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**Investigating the impact of UV-C/H₂O₂ and sunlight/H₂O₂ on the removal of antibiotics,
antibiotic resistance determinants and toxicity present in urban wastewater**

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Abstract

This work aimed at exploring the impact of UV-C/H₂O₂ and sunlight/H₂O₂ processes, applied at pilot scale, on removing: (i) ciprofloxacin and sulfamethoxazole, (ii) cultivable *Escherichia coli* and *Pseudomonas aeruginosa* grown in the presence and absence of sub-minimal inhibitory concentrations of ciprofloxacin and sulfamethoxazole and (iii) the genes 16S rRNA and selected antibiotic resistance genes (ARGs) (i.e., *sul1*, *bla*_{CTX-M}, *qnrS*, *tetM*, etc) from urban wastewater. The major antibiotic transformation products (TPs) formed, were elucidated and the chronic toxicity of the whole effluent mixture against *Vibrio fischeri* was evaluated.

The capability of the processes, in terms of the elimination of the antibiotics present in urban wastewater, varied among the two light sources used: both antibiotics were fully removed during UV-C/H₂O₂, whereas only ciprofloxacin was removed during the sunlight/H₂O₂. The photo-transformation of the antibiotics led to the identification of 21 and 18 TPs of ciprofloxacin and sulfamethoxazole, respectively, while all of them retained their core moiety, responsible for the antibacterial activity. All the UV-C/H₂O₂-treated samples were found to be toxic, whereas the luminescence of *V. fischeri* was not inhibited when tested in the sunlight/H₂O₂-treated samples. During both processes, *E. coli*, *P. aeruginosa* and the colonies of these species still viable in the presence of antibiotics, were successfully inactivated to values below the detection limit. However, sunlight/H₂O₂ has not achieved complete disinfection, as regrowth of *E. coli* and *P. aeruginosa* colonies was observed after 48 h of storage of the treated effluent. Finally, none of the technologies tested was able to completely remove the target ARGs, confirming their inability to prevent the spread of resistance determinants to the environment.

Introduction

Water scarcity in many parts of the world has already caused and continues to cause, various economic problems as well as job losses [1], [2]. This situation is expected to deteriorate due to the increase of the global population and the water use per capita, the climate change and other relevant stress factors [3], [4]. According to the principles of the circular economy, reclaimed water coming from urban wastewater treatment plants (UWTPs), is recognized as one of the main measures for the mitigation of the water crisis [5], as it can be a suitable alternative to water supply for the irrigation of crops. This approach has recently been promoted by the European Union, which has proposed a regulation setting the minimum quality criteria for reclaimed water intended for agricultural irrigation and aquifer recharge [6]. The regulation acknowledges the need of assessing the risk (where relevant) associated with contaminants of emerging concern (CECs) and antimicrobial resistance [7], [8]. The evolution and spread of antimicrobial resistance is recognized as one of the major Global Health challenges of the 21st century by major regulatory, economic and political bodies, including the World Health Organisation (WHO), the United Nations (UN) and the European Commission (EC), and intensive monitoring is recommended for the surveillance of critical hotspots, including UWTPs, aiming at reducing its propagation [9]. In the literature, it is well documented that antibiotic compounds present in concentrations below clinical breakpoints (as is the case in wastewater) may select for resistant bacterial strains [10], while the lateral gene transfer and propagation of antibiotic resistance genes (ARGs), may be favoured in the UWTPs, because of the high microbial density and other selection pressures [11], [12]. In order to combat antimicrobial resistance spread to the environment, it is therefore necessary to identify and/or develop

technologies able to effectively remove both the antibiotics and the resistance determinants when still at the treatment plant, before reuse or disposal of the effluent [12].

Disinfection of wastewater, aiming at the microbial inactivation, may present an opportunity to limit the release of antibiotic-resistant bacteria (ARB) into the environment and contribute to the minimisation of the environmentally-related risk of spreading resistance determinants. The consideration of UV-driven processes, which are commonly applied in UWTPs for disinfection, might be useful towards this objective. Irradiation with either a light source (usually performed with low- or medium-pressure mercury vapor lamps) or natural sunlight, is a potential means of removing microcontaminants and dissolved effluent organic matter (dE_fOM) present in urban wastewater effluents. The UV radiation can damage DNA, resulting in the inhibition of cell replication and, in case of lethal doses, in a loss of reproducibility. Although the adoption of UV for wastewater disinfection has grown significantly over the past few decades, research on the ability of the UV process to remove ARB&ARGs is only advancing during the recent years [13], [14]. To date, there is limited data available on the potential of light-driven processes to simultaneously remove antibiotics, ARB and ARGs from wastewater [15], [16], [17]. More systematic analysis of the operational parameters of the light-driven processes and their impact on the overall efficiency of the technologies to remove such microcontaminants is required. Furthermore, light-driven processes combined with hydrogen peroxide (H₂O₂), yielding additional hydroxyl radicals (HO•), resulting from the dissociation of H₂O₂, can further reduce the microcontaminants present in wastewater effluents, significantly enhancing the efficiency of the process [18]. The superiority of the UV/H₂O₂ over the conventional UV disinfection for the inactivation of ARB in wastewater is clearly evidenced in the scientific literature (HO• can significantly enhance the oxidation potential of the chemical

system, resulting in changes in the bacterial cell structure) [19], while in the case of ARGs, prolonged time of UV/H₂O₂ treatment seems to be required for their effective removal. Also, the possibility of using natural sunlight instead of UV lamps, to stimulate the formation of HO• during the process, may result to a low-cost application.

However, during the oxidation of urban wastewater, transformation products (TPs) of the antibiotics present can be formed, which may be less biodegradable, more toxic and biologically potent, compared to the parent compounds [20]. Thus, efforts should be made to identify the structure of the products, while also determining, whether these retain their core moieties, responsible for the antimicrobial activity of the antibiotic, possibly inducing antimicrobial resistance to the surrounding microorganisms.

Within this context, the possible use of UV-C/H₂O₂ and sunlight/H₂O₂ processes as tertiary treatment of urban wastewater is worthy of examination. Therefore, this work investigated the impact of UV-C/H₂O₂ and sunlight/H₂O₂ oxidation processes on: (a) the degradation of two antibiotics (i.e. ciprofloxacin [CIP] and sulfamethoxazole [SMX]), when present as a mixture in urban wastewater; (b) the inactivation of *Escherichia coli* and *Pseudomonas aeruginosa* including colonies of these species still cultivable in the presence of sub-minimal inhibitory concentrations (sub-MIC) of CIP and SMX and (c) the elimination of the 16S rRNA gene and ARGs encoding resistance to β -lactams (*bla*_{TEM}, *bla*_{OXA-A}, *bla*_{SHV}, *bla*_{CTX-M}, *mecA*), sulphonamides (*sul1*, *sul2*), quinolones (*qnrS*), glycopeptides (*vanA*) and tetracyclines (*tetM*) in urban wastewater. The two processes were investigated at pilot-scale, using actual urban wastewater effluents spiked with the antibiotics, while additional experiments were performed for the elucidation of the major photo-transformation products of CIP and SMX. To evaluate the biological potency of the treated flow, a chronic toxicity test was applied. The

selection of CIP and SMX as the target antibiotics to be investigated, was based on their high consumption, their frequent occurrence in UWTs effluents [21] and the prevalence of bacteria harbouring resistance to these compounds in the wastewater effluents [22], [23]. Fluoroquinolones, including CIP, are recognized by WHO as critically important antibiotics for human medicine [24], while CIP is included in the Watch List of substances for EU-wide monitoring [25], due to its consistency with the European One Health Action Plan against antimicrobial resistance [26]. SMX is a sulphonamide antibiotic widely used as prophylactic and therapeutic medication for treating human and animal diseases and benefiting agricultural productivity. The presence of these compounds in the wastewater has been shown to be potentially associated with increased fluoroquinolone and sulphonamide resistance genes and resistant bacteria in UWTs effluents [27], [28].

