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Continuous ozonation of urban wastewater: Removal of antibiotics, antibiotic-resistant *Escherichia coli* and antibiotic resistance genes and phytotoxicity

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ABSTRACT

This work evaluated the removal of a mixture of eight antibiotics (i.e. ampicillin (AMP), azithromycin (AZM), erythromycin (ERY), clarithromycin (CLA), ofloxacin (OFL), sulfamethoxazole (SMX), trimethoprim (TMP) and tetracycline (TC)) from urban wastewater, by ozonation operated in continuous mode at different hydraulic retention times (HRTs) (i.e. 10, 20, 40 and 60 min) and specific ozone doses (i.e. 0.125, 0.25, 0.50 and 0.75 gO_3 $gDOC^{-1}$). As expected, the efficiency of ozonation was highly ozone dose- and contact time-dependent. The removal of the parent compounds of the selected antibiotics to levels below their detection limits was achieved with HRT of 40 min and specific ozone dose of 0.125 gO_3 $gDOC^{-1}$. The effect of ozonation was also investigated at a microbiological and genomic level, by studying the efficiency of the process with respect to the inactivation of *Escherichia coli* and antibiotic-resistant *E. coli*, as well as to the reduction of the abundance of selected antibiotic resistance genes (ARGs). The inactivation of total cultivable *E. coli* was achieved under the experimental conditions of HRT 40 min and 0.25 gO_3 $gDOC^{-1}$, at which all antibiotic compounds were already degraded. The regrowth examinations revealed that higher ozone concentrations were required for the permanent inactivation of *E. coli* below the Limit of Quantification (<LOQ = 0.01 CFU mL^{-1}). Also, the abundance of the examined ARGs (*intI1*, *aadA1*, *dfrA1*, *qacEΔ1* and *sul1*) was found to decrease with increasing HRT and ozone dose. Despite the fact that the mildest operating parameters were able to eliminate the parent compounds of the tested antibiotics in wastewater effluents, it was clearly demonstrated in this study that higher ozone doses were required in order to confer permanent damage and/or death and prevent potential post-treatment re-growth of both total bacteria and ARB, and to reduce the abundance of ARGs below the LOQ. Interestingly, the mineralization of wastewater, in terms of Dissolved Organic Carbon (DOC) removal, was found to be significantly low even when the higher ozone doses were applied, leading to an increased phytotoxicity towards various plant species. The findings of this study clearly underline the importance of properly optimising the ozonation process (e.g. specific ozone dose and contact time) taking into consideration both the bacterial species and associated ARGs, as well as the wastewater physicochemical properties (e.g. DOC), in order to mitigate the spread of ARB&ARGs, as well as to reduce the potential phytotoxicity.

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1. Introduction

Nowadays, the ecosystems and the human health are threatened by the environmental occurrence of both chemical and biological contaminants of emerging concern (CECs), such as antibiotics and acquired antibiotic resistance genes (ARGs), mobilized by antibiotic-resistant bacteria (ARB). ARB&ARGs are considered as a serious public health problem by various international organizations and the European Union (EU), because of their widespread in the environment, food chain, and even drinking water, among others (Rizzo et al., 2013; Berendonk et al., 2015). Recently, the environmental dimension of antimicrobial resistance has been identified as one of the six emerging issues of environmental concern according to the United Nations Environment (UNEP, 2017; APHA, 2005). In fact, ARB can often do work synergistically by interacting with each other in a variety of mechanisms that enhance remarkably their collective capability to transfer ARGs leading to antibiotic resistance, described two decades ago by Salyers and Amabile-Cuevas (1997) as an '*easy-to-get, hard-to-lose*' phenomenon. In the face of regional and global antibiotic resistance challenges, the European Commission stands at the forefront for intensifying its efforts to shape a relevant legislative instrument with regard to the minimum quality criteria for the assessment of reclaimed wastewater intended for agricultural irrigation and aquifer recharge, including antibiotic resistance and related risks (EU, 2017a).

In the last decade, urban wastewater treatment plants (UWTPs) have been considered as critical hotspots for the propagation of antibiotic resistance in the environment, with the consequences being potentially compounded by the reuse of treated wastewater. The fact that UWTPs are not specifically designed for the elimination of antibiotic compounds (Michael et al., 2013), accompanied by an increase in the prevalence of ARB and their associated ARGs, has triggered an imperative need to improve the efficiency of the existing treatment processes (Pruden, 2013; Karkman et al., 2017; Michael-Kordatou et al., 2018; Manaia et al., 2018). In this sense, various advanced chemical oxidation processes (AOPs) have been extensively investigated to efficiently remove antibiotic residues (Michael et al., 2013), inactivate ARB and remove ARGs from wastewater effluents at bench-, pilot-, and full-scale applications (Zhuang et al., 2015; Alexander et al., 2016; Czekalski et al., 2016; Ferro et al., 2016; Lee and Von Gunten, 2016; Sousa et al., 2017; Karaolia et al., 2018). In a recent review, Michael-Kordatou et al. (2018) investigated the fate of ARB&ARGs during AOPs, the key operating conditions affecting their efficiency to inactivate ARB and remove ARGs, and the main oxidative damage pathways involved in these processes, indicating that besides the operating conditions, the variable behavior observed by the various examined genetic constituents of the microbial community, may be directed by the process distinct oxidative damage mechanisms in place during the application of each treatment technology.

A great deal of interest has been focused on the application of ozone-based systems, which have been demonstrated to be effective in achieving significant abatement of various micro-contaminants, while also providing sufficient disinfection of wastewater at different ozone doses and contact times (Von Gunten, 2003, 2018; Ikehata et al., 2006). In fact, the knowledge on the efficiency of ozonation to eliminate ARB&ARGs is limited since most studies performed so far only focused on the effect of ozone on cultivable bacterial populations, with the effect of the process on ARB or ARGs being still unclear (Michael-Kordatou et al., 2018). Ozone is a powerful oxidant and has been widely used during the last years for the treatment of wastewater. It reacts selectively with organic compounds via a direct reaction with ozone molecules, or through an indirect pathway with hydroxyl radicals ($HO^{\bullet}$) under alkaline pH conditions, which result from ozone decay in water and wastewater. Ozonation has been shown to have a high potential for the oxidation of a variety of pharmaceutical compounds, including antibiotics, and the inactivation of total pathogenic bacteria in drinking water and wastewater (Antoniou et al., 2013; Von Gunten, 2018). The few studies published so far suggest that ozonation is effective to inactivate ARB and remove ARGs and also to eliminate the potential bacterial regrowth, under proper optimized conditions, specific ozone dose and exposure time (Michael-Kordatou et al., 2018). However, further studies are necessary to fill in the gaps of knowledge in relation to the effectiveness of ozonation to the inactivation of ARB and the reduction of the abundance of ARGs.

Within this context, the aim of the present work was to investigate and optimize the efficiency of ozonation operated in continuous mode, for the abatement of a diverse array of antibiotic-related chemical and microbiological microcontaminants from secondary treated wastewater effluents. The objectives of this study were to assess: (i) the removal of a mixture of eight antibiotics belonging to different classes and exhibiting different physicochemical properties, being the macrolides included in the EU watch list of substances for Union-wide monitoring (EU, 2017b, EU, 2018), namely azithromycin (AZM), erythromycin (ERY), clarithromycin (CLA), as well as ampicillin (AMP), ofloxacin (OFL), sulfamethoxazole (SMX), trimethoprim (TMP), and tetracycline (TC); (ii) the inactivation of wastewater autochthonous total and antibiotic-resistant *Escherichia coli* and to further evaluate their regrowth potential after treatment; (iii) the reduction of the abundance of selected genes originally present in wastewater, specifically the bacterial biomarker 16S rRNA, the class 1 integron integrase *intI1* and ARGs (i.e., *qacΔE1*, *sul1*, *aadA1* and *dfrA1*); (iv) the extent of mineralization of the treated wastewater; and (v) the evolution of phytotoxicity against selected plant species. According to the authors' knowledge this work is among the first studies revealing comprehensive data regarding the oxidation of a mixture of antibiotics (rather than one compound) at environmentally low concentrations (rather than high concentrations) during the application of ozonation in continuous mode (rather in batch mode) simulating thus a more realistic scenario from the UWTP full-scale perspective, combined with the assessment of the process in removing ARB&ARGs (rather than evaluating only the disinfection efficiency in relation to the inactivation of various bacterial groups) and phytotoxicity. The results obtained by existing studies performed on the ozonation of wastewater (Table S1) may be sometimes contradictory, while in many cases the lack and/or heterogeneity of the scientific data (e.g. different target micro-contaminants, different experimental configurations, etc), as well as the different methodological approaches applied in the various studies, make difficult the accurate evaluation of the efficiency of the ozonation. *The innovation of this study lies in the fact that it evaluates the efficiency of ozonation operated in continuous mode in relation to the simultaneous removal of both chemical (i.e. antibiotics) and biological (i.e. ARB&ARGs) microcontaminants, as well as phytotoxicity, which is another important parameter when wastewater reuse in crop irrigation is considered. Also, this is one of the very first attempts made to investigate if the variable region of class1 integrons might undergo excision after exposure to ozone (herein indicated by a reduction in aadA1 and dfrA1).*

