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

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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 ($[^1\text{ACY}] = (1.62 \pm 0.07) \times 10^{-3} \text{ mol} \cdot \text{ein}^{-1}$) that further reacted with ACY to form by-products ($k' = (5.64 \pm 0.03) \times 10^{-3} \text{ M}^{-1} \cdot \text{s}^{-1}$). The photolysis of ACY in the presence of hydrogen peroxide accelerated the removal of ACY as a result of formation of hydroxyl radicals. The kinetic constant for the reaction of OH radicals with ACY ($k_{\text{OH/ACY}}$) determined with the kinetic modeling method was $(1.23 \pm 0.07) \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ and with the competition kinetics method was $(2.30 \pm 0.11) \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ with competition kinetics. The acute and chronic effects of the treated aqueous mixtures on different living organisms (*Vibrio fischeri*, *Raphidocelis subcapitata*, *D. magna*) revealed significantly lower toxicity for the samples treated with UV254/H2O2 in comparison to those collected during UV254 treatment. This result suggests that the addition of moderate quantity of hydrogen peroxide (30–150 $\text{mg} \cdot \text{L}^{-1}$) 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_2O_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 UV₂₅₄/H₂O₂ 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 UV₂₅₄ and UV₂₅₄/H₂O₂ 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 UV₂₅₄ and UV₂₅₄/H₂O₂ processes indicated, as main photo-transformation by-products the presence of hydroxylated ACY based intermediates in the UV₂₅₄ treatment process, and hydroxylated imidazole based compounds or species formed by the fragmentation of the pyrimidine ring in the UV₂₅₄/H₂O₂ 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 UV₂₅₄ 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 UV₂₅₄ 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_2O_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 ϵ_{254}^{ACY} , 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 ($1.21 \cdot 10^{-2} M^{-1} \cdot cm^{-1}$), whereas ϕ_{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 to 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 UV₂₅₄ and UV₂₅₄/H₂O₂ 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 UV₂₅₄ treatment process, and hydroxylated imidazole based compounds or species formed by the fragmentation of the pyrimidine ring in the UV₂₅₄/H₂O₂ 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 ($1.62 \cdot 10^{-3} \text{ mol} \cdot \text{ein}^{-1}$)
- Kinetic constant of hydroxyl radical attack to acyclovir was evaluated
- H₂O₂ assisted photo-oxidation process reduces the ecotoxicity of acyclovir

Acyclovir

UV₂₅₄ lamps

Kinetics

Ecotoxicity

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

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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 ($\phi_{ACY} = (1.62 \pm 0.07) \cdot 10^{-3} \text{ mol-ein}^{-1}$) that further reacted with ACY to form by-products ($k' = (5.64 \pm 0.03) \cdot 10^{-3} \text{ M}^{-1} \cdot \text{s}^{-1}$). The photolysis of ACY in the presence of hydrogen peroxide accelerated the removal of ACY as a result of formation of hydroxyl radicals. The kinetic constant for the reaction of OH radicals with ACY ($k_{OH/ACY}$) determined with the kinetic modeling method was $(1.23 \pm 0.07) \cdot 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ and with the competition kinetics method was $(2.30 \pm 0.11) \cdot 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ with competition kinetics. The acute and chronic effects of the treated aqueous mixtures on different living organisms (*Vibrio fischeri*, *Raphidocelis subcapitata*, *D. magna*) revealed significantly lower toxicity for the samples treated with 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⁻¹) might be a useful strategy to reduce the ecotoxicity of UV₂₅₄ 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, $\lambda < 280$ 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 $50 \text{ mJ}\cdot\text{cm}^{-2}$ to $150 \text{ mJ}\cdot\text{cm}^{-2}$ 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 $40 \text{ mJ}\cdot\text{cm}^{-2}$ **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 (**UV₂₅₄/H₂O₂**) **which**

77 **produces highly reactive radical species**, has been proposed as a viable **treatment for effective**
78 removal of micropollutant **and ARB** and, in consequence, **for the reduction of** the ecotoxicity risk
79 (García-Galan et al., 2016; Melo da Silva et al., 2016).

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

90 Acyclovir (ACY) is one of the oldest and most widely used antiviral drug for treating two common
91 viral infections (chickenpox-zoster and herpes simplex) and it is also prescribed to patients with
92 weakened immune systems in order to control viral infections (i.e., viral conjunctivitis) (Bryan-
93 Marrugo et al., 2015). ACY has been recently detected in different WWTP effluents as well as in
94 surface water at level of few nanograms per liter up to over one micrograms per liter (Table 1).

95 **The** photodegradation pathways of ACY under artificial and natural solar light irradiation have
96 been recently investigated (Zhou et al., 2015; Prasse et al., 2015). However, **there is a lack of**
97 investigations on the photochemical transformation of ACY under UV₂₅₄ and UV₂₅₄/H₂O₂
98 treatments and on the **simultaneous** ecotoxicological assessments **of** highly diluted treated solutions
99 containing ACY.

100 More information is needed to determine **the effectiveness** of UV₂₅₄ assisted processes **on the**
101 **removal** of ACY **from** aqueous solutions **and the impact that these processes may have on the**
102 **structure of aquatic communities and on the ecosystem dynamics.**

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

113 **Under this background, in this study we investigated** the degradation kinetics of ACY in
114 **distilled water** under UV₂₅₄ and UV₂₅₄/H₂O₂ irradiation by means of a microcapillary film (MCF)
115 array photoreactor and **we evaluated** the acute and chronic ecotoxicity of highly diluted treated
116 samples using **a range of** selected organisms, to provide important information regarding the
117 photolysis of ACY in UV₂₅₄ based sanitary engineered systems for water reclamation. **The toxicity**
118 **was assessed considering a battery of toxicity tests (*Aliivibrio fischeri*, *Raphidocelis***
119 ***subcapitata*, *Daphnia magna*) and endpoints (bioluminescence, growth inhibition,**
120 **immobilization, survival, reproduction and biomarker) including three trophic and**
121 **phylogenetic levels (Lofrano et al., 2016).**

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

125

126 **2. Materials and methods**

127 *2.1. Materials*

128 Hydrogen peroxide (30% v/v), ACY (pharmaceutical secondary standard), methanol ($\geq 99.9\%$ v/v),
129 formic acid ($>99\%$ w/w), benzoic acid ($\geq 99.5\%$ w/w), orthophosphoric acid (85% w/w in H_2O),
130 sodium hydroxide ($>98\%$ w/w), perchloric acid (70% v/v), catalase from *Micrococcus lysodeikticus*
131 and reagents for ecotoxicity tests were purchased from Sigma-Aldrich. **An aqueous mixture of**
132 **peptone (32 ppm), meat extract (22 ppm), urea (6 ppm), K_2HPO_4 (28 ppm), $\text{CaCl}_2\cdot\text{H}_2\text{O}$ (4**
133 **ppm), NaCl (7 ppm) and Mg_2SO_4 (0.6 ppm) was used for the preparation of a synthetic**
134 **wastewater according to the OECD Guidelines (Organisation for Economic Cooperation and**
135 **development, 1999). The substances were purchased from Sigma-Aldrich and used as**
136 **received.** Milli-Q water was used as solvent in analytical determinations and experiments.

137

138 2.2. Analytical methods

139 The concentration of hydrogen peroxide, ACY, and benzoic acid was measured by HPLC (1100
140 Agilent) equipped with a Gemini 5u C6-Phenyl 110 (Phenomenex) reverse phase column and a
141 diode array detector. The mobile phase was a mixture of 93% aqueous orthophosphoric acid (10
142 mM) and 7% methanol flowing at $8.0 \cdot 10^{-4} \text{ L} \cdot \text{min}^{-1}$. The pH of the aqueous solutions was adjusted
143 with NaOH or HClO_4 and measured with an Accumet Basic AB-10 pH-meter. The molar
144 absorption coefficient of ACY was estimated using a Perkin Elmer UV/VIS spectrometer (mod.
145 Lambda 35). **Total organic carbon (TOC) was monitored by a TOC analyzer (Shimadzu 5000**
146 **A). MS analysis was performed by direct injection on Agilent 6230 TOF LC/MS coupled with**
147 **Agilent HPLC system (1260 Series). The mobile phase was a mixture of methanol (10% v/v)**
148 **and formic acid (0.1% v/v) aqueous solution at flow rate of $0.4 \text{ mL} \cdot \text{min}^{-1}$ and the injection**
149 **volume of samples was 20 μL . The MS source was an electrospray ionization (ESI) interface**
150 **in the positive ion mode with capillary voltage of 3500 V, gas temperature at 325°C , dry gas**
151 **(N_2) flow at $8 \text{ L} \cdot \text{min}^{-1}$ and the nebulizer at 35 psi. The MS spectra were acquired in a mass**
152 **range of 100-3000 m/z with a rate of 1 spectrum/s, time of 1000 ms/spectrum and**
153 **transient/spectrum of 9905.**