According to the authors' knowledge, this is the first study revealing comprehensive data regarding not only the degradation of antibiotics during UV-C/H₂O₂ and sunlight/H₂O₂ processes and the assessment of the treatments in removing resistance determinants (bacteria, completely viable and cultivable in the presence of sub-MIC of the target antibiotics and ARGs), but also the elucidation of the main TPs of CIP and SMX (to examine whether the processes can oxidize the antibacterial moieties of the antibiotics, the quinolone ring and the amino group of CIP and SMX, respectively) and the assessment of the treated effluents with regard to toxicity. This work evaluates UV-C/H₂O₂ and sunlight/H₂O₂ processes in an integrated manner and assesses whether their application enables safe disposal/reuse of treated urban wastewater to the environment.

2. Materials and methods

2.1 Chemicals and reagents

The antibiotics used (CIP, SMX) were of high-purity grade (>99%) and purchased from Sigma-Aldrich (Spain). Stock solutions of CIP and SMX were prepared by dissolving the individual compound (1 g L⁻¹) in ultrapure water and methanol respectively, due to solubility limitations. The stock solutions were kept in the refrigerator and their stability was routinely checked through chromatographic analysis. During one-month period, no statistical differences were observed. Required volumes of the stock solutions were directly added to the aqueous matrix into the reactor to obtain the initial concentration of 100 µg L⁻¹ of each antibiotic. This initial concentration was chosen because it is a sufficiently high concentration to characterize the degradation kinetics using available analytical techniques, and a low enough concentration to simulate real environmental conditions (typical environmental concentrations of antibiotics in the wastewater effluents are in the ng-µg L⁻¹ range). LC-MS grade acetonitrile, methanol, water and formic acid were purchased from Sigma-Aldrich. Ultrapure water was produced using a Milli-Q water purification system from Millipore (Darmstadt, Germany). H₂O₂ (35% w/w, Sigma-Aldrich) was used for the oxidation experiments. For toxicity and microbiological analyses, the residual H₂O₂ was removed from the treated samples by adding bovine liver catalase solution (Sigma-Aldrich). For the determination of the residual H₂O₂ concentration in the treated samples, titanium (IV) oxysulphate solution (Fluka) was used, while its presence in the samples was also checked using Quantofix® strips (Sigma-Aldrich).

Culture collections of *E. coli* K-12 (CECT 4624) and *P. aeruginosa* (CECT 118) strains (Spanish Collection Culture Type) were purchased and stock suspensions were prepared for their spiking into the distilled water during the disinfection experiments. *E. coli* and *P. aeruginosa*

cultures were activated by streaking on Chromocult agar (Merck) and King B medium (Cultimed), respectively, and incubated for 18-24 h at 37 °C. A single colony from *E. coli* plate was inoculated into 14 mL of sterile nutrient broth (Oxoid), while *P. aeruginosa* colony was inoculated into 14 mL of Luria Bertani broth (Sigma-Aldrich). Both suspensions were incubated at 37 °C for 18 h by constant agitation in a rotator shaker to obtain a stationary phase culture. Cells were harvested by centrifugation at 3000 rpm for 10 min and the pellet was re-suspended in 14 mL phosphate buffer saline (PBS) (Oxoid), yielding a final concentration of 10^9 CFU mL⁻¹, approximately. The bacterial suspensions were diluted into the aqueous matrix to reach an initial concentration of 10^6 CFU mL⁻¹. In order to avoid the osmotic stress on the cells during the experiments with distilled water, NaCl (0.9% w/v) was added and therefore the matrix is called ‘saline solution’ for the purposes of this work. Wastewater experiments were performed using bacteria already present in real urban wastewater (indigenous bacteria).

2.2 Water matrices

Both light-driven treatments were carried out using the saline solution (SS), which is distilled water produced at the distillation plant of Plataforma Solar de Almería (PSA) with added NaCl (0.9% w/v) as described in section 2.1. and urban wastewater samples (UWW) collected (every day in batches of 60 L) after the secondary treatment of the UWTP of El Bobar (Almería, Spain) and stored at 4 °C for no more than 2 h before the experiments. Experiments carried out in the SS matrix were performed in order to evaluate the matrix effect on the microorganism’s disinfection, considering that the SS matrix is commonly used for the control experiments. The UWTP (100,000 population equivalents [PE]; 33,000 m³/day) employs conventional activated sludge and decantation as secondary treatment. The main

physicochemical characteristics of the UWW used for the experiments were: pH 7.5, conductivity 1645 $\mu\text{S cm}^{-1}$, turbidity 5.2 NTU, Na^+ 198.18 mg L^{-1} , NH_4^+ 10.18 mg L^{-1} , K^+ 26.95 mg L^{-1} , Mg^{2+} 23.67 mg L^{-1} , Ca^{2+} 82.32 mg L^{-1} , Cl^- 329.60 mg L^{-1} , NO_3^- 28.39 mg L^{-1} and SO_4^{2-} 80.70 mg L^{-1} , Dissolved Organic Carbon (DOC) 15 mg L^{-1} . The most important qualitative characteristics of the distilled water used for the experiments were: pH 5.7, conductivity <10 $\mu\text{S cm}^{-1}$, Cl^- 0.8 mg L^{-1} , NO_3^- 0.5 mg L^{-1} , and DOC <1.5 mg L^{-1} .

2.3 Experimental set-up and procedure

2.3.1 UV-C pilot plant

The UV-C oxidation experiments were performed using a pilot-scale UV-C reactor. The UV-C reactor is equipped with three UV-C lamps (254 nm peak wavelengths, 230 W) connected in series. The configuration allows the system to operate in recirculating batch mode or continuous flow mode. In this study, oxidation experiments were carried out for 90 min in recirculating batch mode, with an illuminated volume of 6.21 L and a total working volume of 80 L. Firstly, the reactor was filled with the aqueous matrix (SS, UWW) and then, the mixture of the two antibiotics (100 $\mu\text{g L}^{-1}$) and the bacterial suspension of *E. coli* and *P. aeruginosa* of an approximate known concentration ($\sim 10^6$ CFU mL^{-1}) were spiked in. After 15 min of homogenization, with the lamp still switched off, an initial sample was taken to ensure the presence of bacteria and antibiotics. Then, H_2O_2 (initial concentration in the range 0.5 - 30 mg L^{-1}) was added to the reactor tank and after 15 min of mixing, the experiment started, and the lamp was switched on. Samples were collected at regular intervals depending on the measurement to be performed. The samples were filtered using 0.22 μm syringe filters (Agilent) before chromatographic analysis. A controller (ProMinent) fixed on the back of the reactor, was

continuously monitoring the water flow rate (46 L min^{-1}) and the UV-C lamp intensity. During the tests, the equipment was registering the sensor measurements in terms of incident irradiation (W m^{-2}), which is the UV-C radiation energy rate incident on a surface per unit area. The accumulated energy was calculated according to Eq. (1):

$$Q_{UV-C} \left(\frac{\text{kJ}}{\text{L}} \right) = Dose \left(\frac{\text{J}}{\text{m}^2} \right) \cdot \frac{A_i}{V_t} \left(\frac{\text{m}^2}{\text{L}} \right) \left(\frac{\text{kJ}}{1000 \text{ J}} \right) \quad (1)$$

where Q_{UV-C} is the accumulated UV-C energy per litre (kJ L^{-1}), Dose is the UV-C ultraviolet irradiation (W m^{-2}) emitted by the lamp multiplied by the illumination time (in seconds), A_i (0.338 m^2) is the irradiated surface, V_t (80 L) is the total volume of the water into the pilot plant and V_i (6.21 L) is the total irradiated volume. Each experiment was performed at least in duplicate and the results were plotted as the average of all replicates.

2.3.2 Compound Parabolic Collector pilot plant

The solar experiments were performed using a pilot-scale compound parabolic collector (CPC) photoreactor, located at PSA ($37^{\circ}84'N$ and $2^{\circ}34'W$), on sunny days between September and October, with a duration of 5 h. The configuration of the CPC photoreactor is described elsewhere [29]. The CPC photoreactor tube module, tilted at an angle of 37° relative to the horizontal plane, was connected to a recirculation tank and a centrifugal pump. The water flow rate was set at 30 L min^{-1} . The total volume of the photoreactor was 60 L, while the illuminated volume and the irradiated collector surface area were 45 L and 4.5 m^2 , respectively. The UV radiation was continuously monitored using a global UVA pyranometer (300-400 nm, Model CUV5, Kipp & Zonen, Netherlands), which provided data in terms of incident irradiation (W m^{-2}) corresponding to the solar radiant energy rate incident per unit of surface area.

