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

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Said Kinani[a], Yasmine Souissi[a], Aziz Kinani[a], Zvetlana Vujović[a], Sélim Aït-Aïssa[b] and Stéphane Bouchonnet[a,*]

[a] Laboratoire de Chimie Moléculaire - École Polytechnique - 91128 Palaiseau, France

[b] Unité Écotoxicologie in vitro et in vivo, Institut National de l'Environnement Industriel et des Risques (INERIS), BP 2, F-60550 Verneuil en Halatte, France

* Corresponding author, e-mail: stephane.bouchonnet@polytechnique.edu

HIGHLIGHTS

Degradation of fluorene under UV-Vis irradiation in water was investigated.

Twenty six photoproducts were characterized using GC-MS coupling.

Competing photodegradation pathways of fluorene under UV-Vis irradiation have been suggested.

Some photoproducts of fluorene are biologically much more active than the parent compound.

ABSTRACT

Degradation of fluorene under UV-Vis irradiation in water was investigated and structural elucidation of the main photoproducts was achieved using gas chromatography coupled with mass spectrometry. Twenty-six photoproducts were structurally identified, mainly on the basis of electron ionization mass spectra interpretation. The main generated transformation products are hydroxy derivatives. Some secondary photoproducts including fluorenone, hydroxy fluorenone, 2-biphenyl carboxylic acid, biphenylene, methanol fluorene congeners and hydroxy fluorene dimers were also observed. A photodegradation pathway was suggested on the basis 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 cytotoxic, estrogenic and dioxin-like activity by using *in vitro* cell-based bioassays. None of the tested compounds was cytotoxic at concentrations up to 100 µM. However, 2-hydroxyfluorene and 3-hydroxyfluorene exerted significant estrogenic and dioxin-like activity on a concentration range of 3-30 µM, while fluorene and 9-hydroxyfluorene were weakly or not active, respectively, in our assays. This supports the view that photodegradation processes can generate by-products of higher toxicological concern than the parent compound and strengthens the need to further identify transformation products in the aquatic environment.

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

1. Introduction

The industrial and urban development which occurred in the second half of the 20th century allowed the emergence of thousands of organic chemicals into aquatic environment without prior study of their toxicity. Several decades later, a large number of these compounds have shown negative impacts on human health and ecosystems even at very low concentrations. Polycyclic Aromatic Hydrocarbons (PAHs) are among the most widespread pollutants in the environment. They are of special concern due to their negative characteristics, including persistence, mobility in the environment and toxicity. The most environmental PAHs result from incomplete combustion of organic matter: forest fires, automobile exhaust, coal, oil refining processes, etc. [1,2,3] Many PAHs are toxic; their mainly adverse health effects are cancers in various tissues such as prostate, breast, pancreatic and cervical [4,5,6,7], cardiovascular diseases [8] and immunosuppression [9]. PAHs are also known to have endocrine disrupting capabilities with consequent alteration of fertility in terrestrial and aquatic organisms [10,11,12]. 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 [13,14,15]. For these reasons, PAHs have been listed as priority pollutants by both the US Environmental Protection Agency (US EPA) and the European Union (EU). Despite a reduction in PAHs emissions from fuel combustion, resulting from the substitution of coal by fossil fuels 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 high.[16] PAHs can be introduced into the aquatic environment through different routes including industrial and municipal wastewater, rainwater runoff, atmospheric deposition and sediment- and air-water exchange [17, 18,16]. The concentration of PAHs in aquatic environments varies widely depending on the nature of water (i.e. ground water *versus* surface 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^{-1} in rainwater [19,20], from 2 to 587 ng.L^{-1} in river water [21, 22,20], from 5 to 1930 ng.L^{-1} in seawater [23,24], from 1.4 to 5 ng.L^{-1} in lake and reservoir water [22,19] and from 4 to 1473 ng.L^{-1} in wastewater [22,20]. In natural water environment, the PAHs are eliminated mainly through processes of sorption to sediments, biodegradation and photodegradation. Photodegradation remains the main route of PAHs degradation, especially 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 oxidation processes involving UV light for water and wastewater purification, the ultimate objective is to achieve complete mineralization of organic pollutants to carbon dioxide, water, or at least to produce small organic intermediates more soluble, readily biodegradable and thus less toxic than their parent compounds [25,26,27]. Numerous studies have been conducted on the photodegradation of PAHs. In most of these studies, much interest has been focused on the optimization of degradation conditions. The evaluation of the degradation efficiency only relies on the disappearance of the initial pollutants. In this way, the kinetics of disappearance are evaluated, as well as the monitoring of the mineralization rate achieved along the process. 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]. For example, studies carried out by Gala *et al*., Bertilsson *et al*., Shemer *et al*. and Woo *et al*. have shown that exposure to UV irradiation may increase the toxicity of many PAHs to a variety of aquatic organisms [30,25,31,27]. However, very little is known about the nature of the substances responsible for this increase in toxicity. In this regard, we were interested in the identification of the phototransformation by-products of PAHs. In the present study, fluorene (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. Second, fluorene is one of the US EPA and EU priority PAHs because it has water solubility significantly higher than those of PAHs with larger molecular weights. Third, a previous study has shown that 2-hydroxyfluorene, which could be a possible photodegradation product of fluorene in water, exhibits estrogenic activity [32]. The primary objectives of the present research were the elucidation of the photoproducts chemical structures and that of the reaction pathways leading to their formation during UV-Vis irradiation of fluorene in aqueous solution. The kinetics of fluorene disappearance and those of appearance of some photoproducts were examined. *In vitro* bioassays based on human MELN and fish PLHC-1 cell lines were carried out to evaluate the estrogenic and dioxin-like potency of photoproducts for which chemical standards were commercially available.

