Photodegradation and ecotoxicology of acyclovir in water under UV254 and UV254/H2O2 processes

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Article Type: Research Paper

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

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Abstract: The photochemical and ecotoxicological fate of acyclovir (ACY) through UV254 direct photolysis and in the presence of hydroxyl radicals (UV254/H2O2 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 [ACY] that further reacted with ACY to form by-products [k' = (5.64 ± 0.03) × 10^-3 M^-1s^-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 (kOH/ACY) determined with the kinetic modeling method was (1.23 ± 0.07) × 10^-3 M^-1s^-1 and with the competition kinetics method was (2.30 ± 0.11) × 10^-3 M^-1s^-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 UV254/H2O2 in comparison to those collected during UV254 treatment. This result suggests that the addition of moderate quantity of hydrogen peroxide (30-150 mg/L) might be a useful strategy to reduce the ecotoxicity of UV254 based sanitary engineered systems for water reclamation.
Dear Editor,

please find enclosed a copy of the paper “Photodegradation and ecotoxicology of acyclovir in water under UV$_{254}$ and UV$_{254}$/H$_2$O$_2$ processes” by Danilo Russo, Antonietta Siciliano, Marco Guida, Emilia Galdiero, Angela Amoresano, Roberto Andreozzi, Nuno M. Reis, Gianluca Li Puma and Raffaele Marotta, which has been revised according to the Referees’ suggestions (all the corrections and new insertions have been printed in red bold typefaces).

We thank the Reviewers for the valuable suggestions that helped us to ameliorate the text. Thank you again for your assistance and cooperation.

Napoli, 3/6/2017

Yours sincerely

The Authors
**Reviewer #1:** The study targets at evaluating the UV and UV/H₂O₂ treatment processes of the antiviral drug acyclovir. A microcapillary film array photoreactor was used to perform part of the experiments to reduce total volumes and time. At the same time 3 well-known bioassays were used to assess the effects of treated acyclovir solutions.

The manuscript is well written. The manuscript should be improved as follows.

**Criticism.** It is not clear how UV is a better treatment process than chlorination to overcome antibiotic resistant bacteria. The strengths and restrictions of the proposed treatment process should be clearly presented to better appreciate the study's findings.

**Response:** We thank the reviewer for the comment. We added some statements in the introduction to clarify this aspect

**Criticism.** A thorough literature review of research related to acyclovir, with the current state of the art as well as gaps-of-knowledge is currently missing from the introduction. Many studies have been done using acyclovir and UV₂⁵⁴ treatment.

**Response:** At the best of our knowledge, as stated in the introduction, no investigations have been reported on the photochemical transformation of ACY under UV₂⁵⁴ and UV₂⁵⁴/H₂O₂ treatments and on the ecotoxicological assessments on highly diluted treated solutions containing ACY. On the contrary, photodegradation pathways of ACY under artificial and natural solar light irradiation have been recently investigated (Zhou et al., Photolysis of three antiviral drugs acyclovir, zidovudine and lamivudine in surface freshwater and seawater. Chemosphere 138 (2015) 792–797; Prasse et al., Co-occurrence of photochemical and microbiological transformation processes in open-water unit process wetlands. Environmental Science & Technology 49 (2015) 14136–14145).

**Criticism.** The rationale for using the specific bioassays, except their wide use and acceptance should be justified.

**Response:** The rationale for using these bioassays is the key information that need to be collected to determine the efficiency of the system investigated. In particular, in order to identify adverse human health and to take into account the ecotoxicity of the treated samples, we decided to use not only bacteria and microcrustacean but also algae and chronic test with Daphnia magna. These data are important because the continuous release of pharmaceuticals in the aquatic environment also at low concentrations and the importance of sublethal effects in the biota should be considered.
**Criticism.** The analytical procedure should be described with greater detail. In some assays more toxic byproducts were observed. An attempt should be made to identify the toxic byproducts in order to increase the study's outcomes.