During the loading of the reactor with the chemicals, the collectors were covered with a thick UV resistant canvas to avoid any photoreaction. The experimental procedure followed was the same as in the UV-C reactor and it is thoroughly described in the previous section. As soon as the appropriate amount of H_2O_2 was added ($2.5\text{--}125 \text{ mg L}^{-1}$) to the reactor and 15 min of mixing in the dark had passed, a sample was taken (zero-illumination time) and the collectors were uncovered. This time was considered as the start of the sunlight/ H_2O_2 process. The inactivation and degradation rates were plotted as a function of the cumulative energy per unit of volume (Q_{UV}) received in the photoreactor, commonly used to compare results under different conditions [30], and calculated by Eq. (2):

$$Q_{\text{UV},n} = Q_{\text{UV},n-1} + \Delta t_n \cdot UV_{\text{G},n} \cdot \frac{A_r}{V_t}; \quad \Delta t_n = t_n - t_{n-1} \quad (2)$$

where $Q_{\text{UV},n}$ and $Q_{\text{UV},n-1}$ are the UV energy accumulated per litre (kJ L^{-1}) at times n and $n-1$, $UV_{\text{G},n}$ is the average incident radiation on the irradiated area, Δt_n is the experimental time of sample, A_r is the illuminated area of the reactor (m^2) and V_t is the total volume of water treated (L). Each experiment was performed at least in duplicate, between 10 a.m. and 16 p.m., and the results were plotted as the average of all the replicates.

2.4 Analytical methods

All analyses for SMX and CIP were performed by means of a reverse-phase liquid chromatography (UPLC) coupled to UV detection (Agilent technologies) using a C-18 column (Luna 5 μ , $3 \times 150 \text{ mm}$, Phenomenex). The mobile phase used was a mixture of Milli-Q water and acetonitrile (70:30, v/v) acidified with 25 mM formic acid. The injected volume was 50 μL and the flow rate was 0.5 mL min^{-1} .

Total carbon (TC), inorganic carbon (IC) and total organic carbon (TOC) were analysed by Shimadzu TOC-5050 (Shimadzu Corporation, Kyoto, Japan). Anions and cations were analysed using ion chromatography, 850 Professional IC - Cation coupled to Metrohm 872 Extension Module. The samples were filtered through 0.22 μm filters prior to analysis.

H_2O_2 was determined by a colorimetric method based on the use of titanium (IV) oxysulfate (Riedel-de Haën, Germany), following DIN 38409 H15, which forms a stable yellow complex with H_2O_2 detected by absorbance measurements at 410 nm. Absorbance was measured using a spectrophotometer (PG Instruments Ltd. T-60-U) and was linearly correlated with H_2O_2 concentration in the range 0.1-100 mg L^{-1} . As mentioned in section 2.1, catalase was added to wastewater samples in order to eliminate residual H_2O_2 prior to toxicity and microbiological analyses: Samples of 1 mL were mixed with 20 μL of 2300 U mg^{-1} bovine liver catalase at 0.1 g L^{-1} .

2.5 Chronic toxicity evaluation

A chronic toxicity assay with *V. fischeri* (NRRL B-11177) was applied. The bacteria were grown in petri dishes with seawater complete medium agar for 72 h at 15.0 ± 0.2 °C. A 500 mL seawater complete medium contained NaCl (15 g), NaH_2PO_4 (3.05 g), K_2HPO_4 (1.049 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.102 g), $(\text{NH}_4)_2\text{HPO}_4$ (0.25 g), bacto peptone (2.5 g), bacto yeast extract (0.25 g) and glycerine (87%, 1.5 mL). A single colony was transferred and grown in suspension (20 mL of growth medium without adding agar) for 12 h in a dark climatic chamber at 15.0 ± 1.0 °C under continuous shaking (180 rpm). A second cultivation of 5 FNU bacteria density was then prepared in 120 mL of medium and incubated in the same conditions for 24 h.

Each experiment consisted of triplicate samples and included a solvent control, a positive control (18% NaCl), a blank series (Milli-Q water) and the treated samples collected through UV-C/H₂O₂ and sunlight/H₂O₂ processes. The salinity of all samples was adjusted to 2% prior testing. The exposure time was 24 h and tests were performed in a dark climatic chamber at 15.0 ± 1.0 °C under continuous shaking. The optical density (OD) at 588 nm measured with a spectrophotometer (PG Instruments Ltd T-60-U) and the bioluminescence using a luminometer (BioFix® Lumi-10, Macherey-Nagel) were the endpoints of bacterial growth recorded. The procedure is described extensively elsewhere [31].

2.6 Enumeration of total and resistant bacteria to sub-MIC of CIP and SMX

Aliquots of samples collected during the treatment were immediately analysed using the spread plate technique. The volume chosen to be plated at each experimental time was dependent on the microbial load expected to be present in the sample. When the bacterial load was expected to be high, 50 µL drop of adequate dilution was plated and when the bacterial load was expected to be low, volume of 500 µL of sample was spread onto prepared dishes. Different selective media were used for each type of microorganism examined. King B agar was prepared for *P. aeruginosa* enumeration and Chromocult Coliform agar was prepared for the *E. coli* enumeration, according to the manufacturers' instructions. The plates were incubated at 37 °C for 24 h. The representative colonies were dark-blue to violet for *E. coli* and fluorescent under UV light for *P. aeruginosa*. For each treated sample and each strain examined, two agar plates were prepared, one only with the proper agar and one with the agar spiked with the mixture of CIP and SMX at 100 µg L⁻¹ each. Although this concentration (100 µg L⁻¹) is far below the clinical MIC of both antibiotics, for *E. coli* and *P. aeruginosa* respectively, it was chosen in

order to investigate whether the resistant bacteria can be selectively enriched even at antibiotic concentrations several hundred-fold lower than previously expected, such as those found in urban wastewater. The colonies grown on the agar containing the antibiotics, were considered more tolerant compared to those that did not grow in the presence of sub-MIC of CIP and SMX [32].

The regrowth of bacteria after the treatment was examined by storing the treated samples in the dark for 48 h (25 °C) and re-plating them for enumeration. The counted colonies during the regrowth experiments were considered as regrowth of damaged/inactivated bacteria that were previously unable to grow on the selective medium. Therefore, bacterial numbers during the regrowth test exceeding the bacterial numbers encountered during the treatment were considered as repaired/reactivated bacteria. It should be noted that all the samples were plated in triplicate.

2.7 DNA extraction and qPCR analysis

UV-C/H₂O₂ and sunlight/H₂O₂ treated samples were filtered through 0.22 µm polycarbonate filter membranes (Merck) and stored at -20 °C until DNA extraction. The DNA was performed using the PowerWater® DNA Isolation Kit (MoBio) and the resulting DNA extracts were shipped at room temperature for further analysis. The extracts were kept refrigerated at -20 °C until analysis. The 16S rRNA gene and ARGs encoding resistance to the antibiotic classes β-lactams (*bla*_{TEM}, *bla*_{OXA-A}, *bla*_{SHV} and *bla*_{CTX-M}, *mecA*), sulfonamides (*sul1* and *sul2*), quinolones (*qnrS*), glycopeptides (*vanA*) and tetracyclines (*tetM*) were quantified using a StepOne™ Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). The DNA extracts were analysed using the standard curve method [33], and based on the criteria established in Rocha et al. [34]. For each sampling point, three independent DNA extracts were

analysed, and each qPCR determination was performed at least in duplicate using the conditions listed in the supplementary information (**Table S1**). The abundance of the 16S rRNA gene and ARGs was expressed as log value per volume of water (gene copy number per mL of sample).

2.8 Transformation products (TPs)

The experiments for the determination of the major TPs generated during UV-C/H₂O₂ and sunlight/H₂O₂ processes were performed in distilled water spiked with 1 mg L⁻¹ of antibiotic concentration. The H₂O₂ dose during these experiments was determined to be 30 mg L⁻¹, which was added to the solution at the beginning of the experiment. The exact protocol followed for the determination of the TPs is provided in the **Text S1**.

