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Removal of antiretroviral drugs stavudine and zidovudine in water under UV254 and UV254/H₂O₂ processes: Quantum yields, kinetics and ecotoxicology assessment

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Abstract: The concentration of antiretroviral drugs in wastewater treatment plants (WWTP) effluents and surface waters of developed and developing countries, especially in the African region more affected by HIV, has increased significantly in recent years due to their widespread use. The presence of antiretroviral in natural water bodies limits the possibility of reuse of such waters, after traditional disinfection process (i.e. UV, chlorine) for civil and irrigation purposes. The removal of stavudine and zidovudine under UV254 or UV254/H₂O₂ irradiation was investigated in distilled water. The quantum yield of direct photolysis and the kinetic constant of reaction of hydroxyl radical with the antiretrovirals at different pH have been evaluated. A battery of ecotoxicological tests (i.e. inhibition growth, bioluminescence, mutagenic and genotoxic activity) using different living organisms belonging to bacteria (*Aliivibrio fischeri*, *Salmonella typhimurium*), crustacean (*Daphnia magna*) and algae (*Raphidocelis subcapitata*) revealed a marked influence of the UV dose absorbed by the solution during the photolytic processes on the ecotoxic activity.

Novelty

We present for the first time a kinetic and ecotoxicological investigation on the removal with UV₂₅₄-assisted processes of two antiretrovirals (stavudine and zidovudine) from milli-Q water through a microphotoreactor, which allows very fast experimentation with minimal sample volumes. Zidovudine and stavudine are new emerging poor biodegradable microcontaminants detected in STP effluents and surface waters, especially in African countries, due to the highest incidence of HIV-positive people. Recently, the level of zidovudine in Kenian surface waters increased up to three order of magnitude. Moreover, these substances have been demonstrated to exert a carcinogenic activity. For this purpose, the ecotoxicity of solutions was evaluated to assess genotoxicity and mutagenicity.

Removal of antiretroviral drugs stavudine and zidovudine in water under UV₂₅₄ and UV₂₅₄/H₂O₂ processes: quantum yields, kinetics and ecotoxicology assessment

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ABSTRACT

The concentration of antiretroviral drugs in wastewater treatment plants (WWTP) effluents and surface waters of developed and developing countries, especially in the African region more affected by HIV, has increased significantly in recent years due to their widespread use. The presence of antiretroviral in natural water bodies limits the possibility of reuse of such waters, after traditional disinfection process (i.e. UV, chlorine) for civil and irrigation purposes. The removal of stavudine and zidovudine under UV₂₅₄ or UV₂₅₄/H₂O₂ irradiation was investigated in distilled water. The quantum yield of direct photolysis and the kinetic constant of reaction of hydroxyl radical with the antiretrovirals at different pH have been evaluated. A battery of ecotoxicological tests (i.e. inhibition growth, bioluminescence, mutagenic and genotoxic activity) using different living organisms belonging to bacteria (*Aliivibrio fischeri*, *Salmonella typhimurium*), crustacean (*Daphnia magna*) and algae (*Raphidocelis subcapitata*) revealed a marked influence of the UV dose absorbed by the solution during the photolytic processes on the ecotoxic activity.

Keywords: photodegradation, microreactor, mutagenicity, genotoxicity, water reuse, zidovudine, stavudine, antiretroviral.

1. Introduction

In the last decades, as a result of the widespread availability of pharmaceutical drugs, occurrence, identification, quantification, removal and environmental fate of these emerging contaminants have received significant critical attention [1-3]. Among this new and increasingly growing class of water microcontaminants, the presence of antiretroviral drugs (ARVs) in wastewater and surface water has been the focus of recent research [4-9]. Since their introduction into the market in the early 90s, ARVs have rapidly spread across the world

1 because of their effectiveness in the treatment of the HIV virus [10]. In fact, ARVs inhibit the
2 reverse transcriptase of the HIV virus, repressing viral replication [11]. The most commonly
3 used ARVs include zidovudine (ZDV), stavudine (STV), lamivudine, abacavir and
4 nevirapine which are usually administered as a combination therapy to increase their
5 effectiveness in preventing HIV reproduction [12]. ZDV was the first marketed antiretroviral
6 [12] and is still one of the most widely used. STV is also one of the most common ARVs,
7 despite presenting several side effects, because of its relatively low price [13]. Collectively,
8 ARVs increase the life expectancy of HIV-positive patients, however, significant concerns
9 have been raised about their simultaneous release to the environment [4,5,9]. New concerns
10 are also related to their consumption in the illicit drugs nyaope [14] and whoonga [15]. As a
11 result, STV and ZDV have been often detected in effluents of wastewater treatment plants
12 (WWTPs) and in natural surface water, in Europe and in Africa, at levels of tens of $\text{ng}\cdot\text{L}^{-1}$ up
13 to hundreds of $\text{ng}\cdot\text{L}^{-1}$ (Table 1).

14 In Europe, the main ARVs contamination route of natural waters is through human body
15 excretion and subsequent release in the sewage system [9]. The further presence of ARVs in
16 the effluents of WWTPs and surface water demonstrates the inefficiency of current WWTPs
17 treatment methods. The highest concentrations have been detected in Kenya and South
18 Africa. The levels of ZDV and STV in these African countries, have been shown to be higher
19 in surface water compared to WWTPs effluents, which contrast with the general trend in
20 Europe. The level of ZDV in Kenian surface waters increased up to three order of magnitude
21 during the period 2012 to 2016 [16,18]. Recently, ZDV has also been detected in
22 groundwater [16] which can be probably ascribed to the illicit use and direct spillage in
23 water.

