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Effect of solar photo-Fenton process in raceway pond reactors at neutral pH on antibiotic resistance determinants in secondary treated urban wastewater

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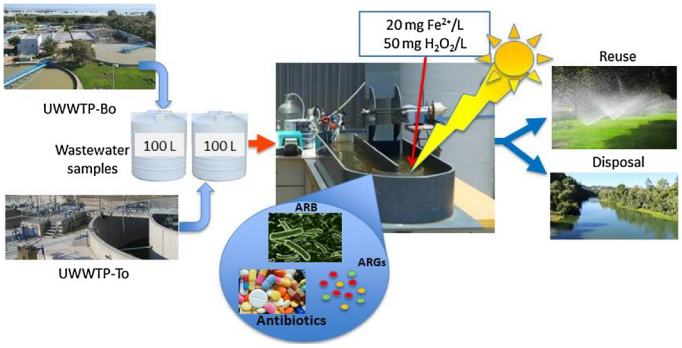
GRAPHICAL ABSTRACT



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ABSTRACT

Solar photo-Fenton process in raceway pond reactors was investigated at neutral pH as a sustainable tertiary treatment of real urban wastewater. In particular, the effect on antibiotic resistance determinants was evaluated. An effective inactivation of different wild bacterial populations was achieved considering total and cefotaxime resistant bacteria. The detection limit (1 CFU mL^{-1}) was achieved in the range 80–100 min (5.4–6.7 kJ L^{-1} of cumulative solar energy required) for Total Coliforms (TC) (40–60 min for resistant TC, 4.3–5.2 kJ L^{-1}), 60–80 min (4.5–5.4 kJ L^{-1}) for *Escherichia coli* (*E. coli*) (40 min for resistant *E. coli*, 4.1–4.7 kJ L^{-1}) and 40–60 min (3.9–4.5 kJ L^{-1}) for *Enterococcus* sp. (*Entero*) (30–40 min for resistant *Entero*, 3.2–3.8 kJ L^{-1}) with 20 mg L^{-1} Fe^{2+} and 50 mg L^{-1} H_2O_2. Under these mild oxidation conditions, 7 out of the 10 detected antibiotics were effectively removed (60–100%). As the removal of antibiotic resistance genes (ARGs) is of concern, no conclusive results were obtained, as sulfonamide resistance gene was reduced to some extent (relative abundance < 1), meanwhile class 1 integron *intI1* and ß-lactam resistance genes were not affected. Accordingly, more research and likely more intensive oxidative conditions are needed for an efficient ARGs removal.

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1. Introduction

Extensive and uncontrolled use of antibiotics for human, veterinary and agriculture purposes has contributed to the emergence of antibiotic resistance in the recent decades and urban wastewater treatment plants (UWWTPs) are among the main anthropogenic sources of antibiotic resistance spread into the environment [1,2]. Un-metabolized antibiotics as well as antibiotic resistant bacteria (ARB) and genes (ARGs), are released into the sewer and can reach UWWTPs [1,2]. Unfortunately, UWWTPs are not designed to remove these contaminants which finally are released into the environment. Moreover, the core of UWWTP, namely the biological process, is designed to promote the exponential growth of bacteria and consequently the biological tank (e.g., aeration tank in activated sludge process) is a potentially suitable environment for antibiotic resistance transfer. Antibiotic resistance transfer in bacterial populations can take place through vertical (i.e., by cell division) and different horizontal gene transfer mechanisms, including conjugation, transduction, and natural transformation [3]. In particular, during conjugation, exchange of genes occurs between active donor (possessing the genetic material to be transferred, in the form of plasmids or transposons) and recipient bacterial cells [4]. Accordingly, the higher the bacterial density the higher the chance that antibiotic resistance transfer through conjugation mechanism can take place. New disinfection methods, as tertiary treatments, are expected to play a crucial role in minimizing the release of antibiotic resistance determinants from UWWTPs into the environment, since consolidated disinfection processes, such chlorination and UV-C radiation, are not effective in controlling antibiotic resistance [2]. As matter of fact, these processes are not able to degrade contaminants of emerging concerns (CECs) (including antibiotics), as well as to inactivate ARB and to remove ARGs under the operating conditions (chlorine and UV-C doses, respectively) typically used in UWWTPs [5–7]. In the last years new advanced treatment methods, such as advanced oxidation processes (AOPs), have been investigated to evaluate the effect on antibiotic resistance. AOPs promote the formation of reactive oxygen species (ROS), such as hydroxyl radicals ($HO^{\bullet}$), that can effectively remove a wide spectrum of CECs, including antibiotics [8–11], as well as inactivate different microorganisms [12–14]. Among photo driven AOPs, photo-Fenton has been increasingly investigated in tertiary treatment of urban wastewater [15–17]. Although photo-Fenton process is typically effective under acidic pH conditions, new methods/approaches have been investigated [17,18] even under almost neutral pH in the removal of CECs [17,19,20]. As matter of fact, CECs occur at really low concentrations in urban wastewater (in the range of ng L^{-1} – a few μg L^{-1}), so even low doses of Fe, while resulting in a poor precipitation, can promote the formation of a sufficient amount of ROS to effectively remove CECs as well as to inactivate microorganisms [15,19]. Moreover, photo-Fenton process can also be operated with solar radiation, thus saving energy costs [21,22]. Water/wastewater treatment by solar photo-Fenton (SPF) has been recently investigated as possible barrier to antibiotic resistance spread into the environment, but only a few papers are available in scientific literature so far. In particular, the effect of SPF on ARB and ARGs was investigated for different bacterial strains (*Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*) using a solar simulator (150-W lamp, 750 W m^{-2} light intensity) [23]. SPF implemented in a compound parabolic collector (CPC) pilot plant was investigated under acidic conditions (pH adjusted to 2.8) in ARB and ARGs removal from the effluent of a Membrane BioReactor [16]. Unfortunately, CPC reactors technology is still quite expensive, being the unit cost estimated as high as 400 € m^{-2} [24]. To cut down solar reactors costs, an alternative (estimated cost 10 € m^{-2}) system, named Raceway Pond Reactor (RPR), has been recently investigated as advanced treatment of urban wastewater by SPF process [22,24,25]. They are channel shaped reactors where water can flow through. It is known that RPRs collect light less efficiently than CPC but they have a larger treated volume/surface ratio considering that it is thoroughly illuminated. In addition, its liquid depth can easily be varied which allow for increasing the treatment capacity [25]. Accordingly, it is of interest to investigate the effect of SPF process on antibiotic resistance in RPR under realistic conditions.

