

Photocatalytic ozonation of urban wastewater and surface water using immobilized TiO₂ with LEDs: Micropollutants, antibiotic resistance genes and estrogenic activity

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Abstract

Photocatalytic ozonation was employed for the first time in continuous mode with TiO₂-coated glass Raschig rings and light emitting diodes (LEDs) to treat urban wastewater as well as surface water collected from the supply area of a drinking water treatment plant (DWTP). Different levels of contamination and types of contaminants were considered in this work, including chemical priority substances (PSs) and contaminants of emerging concern (CECs), as well as potential human opportunistic antibiotic resistant bacteria and their genes (ARB&ARG). Photocatalytic ozonation was more effective than single ozonation (or even than TiO₂ catalytic ozonation) in the degradation of typical reaction by-products (such as oxalic acid), and more effective than photocatalysis to remove the parent micro-pollutants determined in urban wastewater. In fact, only

fluoxetine, clarithromycin, erythromycin and 17- α -ethinylestradiol (EE2) were detected after photocatalytic ozonation, by using solid-phase extraction (SPE) pre-concentration and LC-MS/MS analysis. In surface water, this treatment allowed the removal of all determined micropollutants to levels below the limit of detection (0.01-0.20 ng L⁻¹). The efficiency of this process was then assessed based on the capacity to remove different groups of cultivable microorganisms and housekeeping (16S rRNA) and antibiotic resistance or related genes (*int11*, *blaTEM*, *qnrS*, *sul1*). Photocatalytic ozonation was observed to efficiently remove microorganisms and ARGs. Although after storage total heterotrophic and ARB (to ciprofloxacin, gentamicin, meropenem), fungi, and the genes 16S rRNA and *int11*, increased to values close to the pre-treatment levels, the ARGs (*blaTEM*, *qnrS* and *sul1*) were reduced to levels below/close to the quantification limit even after 3-days storage of treated surface water or wastewater. Yeast estrogen screen (YES), thiazolyl blue tetrazolium reduction (MTT) and lactate dehydrogenase (LDH) assays were also performed before and after photo-catalytic ozonation to evaluate the potential estrogenic activity, the cellular metabolic activity and the cell viability. Compounds with estrogenic effects and significant differences concerning cell viability were not observed in any case. A slight cytotoxicity was only detected for Caco-2 and hCMEC/D3 cell lines after treatment of the urban wastewater, but not for L929 fibroblasts.

1. Introduction

The environmental occurrence of priority substances (PSs), contaminants of emerging concern (CECs), human pathogens and antibiotic resistance genes (ARGs) is a serious issue affecting ecosystem services (such as drinking water supplies) and urban wastewater treatment plants (WWTPs) are hot spots for such type of contamination (Berendonk et al., 2015; Ribeiro et al., 2015a). For instance, conventional WWTPs are not specifically designed to eliminate PSs and CECs occurring at trace concentrations, ending into the environment. Among these, specific pesticides, endocrine disrupting compounds, industrial compounds, flame retardants, polycyclic aromatic hydrocarbons, dioxins and dioxin-like compounds, have already been identified as PSs in Directive 2013/39/EU (Directive, 2013). Furthermore, a watch list of 10 substances/groups of substances that should be monitored within the European Union was recently included in Decision 495/2015/EU (Decision, 2015), namely pharmaceuticals (diclofenac, 17- α -ethinylestradiol (EE2) and three macrolide antibiotics), two natural hormones (17- β -estradiol (E2) and estrone (E1)), some pesticides, an UV filter and a food additive.

Besides the chemical contamination, the biological pollution is also a matter of concern in aquatic environments, in particular in human-impacted areas, calling for adequate responses from WWTPs (Dunlop et al., 2015; Ferro et al., 2015a; Michael et al., 2013; Rizzo et al., 2014). Conventional WWTPs, although able to remove the

bacterial loads up to 2 logarithmic cycles, do not contribute to significant reductions of antibiotic resistance prevalence and, in some cases, can even contribute to its increase (Michael et al., 2013). It is estimated that urban conventional WWTPs using distinct types of biological treatments, and operating to achieve the recommended quality thresholds, release at least 10^9 antibiotic resistant coliforms per minute to the surrounding environment (Vaz-Moreira et al., 2014).

Therefore, the spread of PSs, CECs and antibiotic resistant bacteria and their genes (ARB&ARG) emitted from WWTPs contributes for the contamination of surface and groundwater, existing the possibility that some of these pollutants may reach the drinking water systems (Vaz-Moreira et al., 2014; Xi et al., 2009). This scenario makes also vulnerable the quality of treated wastewater and, thus, its reuse in activities such as irrigation in agriculture when this wastewater is not properly treated (Ferro et al., 2015b). In this context, new treatment options should be capable of eliminating PSs and CECs and reducing ARGs to levels at which further dissemination will be improbable (Berendonk et al., 2015; Dodd, 2012).

Chemical oxidation technologies (COTs) and advanced oxidation processes (AOPs), such as Fenton-based processes, heterogeneous photocatalysis and ozonation-based processes, have been widely studied to eliminate PSs and CECs from different types of waste- water, but most studies are focused on single compounds, normally at concentrations higher than those found in urban wastewater (Ribeiro et al., 2015a). In addition, photocatalysis (with or without H_2O_2) and the photo-Fenton process were already tested for the inactivation of an antibiotic resistant *Escherichia coli* strain (Dunlop et al., 2015; Ferro et al., 2015a; Rizzo et al., 2014). However, more studies are still needed to better understand the effect of the oxidative approaches on the control of ARB&ARG. For instance, photocatalytic ozonation has been studied for the removal of pharmaceuticals in effluents from urban WWTPs (Beltr'an et al., 2012; Encinas et al., 2013; Espejo et al., 2013; Li et al., 2007; Márquez et al., 2014; Moreira et al., 2015; Quinones et al., 2015; Rey et al., 2014; Tanaka et al., 2001), and a couple of reviews were published last year (Mehrjouei et al., 2015; Xiao et al., 2015), but not much is known about the potential of photocatalytic ozonation to remove ARB&ARGs from urban wastewater. Much less information can be found regarding surface water. In fact the literature is still scarce, less than 300 publications being found in Scopus for "photocatalytic ozonation", against more than 35,500 for "photocatalysis" and 12,000 for "ozonation or ozonolysis", in January 2016. The high efficiency of the photocatalytic ozonation treatment has been explained by a synergistic effect between ozonation and photocatalysis. The photogenerated electrons can react with ozone molecules generating ozonide radicals while decreasing the possible recombination of electron-hole pairs (Mehrjouei et al., 2015; Sánchez et al., 1998).

Based on this background information, the present study aimed at performing a comprehensive evaluation of the capacity of photocatalytic ozonation to treat wastewater collected after the secondary treatment of an urban WWTP. These experiments were performed in continuous mode implementing an innovative

approach with TiO₂-coated glass Raschig rings (Sampaio et al., 2013) and light emitting diodes (LEDs). The same process was tested for surface water treatment, aiming at producing water fulfilling the requirements of drinking water. Thus, different levels of contamination and different types of contaminants were considered in this work, including a set of chemical PSs and CECs, some selected from Directive, 2013/39/EU (Directive, 2013) and Decision 495/2015/EU (Decision, 2015), as well as potential human opportunistic pathogens and ARGs. Yeast estrogen screen (YES), thiazolyl blue tetrazolium reduction (MTT) and lactate dehydrogenase (LDH) assays were also performed before and after photocatalytic ozonation to evaluate biological effects, including the potential estrogenic activity, the cellular metabolic activity and the cell viability in fresh and treated waters. Because biological contaminants have the potential to regrow, when the stress conditions imposed by the water treatment processes are relieved, microbiological indicators were also monitored after storage of treated water.

2. Materials and methods

2.1. Chemicals and materials

TiO₂ (80% anatase and 20% rutile crystalline phases) was provided by Evonik Degussa GmbH (P25). Ultrapure water (resistivity > 18 MU cm) was supplied by a Milli-Q water system. Methanol and acetonitrile (MS grade) were acquired from VWR International (Fontenay-sous-Bois, France) whereas methanol and ethanol (HPLC grade) as well as orthophosphoric acid were purchased from Fisher Scientific UK Limited (Leicestershire, UK). Sulphuric acid was purchased from Merck (Darmstadt, Germany). Oxamic acid (>96%) was supplied by Fluka (Buchs, Switzerland).