24 It has been reported [7] that ZDV is not completely removed in conventional treatment
25 plants, a conclusion also shown for an aerobic and anaerobic WWTP in Germany [9],

although, these authors reported 68% of ZDV removal in different German WWTP with an activated sludge system. Further biological treatment studies performed in synthetic wastewater demonstrated that ZDV is non biodegradable, toxic, and inhibitory to activated sludge bacteria [22]. Higher removals have been reported for STV through activated sludge (> 78%) and biological treatment (> 89%) [7,9]. Even though the reported LC_{50} (Daphnid acute 48 h) are $980 \text{ mg}\cdot\text{L}^{-1}$ and higher than $100 \text{ mg}\cdot\text{L}^{-1}$ for STV and ZDV respectively [23-25], synergistic and mutagenic effects on the aquatic fauna and humans cannot be ruled out. For example, ZDV has been demonstrated to have carcinogenic potential [26]. Advanced oxidation processes (AOPs) have increasingly been proposed as effective tertiary treatments for the removal of biorecalcitrant emerging contaminants [27,28]. Among these, the UV_{254}/H_2O_2 is considered one of the most convenient process since it can be simply applied in existing municipal water treatment plants adopting UV_{254} lamps for water disinfection, such as treatment plants for water reuse and tertiary units in conventional STP [29]. Notably, reclaimed water reuse for irrigation is especially suitable in water stressed areas [30], which often also present the highest incidence of HIV-positive people, such as Central and South Africa. In spite of the apparent effectiveness of AOPs in micropollutants removal, the potential for the formation of highly toxic by-products [31,32] calls for longer treatment times and for the further evaluation of the ecotoxicity of the treated water. In this study the kinetics of ZDV and STV direct photolysis under UV_{254} radiation and in the presence of hydrogen (UV_{254}/H_2O_2) was investigated in order to estimate important photo-kinetic parameters, such as the quantum yields and the second-order kinetic constant of reaction between OH radicals and the compounds, which are necessary for design and retrofitting of water treatment plants. The reaction kinetics were investigated by means of a recent developed methodology which used microcapillary photoreactor systems [33], previously adopted for the investigation of the photolytic kinetics of other micropollutants [34-36]. The

1 use of this new microphotoreactor technology has been shown to be particularly suitable for
2 the study on highly priced, hazardous, or poorly available water contaminants since it allows
3 to run the entire experimental campaign using minimal amount of compounds, in this case
4 less than 50 mg of ZDV and STV.
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9 The implementation of water reclamation systems and of the environmental risks posed by
10 the effluents, requires comprehensive ecotoxicological assessment on a set of biological tests
11 on species at different trophic levels [37]. For this purpose, the three most frequently
12 ecotoxicity bioassays in aquatic systems are the assessment on *Aliivibrio fischeri* and
13 *Daphnia magna* tests for acute toxicity and the *Raphidocelis subcapitata* test for chronic
14 toxicity. Although these target organisms have often been used to assess the impact of
15 contaminated water, the main focus of water quality testing should also concern organisms-
16 dependent chemical-physical and biological properties of the target molecules. In particular,
17 several studies have demonstrated that ARVs differ in genotoxic potency, chromosomal
18 damage and aberration types induced *in vitro* and in perinatally exposed mice and infants [38-
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36 In consequence, in the present study we investigated the ecotoxicity of untreated and treated
37 solutions of ZDV and STV using a battery of ecologically relevant testing species to assess
38 the acute and chronic toxicities and genotoxicity and mutagenicity.
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46 **2. Experimental**

47 *2.1. Materials*

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49 Zidovudine (> 99%), stavudine (> 98%), NaOH ($\geq$ 98%), H₂SO₄ (98%), hydrogen peroxide
50 (30% in H₂O), acetonitrile ($\geq$ 99.9%), methanol ($\geq$ 99.9%), phosphoric acid (85% in H₂O),
51 catalase from *Micrococcus lysodeikticus*, CaCl₂·2H₂O ($\geq$ 99.5%), MgSO₄·7H₂O ($\geq$ 98%),
52 NaHCO₃ ($\geq$ 99.5 %), KCl ($\geq$ 99 %), NH₄Cl ($\geq$ 99.9%), MgCl₂·6H₂O ($\geq$ 98%), KH₂PO₄ ($\geq$
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99%), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ($\geq 98\%$), $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ ($\geq 99.9\%$), H_3BO_3 ($\geq 99\%$), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ($\geq 98\%$), ZnCl_2 ($\geq 99\%$), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ($\geq 98\%$), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ($\geq 98\%$) and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ($\geq 98\%$) were all purchased from Sigma-Aldrich and used as received.

Reconstitution solution, osmotic adjusting solution (OAS) and diluent (NaCl 2%) were the reagents used for the *Aliivibrio fischeri* toxicity test (Strategic diagnostics Inc. SDI). All the reacting solutions were prepared adding the contaminants and hydrogen peroxide to milliQ water. When necessary, pH was adjusted by using dilute aqueous solutions of NaOH and H_2SO_4 .