In the present work SPF process in a RPR was investigated in controlling antibiotic resistance in real secondary treated urban wastewater, under natural sunlight and neutral pH conditions. Process efficiency was evaluated in terms of ARB inactivation and ARGs removal, as well as in terms of antibiotics degradation in wastewater samples taken from two different UWWTPs.

2. Material and methods

2.1. Chemicals

Acetonitrile (AcN) and methanol (MeOH) HPLC grade and formic acid (purity, 98%) were supplied by Fluka (Buchs, Germany). Ultrapure water was produced by a Direct-Q Ultrapure Water System from Millipore (Bedford, MA, USA) with a specific resistance of 18.2 MΩ cm^{-1} and total organic carbon (TOC) of 2 mg L^{-1}. Hydrogen peroxide and sulphuric acid were supplied by J.T. Baker (Madrid, Spain) while ferrous sulphate heptahydrate ($FeSO_4{\cdot}7H_2O$) was obtained from Panreac (Barcelona, Spain). Bovine liver catalase, ortho phenanthroline, acetic acid, ammonium nitrate, ascorbic acid and titanium (IV) oxysulfate solution were acquired from Sigma Aldrich (Madrid, Spain).

Analytical standards (purity ≥ 97%) of the antibiotics azithromycin (AZT), ciprofloxacin (CIP), clarithromycin (CLR), clindamycin (CLN), doxycycline (DOX), enrofloxacin (ENR), erythromycin (ERY), levofloxacin (LEV), lincomycin (LIN), metronidazole (MET) and cefotaxime (CFX) were purchased from Sigma-Aldrich (Steinheim, Germany), and norfloxacin (NOR), tetracycline (TET) and trimethoprim (TMP) were purchased from SYMTA (Madrid, Spain). Antibiotic individual stock standard solutions were prepared at 1000 mg L^{-1} in MeOH or ultrapure water. A working standard solution containing all the antibiotics was obtained after mixing individual stock solutions and diluting with AcN. This solution was weekly renewed to avoid degradation of some antibiotic. All standard solutions were stored at −20 °C in amber glass vials.

2.2. Identification and bacterial count

Bacterial count was performed by standard plate counting method after an incubation period of 24 h for Total Coliform (TC) and *Escherichia coli* (*E. coli*) in a culture medium named Chromocult and 48 h for *Enterococcus* sp. (*Entero*) at 37 °C in Enterococcus medium [15], with cefotaxime (CFX) (resistant bacteria, CR) at a concentration of 4 mg L^{-1} and without CFX. CFX is a third generation cephalosporin antibiotic that is on the WHO essential list of medicines and it is a good indicator for human sources of antibiotic resistance. Residual hydrogen peroxide was removed from the samples by bovine liver catalase before microbiological measurements. The microbial detection limit (DL) in all assays was 1 CFU mL^{-1}. Controls were carried out to ensure the bacterial viability along treatment time. To this end, a wastewater aliquot was stored in the dark until the end of assay (180 min) to carry out bacterial count before and after treatment. In all cases, no decrease in CFU mL^{-1} was observed for the controls. Bacterial recovery was checked in the latest samples of each experiment incubating them at 37 °C for 2 day. After, these microbial samples were plated and maintained at the same temperature for 1 day or 2 days for counting TC and *E. coli* or *Entero*, respectively. No regrowth was noticed.

2.3. Wastewater samples

Wastewater samples were regularly collected from the settling tank of the secondary treatment (activated sludge) of two UWWTPs, “El Bobar” (UWWTP-Bo) and “El Toyo” (UWWTP-To), both located in the

Table 1
Average values of the main physical-chemical and microbiological parameters measured on three samples taken from UWWTP-Bo and UWWTP-To.

Parameter	UWWTP-Bo	UWWTP-To
pH	7.7 ± 0.4	7.3 ± 0.1
Conductivity (mS cm^{-1})	1.7 ± 0.25	1.9 ± 0.05
Turbidity (NTU)	7.9 ± 3.5	2.7 ± 0.2
Dissolved Organic Carbon (DOC, mgL^{-1})	19.5 ± 0.5	9.9 ± 1.5
Chemical Oxygen Demand (COD, mg L^{-1})	57.3 ± 5.9	32.1 ± 5.6
Bicarbonates (mg L^{-1})	215 ± 32	157 ± 25
Phosphate (mg L^{-1})	5.6 ± 4.4	7.6 ± 4.2
Chloride (mg L^{-1})	379.1 ± 22.4	471.71 ± 57.2
Sulphate (mg L^{-1})	293.6 ± 72.5	163.5 ± 68.9
Nitrate (mg L^{-1})	3.7 ± 0.5	5.4 ± 0.4
Nitrite (mg L^{-1})	6.7 ± 5.2	3.4 ± 0.7
Bromide (mg L^{-1})	2.9 ± 0.1	3.1 ± 0.3
TC (CFU mL^{-1})	$4.1{\cdot}10^3 \pm 2.7{\cdot}10^3$	$2.2{\cdot}10^3 \pm 1.4{\cdot}10^3$
CR-TC (CFU mL^{-1})	$4.4{\cdot}10^1 \pm 1.4{\cdot}10^1$	$5.2{\cdot}10^1 \pm 2.7{\cdot}10^1$
E. coli (CFU mL^{-1})	$1.9{\cdot}10^3 \pm 1.4{\cdot}10^3$	$4.3{\cdot}10^2 \pm 2.5{\cdot}10^2$
CR-*E. coli* (CFU mL^{-1})	$1.6{\cdot}10^1 \pm 3.6$	$1.7{\cdot}10^1 \pm 5.2$
Entero (CFU mL^{-1})	$1.2{\cdot}10^2 \pm 8.1{\cdot}10^1$	$4.7{\cdot}10^1 \pm 2{\cdot}10^1$
CR-*Entero* (CFU mL^{-1})	$1.3{\cdot}10^1 \pm 6.1$	$2{\cdot}10^1 \pm 1.5{\cdot}10^1$

city of Almeria (south of Spain) during three months (from February to April). The samples were collected manually in 50 L polythene containers, transported from the WWTPs directly to the laboratory and used in the experiments the same day they were collected. UWWTP-Bo was designed to treat urban wastewater for 315,000 population equivalent (11.6 $hm^3\,year^{-1}$). UWWTP-To was designed to treat urban wastewater for 52,000 population equivalent (4.75 $hm^3\,year^{-1}$). Physico-chemical and microbiological average values of all wastewater batches from both UWWTP are included in Table 1.