154

155 3. Experimental apparatus and procedures

156 3.1. MCF array photoreactor

157 The degradation kinetics of ACY by UV_{254} and UV_{254}/H_2O_2 were investigated in a MCF array
158 photoreactor described elsewhere (Reis et al., 2015; Russo et al., 2016). Briefly, the photoreactor
159 (Lamina Dielectrics Ltd) consisted of ten **UV_{254} transparent** microcapillaries of fluorinated
160 polymer characterized by a mean hydraulic diameter of 195 μm . The microcapillaries were coiled
161 around a UV monochromatic (254 nm) lamp (Germicidal G8T5) in the region with uniform
162 emission. Experiments were carried out at room temperature ($\sim 25^\circ C$) in continuous flow through
163 the reactor at different space times, using capillaries of different length exposed to the UV lamp
164 irradiation. The flow rate through the MCF was $6.0 \cdot 10^{-4} L \cdot min^{-1}$. Aqueous samples were collected
165 from the MCF outlet, and rapidly analyzed by HPLC. At the end of each experimental run, the pH
166 of the solutions was unchanged. **The initial concentration of ACY used in the experiments**
167 **ranged between $2.05 \cdot 10^{-5} mol \cdot L^{-1}$ and $4.67 \cdot 10^{-5} mol \cdot L^{-1}$.**

168 The lamp irradiance was varied by changing the nominal power from 4.5 W to 8.0 W using a
169 variable power supply unit. The photon fluxes per unit volume emitted by the UV lamp (P_o) for
170 each power setting, estimated by H_2O_2 actinometry (Nicole et al, 1990; Goldstein et al., 2007), were
171 $1.92 \cdot 10^{-2} ein \cdot (s \cdot L)^{-1}$ (nominal power 8.0 W) and $1.27 \cdot 10^{-2} ein \cdot (s \cdot L)^{-1}$ (nominal power 4.5 W). The
172 MCF average optical path length (l_{MCF}) was 154 μm . All the runs were carried out in duplicate. The
173 data collected were used to estimate the kinetic unknown parameters (quantum yield of direct
174 photolysis at 254 nm of ACY and kinetic constant of hydroxyl radical attack to ACY).

175

176 3.2. Cylindrical batch photoreactor

177 A cylindrical batch photoreactor ($V_b = 0.480 L$), equipped with a low-pressure mercury
178 monochromatic lamp (Helios Italquartz, HGL10T5L, 17W nominal power emitting at 254 nm), was
179 used to provide **large** sample volumes **required for the** ecotoxicity tests at varying treatment times

180 (i.e., different UV_{254} fluence). The UV_{254} dose ($mJ \cdot cm^{-2}$) was calculated as the average photon
181 fluence rate multiplied by the treatment time. The average photon fluence rate emitted by the UV
182 lamp at 254 nm was $4.7 mW \cdot cm^{-2}$ (UVC DELTA OHM radiometer). The experimental device was
183 described elsewhere (Spasiano et al., 2016).

184

185 3.3. Ecotoxicity assessment

186 Reconstituted aqueous solution ($pH = 7.8 \pm 0.2$), was used as dilution water for cladoceran toxicity
187 tests: $CaCl_2 \cdot 2H_2O$ ($290 mg \cdot L^{-1}$), $MgSO_4 \cdot 7H_2O$ ($120 mg \cdot L^{-1}$), $NaHCO_3$ ($65 mg \cdot L^{-1}$), KCl (6
188 $mg \cdot L^{-1}$). Different salts were used for the preparation of algal test medium: $CaCl_2 \cdot 2H_2O$ ($18 mg \cdot L^{-1}$),
189 $MgSO_4 \cdot 7H_2O$ ($15 mg \cdot L^{-1}$), NH_4Cl ($15 mg \cdot L^{-1}$), $MgCl_2 \cdot 6H_2O$ ($12 mg \cdot L^{-1}$), KH_2PO_4 ($1.6 mg \cdot L^{-1}$),
190 $FeCl_3 \cdot 6H_2O$ ($0.08 mg \cdot L^{-1}$), $Na_2EDTA \cdot 2H_2O$ ($0.1 mg \cdot L^{-1}$), H_3BO_3 ($0.185 mg \cdot L^{-1}$), $MnCl_2 \cdot 4H_2O$
191 ($0.415 mg \cdot L^{-1}$), $ZnCl_2$ ($0.003 mg \cdot L^{-1}$), $CoCl_2 \cdot 6H_2O$ ($0.0015 mg \cdot L^{-1}$), $Na_2MoO_4 \cdot 2H_2O$ ($7.0 \cdot 10^{-3}$
192 $mg \cdot L^{-1}$), $CuCl_2 \cdot 2H_2O$ ($1.0 \cdot 10^{-5} mg \cdot L^{-1}$). Reconstitution solution, osmotic adjusting solution (OAS)
193 and diluent (NaCl 2%) were the reagents used in *Vibrio fischeri* toxicity test (Strategic diagnostics
194 Inc. SDI).

195 The enzymatic assays chosen to evaluate oxidative stress were ROS (reactive oxygen species)
196 content using 2,7- dichlorodihydrofluorescein (H_2DCFDA) and activities of SOD (superoxide
197 dismutase), CAT (catalase) and GST (glutathione transferase) that were measured using respective
198 assay kits according to the manufacturer's instruction's (Sigma Aldrich). All determinations were
199 quantified spectrophotometrically.

200 *V. fischeri*, *R. subcapitata* and acute *D. magna* assays were conducted with an initial ACY
201 concentration of $1.2 mg \cdot L^{-1}$ and on its related UV_{254} and UV_{254}/H_2O_2 treated solutions. Chronic
202 toxicity and oxidative stress tests on *Daphnia magna* were performed starting on untreated and
203 treated solutions diluted by 100 fold, in order to assess any differences at sub lethal concentration
204 levels. **Negative and positive controls were included in each experiment. The significance of**

205 **differences of toxicity between the treated samples and controls was assessed by the analysis**
206 **of variance (ANOVA) considering a significance threshold level always set at 5%. For higher**
207 **variance than 5%, post-hoc tests were carried out with Dunnett's method and Tukey's test.**
208 **Whenever possible, toxicity was expressed as median effective concentration (EC₅₀) with 95%**
209 **confidence limit values. Otherwise, toxicity was expressed as percentage of effect (PE, %).**

210

211 3.3.1. *Organisms maintenance and monitoring*

212 Freeze-dried *Vibrio fischeri* (strain NRRL-B-11177) cells were reconstituted with reagent diluent at
213 4 °C. *Raphidocelis subcapitata* were cultured in ISO medium (ISO, 2012) at 23 ± 2 °C with
214 continuous 4500 lux light and aeration (0.2 mm filtered air). *Daphnia magna* were cultured at 20 ±
215 1 °C, with a 16:8 light/dark photoperiod in ISO water (ISO, 2012).

216 Luminescence *V. fischeri* measurements were performed with Microtox® Model 500 Toxicity
217 Analyzer from Microbics Corporation (AZUR Environmental) equipped with a 30 well incubated at
218 15 ± 1 °C and with excitation source at 490 nm wavelength.

219 *R. subcapitata* density was determined by an indirect procedure using a spectrophotometer (Hach
220 Lange DR5000) and cuvette (5 cm). *D. magna* viability, mobility and growth were observed with a
221 stereomicroscope (LEICA EZ4-HD).

222

223 3.3.2. *Bacteria toxicity test*

224 The inhibitory effect of ACY samples on the light emission of *V. fischeri* (strain NRRL-B-11177)
225 was evaluated with the 11348-3:2007 ISO method (ISO, 2007). Tests were carried out on an ACY
226 concentration of 1.2 mg·L⁻¹ and on its related treated by-products solutions. OAS was added to each
227 sample to ensure that the final NaCl concentration was above 2.0%. The initial light output from
228 each cuvette containing reconstituted freeze-dried *V. fischeri* was recorded. The test solutions were
229 then added and after 30 minutes exposure, the final light output was measured. **Positive control**
230 **tests for *V. fischeri* were carried out with C₆H₄Cl₂O (EC₅₀ = 4.1 ± 2.2 mg·L⁻¹).**

231

232 3.3.3. Algae toxicity test

233 The *R. subcapitata* bioassay was conducted following the guidelines ISO 8692 (ISO, 2012). Three
234 replicates were included for each sample. The replicates were inoculated with 10^4 algal cells·mL⁻¹
235 and incubated for 72 h at 23 ± 2 °C under continuous illumination (irradiance range of 120-60
236 $\mu\text{ein}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The algal biomass exposed to the samples was compared with the algal biomass in the
237 negative control. **Positive control tests for *R. subcapitata* were carried out with $\text{K}_2\text{Cr}_2\text{O}_7$ (EC_{50}
238 $= 1 \pm 0.2 \text{ mg}\cdot\text{L}^{-1}$).**

239

240 3.3.4. Crustaceans toxicity test

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

245 The *D. magna* chronic bioassay was carried out according to the guideline OECD 211 (OECD,
246 2012). Ten *D. magna* neonates (< 24 h hold) were used and individually placed for each treatment
247 in beakers containing 50 ml of the test solutions, renewed every two other days. Organisms exposed
248 for 21 days with ACY solutions were then fed one day with *R. subcapitata* (10^7 cell·mL⁻¹).
249 Survival, reproduction and growth were observed daily, and newborns were discarded from
250 beakers.