2.2. Photolytic treatments

A microcapillary film (MCF) array photoreactor was used to perform the UV_{254} photolysis and $\text{UV}_{254}/\text{H}_2\text{O}_2$ experiments. A detailed description and a scheme of the reactor can be found elsewhere [33,34]. Briefly, the polymeric film microreactor consists of ten tubular microcapillaries (Lamina Dielectrics Ltd, Billingshurst, West Sussex, UK) with a mean hydraulic diameter of 195 μm and an average optical path length of 152 μm . The MFC was fed by means of a syringe pump (HA Harvard Apparatus PHD Ultra) and wrapped around a germicidal lamp (Germicidal G8T5, Ge Lighting) emitting at 254 nm. The nominal lamp power could be varied from 8.0 to 4.5 W with the use of a switch power supplier. The emitted photon fluxes per unit volume (I_0/V) were estimated by hydrogen peroxide actinometry [41,42] and were $1.92 \cdot 10^{-2} \text{ ein} \cdot \text{s}^{-1} \cdot \text{L}^{-1}$ and $1.27 \cdot 10^{-2} \text{ ein} \cdot \text{s}^{-1} \cdot \text{L}^{-1}$, respectively. The residence time (space time) through the MFC was varied changing the length of the film exposed to the light. Samples were collected at the outlet of the MFC after reaching steady state conditions, and rapidly analyzed by HPLC. Negligible temperature differences ($\sim 25^\circ\text{C}$) between the inlet and the outlet samples were found in all the experiments. All the experimental runs were

carried out in duplicate. The entire experimental campaign (> 50 runs) was carried out using about 2500 ml of distilled water.

In order to produce relatively larger volumes of treated solutions, necessary to run the ecotoxicology tests, experiments were also carried out in a thermostated (25 °C) stirred glass batch annular photoreactor with a volume of $4.8 \cdot 10^{-1}$ L, housing a low pressure mercury lamp emitting at 254 nm (Helios Italquartz, HGL10T5L, 17 W) in the centre axis of the annulus. In both photoreactor devices, ZDV and STV solutions at an initial concentration of $4.5 \text{ mg} \cdot \text{L}^{-1}$ and $4.35 \text{ mg} \cdot \text{L}^{-1}$ respectively, were treated without and with the addition of hydrogen peroxide (molar ratio $\text{H}_2\text{O}_2/\text{ARV} = 100$) and with a UV_{254} dose corresponding to treatment times or space times necessary to achieve a 45% and 90% conversion of the antiretrovirals and for space times double those needed to achieve a complete conversion. The UV_{254} dose ($\text{mJ} \cdot \text{cm}^{-2}$) was calculated as the average photon fluence rate multiplied by the treatment time (s). The average photon fluence rate emitted by the UV lamp at 254 nm was $4.7 \text{ mW} \cdot \text{cm}^{-2}$ (UVC DELTA OHM radiometer). The experimental device was described elsewhere [36]. Catalase enzyme was also added to both treated and untreated solutions to determine the ecotoxicological effects without the interference given by the presence of H_2O_2 residuals.

2.3. Analytical methods

ZDV, STV, and hydrogen peroxide concentrations were measured by HPLC (1100 Agilent) equipped with a Gemini 5u C6-Phenyl (260 x 4.60 mm) (Phenomenex) column. An isocratic method was used for the simultaneous quantification of ZDV and hydrogen peroxide with mobile phase ($0.8 \text{ mL} \cdot \text{min}^{-1}$) made of 93% aqueous phosphoric acid (10 mM) and 7% acetonitrile. Under these analytical conditions the retention times of hydrogen peroxide and ZDV were 4.1 and 13.7 min, respectively. The mobile phase was changed to 80% water and

20% methanol for the simultaneous identification of H₂O₂ and STV with retention times of 3.7 and 10 min, respectively. The signals were acquired at 266 nm.

The molar absorption coefficients of STV and ZDV were estimated using a Perkin Elmer UV/VIS spectrometer (mod. Lambda 35).

2.4. *Ecotoxicological methods*

2.4.1. *Toxicity test with Daphnia magna*

The test was conducted in accordance with ISO 6341 [43]. Every test was performed in quadruplicate with four control groups. Briefly, neonates aged less than 24 hours were separated into four groups and exposed to different concentrations of untreated and treated solutions of ZDV and STV. All tests were carried out at constant temperature (20±2 °C) and in darkness and organisms were not fed during the experiments. After 48 h exposure, daphnids that were not able to swim within 15 seconds under gentle agitation were considered to be immobilized.

2.4.2. *Bacteria toxicity test*

The Microtox® SPT procedure [44] was used to evaluate the acute toxicity of the samples using as endpoint the bioluminescence inhibition of the naturally emitted by *A. fischeri* (strain NRRL-B-11177) after a contact time of 30 min with the test sample. The samples were serially diluted to a series of four concentrations, then a volume of 10 µL of reconstituted bacterial reagent was added to dilutions series of samples. The emission of bioluminescence was recorded after 30 min of contact time with the bacteria at 15±2 °C.