**Response:** As reported in several sections of the manuscript (abstract, introduction and conclusions), the present paper is a first investigation on the $UV_{254}/H_2O_2$ photodegradation kinetics of ACY in distilled water and on the ecotoxicity of highly diluted treated samples. The analytical analysis of the phototransformation products, which differ among them depending to presence or absence of hydrogen peroxide and the UV-dose adopted, is very complex and requires (i) an accurate investigation about the fragmentation of acyclovir and the intermediates formed during $UV_{254}$ and $UV_{254}/H_2O_2$ processes using different diagnostic techniques (HPLC-MS/MS, GC-MS and NMR); (ii) a separation of the main photoproducts in order to check their ecotoxicity. The results collected from a large experimental campaign, which requires several months of experimental work, would be reasonably discussed in another extended dedicated analytical paper. However, a very preliminary analysis on sample submitted to $UV_{254}$ and $UV_{254}/H_2O_2$ processes indicated, as 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 $UV_{254}/H_2O_2$ treatment process. The results have been inserted in the manuscript in order to support the different trends of ecotoxicity observed on the samples submitted to two photolytic processes.

It is obvious that major efforts will be necessary to better characterize the mixtures treated at different $UV_{254}$ doses.

**Criticism.** It is not clear how the results from the microcapillary reactor and the photoreactor are comparable. The advantages of the microcapillary reactor should be enhanced so that the reader can understand why both type of experiments were needed.

**Response:** A key benefit of the microcapillary flow photoreactor over the batch annular photoreactor is the possibility to carry out the process using very small quantities of reagents in a highly controlled environment (the statement has been introduced in the revised text). Consequently, as stated in the original version of the manuscript, the adoption of microphotoreactor technology is particularly appropriate for investigations on such highly priced or scarcely available commercial substances, including illicit drugs or selected pharmaceuticals. On the contrary, as discussed in section 3.2, a cylindrical batch photoreactor (0.480 L) was used to provide sufficient sample volumes to carry out the ecotoxicity tests and the chemical intermediates analysis at varying treatment times.
Criticism. The experiments should be also performed using real or simulated environmental matrices in order to increase their environmental relevance.

Response: The kinetics of photodegradation of an organic substrate in real matrices (urban wastewater effluent, surface water, etc) are highly influenced by their composition (i.e. nature and concentration of the inorganic and organic species, pH, etc.). These variables change depending on the nature of the influent and the type of the physico-chemical treatment adopted. In the present paper we investigated the kinetics of acyclovir under UV$_{254}$ irradiation in distilled water.

For toxicity tests, neither real nor other simulated environmental matrices were used, considering the large number of chemicals in an effluent and the possibility of interactions (e.g. synergy or additivity) between them and the byproducts of treated solutions.

Moreover, as stated in the conclusions of the manuscript (original version), further efforts are required to evaluate possible cumulative effects of the different species occurring in STP effluents. For this reason, these effects cannot be evaluated and consequently generalized using a single real matrix. However, some additional runs performed in synthetic wastewater were carried out and the results inserted in the text (figures 2l-m) in order to validate the kinetic model proposed.

Criticism. It is not clear what was the initial concentration of acyclovir when the microcapillary experiments were performed.

Response: Required information was added in section 3.1. of the manuscript.

Criticism. No explanation on the statistical analysis of results are presented. For instance the LOD/LOQ, the deviation, the QC/QA are not described in detail.

Response: The required control data were reported in the text and referred to the pertinent paragraphs.

Criticism. The discussion section could be augmented in context and length to increment the study's findings.

Response: Following the reviewer’s suggestion, we added additional information about the mineralization degree, the photodegradation of ACY in synthetic effluents and the proposed structures of main by-products.

Criticism. The methods for evaluating the biochemical endpoints could be described in greater detail. Apart from this, reference on the method used should be given.
Response: Additional information have been added as suggested by the reviewer.

Reviewer #2: The manuscript WR38815 presents an interesting research work related to the photodegradation and ecotoxicology of acyclovir in water under UV$^{254}$ and UV$^{254}$/H$_2$O$_2$ processes. The information gathered in this work is relevant for the scientific community, therefore, the manuscript can be accepted for WR after minor revisions.

Criticism. The standard errors of the kinetic constants must have only one significant number (example: 1.62+-0.07; 5.64+-0.03; 2.3+-0.1; 1.23+-0.07).
Response: Done

Criticism. Please define all the variables used in the kinetic models and include units.
Response: The photon fluxes per unit volume emitted by the UV lamp ($P_o$) and the MCF average optical path length ($l_{MCF}$) have been defined in section 3.1 of the original version of the manuscript (lines 132-136). The term $\varepsilon^{\text{ACY}}_{254}$, as reported in the manuscript (original version, lines 253-254), is the molar absorption coefficient at 254 nm for ACY at pH 4.5, 6.0 and 8.0 ($1.21\cdot10^{-2} \text{ M}^{-1}\text{cm}^{-1}$), whereas $\phi_{\text{ACY}}$ and $k'$ are the quantum yield of photolysis of ACY at 254 nm (lines 248-249) and the kinetic constant for reaction in scheme 1 (lines 249). The respective values and units are reported at lines 260-261 (original version).
Additional information has been added in the revised version of the paper (caption for table 2).