2.4. Solar photo Fenton tests

With the objective of obtaining replicated experiments, two photo Fenton runs were carried out simultaneously each day under solar irradiation, in two twin polyvinylchloride (PVC) RPRs (0.98 m in length and 0.37 m in width) with 5 cm liquid depth and working volume of 15 L during 180 min. This configuration allows keeping the volume of the treated water completely illuminated during the experiments. The RPRs are equipped with temperature (Crison 6050) and pH (Crison 5335) probes and a data acquisition card (LabJack U12) connected to a computer. Sunlight radiation was also measured by a global UV radiometer (Delta Ohm, LP UVA 02 AV) horizontally placed. All experiments were performed at neutral pH. As the RPRs were filled with secondary effluent and covered, 50 mg H_2O_2 L^{-1} was added to each reactor and mixed for 5 min before iron salt (20 mg Fe^{2+} L^{-1}) addition, according to previous studies [15,22]. Each couple of experiments were performed three times on different days, using three different wastewater batches (Day1, Day2 and Day3) to evaluate the effect of the water variability. All tests were started around noon to ensure almost constant values of solar radiation (21 ± 7.9 W m^{-2} with UWWTP-Bo and 26.5 ± 6.8 W m^{-2} with UWWTP-To) and temperature (17.4 ± 6.6 °C with UWWTP-Bo and 23.6 ± 2.6 °C with UWWTP-To) during a run from February to April 2018. ARGs presence was analyzed in five experimental runs, two from UWWTP-Bo (Day2 and Day3) and three from UWWTP-To (Day1, Day2 and Day3); experimental conditions are summarized in supplementary information (SI), Table SI1. In this study, the inactivation rate is calculated also as a function of both experimental time (t) and cumulative energy per unit of volume (Q_{UV}) received in the photo reactor, and calculated by Eq. (1):

$$Q_{UV}\,(kJ/L) = \sum_n \bar{UV}_{n-1}(W/m^2)x\frac{A_r(m^2)}{V_{total}(L)}x\frac{\Delta t_{n-1}(s)}{1000} \quad (1)$$

where $UV_{,n-1}$ is the UV energy accumulated per liter (kJ L^{-1}) at times n and n-1, A_r is the illuminated area of collector (m^2), V_{total} is the total volume of water treated (L), Δtn is the experimental time of sample. 1000 is a conversion factor rom J to kJ. Q_{UV} is commonly used to compare results under different conditions [26].

2.5. Kinetic analysis

The bacteria removal was described by pseudo-first-order kinetics, according to Eqs. (2) and (3).

$$-\,ln\frac{C_{viable,t}}{C_{viable,0}} = k\bullet t \quad (2)$$

$$\frac{C_{viable,t}}{C_{viable,0}} = exp^{-kt} \quad (3)$$

where $C_{viable,t}$ and $C_{viable,0}$ are concentrations of viable bacteria (CFU mg L^{-1}) at time t and t_0, respectively; k is the pseudo-first-order rate constant of bacteria removal (min^{-1}) and t is treatment time (min). The k was determined by Statistica software, version 13.0 (TIBCO Software Inc., USA) using non-linear estimation (Least Squares Estimation).

The half-life time of bacteria inactivation ($t_{1/2}$, min, i.e., the time at which 50% bacteria inactivation was observed) was determined according to the Eq. (4).

$$t_{1/2} = \frac{ln2}{k} \quad (4)$$

2.6. DNA extraction

DNA was extracted from the filtered and concentrated samples using a commercial DNA extraction kit (Norgen Biotek), following the manufacturer's manual. The DNA concentration was quantified by Qubit® 2.0 fluorometer (Invitrogen) using the high sensitivity assay kit for double-stranded DNA. In all cases, total DNA concentrations of 1–10 ng μL^{-1} were obtained. DNA extracts were stored at 4 °C.

2.7. Detection of ARGs using real-time PCR

Two ß-lactam resistance genes (bla_{TEM} *and* $bla_{CTX\text{-}M}$), a quinolone resistance gene (*qnr*) and a sulfonamide resistance gene (*sul1*), were semi-quantitatively evaluated by real time PCR (RT-PCR) by a MyGo Pro® RT-PCR system. The wastewater abundant *intI1* gene, commonly used as a marker for anthropogenic contamination, was also evaluated. Bla_{TEM}, $bla_{CTX\text{-}M}$ and *qnr* genes were evaluated using TaqMan® MGB assays (Applied Biosystems, USA). TaqMan MGB probes incorporate, at one end of the probe, a non-fluorophore quencher, NFQ, which turns off the fluorophore signal at the other end of the probe. The features of NFQ and the short length of the MGB probe result in a very low background signal that improves the sensitivity and accuracy of the PCR. On the other hand, the follow-up reaction for the evaluation of the *sul1* and *intI1* genes was performed by SYBR-Green fluorophore. In each case, one or another technology was used, taking into account the best performance of each one for each specific gene. The specificity of each assay was checked by sequencing the amplicons obtained in a n ABI PRISM® 3100 Genetic Analyzer system. The specific PCR primers shown in Table SI2 were used for the evaluation of these genes. *16S rRNA* bacterial gene was used as housekeeping gene for data normalization. The TaqMan® MGB reactions were performed in a 20 µL reaction mix comprising 1.5 ng of DNA, 1 µL of the TaqMan® Gene Expression Assay, and 10 µL of the SensiFAST Probe No-ROX Kit (Bioline). The SYBR-Green reactions were performed in a 20 µL reaction mix comprising 1.5 ng of DNA, 2 µL of each of the primers (2 µM), and 10 µL of the SensiFAST SYBR No-ROX Kit (Bioline). In both reactions, amplifications were carried out under the following conditions: an initial hold step of 95 °C for 10 min and 45 PCR cycles of 95 °C for 15 s and 60 °C for 1 min. PCR experiments with samples collected before and after SPF

Table 2
Bacterial inactivation by SPF: kinetic parameters and Q_{UV} values corresponding to $t_{1/2}$ (Q_{UV},$t_{1/2}$) and to the time of total inactivation ($Q_{UV,\ tend}$).