2.4.3. Algal growth inhibition test with *Raphidocelis subcapitata*

The growth inhibition test was assessed following the ISO 8692:2012 standard procedure [45]. Exponentially growing algae (10^4 cell·mL⁻¹) were exposed to various concentrations of the test samples in six replicates over a period of 72 h under defined conditions, as described elsewhere [46]. Growth and inhibition were quantified from measurements of the algal biomass density (cell counts) as a function of time. The specific growth rate of *R. subcapitata* in each replicate culture was calculated from the logarithmic increase in cell density in the intervals from 0 to 72 h using the following equation: $\mu = \frac{\ln N_n - \ln N_0}{t_n - t_0}$, where N_0 is the cell concentration at $t = 0$, N_n the final cell concentration after 72 h of exposure, t_0 the time of start measurement, and t_n the time of last measurement (hours from start). The results were expressed as the mean ($\pm$ standard deviation) of the percentage inhibition of the cell growth (% I) of the sample compared with the negative control ($p \leq 0.05$).

2.4.4. Mutagenicity assay with *Salmonella typhimurium*

The Muta-Chromoplate kit was used to evaluate the mutagenicity [47]. The fluctuation tests were performed using *S. typhimurium* strains TA100 and observing the potential *his*- reverse mutation after exposure to mutagens [48].

Bacteria cultures, grown overnight and reaching the exponential growth phase, were exposed for 5 days at 37° C to different samples concentrations, in a liquid medium into 96-well microtiter plates. After this period, the positives samples wells that turned yellow were counted, while the purple wells were considered as negatives. The reversion of mutants (*his*+) exhibited the yellow color due to the acidification of the test medium resulting from the growth of reverse mutants. The number of *his*+ revertant colonies in each sample was determined as a mean value of the three plates. The results were expressed as a mutagenicity ratio (MR), i.e. the ratio of the number of *S. typhimurium* revertants grown in the presence of

the tested sample to the number of spontaneously appeared revertants. The sample was considered mutagenic when $MR \geq 2$ [49]. χ -square analysis was used for statistical evaluation of the treated plates versus the control plates.

2.4.5. Genotoxicity assay with *Salmonella typhimurium*

The umu test [50] was performed according to standard procedure [51], which was developed for the detection of genotoxic materials that cause DNA cell damage. In this assay, a modified strain of *S. typhimurium* TA1535/pSK 1002 bacteria was used, whereby a β -galactosidase gene was linked to SOS-DNA response. Bacterial cultures were grown overnight at 37 °C and then diluted in TGA medium (Tryptone-Glucose-Ampicillin medium) until the cells entered the logarithmic growth phase. The cells were then exposed to the test samples for 2 hours. The induction of genotoxicity (expressed as β -galactosidase activity) was determined colorimetrically at 420 nm after adding o-nitrophenyl galactopyranoside to the samples. Growth was measured as the absorbance at a wavelength of 600 nm. The result was calculated as an induction ratio, $IR = (1/G) \cdot US$, where G was the growth and US the relative enzyme activity. The sample was considered genotoxic when IR was greater than 1.5. The significance of the differences between the mean values of different tests and controls was assessed by Student's test and analysis of variance (ANOVA) with a 0.05 significance level. Moreover, post-hoc analysis were carried out by Tukey's test [52].

3. Results and Discussion

3.1. Absorbance spectra

The absorbance spectra of ZDV (Fig. 1a) and STV (Fig. 1b) at pH 4.0, 6.5 and 8.0 showed invariance in the pH range from 4.0 to 8.0 for ZDV and from 6.5- to 8.0 for STV. Since pH did not affect ZDV and STV degradation kinetics in the pH ranges 4.0-8.0 and 6.0-8.0,

respectively, the reaction kinetics were investigated in the slightly acidic to alkaline pH range from 6.0 to 8.0, which also is more environmentally relevant. The estimated molar absorption coefficients at 254 nm are summarized in Table 2.

3.2. ZDV and STV direct photolysis and quantum yield estimation

The quantum yield (ϕ_i^{254}) of direct photolysis at 254 nm was determined with different sets of experimental runs carried out in the MCF varying the ARV initial concentration, the pH and the lamp power (Table 3). The degradation of the generic ARV by direct photolysis follows the mass balance (eq. 1):

$$\frac{dC_i}{dt} = -\frac{I_0}{V} \cdot \phi_i^{254} \cdot (1 - \exp(-2.3 \cdot l \cdot \varepsilon_i^{254} \cdot C_i)) \quad (1)$$

where C_i is the concentration of ARV, I_0/V is the photon flux per unit volume, l is the average optical length of the reactor (see *Photolytic treatments section*), and ε_i^{254} the molar absorption coefficient of the ARV species (Table 2). A Matlab optimization routine based on the Runge-Kutta method was adopted to determine the value of ϕ_i^{254} which minimized the objective function (optimisation mode):

$$\sum_j^m \sum_i^n (y_{j,i} - c_{j,i})^2 \quad (2)$$

where c and y are the experimental and calculated concentrations at different reaction times (n) and experimental runs (m). Table 3 shows the estimated quantum yields and the 97% interval of confidence of ZDV and STV. The results corroborate with the previous photolytic decomposition investigation [53], that reported higher sensitivity of ZDV to UVA light compared to STV, with a fluorescent irradiation source in the wavelength spectra from 320 nm to 400 nm.

All the adopted experimental runs used for the kinetic modeling along with the experimental conditions and the average standard deviation are reported in Table 4 whereas Fig. 2 (a-d)

show representative examples of the comparison between the experimental and the calculated profiles for ARV degradation by direct photolysis. As reported, the model was validated simulating simultaneously different experimental runs not included in the optimization routine (simulation mode, Fig. 2e-f).