Anhydrous sodium dihydrogen phosphate, sodium chloride and oxalic acid (>99%) were purchased from Sigma-Aldrich (Steinheim, Germany), as well as all reference standards for liquid chromatography (>98% purity): diclofenac sodium, ibuprofen sodium, naproxen, tramadol hydrochloride, azithromycin dihydrate, clarithromycin, erythromycin, sulfamethoxazole, trimethoprim, fluticasone propionate, montelukast sodium, warfarin, clopidogrel hydrogen sulphate, metoprolol tartrate, propranolol hydrochloride, hydrochlorothiazide, atorvastatin calcium salt trihydrate, bezafibrate, simvastatin, carbamazepine, citalopram hydrobromide, fluoxetine hydrochloride, norfluoxetine oxalate, venlafaxine hydrochloride, diphenhydramine, 17- α -ethinylestradiol (EE₂), 17- β -estradiol (E₂), estrone (E₁),alachlor, atrazine, simazine, diuron, isoproturon, chlorfenvinphos, pentachlorophenol, clofibrac acid and perfluorooctanesulfonic acid. Stock solutions of each individual compound (approximately 1000 mg L⁻¹) were prepared in methanol, ethanol or acetonitrile, depending on their solubility, and a working solution (200 mg L⁻¹) was prepared by diluting these solutions in

ethanol. The deuterated compounds used as internal standards were also purchased from Sigma-Aldrich (Steinheim, Germany), namely: ketoprofen-d3, ofloxacin-d3, propranolol-d7 solution, fluoxetine-d5 solution, E2-d5 solution and atrazine-d5. A working solution containing 10 mg L⁻¹ of all internal standards was prepared by diluting individual stock solutions (1000 mg L⁻¹) in ethanol.

For cell culture experiments, dimethyl sulfoxide ($\geq 99.9\%$), Dulbecco's phosphate buffered saline (PBS) pH 7.4, Triton™ X-100, thiazolyl blue tetrazolium (MTT) an trypan blue powder were purchased from Sigma-Aldrich. Dulbecco's modified eagle media (DMEM; ref: 31966-021), heat inactivated fetal bovine serum (FBS), penicillin-streptomycin (PenStrep) and trypsin-EDTA were purchased from Gibco® by Life Technologies™ (UK). EndoGRO™ e MV complete media kit was obtained from Merck Millipore and LDH Cytotoxicity Detection Kit was acquired from Takara Bio Inc. (Shiga, Japan). Caco-2 cell line was purchased to the American Type Culture Collection (ATCC, Wesel, Germany) and used between passage number 35 and 42. hCMEC/D3 cell line was kindly supplied by the Institut National de la Santé et de la Recherche Médicale (INSERM, Paris, France) and used between the passage number 31 to 34. Murine fibroblasts L929 were also obtained from ATCC.

2.2. *Experimental set-up and procedure*

A bubble column reactor (Fig. 1a; 2.2 I.D.x 60 cm height) equipped with a loop column (Fig. 1b; 2.2 I.D.x 15 cm height) was used for all the experiments.

This reactor was designed for ozonation experiments operating in continuous mode (Restivo et al., 2012a) and equipped with LEDs in the present work. Two 10 W UV high intensity LEDs (15.5 mm x 23 mm), with dominant emission line at 382 nm and long service life (intensity remains above 70% after 10,000 h work) (Fig. 1k and m), were placed perpendicularly to each other, irradiating the stream (distance of 5 cm) passing through the loop column, by setting a recirculation flow rate of 60 mL min⁻¹ (Fig. 1g), as performed in a previous work dealing with catalytic ozonation (Restivo et al., 2012b). The wavelength around 382 nm was chosen considering a compromise between the cost of LEDs (increasing when the emission wavelength decreases) and the possibility to achieve an overlapping between the LEDs main emission wave- length and the TiO₂ absorption spectrum. LEDs emitting in UV-C wavelengths can be up to 10 times more expensive than those emitting in near visible wavelengths. In addition, operation at lower wavelengths demands more energy, the LEDs lifetime de- creases significantly (Lui et al., 2014), and energy is wasted in the form of heat. For these reasons, the wavelength near 382 nm was seen as the best option to the system under study, in this way using the minimum energy to activate the TiO₂ photocatalyst.

The loop column was packed with seventy three TiO₂-coated glass Raschig rings (Fig. 1b) that were prepared as described else- where (Sampaio et al., 2013), from a procedure adapted from literature (Quici et al., 2010). Uncoated Raschig

rings were used in non-catalytic assays. In photolysis and photocatalytic experiments, the LEDs were switched on at the beginning of the experiment, with a constant oxygen flow rate ($15 \text{ Ncm}^3 \text{ min}^{-1}$). In ozonation and photocatalytic ozonation assays, a BMT 802X ozone generator (Fig. 1c) was used to produce ozone (from pure oxygen) at a constant inlet concentration (50 g Nm^{-3}) and flow rate ($15 \text{ Ncm}^3 \text{ min}^{-1}$). A BMT 964 ozone analyzer (Fig. 1f) was used to control the concentration of ozone in the gas phase. Gas washing bottles filled with potassium iodide solution were used to remove the ozone leaving the reactor in the gas phase (Fig. 1e).

Regardless of the type of test performed, the reactor was always filled with ultrapure water and the experiment started ($t = 0 \text{ min}$) when the water to be treated (wastewater or surface water) (Fig. 1i) was pumped to the reactor (Fig. 1h), i.e., being diluted until achieving the steady state. Thus, since the reactor was fully filled with ultrapure water before starting the experiment with a realistic matrix, some dilution occurred up to 90 min in every run. This reactor is designed to operate in continuous mode, with a continuous entrance of realistic matrix at the bottom of the biggest column (liquid flow rate of 15 mL min^{-1}), a continuous outlet stream of treated surface water/wastewater at the top of the same column, and a continuous recirculation (60 mL min^{-1}) between the biggest column and the smaller column holding the photocatalyst, in this way simulating a perfectly mixed reactor. Thus, the removal efficiency was inferred when the steady state was achieved, i.e. when the outlet solution had always the same concentration and there was no more dilution effect. For this reason, preliminary studies on hydraulic retention time (HRT) were performed using NaCl as tracer (inlet concentration of 2000 mg L^{-1}) and uncoated Raschig glass rings were placed randomly inside the loop column, measuring the conductivity with a conductimeter Crison GLP 31 (Barcelona, Spain). The gas and liquid streams were maintained at $15 \text{ Ncm}^3 \text{ min}^{-1}$ and 15 mL min^{-1} , having into account preliminary ozonation experiments with different ozone flow rates (using oxalic acid as prove molecule) and that these conditions were optimized for catalytic ozonation in a previous work (Restivo et al., 2012b). The HRT studies allowed to determine the time needed to achieve the steady state and the time spent by the fluid inside the reactor (retention time). Fig. 2 shows the evolution of the normalized conductivity (s/s_t) where s is the conductivity of the outlet solution and s_t the conductivity of the tracer injected in the column. The time needed to achieve the steady state is ca. 90-100 min and the retention time (26 min) was determined from the area under the representation of $1-(s/s_t)$ vs. time. Other run was performed with vertically aligned uncoated glass rings but the results were similar to those obtained with randomly distributed glass rings. Having into account these results, the next experiments were performed during 180 min with randomly distributed Raschig glass rings (retention time = 26 min).

The first set of experiments with chemical pollutants was performed with

ultrapure water spiked with diclofenac (32 mg L^{-1} ; pH 4.5) and the removal of this pharmaceutical was followed over time by analysing the treated effluent (Fig. 1j). In a second set of experiments, wastewater samples collected after the activated sludge biological treatment of an urban WWTP located in Northern Portugal were treated by ozonation, photocatalysis and photocatalytic ozonation ($n = 3$, i.e. a total of three samples for each treatment, each sample collected in a different day). For the catalytic treatments, coated Raschig glass rings were replaced between experiments. The sampling (April-May 2015) was performed using pre-rinsed amber glass bottles (2.5 L). Surface water samples were collected (May-June 2015) in the supply area of a drinking water treatment plant (DWTP) and treated by photocatalytic ozonation ($n = 3$). All samples were transported at $4 \text{ }^{\circ}\text{C}$ to the laboratory and processed immediately.

2.3. Materials characterization

TiO₂-coated glass rings were characterized before and after the photocatalytic ozonation treatment. Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS) were performed in a FEI Quanta 400 FEG ESEM/EDAX Genesis X4M instrument. The materials hydrophilicity was assessed by measuring the water contact angle in an Attension equipment (Theta model) which allows image acquisition and data analysis. The measurements were performed at room temperature using the sessile drop method in five different locations over glass rings. Thermogravimetric (TG) analysis was performed using a STA 490 PC/4/H Luxx Netzsch thermal analyzer by heating the samples from room temperature until $800 \text{ }^{\circ}\text{C}$ at $10 \text{ }^{\circ}\text{C min}^{-1}$ under helium flow, holding this temperature for 7 min and finally changing to air atmosphere during 13 min.