	UWWTP-Bo					UWWTP-To				
	R^2	k min^{-1}	$t_{1/2}$ min	$Q_{UV,\ t1/2}$ (kJ L^{-1})	$Q_{UV,\ tend}$ (kJ L^{-1})	R^2	k min^{-1}	$t_{1/2}$ min	$Q_{UV,\ t1/2}$ (kJ L^{-1})	$Q_{UV,\ tend}$ (kJ L^{-1})
TC	0.962 ± 0.021	0.062 ± 0.027	13.24 ± 5.81	2.5 ± 0.2	6.7 ± 1.2	0.978 ± 0.012	0.064 ± 0.009	10.94 ± 1.55	1.9 ± 0.3	5.4 ± 1.2
E. coli	0.971 ± 0.018	0.075 ± 0.029	10.62 ± 4.53	1.9 ± 0.1	5.4 ± 0.6	0.949 ± 0.016	0.098 ± 0.033	7.79 ± 2.59	1.6 ± 0.1	4.5 ± 1.2
Entero	0.974 ± 0.020	0.085 ± 0.081	16.77 ± 12.21	2.2 ± 0.1	4.5 ± 1.0	0.965 ± 0.022	0.037 ± 0.007	19.44 ± .3.70	1.8 ± 0.2	3.9 ± 0.2
CR-TC	0.958 ± 0.016	0.049 ± 0.020	16.25 ± 6.63	1.2 ± 0.1	5.2 ± 0.5	0.945 ± 0.055	0.084 ± 0.047	10.46 ± 4.87	0.8 ± 0.1	4.3 ± 0.4
CR-*E. coli*	0.885 ± 0.094	0.073 ± 0.044	13.37 ± 8.71	0.9 ± 0.2	4.7 ± 0.8	0.793 ± 0.176	0.062 ± 0.036	14.66 ± 8.00	0.5 ± 0.3	4.1 ± 0.1
CR-*Entero*	0.810 ± 0.109	0.047 ± 0.028	21.60 ± 16.95	1.0 ± 0.1	3.2 ± 1.1	0.829 ± 0.131	0.060 ± 0.028	14.24 ± 7.43	0.5 ± 0.0	3.8 ± 0.5

treatment, were done, each in triplicate, to analyse its effectiveness against the ARG. All *Ct* (cycle threshold) values considered positive range from 18-35. Double delta *Ct* analysis was used for data analysis [27] and the measurement of each gene was normalized with respect to the *16S* gene. Finally, data are plotted in terms of relative abundance (increase/decrease of the target normalized ARG after SPF treatment).

2.8. Analytical determinations

Dissolved iron and hydrogen peroxide were measured using two standard methods, ISO 6332:1988 (LOD 0.1 mg L^{-1}) and DIN 38 402 H15 (LOD 0.3 mg L^{-1}), respectively. Chemical Oxygen Demand (COD) was measured spectrophotometrically with a commercial kit (Handle). Anion determinations were carried out in an ion chromatography (Metrohm 881 Compact IC pro) and dissolved organic carbon (DOC) was analyzed using a TOC analyzer (Shimadzu-V_{CPH}). The samples were filtrated through 0.2 µm syringe-driven filters (Millex®, Millipore) before performing any measurement. Turbidity and conductivity were measured by a turbidity meter (Hanna) and conductivity meter (Phywe), respectively.

2.9. Antibiotic analysis

Antibiotics detection and quantification in wastewater (WW) samples was performed by liquid chromatography, using an Agilent 1200 LC system (Agilent Technologies, Foster city, CA, USA), coupled to mass spectrometry (LC–MS) using a hybrid quadrupole linear ion trap (QqLIT) mass analyser 5500 QTRAP (AB Sciex Instruments, Wilmington, DE, USA). WW samples were collected from the reactor using amber glass bottles pre-rinsed with ultra-pure water, filtrated by 0.45 µm glass microfiber filters (Whatman; Little Chalfont, Buckinghamshire, UK) and analysed by direct injection in the LC–MS system. Prior to analysis, a second filtration was performed by 0.2 µm PTFE syringe filters (AISIMO CORPORATION, London, UK) and AcN containing C13-caffeine as injection control was added to obtain a final proportion of AcN:H2O of 10:90 v/v. Chromatographic separation was achieved using an ACE Excel C18-PFP analytical column (150 × 2.1 mm; 1.7 µm particle size) (Advanced Chromatography Technologies, Scotland). Mobile phases A and B were 0.1% formic acid in ultrapure water and AcN, respectively, at a flow rate of 0.2 mL min^{-1}. Initially, 10% B was maintained for 1 min; then the percentage was increased linearly to 40% between 1 and 9 min and held until 12 min. After that, %B was increased to 100% between 12 and 16 min and kept constant until 25 min. Finally, the mixture was returned to initial conditions within 0.1 min. The column was then re-equilibrated for 7 min before the next injection. Total run time was 32 min and injection volume was 40 µL.

The LC system was coupled to the MS by a TurboIon Spray source operated in positive mode. Operation parameters were: IonSpray Voltage (IS), 5000 V; Source Temperature, 500 °C; CAD Gas, Medium; Ion Source Gas 1, 50 psi; Ion Source Gas 2, 40 psi and Curtain Gas, 25 (arbitrary units). Source dependent parameters were optimized by direct infusion into the MS of individual standard solutions in MeOH (10 µg L^{-1}) using the full scan mode. Data was acquired using Analyst Software 1.5.1 and processed with MultiQuant 3.0.1 software (Applied Biosystems).