3.3. Kinetic modeling of UV_{254}/H_2O_2 process

The second order rate constant $k_{OH,i}$ of the reaction between ZDV and STV with hydroxyl radicals was estimated from a set of experimental runs carried out in the MCF at varying pH, lamp power and $C_{H_2O_2}/C_i$ molar ratio (Table 5). They were modeled according to the following simplified reaction scheme:

Hydroxyl radicals formed by direct photolysis of hydrogen peroxide under UV_{254} radiation (r₁) attack the ARV (r₃), the P and S pseudo by-products formed (r₄-r₅), and the oxidant hydrogen peroxide which generates hydroperoxyl radicals (r₆). The latter recombine according to the termination reaction (r₇) to form H_2O_2 . The simultaneous photolysis of ARV is considered in reaction (r₂). Assuming that hydroxyl radicals attack both substrate and the first generation of chemical intermediates with the same rate constant $k_{OH,i}$ and that at any

reaction time the sum of the unconverted substrate (C_i) and its by-products ($C_{P,S}$) concentration is equal to the initial antiretroviral concentration (C_o):

$$C_o = C_i + C_{P,S}$$

the concentrations of hydroxyl and hydroperoxyl radicals, under the steady state approximation equal:

$$[HO^\bullet]_{ss} = \frac{2F_{H_2O_2}}{k_{OH,i} \cdot C_o + k_H \cdot C_{H_2O_2}} \quad (3)$$

$$[HO_2^\bullet]_{ss}^2 = \frac{F_{H_2O_2} \cdot k_H \cdot C_{H_2O_2}}{k_t(k_{OH,i} \cdot C_o + k_H \cdot C_{H_2O_2})} \quad (4)$$

where

$$F_{H_2O_2} = \frac{I_0}{V} \cdot \phi_{H_2O_2}^{254} \cdot \left(1 - \exp\left(-2.3 \cdot l \cdot (\varepsilon_{H_2O_2}^{254} \cdot C_{H_2O_2} + \varepsilon_i^{254} \cdot C_i)\right)\right) \cdot \frac{\varepsilon_{H_2O_2}^{254} \cdot C_{H_2O_2}}{\varepsilon_{H_2O_2}^{254} \cdot C_{H_2O_2} + \varepsilon_i^{254} \cdot C_i} \quad (5)$$

and the concentration of H_2O_2 and ARVs versus time can be determined by solving the following material balance:

$$\frac{dC_{H_2O_2}}{dt} = -F_{H_2O_2} - \frac{k_H \cdot C_{H_2O_2} \cdot F_{H_2O_2}}{k_{OH,i} \cdot C_o + k_H \cdot C_{H_2O_2}} \quad (6)$$

$$\frac{dC_i}{dt} = -F_i - \frac{2k_{OH,i} \cdot C_i \cdot F_{H_2O_2}}{k_{OH,i} \cdot C_o + k_H \cdot C_{H_2O_2}} \quad (7)$$

where

$$F_i = \frac{I_0}{V} \cdot \phi_i^{254} \cdot \left(1 - \exp\left(-2.3 \cdot l \cdot (\varepsilon_{H_2O_2}^{254} \cdot C_{H_2O_2} + \varepsilon_i^{254} \cdot C_i)\right)\right) \cdot \frac{\varepsilon_{ARV}^{254} \cdot C_{H_2O_2}}{\varepsilon_{H_2O_2}^{254} \cdot C_{H_2O_2} + \varepsilon_i^{254} \cdot C_i} \quad (8)$$

Similarly to the previous analysis, the equations (6-7) were solved by means of an optimization routine to minimize the objective function

$$\sum_{h=1}^2 \sum_j^m \sum_i^n (y_{j,i} - c_{j,i})^2 \quad (9)$$

slightly modified to account for the number of reacting species (h). Selected experimental runs were not included in the optimization procedure to validate the kinetic model without further adjustment of the estimated kinetic parameter $k_{OH,i}$. All the runs adopted, along with their average standard deviation, are summarized in Table 5. Figure 3 shows the comparisons between the experimental and calculated data, both in optimization and simulation modes. The second order rate constant of ARVs investigated with hydroxyl radicals is shown in Table 6. The kinetic constant of reaction of hydroxyl radical with ZDV, determined by the competition kinetics method, in literature varies over a wide range: $(1.3 \pm 0.026) \cdot 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$ determined using para-chlorobenzoic acid as reference compound [57] and $(5.73 \pm 0.76) \cdot 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ determined using acetophenone as reference substance [17]. These values are significant higher than the results obtained in this study. One possible explanation for this discrepancy is that the competition kinetics method is only reliable if the contribution of direct photolysis of the investigated compound is absent or negligible. In the absence of that, the $k_{OH,i}$ value tends to be overestimated ($k'_{OH,i}$) since it also accounts for the contribution of direct photolysis to the degradation. To further clarify this aspect, the competition kinetic method using benzoic acid (BA) as reference compound [58] was also used to estimate the second order rate constant of hydroxyl radicals with ARVs. According to this method the $k'_{OH,i}$ value can be calculated according to (10):

$$\ln \frac{C_i}{C_o} = \frac{k'_{OH,i}}{k_{OH,BA}} \ln \frac{C_{BA}}{C_{BA_o}} \quad (10)$$