251 The amount of ROS produced in *D. magna* was determined using 2,7-dichlorodihydrofluorescein
252 (H_2DCFDA , Sigma Aldrich) using the method previously reported (Galdiero et al., 2016). After 48
253 h of exposure, each exposed and not exposed living daphnids were rinsed with deionized water to
254 remove any excess pharmaceuticals adhered to their body surface and transferred to a 96-well plate.
255 A selected volume (200 μL) of 10 mM H_2DCFDA was added to each well and the plate was then
256 incubated for 4 h in the dark at 20-25°C. Fluorescence was measured using a fluorescence plate

257 reader with an excitation wavelength of 350 nm and an emission of 600 nm. The increase in
258 fluorescence intensity yielded the ROS quantity compared to control.

259 Exposed and not exposed daphnids were homogenized in 1 mL sucrose buffer (0.25 M sucrose, 0.1
260 M Tris-HCl, 1 mM EDTA, pH 7.4) and successively centrifuged at 12.000 g for 15 min at 4°C.
261 Supernatants were collected and used to determinate enzymatic activities. Protein content of the
262 samples was quantified using the protocol described by Bradford (1976) using bovine serum
263 albumin as standard.

264 CAT activity was expressed as H₂O₂ consumed (U·mg⁻¹ of protein) to convert it to H₂O and O₂ per
265 minute, per mg protein at 240 nm (Aebi, 1984).

266 SOD activity was calculated by measuring the decrease in the color development of samples at 440
267 nm with the reference to the xantine oxidase/cithochrome method (Crapo et al., 1978). In
268 particular the superoxide radical, generated from the conversion of xanthine to uric acid and
269 H₂O₂ by xanthine oxidase, reacts with the tetrazolium salt WST-1 forming formazan.

270 One unit of SOD was defined as the amount of enzyme required to produce 50% inhibition in the
271 reaction system.

272 GST was calculated by measuring the changes in absorbance recorded at 340 nm due to the
273 conjugation of glutathione to 1-chloro-2,4-dinitrobenzene (Habig et al., 1974).

274 One unit of enzyme was the quantity necessary for the reduction of 1 µmol·L⁻¹ GSH in 1 min at 37
275 °C.

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

279

280 4. Results and discussion

281 4.1. UV₂₅₄ photolysis: kinetic investigation

282 The results collected from runs of UV₂₅₄ photolysis of ACY in aqueous solution at three different
 283 pH values (4.5, 6.0 and 8.0) in the MCF photoreactor at varying lamp power are reported in Figs.
 284 1a-e as a function of the space time. The results indicate that, for a fixed lamp power, the pH did not
 285 affect the conversion. In fact, for these runs a half-time of about 17 seconds was recorded
 286 independent of the pH. Moreover, **the analysis** of the concentration vs time profile **demonstrated**
 287 that the photolysis of ACY resembled an apparent autocatalytic behavior which suggested the
 288 adoption of an autocatalytic kinetic model to describe the degradation of ACY under the adopted
 289 experimental conditions. Since the destruction of guanine based substrates under UV₂₅₄ irradiation
 290 has been ascribed to both the direct photolysis of guanine derivatives and the reaction of guanine
 291 based molecules with the radical species formed during the photolytic process (Crespo-Hernandez
 292 et al., 2000a,b), the simplified reaction scheme (Scheme 1) was considered for the UV₂₅₄ photolysis
 293 of ACY, which is a guanine derivative:

Scheme 1

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

$$\frac{d[\text{ACY}]}{dt} = -P_o \cdot \phi_{\text{ACY}} \cdot (1 - \exp(-2.3 \cdot l_{\text{MCF}} \cdot \epsilon_{254}^{\text{ACY}} \cdot [\text{ACY}])) - k' \cdot [\text{ACY}] \cdot [\text{B}] \quad (1)$$

$$\frac{d[B]}{dt} = P_o \cdot \phi_{ACY} \cdot (1 - \exp(-2.3 \cdot l_{MCF} \cdot \epsilon_{254}^{ACY} \cdot [ACY])) \quad (2)$$

Where t is the space time in the continuous flow MCF photoreactor (the reaction or exposure time) and the term ϵ_{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 $\sum_j^m \sum_i^n (y_{ACY_{j,i}} - c_{ACY_{j,i}})^2$, 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 $\phi_{ACY} = (1.62 \pm 0.07) \cdot 10^{-3} \text{ mol} \cdot \text{ein}^{-1}$ 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 ϕ_{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.

320

321 4.2. UV_{254}/H_2O_2 oxidation: kinetic investigation

322 The results of a preliminary run carried out in the presence of hydrogen peroxide under darkness
323 indicated that ACY was not degraded in the presence of H_2O_2 alone for reaction times up to 30 min.
324 Photooxidation experiments of ACY by the UV_{254}/H_2O_2 process were carried out under the same

325 experimental conditions (i.e., pH, lamp power and initial concentration of ACY) used in the UV₂₅₄
326 direct photolysis runs.

327 The degradation profiles for ACY and H₂O₂ as a function of space time in the MCF photoreactor
328 were modeled on the basis of a simplified reaction scheme and the mass balances listed in Table 2.

329 The reaction scheme considers the consumption of ACY and hydrogen peroxide by direct
330 photolysis (*reactions 3 and 4*). Hydroxyl radicals generated by UV₂₅₄ photolysis of H₂O₂ can react
331 with hydrogen peroxide (*reaction 5*), ACY (*reaction 6*) and the transformation products (*reaction*
332 *7*). A radical termination of peroxy radicals was considered in the mechanism (*reaction 8*).

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

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

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

345 From this method $k_{OH/ACY}$ was determined as $(1.23 \pm \mathbf{0.07}) \cdot 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$. Graphical examples of the
346 results obtained by the modeling through the optimization procedure are shown in Figures 2a-f
347 (*optimization procedure*). In Figures 2g-i the comparison **is reported** between experimental and

calculated residual ACY and H₂O₂ 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 UV₂₅₄/H₂O₂ 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 ($k'_{sca} = 4.01 \cdot 10^5 \text{ s}^{-1}$) 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 $2.0 \cdot 10^{-5} \text{ M}$) chosen as reference compound (Onstein et al., 1999):

$$\ln\left(\frac{[ACY]}{[ACY]_0}\right) = \frac{k_{OH/ACY}}{k_{OH/BA}} \cdot \ln\left(\frac{[BA]}{[BA]_0}\right) \quad k_{OH/BA} = 5.9 \cdot 10^9 \text{ M}^{-1} \cdot \text{s}^{-1} \quad (\text{pH} = 6.0) \quad (15)$$

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 UV₂₅₄/H₂O₂ experiments carried out at pH = 6.0 and $[H_2O_2]_0/[ACY]_0 = 20$ 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. UV₂₅₄ photolysis and UV₂₅₄/H₂O₂ oxidation: Ecotoxicity assessment

372 A battery of ecotoxicity tests on *V. fischeri*, *D. magna* and *R. subcapitata* were **performed** on
373 untreated and treated aqueous solutions with an initial ACY concentration of $1.2 \text{ mg}\cdot\text{L}^{-1}$. The
374 results showed that the inhibition of *V. fischeri* luminescence remained unchanged in the presence
375 of the UV_{254} and $\text{UV}_{254}/\text{H}_2\text{O}_2$ irradiated solutions, in comparison to the untreated solution (data not
376 shown).

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

386 The inhibition growth of *R. subcapitata* reached 32%, 13% and 20% at UV_{254} doses of 864, 2356
387 and $4712 \text{ mJ}\cdot\text{cm}^{-2}$ respectively (Fig. 4), thus confirming an acute **toxicological** effect on the UV_{254}
388 only treated samples. In contrast, a small reduction of the inhibition growth was observed for the
389 samples **treated with $\text{UV}_{254}/\text{H}_2\text{O}_2$** at increasing UV_{254} doses, **which supported** the beneficial
390 effect of the H_2O_2 assisted photolytic treatment for toxicity reduction.

