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PII: S0043-1354(17)30496-7
DOI: 10.1016/j.watres.2017.06.020
Reference: WR 12975
To appear in: Water Research

Received Date: 06 March 2017
Revised Date: 05 June 2017
Accepted Date: 07 June 2017

Please cite this article as: Danilo Russo, Antonietta Siciliano, Marco Guida, Emilia Galdiero, Angela Amoresano, Roberto Andreozzi, Nuno M. Reis, Gianluca Li Puma, Raffaele Marotta, Photodegradation and ecotoxicology of acyclovir in water under UV_{254} and UV_{254}/H_{2}O_{2} processes, Water Research (2017), doi: 10.1016/j.watres.2017.06.020

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Photodegradation and ecotoxicology of acyclovir in water under UV$_{254}$ and UV$_{254}$/H$_2$O$_2$ processes

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Abstract

The photochemical and ecotoxicological fate of acyclovir (ACY) through UV$_{254}$ direct photolysis and in the presence of hydroxyl radicals (UV$_{254}$/H$_2$O$_2$ process) were investigated in a microcapillary film (MCF) array photoreactor, which provided ultrarapid and accurate photochemical reaction
kinetics. The UVC phototransformation of ACY was found to be unaffected by pH in the range from 4.5 to 8.0 and resembled an apparent autocatalytic reaction. The proposed mechanism included the formation of a photochemical intermediate \( \phi_{ACY} = (1.62 \pm 0.07) \times 10^{-3} \text{ mol ein}^{-1} \) that further reacted with ACY to form by-products \( k' = (5.64 \pm 0.03) \times 10^{-3} \text{ M}^{-1} \text{s}^{-1} \). The photolysis of ACY in the presence of hydrogen peroxide accelerated the removal of ACY as a result of formation of hydroxyl radicals. The kinetic constant for the reaction of OH radicals with ACY \( k_{OH/ACY} \) determined with the kinetic modeling method was \( (1.23 \pm 0.07) \times 10^{9} \text{ M}^{-1} \text{s}^{-1} \) and with the competition kinetics method was \( (2.30 \pm 0.11) \times 10^{9} \text{ M}^{-1} \text{s}^{-1} \) with competition kinetics. The acute and chronic effects of the treated aqueous mixtures on different living organisms \( (Vibrio fischeri, Raphidocelis subcapitata, D. magna) \) revealed significantly lower toxicity for the samples treated with \( \text{UV}_{254}/\text{H}_2\text{O}_2 \) in comparison to those collected during \( \text{UV}_{254} \) treatment. This result suggests that the addition of moderate quantity of hydrogen peroxide \( (30-150 \text{ mg L}^{-1}) \) might be a useful strategy to reduce the ecotoxicity of \( \text{UV}_{254} \) based sanitary engineered systems for water reclamation.

**Keywords**: UVC, hydrogen peroxide photolysis, microreactor, ecotoxicity, water reuse, acyclovir removal.

### 1. Introduction

Water reclamation and water reuse is becoming increasingly common in industrialized countries with high water demands and in water stressed regions characterized by considerable scarcity of freshwater (Hoekstra, 2014). The most common treatment method for water reuse is chlorination at typical dosages ranging from 5 to 20 mg/L with a maximum of two hours of contact time (Asano, 1998). However, concerns related to (i) the adverse impacts of chlorine on irrigated crops, (ii) the high ecotoxicity of chlorinated by-products (DBPs) formed during the chlorination stage (Richardson et al., 2007) and (iii) the survival of antibiotics resistant bacteria during chlorination...
with a possible selection of some antibiotic resistance genes in the wastewater microbial community (Huang et al., 2011) should drive the transition from chlorine disinfection to other more ecofriendly suitable methods. UV radiation treatment (especially UVC, $\lambda < 280$ nm) produces a high sterilization efficiency (Montemayor et al., 2008) and could represent a viable alternative to chlorination for the disinfection and reuse of effluents from wastewater treatment plant (WWTP) for irrigation (i.e., after membrane filtration and/or reverse osmosis) or for aquifer recharge. Numerous wastewater sites have adopted UVC treatment for effluents disinfection. For example, Florida and California have favored wastewater reuse and adopted specific regulations on reclamation technologies through UV disinfection processes. UVC doses (fluence) ranging from 50 mJ·cm$^{-2}$ to 150 mJ·cm$^{-2}$ have been suggested to efficiently inactivate pathogens accounting for the variability in the effluent composition (NWRI, 2012), although German and Austrian regulations (DVGW,1997; ONorm, 2001) suggest the use of 40 mJ·cm$^{-2}$ UVC fluence to eliminate a large variety of bacteria and viruses (Conner-Kerr et al., 1998). Even though UV disinfection has been reported highly effective in the reduction of antibiotic resistance bacteria (ARB), particularly in comparison to chlorination (Shi et al., 2013; Hijnen et al., 2006), other investigations have demonstrated that UV disinfection may not contribute to the significant reduction of selected ARB, such as tetracycline-and sulfonamide-resistant bacteria (Munir et al., 2011; Meckes, 1982) thus indicating a plausible selectivity of UV on ARB (Guo et al., 2013).

Moreover, numerous studies have suggested that under the recommended UVC doses several biorefractory xenobiotics, particularly pharmaceuticals and personal care products generally occurring in municipal discharges and partially removed in WWTPs, may undergo photochemical transformations induced by UVC irradiation (Canonica et al., 2008; Nick et al., 1992; Pereira et al, 2007; Kim et al., 2009; Ma et al., 2016; Kovacic et al, 2016; Liu et al., 2016; Marotta et al., 2013) which may generate by-products with high ecotoxicity (Rozas et al., 2016; Yuan et al., 2011). For these reasons, the use of hydrogen peroxide during UVC disinfection ($\text{UV}_{254}/\text{H}_2\text{O}_2$) which produces highly reactive radical species, has been proposed as a viable treatment for effective removal of
micropollutant and ARB and, in consequence, for the reduction of the ecotoxicity risk (García-Galan et al., 2016; Melo da Silva et al., 2016).