2. Material and methods

2.1. Chemicals and reagents

The antibiotic reference standards for liquid chromatography (>98% purity) for AMP (CAS number 69-53-4), AZM (CAS number

117772-70-0), ERY (CAS number 114-07-8), CLA (CAS number 81103-11-9), OFL (CAS number 82419-36-1), SMX (CAS number 723-46-6), TMP (CAS number 738-70-5), and TC (CAS number 60-54-8), as well as the surrogate standards azithromycin-d3 and ofloxacin-d3 were purchased from Sigma Aldrich (Steinhein, Germany). Methanol (MS grade) and hydrogen peroxide (H_2O_2, 30% w/w) were acquired from VWR International (Fontenay-sous-Bois, France) and Merck (Darmstadt, Germany), respectively. Ethanol (HPLC grade) was purchased from Fisher Scientific (Leicestershire, UK). Formic acid (99%), potassium iodide, sodium dihydrogen phosphate and phosphoric acid (85%) were provided by Fluka (Steinhein, Germany). Catalase from bovine liver powder (2,000–5,000 units/mg protein), supplied by Fluka, was used to quench the reactions in the treated samples prior to chromatographic and toxicity/microbiological analysis. Also, sodium sulphite (Sigma Aldrich) solution was used in the treated samples to remove excess oxidants for the DOC determination. Potassium indigo trisulfonate (Sigma Aldrich), sodium dihydrogen phosphate (Fluka) and phosphoric acid (85%, Fluka) were used for the preparation of the indigo solution. Ultrapure water (resistivity > 18.2 MΩ cm at 25 °C) was supplied by a Milli-Q water system (Millipore). Oasis® HLB (Hydrophilic-Lipophilic Balanced) cartridges (150 mg, 6 mL) used for sample pre-concentration, were supplied by Waters (Milford, MA, USA). Stock solutions of each individual compound (approximately 5,000 mg L^{-1}) were prepared in methanol and working solutions were prepared by diluting these solutions in ultrapure water. The hydrolytic stability of each stock solution was routinely evaluated through chromatographic analysis after storage in a refrigerator for 2 months and it was found to be stable.

2.2. UWTP samples

All experiments were performed using wastewater effluent samples collected between October 2017 and January 2018, from the secondary clarifier of an UWTP located in Northern Portugal. This UWTP, serving a population equivalent of 80,000, has an average flow of 18,000 m^3 day^{-1} and employs conventional activated sludge (CAS) as secondary treatment. Grab samples were collected in sterile glass bottles and processed immediately after being transferred to the laboratory. The qualitative parameters (pH, conductivity, DOC, chemical oxygen demand (COD), and total suspended solids (TSS)) of the secondary-treated wastewater samples used during the experimental period were determined via routine analysis (Table S2). The experiments were performed with secondary-treated effluents, as collected or spiked with a mixture of the target antibiotics (AMP, AZM, CLA, ERY, OFL, SMX, TMP and TC) with an initial concentration of 100 μg L^{-1}, without prior pH adjustment (the inherent wastewater pH values ranged between 7.2 and 7.8) and at the inherent temperature values (22–25 °C). It is noted that the inherent concentrations of the antibiotics in the wastewater samples were, for half of the examined antibiotics, below the method's detection limit (MDL): AMP ≤ MDL, AZM = 184.5–358.8 ng L^{-1}, CLA = 433.2–474.8 ng L^{-1}, ERY ≤ MDL, OFL ≤ MDL-6.12 ng L^{-1}, SMX ≤ MDL, TC ≤ MDL, and TMP = 129.5–334.1 ng L^{-1}. The MDL values for each compound are provided in Table 1.

2.3. Ozonation set-up and procedure

Ozonation experiments were performed in a continuous mode, through a flow-through reactor where untreated wastewater effluents were pumped on a continuous basis, and treated samples were exiting continuously. A cylindrical borosilicate bubble column reactor (Fig. 1a; 3.0 cm internal diameter (I.D.) × 70 cm height, useful volume of approximately 310 mL) packed with ca. 400 glass rings, was used in order to promote the contact between the gas phase and wastewater, increasing the mass transfer of ozone. The use of borosilicate in the reactor column ensured that no adsorption/desorption of the examined compounds on the reactor walls occurred. This material has been widely used in ozonation reactors (Mecha et al., 2017; Orhon et al., 2017; Rozas et al., 2017). Also, the borosilicate bubble column provides the possibility of visual contact in the reactor to avoid any malfunctions during the performance of the experiments. Regardless of the type of experiment performed, the reactor was always filled with ultrapure water and the experiments commenced ($t_0 = 0$ min) when the wastewater to be treated was pumped into the reactor through the peristaltic pump (Fig. 1b), being diluted until achieving steady-state conditions. Preliminary experiments, through conductivity measurements with a tracer solution of NaCl (concentration of 2,000 mg L^{-1}), were performed to determine the time required to achieve the steady state for each HRT (Fig. S1). For the examined HRTs (i.e. 10, 20, 40 and 60 min), the inlet liquid flow rate varied between 40 and 6 mL min^{-1}. According to Fig. 1, the wastewater effluents were continuously entering the reactor by the bottom of the column (Fig. 1c) and leaving it from its top (Fig. 1d). The efficiency of the process was estimated after achieving the steady state for each selected HRT. A BMT 802X ozone generator (Fig. 1e) was used to produce ozone from pure oxygen. The desired ozone concentration was produced through the adjustment of the O_2 gas flow rate, which varied from 20 to 160 mL min^{-1} (adjusted by a mass flow controller (Fig. 1f) and the electric intensity of the ozone generator). The ozone concentration was normalised to the DOC values of each sample at each treatment time point. The examined specific ozone doses were 0.125, 0.25, 0.50 and 0.75 gO_3 $gDOC^{-1}$. The ozone concentration in the liquid phase was measured by the Indigo colorimetric method (Bader and Hoigné, 1981). The ozone escaping the reactor in the gas phase was carefully removed by gas washing bottles filled with a KI solution (Fig. 1g). Two sets of experiments were performed using: (1) wastewater effluents as collected and (2) wastewater effluents spiked with the mixture of the selected antibiotics. Moreover, experiments with the addition

Table 1
Occurrence of antibiotics (expressed as a concentration range) in secondary-treated wastewater effluent samples taken from the secondary clarifier of the UWWTP. The concentration range refers to the samples taken between October 2017 and January 2018.

Compounds	Method detection limit (MDL) (ng L^{-1})	Concentration range (ng L^{-1})
Ampicillin (AMP)	0.97	<MDL
Azithromycin (AZM)	0.15	184.5–358.8
Clarithromycin (CLA)	0.01	433.2–474.8
Erythromycin (ERY)	0.74	<MDL
Ofloxacin (OFL)	1.01	<MDL - 6.12
Sulfamethoxazole (SMX)	0.18	<MDL
Tetracycline (TC)	1.12	<MDL
Trimethoprim (TMP)	0.41	129.5–334.1

MDL = Method Detection Limit.