Criticism. Please include the kinetics of acyclovir degradation in a real matrix (urban wastewater after secondary treatment).
Response: We thank the reviewer for the suggestion, however, on the basis of our experience, the kinetics of the direct and indirect photodegradation of an organic substrate in real matrices (urban wastewater effluent, surface water, etc) are strongly affected by the nature and the concentration of the inorganic and organic substances contained. These variables change depending on the nature of the influent and the type of the physico-chemical treatment adopted. The present paper is a first preliminary investigation on the kinetics of acyclovir under UV$^{254}$ irradiation in distilled water. Moreover, as stated in the conclusions of the manuscript (original version), further efforts are required to evaluate possible cumulative effects of the different species occurring in STP effluents. For these reasons, these effects cannot be evaluated and consequently generalized using a single real matrix.
Criticism. Please include some information about the mineralization of acyclovir and degradation by-products.

Response: The analytical analysis of the phototransformation products, which differ among them depending on presence or absence of hydrogen peroxide and the UV-dose adopted, is very complex and it requires (i) an accurate investigation about the fragmentation of acyclovir and the intermediates formed during UV$_{254}$ and UV$_{254}$/H$_2$O$_2$ processes using different diagnostic techniques (HPLC-MS/MS, GC-MS and NMR); (ii) a separation of the main photoproducts in order to check their ecotoxicity. The results collected from a large experimental campaign, which requires several months of experimental work, would be reasonably discussed in another extended dedicated analytical paper. However, a very preliminary analysis on treated samples (reported in the revised version of the manuscript) indicated, as 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. Moreover, TOC removal degrees were inserted in table 3 and in the revised text.
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
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
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 ($\phi_{ACY} = (1.62 \pm 0.07) \times 10^{-3}$ mol-ein$^{-1}$) that further reacted with ACY to form by-products ($k' = (5.64 \pm 0.03) \times 10^{-3}$ M$^{-1}$·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 $(1.23 \pm 0.07) \times 10^{9}$ M$^{-1}$·s$^{-1}$ and with the competition kinetics method was $(2.30 \pm 0.11) \times 10^{9}$ M$^{-1}$·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 UV$_{254}$/H$_2$O$_2$ in comparison to those collected during UV$_{254}$ treatment. This result suggests that the addition of moderate quantity of hydrogen peroxide (30-150 mg·L$^{-1}$) might be a useful strategy to reduce the ecotoxicity of 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, $\lambda < 280 \text{ nm}$) 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; Hijnens 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 H₂O), 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), K₂HPO₄ (28 ppm), CaCl₂·H₂O (4 ppm), NaCl (7 ppm) and Mg₂SO₄ (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 HClO₄ 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 (N₂) 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\textsubscript{254} and UV\textsubscript{254}/H\textsubscript{2}O\textsubscript{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\textsubscript{254} transparent microcapillaries of fluorinated polymer characterized by a mean hydraulic diameter of 195 \( \mu \)m. 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 (\( \sim 25 \) °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\cdot10^{-4} \text{ L}\cdot\text{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\cdot10^{-5} \text{ mol}\cdot\text{L}^{-1} and 4.67\cdot10^{-5} \text{ mol}\cdot\text{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\textsubscript{2}O\textsubscript{2} actinometry (Nicole et al, 1990; Goldstein et al., 2007), were 1.92\cdot10^{-2} \text{ ein-(s} \cdot\text{L)}^{-1} (nominal power 8.0 W) and 1.27\cdot10^{-2} \text{ ein-(s} \cdot\text{L)}^{-1} (nominal power 4.5 W). The MCF average optical path length \((l_{MCF})\) was 154 \( \mu \)m. 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 \((V_b = 0.480 \text{ L})\), 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 *K₂Cr₂O₇* (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 *K₂Cr₂O₇* (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 determinate 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 xantine 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:

\[
\text{ACY} \xrightarrow{hv} \phi \xrightarrow{k'} B \\
\text{By-products}
\]

\[
\text{Scheme 1}
\]