Among the emerging Pharmaceuticals and Personal Care Products detected in WWTP effluents, antiviral drugs play a leading role (Richardson, 2012; Jain et al., 2013) due to their scarce biodegradability (Funke et al., 2016) and increased usage during the last decade, particularly for the treatment of viral diseases and for the prevention of pandemic outbreaks (Hill et al., 2014). Moreover, antiviral drugs have been considered as some of the most hazardous therapeutic substances exerting high toxicity towards biota, such as crustaceans, fish and algae (Sanderson et al., 2004). The presence of antiviral drugs in the environment raises considerable concern regarding their potential effect on the ecosystem, with the potential of developing antiviral drug resistance, in analogy to the development of antibiotic resistant bacteria (Singer et al., 2007; Gillman et al., 2015).

Acyclovir (ACY) is one of the oldest and most widely used antiviral drug for treating two common viral infections (chickenpox-zoster and herpes simplex) and it is also prescribed to patients with weakened immune systems in order to control viral infections (i.e., viral conjunctivitis) (Bryan-Marrugo et al., 2015). ACY has been recently detected in different WWTP effluents as well as in surface water at level of few nanograms per liter up to over one micrograms per liter (Table 1). The photodegradation pathways of ACY under artificial and natural solar light irradiation have been recently investigated (Zhou et al., 2015; Prasse et al., 2015). However, there is a lack of investigations on the photochemical transformation of ACY under UV$_{254}$ and UV$_{254}$/H$_2$O$_2$ treatments and on the simultaneous ecotoxicological assessments of highly diluted treated solutions containing ACY.

More information is needed to determine the effectiveness of UV$_{254}$ assisted processes on the removal of ACY from aqueous solutions and the impact that these processes may have on the structure of aquatic communities and on the ecosystem dynamics.

The use of microcapillary flow photoreactors has recently been proposed to intensify the treatment of substances that are either highly priced, scarcely commercially available or controlled substances
such as illicit drugs or selected pharmaceuticals (Reis and Li Puma, 2015; Russo et al., 2016). In contrast to conventional laboratory photochemical systems which require relatively larger volume of liquid, photochemical treatments in microphotoreactors are carried out in a highly controlled environment with minimal sample volumes (of the order of few mL), the sufficient amount to generate samples for subsequent analysis. Furthermore, photochemical transformations in microphotoreactors are executed at extremely short residence times (of the order of seconds) in comparison to conventional laboratory photoreactors, resulting in an efficient use of time and resources.

Under this background, in this study we investigated the degradation kinetics of ACY in distilled water under UV$_{254}$ and UV$_{254}$/H$_2$O$_2$ irradiation by means of a microcapillary film (MCF) array photoreactor and we evaluated the acute and chronic ecotoxicity of highly diluted treated samples using a range of selected organisms, to provide important information regarding the photolysis of ACY in UV$_{254}$ based sanitary engineered systems for water reclamation. The toxicity was assessed considering a battery of toxicity tests (Aliivibrio fischeri, Raphidocelis subcapitata, Daphnia magna) and endpoints (bioluminescence, growth inhibition, immobilization, survival, reproduction and biomarker) including three trophic and phylogenetic levels (Lofrano et al., 2016).

The battery of toxicity tests proposed were sensitive indicators of toxic pollutants, and also determined the great diversity of potential stress-receptor that could result from pharmaceuticals and their byproducts entering the environment (FDA, 1998).

2. Materials and methods

2.1. Materials

Hydrogen peroxide (30% v/v), ACY (pharmaceutical secondary standard), methanol (≥99.9% v/v), formic acid (>99% w/w), benzoic acid (≥99.5% w/w), orthophosphoric acid (85% w/w in H$_2$O), sodium hydroxide (>98% w/w), perchloric acid (70% v/v), catalase from Micrococcus lysodeikticus and reagents for ecotoxicity tests were purchased from Sigma-Aldrich. An aqueous mixture of
peptone (32 ppm), meat extract (22 ppm), urea (6 ppm), K$_2$HPO$_4$ (28 ppm), CaCl$_2$·H$_2$O (4 ppm),
NaCl (7 ppm) and Mg$_2$SO$_4$ (0.6 ppm) was used for the preparation of a synthetic wastewater
according to the OECD Guidelines (Organisation for Economic Cooperation and
development, 1999). The substances were purchased from Sigma-Aldrich and used as received.
Milli-Q water was used as solvent in analytical determinations and experiments.

2.2. Analytical methods
The concentration of hydrogen peroxide, ACY, and benzoic acid was measured by HPLC (1100
Agilent) equipped with a Gemini 5u C6-Phenyl 110 (Phenomenex) reverse phase column and a
diode array detector. The mobile phase was a mixture of 93% aqueous orthophosphoric acid (10
mM) and 7% methanol flowing at 8.0·10$^{-4}$ L·min$^{-1}$. The pH of the aqueous solutions was adjusted
with NaOH or HClO$_4$ and measured with an Accumet Basic AB-10 pH-meter. The molar
absorption coefficient of ACY was estimated using a Perkin Elmer UV/VIS spectrometer (mod.
Lambda 35). Total organic carbon (TOC) was monitored by a TOC analyzer (Shimadzu 5000 A).
MS analysis was performed by direct injection on Agilent 6230 TOF LC/MS coupled with Agilent
HPLC system (1260 Series). The mobile phase was a mixture of methanol (10% v/v) and formic
acid (0.1% v/v) aqueous solution at flow rate of 0.4 mL·min$^{-1}$ and the injection volume of samples
was 20 µL. The MS source was an electrospray ionization (ESI) interface in the positive ion mode
with capillary voltage of 3500 V, gas temperature at 325 °C, dry gas (N$_2$) flow at 8 L·min$^{-1}$ and the
nebulizer at 35 psi. The MS spectra were acquired in a mass range of 100-3000 m/z with a rate of 1
spectrum/s, time of 1000 ms/spectrum and transient/spectrum of 9905.

3. Experimental apparatus and procedures
3.1. MCF array photoreactor
The degradation kinetics of ACY by UV$_{254}$ and UV$_{254}$/H$_2$O$_2$ were investigated in a MCF array
photoreactor described elsewhere (Reis et al., 2015; Russo et al., 2016). Briefly, the photoreactor
(Lamina Dielectrics Ltd) consisted of ten UV$_{254}$ transparent microcapillaries of fluorinated polymer characterized by a mean hydraulic diameter of 195 μm. The microcapillaries were coiled around a UV monochromatic (254 nm) lamp (Germicidal G8T5) in the region with uniform emission. Experiments were carried out at room temperature (~25 °C) in continuous flow through the reactor at different space times, using capillaries of different length exposed to the UV lamp irradiation. The flow rate through the MCF was 6.0·10^{-4} L·min^{-1}. Aqueous samples were collected from the MCF outlet, and rapidly analyzed by HPLC. At the end of each experimental run, the pH of the solutions was unchanged. The initial concentration of ACY used in the experiments ranged between 2.05·10^{-5} mol·L^{-1} and 4.67·10^{-5} mol·L^{-1}.