2.4. Chemical analysis

High performance liquid chromatography with diode array detection (HPLC-DAD) was performed to analyze the removal of diclofenac, using a Hitachi Elite Lachrom apparatus equipped with a YMC Hydrosphere C18 column (250 mm x 4.6 mm i.d.), working at room temperature under isocratic elution. The mobile phase consisted in a mixture of phosphate buffer solution (pH 2.8) and methanol (30/70, v/v). The flow rate was 1 mL min^{-1} and the volume of injection was 15 mL. A pH meter pHenomenal[®] pH 1100L (VWR, Germany) was used for all pH adjustments. The oxalic and oxamic acid concentrations were determined by HPLC-UV, using an Altech AO-1000 column (300 mm x 6.5 mm i.d.) operating under isocratic elution with 5 mM H₂SO₄ at 0.5 mL min^{-1} . The total organic carbon (TOC) content was determined using a Shimadzu TOC-5000A analyzer. The concentrations of the target micro-pollutants (listed in section 2.1) were determined in urban wastewater and surface water samples, before and after the

treatment by the advanced oxidation processes, using a fully validated method of solid phase extraction (SPE) and ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS) in a Shimadzu Corporation apparatus (Tokyo, Japan), as described elsewhere (Ribeiro et al., 2015b). Wastewater (100 mL) and surface water (250 mL) samples were acidified to pH 3 with sulphuric acid, followed by extraction of the micropollutants using Oasis® HLB (Hydrophilic-Lipophilic-Balanced sorbent, 150 mg, 6 mL) cartridges (Waters, Milford, Massachusetts, USA). For internal calibration, isotopically labeled internal standards were added to the samples before SPE.

25. Microbiological characterization, DNA extraction and qPCR analysis

Wastewater and surface water samples were characterized for the abundance of different groups of cultivable microorganisms, quantification of selected antibiotic resistance and related genes (herein referred to ARG), before and immediately after photo-catalytic ozonation, and after three days of water storage at room temperature. For each photocatalytic ozonation experiment, all assays were performed in triplicate. The membrane filtration method was used for the enumeration of cultivable microorganisms. Briefly, adequate serial dilutions of water samples were filtered through cellulose membrane filters (0.22 µm porosity; Whatman, UK) and incubated on culture media targeting different microbial groups: Plate Count Agar (PCA) (Merck, 30 °C, 1e7 days) for total heterotrophs; membrane Fecal Coliforms (Difco, 37 °C, 1 day) for enterobacteria; m-Enterococcus agar (Difco, 37 °C, 2 days) for enterococci; and Rose Bengal Chloramphenicol agar (VWR International, 30 °C, 7 days) for fungi. Additionally, PCA supplemented with ciprofloxacin (4 mg L⁻¹), gentamicin (16 mg L⁻¹) or meropenem (4 mg L⁻¹) were used to assess resistance prevalence in surface water before and after treatment. These antibiotics were selected because resistance to carbapenems (e.g., meropenem) and aminoglycosides (e.g., gentamicin) is commonly found in drinking water bacteria or because selection of fluoroquinolone resistance (e.g., ciprofloxacin) is sometimes observed in aquatic environments (Vaz-Moreira et al., 2014). For culture-independent assays, total DNA was extracted from water samples with volumes ranging from 100 mL to 1 L, after filtration through polycarbonate membranes (0.22 µm porosity; Whatman, UK), using the commercial kit PowerWater® DNA Isolation (MO BIO Laboratories, Inc., USA). DNA extracts were cryopreserved at 20 °C until the analyses of the abundance of ARG. Quantitative PCR (qPCR) was used according to the conditions indicated in Table 1, as described elsewhere (Narciso-da-Rocha et al., 2014).

26. Biological assays

26.1. Yeast estrogen screen (YES) assay

The YES assay was performed using the commercially available kit XenoScreen

YES/YAS assay (Xenometrix, Hégenheim, France).

Water samples (wastewater and surface water) collected before and after photocatalytic ozonation were filtered through 0.45 µm hydrophilic membranes and directly analyzed, without any pre-concentration. The assay and potential estrogenic activity calculations were carried out following the manufacturer's instructions. Briefly, samples were transferred to a 96-well microplate, mixed with assay medium and inoculated with the transformed yeast cells. Assay mixture consisted of growth medium containing the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG). The natural estrogen E2, at concentrations between 10^{-6} - 10^{-9} M, was used as positive control and standard. Ultrapure water was used as negative control. A solvent blank was also assayed. The plate was incubated for 48 h at 31 °C with orbital shaking. After incubation, spectrophotometric measurements were performed in a microplate reader (Synergy HT, Bio-Tek Instruments, Winooski, USA) at 690 nm for growth and at 570 nm for expression of β-galactosidase. The potential estrogenic activity was determined through the calculation of the parameters growth factor (G) and induction ratio (IR). The G parameter was calculated as the ratio of absorbance at 690 nm for the sample $(A_{690})_{\text{sample}}$ and the absorbance at 690 nm for the solvent $(A_{690})_{\text{solvent}}$. The IR parameter was calculated as $(1/G) \times ((A_{570}-A_{690})_{\text{sample}}/(A_{570}-A_{690})_{\text{solvent}})$.

2.62 Thiazolyl blue tetrazolium reduction (MTT) and lactate dehydrogenase (LDH) assays

Thiazolyl blue tetrazolium reduction (MTT) assay evaluates cellular metabolic activity while lactate dehydrogenase (LDH) assay provides information about cell viability through evaluation of membrane integrity. Cell culture procedures are described in detail elsewhere (Ferreira et al., 2015). Briefly, samples were diluted 1:2.5 in culture medium. Afterwards, 100 µL of diluted sample were added to cell layers or suspension, containing 100 µL of culture medium. After 24 h, the supernatant was removed for LDH assay, while the remaining content was used for MTT assay. For MTT assay, absence of cytotoxicity (100%) was evaluated by replacing test sample by culture medium. For LDH assay, absence of cell viability (100%) was evaluated by replacing test sample by 1% (v/v) Triton X-100 solution in PBS.

3. Results and discussion

3.1 Degradation of organic micropollutants

The first set of experiments with chemical pollutants was performed using ultrapure water spiked with diclofenac as model compound, due to the known high occurrence of this pharmaceutical in the environment, its recalcitrance, and inclusion in the watch list for European Union monitoring in the field of water

policy (Décision, 2015). The evolution of the normalized concentration of diclofenac is shown in Fig. 3a, the time needed to achieve the steady state in all the experiments being around 90-100 min, as concluded from HRT studies (Fig. 2). Fig. 3a shows that complete removal of this compound was achieved in all the experiments where ozone was employed (ozonation, photolysis assisted ozonation, catalytic ozonation, and photocatalytic ozonation). For (non-catalytic) photolysis, the outlet and inlet diclofenac concentrations were practically the same in steady state. This result was expected since this model compound absorbs maximally radiation at wavelengths below 325 nm while the radiation peak of the used LEDs was centered at 382 nm, above 367 and below 410 nm (Fig. 1m). In the case of TiO₂-coated glass rings photocatalysis, a modest diclofenac removal in steady state was observed (ca. 26%; i.e. $C/C_0=0.74$).

Oxalic and oxamic acids are well known reaction products during ozonation of larger molecules (Faria et al., 2008), and thus they were also analyzed, but only oxalic acid was detected above the limit of detection ϵ LOD (Fig. 3b). As expected, oxalic acid was not formed during photolysis, while photocatalysis originated a low amount of this acid in steady state (ca. 2.0 mg L⁻¹), which is probably related to the poor efficiency of this process for the degradation of diclofenac. In contrast, ozonation alone led to the largest amount of oxalic acid (ca. 13.0 mg L⁻¹), followed by photolysis assisted ozonation and catalytic ozonation (ca. 10.5 mg L⁻¹), and then by photocatalytic ozonation (ca. 9.0 mg L⁻¹). TOC analysis was performed to account for the presence of other organic compounds that can be formed during the degradation of diclofenac (Fig. 3c). Once again, photolysis and photocatalysis were the least effective treatments, with TOC removals of ca. 3 and 20% in steady state, respectively, while photolysis assisted ozonation and photocatalytic ozonation were the best performing options in terms of TOC removal (ca. 51% and 56%, respectively).