2. Materials and methods

2.1 Chemicals, reagents and sample preparation

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

2.2 Irradiation device

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

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

2.3 Extraction of residual fluorene and its photoproducts

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

2.4 GC-MS analysis

Residual fluorene and generated photoproducts (derivatized and non-derivatized extracts) were analysed using a “450-GC” gas chromatograph equipped with a “CP-8400” autosampler and 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, 90% methylpolysiloxane) capillary column (internal diameter: 0.25 mm, film thickness: 0.25 µm) from Varian. All experiments were performed by automatically injecting 1.0 µL of sample in the splitless mode at a rate of 50 $\mu L.s^{-1}$. High purity (99.999%) helium was used as the carrier gas at a constant flow of 1.4 $mL.min^{-1}$ hold by electronic pressure control. The injector 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 $°C.min^{-1}$ up to 320 °C where it was held for 7.5 min. The total duration of GC analysis was 35.0

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

2.5 In vitro bioassays

2.5.1 Estrogenic activity

The MELN reporter cell line has been used in order to assess the estrogenic activity of commercially available photoproducts of fluorene. This cell line was obtained by stable transfection of MCF-7 human breast cancer cells by an ERE-bGlob-Luc-SVNeo plasmid [34]. The cells were routinely cultured in phenol red containing Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 5% foetal calf serum (FCS), 1% nonessential amino acids and penicillin/streptomycin (50 U/mL each) in a 5% CO_2 humidified atmosphere at 37 °C. For experiments, cells were left to incubate for 2 days in phenol red free DMEM supplemented with 3% dextran charcoal coated-FCS (DCC medium) before seeded in white opaque 96-wells culture plates at a density of 50,000 cells per well. Serial dilutions of reference or test chemicals were added in triplicates 24 h later and then left to incubate for 16 h. In all assays, DMSO in the culture medium was always at 0.1% v/v, including in cell controls. At this concentration, no effect on cell viability or luciferase activity was observed. After cell exposure,

the medium was removed and replaced by 50 µL of DCC medium containing 0.3 mM of D-luciferin 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).

2.5.2 Dioxin-like activity

The dioxin-like activity was monitored by using the fish hepatic PLHC-1 cell line (ATCC, #CRL-2406) as described by Louiz *et al*. [15]. The cells were routinely grown at 30 °C in E-MEM culture media supplemented with 10% FCS and 1% antibiotics in a 5% CO_2 humidified atmosphere. For experiments, cells were seeded in 96-well plates at a density of approximately 50,000 cells per well. After 24 h of incubation, cells were exposed to test chemicals or sample extracts for 4 h incubation period. Then, plates were processed for 7-ethoxyresorufin-O-deethylase (EROD) activity in intact cells, as previously described.[15] Results were expressed as percent of EROD activity induced by the positive control (tetrachlorodibenzodioxin (TCDD) at 0.3 nM).

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 [35], as previously described [36]. The results were expressed as relative to DMSO treated control cells.

3. Results and discussion

3.1 Identification of fluorene photoproducts by GC-MS

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

Table 1

Six products with M = 182 were detected with close retention times (ranging from 25.7 to 29.3 min.) and identical mass spectra. Compared to fluorene mass weight, those compounds are shifted by + 16 amu, suggesting addition of an oxygen atom. In derivatized extracts, the corresponding molecular and pseudo molecular ions are shifted by + 72 amu (one hydrogen atom replaced by $Si(CH_3)_3$) in EI and CI, leading to the conclusion that these compounds are hydroxylated derivatives of fluorene (OH-Flu). Owing to the analysis of standard solutions, the chromatographic peaks at 26.4, 26.5 and 29.1 were unambiguously attributed to, 9OH-Flu, 2OH-Flu and 3OH-Flu, respectively, and both other peaks were logically assumed to correspond to 1-hydroxyfluorene (1OH-Flu) and 4-hydroxyfluorene (4OH-Flu).

A product is detected at 26.5 min. Its main ions in EI are m/z 180 and m/z 152, corresponding to the molecular ion $M^{+\cdot}$ and to $[M\text{-}CO]^{+\cdot}$, respectively. The EI and CI mass spectra and the retention time of this product were consistent with those observed for the authentic standard of 9-fluorenone. This result suggests that a CH_2 group of fluorene has been oxidized into CO, as depicted in Figure 1. A similar oxidation was reported by Rivas et al. who proposed a detailed mechanism of oxidation of fluorene by hydroxyl radicals. 9-fluorenone, 9-fluorenol and dibenzofuran were formed during the first step of the reaction; further oxidation of these products proceeded via hydroxylation and cleavage of the fluorene ring [37].