The lamp irradiance was varied by changing the nominal power from 4.5 W to 8.0 W using a variable power supply unit. The photon fluxes per unit volume emitted by the UV lamp ($P_o$) for each power setting, estimated by H$_2$O$_2$ actinometry (Nicole et al, 1990; Goldstein et al., 2007), were 1.92·10^{-2} ein·(s·L)$^{-1}$ (nominal power 8.0 W) and 1.27·10^{-2} ein·(s·L)$^{-1}$ (nominal power 4.5 W). The MCF average optical path length ($l_{MCF}$) was 154 μm. All the runs were carried out in duplicate. The data collected were used to estimate the kinetic unknown parameters (quantum yield of direct photolysis at 254 nm of ACY and kinetic constant of hydroxyl radical attack to ACY).

### 3.2. Cylindrical batch photoreactor

A cylindrical batch photoreactor ($V_b = 0.480$ L), equipped with a low-pressure mercury monochromatic lamp (Helios Italquartz, HGL10T5L, 17W nominal power emitting at 254 nm), was used to provide large sample volumes required for the ecotoxicity tests at varying treatment times (i.e., different UV$_{254}$ fluence). The UV$_{254}$ dose (mJ·cm$^{-2}$) was calculated as the average photon fluence rate multiplied by the treatment time. The average photon fluence rate emitted by the UV lamp at 254 nm was 4.7 mW·cm$^{-2}$ (UVC DELTA OHM radiometer). The experimental device was described elsewhere (Spasiano et al., 2016).
3.3. Ecotoxicity assessment

Reconstituted aqueous solution (pH = 7.8 ± 0.2), was used as dilution water for cladoceran toxicity tests: CaCl$_2$·2H$_2$O (290 mg·L$^{-1}$), MgSO$_4$·7H$_2$O (120 mg·L$^{-1}$), NaHCO$_3$ (65 mg·L$^{-1}$), KCl (6 mg·L$^{-1}$). Different salts were used for the preparation of algal test medium: CaCl$_2$·2H$_2$O (18 mg·L$^{-1}$), MgSO$_4$·7H$_2$O (15 mg·L$^{-1}$), NH$_4$Cl (15 mg·L$^{-1}$), MgCl$_2$·6H$_2$O (12 mg·L$^{-1}$), KH$_2$PO$_4$ (1.6 mg·L$^{-1}$), FeCl$_3$·6H$_2$O (0.08 mg·L$^{-1}$), Na$_2$EDTA·2H$_2$O (0.1 mg·L$^{-1}$), H$_3$BO$_3$ (0.185 mg·L$^{-1}$), MnCl$_2$·4H$_2$O (0.415 mg·L$^{-1}$), ZnCl$_2$ (0.003 mg·L$^{-1}$), CoCl$_2$·6H$_2$O (0.0015 mg·L$^{-1}$), Na$_2$MoO$_4$·2H$_2$O (7.0·10$^{-3}$ mg·L$^{-1}$), CuCl$_2$·2H$_2$O (1.0·10$^{-5}$ mg·L$^{-1}$). Reconstitution solution, osmotic adjusting solution (OAS) and diluent (NaCl 2%) were the reagents used in Vibrio fischeri toxicity test (Strategic diagnostics Inc. SDI).

The enzymatic assays chosen to evaluate oxidative stress were ROS (reactive oxygen species) content using 2,7- dichlorodihydrofluorescein (H$_2$DCFDA) and activities of SOD (superoxide dismutase), CAT (catalase) and GST (glutathione transferase) that were measured using respective assay kits according to the manufacturer’s instruction’s (Sigma Aldrich). All determinations were quantified spectrophotometrically.

V. fischeri, R. subcapitata and acute D. magna assays were conducted with an initial ACY concentration of 1.2 mg·L$^{-1}$ and on its related UV$_{254}$ and UV$_{254}$/H$_2$O$_2$ treated solutions. Chronic toxicity and oxidative stress tests on Daphnia magna were performed starting on untreated and treated solutions diluted by 100 fold, in order to assess any differences at sub lethal concentration levels. Negative and positive controls were included in each experiment. The significance of differences of toxicity between the treated samples and controls was assessed by the analysis of variance (ANOVA) considering a significance threshold level always set at 5%. For higher variance than 5%, post-hoc tests were carried out with Dunnett’s method and Tukey’s test. Whenever
possible, toxicity was expressed as median effective concentration (EC$_{50}$) with 95% confidence limit values. Otherwise, toxicity was expressed as percentage of effect (PE, %).

### 3.3.1. Organisms maintenance and monitoring

Freeze-dried *Vibrio fischeri* (strain NRRL-B-11177) cells were reconstituted with reagent diluent at 4 °C. *Raphidocelis subcapitata* were cultured in ISO medium (ISO, 2012) at 23 ± 2 °C with continuous 4500 lux light and aeration (0.2 mm filtered air). *Daphnia magna* were cultured at 20 ± 1 °C, with a 16:8 light/dark photoperiod in ISO water (ISO, 2012).

Luminescence *V. fischeri* measurements were performed with Microtox® Model 500 Toxicity Analyzer from Microbics Corporation (AZUR Environmental) equipped with a 30 well incubated at 15 ± 1 °C and with excitation source at 490 nm wavelength. *R. subcapitata* density was determined by an indirect procedure using a spectrophotometer (Hach Lange DR5000) and cuvette (5 cm). *D. magna* viability, mobility and growth were observed with a stereomicroscope (LEICA EZ4-HD).