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 ($\phi_{ACY}$) 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:

\[
\frac{d[ACY]}{dt} = -P_o \cdot \phi_{ACY} \cdot (1 - \exp(-2.3 \cdot l\text{MCF} \cdot e_{254} \cdot [ACY])) - k' \cdot [ACY] \cdot [B]
\]
\[
\frac{d[B]}{dt} = P_o \cdot \phi_{ACY} \cdot (1 - \exp(-2.3 \cdot l_{MCF} \cdot \varepsilon_{254}^{ACY} \cdot [ACY]))
\]  
(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 \((1.21 \cdot 10^{-2} \text{ M}^{-1} \cdot \text{cm}^{-1})\). 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 \[\sum_j \sum_i \left(y_{ACY,j,i} - c_{ACY,j,i}\right)^2,\] 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 \( \phi_{ACY} = (1.62 \pm 0.07) \cdot 10^{-3} \text{ mol-ein}^{-1} \) and \( k' = (5.64 \pm 0.03) \cdot 10^{-3} \text{ M}^{-1} \cdot \text{s}^{-1} \). 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 \( UV_{254}/H_2O_2 \) 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 kinetic constant of the reaction between hydroxyl radical and ACY ($k_{OH/ACY}$): $5.0 \times 10^9$ M$^{-1}$·s$^{-1}$ (pH=9, T=18 °C, solar simulator $\lambda$> 320 nm) (Prasse et al., 2015) and $1.19 \times 10^{10}$ M$^{-1}$·s$^{-1}$ (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 $k_{OH/ACY}$ values differed by more than 50%, $k_{OH/ACY}$ 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 $k_{OH/ACY}$. The iterative method minimized the objective function (Eq. 14) that in this case was slightly modified to include the number of the reacting species ($h$):

$$\Phi = \sum_{g}^{h} \sum_{j}^{m} \sum_{i}^{n} (y_{g,j,i} - c_{g,j,i})^2$$ (14)

From this method $k_{OH/ACY}$ was determined as $(1.23 \pm 0.07) \times 10^9$ M$^{-1}$·s$^{-1}$. 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 ($k'_{sca} = 4.01 \cdot 10^{-1} \text{ s}^{-1}$) 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):

$$\ln \left( \frac{[ACY]}{[ACY]_0} \right) = k_{OH/ACY} \cdot \frac{[BA]}{[BA]_0}$$

$$k_{OH/BA} = 5.9 \cdot 10^{9} \text{ M}^{-1} \cdot \text{s}^{-1} \quad (\text{pH} = 6.0) \quad (15)$$

An average value $k_{OH/ACY} = (2.30 \pm 0.11) \cdot 10^{9} \text{ M}^{-1} \cdot \text{s}^{-1}$ was thus calculated from UV$_{254}$/H$_2$O$_2$ experiments carried out at pH = 6.0 and [H$_2$O$_2$]/[ACY]$_0 = 20$ 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$_{254}$ photolysis and UV$_{254}$/H$_2$O$_2$ oxidation: Ecotoxicity assessment
A battery of ecotoxicity tests on *V. fischeri*, *D. magna* and *R. subcapitata* were performed on untreated and treated aqueous solutions with an initial ACY concentration of 1.2 mg·L\(^{-1}\). The results showed that the inhibition of *V. fischeri* luminescence remained unchanged in the presence of the UV\(_{254}\) and UV\(_{254}/\text{H}_2\text{O}\) irradiated solutions, in comparison to the untreated solution (data not shown).

The results obtained for *D. magna* (exposure time = 24 and 48 h) for the UV\(_{254}\) and UV\(_{254}/\text{H}_2\text{O}_2\) treated and untreated samples are reported in Figures 3A,B. The samples treated with UV\(_{254}\) irradiation in the absence of hydrogen peroxide, initially showed an increase of immobility of daphnids at increasing UV\(_{254}\) dose and consequently at higher ACY conversion, suggesting an increase in acute ecotoxicity, although, this eventually decreased significantly at the highest UV\(_{254}\) dose. On the other hand, the acute ecotoxicity of the UV\(_{254}/\text{H}_2\text{O}_2\) treated solutions toward *D. magna* was significantly lower in comparison to the samples treated with UV\(_{254}\) only, even at much lower UV doses. It is important to note that the acute ecotoxicity of the UV\(_{254}\) sample after complete conversion of ACY was higher than the value for the un-irradiated control sample.