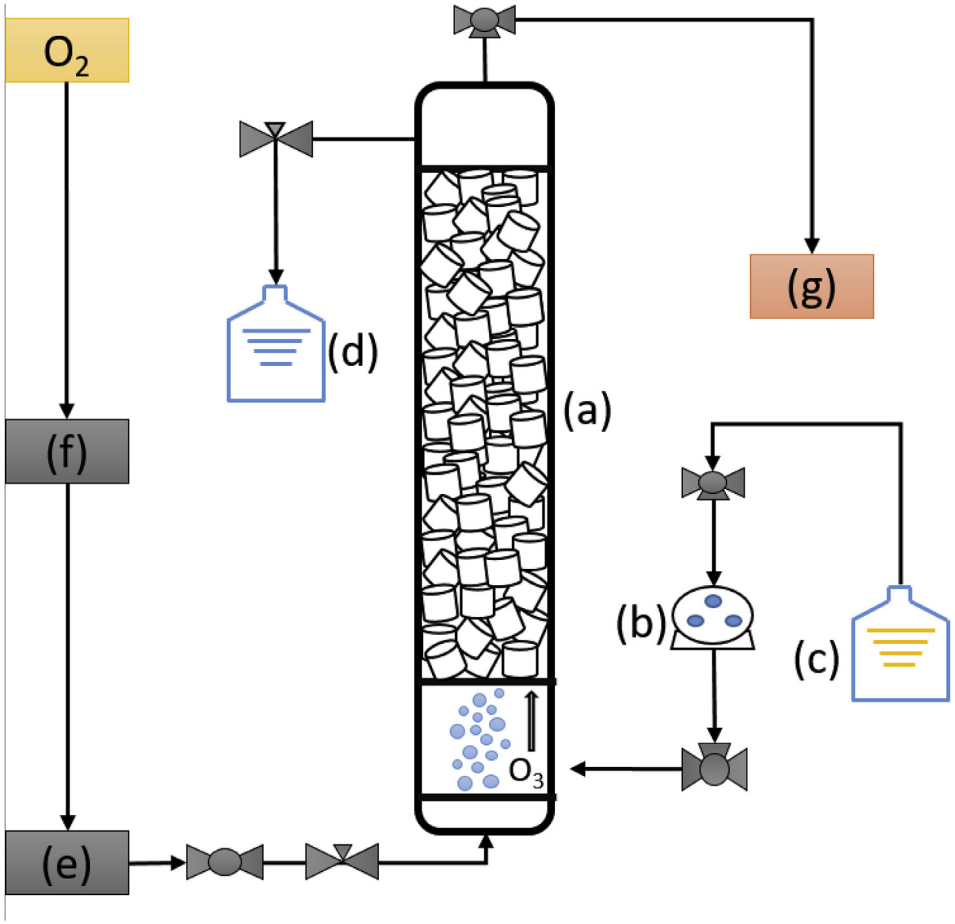

Fig. 1. Experimental set-up of the ozonation reactor operating in continuous mode: (a) bubble column reactor filled with glass rings for gas-liquid-solid contact, (b) peristaltic pump, (c) inlet solution (secondary-treated effluents), (d) outlet solution, (e) ozone generator, (f) mass flow controller and (g) ozone gas destroyer.

of H_2O_2 at different concentrations (i.e. 0, 0.04, 0.08 and 0.12 mM) were also performed. The selected concentrations were based on previous studies (Valero et al., 2015), in an attempt to optimize the concentration of H_2O_2 able to enhance the degradation efficiency of pollutants by catalytic decomposition of O_3, generating $HO^{\bullet}$, avoiding the use of an excessive amount which could favour the reaction with $HO^{\bullet}$ to form a weaker hydroperoxyl radical ($HO_2^{\bullet}$) (Ribeiro et al., 2019).

Prior to chromatographic analysis, the obtained samples were transferred immediately to glass vials with a stoichiometric excess of catalase solution in order to remove any residual oxidants. All samples were subsequently filtered through 1.2-μm glass-fiber filters (47 mm GF/C, Whatman™; Maidstone, United Kingdom). The non-spiked wastewater samples were pre-concentrated by solid phase extraction (SPE) using Oasis® HLB cartridges, as described elsewhere (Ribeiro et al., 2015). Each experiment was performed in triplicate and average values are quoted as results. The error bars depicted in the figures represent the relative standard deviation (RSD) of three independent measurements derived from three separate experimental runs. RSD values were always below 10%.

2.4. Analytical methods

For Ultra-High Performance Liquid Chromatography with tandem Mass Spectrometry (UHPLC–MS/MS) analysis, a Shimadzu Corporation apparatus (Tokyo, Japan) was used, consisting of a UHPLC equipment (Nexera) with two pumps (LC-30AD), an autosampler (SIL-30AC), an oven (CTO-20AC), a degasser (DGU-20A 5R) and a system controller (CBM-20A), coupled to a triple quadrupole mass spectrometer detector (Ultra-Fast Mass Spectrometry series LCMS-8040) with an ESI source operating in both positive and negative ionization modes. An analytical method described elsewhere was used (Barbosa et al., 2018) for the detection and quantification of the target antibiotics. A Kinetex™ 1.7 μm XB-C18 100 Å column (100 × 2.1 mm i.d.) supplied by Phenomenex, Inc. (California, USA) was employed. The mobile phase consisted of ultrapure water and methanol, both acidified with 0.1% formic acid, operating at gradient mode, with a flow rate of 0.25 mL min^{-1}. Column oven and autosampler temperatures were set at 30 °C and 4 °C, respectively. The volume of injection was 5 μL. The optimized ESI parameters were the following: nebulizing gas flow, 3.0 L min^{-1}; drying gas flow, 15 L min^{-1}, capillary voltage, 4.5 kV; desolvation temperature, 400 °C; and source temperature, 250 °C. Argon at 230 kPa was the collision induced dissociation gas (CID).

The two selected reaction-monitoring (SRM) transitions (Table S3) between the precursor ion and the two most abundant fragment ions were employed for quantification (SRM1) and identity confirmation (SRM1/SRM2 ratio). All MS data was processed using the software package LC Solution Version 5.41SP1.

A Shimadzu TOC-5000A instrument (Shimadzu Scientific Instruments, Japan) was employed in order to monitor the DOC in the samples before and after treatment and therefore, to assess the extent of the mineralization. The pH and conductivity were measured with a pH meter pHenomenal® pH 1100 L (VWR, Germany).

2.5. Enumeration of total and antibiotic-resistant Escherichia coli

Bacteria enumeration was performed using the membrane filtration method, as described elsewhere (Novo and Manaia, 2010; Michael et al., 2012). The chromogenic agar of tryptone bile X-glucuronide (t-BX) (Sigma Aldrich, Steinhein, Germany) was used for the detection and enumeration of *Escherichia coli*. For the enumeration of ARB, the medium was spiked with TMP or SMX, at concentrations of 16 or 516 mg L^{-1}, respectively. These concentrations were chosen based on the minimum inhibitory concentration (MIC) of each of the examined antibiotics, as determined in the Clinical and Laboratory Standards Institute (CLSI) (Wayne, 2007). Serial dilutions of each sample were prepared in a saline solution (NaCl 0.85%) and filtered through mixed cellulose ester membranes (0.22 μm pore size, Millipore), which were placed onto the culture medium and incubated for 24 h at 44 ± 1 °C for *E. coli*. Bacteria counts, expressed as Colony Forming Units per mL (CFU/mL), were enumerated on the respective culture media, with the Limit of Quantification (LOQ) of *E. coli* being equal to 1 CFU 100 mL^{-1}, since 100 mL was the maximum volume filtered. The prevalence of antibiotic resistant *E. coli* was assumed as the ratio of CFU mL^{-1} observed on the medium containing the antibiotic and that CFU mL^{-1} on the medium without antibiotic, as described elsewhere (Novo and Manaia, 2010; Karaolia et al., 2017). Additionally, the regrowth potential of the examined bacterial groups was investigated in ozonated samples in which the examined bacterial groups were at levels below LOQ. Regrowth was examined after 24, 48 or 72 h of storage in the dark at both 24 ± 1 °C and 44 ± 1 °C. This investigation took place in order to examine whether the reduction of the examined bacterial groups denoted their permanent inactivation, or if it was a transient effect of injury and temporary inactivation, with regrowth after some time for repair/reactivation. The examination of the ozonation regrowth capacity aimed at giving insights on the potential bacterial regrowth after treatment, in order to have an indication of what could happen in terms of faecal contamination, if the treated wastewater was discharged in a natural receiving environment or stored to be used for reuse purposes.

