducers of EROD activity in the PLHC-407 1 cell line (Figure 5), with EC50s around 10 and 3  $\mu$ M, respectively. This induction was marked 408 after 4 hours of cell exposure but it was strongly reduced after 24 h, only a weak induction (i.e. 409 10% that of the positive control) remaining at that time point. Similar patterns of transient 410 EROD induction were previously reported with weakly persistent AhR agonists such as various

PAHs, which suggested that the compounds were metabolized by cellular systems [<sup>49</sup>,15].
Fluorene and 9OH-Flu were found weakly or non-active in this bioassay.

413

#### Figure 5

414 Overall, our results indicate that Flu photolysis could generate hydroxylated degradation 415 products that are biologically much more active than is the parent compound. Further 416 experiments assessing the biological activity of photolyzed aqueous solutions of fluorene will 417 be necessary to characterize the whole toxicity of the mixture of photoproducts and thus to 418 provide a more comprehensive identification of active fluorene by-products. For instance, the 419 use of effect-directed analysis approach (EDA) [<sup>50</sup>], e.g. based on HPLC fractionation of the chemical mixture and chemical identification of bioactive fractions could nicely provide new 420 421 information on active photoproducts.<sup>51</sup> Such approach has already proven useful to identify phototransformation products of key emerging aquatic pollutants such as diclofenac [<sup>52</sup>] or 422 423 estrone [<sup>53</sup>]. Identifying biologically active oxygenated PAHs using EDA would doubtless 424 provide crucial basic knowledge that is still needed, for instance, to better interpret estrogenic 425 and dioxin-like activities of PAHs-associated fractions in environmental complex samples.

426

#### 427 **4. Conclusion**

428 Studies aiming to identify transformation products of pollutants are necessary to understand 429 their behavior and impact on the environment, as well as their effects on human health. In the present work, the photodegradation of fluorene (Flu) under UV-Vis irradiation in water was 430 431 investigated. The complete mineralization of Flu was not observed under our conditions. Using 432 gas chromatography coupled with mass spectrometry, twenty-six photoproducts were 433 characterized, mainly on the basis of electron and chemical ionization mass spectra 434 interpretation. The main generated transformation products are monohydroxy derivatives. 435 Some secondary photoproducts were also observed: fluorenone, hydroxy fluorenone, 2-436 biphenyl carboxylic acid, biphenylene, methanol fluorene congeners and hydroxy fluorene 437 dimers. Structural elucidation of photoproducts led to suggest photodegradation pathways.

Fluorene and the main photoproducts for which chemical standards were commercially available were tested for their ability to elicit cytotoxic, estrogenic and dioxin-like activity by using *in vitro* cell-based bioassays. None of the tested compounds was cytotoxic at

- 441 concentrations up to 100 µM. However, 2-hydroxyfluorene and 3-hydroxyfluorene exerted
- 442 significant estrogenic and dioxin-like activity on a concentration range of 3-30  $\mu$ M. This study
- 443 demonstrates that UV-Vis irradiation of fluorene generates hydroxylated photoproducts that are
- biologically much more active than is the parent compound and strengthens the need to consider
- 445 transformation products and not only identified pollutants in the environment.
- 446

447

### 448 **Figure captions**

- 449 Figure 1. Reaction pathways proposed for the oxidation of fluorene under UV-Vis irradiation
- 450 in water. BPCA: Biphenyl carboxylic acid.



- 452 Figure 2. Reaction pathways proposed for the formation of OH-diFlu dimers and 9MeOH-Flu
- 453 under UV-Vis irradiation in water
- 454 Figure 2. The relative amounts reported correspond to the areas of chromatographic peak
- 455 integrated on the total ionic current (TIC) in the EI mode and normalized to the initial TIC
- 456 value which corresponds to the fluorene peak area at  $t_0$ .
- 457





HO.



462 Figure 3. Relative amounts of fluorene photoproducts as a function of the irradiation time.







Figure 4. *In vitro* estrogenic activity of Flu, 2OH-Flu, 3OH-Flu and 9OH-Flu in the MELN
reporter cell line. Results are expressed as % of luciferase induction relative to the positive
control estradiol (E2, 10 nM)



470

471 Figure 5. *In vitro* dioxin-like activity of Flu, 2OH-Flu, 3OH-Flu and 9OH-Flu in the PLHC-1

472 cell line after cell exposure for a) 4 h and b) 24 h. Results are expressed as % of EROD activity

473 induction relative to the positive control dioxin (TCDD, 0.3 nM)





MW	Rt	Main ions in mass spectra <sup>a</sup>			Identified compounds	
	(min.)	EI	CI	CI (DC) <sup>b</sup>	Structure	Name

152	18.0	152 (100), 150 (90), 122 (45), 133 (27), 91 (27)	nd <sup>c</sup>	nd		Biphenylene
180	26.5	180 (100), 152 (59), 151 (19)	181 (100)	nd		9-Fluorenone
182	26.4	182 (100), 181 (79), 165 (23), 153 (34),152 (43)	183 (100)	255 (100)	OH D	9-Fluorenol
182	25.7 28.7 28.9 29.1 29.3	182 (100),181 (49), 165 (9), 164 (18), 153 (21), 152 (36), 151 (11)	183 (100)	255 (100)		Hydroxy-fluorene congeners
196	27.8 29.8 31.8 31,9	196 (100), 168 (98), 140 (30), 139 (71), 117 (17), 91 (17)	197 (100)	269 (100)	O OH	Hydroxy-9- fluorenone congeners
196	30.1	196 (66), 178 (82), 166 (58), 165 (100), 152 (16)	197 (100)	269 (100)	HO	9-Methanol- fluorene

196	30.2 30.3 30.4 30.5	196 (55), 178 (24), 166 (55), 165 (100), 152 (15)	197 (100)	269 (100)	ОН	Methanol-fluorene congeners
198	<ul><li>31.2</li><li>31.3</li><li>31.4</li><li>31.6</li></ul>	198 (100), 197 (78), 181 (69), 162 (15), 153 (42), 152 (45), 141 (25), 139 (22) 115 (36)	199 (100)	343 (100)	OH OH OH	Hydroxy-9-fluorenol
198	28.5	198 (55), 197 (41), 181 (100), 169 (13), 153 (19), 152 (18), 141 (26), 139 (12), 115 (26)	199 (100)	271 (100)	OH OH	2-Biphenyl carboxylic acid
348	37.1	348 (33), 174 (100)	349 (100)	421 (100)	ОН	Hydroxy fluorene dimer
348	38.4 38.5 38.6	348 (62), 174 (100)	349 (100)	421 (100)	ОН	Hydroxy fluorene dimer isomers

480 <sup>a</sup> relative abundances (%) are given in parentheses - <sup>b</sup> DC: Derivatized compound - <sup>c</sup> nd: non
481 detected

#### 483

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