The inhibition growth of *R. subcapitata* reached 32%, 13% and 20% at UV\(_{254}\) doses of 864, 2356 and 4712 mJ·cm\(^{-2}\) respectively (Fig. 4), thus confirming an acute toxicological effect on the UV\(_{254}\) only treated samples. In contrast, a small reduction of the inhibition growth was observed for the samples treated with UV\(_{254}/\text{H}_2\text{O}_2\) at increasing UV\(_{254}\) doses, which supported the beneficial effect of the \text{H}_2\text{O}_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\(_{254}\) only samples was observed in comparison to the those treated with the UV\(_{254}/\text{H}_2\text{O}_2\) samples (Fig. 5A). The increase was
recorded for UVC doses of 864 and 2356 mJ-cm\(^{-2}\) for the UV\(_{254}\) process and at 280 mJ-cm\(^{-2}\) for the samples treated with UV\(_{254}/H_2O_2\).

The SOD activity resulted in significant alterations only for samples treated by UV\(_{254}\) (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 UV\(_{254}/H_2O_2\) 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 \(\mu g\cdot L^{-1}\)) and its treated samples on \(D.\ magn\)a reproduction and survival are reported in Figure 6A,B.

The results of chronic toxicity showed that the UV\(_{254}\) 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 UV\(_{254}\) 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 UV\(_{254}\) 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 UV\(_{254}/H_2O_2\) 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.

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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 ($\phi_{ACY} = (1.62 \pm 0.07) \cdot 10^{-3}$ mol-ein$^{-1}$) that further reacted with ACY to form by-products ($k' = (5.64 \pm 0.03) \cdot 10^{-3}$ M$^{-1}$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 $(1.23 \pm 0.07) \cdot 10^{9}$ M$^{-1}$s$^{-1}$ and with the competition kinetics method was $(2.30 \pm 0.11) \cdot 10^{9}$ M$^{-1}$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 UV$_{254}$/H$_2$O$_2$ in comparison to those collected during UV$_{254}$ treatment. This result suggests that the addition of moderate quantity of hydrogen peroxide (30-150 mg·L$^{-1}$) might be a useful strategy to reduce the ecotoxicity of 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
(Khan et al., 2016) 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, \( \lambda < 280 \text{ nm} \)) 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 (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\textsubscript{254} and UV\textsubscript{254}/H\textsubscript{2}O\textsubscript{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\textsubscript{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), K₂HPO₄ (28 ppm), CaCl₂·H₂O (4 ppm), NaCl (7 ppm) and MgSO₄ (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 HClO₄ 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 (N₂) 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 UV₂₅₄ and UV₂₅₄/H₂O₂ 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\textsubscript{254} transparent microcapillaries of fluorinated polymer characterized by a mean hydraulic diameter of 195 \(\mu\text{m}\). 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 (\(\sim\)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\(\times\)10\textsuperscript{-4} L·min\textsuperscript{-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\(\times\)10\textsuperscript{-5} mol·L\textsuperscript{-1} and 4.67\(\times\)10\textsuperscript{-5} mol·L\textsuperscript{-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\textsubscript{2}O\textsubscript{2} actinometry (Nicole et al, 1990; Goldstein et al., 2007), were 1.92\(\times\)10\textsuperscript{-2} ein·(s·L\textsuperscript{-1}) (nominal power 8.0 W) and 1.27\(\times\)10\textsuperscript{-2} ein·(s·L\textsuperscript{-1}) (nominal power 4.5 W). The MCF average optical path length \((l_{MCF})\) was 154 \(\mu\text{m}\). 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 \((V_b = 0.480 \text{ L})\), 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\textsubscript{254} fluence). The UV\textsubscript{254} dose (mJ·cm\textsuperscript{-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\textsuperscript{-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\textsubscript{50}) with 95% confidence limit values. Otherwise, toxicity was expressed as percentage of effect (PE, %).

3.3.1. Organisms maintenance and monitoring

Freeze-dried \textit{Vibrio fischeri} (strain NRRL-B-11177) cells were reconstituted with reagent diluent at 4 °C. \textit{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). \textit{Daphnia magna} were cultured at 20 ± 1 °C, with a 16:8 light/dark photoperiod in ISO water (ISO, 2012).