2.6. Quantification of selected ARGs

Total genomic DNA was used to monitor selected genes and ARGs in the treated samples obtained from different experimental runs performed at different operating conditions. The genes examined to this study were the four ARGs of *aad*A1, *dfr*A1, *qacEΔ*1 and *sul1*, the class 1 integron − integrase (*intI*1) and the housekeeping gene of 16S rRNA (the reasons of selecting these genes/ARGs are provided in Section 3.3). Three sets of experimental conditions were selected to assess the effect of the experimental parameters (i.e. HRT and specific ozone dose) on the reduction of the abundance of the selected genes: (i) 0.25 g O_3 gDOC $^{-1}$ and HRT of 10 min, which can be considered as a realistic scenario in a full-scale UWTP, with many studies in the literature reporting these conditions; (ii) 0.75 gO_3 gDOC $^{-1}$ and HRT of 40 min, which were the optimum conditions determined in this study for the degradation of the target antibiotics and the bacterial inactivation; and (iii) 0.25 gO_3 gDOC $^{-1}$ and HRT of 40 min, in order to examine the influence of lower ozone dose along with higher contact time.

The total genomic DNA extraction was performed using the PowerWater® DNA Isolation Kit (MOBIO Laboratories Inc.) following filtration of the samples (volume 900–1000 mL) through polycarbonate filter membranes (45 mm diameter, 0.22 μm pore size, Millipore). DNA quantification was assessed using a Qubit 3.0 Fluorometer (ThermoFIsher Scientific). Quantitative polymerase chain reaction (qPCR, StepOne TM Real-Time PCR System, Life-Technologies, Carlsbad, CA, USA) using SYBR Green chemistry was employed to measure the abundance (per mL of sample) of *aad*A1, *dfr*A1, *qacEΔ*1 and *sul1*, *intI*1 and 16S rRNA. Calibration curves based on adequate ten-fold dilution series of the standards for the respective gene were run along with the test samples. Primers information and qPCR conditions are listed in Table 2.

2.7. Phytotoxicity assays

The phytotoxicity assessment of samples collected before and after the ozonation treatment (at the same experimental conditions mentioned in Section 2.6) was carried out in triplicate using the Phytotestkit microbiotest (MicroBioTests Inc.). The selection of this phytotoxicity assay among various toxicity tests, aimed at providing evidence on the suitability of the ozonation process as a tertiary treatment for wastewater effluents intended for reuse in agricultural irrigation. The following plants were used in this assay, due to their rapid germination and growth of roots and shoots, and their sensitivity to low concentrations of phytotoxic substances: monocotyl Sorgho (*Sorghum saccharatum*), dicotyl garden cress (*Lepidium sativum*), and dicotyl mustard (*Sinapis alba*). A control test was performed using tap water. The data interpretation is extensively described elsewhere (Michael et al., 2012).

3. Results and discussion

3.1. Degradation of antibiotics

The efficiency of the ozonation process, operated in continuous

Table 2
List of primers and conditions used to detect the target genes.

Target gene	Primers	Primers sequence	Conditions	Primers reference
16S rRNA	1114F 1275R	CGGCAACGAGCGCAACCC CCATTGTAGCACGTGTGTAGCC	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)	(Denman and Mcsweeney, 2006)
*aad*A1	aadA-01F aadA-01R	GTTGTGCACGACGACATCATT GGCTCGAAGATACCTGCAAGAA	95 °C 2 min (1 cycle), 95 °C 15 s - 60 °C 1 min (40 cycles)	Zhu et al. (2013)
*dfr*A1	dfA1F dfA1R	GGAATGGCCCTGATATTCCA AGTCTTGCGTCCAACCAACAG	95 °C 10 min (1 cycle), 95 °C 15 s - 60 °C 1 min (40 cycles)	Zhu et al. (2013)
*qacEΔ*1	qacEΔ1-02F qacEΔ1-02R	CCCCTTCCGCCGTTGT CGACCAGACTGCATAAGCAACA	95 °C 5 min (1 cycle), 95 °C 10 s - 60 °C 30 s (35 cycles)	Zhu et al. (2013)
*sul*1	sul1-FW sul1-RV	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	95 °C 5 min (1 cycle), 95 °C 15 s - 60 °C 30 s (35 cycles)	Pei et al. (2006)
*intI*1	intI1-LC1 intI1-LC5	GCCTTGATGTTACCCGAGAG GATCGGTCGAATGCGTGT	95 °C 10 min (1 cycle), 95 °C 15 s - 60 °C 1 min (40 cycles)	Barraud et al. (2010)

mode, in degrading the target antibiotics was assessed under various HRTs (i.e. 10, 20, 40 and 60 min) and specific ozone concentrations (i.e. 0.125, 0.25, 0.50 and 0.75 gO_3 $gDOC^{-1}$). These conditions (i.e. HRTs and ozone doses) were selected based on the findings of previous studies dealing with the application of ozonation for the removal of various organic microcontaminants (Reungoat et al., 2012; Prieto-Rodríguez et al., 2013; Margot et al., 2013; Marce et al., 2016; Sousa et al., 2017), and taking into account the full-scale applicability of the process in relation to the operating cost. The latter was taken into consideration by applying the lowest ozone dose able to effectively eliminate the examined compounds under continuous mode operation. The results revealed that the spiked antibiotics, in mixture, were efficiently degraded (>99%) by ozonation applying both HRTs of 40 and 60 min, at all examined ozone concentrations (Figs. S2c and d). For the lower HRTs of 10 min and 20 min with an ozone dose of 0.125 gO_3 $gDOC^{-1}$ (Figs. S2a and b), only SMX, OFL, and TC were degraded rapidly below the LOD, while AMP, AZM, CLA, ERY and TMP were degraded up to 84, 73, 98, 70 and 96%, respectively. When gradually increasing the ozone dose, the elimination of the parent compounds of all antibiotics was shown to slightly increase for both HRTs of 10 and 20 min (10 min).

This behaviour was expected even at low ozone doses, since this oxidant is able to either selectively attack double bonds and molecules containing electron rich moieties present in the different classes of the examined antibiotic compounds (e.g., macrolides, sulfonamides and fluoroquinolones) or develop a radical oxidation pathway due to its self-decomposition at the natural pH of the wastewater (pH 7.3–7.8). Considering that the experiments were conducted at the inherent pH of the wastewater effluents, it can be assumed that the elimination of the parent compounds was predominantly driven by HO•, rather than the direct reaction with molecular O_3 (Michael-Kordatou et al., 2017). The results presented herein are consistent with those reported in other relevant scientific studies using different reactor configurations and applying different ozone concentrations (Reungoat et al., 2012; Lee et al., 2013; Margot et al., 2013; Prieto-Rodríguez et al., 2013). For instance, Lee et al. (2013) reported a degradation higher than 90% for TMP and SMX in secondary-treated effluents treated by a bench-scale semi-continuous ozonation. Another similar bench-scale study using ozonation at semi-operation mode demonstrated that the elimination of the parent compounds of sulfonamide and macrolide antibiotics spiked in ultrapure water and in pharmaceutical wastewater effluents was achieved in less than 20 min (Lin et al., 2009). In a pilot-scale ozonation study (0.32–1.23 gO_3 $gDOC^{-1}$; HRTs up to 20 min), Margot et al. (2013) reported the removal of 40 microcontaminants (including AZM, CLA, TMP, and SMX) over 80% from secondary-treated wastewater effluents. A removal of 90% of microcontaminants (including CLA, ERY, OFL, SMX and TMP) from secondary-treated effluents was reported after a treatment time of 20 min using pilot-scale ozonation operating in a semi-continuous mode (0.19 gO_3 $gDOC^{-1}$) (Prieto-Rodríguez et al., 2013). Similar results were reported by Reungoat et al. (2010), in a study dealing with full-scale ozonation (5 mgO_3 L^{-1} and HRT 15 min) of secondary-treated wastewater. Among the 25 selected microcontaminants quantified in the samples, 9 showed a reduction of more than 90% (including ERY, TMP, and SMX) and 13 others were reduced by more than 70%.