391 The results showed an increase of **the production of ROS** in all samples, that **could** enhance **the**
392 sublethal toxicity in daphnids. **Aquatic organisms can in fact adapt to an increase of ROS**
393 **production by upregulating the activity of their antioxidant enzymes, particularly of CAT and**
394 **SOD which represent** the first and the second line of defense against ROS (Oexle et al., 2016). An
395 evident increase of ROS production in **the daphnids treated with UV_{254} only samples was observed**
396 **in comparison to the those treated with the $\text{UV}_{254}/\text{H}_2\text{O}_2$ samples** (Fig. 5A). The increase was

397 recorded for UVC doses of 864 and 2356 $\text{mJ}\cdot\text{cm}^{-2}$ **for the** UV_{254} process and **at** 280 $\text{mJ}\cdot\text{cm}^{-2}$ **for the**
398 samples treated **with** $\text{UV}_{254}/\text{H}_2\text{O}_2$.

399 The SOD activity resulted in significant alterations only for samples treated by UV_{254} (Fig. 5B).
400 The enzyme inhibition increased when **the** UVC dose was increased **and reached** the highest
401 inhibition at 2356 $\text{mJ}\cdot\text{cm}^{-2}$. No effect was observed in **the** samples treated **with** $\text{UV}_{254}/\text{H}_2\text{O}_2$ except
402 for samples treated with **a** UVC dose of 280 $\text{mJ}\cdot\text{cm}^{-2}$ (**TOC removal degree: 28%**).

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

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

411 A 21 days chronic exposure experiment was performed to determine the toxicity of 100 fold diluted
412 untreated and treated solutions. The effects of ACY (120 $\mu\text{g}\cdot\text{L}^{-1}$) and its treated samples on *D.*
413 *magna* reproduction and survival are reported in Figure 6A,B.

414 The results of chronic toxicity showed that the UV_{254} treatment, even at such low concentrations of
415 ACY, significantly decreased the survival of *D. magna* compared to the control group. A decrease
416 of survival was further recorded for samples exposed at a **TOC removal of less than 5%** (ACY
417 conversion degree: 45%), probably due the presence of unconverted ACY, and at UV_{254} dose of
418 2356 $\text{mJ}\cdot\text{cm}^{-2}$ (ACY conversion: 90%), due to the formation of first-generation-transformation by-
419 products **structurally similar to ACY**. At higher UV_{254} doses (4712 $\text{mJ}\cdot\text{cm}^{-2}$, **TOC removal ~**
420 **5%**), the survival percentage was similar to that of the control samples and always higher to that of
421 the untreated sample. On the contrary, the ecotoxicity assessment for the $\text{UV}_{254}/\text{H}_2\text{O}_2$ treated

422 solutions reflected the results already recorded in the acute tests, revealing a marked reduction of
423 chronic toxic effects for the exposures of the daphnids to the UV₂₅₄/H₂O₂ samples, especially the
424 highest UV₂₅₄ doses **(950 mJ·cm⁻², TOC removal 77% and 1900 mJ·cm⁻², TOC removal higher**
425 **than 95%).**

426 As reported in Table 3, the reproduction of *D. magna* was completely inhibited in the organisms
427 contacted with samples exposed to UV₂₅₄ doses of 864 mJ·cm⁻² and 2356 and in absence of H₂O₂.
428 These results revealed that all the endpoints were different than the control solutions with an
429 extended exposure to the treatment, thus confirming that the photoproducts formed during UV₂₅₄
430 irradiation of aqueous ACY solutions exerted significant chronic adverse effects to *D. magna* at the
431 population level. On the contrary, the total number of neonates and the number of first-brood were
432 not statistically different among the samples untreated and treated by UV₂₅₄/H₂O₂.

433 **The different chemical species formed during the UV₂₅₄ and the UV₂₅₄/H₂O₂ photochemical**
434 **processes could reasonably explain the observed toxicological effects. To provide a**
435 **preliminary validation of this hypothesis, two samples, one from UV₂₅₄ photolysis and the**
436 **second from UV₂₅₄/H₂O₂ treatment, were directly analyzed with MS-spectrometer to identify**
437 **the main chemical intermediates formed, with the knowledge that a thorough identification of**
438 **the transformation by-products required more sophisticated diagnostic techniques**
439 **(Buchberger, 2011).**

440 **A list of molecular structures of the main intermediates that could be attributed to some**
441 **peaks detected in the mass spectra for two samples is reported in Table 4. Some of the**
442 **structures shown in Table 4 correspond to the chemical intermediates previously detected and**
443 **reported in literature. In particular, for the UV₂₅₄ photolysis, the structures II, IV and V**
444 **were observed during the degradation of ACY by TiO₂ photocatalysis at 365 nm (An et al.,**
445 **2015) whereas the by-products VII and X proposed for UV₂₅₄/H₂O₂ were the same of those**
446 **observed during the photooxidation of ACY in phosphate buffer at wavelength higher than**
447 **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, UV₂₅₄/H₂O₂ 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 UV₂₅₄ treated sample.

5. Conclusion

The photodegradation of ACY was investigated under UV₂₅₄ 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 $(1.62 \pm 0.073) \cdot 10^{-3} \text{ mol} \cdot \text{ein}^{-1}$ in the pH range 4.5 – 8.0. An average value of $1.76 \cdot 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ was calculated for the kinetic constant of reaction between hydroxyl radical and ACY. Considering (i) the UV₂₅₄ 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 UV₂₅₄ disinfection stage would reduce this risk.

The results obtained contribute to provide useful information for a vision about the fate of ACY during the UV₂₅₄ and UV₂₅₄/H₂O₂ 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

474 preliminary analysis on the treated samples indicated, as the main photo-transformation by-
475 products, the presence of hydroxylated ACY based intermediates in the UV₂₅₄ treatment
476 process, and hydroxylated imidazole based compounds or species formed by the
477 fragmentation of the pyrimidine ring in the UV₂₅₄/H₂O₂ treatment process.

478 Further efforts are required to identify the main photoproducts, to elucidate the mechanism of ACY
479 photodegradation under UVC radiation and to evaluate possible cumulative effects of the different
480 species occurring in STP effluents.

481

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485

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1 Photodegradation and ecotoxicology of acyclovir in water under UV₂₅₄ and 2 UV₂₅₄/H₂O₂ processes

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21 22 Abstract

23 The photochemical and ecotoxicological fate of acyclovir (ACY) through UV₂₅₄ direct photolysis
24 and in the presence of hydroxyl radicals (UV₂₅₄/H₂O₂ process) were investigated in a microcapillary
25 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 ($\phi_{ACY} = (1.62 \pm 0.07) \cdot 10^{-3} \text{ mol-ein}^{-1}$) that further reacted with ACY to form by-products ($k' = (5.64 \pm 0.03) \cdot 10^{-3} \text{ M}^{-1} \cdot \text{s}^{-1}$). The photolysis of ACY in the presence of hydrogen peroxide accelerated the removal of ACY as a result of formation of hydroxyl radicals. The kinetic constant for the reaction of OH radicals with ACY ($k_{OH/ACY}$) determined with the kinetic modeling method was $(1.23 \pm 0.07) \cdot 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ and with the competition kinetics method was $(2.30 \pm 0.11) \cdot 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ with competition kinetics. The acute and chronic effects of the treated aqueous mixtures on different living organisms (*Vibrio fischeri*, *Raphidocelis subcapitata*, *D. magna*) revealed significantly lower toxicity for the samples treated with 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⁻¹) might be a useful strategy to reduce the ecotoxicity of UV₂₅₄ 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, $\lambda < 280$ 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 $\text{mJ}\cdot\text{cm}^{-2}$ to 150 $\text{mJ}\cdot\text{cm}^{-2}$ 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 $\text{mJ}\cdot\text{cm}^{-2}$ UVC fluence to eliminate a large variety of bacteria and viruses (Conner-Kerr et al., 1998). Even though UV disinfection has been reported highly effective in the reduction of antibiotic resistance bacteria (ARB), particularly in comparison to chlorination (Shi et al., 2013; Hijnen et al., 2006), other investigations have demonstrated that UV disinfection may not contribute to the significant reduction of selected ARB, such as tetracycline- and sulfonamide-resistant bacteria (Munir et al., 2011; Meckes, 1982) thus indicating a plausible selectivity of UV on ARB (Guo et al., 2013).

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

micropollutant and ARB and, in consequence, for the reduction of the ecotoxicity risk (García-Galan et al., 2016; Melo da Silva et al., 2016).