Since oxalic acid was the main by-product detected from diclofenac degradation, additional experiments were performed having oxalic acid only (90 mg L⁻¹) in ultrapure water as feed (Fig. 3d). Oxalic acid was completely recalcitrant in the photolysis assay, while low removal values were obtained with ozonation (8%) and catalytic ozonation (11%). All the other processes (photocatalysis, photolysis assisted ozonation and photocatalytic ozonation) were quite effective in the removal of oxalic acid (55%, 58% and 66%, respectively). Even so, photocatalytic ozonation was more efficient than photolysis assisted ozonation for the degradation of oxalic acid, probably due to the overlap between the absorption spectrum of TiO₂ and the emission spectrum of the LEDs, enough to get the minimum energy needed to surpass the energy band gap of TiO₂. In batch conditions (Moreira et al., 2015), it was already observed that oxalic acid (one of the main reaction by-products in ozonation-based processes) is oxidized efficiently by photocatalytic ozonation, while photolysis assisted ozonation was not able to remove the oxalic acid accumulated. In addition, it is known that UV at 382 nm (far from UV-C) is inefficient for disinfection in the absence of a photocatalyst (Nelson et

al., 2013). Thus, considering the targeted simultaneous treatment of both chemical and biological contaminants, and since a photocatalyst can increase the process disinfection efficiency by the formation of highly reactive radicals, photocatalytic ozonation was applied to treat wastewater samples collected after the secondary biological treatment of an urban WWTP. Ozonation and photocatalysis experiments were also performed in order to assess the individual contribution of each process on their own.

The micropollutants (pharmaceuticals, a metabolite, hormones, pesticides and an industrial compound listed in section 2.1) were analyzed (before and after the treatment) by UHPLC-MS/MS, after pre-concentration of the samples by SPE. Fig. 4 shows the concentration range for the detected micropollutants in the effluent of the biological treatment used in the urban WWTP during the sampling campaign (April-May 2015). The highest concentrations were observed for the following compounds with a frequency of detection of 100% (9/9): the anti-inflammatory naproxen, the diuretic hydrochlorothiazide, the lipid regulator bezafibrate and the synthetic hormone EE2 included in the watch list of the Decision 495/2015/EU (Decision, 2015). Diclofenac, azithromycin, clarithromycin and erythromycin, also included in the watch list, as well as ibuprofen, clopidogrel, propranolol, atorvastatin, carbamazepine, fluoxetine and norfluoxetine, were quantified in all samples. Citalopram and venlafaxine were also detected, but not always above the limit of quantification (LOQ), whereas sulfamethoxazole, trimethoprim, diphenhydramine, E2 and isoproturon were not detected in some samples.

Fig. 5 shows that the higher efficiency to remove the detected micropollutants was found for ozonation and photocatalytic ozonation. Photocatalysis was comparatively less effective in the degradation of these micropollutants. Only fluoxetine, clarithromycin, erythromycin and EE2 were detected in the outlet solution after photocatalytic ozonation. While both antibiotics were only detected in the final effluent of one of the triplicate experiments, fluoxetine and EE2 were detected twice. Naproxen was only quantified in one replicate assay. It is also important to note that samples were collected daily for each experiment. Regarding the TOC removal, the initial TOC of ca. 25 mg L^{-1} decreased to 19 mg L^{-1} in steady state conditions of photocatalytic ozonation. The initial and final pH values were quite similar (7.4 and 7.2, respectively) while the low weight carboxylic acids and ions were below the LOQs. Thus, both single ozonation and photocatalytic ozonation treatments were quite effective to remove the chemical organic micropollutants (Fig. 5), but it should be also taken into account that ozonation is typically much less effective than photocatalytic ozonation in the mineralization (Fig. 3c) and degradation of typical reaction by-products (Fig. 3b). Among different treatments described elsewhere in batch conditions (Moreira et al., 2015), photocatalytic ozonation was considered the best performing process: a fast mineralization of amoxicillin and diclofenac was observed in spiked urban wastewater; toxic intermediate products for *Escherichia coli* and *Staphylococcus aureus* were not detected; and the parent micropollutants

determined were removed from an urban wastewater.

Photocatalytic ozonation, the most efficient process for the degradation and mineralization of chemical pollutants in urban wastewater, was also tested to treat surface water samples collected near a DWTP. The micropollutants detected after pre-concentration of the samples by SPE were: clarithromycin, fluoxetine, nor-fluoxetine, carbamazepine, EE2, E2 and isoproturon (Table 2). The antibiotic clarithromycin, recently included in the watch list of substances for monitoring (Decision, 2015), was quantified at trace concentrations (up to 4.13 ng L^{-1}). Fluoxetine was also detected in all samples, but only quantified in one sample (at 5.72 ng L^{-1}), while its human metabolite norfluoxetine was detected once in the same sample, with a higher concentration (7.66 ng L^{-1}) than the parent compound. The highest levels were obtained for the anti-epileptic carbamazepine (up to 56.2 ng L^{-1}) and for the pesticide isoproturon (up to 24.3 ng L^{-1}), the last considered as PS (Directive, 2013). All these micropollutants were completely removed by photocatalytic ozonation. Although not quantifiable, EE2 and E2 were detected before photocatalytic ozonation and completely removed. Therefore, photocatalytic ozonation was observed as an efficient treatment for the degradation of organic micropollutants in both urban wastewater and surface water.

32. *TiO₂-coated glass rings characterization*

The TiO₂-coated glass rings (shown in Fig. 6a) were characterized by different techniques before and after photocatalytic ozonation of the urban wastewater samples. A negligible weight loss (WL) was obtained by TG analysis of the fresh TiO₂-coated glass rings (not shown), regardless of the atmosphere employed (helium or air). In the case of the rings recovered after photocatalytic ozonation, desorption of water occurred below $100 \text{ }^{\circ}\text{C}$ under helium atmosphere while 4% of the weight decreased when the gas phase was shifted from helium to air at $800 \text{ }^{\circ}\text{C}$, most probably due to the oxidation of some organic matter remaining in the surface of these rings after photocatalytic ozonation. SEM micrographs, EDS spectra and contact angles of water droplets over the glass rings are shown in Fig. 6b and c, respectively before and after the photocatalytic ozonation treatment. TiO₂ presence is evident in both samples, with Fig. 6d showing a higher SEM magnification of the TiO₂ particles. It is also possible to conclude that TiO₂-coated glass rings are highly hydrophilic since very low contact angles were obtained ($<15^{\circ}$). Overall, no significant differences were observed between the fresh and used samples, even considering the high matrix complexity of effluents from urban WWTPs.

33. *Microbial inactivation and reactivation*

The photocatalytic ozonation of secondarily treated wastewater showed the

reduction of enterococci, enterobacteria, and fungi from $\sim 10^5$ e 10^6 colony forming units (CFU) per 100 mL to values around or below $\sim 10^1$ CFU/100 mL. In contrast, total heterotrophs that may comprise bacteria with different levels of endurance, such as endospore producers, presented lower reductions, with about $\sim 10^2$ CFU/100 mL being detected after the advanced treatment (Fig. 7a). These results were confirmed in the assays testing the regrowth capacity, with significantly higher counts of total heterotrophic populations and fungi than of the other microbial groups analyzed. Regrowth may have resulted from the combination of treatment survival and capacity to use any available carbon and energy sources. Indeed, the occurrence of microbial regrowth after water treatment has been attributed to the presence of assimilable organic carbon, generated during the oxidation of recalcitrant organic matter (Thayanukul et al., 2013; Zhao et al., 2014). The comparison of the regrowth capacity in the presence of light or in the dark, suggested that light may have facilitated the recovery of cell injuries, mainly in bacterial heterotrophs and fungi (Fig. 7a), as previously described (Hijnen et al., 2006).

Since most of the microorganisms in water are not cultivable, and advanced water treatment may enhance the fraction of non-cultivable populations, the use of culture-independent methods was necessary in this study. In spite of their stress status, some bacteria maintain viability, being able to regrow when the disinfection stress is relieved. From some of these bacterial cells, DNA extraction may be not possible immediately after surface water/ wastewater treatment, therefore the measurement of the same target genes after three days of incubation was a way to assess the abundance of bacteria fitted to survive after disinfection. Of the analyzed genes, 16S rRNA, *intI1*, *blaTEM*, *qnrS* and *sul1* were above the LOQ in the secondarily treated wastewater samples analyzed. The gene *vanA* was below the LOD (Fig. 7c). Photocatalytic ozonation led to a significant reduction of total prokaryotic cells (assessed based on the abundance of the 16S rRNA gene) and of the gene *intI1*, associated with mobile gene cassettes, also with widespread distribution in different bacterial groups. In addition, also the antibiotic resistance genes *blaTEM*, *qnrS* and *sul1* were significantly reduced after the photocatalytic ozonation. These genes, although highly prevalent in human impacted environments were originally at lower abundance than 16S rRNA and *intI1* genes, and were reduced to levels below or close the LOQ (~ 10 gene copies per mL) after photocatalytic ozonation. For the genes 16S rRNA and *intI1*, the reduction observed after treatment was apparently a transient effect. After storage in the dark or presence of light, the gene copy numbers per 100 mL of 16S rRNA and *intI1* genes were about 1 logarithmic cycle below the initial value (Fig. 7c). The other analyzed genes did not yield a significant increase in their relative abundance during storage (Fig. 7c). These results were also useful to measure the effectiveness of the treatment process, with the regrowth capacity representing a good indication of the fraction of live cells remaining immediately after treatment.