Figure 1

Nine products with M = 196 were detected. They were divided into two groups on the basis of 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 of OH-Flu-one are displayed in the NIST mass spectra database [38]. These spectra are identical so that the different isomers could not be determined owing to the database. It is to be noted that 2-benzyl-1,4-benzoquinone provides the same EI mass spectrum [38]. Its formation has 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 of interest led to the addition of one trimethylsilyl (TMS) group to each compound (M = 268), showing the presence of one exchangeable hydrogen atom, in agreement with OH-Flu-one isomers. The five other products with M = 196 (RT = 30.1, 30.2, 30.3, 30.4 and 30.5 minutes) displayed EI mass spectra with ions at m/z 178, m/z 166 and m/z 165, resulting from

eliminations of H_2O, $H_2C{=}O$ and $CH_3O^{\cdot}$, respectively. After many attempts to find likely chemical structures corresponding to $C_{13}H_8O_2$, we finally concluded that the only possibilities corresponding to these EI mass spectra were structures of fluorene methanol (MeOH-Flu), in agreement with the solution suggested by the NIST database. Since photolysis had been performed in pure water, the only explanation for the addition of a carbon atom to Flu was through the formation of a dimer species, for which a mechanism of formation is proposed in Figure 2. The first eluted isomer (RT = 30.1 min.) was determinated to be 9MeOH-Flu. As a matter of fact, its EI mass spectrum displays a m/z 178 ion (loss of H_2O from $M^{+\cdot}$) in greater abundance than for other isomers (82% vs 24%). The supporting information file SI-1 shows the dissociation pathways proposed to interpret the EI mass spectrum of 9MeOH-Flu. The easier loss of water from 9MeOH-Flu than for other MeOH-Flu isomers is easily rationalized on the basis of the α,β concerted elimination of H_2O involving a hydrogen atom of the sp3 carbon, which cannot occur for other isomers.

Figure 2

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

3-2 Mechanistic approach of the photodegradation of fluorene

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

fluorene not in water but in methylene chloride and hexane [45]. Sabaté et al. reached the same conclusion performing irradiation in an ethanol/water mixture [46]. Both studies focused on the stability of fluorene under photolysis and only Flu, OH-Flu and Flu-one were detected owing to a UV-vis spectrophotometer; other by-products were not detected or not considered. The formation of BPCA is assumed to result from oxidation of Flu-one through addition of a hydroxy radical. Since dihydroxyfluorene (diOH-Flu) isomers could not be identified, their formation has been assumed to result from a hydroxy radical addition onto 9OH-Flu and/or onto another OH-Flu isomer. Finally, the formation of hydroxy-9-fluorenone may result either from dehydrogenation of hydroxy-9-fluorenol, via a mechanism analogous to that involved in 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_2OH 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.

With the aim to establish how photolysis products of Flu are related to each other and if the evolutions of relative amounts are in agreement with the proposed reaction pathways, we followed relative concentrations as a function of the irradiating time up to 120 minutes. A "real" kinetic study was not the purpose of its work; it would have required possessing standard compounds for all the photolysis products and would have been very complicated to achieve since several photolysis pathways are undoubtedly in competition. Given the unexpected evolution of the fluorene concentration as a function of the irradiation time, kinetics experiments were carried out three times and provided results with a good repeatability (RSD < 15%)". Figure 3 displays the evolution of the relative amounts of fluorene and its photoproducts. Since concentrations of isomeric compounds undergo the same evolution, sums of amounts were plotted for much clarity. It is to be noted that the amounts reported in figure 3 correspond to the areas of chromatographic peak integrated on the total ionic current (TIC) in the EI mode and normalized to the initial TIC value which corresponds to the fluorene peak area at t_0. Considering the likely differences in response factors between compounds, the relative amounts plotted allow the monitoring of a given photoproduct but they cannot be used to compare concentrations of different photoproducts.

The concentration of Flu decreases by a factor four during the first fifteen minutes of irradiation, then increases until one hour of irradiation and decreases again during the following hour. Figure 3 shows evidence that the fast decrease in Flu concentration corresponds to dimers formation. Dimers partially dissociate in turn to give biphenylene and MeOH-Flu isomers, which are assumed to eliminate $\cdot CH_2OH$ radicals under irradiation to provide fluorene again (Figure 2), thus explaining the increasing relative amount of Flu after the first fifteen minutes of irradiation. In competition with this reaction pathway, the mechanisms suggested in Figure 1 appear to be slower. The kinetics features are in good agreement with the proposed mechanisms since the concentrations in Flu-one and BCPA increase as that in 9OH-Flu decreases. Amounts of diOH-Flu isomers increase then slowly decrease as those of OH-Flu isomers increase.