Overall, the degradation of the parent compounds of the antibiotics was improved with increasing the HRT and ozone dose, as expected. According to the aforementioned results obtained from the spiked experiments, the HRT of 40 min could be considered as optimum, since it allowed to achieve a rapid and complete elimination of the parent compounds of all the examined antibiotics at μg L^{-1} level, even when using the lowest applied ozone dose of 0.125 gO_3 $gDOC^{-1}$ (Fig. S2). However, considering that the application of ozonation at a full-scale UWTP for 40 min is not cost-effective and that no significant differences were found between the different applied ozone concentrations for the HRTs of 10 and 20 min, a new set of experiments was carried out to determine the optimum experimental conditions with respect to the antibiotics' degradation at the lowest HRTs of 10 and 20 min, by examining the contribution of the addition of hydrogen peroxide (H_2O_2) to the overall process efficiency.

The degradation of the target antibiotics was investigated by comparing single ozonation (absence of H_2O_2) and ozonation assisted by the addition of H_2O_2 at different concentrations (i.e. 0.04, 0.08 and 0.12 mM), which were selected according to the findings reported in previous studies (Cho and Yoon, 2006; Lanao et al., 2008; Valero et al., 2015). The addition of H_2O_2 to the ozonation process leads to the so-called peroxone process (O_3/H_2O_2), which is expected to promote higher removals of organic compounds than single ozonation, due to the enhanced ozone decay and production rate of HO• (Von Gunten, 2003; Von Sonntag and Von Gunten, 2012). Fig. 2 shows the effect of the addition of H_2O_2 on the degradation of each antibiotic by ozonation for the HRT of 10 min, as a function of the applied ozone concentration.

It can be observed in Fig. 2b–d that the increase of the oxidant concentration had a marginal influence on the degradation of the examined antibiotics. The results obtained in this study revealed that the removal of the antibiotics which were not completely degraded by single ozonation (AMP, AZM, and ERY), was similar with that observed when applying the O_3/H_2O_2 process for all the tested concentrations of H_2O_2. AMP, AZM and ERY were not completely removed after 10 min of treatment under all the tested ozone doses and H_2O_2 concentrations, and their removal percentages by single (O_3) and combined (O_3/H_2O_2) treatments was differed only by 2–3%. This may be caused by the rapid consumption of ozone by dissolved effluent organic matter (dE_fOM), when using low specific ozone doses (gO_3 $gDOC^{-1} < 0.5$). This reaction outcompetes the relatively slow reaction of ozone with H_2O_2 (Von Sonntag and Von Gunten, 2012; Lee et al., 2014; Miklos et al., 2018). Similarly, Acero and Von Gunten (2001) also reported the effect of the wastewater qualitative parameters on the efficiency of ozonation and O_3/H_2O_2.

Several studies have reported similar findings, indicating that the enrichment of the ozonation reaction with H_2O_2 slightly affected the degradation of microcontaminants. For instance, Lee et al. (2013) reported negligible variations when adding H_2O_2 in wastewater (the H_2O_2/O_3 molar ratio and ozone doses ranged between 0.5-1.0 and 0.25–1.5 gO_3 $gDOC^{-1}$, respectively), when using a lab-scale ozone reactor operating in batch mode to examine the degradation of 16 microcontaminants, including TMP and SMX, in secondary-treated effluents. Similar results were obtained in the study of Lee et al. (2014) who reported that the removal of the tested microcontaminants during O_3/H_2O_2 process increased only slightly (<10%) by the addition of H_2O_2 (molar ratio H_2O_2/O_3: 0, 0.25, and 0.5). In a pilot-scale ozonation (0.64–1.08 gO_3 $gDOC^{-1}$; HRTs 12–23 min; addition of H_2O_2 up to 2.5 mg L^{-1}), Kovalova et al. (2013) reported a negligible improvement of ±10% for microcontaminants elimination by O_3/H_2O_2 in comparison to single ozonation.

Therefore, these results showed that the addition of H_2O_2, which would increase the operating cost of the process, did not enhance remarkably the degradation of the examined compounds. However, it is important to mention that in the experiments performed with non-spiked wastewater effluents (inherent antibiotics' concentrations), even the mildest experimental conditions tested with single ozonation (HRT of 10 min and 0.125 gO_3 $gDOC^{-1}$) were found to be able to provide a removal of the examined microcontaminants

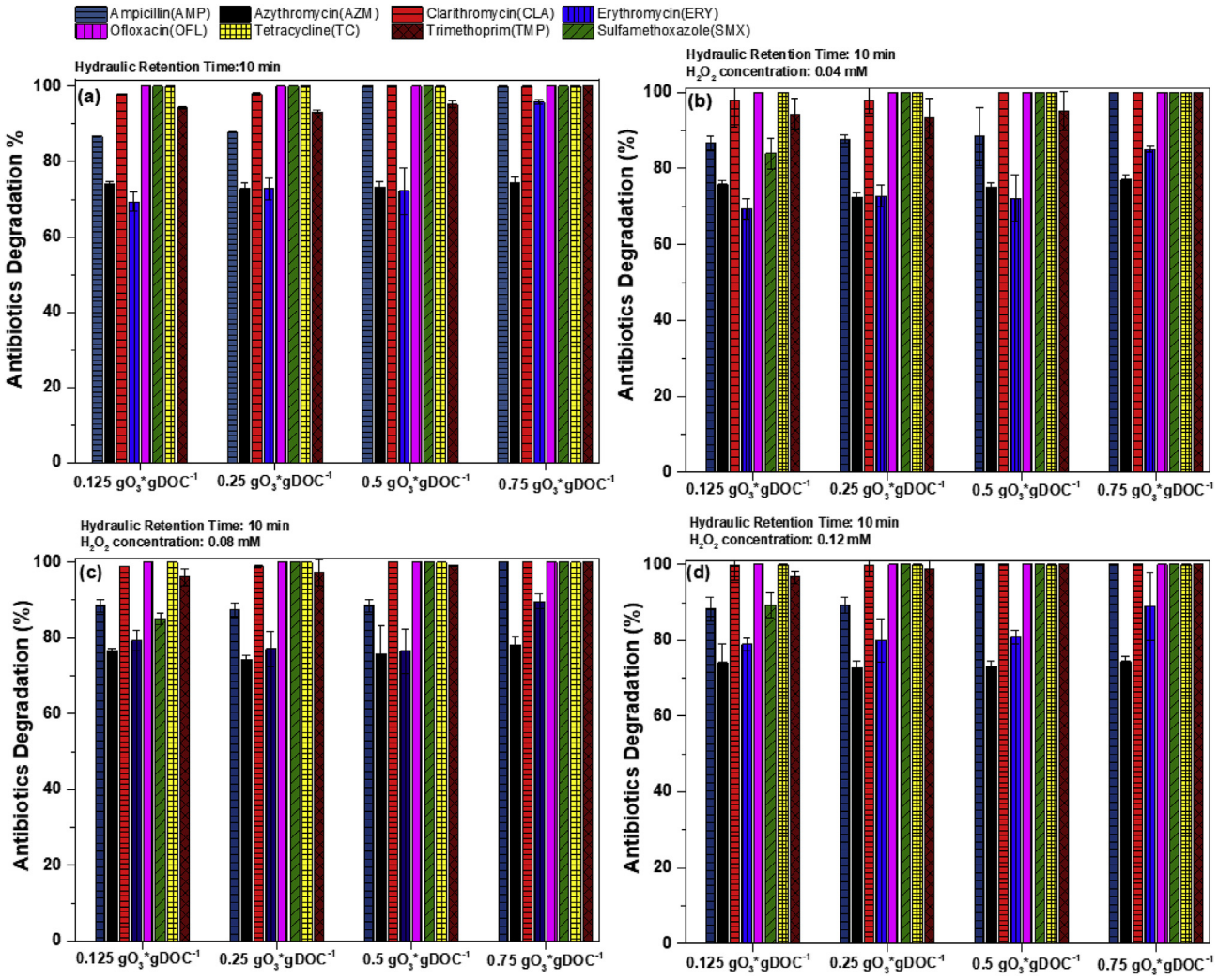

Fig. 2. Degradation of the target antibiotics by continuous mode ozonation with an HRT of 10 min, varying the O_3 doses (0.125, 0.25, 0.5 and 0.75 gO_3 $gDOC^{-1}$) and the H_2O_2 concentration: (a) 0 mM, (b) 0.04 mM, (c) 0.08 mM, and (d) 0.12 mM of H_2O_2). Experimental conditions: $[A_0] = 100\ \mu g\ L^{-1}$; matrix: secondary treated effluents; pH 7.3–7.8; $T = 24 \pm 1$ °C.