3. Results and discussion

3.1 Degradation of antibiotics during UV-C/H₂O₂ and sunlight/H₂O₂ processes

3.1.1 UV-C reactor

Prior to the UV-C/H₂O₂ oxidation experiments, preliminary trials were performed to determine the effect of dark recirculation of the aqueous matrices in the UV-C reactor, on the degradation of spiked antibiotics (hydrolysis) at a concentration of 100 µg L⁻¹, the effect of H₂O₂ on the degradation of antibiotics in the absence of light (direct oxidation), as well as the effect of UV-C light alone on the antibiotics (photolysis). Dark recirculation of SS and UWW in the UV-C reactor showed no significant effect on the spiked antibiotics (100 µg L⁻¹) for a total of 2 h in the dark (removal <2%). Furthermore, the effect of the oxidant alone in the dark, caused 41% removal of CIP and only 5% removal of SMX after 300 min. Photolytic experiments performed with SS showed that UV-C light alone could completely degrade the two antibiotics (>99%

degradation of CIP was observed after 45 min and 1.03 kJ L⁻¹, while the degradation of SMX took place after 20 min and 0.37 kJ L⁻¹) (**Fig.1a**). In the UWW matrix, CIP reduction reached >99% after irradiation of 90 min and 1.15 kJ L⁻¹, whereas the removal of SMX was achieved in half the time, after 45 min and 0.52 kJ L⁻¹ (**Fig.1a**).

In order to determine the optimum H₂O₂ dose, at which the highest removal of the target antibiotics could be accomplished during the oxidation experiments, several oxidant concentrations were tested (ranging from 0.5 to 30 mg L⁻¹). The range of H₂O₂ concentrations examined was based on the findings of previous studies [35], [36], [37]. In order to compare the amount of H₂O₂ theoretically required for total mineralization of the antibiotics (calculated based on the stoichiometric reaction of the antibiotic with the oxidant), with the actual oxidant concentration used for the experiments, the following were considered: The stoichiometric ratio of H₂O₂ required for total mineralization of CIP, according to the following Eq. (3), is 1:47.

Therefore, the minimum concentration of H₂O₂ required to achieve total mineralization of the solution of 100 µg L⁻¹ CIP (0.0003018 mmol L⁻¹) is 0.01418 mmol L⁻¹ (0.0003018 × 47) that corresponds to 0.48212 mg L⁻¹ of H₂O₂. Similarly, the stoichiometric ratio of H₂O₂ required for total mineralization of SMX, according to the following Eq. (4), is 1:33.

Therefore, the minimum concentration of H₂O₂ required to achieve total mineralization of the solution of 100 µg L⁻¹ SMX (0.00039482 mmol L⁻¹) is 0.01303 mmol L⁻¹ (0.00039482 × 33) that corresponds to 0.44319 mg L⁻¹ of H₂O₂.

The optimum concentration was found to be 5 mg L⁻¹ of H₂O₂ for the UV-C oxidation process (*results not shown*), more than one order of magnitude higher than the H₂O₂ stoichiometric

concentration calculated here, indicating the recalcitrance of the antibiotics as well as the complexity of the wastewater matrix (presence of dE_fOM).

Both antibiotics were degraded by more than 99% during the UV-C/ H_2O_2 treatment (SS: >99% degradation of CIP after 25 min and 0.50 kJ L^{-1} and SMX after 20 min and 0.38 kJ L^{-1} ; UWW: >99% removal of CIP after 90 min and 0.9 kJ L^{-1} and >99% degradation of SMX under the same conditions). Similar observations were also made in the study of Boudriche et al. [35], where complete degradation of CIP was achieved in the first 30 min of UV-C/ H_2O_2 treatment at the same oxidant concentration ($[H_2O_2]=5\text{ mg L}^{-1}$). The lower energy needed for the complete degradation of CIP in UWW during the experiments with H_2O_2 (0.9 kJ L^{-1}) compared to the energy needed (1.15 kJ L^{-1}) in the photolytic experiments, can be attributed to the generation of highly reactive $HO^\bullet$, that enhance the rate of CIP degradation in the first case. However, the case of SMX differs, since the presence or the absence of H_2O_2 led to no distinct difference in its behaviour, indicating that SMX undergoes photolysis.

3.1.2 CPC reactor

In the case of the CPC reactor, the duration of the hydrolysis and photolysis experiments, was the same as in the actual sunlight/ H_2O_2 experiments (5h), in order to establish the contribution of their effect on the overall antibiotic removal. Hydrolysis experiments showed no significant effect on the spiked antibiotics for a total of 5 h in the dark (removal <2%), which is in agreement with the findings of previous studies for both antibiotics [38], [39].

Photolytic experiments using SS, resulted in the reduction of CIP to the detection limit (>99% removal), after 60 min of irradiation and 8 kJ L^{-1} , while the degradation of SMX was moderate, only 46% after 300 min of irradiation and 54 kJ L^{-1} (**Fig.1b**). Similarly, the photolysis

of CIP in the UWW resulted in the removal of the compound (>99%) after 60 min and 8 kJ L⁻¹. However, only 14% reduction was observed for SMX, after 300 min of irradiation 46 kJ L⁻¹ (**Fig.1b**). The results obtained are consistent with the findings existing in the literature, confirming that the fluoroquinolones, including CIP, degrade very quickly when exposed to sunlight [38]. Also, it is known that in slightly basic pH, CIP exists predominantly in cationic form (the pH of both matrices falls in the range of the dissociation constants of CIP, $pK_{a,1}=5.9$ and $pK_{a,2}=8.9$), which favours the photodegradation of the compound [40]. On the other hand, SMX was poorly degraded under sunlight (only 14%) confirming that the neutral pH conditions (pH~7.5) prevailing in the reactor, in combination with the limited photolysis of SMX under sunlight (according to the UV spectra, only a very small fraction of sunlight [i.e., UV-A and UV-B] is expected to promote direct photolysis of SMX, [41]), affected the degradation of the compound negatively [42]. This is in accordance with the study of Niu et al. [43], where the photolysis of SMX was shown to be favoured in acidic conditions, but the degradation rate decreased at near-neutral or alkaline conditions. This can be explained by the pK_a values of the SMX molecule which are $pK_{a,1}=1.85$ and $pK_{a,2}=5.60$ [44]. When the pH values are between the $pK_{a,1}$ and $pK_{a,2}$ values, SMX exists mainly in the form of a neutral molecule [45], [39] which has stronger light absorption and higher photochemical reactivity, leading to shorter half-lives and higher degradation efficiency. When the pH value is higher than $pK_{a,2}$, SMX is mainly negatively charged and has lower photochemical reactivity [45], [39] and greater stability [46]. In a previous study of Rizzo et al. [47], SMX was moderately degraded during photolysis (43% after 300 min irradiation and 53.7 kJ L⁻¹).

Obviously, the matrix composition is also a factor affecting the degradation of the compounds, as the reduction of SMX in the SS was 46 % and in the UWW 14%, which might be

ascribed to the higher organic content of the UWW compared to the SS. The presence of natural organic compounds occurring in the wastewater matrix can affect the degradation rate of the target compounds, due to the competitive absorption of photons by other light absorbing species [30].

In order to determine the optimum H_2O_2 dose, in which sufficient degradation of antibiotics could be accomplished during the sunlight/ H_2O_2 oxidation experiments, several oxidant concentrations were tested (ranging from 2.5 to 125 mg L^{-1}). The range of H_2O_2 concentrations examined was based on the findings of previous relevant studies [48], [49], [17]. The optimum concentration was found to be 30 mg L^{-1} of H_2O_2 .