Figure 3

3.3 Irradiation in more realistic conditions

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

3.4 In vitro activity of hydroxyfluorenes

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

Figure 4

Interestingly, 2OH-Flu and 3OH-Flu were also potent inducers of EROD activity in the PLHC-1 cell line (Figure 5), with EC50s around 10 and 3 µM, respectively. This induction was marked after 4 hours of cell exposure but it was strongly reduced after 24 h, only a weak induction (i.e. 10% that of the positive control) remaining at that time point. Similar patterns of transient EROD induction were previously reported with weakly persistent AhR agonists such as various

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

Figure 5

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

4. Conclusion

Studies aiming to identify transformation products of pollutants are necessary to understand 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 investigated. The complete mineralization of Flu was not observed under our conditions. Using gas chromatography coupled with mass spectrometry, twenty-six photoproducts were characterized, mainly on the basis of electron and chemical ionization mass spectra interpretation. The main generated transformation products are monohydroxy derivatives. Some secondary photoproducts were also observed: fluorenone, hydroxy fluorenone, 2-biphenyl carboxylic acid, biphenylene, methanol fluorene congeners and hydroxy fluorene 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 µM. This study
443 demonstrates that UV-Vis irradiation of fluorene generates hydroxylated photoproducts that are
444 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.
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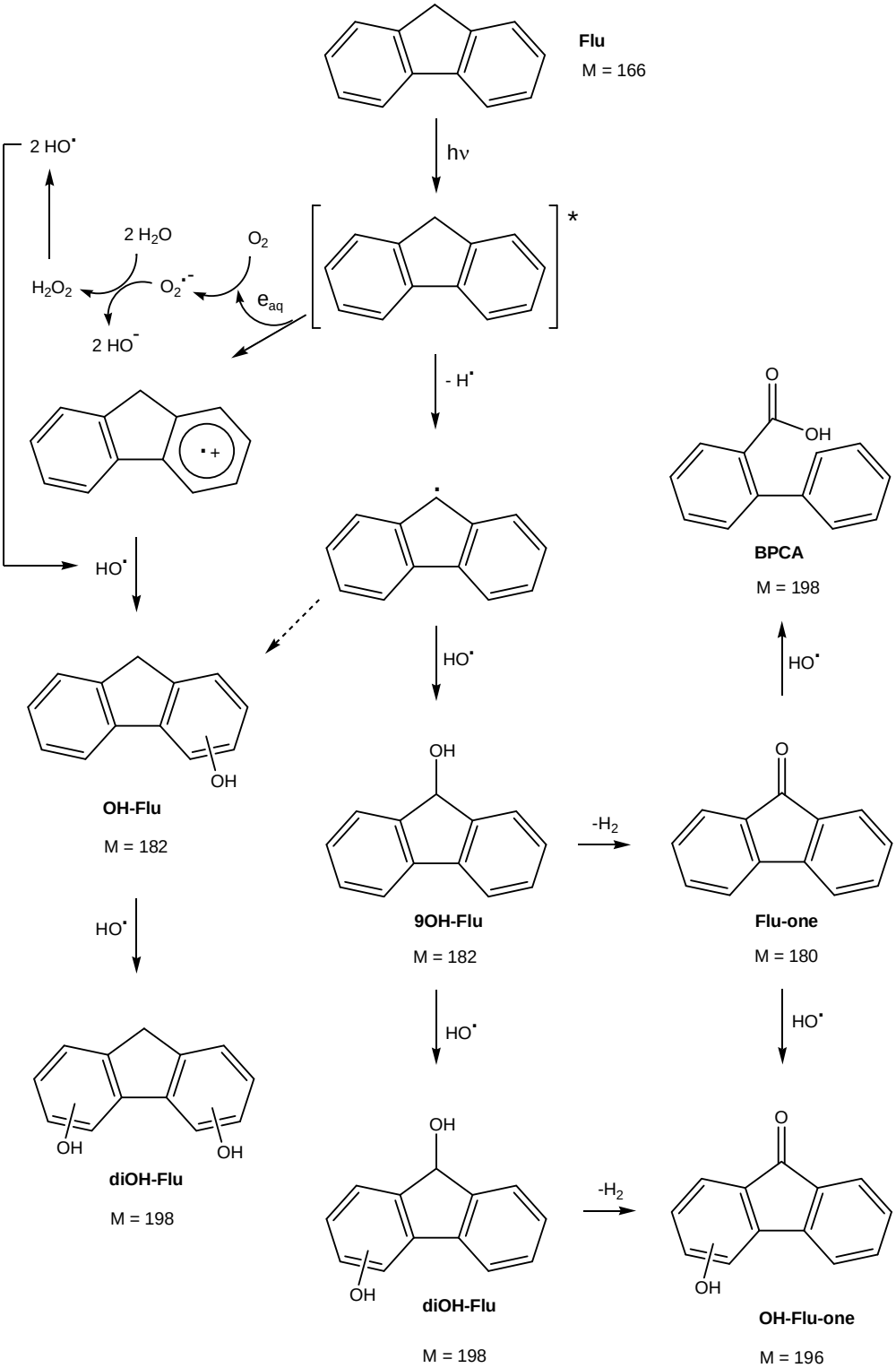
447

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.