### 3.3.2. Bacteria toxicity test

The inhibitory effect of ACY samples on the light emission of *V. fischeri* (strain NRRL-B-11177) was evaluated with the 11348-3:2007 ISO method (ISO, 2007). Tests were carried out on an ACY concentration of 1.2 mg·L$^{-1}$ and on its related treated by-products solutions. OAS was added to each sample to ensure that the final NaCl concentration was above 2.0%. The initial light output from each cuvette containing reconstituted freeze-dried *V. fischeri* was recorded. The test solutions were then added and after 30 minutes exposure, the final light output was measured. Positive control tests for *V. fischeri* were carried out with C$_6$H$_4$Cl$_2$O (EC$_{50}$ = 4.1 ± 2.2 mg·L$^{-1}$).

### 3.3.3. Algae toxicity test
The *R. subcapitata* bioassay was conducted following the guidelines ISO 8692 (ISO, 2012). Three replicates were included for each sample. The replicates were inoculated with \(10^4\) algal cells·mL\(^{-1}\) and incubated for 72 h at 23 ± 2 °C under continuous illumination (irradiance range of 120-60 μein·m\(^{-2}\)·s\(^{-1}\)). The algal biomass exposed to the samples was compared with the algal biomass in the negative control. Positive control tests for *R. subcapitata* were carried out with K\(_2\)Cr\(_2\)O\(_7\) (EC\(_{50}\) = 1 ± 0.2 mg·L\(^{-1}\)).

### 3.3.4. Crustaceans toxicity test

Acute toxicity tests with *D. magna* were carried out according to ISO 6341 (ISO, 2013). Newborn daphnids (<24 h old) were exposed in four replicates for 24 h and 48 h at 20 ± 1 °C. Toxicity was expressed as percentage of immobilized organisms. Positive control tests for *D. magna* were carried out with K\(_2\)Cr\(_2\)O\(_7\) (48h, EC\(_{50}\) = 0.6 ± 0.1 mg·L\(^{-1}\)).

The *D. magna* chronic bioassay was carried out according to the guideline OECD 211 (OECD, 2012). Ten *D. magna* neonates (< 24 h hold) were used and individually placed for each treatment in beakers containing 50 ml of the test solutions, renewed every two other days. Organisms exposed for 21 days with ACY solutions were then fed one day with *R. subcapitata* (10\(^7\) cell·mL\(^{-1}\)). Survival, reproduction and growth were observed daily, and newborns were discarded from beakers.

The amount of ROS produced in *D. magna* was determined using 2,7-dichlorodihydrofluorescein (H\(_2\)DCFDA, Sigma Aldrich) using the method previously reported (Galdiero et al., 2016). After 48 h of exposure, each exposed and not exposed living daphnids were rinsed with deionized water to remove any excess pharmaceuticals adhered to their body surface and transferred to a 96-well plate. A selected volume (200 μL) of 10 mM H\(_2\)DCFDA was added to each well and the plate was then incubated for 4 h in the dark at 20-25°C. Fluorescence was measured using a fluorescence plate...
reader with an excitation wavelength of 350 nm and an emission of 600 nm. The increase in fluorescence intensity yielded the ROS quantity compared to control.

Exposed and not exposed daphnids were homogenized in 1 mL sucrose buffer (0.25 M sucrose, 0.1 M Tris-HCl, 1 mM EDTA, pH 7.4) and successively centrifuged at 12,000 g for 15 min at 4°C. Supernatants were collected and used to determine enzymatic activities. Protein content of the samples was quantified using the protocol described by Bradford (1976) using bovine serum albumin as standard.

CAT activity was expressed as H$_2$O$_2$ consumed (U·mg$^{-1}$ of protein) to convert it to H$_2$O and O$_2$ per minute, per mg protein at 240 nm (Aebi, 1984).

SOD activity was calculated by measuring the decrease in the color development of samples at 440 nm with the reference to the xanthine oxidase/cithocrome method (Crapo et al., 1978). In particular the superoxide radical, generated from the conversion of xanthine to uric acid and H$_2$O$_2$ by xanthine oxidase, reacts with the tetrazolium salt WST-1 forming formazan. One unit of SOD was defined as the amount of enzyme required to produce 50% inhibition in the reaction system.

GST was calculated by measuring the changes in absorbance recorded at 340 nm due to the conjugation of glutathione to 1-chloro-2,4-dinitrobenzene (Habig et al., 1974). One unit of enzyme was the quantity necessary for the reduction of 1 µmol·L$^{-1}$ GSH in 1 min at 37°C.

Test runs were performed in triplicate with additional controls including on aqueous solutions containing hydrogen peroxide supplemented with catalase, used to destroy the residual hydrogen peroxide.

4. Results and discussion

4.1. UV$_{254}$ photolysis: kinetic investigation
The results collected from runs of UV$_{254}$ photolysis of ACY in aqueous solution at three different pH values (4.5, 6.0 and 8.0) in the MCF photoreactor at varying lamp power are reported in Figs. 1a-e as a function of the space time. The results indicate that, for a fixed lamp power, the pH did not affect the conversion. In fact, for these runs a half-time of about 17 seconds was recorded independent of the pH. Moreover, the analysis of the concentration vs time profile demonstrated that the photolysis of ACY resembled an apparent autocatalytic behavior which suggested the adoption of an autocatalytic kinetic model to describe the degradation of ACY under the adopted experimental conditions. Since the destruction of guanine based substrates under UV$_{254}$ irradiation has been ascribed to both the direct photolysis of guanine derivatives and the reaction of guanine based molecules with the radical species formed during the photolytic process (Crespo-Hernandez et al., 2000a,b), the simplified reaction scheme (Scheme 1) was considered for the UV$_{254}$ photolysis of ACY, which is a guanine derivative:

$$\text{ACY} \xrightarrow{\text{hv}} \phi \xrightarrow{k'} B$$

where B indicates a pseudo intermediate (hydrated electron, oxygen reactive species, etc.) capable of reacting with ACY molecules according to a simple autocatalytic-type kinetics. The quantum yield of photolysis of ACY at 254 nm ($\phi_{\text{ACY}}$) and the kinetic constant $k'$ were estimated through an iterative method, using simultaneously the concentration data reported in Figures 1a,e to solve ODE equations 1 and 2:

$$\frac{d[\text{ACY}]}{dt} = -P_o \phi_{\text{ACY}} \cdot \left(1 - \exp\left(-2.3.l_{\text{MCF}} \cdot \varepsilon_{254}^{\text{ACY}} [\text{ACY}]\right)\right) \cdot k' \cdot [\text{ACY}] \cdot [B]$$  \hspace{1cm} (1)
\[
\frac{d[B]}{dt} = P_o \phi_{ACY} \cdot \left(1 - \exp\left(-2.3 \cdot I_{MCF} \cdot \varepsilon_{254}^{ACY} \cdot [ACY]\right)\right)
\]  \hspace{1cm} (2)

Where \(t\) is the space time in the continuous flow MCF photoreactor (the reaction or exposure time) and the term \(\varepsilon_{254}^{ACY}\) is the molar absorption coefficient at 254 nm for ACY at pH 4.5, 6.0 and 8.0 (1.21 \times 10^{-2} \text{ M}^{-1} \cdot \text{cm}^{-1}). This result is in agreement with the pKa values of ACY (2.27 and 9.25) (Florence, 2010).