Fig.1b depicts the solar oxidation of CIP and SMX when present as a mixture in SS and UWW, during the experiments performed with and without H_2O_2 . Considering the effect of photolysis on the degradation of CIP during control experiments, the rapid elimination of the antibiotic in SS was attributed to its photodegradation properties. The phenomenon was fast, as expected, and the degradation of CIP took place after 10 min and 1 kJ L^{-1} . In UWW, 60 min of treatment and 8 kJ L^{-1} were needed for the same level of degradation of CIP. On the other hand, SMX was shown to be poorly degraded in both matrices (50% reduction and 53 kJ L^{-1} in SS, 46% reduction and 42 kJ L^{-1} in UWW, after 300 min of treatment), affirming its strong persistence towards sunlight/ H_2O_2 treatment. Comparing the degradation times of the antibiotics with and without the oxidant, it can be observed that the addition of H_2O_2 made the degradation of both antibiotics more rapid both in SS and UWW. The fact that SMX was not degraded to a great extent, may be related to the pH conditions prevailing in the reactor and the limited UV fraction of the sunlight, as explained in the previous paragraph. In other similar studies, this phenomenon was also observed. In the study of Ao et al. [50], it was shown that the

decomposition of SMX was favoured in acidic conditions (pH 3-5), while an increase in pH (7-11), noticeably reduced the degradation rate of SMX. Elmolla and Chauduri [51] reported that during oxidation processes with the use of H_2O_2 , the latter seems to be more stable at low pH due to the generation of oxonium ions (H_3O^+). The improvement of the H_2O_2 stability is conducive to the SMX removal. Under alkaline conditions, hydroperoxide anion (HO_2^-) is generated in UV/ H_2O_2 system. It is a robust scavenger of $\text{HO}^\bullet$ and can cause the decomposition of H_2O_2 [52]. The decomposition of H_2O_2 decreases the amount of the available $\text{HO}^\bullet$ and eventually reduces the degradation rate. In addition, H_2O_2 shows an elevated self-decomposition rate at higher pH [53] which makes the molecules of H_2O_2 lose their oxidation capacity.

3.2 Kinetics

Degradation data obtained during light-driven oxidation experiments were fitted to a pseudo-first-order kinetics model ($R^2 > 0.95$), and apparent rate constants were calculated for each process for both compounds (**Fig.2**). The degradation of antibiotics confirmed the linear expression of $-\ln(C/C_0)$ (where C_0 and C refer to the concentrations of the antibiotics at times 0 and t [min], respectively), as a function of the treatment time. The apparent rate constant k_{app} was obtained from the slope of the linear plots. As expected, the antibiotic degradation rate differed greatly in the two matrices, being much lower in UWW than in SS. This was attributed to the high concentration of dE/OM and inorganic ions present in wastewater, which both act as scavengers of $\text{HO}^\bullet$, slowing down the degradation of antibiotics [54], [36]. It is notable that the consumption of H_2O_2 during the processes was minimal (**Fig.2**) and thus, no supplementary oxidant was added to the reactor.

The presence of microorganisms in the UWW may induce competition between organic matter and bacteria for radicals, contributing thereby to the slower degradation of the antibiotics [55]. The slower photodegradation of the antibiotics in the saline solution compared to the distilled water (*data not shown*) was attributed to the presence of Cl^- anions (deriving from the NaCl added to the distilled water), possibly acting as $\text{HO}^\bullet$ scavengers, reducing thus photodegradation efficiency.

3.3 Mineralisation and photo-transformation of CIP and SMX

3.3.1 Mineralisation

The DOC of the reaction solution (corresponding to the inherent DOC of the wastewater) decreased by 8.2% and only 3.5% during the UV-C/ H_2O_2 and sunlight/ H_2O_2 oxidation, respectively. The relatively low DOC removal compared to the antibiotics' depletion confirms the formation of recalcitrant organic intermediates deriving from the oxidation of the dE₇OM and the antibiotics, as complete mineralisation was not attained in any case.

3.3.2 Elucidation of the main TPs of CIP and SMX

The TPs generated during the UV-C/ H_2O_2 and sunlight/ H_2O_2 treatment of CIP and SMX were tentatively identified and the degradation pathway was proposed. For this purpose, a suspect screening strategy was applied. Two suspect lists were used, including accurate masses of thirty and twenty-two TPs of CIP and SMX, respectively. The lists were elaborated considering the TPs generated by diverse photo- and bio-chemical degradation processes, previously reported in literature. Samples were screened for the selected masses and identification criteria were set to reduce false negative findings (peak intensity threshold 1000

cps, signal-to-noise ratio >10, mass error <5 ppm and isotope ratio difference <10% for the precursor ion). The tentative assignment of the structures was based on the comparison of the acquired MS/MS spectra and retention behaviour with the information reported in literature, along with interpretation of the fragmentation pathways when this information was not available. The number of identified TPs of CIP and SMX revealed the complexity involved with the UV-C- and solar-driven processes and proposed the existence of various degradation routes resulting in multi-step and interconnected pathways.

An accurate quantification of the TPs identified was not possible due to the lack of pure analytical standards. Thus, the formation profile of the TPs was interpreted based on their relative intensity, which is expressed as the chromatographic peak area of the corresponding TP vs treatment time (**Fig.3** and **Fig.4**).

3.3.2.1 TPs of CIP

Table S2 depicts the proposed chemical structures and the exact mass information of the TPs identified during the degradation of CIP by the photo-oxidation processes (products numbered sequentially based on molecular weight). Up to twenty-one TPs could be identified. Although most of the TPs (fifteen compounds) were common in both treatments, five of them were found only in the solar-treated samples and one was found solely in the UV-C/H₂O₂ treated samples (**Table S4**). The proposed structures are consistent with the expected transformations of CIP, which were found to occur through hydroxy substitution of fluorine (**TP330**, **TP362B**, **TP344A/B/C** and **TP346**), defluorination (**TP344D**, **TP288A/B** and **TP263**), hydroxylation of the quinolone core (**TP348A/B/C**, **TP362B**) and mainly by oxidation and partial or total elimination of the piperazine ring [56], [57], [58], which has been reported through the formation of keto-

derivative intermediates (**TP344A/B**, **TP362**, **TP360**), ring cleavage (**TP344D**, **TP334**), dealkylation (**TP306B**, **TP288A**, **TP263**, **TP245**) and formation of an enamine derivative (**TP288B**, **TP346**, **TP304**, **TP306A**). Finally, the oxidation of the cyclopropyl group leading to ring cleavage was observed only in **TP288B** and **TP306A**. **Fig.5a** shows a tentative route that agrees with the ones proposed in the literature.

Total removal of CIP TPs was obtained when UV-C/H₂O₂ was applied (**Fig.3a**), but sunlight/H₂O₂ proved to be a less efficient process. It was found that during the solar oxidation of CIP most TPs emerged during the first stages of the treatment, remained in the reaction solution after the total degradation of CIP (30 min). This finding is in accordance with the fact that DOC was not efficiently reduced. **Fig.3b** shows the profile of the abundance of the main TPs during the treatment. **TP245**, **TP288A** and **TP348A** were the most predominant at the end of the experiment, showing an increase-decrease profile with the reaction time with a maximum at 15 min of treatment. On the other hand, **TP330** and **TP344D** were generated very quickly at the beginning of the treatment, experiencing a gradual decrease over time. In the case of UV-C treatment (**Fig.3a**), the most abundant and persistent TPs were **TP263** followed by **TP334**, which however degraded after 14 min of UV-C/H₂O₂ treatment.

Considering that the fluoroquinolone structure of CIP, responsible for the biological activity of the compound, is present in all the TPs identified, the formation of stable TPs can represent a potential problem. The TPs of CIP may retain toxicity to the aquatic organisms, while maintaining their antibacterial activity which can potentially result in antimicrobial resistance, when released in the environment.

3.3.2.2 TPs of SMX

The main TPs identified during the degradation of SMX are shown in **Table S3**. Degradation pathways of SMX include the rearrangement of the isoxazole ring (**TP254**), cleavage of the sulphonamide bond (**TP99**, **TP174**), hydroxylation of benzene (**TP270 B/D**, **TP253 A**) and isoxazole (**TP270 A/C**) rings, oxidation of the amine group at the benzene ring (**TP268**, **TP284**, **TP296**, **TP312**) and oxidation of the double bond at the isoxazole ring and further ring opening (**TP216 A/B**, **TP272 A/B/C**, **TP288**). A transformation pathway is proposed in **Fig. S2**.

From the eighteen TPs identified, thirteen were common to both light-driven oxidation processes. However, some differences were also observed (**Table S5**).