451

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.

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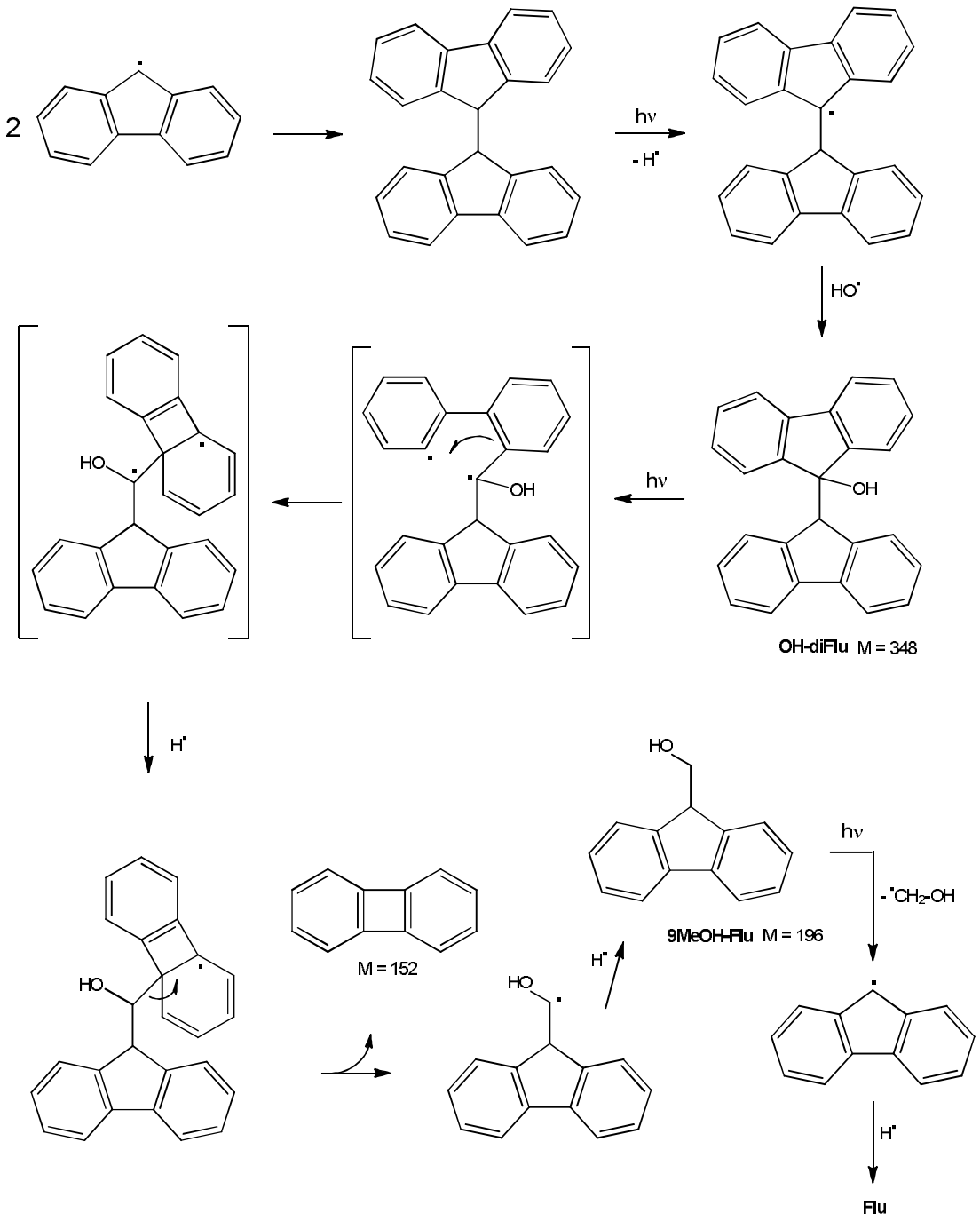