The MATLAB routine “ode45”, based on the Runge-Kutta method with adaptive step-size, was used for the optimization procedure which minimized the objective function

\[
\sum_{j}^{m} \sum_{i}^{n} (y_{ACY}^{j,i} - c_{ACY}^{j,i})^2,
\]

made by the squares of the differences between the calculated “\(y\)” and experimental “\(c\)” concentrations of ACY, varying the reaction time “\(n\)” and for different experimental photolytic runs “\(m\)”. The determined kinetic parameters that minimized the objective function were \(\phi_{ACY} = (1.62 \pm 0.07) \times 10^{-3} \text{ mol} \cdot \text{ein}^{-1}\) and \(k' = (5.64 \pm 0.03) \times 10^{-3} \text{ M}^{-1} \cdot \text{s}^{-1}\). The comparison between experimental and calculated data, reported in Figures 1a-e including the percentage standard deviations, demonstrated close prediction of the concentration profiles of ACY in the MCF photoreactor.

The \(\phi_{ACY}\) value reported above has the same order of magnitude as the quantum yield of photodecomposition of other guanine derivatives, such as guanosine and 9-ethyl-guanine at similar concentrations (Crespo-Hernandez et al., 2000a), thus suggesting that the purine structure could play a fundamental role in the UV photolysis of guanine derivatives. The differences could be ascribed to a slight effect of the nature of the group attached to the 9-N on the UV-photolysis kinetics.

4.2. \(UV_{254}/H_2O_2\) oxidation: kinetic investigation

The results of a preliminary run carried out in the presence of hydrogen peroxide under darkness indicated that ACY was not degraded in the presence of \(H_2O_2\) alone for reaction times up to 30 min.
Photooxidation experiments of ACY by the UV\textsubscript{254}/H\textsubscript{2}O\textsubscript{2} process were carried out under the same experimental conditions (i.e., pH, lamp power and initial concentration of ACY) used in the UV\textsubscript{254} direct photolysis runs.

The degradation profiles for ACY and H\textsubscript{2}O\textsubscript{2} as a function of space time in the MCF photoreactor were modeled on the basis of a simplified reaction scheme and the mass balances listed in Table 2. The reaction scheme considers the consumption of ACY and hydrogen peroxide by direct photolysis (reactions 3 and 4). Hydroxyl radicals generated by UV\textsubscript{254} photolysis of H\textsubscript{2}O\textsubscript{2} can react with hydrogen peroxide (reaction 5), ACY (reaction 6) and the transformation products (reaction 7). A radical termination of peroxyl radicals was considered in the mechanism (reaction 8).

The literature reports two different values of the the kinetic constant of the reaction between hydroxyl radical and ACY ($k_{\text{OH}/\text{ACY}}$): 5.0-10\textsuperscript{9} M\textsuperscript{-1}.s\textsuperscript{-1} (pH=9, T=18 °C, solar simulator $\lambda >$ 320 nm) (Prasse et al., 2015) and 1.19-10\textsuperscript{10} M\textsuperscript{-1}.s\textsuperscript{-1} (pH= 6-9, lamp $\lambda >$ 340 nm) (Zhou et al., 2015) which were determined with competition kinetics in the presence of a reference compound (i.e., acetophenone, Zhou et al., 2015, and p-chloro-benzoic acid, Prasse et al., 2015). Since these $k_{\text{OH}/\text{ACY}}$ values differed by more than 50%, $k_{\text{OH}/\text{ACY}}$ was determined using both numerical optimization and competition kinetics.

Specifically, the same iterative optimization procedure reported in section 4.1, using simultaneously a set of 9 photodegradation runs in distilled water, at different initial concentrations of ACY and hydrogen peroxide, pH and lamp power, was used for the estimation of $k_{\text{OH}/\text{ACY}}$. The iterative method minimized the objective function (Eq. 14) that in this case was slightly modified to include the number of the reacting species ($h$):

$$\Phi = \sum_{g} \sum_{j} \sum_{i} (y_{g,j,i} - c_{g,j,i})^2$$  \hspace{1cm} (14)

From this method $k_{\text{OH}/\text{ACY}}$ was determined as (1.23 ± 0.07)-10\textsuperscript{9} M\textsuperscript{-1}.s\textsuperscript{-1}. Graphical examples of the results obtained by the modeling through the optimization procedure are shown in Figures 2a-f.
(optimization procedure). In Figures 2g-i the comparison is reported between experimental and calculated residual ACY and H$_2$O$_2$ concentration, when the model was used in simulation mode without any further parameter adjustment (simulation mode), using the $k_{OH/ACY}$ kinetic constant above estimated. It can be noted a good capability of the model of predicting the experimental data under the adopted conditions.

Two additional UV$_{254}$/H$_2$O$_2$ runs (Figs. 2l-m) were carried out using synthetic wastewater to further validate the kinetic results obtained. The photolytic runs were simulated using the proposed kinetic model properly modified to include the HO radical scavenging effect of the species forming the synthetic matrix (Spasiano et al., 2016). For this purpose, the pseudo-first order rate constant ($k_{sca} = 4.01 \cdot 10^4$ s$^{-1}$) was considered for the reaction between the hydroxyl radicals and the scavenger species (Spasiano et al., 2016). Also in this case, a good capability of the model was still observed to predict the experimental data under the adopted conditions.