2.10. Statistic analysis

Statistically significant differences between (i) total and antibiotic resistant bacteria, (ii) bacterial species and (iii) UWWTP-To and UWWTP-Bo were investigated. Statistical analyses were performed by Statistica software, version 13.0 (TIBCO Software Inc., USA). Normality of the data was verified with Shapiro-Wilk test. Due to normal distribution of $t_{1/2}$-values (SI Table SA), parametrical *t*-test (homogeneous variances) and Cochran–Cox test (non-homogeneous variances) were used. The variance homogeneity was assessed with Levene's test. The statistical significance was assumed at level $\alpha = 0.05$.

3. Results and discussion

3.1. Bacterial inactivation by solar photo-Fenton in RPRs

The main target of tertiary treatments for wastewater reuse is water disinfection; that is the inactivation of pathogenic bacteria, mainly. In this study, inactivation of total and CR TC, *E. coli* and *Entero* by solar photo-Fenton was investigated under the best operation conditions (20 mg Fe^{2+} L^{-1} and 50 mg H_2O_2 L^{-1}) established in a previous work [22].

The plots of all inactivation kinetics are available in SI file (Figs. SI1–SI12). Kinetics parameters (k and $t_{1/2}$) and R^2 values are summarized in Table 2. Figs. 1 and 2 show the inactivation of total and antibiotic resistant (CR-) TC, *E. coli* and *Entero* by SPF in RPRs, for wastewater samples taken from UWWTP-Bo and UWWTP-To, respectively. Sunlight and Sunlight/H_2O_2 tests did result in lower inactivation rates (UVA: k = 0.022 ± 0.0015 min^{-1}, R^2 = 0.952 ± 0.016 for TC; k = 0.033 ± 0.004 min^{-1}, R^2 = 0.937 ± 0.004 for *E. coli*; k = 0.001 ± 0.001 min^{-1}, R^2 = 0.893 ± 0.017 for *Entero*. UVA/H_2O_2: k = 0.042 ± 0.001 min^{-1}, R^2 = 0.895 ± 0.002 for TC; k = 0.060 ± 0.002 min^{-1}, R^2 = 0.843 ± 0.011 for *E. coli*; k = 0.002 ± 0.001 min^{-1}, R^2 = 0.902 ± 0.045 for *Entero*) compared to solar photo-Fenton after 120 min. The values of Quv were 0.8 ± 0.5 kJ L^{-1} and 0.85 ± 0.2 kJ L^{-1} for sunlight and sunlight/H_2O_2 assays, respectively.

As expected, due to the higher initial concentration, the inactivation of TC took longer time (between 80 and 100 min) compared to the other tested bacterial species (Figs. 1 and 2). These treatment times are in agreement with those published in a previous work [22], pointing out the repetitiveness and robustness of the treatment. It is noteworthy that $t_{1/2}$ values were shorter for *E. coli* compared to TC and *Entero*, which is consistent with the corresponding values of Q_{UV} at $t_{1/2}$ ($Q_{UV,t1/2}$) (Table 2). The percentage of CR-*E. coli* and CR-*Entero* increased during the first 20–30 min of treatment in both UWWTPs (Figs. 1B, C, 2 B and C), although antibiotic resistant population was inactivated in a shorter