(Table 1) to concentrations below their limit of detection. In order to investigate whether the mildest ozonation conditions were adequate towards the inactivation of ARB and removal of ARGs, a new series of optimization experiments was performed with varying ozone doses and HRTs, as discussed in the following Sections.

3.2. Inactivation of total and antibiotic-resistant cultivable *Escherichia coli*

The ozonation process operated in continuous mode was investigated with regard to its efficiency to inactivate the total and antibiotic-resistant cultivable *E. coli*. The selection of *E. coli* was based on its role as indicator of microbiological contamination. *E. coli* and faecal coliforms in general, are used worldwide as indicators of faecal contamination, in particular to assess the microbiological water/wastewater quality and are included in the wastewater legislation for reuse purposes (APHA 2005, ISO9308-1:2000). *E. coli* are excreted in faeces and consequently they reach UWTPs, where they can survive and thrive throughout the CAS treatment (Forster et al., 2002; Wellington et al., 2013; Karaolia et al., 2017). Therefore, the ubiquity of these bacteria in human-impacted environments, together with their persistence in the environment, as well as genome plasticity, make them important tracers to assess the antibiotic resistance status of environmental samples. Additionally, in previous studies including both Gram-negative and Gram-positive faecal indicator bacteria (*E. coli* and *Enterococcus* spp., respectively), noteworthy differences were not observed regarding their inactivation. Comparing both groups, *Enterococcus* spp. has usually lower abundance and suffers higher inactivation than *E. coli* (Moreira et al., 2016, 2018; Biancullo et al., 2019). It is also noted that the selection of *E. coli* as the target bacterium in this study was based on the fact that the latter is included in the Proposal for a "Regulation of the European Parliament and of the Council", on minimum requirements for water reuse (EU, 2019). The selection of SMX and TMP to investigate the antibiotic resistance was based on their wide use and frequent detection in wastewater and aquatic environments.

The initial abundance of *E. coli* in the secondary-treated effluents was up to 8×10^3 CFU mL^{-1}. The experiments were performed in triplicate for all the examined HRTs and ozone concentrations. The obtained results regarding the inactivation of total *E. coli* (Fig. 3a), indicated by absence of colonies in 100 mL of treated wastewater (<LOD = 0.01 CFU mL^{-1}), were found using an HRT of

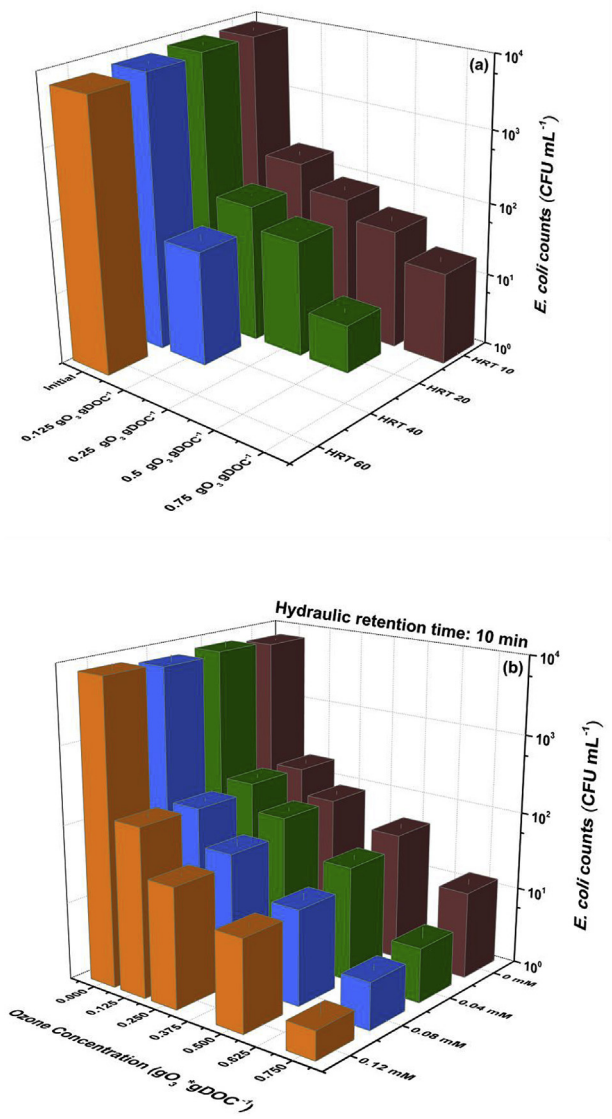

Fig. 3. Inactivation profile of *E. coli* (expressed as *E. coli* CFU mL^{-1}) by continuous mode ozonation (a) varying the O_3 doses (0.125, 0.25, 0.50 and 0.75 gO_3 $gDOC^{-1}$) and HRTs (10, 20, 40, 60 min); and (b) using an HRT of 10 min and varying the O_3 doses and H_2O_2 concentrations (0 mM, 0.04 mM, 0.08 mM, 0.12 mM of H_2O_2). Experimental conditions: $[A_0] = 100\ \mu g\ L^{-1}$; matrix: secondary treated effluents; pH 7.3–7.8; $T = 24 \pm 1$ °C.

60 min for all the examined ozone concentrations. Similarly, an HRT of 40 min was found to be adequate to inactivate *E. coli* to values below LOD for almost all tested ozone concentrations, except 0.125 gO_3 g DOC^{-1}. For the HRT of 20 min, the higher ozone concentration tested (0.75 gO_3 $gDOC^{-1}$) was the only one able to inactivate *E. coli* to values below the LOD, whereas inactivation was not attained when using an HRT of 10 min. One study dealing with similar experimental conditions, reported a 1-log reduction of *E. coli* in a pilot-scale ozonation reactor treating secondary-treated effluents with an ozone dose up to 0.73 gO_3 $gDOC^{-1}$ and a contact time of 20 min (Lüddeke et al., 2015). Also, Ostoich et al. (2013) reported the complete inactivation (<LOD = 0.01 CFU mL^{-1}), of faecal coliforms by ozonation in a UWTP of Italy, using ozone doses varying from 10 to 15 mgO_3 L^{-1} and a contact time of 30 min. Another study focusing on the inactivation of cultivable bacterial populations after batch-mode ozonation under different contact times and ozone doses up to 50 g $(Nm^3)^{-1}$, showed that the removal of total heterotrophs, enterococci and coliforms (including of antibiotic resistant *E. coli* strain A2FCC14) was higher than 99%, within 30 min of ozonation (Sousa et al., 2017).

The capacity of ozonation operated in continuous mode, was also investigated in terms of its efficiency to inactivate antibiotic-resistant *E. coli*. The obtained results (Fig. S3) demonstrated the inactivation, below LOD, of *E. coli* harbouring resistance to TMP and SMX, by increasing HRTs and applied ozone doses. The inactivation of the examined ARB occurred for HRTs of 10 and 20 min and for ozone doses of 0.5 and 0.25 gO_3 $gDOC^{-1}$, respectively. The apparent observation that cultivable TMP- and SMX-resistant *E. coli* was lost earlier than the total cultivable *E. coli* is probably due to the lower abundance of the first, which had already reached the LOQ value. Indeed, those results were expected due to the lower number of bacteria exhibiting resistance to TMP and SMX. It should be noted however that oxidizing agents (i.e. O_3 and $HO^{\bullet}$) might have led to the induction of a 'Viable But Not Cultivable state' (VBNC) in the bacteria, explaining that non-cultivable bacteria were detected in the presence of antibiotics. Michael-Kordatou et al. (2017) observed that total *E. coli* and ERY-resistant *E. coli* were inactivated to values below LOQ within 15 min, using an ozone dose of 0.3 mgO_3 L^{-1} during batch-mode ozonation. Oh et al. (2016) investigated ozonation at batch mode with an ozone dose of 3 mgO_3 L^{-1}, for the potential inactivation of *E. coli* containing the 64,508 bp nucleotide sequence of the Inc-P-1beta antibiotic resistance plasmid pB10, which has multiple resistance to a number of antibiotics. It was shown that after 15 min of contact time, the total and resistant *E. coli* reduced by more than 90%. Alexander et al. (2016) investigated the abundance of ARB in wastewater effluents treated by ozonation (0.9 gO_3 $gDOC^{-1}$ and HRT of 18 ± 2 min), with *Enterococcus* spp. exhibiting the highest susceptibility to ozone (98% reduction), and *P. aeruginosa* the highest tolerance. Moreira et al. (2016) reported the inactivation to vales below LOQ of total heterotrophs and *Enterococcus* spp., as well as of bacteria harbouring resistance to ciprofloxacin, gentamicin and meropenem, after 26 min of treatment with ozone dose of 50 g $(Nm^3)^{-1}$ in a lab-scale continuous mode ozonation system.