The time-evolution profile of the TPs of SMX (**Fig.4**) showed that during the solar oxidation of SMX the most predominant TP was **TP254**, which increased its concentration along the process thus pointing out the rearrangement of the isoxazole ring as the main transformation route. The same behaviour was observed in the treatment with UV-C/H₂O₂, but in this case a concomitant degradation of **TP254** occurred after 10 min of reaction time, showing again the greater efficiency of this treatment in TPs removal.

In a previous study of Majewsky et al. [59] it was shown that the SMX TPs retaining the sulphonamide toxicophore (amino group) exhibited residual antibacterial properties, whereas TPs resulted from breakdown of the SMX structure lost this mechanism of action. Considering these results, it can be anticipated that all the TPs of SMX identified in this study, which retain the amino group in their molecule, will possibly be biologically active and may induce antimicrobial resistance.

3.4 Chronic toxicity assessment

The inhibition results for *V. fischeri* with the bioluminescence induction as the endpoint monitored, followed a different trend along the two treatments (**Fig.6a and 6b**).

During the UV-C/H₂O₂ process, it was shown that it has not been able to eliminate toxicity even after 90 min of treatment (**Fig.6a and 6b**). No reduction in inhibition of bioluminescence was observed in the samples collected after 30 min of UV-C/H₂O₂ treatment, nor in the samples treated for 60 min. A further increase in irradiation to 90 min though, led to a noticeably reduction in the inhibition (a decrease to $15 \pm 1\%$ of inhibition). The positive control caused toxicity with values $99 \pm 1\%$, whereas the blank tests, showed no inhibition to the luminescence of the *V. fischeri* ($4 \pm 3\%$) (*data not shown*).

Regarding the sunlight/H₂O₂ process, it was shown that the untreated effluents caused an inhibition of $99 \pm 1\%$, but after 120 min of sunlight/H₂O₂ treatment the toxicity was eliminated.

Since the TPs of CIP (e.g. **TP245**, **TP330**, **TP344 B**, **TP344** and **TP348 A**), present in the reaction solution at the first stages of oxidation (5 min) until the complete degradation of CIP (30 min) (**Fig.6a**), and the TPs of SMX (**TP270 D**, **TP284**, **TP348 A**, **TP254**, **TP272 A** and **TP296**) (**Fig.6b**) remained in the reaction solution until the end of the treatment, whereas the toxicity was eliminated, indicates that the TPs of the two antibiotics, are not the ones exerting toxicological effects towards *V. fischeri*.

The luminescence of *V. fischeri* was only inhibited in the UV-C/H₂O₂ treated samples, indicating that under irradiation of a lower wavelength, oxidation of both the antibiotics and the dE_{OM} existing in wastewater may lead to more toxic TPs. As mentioned before, the TPs of SMX remained in the solution until the end of the sunlight/H₂O₂ treatment, while the toxicity of the treated samples was eliminated. Also, although the TPs of CIP degraded after 14 min of UV-

C/H₂O₂ treatment, the solution was still toxic after 90 min of treatment. Therefore, the toxic effects observed can be ascribed to the dE_fOM and its associated oxidation products.

3.5 Inactivation and post-treatment regrowth of total and susceptible to sub-MIC of CIP and SMX *E. coli* and *P. aeruginosa*

The selection of *E. coli*, was based on its well-known role as indicator of microbiological contamination for the assessment of the microbiological water/wastewater quality, while that of *P. aeruginosa*, on its inclusion in the “critical” category of the WHO’s priority list of bacterial pathogens for which research and development of new antibiotics is urgently required [60]. Also, the fact that nosocomial infections caused by *P. aeruginosa* (i.e. pneumonia, bloodstream infection, urinary tract infection) have become a healthcare concern, mainly due to the high level of resistance to several antibiotics [61], led the authors select the investigation of this species.

The performance of the two light-driven oxidation processes during the treatment of SS and UWW was assessed with respect to the removal of cultivable *E. coli* and *P. aeruginosa* bacteria and to the inactivation of colonies of these species still viable in the presence of sub-MIC of CIP and SMX (100 µg L⁻¹) (**Fig.7**). Inactivation of the bacterial populations to values below or close to the detection limit (detection limit [DL]: 2 CFU mL⁻¹) was observed at the end of both treatments.

As expected, the inactivation curves and rates of *E. coli* and *P. aeruginosa* in the two matrices were quite different, exhibiting lower reduction rates in UWW compared to those in SS. In the case of UV-C/H₂O₂ process, the inactivation of bacteria was fast (after 3 min and 0.003 kJ L⁻¹ in SS and after 8 min and 0.06 kJ L⁻¹ in UWW). In UWW, *E. coli* and *P. aeruginosa* reached the DL after 150 min (Q_{UV}=20.2 kJ L⁻¹) and 120 min (Q_{UV}=15.9 kJ L⁻¹) of sunlight/H₂O₂

treatment, respectively. In SS, although the initial concentration of the bacterial population was similar, their inactivation reached the DL faster, after 30 min (4.1 kJ L^{-1}) and 60 min (8.8 kJ L^{-1}) of sunlight/ H_2O_2 treatment for *E. coli* and *P. aeruginosa* respectively. This suggests that the inactivation mechanism, based on the stepwise damage induced by the UV irradiation which starts at the bacterial cell wall and gradually proceeds to the inner cell components, eventually causing permanent lethal damage [62], is strongly sensitive to the chemical composition of water. In addition, the presence of dE_fOM in UWW that can act as scavenger of the $\text{HO}^\bullet$ generated during photo-driven oxidation processes, may induce competition between bacteria and organic matter for radicals and thus, reduce the inactivation efficiency [55]. The marked effect of the presence of dE_fOM in UWW effluents was also observed on the disinfection efficiency by sunlight/ H_2O_2 in a previous study of Bichai et al. [63].

As mentioned previously, the bacterial inactivation profiles during the UV-C/ H_2O_2 and sunlight/ H_2O_2 treatments, differed greatly in terms of the time required and hence the accumulative energy needed for the inactivation to the DL of the microorganisms; the inactivation of the bacterial populations in the UV-C reactor, was much more rapid compared to that observed when the irradiation used was natural sunlight (UV-C/ H_2O_2 : 8 min; sunlight/ H_2O_2 : 120 - 150 min in UWW), while the accumulated energy required was almost 3 order of magnitude higher (UV-C/ H_2O_2 : 0.06 kJ L^{-1} ; sunlight/ H_2O_2 : $15.9 - 20.2 \text{ kJ L}^{-1}$ in UWW). This observation may be attributed to the fact that irradiation of H_2O_2 with UV-C photons generates more $\text{HO}^\bullet$ compared to the sunlight (since only a small amount of sunlight is UV, under which the photodecomposition of H_2O_2 to $\text{HO}^\bullet$ is favoured [64]), and thus, UV-C/ H_2O_2 would be more efficient than the sunlight/ H_2O_2 process. The results were also correlated to the UV dose/fluence in order to compare the results of this study with the real-scale conditions. Here, it should be

noted that the UV dose/fluence commonly applied in UWWTPs, is often less than 100 mJ cm^{-2} , much lower than that applied in this study. In this study, the fluence rate was 26 W m^{-2} in average, which corresponds to doses/fluence of approximately 24 mJ cm^{-2} and 1806 mJ cm^{-2} for reaction times of 2 to 10 min, respectively. The strong germicidal power of UV-C radiation observed for bacterial inactivation is based on the generation of very specific damages in the bacterial DNA that inhibit its duplication and consequently bacterial reproduction.

In general, no significant differences in treatment times and inactivation rates were observed among the two species tested. In addition, *E. coli* and *P. aeruginosa* colonies still viable in the presence of sub-MIC of CIP and SMX reached the DL as well, in a comparable period to the susceptible ones. It is important to note that the difference in the susceptibility of *E. coli* and *P. aeruginosa* to the applied treatments is clearer in the case of UWW compared to the SS, which is attributed to the competition of dEOM and bacteria for the radicals generated.