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

Acyclovir (ACY) is one of the oldest and most widely used antiviral drug for treating two common viral infections (chickenpox-zoster and herpes simplex) and it is also prescribed to patients with weakened immune systems in order to control viral infections (i.e., viral conjunctivitis) (Bryan-Marrugo et al., 2015). ACY has been recently detected in different WWTP effluents as well as in surface water at level of few nanograms per liter up to over one micrograms per liter (Table 1).

The photodegradation pathways of ACY under artificial and natural solar light irradiation have been recently investigated (Zhou et al., 2015; Prasse et al., 2015). However, there is a lack of investigations on the photochemical transformation of ACY under UV_{254} and UV_{254}/H_2O_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

103 such as illicit drugs or selected pharmaceuticals (Reis and Li Puma, 2015; Russo et al., 2016). In
104 contrast to conventional laboratory photochemical systems which require relatively larger volume
105 of liquid, photochemical treatments in microphotoreactors are carried out in a highly controlled
106 environment with minimal sample volumes (of the order of few mL), the sufficient amount to
107 generate samples for subsequent analysis. Furthermore, photochemical transformations in
108 microphotoreactors are executed at extremely short residence times (of the order of seconds) in
109 comparison to conventional laboratory photoreactors, resulting in an efficient use of time and
110 resources.

111 Under this background, in this study we investigated the degradation kinetics of ACY in distilled
112 water under UV₂₅₄ and UV₂₅₄/H₂O₂ irradiation by means of a microcapillary film (MCF) array
113 photoreactor and we evaluated the acute and chronic ecotoxicity of highly diluted treated samples
114 using a range of selected organisms, to provide important information regarding the photolysis of
115 ACY in UV₂₅₄ based sanitary engineered systems for water reclamation. The toxicity was assessed
116 considering a battery of toxicity tests (*Aliivibrio fischeri*, *Raphidocelis subcapitata*, *Daphnia*
117 *magna*) and endpoints (bioluminescence, growth inhibition, immobilization, survival, reproduction
118 and biomarker) including three trophic and phylogenetic levels (Lofrano et al., 2016).

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

122

123 **2. Materials and methods**

124 *2.1. Materials*

125 Hydrogen peroxide (30% v/v), ACY (pharmaceutical secondary standard), methanol ($\geq 99.9\%$ v/v),
126 formic acid ($>99\%$ w/w), benzoic acid ($\geq 99.5\%$ w/w), orthophosphoric acid (85% w/w in H₂O),
127 sodium hydroxide ($>98\%$ w/w), perchloric acid (70% v/v), catalase from *Micrococcus lysodeikticus*
128 and reagents for ecotoxicity tests were purchased from Sigma-Aldrich. An aqueous mixture of

129 peptone (32 ppm), meat extract (22 ppm), urea (6 ppm), K_2HPO_4 (28 ppm), $CaCl_2 \cdot H_2O$ (4 ppm),
130 $NaCl$ (7 ppm) and Mg_2SO_4 (0.6 ppm) was used for the preparation of a synthetic wastewater
131 according to the OECD Guidelines (Organisation for Economic Cooperation and
132 development, 1999). The substances were purchased from Sigma-Aldrich and used as received.
133 Milli-Q water was used as solvent in analytical determinations and experiments.

134

135 2.2. Analytical methods

136 The concentration of hydrogen peroxide, ACY, and benzoic acid was measured by HPLC (1100
137 Agilent) equipped with a Gemini 5u C6-Phenyl 110 (Phenomenex) reverse phase column and a
138 diode array detector. The mobile phase was a mixture of 93% aqueous orthophosphoric acid (10
139 mM) and 7% methanol flowing at $8.0 \cdot 10^{-4} \text{ L} \cdot \text{min}^{-1}$. The pH of the aqueous solutions was adjusted
140 with $NaOH$ or $HClO_4$ and measured with an Accumet Basic AB-10 pH-meter. The molar
141 absorption coefficient of ACY was estimated using a Perkin Elmer UV/VIS spectrometer (mod.
142 Lambda 35). Total organic carbon (TOC) was monitored by a TOC analyzer (Shimadzu 5000 A).
143 MS analysis was performed by direct injection on Agilent 6230 TOF LC/MS coupled with Agilent
144 HPLC system (1260 Series). The mobile phase was a mixture of methanol (10% v/v) and formic
145 acid (0.1% v/v) aqueous solution at flow rate of $0.4 \text{ mL} \cdot \text{min}^{-1}$ and the injection volume of samples
146 was 20 μL . The MS source was an electrospray ionization (ESI) interface in the positive ion mode
147 with capillary voltage of 3500 V, gas temperature at 325 $^{\circ}\text{C}$, dry gas (N_2) flow at $8 \text{ L} \cdot \text{min}^{-1}$ and the
148 nebulizer at 35 psi. The MS spectra were acquired in a mass range of 100-3000 m/z with a rate of 1
149 spectrum/s, time of 1000 ms/spectrum and transient/spectrum of 9905.

150

151 3. Experimental apparatus and procedures

152 3.1. MCF array photoreactor

153 The degradation kinetics of ACY by UV_{254} and UV_{254}/H_2O_2 were investigated in a MCF array
154 photoreactor described elsewhere (Reis et al., 2015; Russo et al., 2016). Briefly, the photoreactor

(Lamina Dielectrics Ltd) consisted of ten UV₂₅₄ transparent microcapillaries of fluorinated polymer characterized by a mean hydraulic diameter of 195 µm. The microcapillaries were coiled around a UV monochromatic (254 nm) lamp (Germicidal G8T5) in the region with uniform emission. Experiments were carried out at room temperature (~25 °C) in continuous flow through the reactor at different space times, using capillaries of different length exposed to the UV lamp irradiation. The flow rate through the MCF was $6.0 \cdot 10^{-4} \text{ L} \cdot \text{min}^{-1}$. 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 $2.05 \cdot 10^{-5} \text{ mol} \cdot \text{L}^{-1}$ and $4.67 \cdot 10^{-5} \text{ mol} \cdot \text{L}^{-1}$. 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 H₂O₂ actinometry (Nicole et al, 1990; Goldstein et al., 2007), were $1.92 \cdot 10^{-2} \text{ ein} \cdot (\text{s} \cdot \text{L})^{-1}$ (nominal power 8.0 W) and $1.27 \cdot 10^{-2} \text{ ein} \cdot (\text{s} \cdot \text{L})^{-1}$ (nominal power 4.5 W). The MCF average optical path length (l_{MCF}) was 154 µm. All the runs were carried out in duplicate. The data collected were used to estimate the kinetic unknown parameters (quantum yield of direct photolysis at 254 nm of ACY and kinetic constant of hydroxyl radical attack to ACY).

171

172 3.2. Cylindrical batch photoreactor

173 A cylindrical batch photoreactor ($V_b = 0.480 \text{ L}$), equipped with a low-pressure mercury
174 monochromatic lamp (Helios Italquartz, HGL10T5L, 17W nominal power emitting at 254 nm), was
175 used to provide large sample volumes required for the ecotoxicity tests at varying treatment times
176 (i.e., different UV₂₅₄ fluence). The UV₂₅₄ dose ($\text{mJ} \cdot \text{cm}^{-2}$) was calculated as the average photon
177 fluence rate multiplied by the treatment time. The average photon fluence rate emitted by the UV
178 lamp at 254 nm was $4.7 \text{ mW} \cdot \text{cm}^{-2}$ (UVC DELTA OHM radiometer). The experimental device was
179 described elsewhere (Spasiano et al., 2016).

180

181 3.3. Ecotoxicity assessment

182 Reconstituted aqueous solution ($\text{pH} = 7.8 \pm 0.2$), was used as dilution water for cladoceran toxicity
183 tests: $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ($290 \text{ mg} \cdot \text{L}^{-1}$), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ($120 \text{ mg} \cdot \text{L}^{-1}$), NaHCO_3 ($65 \text{ mg} \cdot \text{L}^{-1}$), KCl (6
184 $\text{mg} \cdot \text{L}^{-1}$). Different salts were used for the preparation of algal test medium: $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ($18 \text{ mg} \cdot \text{L}^{-1}$),
185 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ($15 \text{ mg} \cdot \text{L}^{-1}$), NH_4Cl ($15 \text{ mg} \cdot \text{L}^{-1}$), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ($12 \text{ mg} \cdot \text{L}^{-1}$), KH_2PO_4 ($1.6 \text{ mg} \cdot \text{L}^{-1}$),
186 $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ($0.08 \text{ mg} \cdot \text{L}^{-1}$), $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ ($0.1 \text{ mg} \cdot \text{L}^{-1}$), H_3BO_3 ($0.185 \text{ mg} \cdot \text{L}^{-1}$), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$
187 ($0.415 \text{ mg} \cdot \text{L}^{-1}$), ZnCl_2 ($0.003 \text{ mg} \cdot \text{L}^{-1}$), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ($0.0015 \text{ mg} \cdot \text{L}^{-1}$), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ($7.0 \cdot 10^{-3}$
188 $\text{mg} \cdot \text{L}^{-1}$), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ($1.0 \cdot 10^{-5} \text{ mg} \cdot \text{L}^{-1}$). Reconstitution solution, osmotic adjusting solution (OAS)
189 and diluent (NaCl 2%) were the reagents used in *Vibrio fischeri* toxicity test (Strategic diagnostics
190 Inc. SDI).