The competition kinetic method was used to estimate the $k_{OH/ACY}$ constant in the same MCF photoreactor, to further validate the kinetic model proposed above. The method compares the ACY concentration decay to that of benzoic acid (BA) (initial concentration $2.0 \cdot 10^{-5}$ M) chosen as reference compound (Onstein et al., 1999):

$$\frac{\text{Ln}([\text{ACY}])}{\text{Ln}([\text{BA}])} = \frac{k_{OH/ACY}}{k_{OH/BA}} \cdot \frac{[\text{BA}]}{[\text{ACY}]}$$

$$k_{OH/BA} = 5.9 \cdot 10^9 \text{M}^{-1} \cdot \text{s}^{-1}$$

(pH = 6.0)  

(15)

An average value $k_{OH/ACY} = (2.30 \pm 0.11) \cdot 10^9$ M$^{-1}$·s$^{-1}$ was thus calculated from UV$_{254}$/H$_2$O$_2$ experiments carried out at pH = 6.0 and [H$_2$O$_2$]/[ACY]$_o$ = 20 and at different lamp power (4.5 W and 8.0 W). The difference of this from the value estimated with kinetic modeling may be ascribed to the intrinsic limitations of the competition kinetics method that does not include the contribution of ACY consumption by direct photolysis. However, both $k_{OH/ACY}$ values estimated in the present investigation were significantly lower than those previously reported in the literature (Zhou et al., 2015; Prasse et al., 2015).
4.3. UV\textsubscript{254} photolysis and UV\textsubscript{254}/H\textsubscript{2}O\textsubscript{2} oxidation: Ecotoxicity assessment

A battery of ecotoxicity tests on \textit{V. fischeri}, \textit{D. magna} and \textit{R. subcapitata} were performed on untreated and treated aqueous solutions with an initial ACY concentration of 1.2 mg·L\textsuperscript{-1}. The results showed that the inhibition of \textit{V. fischeri} luminescence remained unchanged in the presence of the UV\textsubscript{254} and UV\textsubscript{254}/H\textsubscript{2}O\textsubscript{2} irradiated solutions, in comparison to the untreated solution (data not shown).

The results obtained for \textit{D. magna} (exposure time = 24 and 48 h) for the UV\textsubscript{254} and UV\textsubscript{254}/H\textsubscript{2}O\textsubscript{2} treated and untreated samples are reported in Figures 3A,B. The samples treated with UV\textsubscript{254} irradiation in the absence of hydrogen peroxide, initially showed an increase of immobility of daphnids at increasing UV\textsubscript{254} dose and consequently at higher ACY conversion, suggesting an increase in acute ecotoxicity, although, this eventually decreased significantly at the highest UV\textsubscript{254} dose. On the other hand, the acute ecotoxicity of the UV\textsubscript{254}/H\textsubscript{2}O\textsubscript{2} treated solutions toward \textit{D. magna} was significantly lower in comparison to the samples treated with UV\textsubscript{254} only, even at much lower UV doses. It is important to note that the acute ecotoxicity of the UV\textsubscript{254} sample after complete conversion of ACY was higher than the value for the un-irradiated control sample.

The inhibition growth of \textit{R. subcapitata} reached 32%, 13% and 20% at UV\textsubscript{254} doses of 864, 2356 and 4712 mJ·cm\textsuperscript{-2} respectively (Fig. 4), thus confirming an acute toxicological effect on the UV\textsubscript{254} only treated samples. In contrast, a small reduction of the inhibition growth was observed for the samples treated with UV\textsubscript{254}/H\textsubscript{2}O\textsubscript{2} at increasing UV\textsubscript{254} doses, which supported the beneficial effect of the H\textsubscript{2}O\textsubscript{2} assisted photolytic treatment for toxicity reduction.

The results showed an increase of the production of ROS in all samples, that could enhance the sublethal toxicity in daphnids. Aquatic organisms can in fact adapt to an increase of ROS production by upregulating the activity of their antioxidant enzymes, particularly of CAT and SOD which represent the first and the second line of defense against ROS (Oexle et al., 2016). An evident increase of ROS production in the daphnids treated with UV\textsubscript{254} only samples was observed in comparison to the those treated with the UV\textsubscript{254}/H\textsubscript{2}O\textsubscript{2} samples (Fig. 5A). The increase was
recorded for UVC doses of 864 and 2356 mJ·cm⁻² for the UV₂₅₄ process and at 280 mJ·cm⁻² for the samples treated with UV₂₅₄/H₂O₂.

The SOD activity resulted in significant alterations only for samples treated by UV₂₅₄ (Fig. 5B). The enzyme inhibition increased when the UVC dose was increased and reached the highest inhibition at 2356 mJ·cm⁻². No effect was observed in the samples treated with UV₂₅₄/H₂O₂ except for samples treated with a UVC dose of 280 mJ·cm⁻² (TOC removal degree: 28%).

Both processes led to a significant increase of CAT activity compared to the control (Fig. 5C), since CAT is responsible for the detoxification of high levels of hydrogen peroxide, one of the most important ROS producers under oxidative stress conditions.

On the contrary, GST activity remained unchanged or decreased with both treatments as shown in Figure 5D. Probably the response patterns may be species-specific in nature, while varying in intensity response. The antioxidant enzymes can maintain cellular redox balance, alleviate the toxicological effects of ROS and protect the cells against the oxidative damage of their structures including lipid, membranes, proteins and nucleic acids (Oropesa et al., 2017).

A 21 days chronic exposure experiment was performed to determine the toxicity of 100 fold diluted untreated and treated solutions. The effects of ACY (120 μg·L⁻¹) and its treated samples on D. magna reproduction and survival are reported in Figure 6A,B.