Finally, the regrowth potential of the examined types of bacteria was investigated after 24 and 48 h of storage of the treated samples at $25 \pm 2 \text{ }^{\circ}\text{C}$ in the dark. After 48 h of incubation, the re-plated sunlight/ H_2O_2 treated samples showed 8 CFU mL^{-1} of *E. coli* and 50 CFU mL^{-1} of *P. aeruginosa* grown on the corresponding medium. The results indicate the existence of repair mechanisms of *E. coli* and *P. aeruginosa* after the oxidation process (even at high fluence values, 1806 mJ cm^{-2}), while the fact that all the regrown colonies were observed on the plates containing antibiotics at sub-MIC, implies that when the stress factor (which in this case is the sunlight/ H_2O_2 treatment) is gone, the microorganisms might become more tolerant. A similar repair of the examined bacteria was observed by Fiorentino et al. (2015), where after H_2O_2 /sunlight treatment of a secondary effluent, inactivation of total and multidrug resistant *E. coli* to below the quantification limits was achieved. Despite the accomplished inactivation of the

H₂O₂/sunlight process, 48 h after the treatment there were 0.3×10^2 CFU mL⁻¹ *E. coli* re-growing in treated samples with 3% of the enumerated *E. coli* population being resistant to the examined mixture of antibiotics, namely ampicillin, ciprofloxacin and tetracycline.

On the other hand, no regrowth was observed in the UV-C/H₂O₂ treated samples. This might be an indication of a permanent damage to the cellular functions of the examined species through the UV-C/H₂O₂ oxidation, disabling thus their repair and highlighting the capacity of the process to inactivate these bacteria. When UV-C irradiation is applied, the damage is mainly at the genome level, due to the high absorption by the thymine and cytosine bases. This stress induces responses of chaperones to repair the DNA damages, but soon this response is surpassed [65]. Similarly, during the UV-C disinfection, the regrowth is influenced by the dose of UV-C received by the microorganisms. The stepwise damage induced by the UV irradiation starts at the bacterial cell wall and gradually proceeds to the inner cell components, eventually causing permanent lethal damage [66]. Also, according to Dodd, [67], UV radiation interacts with target moieties in bacterial cells by physical processes first, such as light absorption by certain chromophores (e.g L-tryptophan), which may subsequently lead to photochemical reactions that can change the genetic information of the cell, contributing thus to the inactivation, mutagenesis and death of the cell.

3.6 ARGs elimination during the light-driven oxidation processes

The long-term application of antibiotics in the protection of humans and animals has resulted in bacteria possessing various resistances to antibiotics that are generally controlled by ARGs. Several types of ARGs have been found in the environment, including genes that confer resistant to tetracycline, sulphonamide, aminoglycoside, vancomycin, β -lactam antibiotics etc.

Tetracycline-resistant bacteria emerge in environments with the introduction of tetracycline, and at least 40 different tetracycline resistance genes (*tet*) have been characterized to date. Resistance to tetracycline is governed by *tet* genes, which are involved in either active efflux of the drug, ribosomal protection or enzymatic drug modification. Sulphonamide resistance is primarily mediated by the *sul1*, *sul2* and *sul3* genes encoding dihydropteroate synthetase (DHPS) with a low affinity for sulphonamide antibiotics [68], [69]. *Sul1* and *sul2* have been detected in bacteria isolates from faecal slurry of dairy farms, wastewater treatment facilities, water or sediments of aquaculture areas, and even from the river or seawater without evidence pollution [69], suggesting that sulphonamide resistance genes are worthy of concern. As the largest group of diverse and specific resistance determinants in bacteria, β -lactamase resistance genes are intensively explored in terms of dissemination mechanisms in the environment. B-lactamase resistance genes are unique because of their broad spectrum of activity against β -lactams and consequently, very high mutation frequency [70], [71]. For these reasons, it was decided to investigate the abundance of the 16S rRNA, *sul1*, *sul2*, *qnrS*, *bla*_{TEM}, *bla*_{OXA}, *bla*_{CTX-M}, *bla*_{SHV}, *mecA*, *vanA* and *tetM* genes, in the samples collected during the light-driven oxidation processes. These genes were selected considering their wide occurrence in wastewater [72] and their ability to confer resistance against essential classes of antibiotics used for the treatment of serious infectious diseases [73], [74], [75], [76], [77], [78]. The results, expressed in values of gene copy number mL⁻¹ of sample, are presented in **Fig.7**.

The abundance of the *vanA* and *mecA* genes, was found to be below the quantification limit in all samples. The 16S rRNA gene, a housekeeping gene used to measure the bacterial abundance, was the most abundant gene in all samples (from 6.50 to 4.11 log gene copies mL⁻¹ of sample during the UV-C/H₂O₂ and 6.50 to 5.71 during the sunlight/H₂O₂ processes). During

the UV-C/H₂O₂ oxidation, the 16S rRNA gene was reduced for about 2.4 logs, whereas during the sunlight/H₂O₂ treatment the gene was slightly decreased (0.8 logs), indicating a greater sensitivity of the bacteria population to the UV-C radiation.

Both processes seemed to be insufficient in reducing ARGs to levels below the quantification limit. Particularly, the occurrence of most of the tested ARGs was not remarkably affected after 300 min of sunlight/H₂O₂ treatment (≤ 0.7 log reduction). While no differences were observed for *sul1*, *sul2*, *qnrS*, *bla*_{OXA-A} and *bla*_{TEM} between the untreated and the samples collected after treatment, the *tetM* and *bla*_{CTX-M} genes were reduced after the treatment (~ 1.4 logs).

UV-C/H₂O₂ process was demonstrated to be effective in reducing *sul1*, *sul2*, *tetM*, *bla*_{OXA-A} and *bla*_{TEM} genes for 2.0-3.7 logs and *qnrS* and *bla*_{SHV} genes to values below the quantification limits (within 90 min of treatment and 0.8 kJ L⁻¹).

In the studies of Ferro et al. [13] and [79] which deal with the removal of selected ARGs by UV/H₂O₂ process at a dose of 20 mg L⁻¹ H₂O₂ in deionized water, the process did not affect the *bla*_{TEM} gene copy number and poorly affected the *qnrS* copy number of (4.3×10^4 copies mL⁻¹) after 240 min of treatment (accumulated energy in kJ L⁻¹ is not calculated in this study). Moreover, *bla*_{TEM} gene was still present even when the treatment time was extended to 300 min [79].

In another study of Moreira et al. [49], the efficiency of sunlight/H₂O₂ to reduce the 16S rRNA gene and *intI1* genes, and selected ARGs (*bla*_{TEM}, *qnrS*, *sul1* and *bla*_{CTX-M}) present in wastewater effluents was investigated under various operating parameters. The results obtained, showed that the sunlight/H₂O₂ treatment was able to reduce the abundance of the ARGs for 1 log in average, except for *bla*_{CTX-M} (3 log reduction).

Based on the results presented in this study, as well as in previous studies regarding the removal of ARGs through the light-driven oxidation processes, it can be inferred that the treatments are unable to completely remove resistance determinants from urban wastewater.

A summary of the results obtained in both processes, using the UWW matrix, is provided in **Table 1**.

4. Conclusions

This study investigated two light-driven oxidation processes as possible tertiary treatment methods of urban wastewater, by evaluating their efficiency to perform both chemical decontamination and disinfection. The UV-C/H₂O₂ treatment was found capable of eliminating CIP and SMX (90 min, 0.9 kJ L⁻¹), whereas sunlight/H₂O₂ process was only able to eliminate CIP (CIP was eliminated in 60 min and 8 kJ L⁻¹, while SMX was removed only by 46% after 300 min and 42 kJ L⁻¹). Similar results were obtained for the two processes, when the matrix was SS, except from the shorter times required for antibiotics' elimination (due to absence of dE_{OM} in the SS). This reveals the superiority of UV-C/H₂O₂ over sunlight/H₂O₂ process for the removal of antibiotics, regardless of the matrix used. The formation of recalcitrant organic intermediates was evident from the fact that complete mineralization was not achieved by any treatment. The results of the chronic toxicity bioassay applied, using the *V. fischeri* bacterium, showed that the toxicity is probably derived from the oxidation of the dE_{OM} itself. So, in terms of toxicity, which seemed to be greater throughout UV-C/H₂O₂, the process is accused with a drawback.