191 The enzymatic assays chosen to evaluate oxidative stress were ROS (reactive oxygen species)
192 content using 2,7- dichlorodihydrofluorescein (H_2DCFDA) and activities of SOD (superoxide
193 dismutase), CAT (catalase) and GST (glutathione transferase) that were measured using respective
194 assay kits according to the manufacturer's instruction's (Sigma Aldrich). All determinations were
195 quantified spectrophotometrically.

196 *V. fischeri*, *R. subcapitata* and acute *D. magna* assays were conducted with an initial ACY
197 concentration of $1.2 \text{ mg} \cdot \text{L}^{-1}$ and on its related UV_{254} and $\text{UV}_{254}/\text{H}_2\text{O}_2$ treated solutions. Chronic
198 toxicity and oxidative stress tests on *Daphnia magna* were performed starting on untreated and
199 treated solutions diluted by 100 fold, in order to assess any differences at sub lethal concentration
200 levels. Negative and positive controls were included in each experiment. The significance of
201 differences of toxicity between the treated samples and controls was assessed by the analysis of
202 variance (ANOVA) considering a significance threshold level always set at 5%. For higher variance
203 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 $1.2 \text{ mg} \cdot \text{L}^{-1}$ 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 $\text{C}_6\text{H}_4\text{Cl}_2\text{O}$ ($EC_{50} = 4.1 \pm 2.2 \text{ mg} \cdot \text{L}^{-1}$).

3.3.3. Algae toxicity test

229 The *R. subcapitata* bioassay was conducted following the guidelines ISO 8692 (ISO, 2012). Three
230 replicates were included for each sample. The replicates were inoculated with 10^4 algal cells·mL⁻¹
231 and incubated for 72 h at 23 ± 2 °C under continuous illumination (irradiance range of 120-60
232 $\mu\text{ein}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The algal biomass exposed to the samples was compared with the algal biomass in the
233 negative control. Positive control tests for *R. subcapitata* were carried out with K₂Cr₂O₇ ($\text{EC}_{50} = 1 \pm$
234 $0.2 \text{ mg}\cdot\text{L}^{-1}$).

235

236 3.3.4. Crustaceans toxicity test

237 Acute toxicity tests with *D. magna* were carried out according to ISO 6341 (ISO, 2013). Newborn
238 daphnids (<24 h old) were exposed in four replicates for 24 h and 48 h at 20 ± 1 °C. Toxicity was
239 expressed as percentage of immobilized organisms. Positive control tests for *D. magna* were carried
240 out with K₂Cr₂O₇ (48h, $\text{EC}_{50} = 0.6 \pm 0.1 \text{ mg}\cdot\text{L}^{-1}$).

241 The *D. magna* chronic bioassay was carried out according to the guideline OECD 211 (OECD,
242 2012). Ten *D. magna* neonates (< 24 h hold) were used and individually placed for each treatment
243 in beakers containing 50 ml of the test solutions, renewed every two other days. Organisms exposed
244 for 21 days with ACY solutions were then fed one day with *R. subcapitata* (10^7 cell·mL⁻¹).
245 Survival, reproduction and growth were observed daily, and newborns were discarded from
246 beakers.

247 The amount of ROS produced in *D. magna* was determined using 2,7-dichlorodihydrofluorescein
248 (H₂DCFDA, Sigma Aldrich) using the method previously reported (Galdiero et al., 2016). After 48
249 h of exposure, each exposed and not exposed living daphnids were rinsed with deionized water to
250 remove any excess pharmaceuticals adhered to their body surface and transferred to a 96-well plate.
251 A selected volume (200 μL) of 10 mM H₂DCFDA was added to each well and the plate was then
252 incubated for 4 h in the dark at 20-25°C. Fluorescence was measured using a fluorescence plate

253 reader with an excitation wavelength of 350 nm and an emission of 600 nm. The increase in
254 fluorescence intensity yielded the ROS quantity compared to control.

255 Exposed and not exposed daphnids were homogenized in 1 mL sucrose buffer (0.25 M sucrose, 0.1
256 M Tris-HCl, 1 mM EDTA, pH 7.4) and successively centrifuged at 12.000 g for 15 min at 4°C.

257 Supernatants were collected and used to determinate enzymatic activities. Protein content of the
258 samples was quantified using the protocol described by Bradford (1976) using bovine serum
259 albumin as standard.

260 CAT activity was expressed as H_2O_2 consumed ($\text{U}\cdot\text{mg}^{-1}$ of protein) to convert it to H_2O and O_2 per
261 minute, per mg protein at 240 nm (Aebi, 1984).

262 SOD activity was calculated by measuring the decrease in the color development of samples at 440
263 nm with the reference to the xantine oxidase/cithochrome method (Crapo et al., 1978). In particular
264 the superoxide radical, generated from the conversion of xanthine to uric acid and H_2O_2 by xanthine
265 oxidase, reacts with the tetrazolium salt WST-1 forming formazan.

266 One unit of SOD was defined as the amount of enzyme required to produce 50% inhibition in the
267 reaction system.

268 GST was calculated by measuring the changes in absorbance recorded at 340 nm due to the
269 conjugation of glutathione to 1-chloro-2,4-dinitrobenzene (Habig et al., 1974).

270 One unit of enzyme was the quantity necessary for the reduction of $1\ \mu\text{mol}\cdot\text{L}^{-1}$ GSH in 1 min at 37
271 °C.

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

275

276 **4. Results and discussion**

277 *4.1. UV₂₅₄ photolysis: kinetic investigation*

278 The results collected from runs of UV₂₅₄ photolysis of ACY in aqueous solution at three different
 279 pH values (4.5, 6.0 and 8.0) in the MCF photoreactor at varying lamp power are reported in Figs.
 280 1a-e as a function of the space time. The results indicate that, for a fixed lamp power, the pH did not
 281 affect the conversion. In fact, for these runs a half-time of about 17 seconds was recorded
 282 independent of the pH. Moreover, the analysis of the concentration vs time profile demonstrated
 283 that the photolysis of ACY resembled an apparent autocatalytic behavior which suggested the
 284 adoption of an autocatalytic kinetic model to describe the degradation of ACY under the adopted
 285 experimental conditions. Since the destruction of guanine based substrates under UV₂₅₄ irradiation
 286 has been ascribed to both the direct photolysis of guanine derivatives and the reaction of guanine
 287 based molecules with the radical species formed during the photolytic process (Crespo-Hernandez
 288 et al., 2000a,b), the simplified reaction scheme (Scheme 1) was considered for the UV₂₅₄ photolysis
 289 of ACY, which is a guanine derivative:

Scheme 1

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

$$\frac{d[\text{ACY}]}{dt} = -P_o \cdot \phi_{\text{ACY}} \cdot (1 - \exp(-2.3 \cdot l_{\text{MCF}} \cdot \epsilon_{254}^{\text{ACY}} \cdot [\text{ACY}])) - k' \cdot [\text{ACY}] \cdot [\text{B}] \quad (1)$$

$$\frac{d[B]}{dt} = P_o \cdot \phi_{ACY} \cdot (1 - \exp(-2.3 \cdot l_{MCF} \cdot \epsilon_{254}^{ACY} \cdot [ACY])) \quad (2)$$

Where t is the space time in the continuous flow MCF photoreactor (the reaction or exposure time) and the term ϵ_{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 $\sum_j^m \sum_i^n (y_{ACY_{j,i}} - c_{ACY_{j,i}})^2$, 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 $\phi_{ACY} = (1.62 \pm 0.07) \cdot 10^{-3} \text{ mol} \cdot \text{ein}^{-1}$ 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 ϕ_{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

321 experimental conditions (i.e., pH, lamp power and initial concentration of ACY) used in the UV₂₅₄
322 direct photolysis runs.

323 The degradation profiles for ACY and H₂O₂ as a function of space time in the MCF photoreactor
324 were modeled on the basis of a simplified reaction scheme and the mass balances listed in Table 2.