Luminescence \textit{V. fischeri} measurements were performed with Microtox\textsuperscript{®} 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.

\textit{R. subcapitata} density was determined by an indirect procedure using a spectrophotometer (Hach Lange DR5000) and cuvette (5 cm). \textit{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 \textit{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\textsuperscript{-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 \textit{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 \textit{V. fischeri} were carried out with C\textsubscript{6}H\textsubscript{4}Cl\textsubscript{2}O (EC\textsubscript{50} = 4.1± 2.2 mg·L\textsuperscript{-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 old) 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 determinate 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 xantine oxidase/cithocrome method (Crapo et al., 1978). In particular
the superoxide radical, generated from the conversion of xanthisme 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:

\[
\begin{align*}
\text{ACY} & \xrightarrow{\text{hv}} B \\
& \xrightarrow{k'} B \\
\text{By-products}
\end{align*}
\]

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 ($\Phi_{ACY}$) 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:

\[
\frac{d[ACY]}{dt} = -P_o \cdot \Phi_{ACY} \cdot (1 - \exp(-2.3 \cdot l_{MCF} \cdot \varepsilon_{254} \cdot [ACY])) - k' \cdot [ACY] \cdot [B] \tag{1}
\]
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$^{-2}$ M$^{-1}$·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 $\phi_{ACY} = (1.62 \pm 0.07) \times 10^{-3}$ mol·ein$^{-1}$ and $k' = (5.64 \pm 0.03) \times 10^{-3}$ M$^{-1}$·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$_2$O$_2$ 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$_2$O$_2$ alone for reaction times up to 30 min. Photooxidation experiments of ACY by the UV$_{254}$/H$_2$O$_2$ process were carried out under the same conditions, and the results are shown in Figures 1a-e.
experimental conditions (i.e., pH, lamp power and initial concentration of ACY) used in the UV254 direct photolysis runs.

The degradation profiles for ACY and H₂O₂ 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 UV₂₅₄ photolysis of H₂O₂ 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 kinetic constant of the reaction between hydroxyl radical and ACY (kₐₒ/ₐᶜ): 5.0·10⁹ M⁻¹·s⁻¹ (pH=9, T=18 °C, solar simulator λ>320 nm) (Prasse et al., 2015) and 1.19·10¹⁰ M⁻¹·s⁻¹ (pH=6-9, lamp λ>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 kₐₒ/ₐᶜ values differed by more than 50%, kₐₒ/ₐᶜ 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 kₐₒ/ₐᶜ. 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):

\[ \Phi = \sum_{g} \sum_{j} \sum_{i} (y_{g,i} - c_{g,i,i})^2 \]  

From this method kₐₒ/ₐᶜ was determined as (1.23 ± 0.07)·10⁹ M⁻¹·s⁻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 ($k_{sca} = 4.01 \cdot 10^{-1}$ 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 2.0·10$^{-5}$ M) chosen as reference compound (Onstein et al., 1999):

$$\frac{\text{Ln}\left(\frac{[\text{ACY}]}{[\text{ACY}]_0}\right)}{\text{Ln}\left(\frac{[\text{BA}]}{[\text{BA}]_0}\right)} = \frac{k_{OH/ACY}}{k_{OH/BA}}$$

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]$_0$ = 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$_{254}$ photolysis and UV$_{254}$/H$_2$O$_2$ oxidation: Ecotoxicity assessment
A battery of ecotoxicity tests on *V. fischeri*, *D. magna* and *R. subcapitata* were performed on untreated and treated aqueous solutions with an initial ACY concentration of 1.2 mg·L$^{-1}$. The results showed that the inhibition of *V. fischeri* luminescence remained unchanged in the presence of the UV$_{254}$ and UV$_{254}$/H$_2$O$_2$ irradiated solutions, in comparison to the untreated solution (data not shown).

The results obtained for *D. magna* (exposure time = 24 and 48 h) for the UV$_{254}$ and UV$_{254}$/H$_2$O$_2$ treated and untreated samples are reported in Figures 3A,B. The samples treated with UV$_{254}$ irradiation in the absence of hydrogen peroxide, initially showed an increase of immobility of daphnids at increasing UV$_{254}$ dose and consequently at higher ACY conversion, suggesting an increase in acute ecotoxicity, although, this eventually decreased significantly at the highest UV$_{254}$ dose. On the other hand, the acute ecotoxicity of the UV$_{254}$/H$_2$O$_2$ treated solutions toward *D. magna* was significantly lower in comparison to the samples treated with UV$_{254}$ only, even at much lower UV doses. It is important to note that the acute ecotoxicity of the UV$_{254}$ sample after complete conversion of ACY was higher than the value for the un-irradiated control sample.