where C_{BA} and C_{BA_o} are the unconverted and initial concentration of benzoic acid, and $k_{OH,BA}$ the kinetic constant of reaction between benzoic acid and hydroxyl radical ($5.9 \cdot 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ [55]). Duplicate experimental runs were carried out under the following conditions: $C_{ZDV_o} = 2.46 \cdot 10^{-5} \text{ M}$, $C_{BA_o} = 2.62 \cdot 10^{-5} \text{ M}$, $C_{H_2O_2_o} = 1.41 \cdot 10^{-3} \text{ M}$, and $C_{STV_o} = 2.21 \cdot 10^{-5} \text{ M}$, $C_{BA_o} = 2.15 \cdot 10^{-5} \text{ M}$, $C_{H_2O_2_o} = 9.8 \cdot 10^{-4} \text{ M}$. Plotting $\ln C_i/C_o$ vs $\ln C_{BA}/C_{BA_o}$, $k'_{OH,i}$ was estimated as

6.39·10⁹ M⁻¹ s⁻¹ and 5.33·10⁹ M⁻¹ s⁻¹ for ZDV and STV, respectively. As expected both values overestimate the results reported in Table 6. Moreover, the difference is higher for ZDV, in agreement with the higher significance of direct photolysis in the degradation of this compound compared to STV. It is also important to notice that the $k'_{OH,ZDV}$ value estimated in the present study, adopting the competition kinetics method, was within those reported in the literature [17,57].

3.4. Ecotoxicological assessment

The inhibition of *A. fischeri* luminescence and *D. magna* immobility was not observed on untreated and UV₂₅₄ or UV₂₅₄/H₂O₂ treated solutions (data not shown). The growth inhibition of algae *R. subcapitata* exposed to ZDV and STV containing solutions before and during the photolytic processes (UV₂₅₄ or UV₂₅₄/H₂O₂) is shown in Figures 4a-f. A marked different trend in effects on algae growth according to the dose–response correlation was observed. ZDV inhibited slightly algal growth, while STV had insignificant effect (less than 20%). The toxicity of the treated solutions in both processes slightly increased increasing the UV dose, also for treatment times corresponding to the complete removal of the antiretroviral drugs. In particular, the inhibited algal growth for the ZDV treated solutions increased by 36% and 44% when the conversion of ZDV was 90% and 100% respectively in both UV₂₅₄ and UV₂₅₄/H₂O₂ treatments (Fig. 4a). The same increasing trend of ecotoxicity was observed with the STV treated solutions, although the increase in ecotoxicity to *R. subcapitata* was less significant in comparison to the ZDV treated solutions. The inhibition was 20% and 30% when the STV conversions was 90% and 100% respectively (Fig. 4b).

The antiretroviral drugs showed marked algae growth activity when the dilution factor of untreated and treated solutions was increased from 1:10 (Figs. 4c-d) to 1:100 (Figs. 4e-f), which appeared to stimulate algal growth with a statistically significant extent. This

uncommon “apparently beneficial effect” observed at low doses, known as hormesis, has been previously reported for some bioindicators such as crustaceans [59,60] and plants and algae [61] in the presence of nitrogen-containing organic molecules (trinitrotoluene, triazine herbicides, etc.) such as are STV and ZDV.

Tables 7 and 8 summarize the mutagenicity and genotoxicity results. Both antiretrovirals were not able to determine a significant SOS system induction, while variability among the mutagenic responses was observed in the Salmonella mutagenicity assay (threshold value: 2.0). The mutagenicity results indicated that (i) potential mutagenic degradation intermediates could have been formed at significant levels during the photolysis of ZDV than STV, (ii) the mutagenicity of the samples further increased at increasing UV_{254} doses and (iii) the UV_{254}/H_2O_2 treatment produced less mutagenic intermediate products than those formed during UV_{254} photolysis. Residual mutagenic activity was also observed on the ZDV samples treated by UV_{254} photolysis for conversions higher than 90%, after a 1:1000 dilution (Table 7). It is useful to point out that the concentration of ZDV in these samples was of the same order of magnitude as the values detected in African surface water (Table 1).

During the UV_{254}/H_2O_2 process it was observed a slight increase of revertants followed by disappearance of revertants at the highest UV_{254} exposure.

Investigations of the genotoxic endpoints demonstrated that the untreated solutions could not be classified as genotoxic since the induction ratio was below the threshold value of 1.5 (Tables 7-8). On the contrary, a statistically significant increase in umuC induction was recorded for ZDV undiluted solutions for UV_{254} doses corresponding to ZDV conversions of 45% and 90% for both UV_{254} and UV_{254}/H_2O_2 processes (Table 7). The genotoxic activity showed a decreasing trend as the dilution factor of the solutions increased.

STV genotoxic data demonstrated (Table 8) that only the treated solutions were able to induce a significant SOS response with IF higher than 2 also observed for the highest dilution

factor (1:1000), which corresponds to an initial concentration of STV of $4.3 \mu\text{g}\cdot\text{L}^{-1}$. The results collectively may indicate that some photoproducts generated during the photolytic processes could have genotoxic activity. However, genotoxic activity was not observed in the solutions treated for prolonged treatment times, thus suggesting that the genotoxic transformation products observed in earlier times might have further evolved to non-genotoxic metabolites.