325 The reaction scheme considers the consumption of ACY and hydrogen peroxide by direct
326 photolysis (*reactions 3 and 4*). Hydroxyl radicals generated by UV₂₅₄ photolysis of H₂O₂ can react
327 with hydrogen peroxide (*reaction 5*), ACY (*reaction 6*) and the transformation products (*reaction*
328 *7*). A radical termination of peroxy radicals was considered in the mechanism (*reaction 8*).

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

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

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

341 From this method $k_{OH/ACY}$ was determined as $(1.23 \pm 0.07) \cdot 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$. Graphical examples of the
342 results obtained by the modeling through the optimization procedure are shown in Figures 2a-f
343 (*optimization procedure*). In Figures 2g-i the comparison is reported between experimental and

344 calculated residual ACY and H₂O₂ concentration, when the model was used in simulation mode
 345 without any further parameter adjustment (*simulation mode*), using the $k_{OH/ACY}$ kinetic constant
 346 above estimated. It can be noted a good capability of the model of predicting the experimental data
 347 under the adopted conditions.

348 Two additional UV₂₅₄/H₂O₂ runs (Figs. 2l-m) were carried out using synthetic wastewater to further
 349 validate the kinetic results obtained. The photolytic runs were simulated using the proposed kinetic
 350 model properly modified to include the HO radical scavenging effect of the species forming the
 351 synthetic matrix (Spasiano et al., 2016). For this purpose, the pseudo-first order rate constant ($k'_{sca} =$
 352 $4.01 \cdot 10^{-1} \text{ s}^{-1}$) was considered for the reaction between the hydroxyl radicals and the scavenger
 353 species (Spasiano et al., 2016). Also in this case, a good capability of the model was still observed
 354 to predict the experimental data under the adopted conditions.

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

$$\ln\left(\frac{[ACY]}{[ACY]_0}\right) = \frac{k_{OH/ACY}}{k_{OH/BA}} \cdot \ln\left(\frac{[BA]}{[BA]_0}\right) \quad k_{OH/BA} = 5.9 \cdot 10^9 \text{ M}^{-1} \cdot \text{s}^{-1} \quad (\text{pH} = 6.0) \quad (15)$$

359 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 UV₂₅₄/H₂O₂
 360 experiments carried out at pH = 6.0 and $[H_2O_2]_0/[ACY]_0 = 20$ and at different lamp power (4.5 W
 361 and 8.0 W). The difference of this from the value estimated with kinetic modeling may be ascribed
 362 to the intrinsic limitations of the competition kinetics method that does not include the contribution
 363 of ACY consumption by direct photolysis. However, both $k_{OH/ACY}$ values estimated in the present
 364 investigation were significantly lower than those previously reported in the literature (Zhou et al.,
 365 2015; Prasse et al., 2015).

366

367 4.3. UV₂₅₄ photolysis and UV₂₅₄/H₂O₂ oxidation: Ecotoxicity assessment

368 A battery of ecotoxicity tests on *V. fischeri*, *D. magna* and *R. subcapitata* were performed on
369 untreated and treated aqueous solutions with an initial ACY concentration of $1.2 \text{ mg}\cdot\text{L}^{-1}$. The
370 results showed that the inhibition of *V. fischeri* luminescence remained unchanged in the presence
371 of the UV_{254} and $\text{UV}_{254}/\text{H}_2\text{O}_2$ irradiated solutions, in comparison to the untreated solution (data not
372 shown).

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

382 The inhibition growth of *R. subcapitata* reached 32%, 13% and 20% at UV_{254} doses of 864, 2356
383 and $4712 \text{ mJ}\cdot\text{cm}^{-2}$ respectively (Fig. 4), thus confirming an acute toxicological effect on the UV_{254}
384 only treated samples. In contrast, a small reduction of the inhibition growth was observed for the
385 samples treated with $\text{UV}_{254}/\text{H}_2\text{O}_2$ at increasing UV_{254} doses, which supported the beneficial effect
386 of the H_2O_2 assisted photolytic treatment for toxicity reduction.

387 The results showed an increase of the production of ROS in all samples, that could enhance the
388 sublethal toxicity in daphnids. Aquatic organisms can in fact adapt to an increase of ROS
389 production by upregulating the activity of their antioxidant enzymes, particularly of CAT and SOD
390 which represent the first and the second line of defense against ROS (Oexle et al., 2016). An
391 evident increase of ROS production in the daphnids treated with UV_{254} only samples was observed
392 in comparison to the those treated with the $\text{UV}_{254}/\text{H}_2\text{O}_2$ samples (Fig. 5A). The increase was

393 recorded for UVC doses of 864 and 2356 $\text{mJ}\cdot\text{cm}^{-2}$ for the UV_{254} process and at 280 $\text{mJ}\cdot\text{cm}^{-2}$ for the
 394 samples treated with $\text{UV}_{254}/\text{H}_2\text{O}_2$.

395 The SOD activity resulted in significant alterations only for samples treated by UV_{254} (Fig. 5B).
 396 The enzyme inhibition increased when the UVC dose was increased and reached the highest
 397 inhibition at 2356 $\text{mJ}\cdot\text{cm}^{-2}$. No effect was observed in the samples treated with $\text{UV}_{254}/\text{H}_2\text{O}_2$ except
 398 for samples treated with a UVC dose of 280 $\text{mJ}\cdot\text{cm}^{-2}$ (TOC removal degree: 28%).

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

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

407 A 21 days chronic exposure experiment was performed to determine the toxicity of 100 fold diluted
 408 untreated and treated solutions. The effects of ACY (120 $\mu\text{g}\cdot\text{L}^{-1}$) and its treated samples on *D.*
 409 *magna* reproduction and survival are reported in Figure 6A,B.

410 The results of chronic toxicity showed that the UV_{254} treatment, even at such low concentrations of
 411 ACY, significantly decreased the survival of *D. magna* compared to the control group. A decrease
 412 of survival was further recorded for samples exposed at a TOC removal of less than 5% (ACY
 413 conversion degree: 45%), probably due the presence of unconverted ACY, and at UV_{254} dose of
 414 2356 $\text{mJ}\cdot\text{cm}^{-2}$ (ACY conversion: 90%), due to the formation of first-generation-transformation by-
 415 products structurally similar to ACY. At higher UV_{254} doses (4712 $\text{mJ}\cdot\text{cm}^{-2}$, TOC removal $\sim 5\%$),
 416 the survival percentage was similar to that of the control samples and always higher to that of the
 417 untreated sample. On the contrary, the ecotoxicity assessment for the $\text{UV}_{254}/\text{H}_2\text{O}_2$ 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 UV₂₅₄/H₂O₂ samples, especially the highest UV₂₅₄ 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 UV₂₅₄ doses of 864 mJ·cm⁻² and 2356 and in absence of H₂O₂. 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 UV₂₅₄ 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 UV₂₅₄/H₂O₂. The different chemical species formed during the UV₂₅₄ and the UV₂₅₄/H₂O₂ photochemical processes could reasonably explain the observed toxicological effects. To provide a preliminary validation of this hypothesis, two samples, one from UV₂₅₄ photolysis and the second from UV₂₅₄/H₂O₂ 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 UV₂₅₄ photolysis, the structures II, IV and V were observed during the degradation of ACY by TiO₂ photocatalysis at 365 nm (An et al., 2015) whereas the by-products VII and X proposed for UV₂₅₄/H₂O₂ 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

444 significantly different in the two samples. In particular, UV₂₅₄/H₂O₂ process seems to lead mainly to
445 the formation of hydroxylated imidazole-based compounds or species formed by the fragmentation
446 of the pyrimidine ring whereas some hydroxylated ACY based intermediates are detected in the
447 UV₂₅₄ treated sample.

448

449 **5. Conclusion**

450 The photodegradation of ACY was investigated under UV₂₅₄ irradiation in the absence and in the
451 presence of hydrogen peroxide. A moderate rate of direct photolysis at 254 nm for ACY was
452 observed with a quantum yield of $(1.62 \pm 0.073) \cdot 10^{-3} \text{ mol} \cdot \text{ein}^{-1}$ in the pH range 4.5 – 8.0. An
453 average value of $1.76 \cdot 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ was calculated for the kinetic constant of reaction between
454 hydroxyl radical and ACY. Considering (i) the UV₂₅₄ doses typically used for the disinfection of
455 municipal sewage treatment plant effluents, (ii) the concentration values of ACY measured in
456 WWTP effluents, and (iii) the results collected during the kinetic and ecotoxicity assessment, the
457 occurrence of residual photodecomposition by-products in treated effluents is very likely, and these
458 are likely to have a high ecotoxicological index. However, the addition of appropriate amount of
459 hydrogen peroxide during the UV₂₅₄ disinfection stage would reduce this risk.