The inhibition growth of *R. subcapitata* reached 32%, 13% and 20% at UV$_{254}$ doses of 864, 2356 and 4712 mJ·cm$^{-2}$ respectively (Fig. 4), thus confirming an acute toxicological effect on the UV$_{254}$ only treated samples. In contrast, a small reduction of the inhibition growth was observed for the samples treated with UV$_{254}$/H$_2$O$_2$ at increasing UV$_{254}$ doses, which supported the beneficial effect of the H$_2$O$_2$ 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$_{254}$ only samples was observed in comparison to the those treated with the UV$_{254}$/H$_2$O$_2$ samples (Fig. 5A). The increase was
recorded for UVC doses of 864 and 2356 mJ-cm$^{-2}$ for the \( \text{UV}_\text{254} \) process and at 280 mJ-cm$^{-2}$ for the samples treated with \( \text{UV}_\text{254}/\text{H}_2\text{O}_2 \).

The SOD activity resulted in significant alterations only for samples treated by \( \text{UV}_\text{254} \) (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 \( \text{UV}_\text{254}/\text{H}_2\text{O}_2 \) 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 \( D. \text{magna} \) reproduction and survival are reported in Figure 6A,B.

The results of chronic toxicity showed that the \( \text{UV}_\text{254} \) treatment, even at such low concentrations of ACY, significantly decreased the survival of \( D. \text{magna} \) 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 \( \text{UV}_\text{254} \) 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 \( \text{UV}_\text{254} \) 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 \( \text{UV}_\text{254}/\text{H}_2\text{O}_2 \) 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\textsubscript{254}/H\textsubscript{2}O\textsubscript{2} samples, especially the highest
UV\textsubscript{254} doses (950 mJ cm\textsuperscript{-2}, TOC removal 77% and 1900 mJ cm\textsuperscript{-2}, TOC removal higher than 95%).

As reported in Table 3, the reproduction of \textit{D. magna} was completely inhibited in the organisms
contacted with samples exposed to UV\textsubscript{254} doses of 864 mJ cm\textsuperscript{-2} and 2356 and in absence of H\textsubscript{2}O\textsubscript{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\textsubscript{254}
irradiation of aqueous ACY solutions exerted significant chronic adverse effects to \textit{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\textsubscript{254}/H\textsubscript{2}O\textsubscript{2}.

The different chemical species formed during the UV\textsubscript{254} and the UV\textsubscript{254}/H\textsubscript{2}O\textsubscript{2} photochemical
processes could reasonably explain the observed toxicological effects. To provide a preliminary
validation of this hypothesis, two samples, one from UV\textsubscript{254} photolysis and the second from
UV\textsubscript{254}/H\textsubscript{2}O\textsubscript{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\textsubscript{254} photolysis, the structures II, IV and V were observed during the
degradation of ACY by TiO\textsubscript{2} photocatalysis at 365 nm (An et al., 2015) whereas the by-products
VII and X proposed for UV\textsubscript{254}/H\textsubscript{2}O\textsubscript{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**


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


<table>
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<tr>
<th>WWTP effluent (ng/L)</th>
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<th>Location</th>
<th>Ref</th>
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<td>27.3 – 53.3</td>
<td>2.2 - 190</td>
<td>Germany</td>
<td>(Prasse et al., 2010)</td>
</tr>
<tr>
<td>121 - 148</td>
<td>5 - 25</td>
<td>Germany</td>
<td>(Prasse et al., 2011)</td>
</tr>
<tr>
<td>44.0 - 650</td>
<td>--</td>
<td>Germany</td>
<td>(Funke et al., 2016)</td>
</tr>
<tr>
<td>114 - 205</td>
<td>8.9 – 112.6</td>
<td>China</td>
<td>(Peng et al., 2014)</td>
</tr>
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<td>12 - 50</td>
<td>10 - 23</td>
<td>Japan</td>
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<td>154</td>
<td>--</td>
<td>USA</td>
<td>(McCurry et al., 2014)</td>
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</table>

Table 1
3) \[ \text{ACY} \xrightarrow{h\nu} \text{TPs} \quad \varnothing_{\text{ACY}} \quad (\text{estimated in this study}) \]