Regarding surface water, the photocatalytic ozonation treatment produced similar results to those obtained for the secondarily treated wastewater. Except for total heterotrophs, the photocatalytic ozonation treatment led to the removal of the analyzed microbial groups (Fig. 7b) to values close to or below the LOD (10^{-2} CFU/100 mL). However, regrowth was observed for fungi and was particularly enhanced for total heterotrophs. In addition, in this case, the analysis of specific potential antibiotic resistant populations suggested that bacteria with acquired anti-biotic resistance traits may have the capacity to regrow in photocatalytic ozonated water up to values found in non-treated surface water (Fig. 8). Similar conclusions were retrieved in a study where the effect of chlorination and H₂O₂/sunlight processes on the regrowth of a multidrug *Escherichia coli* strain was investigated (Fiorentino et al., 2015).

In surface water the genes *qnrS*, *vanA* and *sul1* were below the LOQ, while *blaTEM* presented 10^4 gene copy number per 100 mL (Fig. 7d). Photocatalytic ozonation treatment led to reductions of around 3 logarithmic cycles of the 16S rRNA and *int11* genes, and the gene *blaTEM* was reduced to levels close to the LOQ. As for wastewater, for 16S rRNA and *int11* genes, the inactivation effect was apparently transient (Fig. 7d), corroborating the cultivable-dependent data. Although these findings may suggest a potential increase of microbiological risks, it would be necessary to study the diversity of the populations and/or the prevalence of resistance genes among them to have a better estimation of potential risks.

3.4. Biological effects

YES assay results revealed that no agonistic estrogenic activity was found for samples collected before and after photocatalytic ozonation treatment of the WWTP effluent, along with no growth inhibition. The IR values determined for both samples were between 0.804 and 1.074, which is below IR₁₀ (1.487 for E₂). These observations indicate that the proposed treatment strategy did not originate compounds with estrogenic effects. Furthermore, cytotoxicity and cellular viability assays using cell models for digestive epithelium (Caco-2), blood-brain barrier (hCMEC/D3), and skin (L929) showed no significant difference concerning cell viability (Table 3, LDH assay). MTT assay is a suitable tool for assessment of cytotoxic effects from wastewater, surface water and drinking water samples as reported before (Trintinaglia et al., 2015; Negura et al., 2009). Slight cytotoxicity was observed for Caco-2 and hCMEC/D3 cell lines after treatment, but not for L929 fibroblasts (Table 3, MTT assay). The first two cell lines are human-derived, while L929 fibroblasts were originated in mouse, accounting for their different susceptibility. Furthermore, as reported before (Trintinaglia et al., 2015), the exposure to water testing sample mixed with culture media can originate different results, depending upon the final growth media composition.

Regarding the surface water, no agonistic estrogenic activity was found for these

samples when using the YES assay, along with no growth inhibition. The IR values determined for both samples were between 0.681 and 0.962, which is also below IR10 (1.487 for E2). These observations corroborate that the proposed treatment strategy did not originate compounds with estrogenic effects. Similar results were obtained when applying recombinant yeasts expressing estrogen receptor alpha to evaluate the estrogenic activity of wastewater, bottled waters and tap water in Finnish samples (Omoruyi and Pohjanvirta, 2015). For LDH assay, cellular viability was maintained after treatment for both Caco-2 and hCMEC/D3 cells, providing values similar to those obtained for culture media.

4. Conclusions

Among different oxidative treatments tested in continuous mode, photocatalytic ozonation using TiO₂-coated glass Raschig rings with LEDs irradiation was the most efficient for the removal of PSs and CECs from urban wastewater. In these experiments, fluoxetine, clarithromycin, erythromycin and EE2 were more refractory to oxidation than the other compounds. The same process completely removed all the micropollutants detected in surface water (carbamazepine, isoproturon, clarithromycin, norfluoxetine, fluoxetine, E2 and EE2). In addition, the efficiency to remove different groups of cultivable microorganisms and housekeeping (16S rRNA) and antibiotic resistance or related genes (*intI1*, *blaTEM*, *qnrS*, *sul1*) was studied, with photocatalytic ozonation showing high efficiency on the removal of microbial loads, including ARG, from both urban wastewater and surface water. The tested ARGs were successfully removed to levels below/close to the threshold of quantification. Moreover, after the 3-days storage of treated surface water/wastewater at room temperature, there was no evidence of regrowth of the host, as inferred from the absence of an increase in the relative abundance of the respective ARG. However, both culture-dependent and -independent methods demonstrated that part of the surface water/wastewater microbiota was viable, able to regrow, and the potential risk of this regrowth is still to be assessed. From YES, MTT and LDH assays it follows that compounds with estrogenic effects were not formed and that differences concerning cell viability were not statistically significant for both urban wastewater and surface water. Therefore, photocatalytic ozonation proved to be a potential solution for simultaneous removal of organic micropollutants, different microbial groups of potential human pathogens, ARB and ARG present in wastewater or surface water. However, more studies are still required as well as the evaluation of the process associated costs.

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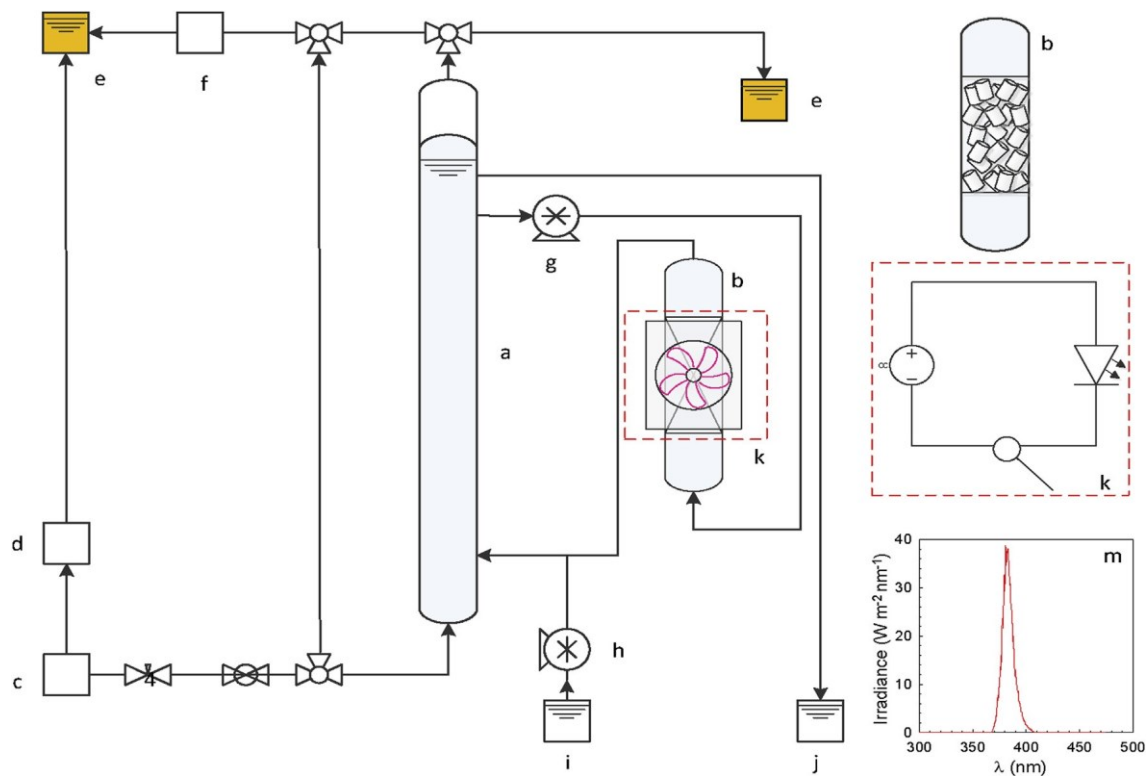


Fig. 1. Experimental set-up of the homemade photocatalytic reactor able to work in continuous mode: (a) Column for gas-liquid contact; (b) Column for liquid-solid contact (Raschig rings); (c) Ozone generator; (d) Mass flow controller; (e) Ozone gas destroyer; (f) Ozone gas monitor; (g) Peristaltic pump for recirculation; (h) Peristaltic pump; (i) Inlet solution; (j) Outlet solution; (k) LEDs; (m) LEDs irradiance spectrum