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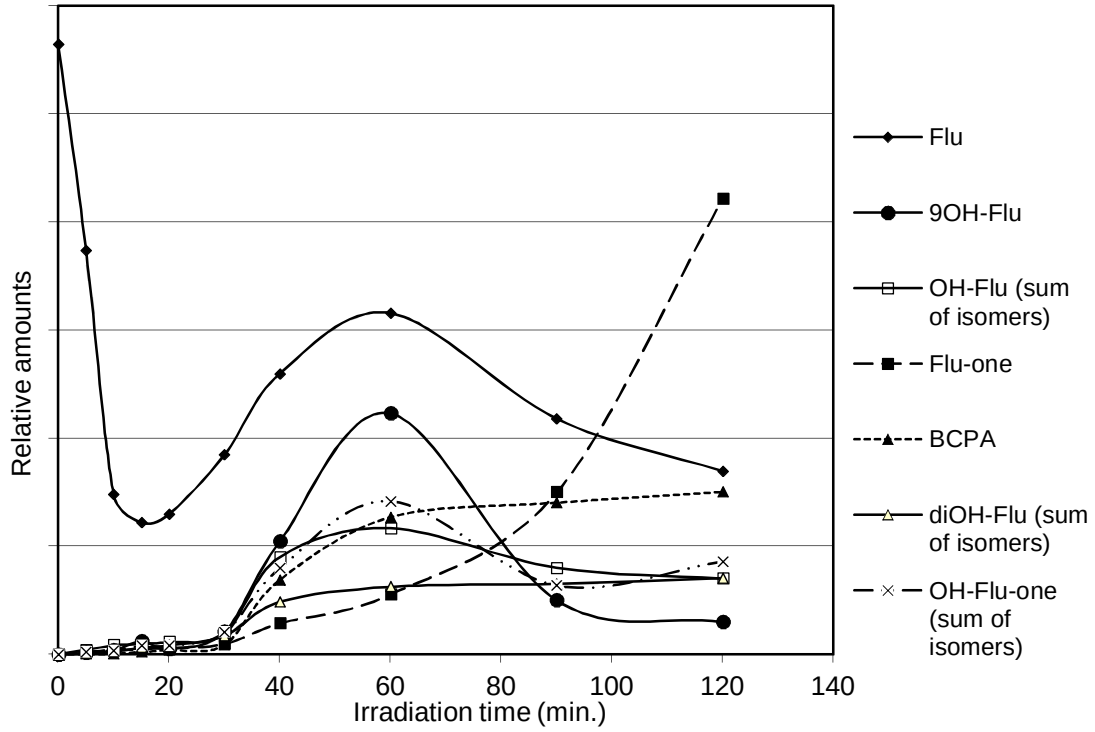
462 Figure 3. Relative amounts of fluorene photoproducts as a function of the irradiation time.
463 Upper part involves photoproducts displayed in Figure 1while lower part involves those in



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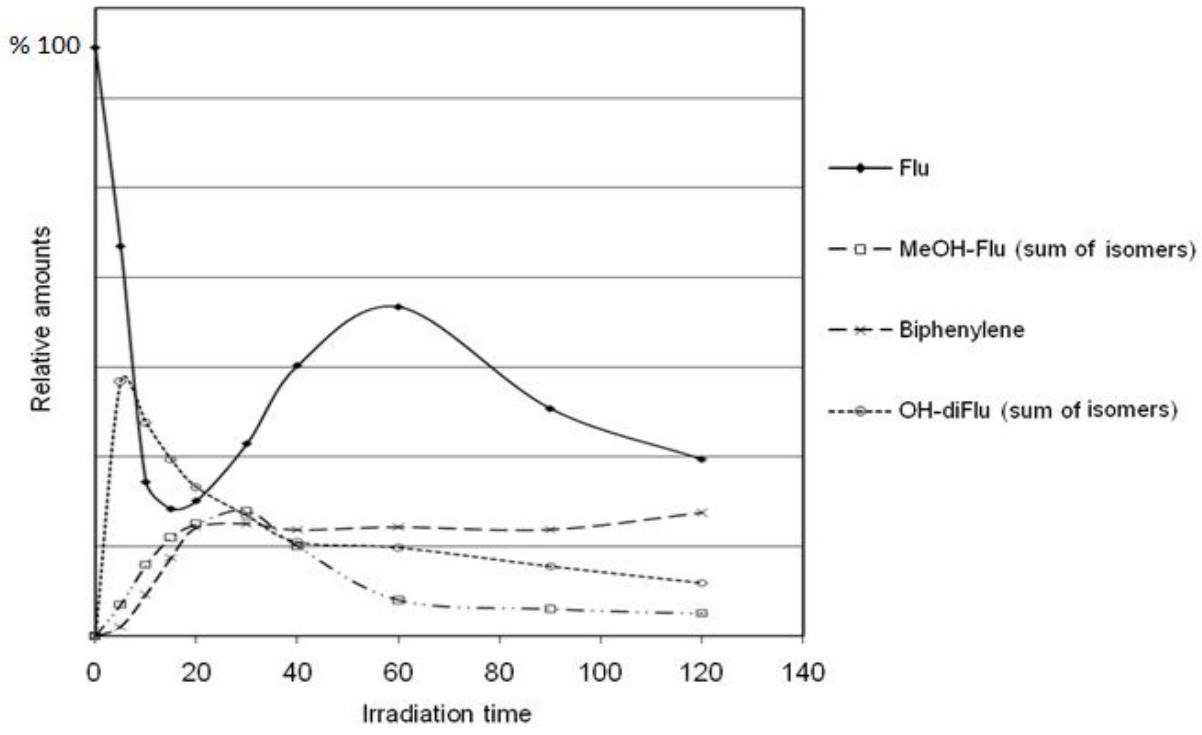

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Figure 4. *In vitro* estrogenic activity of Flu, 2OH-Flu, 3OH-Flu and 9OH-Flu in the MELN reporter cell line. Results are expressed as % of luciferase induction relative to the positive control estradiol (E2, 10 nM)