The pathways of the photo-transformations of the two antibiotics determined, showed that all the TPs identified for CIP and SMX still retain the core quinolone and amino moieties, respectively, which are responsible for the antibacterial activity of the compounds. This is an

interesting observation, as further investigations should be carried out in order to determine the appropriate dose or accumulated energy, which will be capable to oxidize the antimicrobial moiety of the TPs, evaluating at the same time the impact of the TPs on antimicrobial resistance spread.

Both treatments were found able to inactivate *E. coli* and *P. aeruginosa* in SS and UWW, including the colonies of these species cultivable in the presence of sub-MIC of CIP and SMX, noting though, a quite big difference in the dose/accumulated energy required by each process (UV-C/H₂O₂: 8 min, 0.1 kJ L⁻¹; sunlight/H₂O₂: 120-150 min, 16-20 kJ L⁻¹). Moreover, after 48 h of post-treatment storage of the sunlight/H₂O₂ treated samples, bacterial regrowth occurred, suggesting that the treatment was not only longer, but also it did not provide complete and permanent disinfection. ARGs exhibited different behaviour during the two treatments, as specific genes were removed to values below the quantification limits and others were persistent throughout the treatment. Again, the UV-C/H₂O₂ showed its superiority over the sunlight/H₂O₂ process, as during the application of the former, all the *bla* and *qnrS* genes were eliminated to the LOQ, while in the application of the latter, none of the examined genes were removed. However, the obtained findings confirmed the inability of both processes to prevent the spread of ARGs to the environment. The inactivation of the studied bacteria and the removal of ARGs, were faster than the degradation of the target antibiotics. As more knowledge is being gathered with respect to the potential adverse effects of the ARB and ARGs after their release to the environment, attention should be given so that the technologies applied at the UWTPs achieve both the removal of antibiotics and their TPs, and the elimination of the antimicrobial resistant bacterial and gene loads, while suppressing post-treatment bacterial regrowth.

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(a) UV-C/H₂O₂

(b) Sunlight/H₂O₂

Fig.1 - Photodegradation of target antibiotics in saline solution (SS) and urban wastewater (UWW) matrices under UV-C light (a) and sunlight (b), shown as functions of the cumulative energy per unit of volume, Q_{UV} (kJ L^{-1}). The inset graph in (a) shows clearly the degradation of SMX as a function of treatment time in the first 20 min of treatment. *Experimental conditions:* $[Antibiotics]_0 = 100 \mu\text{g L}^{-1}$; $[H_2O_2]_{0,a} = 5 \text{ mg L}^{-1}$; $[H_2O_2]_{0,b} = 30 \text{ mg L}^{-1}$; $pH = 8$; $T = 24 \pm 2 \text{ }^\circ\text{C}$.

Fig.2 - Degradation rate of the target antibiotics at the optimum H₂O₂ concentration during (a) UV-C/H₂O₂ and (b) sunlight/H₂O₂ treatment along with the consumption of H₂O₂ during the processes. *Experimental conditions:* $[A]_0=100 \mu g L^{-1}$; $[H_2O_2]_{0,a}=5 mg L^{-1}$; $[H_2O_2]_{0,b}=30 mg L^{-1}$; $pH=8$; $T=24 \pm 2 ^\circ C$.

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Fig.3 - Profile of the main TPs of CIP generated during (a) sunlight/H₂O₂ and (b) UV-C/H₂O₂ treatment.

Fig.4 - Intensity profile of the main TPs of SMX generated during (a) sunlight/H₂O₂ and (b) UV-C/H₂O₂ treatment.

Fig.5a - Tentative transformation pathway of CIP degradation by UV-C/H₂O₂ and sunlight/H₂O₂ processes.

Fig.5b - Tentative transformation pathway of SMX degradation by UV-C/H₂O₂ and sunlight/H₂O₂ and processes.

Fig.6a - Main CIP transformation products profiles and chronic toxicity assessment towards *V. fischeri* (exposure time=24 h) during the UV-C/H₂O₂ and sunlight/H₂O₂ oxidation processes, T=20±0.5°C. The initial spiked concentrations of antibiotic samples were 100 µg L⁻¹. Positive control was NaCl (18%). The values are the mean of three independent experiments ± SD.

Sunlight/H₂O₂ (SMX TPs)

Fig.6b - Main SMX transformation products profiles and chronic toxicity assessment towards *V. fischeri* (exposure time=24 h) during the UV-C/H₂O₂ and sunlight/H₂O₂ oxidation processes, T=20±0.5°C. The initial spiked concentrations of antibiotic samples were 100 µg L⁻¹. Positive control was NaCl (18%). The values are the mean of three independent experiments ± SD.

Fig.7 - Inactivation of *E. coli* and *P. aeruginosa* strains by UV-C/H₂O₂ and sunlight/H₂O₂ treatment. Experimental conditions: $[A]_0 = 100 \mu\text{g L}^{-1}$; $[\text{H}_2\text{O}_2]_{0,a,b} = 5 \text{ mg L}^{-1}$; $[\text{H}_2\text{O}_2]_{0,c,d} = 30 \text{ mg L}^{-1}$; $\text{pH} = 8$; $T = 24 \pm 2 \text{ }^\circ\text{C}$.

Fig.8 - Abundance of 16S rRNA and ARGs (copies per mL of sample) in total DNA as a function of accumulated energy (a) UV-C/H₂O₂ and (b) sunlight/H₂O₂ oxidation of urban wastewater. *Experimental conditions:* $[A]_0=100 \mu\text{g L}^{-1}$; $[H_2O_2]_{0,a}=5 \text{ mg L}^{-1}$; $[H_2O_2]_{0,b}=30 \text{ mg L}^{-1}$; $pH_0=7.5-8.0$.

Treatment process and conditions of operation	Removal of antibiotics	Mineralization (DOC removal)	Generation of TPs and maintenance of the antibacterial active moiety in the TPs		Removal of toxicity
UV-C/H₂O₂ <ul style="list-style-type: none"> Pilot-scale setup UV-C irradiation ($\lambda=254$ nm, 230 W) 80 L of CAS effluents spiked with CIP&SMX (100 $\mu\text{g L}^{-1}$) [H₂O₂]=5 mg L⁻¹ pH: inherent, 7.5-8.0 	<p>CIP: >99% 90 min, Q_{UV}=0.9 kJ L⁻¹</p> <p>SMX: >99% 90 min, Q_{UV}=0.9 kJ L⁻¹</p>	8.2 $\pm$ 0.7 %	<p>CIP: 16 TPs</p> <p>All the TPs retained the antibacterial moiety of the parent compound in their molecule</p>	<p>SMX: 16 TPs</p> <p>All the TPs retained the antibacterial moiety of the parent compound in their molecule</p>	<p>85 $\pm$ 1% 90 min Q_{UV}=0.8 kJ L⁻¹</p>
sunlight/H₂O₂ <ul style="list-style-type: none"> Pilot-scale setup Natural solar irradiation 60 L of CAS effluents spiked with CIP&SMX (100 $\mu\text{g L}^{-1}$) [H₂O₂]=30 mg L⁻¹ pH: inherent, 7.5-8.0 	<p>CIP: >99% 60 min, Q_{UV}=8 kJ L⁻¹</p> <p>SMX: ~46% 300 min, Q_{UV}=42 kJ L⁻¹</p>	3.5 $\pm$ 0.1 %	<p>CIP: 20 TPs</p> <p>All the TPs retained the antibacterial moiety of the parent compound in their molecule</p>	<p>SMX: 15 TPs</p> <p>All the TPs retained the antibacterial moiety of the parent compound in their molecule</p>	<p>99 $\pm$ 1% 120 min Q_{UV}=16 kJ L⁻¹</p>

Table 1 - Table summarizing the performance of the processes examined using the UWW matrix

Highlights

- Oxidation processes showed different treatability for microcontaminants
- UV-C/H₂O₂ proved to be more efficient in terms of time and energy required

- Resulting ecotoxicity can be attributed to the dE₇OM and its oxidation products
- All the TPs identified retain the antibacterial moiety in their molecules
- *bla*_{OXA-A}, *bla*_{SHV}, *bla*_{TEM} and *qnrS* resistance genes were eliminated by UV-C/H₂O₂

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Declaration of Interest Statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

We remain at your disposal for any further information.

Yours sincerely,

Dr. D. Fatta-Kassinou