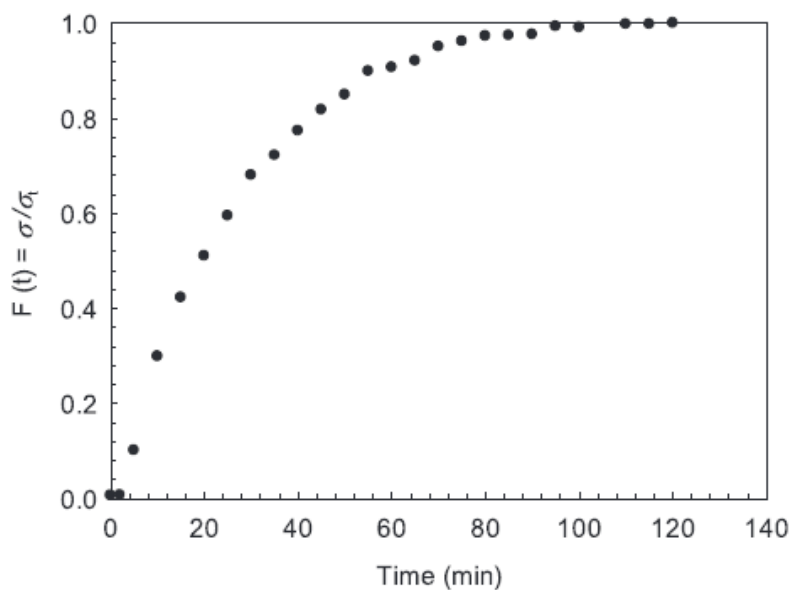


Fig. 2. Normalized conductivity (s/s_t) of the outlet solution, introducing an inlet NaCl solution (2000 mg L^{-1}). s is the conductivity of the outlet solution and s_t the conductivity of the tracer injected in the column.

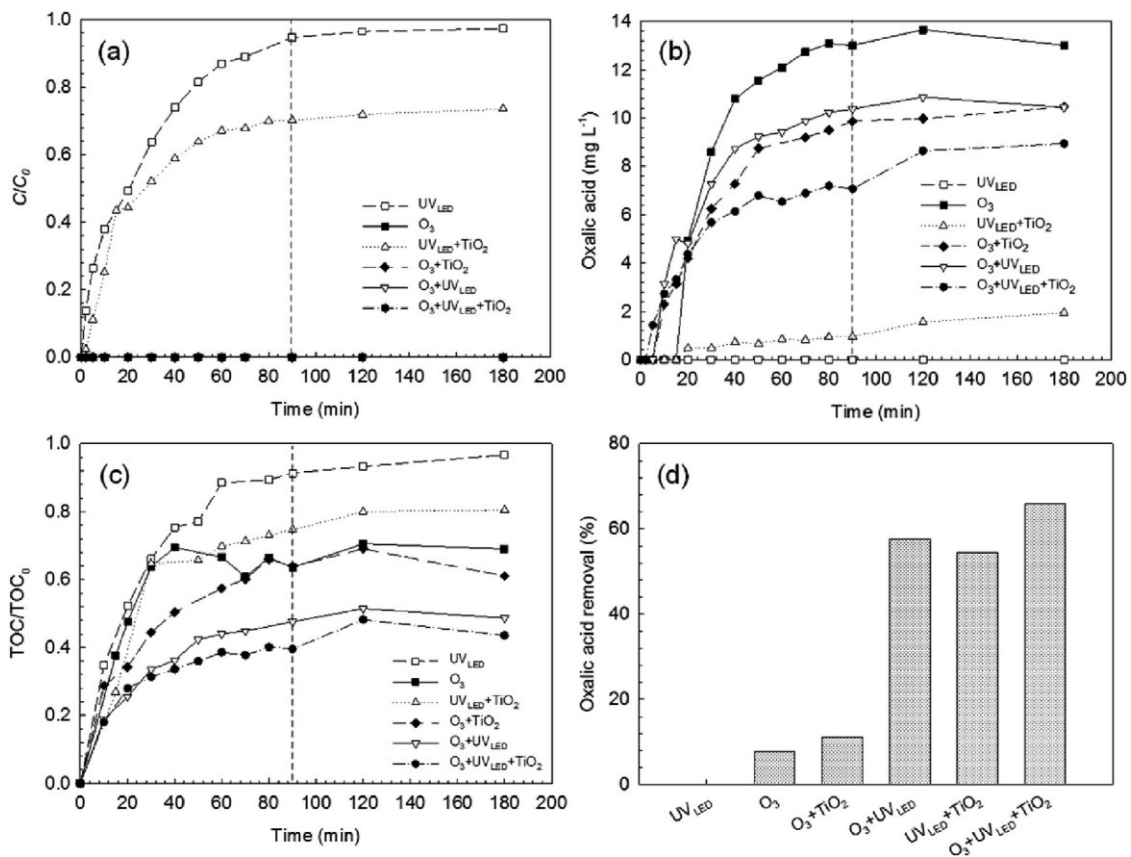


Fig. 3. (a) Normalized concentration (C/C_0) of diclofenac in ultrapure water ($C_0 = 32 \text{ mg L}^{-1}$), (b) evolution of its oxalic acid by-product concentration, and (c) respective normalized TOC content (TOC/TOC_0) under different advanced processes. (d) Removal of oxalic acid when used as feed ($C_0 = 90 \text{ mg L}^{-1}$). Experiments started with the column filled with ultrapure water only (without pollutant at $t = 0 \text{ min}$), the steady state was obtained at ca. 90 min and the retention time is 26 min.

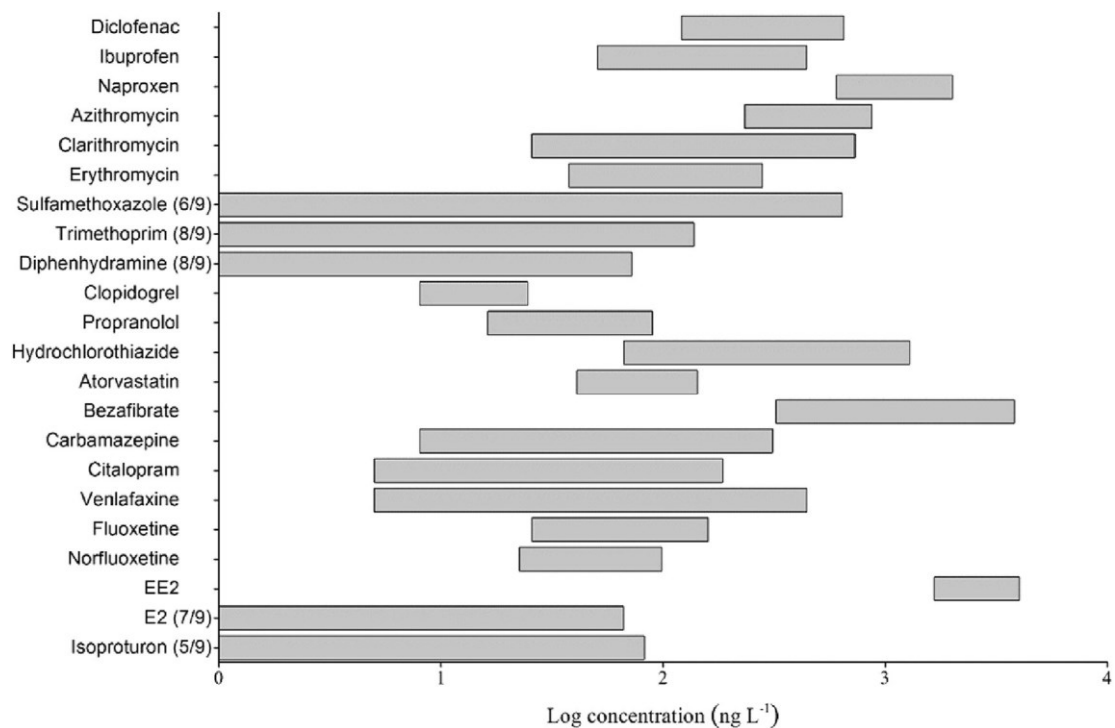


Fig. 4. Logarithmic range of concentrations (ng L^{-1}) of the detected micropollutants in WWTP samples collected after the secondary biological treatment. The frequency of occurrence was 100% (9/9) for all compounds, except when indicated.