4) \[ \text{H}_2\text{O}_2 \xrightarrow{h\nu} 2\text{HO}^* \quad \varnothing_{\text{H}_2\text{O}_2} = 0.55 \text{ mol} \cdot \text{e}^{-1} \quad \varepsilon_{254}^\text{H}_2\text{O}_2 = 18.6 \text{ M}^{-1} \cdot \text{cm}^{-1} \quad (\text{Goldstein et al., 2007}) \]

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

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

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

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

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

10) \[ F_{\text{H}_2\text{O}_2} = \varnothing_{\text{H}_2\text{O}_2} \cdot P_0 \cdot \left(1 - \exp\left(-2.3 \cdot l_{\text{MCF}} \cdot \left(\varepsilon_{254}^{\text{ACY}} \cdot [\text{ACY}] + \varepsilon_{254}^{\text{H}_2\text{O}_2} \cdot [\text{H}_2\text{O}_2]\right)\right)\right) \cdot f_{\text{H}_2\text{O}_2} \]

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

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

13) \[ F_{\text{ACY}} = \varnothing_{\text{ACY}} \cdot P_0 \cdot \left(1 - \exp\left(-2.3 \cdot l_{\text{MCF}} \cdot \left(\varepsilon_{254}^{\text{ACY}} \cdot [\text{ACY}] + \varepsilon_{254}^{\text{H}_2\text{O}_2} \cdot [\text{H}_2\text{O}_2]\right)\right)\right) \cdot f_{\text{ACY}} \]

Table 2
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<th>Sample</th>
<th>First brood (day)</th>
<th>Number of Living offspring per parent animal</th>
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<tr>
<td>Control solution</td>
<td>8</td>
<td>78 ± 5</td>
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<tr>
<td>$\text{UV}_{254}$ dose: 0 mJ·cm$^{-2}$</td>
<td>10</td>
<td>72 ± 3</td>
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<tr>
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<td>42 ± 3</td>
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<td>$\text{TOC removal degree: ~ 5%}$</td>
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<td>$\text{TOC removal degree: 28 %}$</td>
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Table 3
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Table 4
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
**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$_2$]$_o$/[ACY]$_o$ = 20); (b): pH = 6.0 (8.0 W, [H$_2$O$_2$]$_o$/[ACY]$_o$ = 50);  
(c): pH = 8.0 (8.0 W, [H$_2$O$_2$]$_o$/[ACY]$_o$ = 50); (d): pH = 6.0 (4.5 W, [H$_2$O$_2$]$_o$/[ACY]$_o$ = 50);  
(e): pH = 6.0 (4.5 W, [H$_2$O$_2$]$_o$/[ACY]$_o$ = 70); (f): pH = 6.0 (4.5 W, [H$_2$O$_2$]$_o$/[ACY]$_o$ = 100);  
(g): pH = 4.0 (8.0 W, [H$_2$O$_2$]$_o$/[ACY]$_o$ = 100); (h): pH = 8.2 (8.0 W, [H$_2$O$_2$]$_o$/[ACY]$_o$ = 100);  
(i): pH = 4.0 (4.5 W, [H$_2$O$_2$]$_o$/[ACY]$_o$ = 20); (l) pH = 6.0 (8.0 W, [H$_2$O$_2$]$_o$/[ACY]$_o$ = 60);  
(m) pH = 6.0 (8.0 W, [H$_2$O$_2$]$_o$/[ACY]$_o$ = 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\textsubscript{254} (A) and UV\textsubscript{254}/H\textsubscript{2}O\textsubscript{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\textsubscript{254}/H\textsubscript{2}O\textsubscript{2} process and mass balance equations. The terms $f_{H_2O_2}$ and $f_{ACY}$ indicate the fraction of UV\textsubscript{254} radiation absorbed by hydrogen peroxide and ACY respectively. The TPs concentration was assumed equal to the amount of ACY consumed ($[ACY]_0 - [ACY]$).

$\epsilon_{254}^{H_2O_2}$ 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\textsubscript{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\textsubscript{254} and UV\textsubscript{254}/H\textsubscript{2}O\textsubscript{2} photolysis.

$^{(\circ)}$ The structures proposed on the basis of the pseudo-molecular [M+H]$^+$ ion due to the low intensity of the MS/MS fragmentation signals.