The results of chronic toxicity showed that the UV₂₅₄ treatment, even at such low concentrations of ACY, significantly decreased the survival of D. magna compared to the control group. A decrease of survival was further recorded for samples exposed at a TOC removal of less than 5% (ACY conversion degree: 45%), probably due the presence of unconverted ACY, and at UV₂₅₄ dose of 2356 mJ·cm⁻² (ACY conversion: 90%), due to the formation of first-generation-transformation by-products structurally similar to ACY. At higher UV₂₅₄ doses (4712 mJ·cm⁻², TOC removal ~ 5%), the survival percentage was similar to that of the control samples and always higher to that of the untreated sample. On the contrary, the ecotoxicity assessment for the UV₂₅₄/H₂O₂ treated solutions
reflected the results already recorded in the acute tests, revealing a marked reduction of chronic
toxic effects for the exposures of the daphnids to the UV$_{254}$/H$_2$O$_2$ samples, especially the highest
UV$_{254}$ doses (950 mJ·cm$^{-2}$, TOC removal 77% and 1900 mJ·cm$^{-2}$, TOC removal higher than 95%).
As reported in Table 3, the reproduction of *D. magna* was completely inhibited in the organisms
contacted with samples exposed to UV$_{254}$ doses of 864 mJ·cm$^{-2}$ and 2356 and in absence of H$_2$O$_2$.
These results revealed that all the endpoints were different than the control solutions with an
extended exposure to the treatment, thus confirming that the photoproducts formed during UV$_{254}$
irradiation of aqueous ACY solutions exerted significant chronic adverse effects to *D. magna* at the
population level. On the contrary, the total number of neonates and the number of first-brood were
not statistically different among the samples untreated and treated by UV$_{254}$/H$_2$O$_2$.
The different chemical species formed during the UV$_{254}$ and the UV$_{254}$/H$_2$O$_2$ photochemical
processes could reasonably explain the observed toxicological effects. To provide a preliminary
validation of this hypothesis, two samples, one from UV$_{254}$ photolysis and the second from
UV$_{254}$/H$_2$O$_2$ treatment, were directly analyzed with MS-spectrometer to identify the main chemical
intermediates formed, with the knowledge that a thorough identification of the transformation by-
products required more sophisticated diagnostic techniques (Buchberger, 2011).
A list of molecular structures of the main intermediates that could be attributed to some peaks
detected in the mass spectra for two samples is reported in Table 4. Some of the structures shown in
Table 4 correspond to the chemical intermediates previously detected and reported in literature. In
particular, for the UV$_{254}$ photolysis, the structures II, IV and V were observed during the
degradation of ACY by TiO$_2$ photocatalysis at 365 nm (An et al., 2015) whereas the by-products
VII and X proposed for UV$_{254}$/H$_2$O$_2$ were the same of those observed during the photooxidation of
ACY in phosphate buffer at wavelength higher than 270 nm (Iqbal et al., 2005). The attribution of
reliable structures to the remaining recorded MS signals not previously observed by others, needs
further analytical assessments. However, although an uncomplete analysis is available for the
products of degradation of ACY, the data collected indicated the presence of chemical species
significantly different in the two samples. In particular, UV$_{254}$/H$_2$O$_2$ process seems to lead mainly to the formation of hydroxylated imidazole-based compounds or species formed by the fragmentation of the pyrimidine ring whereas some hydroxylated ACY based intermediates are detected in the UV$_{254}$ treated sample.

5. Conclusion

The photodegradation of ACY was investigated under UV$_{254}$ irradiation in the absence and in the presence of hydrogen peroxide. A moderate rate of direct photolysis at 254 nm for ACY was observed with a quantum yield of $(1.62 \pm 0.073) \times 10^{-3}$ mol-ein$^{-1}$ in the pH range 4.5 – 8.0. An average value of $1.76 \times 10^9$ M$^{-1}$s$^{-1}$ was calculated for the kinetic constant of reaction between hydroxyl radical and ACY. Considering (i) the UV$_{254}$ doses typically used for the disinfection of municipal sewage treatment plant effluents, (ii) the concentration values of ACY measured in WWTP effluents, and (iii) the results collected during the kinetic and ecotoxicity assessment, the occurrence of residual photodecomposition by-products in treated effluents is very likely, and these are likely to have a high ecotoxicological index. However, the addition of appropriate amount of hydrogen peroxide during the UV$_{254}$ disinfection stage would reduce this risk.

The results obtained contribute to provide useful information for a vision about the fate of ACY during the UV$_{254}$ and UV$_{254}$/H$_2$O$_2$ treatment processes and the eventual associated risks for living organisms (animals and plants) in the aquatic environment.

The results collected confirm the use of oxidative stress biomarkers as promising tool in order to evaluate the toxicological effects of environmental pollutants as early indicators in ecotoxicology. Exposure to environmental pollutants may disrupt the balance of biological oxidant-to-antioxidant ratio in aquatic species leading to elevated levels of ROS and resulting in oxidative stress. A preliminary analysis on the treated samples indicated, as the main photo-transformation by-products, the presence of hydroxylated ACY based intermediates in the UV$_{254}$ treatment process,
and hydroxylated imidazole based compounds or species formed by the fragmentation of the pyrimidine ring in the UV_{254}/H_{2}O_{2} treatment process. Further efforts are required to identify the main photoproducts, to elucidate the mechanism of ACY photodegradation under UVC radiation and to evaluate possible cumulative effects of the different species occurring in STP effluents.

Acknowledgements

The Authors are grateful to ERASMUS-Mobility Student Program, and to Ing. Giulio Di Costanzo for his precious support during the experimental campaign.

References


Paris, France.


Organisation for Economic Cooperation and development (OECD), 1999. Guidelines for testing of chemicals, simulation test-aerobic sewage treatment, 303A.


Figure 1: Comparison between experimental (circle) and predicted (line) data for UV$_{254}$ photolysis of ACY at different pH and power of lamp in the MCF photoreactor. 

(a) pH = 6.0 (8.0 W); (b) pH = 4.0 (8.0 W); (c,d) pH = 6.0 (4.5 W); (e) pH = 8.5 (8.0 W).

Figure 2: Comparison between experimental (circle) and predicted (line) data for UV$_{254}$/H$_2$O$_2$ photodegradation of ACY (●) and hydrogen peroxide (○) in the MCF photoreactor at different pH, power of lamp and starting H$_2$O$_2$ load. Optimization mode (a-f), simulation mode (g-m).

(a): pH = 6.0 (8.0 W, [H$_2$O$_2$]/[ACY] = 20); (b): pH = 6.0 (8.0 W, [H$_2$O$_2$]/[ACY] = 50); (c): pH = 8.0 (8.0 W, [H$_2$O$_2$]/[ACY] = 50); (d): pH = 6.0 (4.5 W, [H$_2$O$_2$]/[ACY] = 50); (e): pH = 6.0 (4.5 W, [H$_2$O$_2$]/[ACY] = 70); (f): pH = 6.0 (4.5 W, [H$_2$O$_2$]/[ACY] = 100); (g): pH = 4.0 (8.0 W, [H$_2$O$_2$]/[ACY] = 100); (h): pH = 8.2 (8.0 W, [H$_2$O$_2$]/[ACY] = 100); (i): pH = 4.0 (4.5 W, [H$_2$O$_2$]/[ACY] = 20); (l) pH = 6.0 (8.0 W, [H$_2$O$_2$]/[ACY] = 60); (m) pH = 6.0 (8.0 W, [H$_2$O$_2$]/[ACY] = 142).