460 The results obtained contribute to provide useful information for a vision about the fate of ACY
461 during the UV₂₅₄ and UV₂₅₄/H₂O₂ treatment processes and the eventual associated risks for living
462 organisms (animals and plants) in the aquatic environment.

463 The results collected confirm the use of oxidative stress biomarkers as promising tool in order to
464 evaluate the toxicological effects of environmental pollutants as early indicators in ecotoxicology.
465 Exposure to environmental pollutants may disrupt the balance of biological oxidant-to-antioxidant
466 ratio in aquatic species leading to elevated levels of ROS and resulting in oxidative stress. A
467 preliminary analysis on the treated samples indicated, as the main photo-transformation by-
468 products, the presence of hydroxylated ACY based intermediates in the UV₂₅₄ treatment process,

469 and hydroxylated imidazole based compounds or species formed by the fragmentation of the
470 pyrimidine ring in the UV₂₅₄/H₂O₂ treatment process.

471 Further efforts are required to identify the main photoproducts, to elucidate the mechanism of ACY
472 photodegradation under UVC radiation and to evaluate possible cumulative effects of the different
473 species occurring in STP effluents.

474

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478

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WWTP effluent (ng/L)	Surface water (ng/L)	Location	Ref
27.3 – 53.3	2.2 - 190	Germany	(Prasse et al., 2010)
121- 148	5 - 25	Germany	(Prasse et al., 2011)
44.0 - 650	--	Germany	(Funke et al., 2016)
114 - 205	8.9 – 112.6	China	(Peng et al., 2014)
12 - 50	10 - 23	Japan	(Azuma et al., 2016)
947	738 - 1590	USA	(Bradley et al., 2014)
154	--	USA	(McCurry et al., 2014)

Table 1

3)	$ACY \xrightarrow{h\nu} \text{TPs}$	Φ_{ACY}	(estimated in this study)
4)	$\text{H}_2\text{O}_2 \xrightarrow{h\nu} 2\text{HO}^\bullet$	$\Phi_{\text{H}_2\text{O}_2} = 0.55 \text{ mol} \cdot \text{ein}^{-1}$ $\varepsilon_{254}^{\text{H}_2\text{O}_2} = 18.6 \text{ M}^{-1} \cdot \text{cm}^{-1}$	(Goldstein et al., 2007)
5)	$\text{HO}^\bullet + \text{H}_2\text{O}_2 \xrightarrow{k_h} \text{H}_2\text{O} + \text{HO}_2^\bullet$	$k_h = 2.7 \cdot 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$	(Buxton et al., 1988)
6)	$ACY + \text{HO}^\bullet \xrightarrow{k_{OH/ACY}} \text{TPs}$	$k_{OH/ACY}$	(estimated in this study)
7)	$\text{TPs} + \text{HO}^\bullet \xrightarrow{k_{OH/TP}} \text{TP}$	$k_{OH/TP}$	(estimated in this study)
8)	$2\text{HO}_2^\bullet \xrightarrow{k_t} \text{H}_2\text{O}_2 + \text{O}_2$	$k_t = 8.3 \cdot 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$	(Bielski et al., 1985)
9)	$\frac{d[\text{HO}^\bullet]}{d\tau} = 2F_{\text{H}_2\text{O}_2} - [\text{HO}^\bullet] \cdot (k_h \cdot [\text{H}_2\text{O}_2] - k_{OH/ACY} \cdot [ACY] - k_{OH/TP} \cdot [\text{TPs}])$		
10)	$F_{\text{H}_2\text{O}_2} = \Phi_{\text{H}_2\text{O}_2} \cdot P_o \cdot \left(1 - \exp\left(-2.3 \cdot l_{MCF} \cdot (\varepsilon_{254}^{ACY} \cdot [ACY] + \varepsilon_{254}^{\text{H}_2\text{O}_2} \cdot [\text{H}_2\text{O}_2])\right)\right) \cdot f_{\text{H}_2\text{O}_2}$		
11)	$\frac{d[\text{HO}_2^\bullet]}{d\tau} = k_h \cdot [\text{HO}^\bullet] \cdot [\text{H}_2\text{O}_2] - 2k_t \cdot [\text{HO}_2^\bullet]^2$		
12)	$\frac{d[ACY]}{d\tau} = -F_{ACY} - k_{OH/ACY} \cdot [ACY] \cdot [\text{HO}^\bullet]$		
13)	$F_{ACY} = \Phi_{ACY} \cdot P_o \cdot \left(1 - \exp\left(-2.3 \cdot l_{MCF} \cdot (\varepsilon_{254}^{ACY} \cdot [ACY] + \varepsilon_{254}^{\text{H}_2\text{O}_2} \cdot [\text{H}_2\text{O}_2])\right)\right) \cdot f_{ACY}$		

Table 2

UV ₂₅₄		
Sample	First brood (day)	Number of Living offspring per parent animal
Control solution	8	78 ± 5
UV ₂₅₄ dose: 0 mJ·cm ⁻²	10	72 ± 3
UV ₂₅₄ dose: 864 mJ·cm ⁻² TOC removal degree: < 5%	15	42 ± 3
UV ₂₅₄ dose: 2356 mJ·cm ⁻² TOC removal degree: < 5%	17	37 ± 6
UV ₂₅₄ dose: 4712 mJ·cm ⁻² TOC removal degree: ~ 5%	11	68 ± 5
UV ₂₅₄ /H ₂ O ₂		
Sample	First brood (day)	Number of Living offspring per parent animal
Control solution	8	75 ± 1
UV ₂₅₄ dose: 0 mJ·cm ⁻²	10	74 ± 4
UV ₂₅₄ dose: 280 mJ·cm ⁻² TOC removal degree: 28 %	9	69 ± 5
UV ₂₅₄ dose: 950 mJ·cm ⁻² TOC removal degree: 77 %	9	65 ± 2
UV ₂₅₄ dose: 1900 mJ·cm ⁻² TOC removal degree: > 95 %	9	70 ± 2

Table 3

UV₂₅₄

n°	Previously reported	Measured mass [M+H] ⁺ (Da)	Structure ^(c)
I	NO	136.93
II	YES, An et al, 2015	158.15
III	NO	202.18
IV	YES, An et al, 2015	242.98
V	YES, An et al, 2015	258.98

UV₂₅₄/H₂O₂

VI	NO	103.08
VII	YES, Iqbal et al., 2005	113.07
VIII	NO	129.07
IX	NO	146.09
X	YES, Iqbal et al., 2005	156.08
XI	NO	214.17
VI	NO	230.12

Table 4

Figure

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 1: Comparison between experimental (circle) and predicted (line) data for UV₂₅₄ 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 UV₂₅₄/H₂O₂ photodegradation of ACY (●) and hydrogen peroxide (○) in the MCF photoreactor at different pH, power of lamp and starting H₂O₂ load. *Optimization mode (a-f), simulation mode (g-m).*

(a): pH = 6.0 (8.0 W, [H₂O₂]_o/[ACY]_o = 20); (b): pH = 6.0 (8.0 W, [H₂O₂]_o/[ACY]_o = 50);

(c): pH = 8.0 (8.0 W, [H₂O₂]_o/[ACY]_o = 50); (d): pH = 6.0 (4.5 W, [H₂O₂]_o/[ACY]_o = 50);

(e): pH = 6.0 (4.5 W, [H₂O₂]_o/[ACY]_o = 70); (f): pH = 6.0 (4.5 W, [H₂O₂]_o/[ACY]_o = 100);

(g): pH = 4.0 (8.0 W, [H₂O₂]_o/[ACY]_o = 100); (h): pH = 8.2 (8.0 W, [H₂O₂]_o/[ACY]_o = 100);

(i): pH = 4.0 (4.5 W, [H₂O₂]_o/[ACY]_o = 20); (l) pH = 6.0 (8.0 W, [H₂O₂]_o/[ACY]_o = 60);

(m) pH = 6.0 (8.0 W, [H₂O₂]_o/[ACY]_o = 142).

Figure 3: Evolution of acute toxicity with *D. magna* (24 h and 48h) during the UV₂₅₄ (A) and UV₂₅₄/H₂O₂ (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 UV₂₅₄ and UV₂₅₄/H₂O₂ 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 UV₂₅₄ (A) and UV₂₅₄/H₂O₂ (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 photooxidation by UV₂₅₄/H₂O₂ process and mass balance equations. The terms $f_{H_2O_2}$ and f_{ACY} indicate the fraction of UV₂₅₄ 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 for hydrogen peroxide at 254 nm respectively.

Table 3: First brood and live offspring after 21 days of *D. magna* exposure for different UV₂₅₄ doses (with and without hydrogen peroxide).

Table 4: Molecular structures of the chemical species identified from the MS spectra of samples submitted to UV₂₅₄ and UV₂₅₄/H₂O₂ photolysis.

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