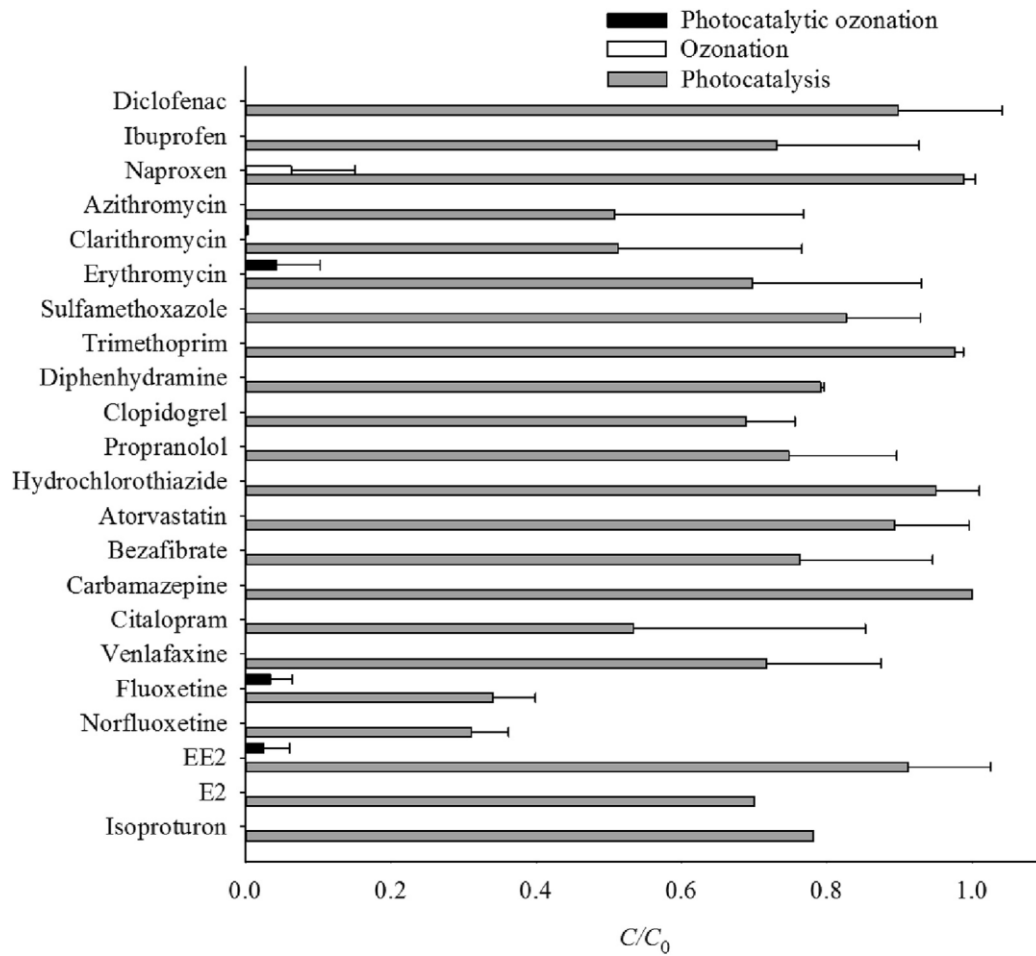


Fig. 5. Normalized concentration of micropollutants (C/C_0) in the effluents from urban WWTP treated by photocatalysis, ozonation and photocatalytic ozonation; C_0 refers to the concentration before treatment and C to that after treatment (retention time: 26 min); compounds with C_0 below the LOD before treatment are not shown.

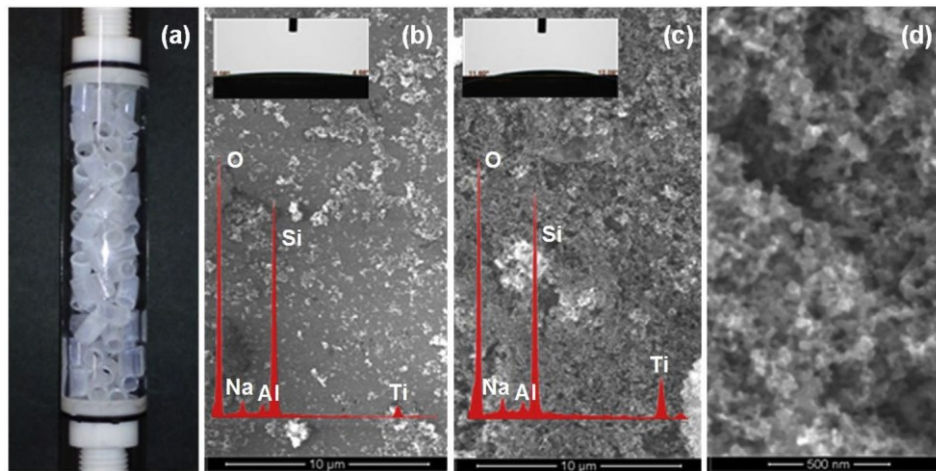
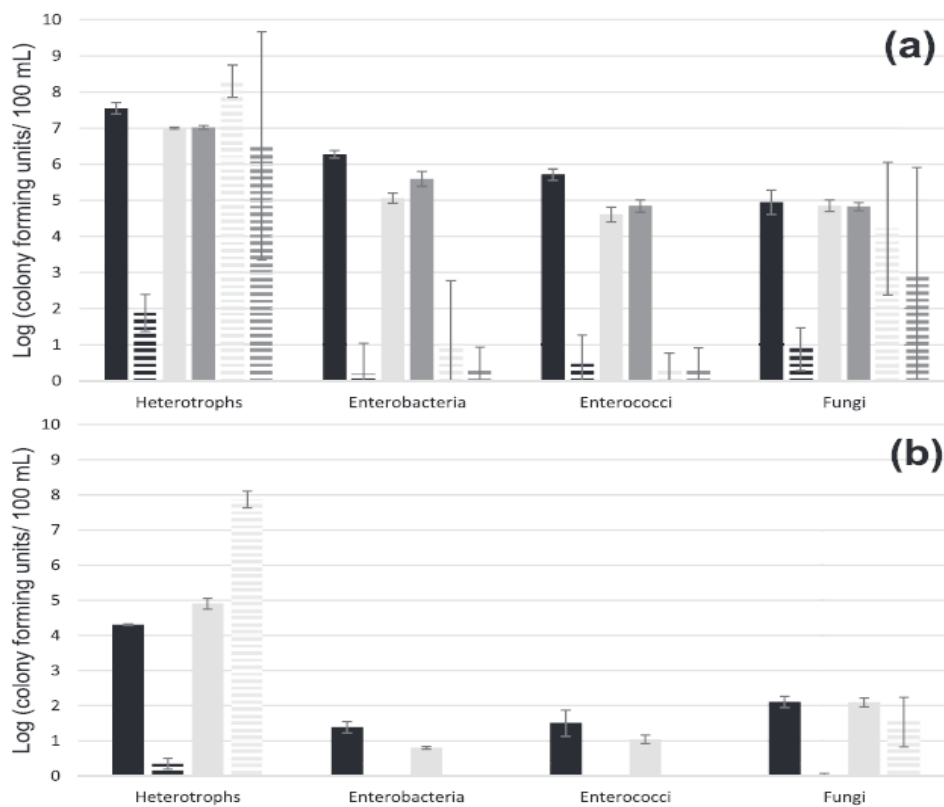


Fig. 6. (a) TiO₂-coated glass Raschig rings and respective (b, c, d) SEM micrographs, EDS and contact angles (b) before and (c, d) after photocatalytic ozonation.