4. Conclusions

The removal of stavudine and zidovudine by UV_{254} radiation without and with hydrogen peroxide was investigated in a microcapillary film photoreactor using minimal quantities of water samples. Higher UV_{254} -photolysis quantum yields were observed for zidovudine, $(2.357 \pm 0.0589) \cdot 10^{-2} \text{ mol}\cdot\text{ein}^{-1}$ in the pH range from 4.0 to 8.0, while stavudine quantum yield was 28-fold lower $(8.34 \pm 0.334) \cdot 10^{-4} \text{ mol}\cdot\text{ein}^{-1}$ in the pH range from 6.0 to 8.0. The second-order rate constant of reaction with hydroxyl radicals was $(9.98 \pm 0.68) \cdot 10^8 \text{ M}^{-1}\cdot\text{s}^{-1}$ (pH range 4.0 – 8.0) for zidovudine and $(2.03 \pm 0.18) \cdot 10^9 \text{ M}^{-1}\cdot\text{s}^{-1}$ (pH: 6.0 – 8.0) for stavudine. The well known ecotoxicological tests using *A. fischeri* and *D. magna* as bioindicators did not evidenced acute or chronic effects. A hormetic effect was observed for the first time in *R. subcapitata* for ZDV and STV treated solutions at different UV_{254} doses after a dilution from 1:10 to 1:100.

On the contrary, specific tests using *Salmonella t.* revealed mutagenic and genotoxic activity of the ZDV and STV samples also at high dilution factors depending on the type of the photolytic treatment and substrate conversion.

Generally, UV_{254} photolysis in the presence of hydrogen peroxide reduces the ecotoxicological risk associated to direct photolysis of the aqueous solutions containing the antiretrovirals, but for this purpose UV_{254} doses ($\geq 2000 \text{ mJ}\cdot\text{cm}^{-2}$), significantly higher than

the levels suggested for the water UV disinfection processes ($50 - 200 \text{ mJ}\cdot\text{cm}^{-2}$) are necessary. This study pointed out the critical importance of selecting suitable bioindicators depending on chemical and biological properties of the selected xenobiotics detected in STP effluents and in surface waters.

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Removal kinetics of Zidovudine and Stavudine under UV_{254} and UV_{254}/H_2O_2 were studied

Zidovudine and Stavudine quantum yields at 254 nm were estimated

Kinetic constants of reaction between OH radicals and antiretrovirals were estimated

No acute/chronic effects of treated and untreated samples on *Vibrio f.* and *Daphnia m.*

Mutagenic/genotoxic activity of treated and untreated diluted samples on *Salmonella t.*

<div><div><div>Zidovudine</div><div> </div><div>pK_a = 9.8 [17]</div></div></div>				
WWTP effluent (ng/L)	Surface water (ng/L)	Groundwater (ng/L)	Location	Ref
--	18.3	--	Kenya	[18]
110 – 90	17410 – 50	30 – 20	Kenya	[16]
513	7684	--	Kenya	[5]
--	973 – 51.7	--	South Africa	[7]
564 – 98.2	170 – 1.2	--	Germany	[9]
180 – 57	30 – 22	--	Germany	[19]
37 – 22	--	--	Finland	[6]
191 – 154	--	--	France	[20]
<div><div><div>Stavudine</div><div> </div><div>pK_a = 10 [21]</div></div></div>				
WWTP effluent (ng/L)	Surface water (ng/L)	Location		Ref
--	778 – 102	Kenya		[18]
--	4.3 – 1.3	Germany		[9]

Table 1

	$\varepsilon_i^{254nm} (M^{-1} \cdot cm^{-1})$	pH range
ZDV	$1.19 \cdot 10^4$	4 - 8
STV	$7.81 \cdot 10^3$	6 - 8

Table 2

	$\phi_i^{254} (mol \cdot ein^{-1})$	pH range
ZDV	$(2.357 \pm 0.0589) \cdot 10^{-2}$	4 - 8
STV	$(8.34 \pm 0.334) \cdot 10^{-4}$	6 - 8

Table 3

ZDV (optimization mode)				
Run	C_o·10⁵ (M)	pH	lamp nominal power (W)	σ (%)
<i>1</i>	3.76	6	8	1.27
<i>2</i>	3.67	4	8	0.55
<i>3</i>	1.86	6	8	1.22
<i>4</i>	1.83	4	8	1.57
<i>5</i>	1.86	6	4.5	1.98
ZDV (simulation mode)				
<i>6</i>	3.65	8	8	0.48
<i>7</i>	3.76	6	4.5	1.14
STV (optimization mode)				
<i>8</i>	4.49	6	8	1.65
<i>9</i>	4.46	8	8	1.39
<i>10</i>	2.27	6	8	3.87
STV (simulation mode)				
<i>11</i>	4.49	6	4.5	2.41

Table 4

ZDV (optimization mode)					
Run	$C_o \cdot 10^5$ (M)	$[H_2O_2]_o \cdot 10^3$ (M)	pH	lamp nominal power (W)	σ (%)
1bis	4.89	0.7	6	4.5	1.60
2bis	4.69	1.61	6	4.5	0.90
3bis	4.78	2.02	4	4.5	0.33
4bis	4.86	2.69	6	4.5	0.36
5bis	4.87	3.49	6	4.5	0.63
ZDV (simulation mode)					
6bis	4.74	1.79	8	4.5	0.83
7bis	4.80	1.87	6	8	1.59
STV (optimization mode)					
8bis	4.47	1.83	6	4.5	3.74
9bis	4.48	2.99	6	4.5	2.58
10bis	4.47	4.17	6	4.5	3.74
11bis	4.43	4.16	8	4.5	2.57
12bis	4.47	1.83	6	8	1.82
STV (simulation mode)					
13bis	4.38	4.09	8	8	1.17