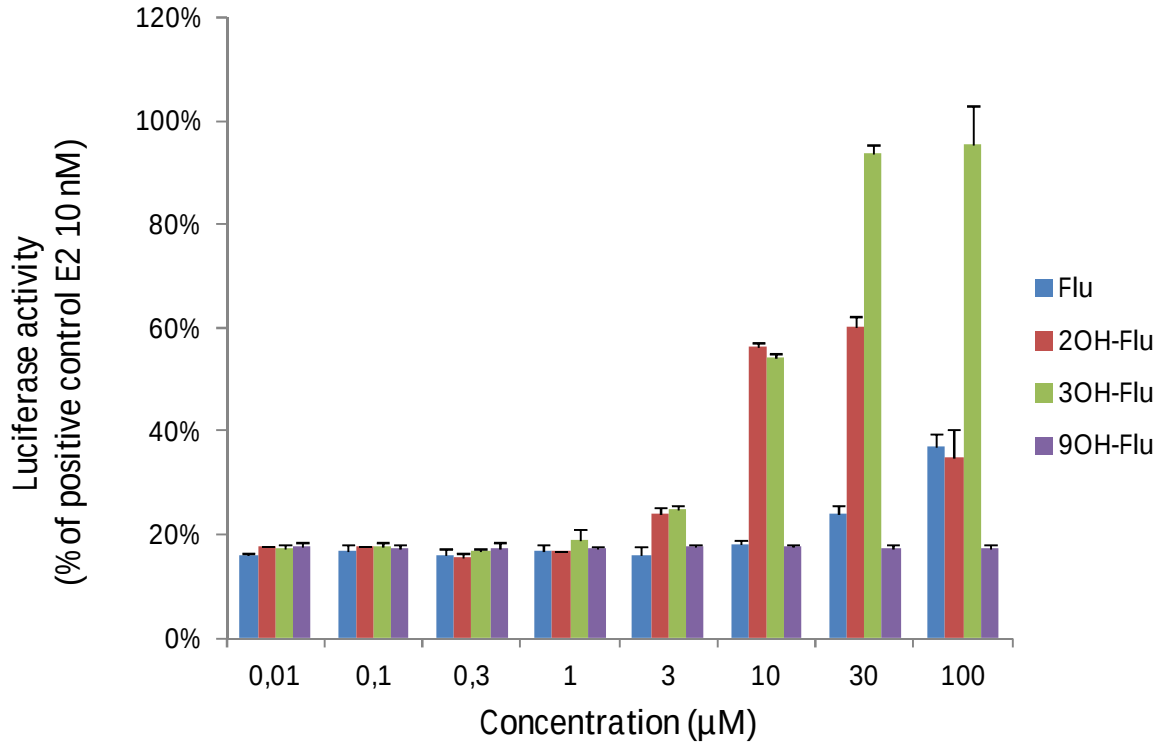
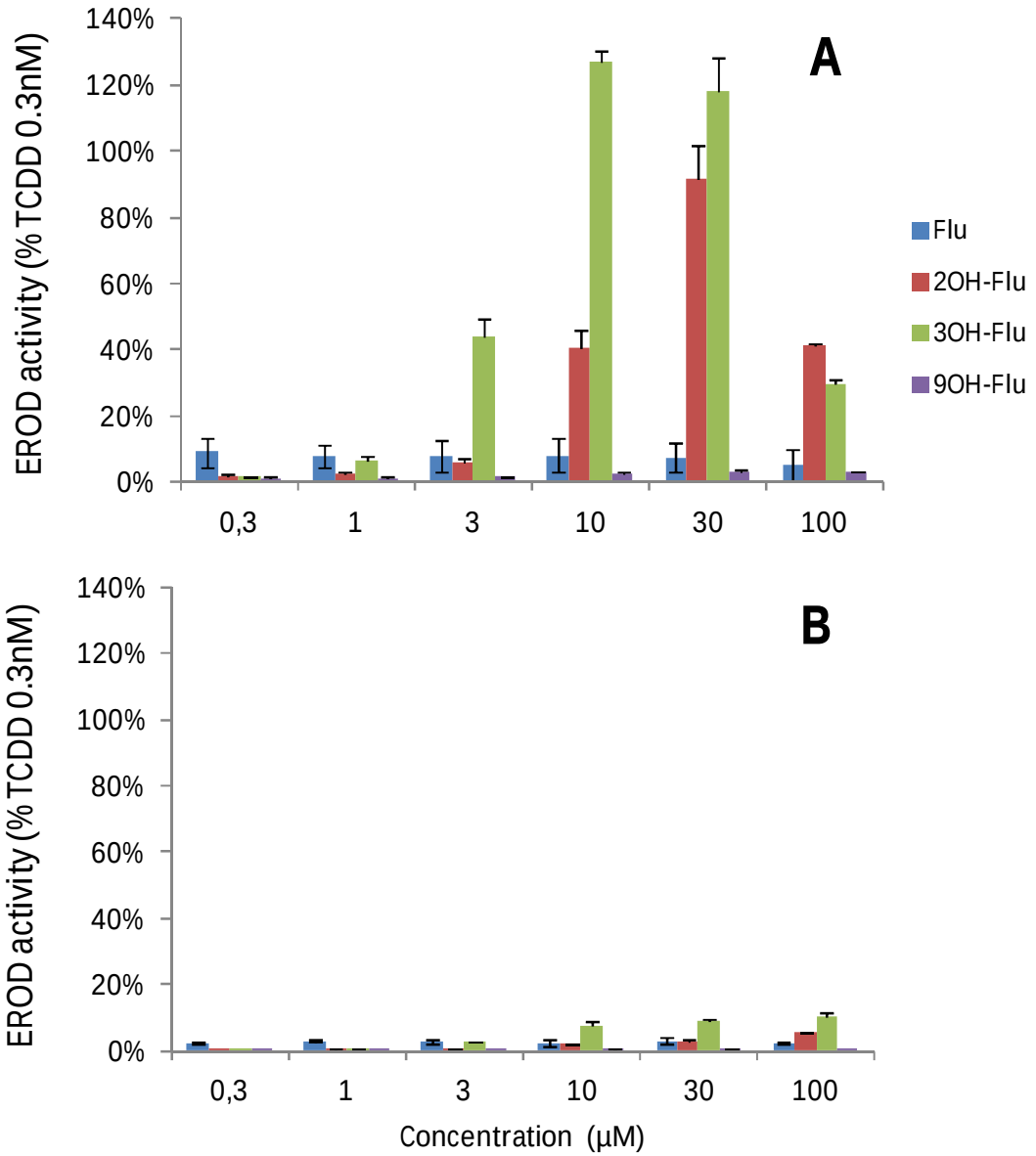