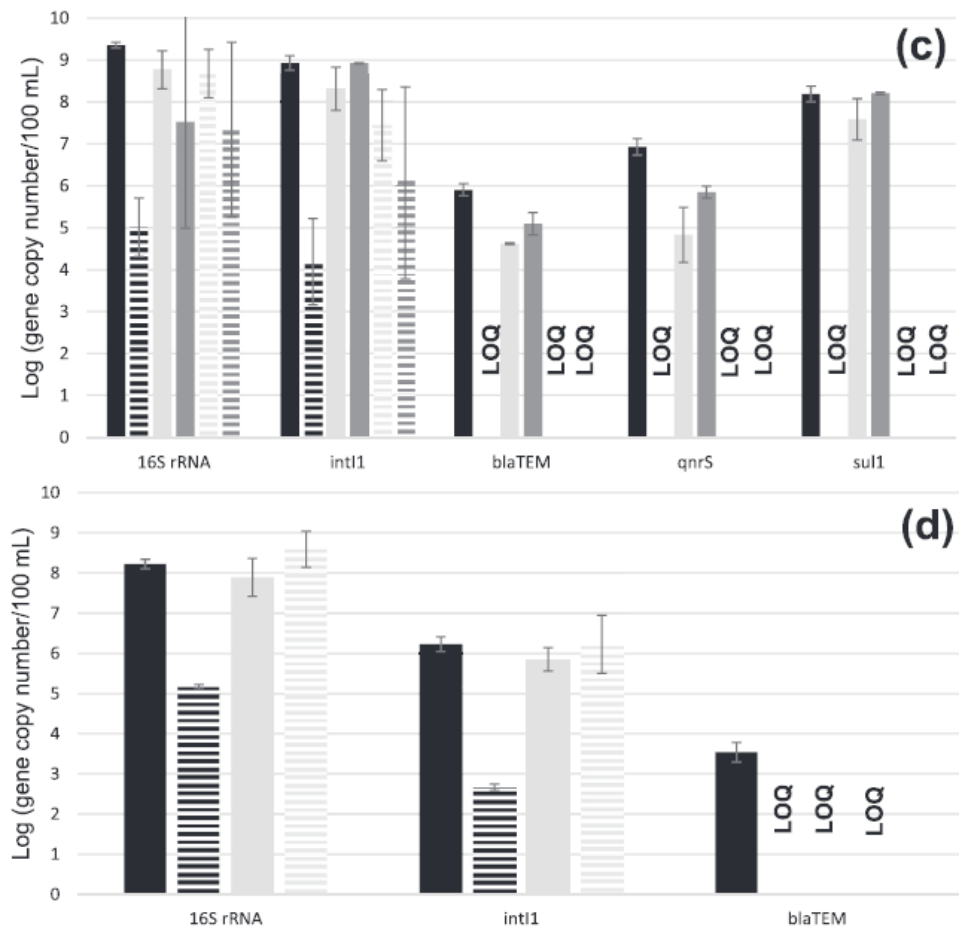


Fig. 7. Microbial inactivation by photocatalytic ozonation in continuous reaction performed on wastewater effluents (a, c) and surface water (b, d) was assessed based on the quantification of selected microbial groups using culture-dependent methods (a, b) and qPCR of selected genes (c, d), in non-treated control samples (filled bars) and after a treatment retention time of 26 min (striped bars), at the initial time (black) and after 3-days storage at room temperature with light (grey) or in the dark (dark grey). LOQ, below or close to the limit of quantification (at least more than half of the quantification reactions were below the quantification limit and none was more than 3 times above that value).

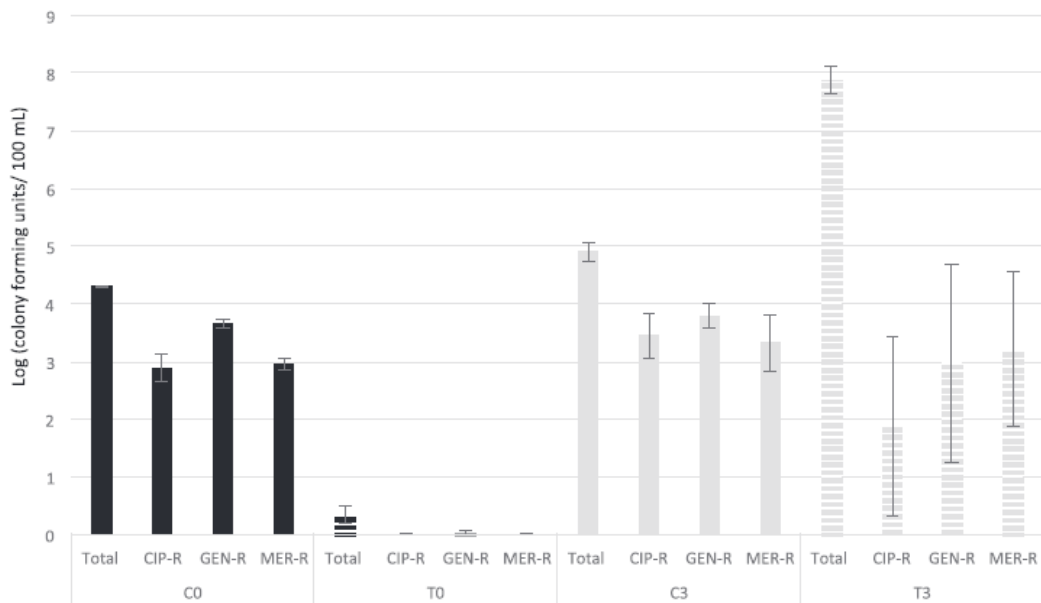


Fig. 8. Total, ciprofloxacin resistant (CIP-R), gentamicin resistant (GEN-R) and meropenem resistant (MER-R) heterotrophic counts before (C0), after photocatalytic ozonation treatment (T0) and after 3-days incubation of untreated surface water (C3) and treated surface water (T3) at dark conditions for microbial reactivation assessment.

Table 1
Conditions used in quantitative PCR assays

Gene	Primers	Reference	Efficiency (%)	Conditions	Reference
16S rRNA	1114F (CGGCAACGAGCGCAACCC) 1275R (CCATTGTAGCACGTGTGTAGCC)	<i>Escherichia coli</i> ATCC 25922	100	95 °C for 10 min (1 cycle) 95 °C for 15 s, 55 °C for 20 s and 72 °C for 10 s (35 cycles) Other: 1a	(Denman and McSweeney, 2006)
<i>bla</i> TEM	<i>bla</i> TEM-F (TTCCTGTTTTGCTCACCCAG) <i>bla</i> TEM-R (CTCAAGGATCTTACCGCTGTTG)	<i>Escherichia coli</i> (A2FCC14)	96	95 °C for 10 min (1 cycle) 95 °C for 15 s, 60 °C for 30 s and 72 °C for 10 s (40 cycles) Other: 2a	(Bibbal et al., 2007)
<i>int1</i>	<i>int1</i> -F (CCTCCCGCACGATGATC) <i>int1</i> -R (TCCACGCATCGTCAGGC)	<i>Escherichia coli</i> (A2FCC14)	94	95 °C for 10 min (1 cycle) 95 °C for 15 s, 55 °C for 30 s and 72 °C for 10 s (40 cycles) Other: 1a	(Goldstein et al., 2001)
<i>qnrS</i>	<i>qnrS</i> rF11 (GACGTGCTAACTTGCGTGAT) <i>qnrS</i> rR11 (TGGCATTGTTGGAACTTG)	<i>Enterobacter cloacae</i> (S1+)	95	95 °C for 5 min (1 cycle) 95 °C for 15 s, 60 °C for 1 min (40 cycles) Other: 2c	(Marti and Balcázar, 2013)
<i>sul1</i>	<i>sul1</i> -FW (CGCACCGGAAACATCGCTGCAC) <i>sul1</i> -RV (TGAAGTTCCGCCGCAAGGCTCG)	<i>Achromobacter</i> sp.	94	95 °C for 5 min (1 cycle) 95 °C for 15 s, 60 °C for 1 min (40 cycles) Other: 2b	(Pei et al., 2006)
<i>vanA</i>	<i>vanA</i> 3FP (CTGTGAGGTCGGTTGTGCG) <i>vanA</i> 3RP (TTTGGTCCACCTCGCCA)	<i>Enterococcus faecalis</i> (H1EV23)	98	95 °C for 5 min (1 cycle) 95 °C for 15 s, 60 °C for 1 min (40 cycles) Other: 2a	(Volkman et al., 2004)

1) KAPA SYBR[®] FAST ABI Prism[®] qPCR Master Mix; 2) SYBR[®] Select Master Mix; a) 200 nM of primer; b) 400 nM of primer; c) 600 nM of primer.

Table 2

Concentration of the micropollutants detected in surface water collected during three sampling campaigns, between May and June 2015, and normalized concentration after treatment (C/C_0).

	C_0 (ng L ⁻¹)	C/C_0	C_0 (ng L ⁻¹)	C/C_0	C_0 (ng L ⁻¹)	C/C_0
Clarithromycin	4.13	0	1.36	0	2.11	0
Fluoxetine	5.72	0	<LOQ	0	<LOQ	0
Norfluoxetine	7.66	0	<LOD	n.a.	<LOD	n.a.
Carbamazepine	<LOQ	0	35.1	0	56.2	0
E2	<LOQ	0	<LOQ	0	<LOQ	0
EE2	<LOD	n.a.	<LOQ	0	<LOQ	0
Isoproturon	<LOQ	0	24.3	0	21.3	0

LOD: limit of detection; LOQ: limit of quantification; n.a.: not applicable.

Table 3

Results (percentage) from MTT^a and LDH^b assays before and after treatment of urban wastewater sample

Cell line	MTT assay		LDH assay ^c	
	Before treatment	After treatment	Before treatment	After treatment
Caco-2	92 ± 20	59 ± 11	55.3 ± 3.1 (51.5 ± 4.6)	49.0 ± 3.3 (49.5 ± 4.0)
hCMEC/D3	106 ± 10	58 ± 21	61.1 ± 3.7 (66.6 ± 7.0)	60.3 ± 4.2 (67.4 ± 5.5)
L929	120 ± 19	91 ± 10	50.7 ± 2.5 (51.6 ± 3.0)	55.7 ± 3.2 (56.7 ± 3.3)

^a Values for culture media were 100% (RSD < 20%) and between 1.3 and 10% for Triton X-100 (total disruption of cells).

^b Values for Triton X-100 (total disruption of cells) were 100% (RSD < 8%).

^c Values in parentheses correspond to blank values obtained in culture media only (intact cells).