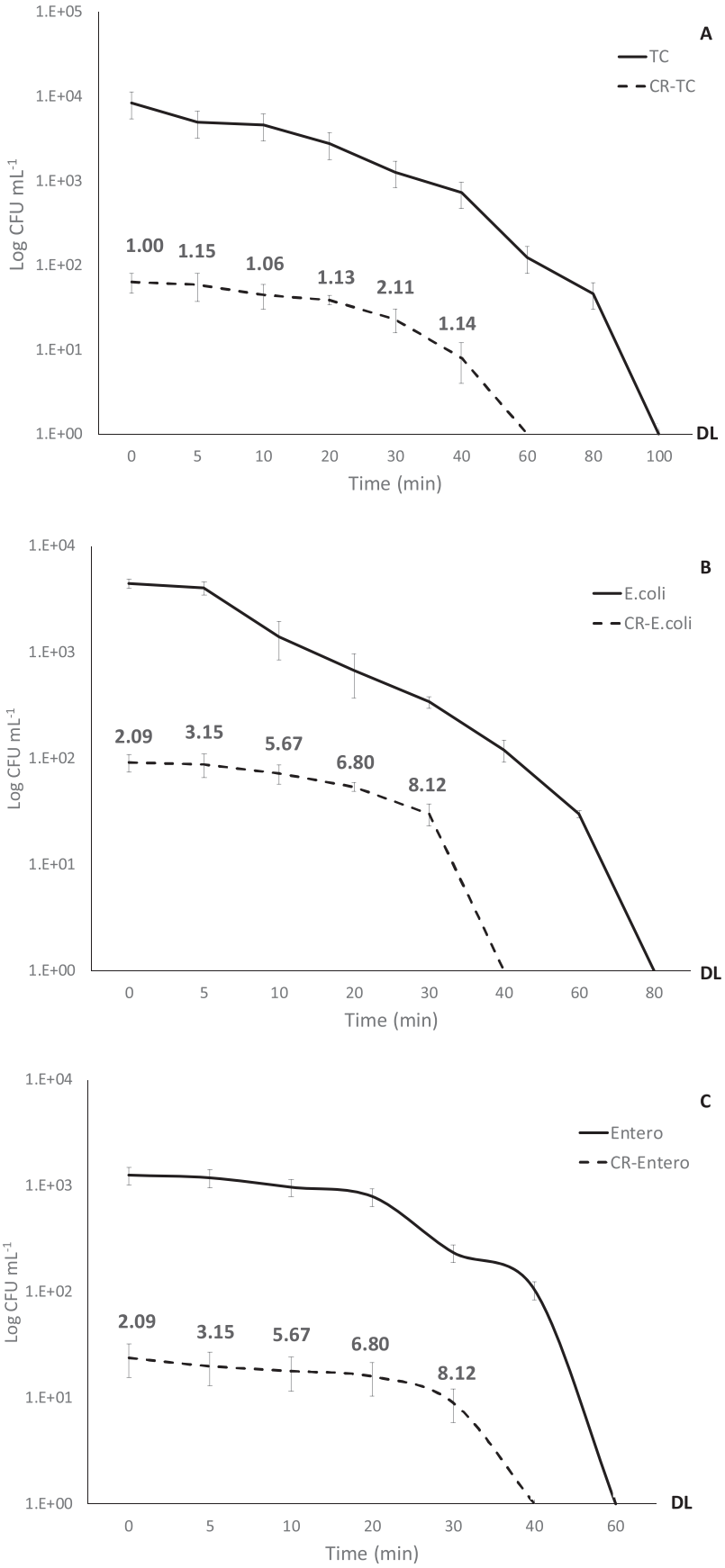

Fig. 1. Inactivation by SPF (20 mg Fe^{2+} L^{-1} and 50 mg H_2O_2 L^{-1}) of total and CFX resistant (CR-) TC (A), *E. coli* (B) and *Entero* (C), in UWWTP-Bo wastewater samples. The percentages values of the resistant bacteria with respect to the total are indicated close to the corresponding experimental data.

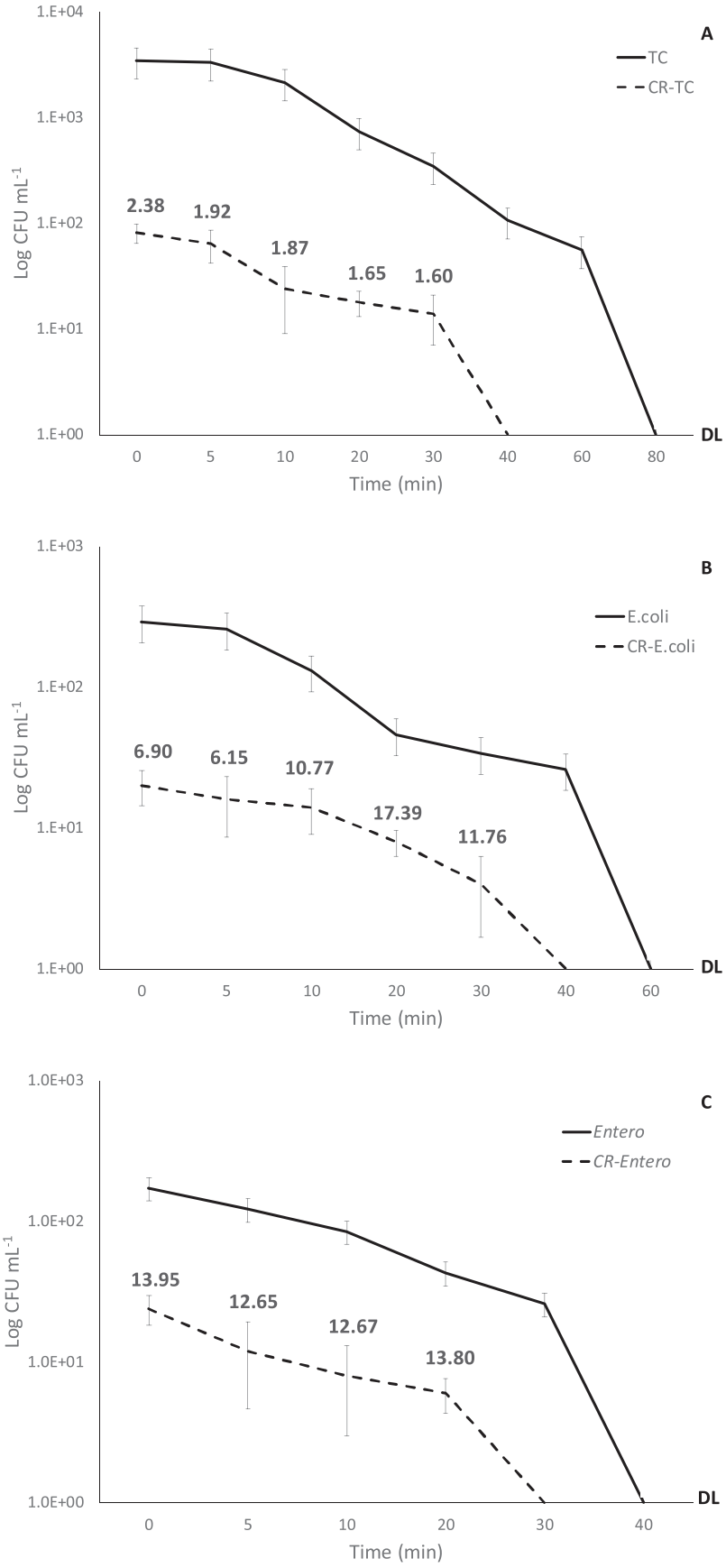

Fig. 2. Inactivation by SPF (20 mg Fe^{2+} L^{-1} and 50 mg H_2O_2 L^{-1}) of total and CFX resistant (CR-) TC (A), *E. coli* (B) and *Entero* (C), in UWWTP-To wastewater samples. The percentages values of the resistant bacteria with respect to the total are indicated close to the corresponding experimental data.

Table 3
Antibiotic concentrations before and after three hours of SPF treatment in the RPR for both WWs (El Bobar and El Toyo) and removal rate obtained.