Figure 3: Evolution of acute toxicity with D. magna (24 h and 48h) during the UV$_{254}$ (A) and UV$_{254}$/H$_2$O$_2$ (B) treatments. Data with different letters (a-b) are significantly different (Tukey’s, p<0.05).

Figure 4: Toxicity data with R. subcapitata (72 h). Data with different letters (a–c) are significantly different (Tukey’s, p<0.05).

Figure 5: Effects of UV$_{254}$ and UV$_{254}$/H$_2$O$_2$ processes on (A) ROS production, (B) SOD, (C) Cat, (D) GST in Daphnia magna after 48 h of exposure. For each parameter, mean and standard deviation are shown. Data with different letters (a-d) are significantly different (Tukey’s, p<0.05).

*Ctr- (negative control)
Figure 6: Survival curves of *D. magna* during the time of exposure (21 days) for UV$_{254}$ (A) and UV$_{254}$/H$_2$O$_2$ (B) treated solutions. Data with different letters (a-b) are significantly different (Tukey’s, p<0.05). Dilution: 1:100.

Table 1: Occurrence of ACY in WWTP effluents and in surface waters.

Table 2: Reaction kinetics mechanism of ACY photoxidation by UV$_{254}$/H$_2$O$_2$ process and mass balance equations. The terms $f_{H_2O_2}$ and $f_{ACY}$ indicate the fraction of UV$_{254}$ radiation absorbed by hydrogen peroxide and ACY respectively. The TPs concentration was assumed equal to the amount of ACY consumed ([ACY]$_0$ – [ACY]).

$e^{H_2O_2}_{254}$ and $\phi^{H_2O_2}$ are the molar absorption coefficient and the quantum yield of photolysis of hydrogen peroxide at 254 nm respectively.

Table 3: First brood and live offspring after 21 days of *D. magna* exposure for different UV$_{254}$ doses (with and without hydrogen peroxide).

Table 4: Molecular structures of the chemical species identified from the MS spectra of samples submitted to UV$_{254}$ and UV$_{254}$/H$_2$O$_2$ photolysis.

© The structures proposed on the basis of the pseudo-molecular [M+H]$^+$ ion due to the low intensity of the MS/MS fragmentation signals.
Figure 1
Figure 2
Figure 3
Figure 4

[Diagram showing ACY conversion degree and inhibition of algal growth with UV-C doses.]

- UV$_{254}$
- UV$_{254}$/H$_2$O$_2$
Figure 5
Figure 6
- Photolysis and UV/H₂O₂ degradation of acyclovir were studied in a microphotoreactor
- UV₂₅⁴ photolysis quantum yield of acyclovir was estimated (1.62·10⁻³ mol·ein⁻¹)
- Kinetic constant of hydroxyl radical attack to acyclovir was evaluated
- H₂O₂ assisted photo-oxidation process reduces the ecotoxicity of acyclovir
Acyclovir

UV$_{254}$ lamps

Kinetics

$+\ H_2O_2$

Ecotoxicity
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Table 1
3) \( h_\nu \) 
\[ \text{ACY} \rightarrow \text{TPs} \] 
\[ \Phi_{\text{ACY}} \] (estimated in this study)

4) \( h_\nu \) 
\[ \text{H}_2\text{O}_2 \rightarrow 2\text{HO}^\bullet \] 
\[ \Phi_{\text{H}_2\text{O}_2} = 0.55 \text{ mol-ein}^{-1} \] (Goldstein et al., 2007)

5) \( \text{HO}^\bullet + \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{HO}_2^\bullet \) 
\[ k_h = 2.7 \cdot 10^7 \text{ M}^{-1} \cdot \text{s}^{-1} \] (Buxton et al., 1988)

6) \( \text{ACY} + \text{HO}^\bullet \rightarrow \text{TPs} \) 
\[ k_{\text{OH/ACY}} \] (estimated in this study)

7) \( \text{TPs} + \text{HO}^\bullet \rightarrow \text{TP} \) 
\[ k_{\text{OH/TP}} \] (estimated in this study)

8) \( 2\text{HO}_2^\bullet \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \) 
\[ k_t = 8.3 \cdot 10^5 \text{ M}^{-1} \cdot \text{s}^{-1} \] (Bielski et al., 1985)

9) 
\[ \frac{d[\text{HO}^\bullet]}{d\tau} = 2F_{\text{H}_2\text{O}_2} \cdot [\text{HO}^\bullet] \cdot \left( k_h \cdot [\text{H}_2\text{O}_2] - k_{\text{OH/ACY}} \cdot [\text{ACY}] - k_{\text{OH/TP}} \cdot [\text{TPs}] \right) \]

10) 
\[ F_{\text{H}_2\text{O}_2} = \Phi_{\text{H}_2\text{O}_2} \cdot P_o \cdot \left( 1 - \exp \left( -2.3 \cdot l_{\text{MCF}} \cdot (\varepsilon_{\text{ACY}, 254} \cdot [\text{ACY}] + \varepsilon_{\text{H}_2\text{O}_2, 254} \cdot [\text{H}_2\text{O}_2]) \right) \right) \cdot f_{\text{H}_2\text{O}_2} \]

11) 
\[ \frac{d[\text{HO}_2^\bullet]}{d\tau} = k_h \cdot [\text{HO}_2^\bullet] \cdot [\text{H}_2\text{O}_2] - 2k_t \cdot [\text{HO}_2]^2 \]

12) 
\[ \frac{d[\text{ACY}]}{d\tau} = -F_{\text{ACY}} - k_{\text{OH/ACY}} \cdot [\text{ACY}] \cdot [\text{HO}_2^\bullet] \]

13) 
\[ F_{\text{ACY}} = \Phi_{\text{ACY}} \cdot P_o \cdot \left( 1 - \exp \left( -2.3 \cdot l_{\text{MCF}} \cdot (\varepsilon_{\text{ACY}, 254} \cdot [\text{ACY}] + \varepsilon_{\text{H}_2\text{O}_2, 254} \cdot [\text{H}_2\text{O}_2]) \right) \right) \cdot f_{\text{ACY}} \]

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