Figure 5. *In vitro* dioxin-like activity of Flu, 2OH-Flu, 3OH-Flu and 9OH-Flu in the PLHC-1 cell line after cell exposure for a) 4 h and b) 24 h. Results are expressed as % of EROD activity induction relative to the positive control dioxin (TCDD, 0.3 nM)



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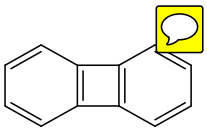
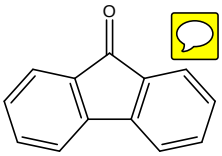
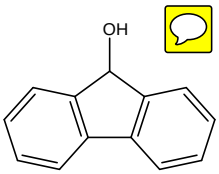
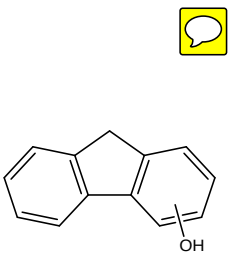
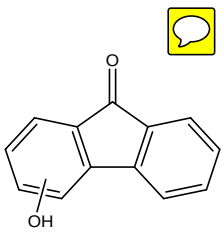
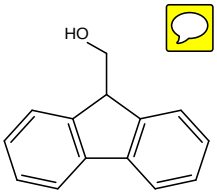
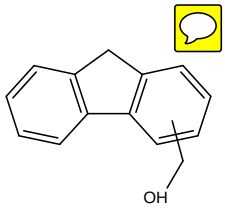
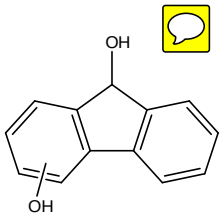
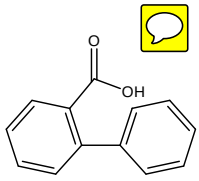
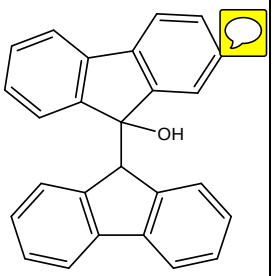
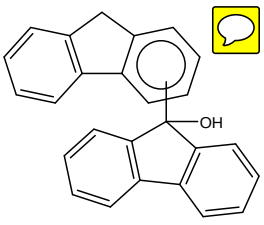
479 Table 1. Identified photoproducts resulting from UV-Vis irradiation of fluorene in water

MW	Rt (min.)	Main ions in mass spectra [a]			Identified compounds	
		EI	CI	CI (DC)[b]	Structure	Name

152	18.0	152 (100), 150 (90), 122 (45), 133 (27), 91 (27)	nd[c]	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)		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)		Hydroxy-9-fluorenone congeners
196	30.1	196 (66), 178 (82), 166 (58), 165 (100), 152 (16)	197 (100)	269 (100)		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)		Methanol-fluorene congeners
198	31.2 31.3 31.4 31.6	198 (100), 197 (78), 181 (69), 162 (15), 153 (42), 152 (45), 141 (25), 139 (22) 115 (36)	199 (100)	343 (100)		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)		2-Biphenyl carboxylic acid
348	37.1	348 (33), 174 (100)	349 (100)	421 (100)		Hydroxy fluorene dimer
348	38.4 38.5 38.6	348 (62), 174 (100)	349 (100)	421 (100)		Hydroxy fluorene dimer isomers

[a] relative abundances (%) are given in parentheses - [b] DC: Derivatized compound – [c] nd: non detected

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484 **References**

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