Antibiotic	El Toyo WW			El Bobar WW		
	Ci (ng/L)	Cf (ng/L)	Removal (%)	Ci (ng/L)	Cf (ng/L)	Removal (%)
Azithromycin	25	19	**24**	nd	–	–
Ciprofloxacin	460	nd	**100**	1550	nd	**100**
Clarithromycin	22	17	**24**	50	46	**8**
Erithromycin	14	9	**36**	177	138	**22**
Lincomycin	nd	–	–	126	20	**84**
Levofloxacin	706	278	**61**	1540	388	**75**
Enrofloxacin	23	nd	**100**	nd	–	–
Doxycycline	25	nd	**100**	nd	–	–
Clindamycin	119	52	**57**	60	8	**86**
Metronidazole	nd	–	–	128	38	**70**
TOTAL:	**1393**	**374**	**73%**	**3631**	**638**	**82%**

C_i: initial concentration; C_f: final concentration; nd: not detected.

time than the total population. According to the results of *t*-test/Cochran-Cox test (SI, Tables SI3–SI6), no statistically significant difference in terms of $t_{1/2}$ (n = 6) were observed: (i) for bacteria inactivation between UWWTP-Bo and UWWTP-To (SI Table SB); (ii) between TC and CR-TC (in UWWTP-To: t = 0.230, p = 0.826 and in UWWTP-Bo: t = −0.838, p = 0.422) and between *E. coli* and CR-*E. coli* (in UWWTP-To: t = −2.001, p = 0.092, and in UWWTP-Bo: t = −0.686, p = 0.508); and (iii) between CR-TC and CR-*E. coli* in both UWWTP-To (t = −1.099, p = 0.297) and UWWTP-Bo (t = 0.645, p = 0.533). Unfortunately, due to the lower initial bacterial density, statistical comparisons for *Entero* population were not possible. Therefore, as antibiotic resistant bacteria are inactivated with the same kinetics than non-resistant bacteria, but at shorter times (due to the lower initial concentration), the removal of total bacteria of a given species is enough to accept that CR bacteria were removed up to the detection limit.

The bacterial inactivation results observed in these experiments are in agreement with a previous work where the inactivation of wild bacteria by SPF was investigated using the same reactor (RPR) and operating conditions [22]. As a matter of fact, inactivation below 1 CFU mL^{-1} of TC, *E. coli* and *Entero* as well as their resistant counterpart, took place within treatment times consistent with those observed in our work (80–100 min). It is worthy to note that, although water turbidity increased because of iron precipitation (dissolved iron was not detected in collected samples during all assays), a sufficient amount of ROS was produced to effectively inactivate target bacteria (on average an increase of 4.5 NTU was detected as iron was added). This increase in turbidity is due to the fact that once Fe^{2+} is added to the reactor, it almost instantaneously reacts with hydrogen peroxide giving $HO^{\cdot}$ and Fe^{3+}. Regarding the H_2O_2 concentration, an initial drop (12 mg L^{-1}) could be observed in both cases when the ferrous iron was added due to the Fenton reaction. After, a progressive decrease of H_2O_2 was detected during each assay due to the activity of the ferric hydroxides formed at neutral pH in the heterogeneous photo-Fenton process [28]. The total consumption of hydrogen peroxide in both wastewaters was around 60%. Fiorentino et al. [29] investigated the inactivation of CR-*E. coli* in a CPC system operated as SPF process. The Q_{UV} required to achieve the inactivation below DL of CR-*E. coli* was higher (16–24 kJ L^{-1}) compared to that one observed in the present study (4.1 and 4.7 kJ L^{-1} for UWWTP-Bo and UWWTP-To, respectively), but in the quoted work the resistant species were inoculated into the wastewater at higher concentrations (10^6 CFU mL^{-1}). In addition, in RPRs there is no dark volume avoiding any mechanism of bacterial cellular reparation during the solar photocatalytic treatment [30].

Depending on the purpose of treated water and of each country, its microbiological quality must meet specific requeriments. *E. coli*, as faecal indicator, is the most widely considered to reuse treated wastewater for irrigation in agriculture although its limit concentration ranges from 10 CFU mL^{-1} in the World Health Organization guideline [31], 10 CFU 100 m L^{-1} in the Italian Legislation (Italian Technical Guidelines for Wastewater Reuse, 2003) or up to 1 CFU mL^{-1} in the Spanish reuse law (Royal decree 1620 /2007). In all cases disinfection by SPF process satisfied these legislations. Additionally, the residual H_2O_2 concentrations are not toxic for cultivated plants since the farmers use this reactant in concentrations around 50 mg L^{-1} for maintaining the crops disinfected [32].

3.2. Effect of solar photo-Fenton on antibiotics removal

The occurrence of antibiotics in the effluent of UWWTPs is of concern because, although they are released into the environment at low concentrations (from ng L^{-1} to a few μg L^{-1}), the selection of ARB and ARGs can take place at extremely low antibiotic concentrations [33,34]. In the present study, a set of 14 antibiotics of different therapeutic classes (Section 2.1) were investigated. They were selected based on their reported occurrence in WW and relevance. As matter of fact, ERY, CLR and AZT were included in the first EU Watch list (Decision 2015/495/EU), while CIP and ENR have been recommended by COST Action ES 1403 - NEREUS, due to their potential for crop uptake [35]. 10 out of the 14 antibiotics investigated could be detected in any of the samples, being CIP and LEV the most abundant and recurrent (Table 3). The concentration of antibiotics was evaluated before and after three hours of SPF treatment in the RPR for both WWs (El Bobar and El Toyo). Table 3 shows the percentages of removal for each antibiotic in El Bobar and El Toyo wastewater samples.

Removal rates ≥70% were observed for 5 out of 7 antibiotics detected in El Bobar WW (Table 3). CIP and LEV, present at the highest initial concentrations (> 1000 ng L^{-1}), reached removal percentages of 100% and 75%, respectively. On the opposite, CLR and ERY were degraded at low rates (8 and 22%, respectively).

Up to 8 antibiotics were detected in El Toyo WW (Table 3). Five of them, CIP, CLR, ERY, LEV and CLN, had been also identified in El Bobar WW but at considerably lower initial concentrations, from 2 (CLR), to 13 (ERY) times lower, in most cases. Only CLN was present at a double concentration compared to El Bobar (119 and 60 ng L^{-1}, respectively). Total removal was observed for CIP, ENR and DOX, while the remaining 5 antibiotics were removed at low rates (from 24% to 61%). Overall, the total charge of antibiotics detected in El Toyo WW (1393 ng L^{-1}) was lower than in El Bobar (3631 ng L^{-1}). However, similar total removal of antibiotics were obtained (73% in El Toyo WW and 82% in El Bobar WW). The pH is a relevant parameter to explain the results, since photo-Fenton at neutral pH is less efficient for antibiotic removal than photo-Fenton at acidic pH. Accordingly, [36] observed 99.9% removal of ERY by SPF process in the same RPR but operated in continuous flow mode and at pH 2.8. The same was reported by Karaolia et al. [16], who investigated the removal of antibiotics by SPF in a compound parabolic collector-based reactor at an initial acidic pH.