The results obtained for the HRTs of 10 and 20 min, revealed the sensitivity of the indigenous *E. coli* (total cultivable and antibiotic-resistant *E. coli*) towards the oxidizing agents (O_3 and $HO^{\bullet}$). This could be attributed to the physical vulnerability and the damage of the cell wall and membrane, which may then lead to the cell lysis and consequent leakage of cell components to the external environment (Zuma et al., 2009; Dodd, 2012; Von Sonntag and Von Gunten, 2012; Pak et al., 2016).

The regrowth potential of *E. coli* was investigated for the two mildest operating conditions, which led to their inactivation to levels below the LOQ just after treatment (i.e., according to Fig. 3a: HRT of 20 min and 0.75 gO_3 g DOC^{-1}; HRT of 40 min and 0.25 gO_3 g DOC^{-1}). The idea behind this approach was to understand the alterations on the faecal microbiota occurring in treated wastewater stored for reuse purposes. Incubation periods of 24, 48, and 72 h and temperatures set at 24 ± 1 °C and 44 ± 1 °C were employed. The selection of the incubation temperatures was made according to the environmental conditions (environmental temperature: 22–25 °C), and the maximal temperature tolerated by faecal coliforms, namely *E. coli* (44–45 °C). The environmental incubation temperature constitutes a more realistic approach concerning the regrowth potential of the treated samples, whereas incubation at 44 °C provided an indication on the potential of regrowth under a stressful temperature value. Fig. 4 shows that ozonation operated under low ozone dose and high HRT was efficient to avoid the regrowth at 24 ± 1 °C, whereas the experiments using higher ozone dose and lower HRT led to the regrowth of *E. coli* at both temperatures, suggesting that shorter contact time is a key factor

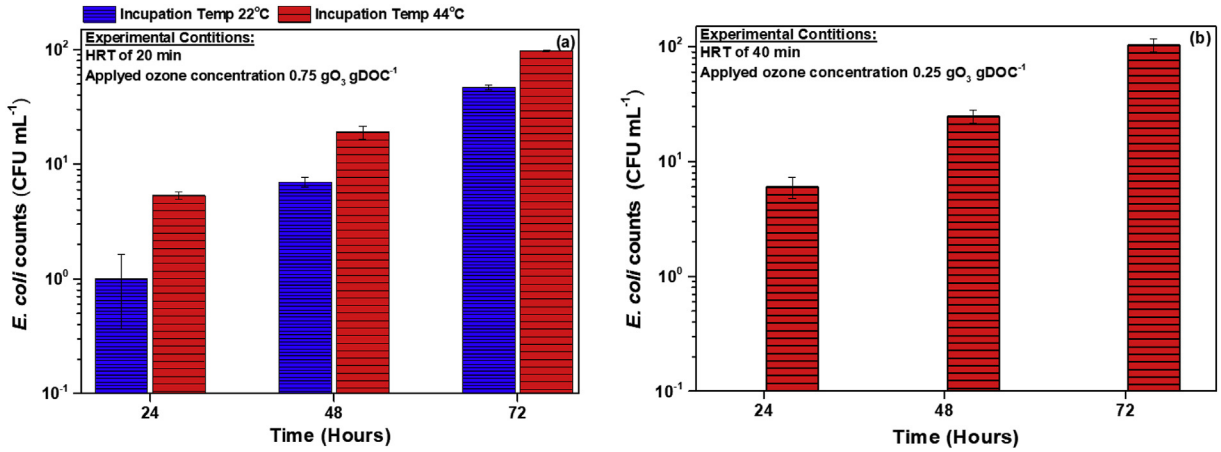

Fig. 4. (a) Regrowth profile of *E. coli* (expressed as CFU mL^{-1}) after continuous mode ozonation of wastewater samples (a) using a HRT of 20 min and an O_3 dose of 0.75 gO_3 $gDOC^{-1}$ and (b) using an HRT of 40 min and an O_3 dose of 0.25 gO_3 $gDOC^{-1}$. Experimental conditions: $[A_0] = 100\ \mu g\ L^{-1}$; matrix: secondary treated effluents; pH 7.3–7.8; $T = 24 \pm 1$ °C.

determining the observed regrowth. The results of this study indicated that low contact times at high ozone doses were not sufficient to induce non-cultivability in the *E. coli* wastewater population. As consequence, the microorganisms affected by ozone and HO• maintained viability, and therefore exhibited regrowth after a certain time period, when the stress was relieved. It is important to mention here that a high number of cells present in wastewater may be aggregated in flocs of different sizes. Hence, inner bacterial cells may be protected from the deleterious effects of ozone and radicals. Therefore, after treatment some cells can be injured, and may need time to repair the damages. Depending on the extent of cell damage, the time needed to recover may vary among surviving cells. Equally, it is expected that they will not grow at the same rate. According to the scientific literature (Lüddeke et al., 2015; Pak et al., 2016; Marce et al., 2017; Jäger et al., 2018), ozonation time, the applied ozone concentration, the physical characteristics of the examined organisms and the wastewater composition, are the major parameters affecting the bacterial regrowth after ozonation.

Further experiments in the presence of ozone and H_2O_2 (0.04,

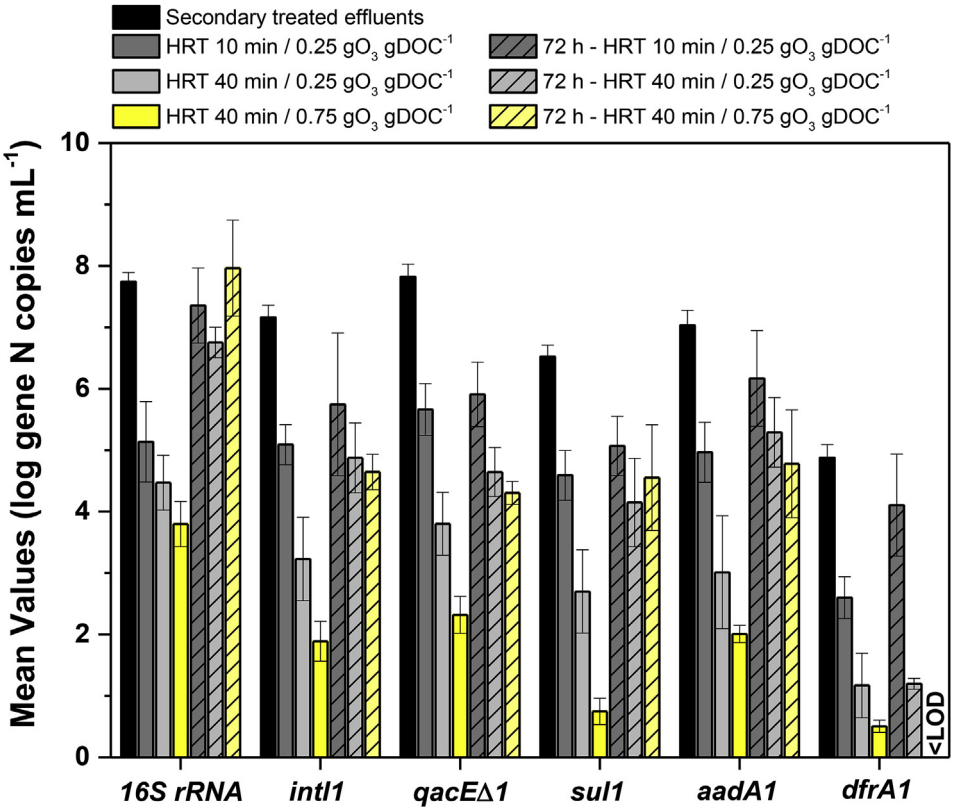

Fig. 5. Examined genes, expressed as log_{10} mean values, for the examined experimental conditions of HRT of 10 min with 0.25 gO_3 $gDOC^{-1}$, HRT of 40 min with 0.25 gO_3 $gDOC^{-1}$ and HRT of 40 min with 0.75 gO_3 $gDOC^{-1}$ and their mean values after 72 h of incubation in the dark at room temperature (22–25 °C). Mean values are represented in log_{10} copies number per 1 mL of sample. Experimental conditions: matrix: secondary treated effluents; pH 7.3–7.8.