Table 5

	$k_{OH,i} (M^{-1} \cdot s^{-1})$	pH range
ZDV	$(9.98 \pm 0.68) \cdot 10^8$	4 - 8
STV	$(2.03 \pm 0.18) \cdot 10^9$	6 - 8

Table 6

		Mutagenic ratio				Induction ratio			
	Dilution factor	Conversion degree (%)				Conversion degree (%)			
		0	45	90	100	0	45	90	100
UV ₂₅₄	1	8.1	2.4	9.1	9.3	1.2	2.1	2.9	1.0
	10 ⁻¹	4.3	1.5	5.5	5.9	0.2	1.5	1.7	0.8
	10 ⁻²	2.7	1	3.3	3.7	ND	1.9	1.5	0.2
	10 ⁻³	2	ND	2.6	2.8	ND	ND	ND	ND
UV ₂₅₄ /H ₂ O ₂	1	7.9	6.5	2.9	0.8	1.5	0.5	3.1	0.9
	10 ⁻¹	6	4.9	0.5	0.3	0.7	ND	1.6	0.6
	10 ⁻²	2.1	0.7	ND	ND	ND	ND	1.6	ND
	10 ⁻³	ND	ND	ND	ND	ND	ND	ND	ND

Table 7

		Mutagenic ratio				Induction ratio			
	Dilution factor	Conversion degree (%)				Conversion degree (%)			
		0	45	90	100	0	45	90	100
UV ₂₅₄	1	4.2	4.4	5.1	2.1	0.2	2.58	2.68	ND
	10 ⁻¹	2.2	2.7	2.5	1.6	ND	2.02	2.22	ND
	10 ⁻²	2	2.1	2.1	1.5	ND	2.52	2.20	ND
	10 ⁻³	ND	ND	ND	ND	ND	2.87	2.26	ND
UV ₂₅₄ /H ₂ O ₂	1	4.1	4.6	ND	ND	0.5	2.68	2.50	ND
	10 ⁻¹	1.2	1.0	ND	ND	ND	2.22	2.28	ND
	10 ⁻²	ND	ND	ND	ND	ND	2.20	2.05	ND
	10 ⁻³	ND	ND	ND	ND	ND	2.20	2.22	ND

Table 8

Figure 1

OPTIMIZATION MODE

SIMULATION MODE

Figure 2

OPTIMIZATION MODE

SIMULATION MODE

Figure 3

Figure 4

Table 1 - Levels of zidovudine and stavudine in WWTPs effluents, surface water, and ground water.

Table 2 - Estimated molar absorption coefficient of ZDV and STV.

Table 3 - Estimated direct photolysis quantum yields of ZDV and STV at 254 nm.

Table 4 - Experimental runs and experimental conditions used for the estimation of quantum yields photolysis at 254 nm of ZDV and STV along with percentage standard deviation (% σ).

Table 5 - Experimental runs and experimental conditions for $k_{OH,i}$ estimation of ZDV and STV along with percentage standard deviation (% σ).

Table 6 - Estimated kinetic constant of hydroxyl radical attack to ZDV and STV with 97% confidence interval.

Table 7 - Mutagenic results from Ames and Umu tests for ZDV treated solutions. Starting concentration: $4.5 \text{ mg}\cdot\text{L}^{-1}$.

Table 8 - Mutagenic results from Ames and Umu tests for STV treated solutions. Starting concentration: $4.35 \text{ mg}\cdot\text{L}^{-1}$.

Fig. 1 - Absorbance spectra of ZDV (a) and STV (b). $C_{\text{init}} = 5\cdot 10^{-5} \text{ M}$. (—) pH = 4.0; (- - -) pH = 6.5; (···) pH = 8.0.

Fig. 2 - Comparison between experimental (symbols) and calculated (continuous lines) data for the direct photolysis of ZDV (a, b, e) and STV (c, d, f) under UV_{254} irradiation. Experimental conditions are summarized in Table 4 (a-1; b-4; c-9; d-10; e-6; f-11).

Fig. 3 - Comparison between experimental (symbols) and calculated (continuous lines) data for the $\text{UV}_{254}/\text{H}_2\text{O}_2$ degradation of ZDV (a, b, e) and STV (c, d, f). Experimental conditions are summarized in Table 5 (a-3bis; b-5bis; c-9bis; d-11bis; e-7bis; f-13bis). ZDV and STV concentrations are multiplied by 160 for visual convenience.

Fig. 4: Inhibition algal growth (*R. subcapitata*) for solutions containing the selected antiretrovirals by UV_{254} and $\text{UV}_{254}/\text{H}_2\text{O}_2$. Starting concentration: $4.5 \text{ mg}\cdot\text{L}^{-1}$ for ZDV, $4.35 \text{ mg}\cdot\text{L}^{-1}$ for STV. Data with different letters (a–d) are significantly different ($p < 0.05$).