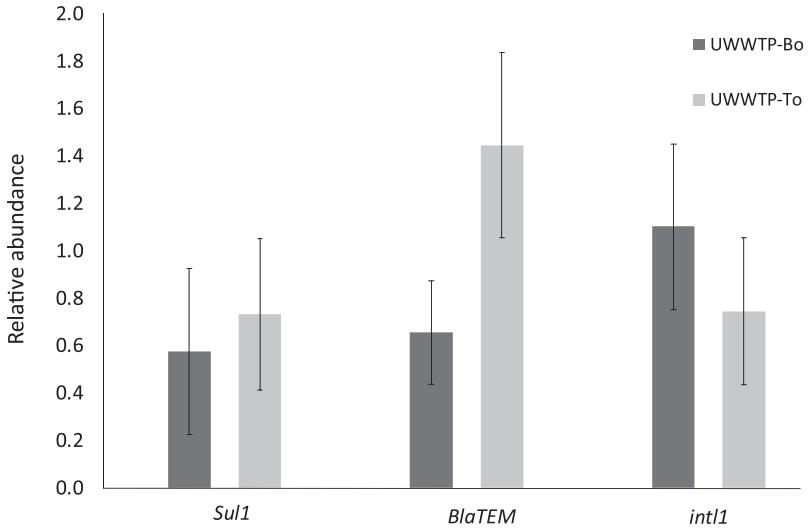

Fig. 3. Relative abundance of *intI1*, bla_{TEM} and sul1 genes after SPF treatment (20 mg Fe^{2+} L^{-1} and 50 mg H_2O_2 L^{-1}) in wastewater samples taken from UWWTP-Bo and UWWTP-To.

3.3. Effect of solar photo-Fenton on ARGs

Total bacterial genomic DNA was extracted from secondary effluent of both UWWTPs (El Bobar and El Toyo) before and after treatment by SPF process and subsequently analyzed using semiquantitative Real-Time PCR. Five clinically relevant genes related to the resistance of microorganisms to antibiotics were measured: *sul1, qnr,* bla_{TEM}, $bla_{CTX\text{-}M}$, and *intI1* [37,38]. The measurement of each gene was normalized with respect to the *16S* gene and the resulting values were plotted in terms of relative abundance (Fig. 3).

The *intI1* gene was detected in all wastewater samples collected. This result is consistent with the scientific literature, where *intI1* is typically detected in wastewater and commonly used as a marker for anthropogenic contamination [38,39]. Moreover, *intI1* gene is linked to genes that confer antibiotic resistance and it is found in pathogenic bacteria, humans and domestic animals [40]. bla_{TEM} and *sul1* genes were also detected in all the untreated wastewater samples analyzed in this work. This is consistent with the literature, being these genes among the most prevalent ARGs in UWWTPs [41]. Unlike of the three above quoted genes, $bla_{CTX\text{-}M}$ and *qnr* were only detected in a few raw wastewater samples (data not shown). Quinolone resistant genes, such as *qnr*, are scanty in urban wastewater being its removal easier [42]. Unlike of that observed for bacteria inactivation and antibiotic removal, the effect of SPF process on *intI1*, bla_{TEM} and *sul1* genes is not so evident and differences before and after treatment (measured as relative abundance) depended on the target ARG and type of wastewater (Fig. 3). While SPF process reduced to some extent *sul1* gene (relative abundance < 1), it did not particularly affect *intI1* and bla_{TEM} genes, being some increases also observed (relative abundance > 1). The effect of AOPs on ARGs has been scarcely investigated so far. In particular, to author's knowledge, only one paper is available in scientific literature where the effect of SPF process on ARGs was investigated [16]. In this work, the effluent of a membrane biological reactor was treated by SPF ($[Fe_2{}^+]_0 = 5\ mg\ L^{-1}$, $[H_2O_2]_0 = 50\ mg\ L^{-1}$, pH 2.8) at pilot scale in a CPC based reactor. Although total DNA concentration was reduced by 97%, ARGs as *sul1* and *ermB* were still present in the remaining total DNA determined after SPF treatment [16]. These results are consistent with a previous work where the effect of UV/H_2O_2 (wide spectrum UV lamp with main emission in the range 320–450 nm) on ARGs (namely, bla_{TEM}, *qnrS* and *tetW*) was investigated under realistic conditions for wastewater treatment (natural pH (7.6) and 20 H_2O_2 mg L^{-1}) [43]. In spite of the bacterial inactivation and a decrease of ARGs in intracellular DNA after 60 min treatment, the authors did not observe any ARGs removal from water suspension. Differently, Zhang et al. [44] showed that UV-C/H_2O_2 can effectively remove ARGs (2.8–3.5 logs removal of *sul*1, *tet*X, and *tet*G, within 30 min treatment) but only under not feasible conditions in real UWWTPs (pH 3.5 and 340 mg H_2O_2 L^{-1}). The differences among the works available in the scientific literature can be explained by (i) the different AOP investigated, (ii) the different experimental set-up and operating conditions, (iii) the different target ARGs and (iv) ARGs measurement methods and instruments.

4. Conclusions

The SPF process in RPR at neutral pH was effective for bacterial inactivation and antibiotics removal from real secondary effluents of two different UWWTPs. The DL in the inactivation of total and resistant bacterial populations (TC, *E. coli* and *Entero*) was reached in all experiments, within the range 30–100 min (3.2–6.7 kJ L^{-1}), depending on the target bacterial family and wastewater sample. Additionally, 7 out of 10 antibiotics detected in the investigated wastewater samples were effectively removed (60–100%). However, SPF process in the tested conditions was poorly effective in the removal of the ARGs, because only *sul1* gene was reduced to some extent (relative abundance < 1). According to these results, it is not possible to state that the process can effectively minimize the risk of antibiotic resistance transfer into the environment and additional research on more intensive oxidative conditions is needed.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2019.06.014.

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