0.08 and 0.12 mM), did not reveal significant differences in terms of *E. coli* inactivation (Fig. 3b), in comparison to the experiments performed in the absence of H_2O_2 at the same experimental conditions (HRT of 10 min, ozone doses: 0.125, 0.25, 0.5 and 0.75 gO_3 $gDOC^{-1}$). Specifically, a slight reduction (<5%) was observed as the H_2O_2 concentration increased. However, this reduction falls within the statistical error of the measurements. Similar results were obtained for the inactivation of TMP- and SMX-resistant *E. coli* (Fig. S4). It is important to note that the results obtained in the presence of H_2O_2 for the inactivation of total and resistant bacteria were in agreement with those observed in the case of the degradation of antibiotics, i.e. the process efficiency was not affected by the addition of H_2O_2.

Hübner et al. (2012) investigated the bacteria inactivation in secondary-treated effluents by ozonation with the addition of H_2O_2 and reported no differences after the addition of H_2O_2. Similar results were also found by Valero et al. (2015), during the inactivation of *Enterococcus* spp. through ozonation in the presence and absence of H_2O_2. These findings can be attributed to the strong competition of H_2O_2 with the highly reactive moieties present in the dE_fOM occurring in the secondary-treated effluents, for consumption of ozone molecules (Pocostales et al., 2010; Miklos et al., 2018).

3.3. Removal of selected genes and ARGs

Continuous ozonation was evaluated with regard to its efficiency to remove selected genes and ARGs. Three experimental conditions as mentioned previously (Section 2.7) were selected to assess the influence of the experimental parameters (i.e. HRT and ozone dose) towards the selected genes. The housekeeping 16S rRNA gene was used as a biomarker for bacteria, aiming at assessing the reduction in the load of total bacteria. Class 1 integrons have been suggested as proxies for acquired antibiotic resistance in the environment (Gillings et al., 2015). These genetic elements have conserved regions, namely *intI1, sul1* and *qacΔE1*, and a variable region which often includes genes such as *aadA1* or *dfrA1* (Ferreira Da Silva et al., 2007, Moura et al., 2012).This study aimed at assessing the reduction of the abundance of *intI1* and the conserved genes of the class 1 integrons and at investigating if ozonation led to gene excision, herein indicated by a reduction in *aadA1* and *dfrA1*. Genes quantification was performed to understand how the produced oxidizing agents (i.e. O_3 and $HO^{\bullet}$) interact with the integrons and if the ozonation treatment is capable of efficiently disintegrating this mobile genetic element (Zhang et al., 2009).

The comparison between secondary-treated effluent and ozonated samples showed that the reduction of the abundance of ARGs, bacterial load and *intI1* were HRT- and dose-dependent. An HRT of 40 min and 0.75 gO_3 $gDOC^{-1}$ were found to be the optimum operating conditions for the reduction of the genes abundance (Fig. 5). The log reduction varied between 2 and 3, 3–4 and 4–6, for an HRT of 10 min and 0.25 gO_3 $gDOC^{-1}$, HRT of 40 min and 0.25 gO_3 $gDOC^{-1}$ and HRT of 40 min with 0.75 gO_3 $gDOC^{-1}$, respectively. The results revealed that when using an HRT of 40 min, even when applying a low ozone dose, the reduction of the abundance of the examined genes was similar to that achieved when using the higher ozone dose, suggesting that an appropriate HRT would lead to the desired disintegration levels of the examined ARGs, even with low ozone doses. Ozone disinfection, with an appropriate HRT and ozone dose, is apparently enough to compromise DNA integrity into a condition in which it could not act as PCR template. These results are in agreement with the fact that once the bacteria cell surface is compromised by oxidizing agents, and the cell interior is exposed to the external environment, O_3 and $HO^{\bullet}$ may interfere with DNA (Von Sonntag and Von Gunten, 2012; Dodd, 2012; Pak

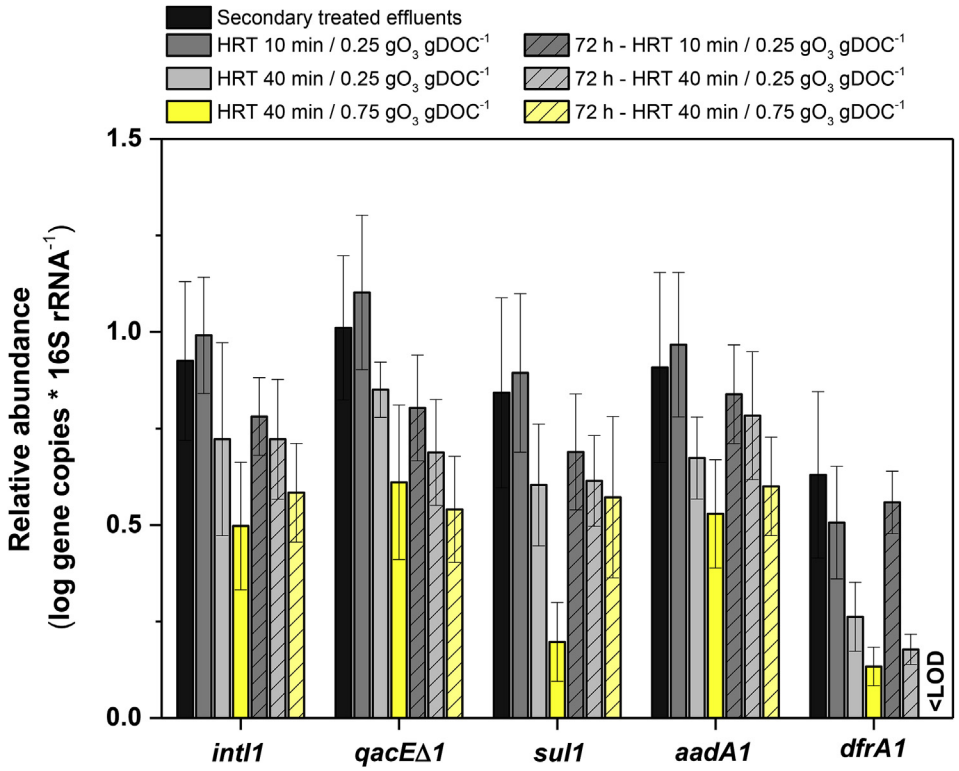

Fig. 6. Examined genes, expressed as the relative abundance of the log_{10} mean values, for the examined experimental conditions of HRT of 10 min with 0.25 gO_3 $gDOC^{-1}$, HRT of 40 min with 0.25 gO_3 $gDOC^{-1}$ and HRT of 40 min with 0.75 gO_3 $gDOC^{-1}$ and their mean values after 72 h of incubation in the dark at room temperature (22–25 °C). Mean values are represented in log_{10} copies number per 1 mL of sample. Experimental conditions: matrix: secondary treated effluents; pH 7.3–7.8.

et al., 2016).

One important observation made in this study was that the resistant *E. coli* decreased throughout ozonation, whereas the reduction of the abundance of the selected genes observed after treatment was apparently transient. The reactivation of the respective bacterial hosts may explain that in the samples treated with 0.25 gO_3 $gDOC^{-1}$ and an HRT of 10 min, the examined genes were detected after 72 h at almost the same levels as the initial values (1-log cycle below the initial), under dark and ambient temperature (22–25 °C) (Fig. 5). Additionally, from Fig. 6, which presents the relative abundance of the examined genes, it can be observed that despite the detected reduction of the abundance of the examined ARGs and integrase genes after the treatment, an increase in the prevalence of these genetic determinants was detected 72 h after the treatment. These observations were expected and are in accordance with the results presented in Section 3.2, where no total inactivation of the examined bacteria was obtained for the same samples. Having in mind that the 16S rRNA gene is a biomarker of the load of total bacteria, its increase in the treated samples under all operating conditions tested implies that other bacterial groups thriving in wastewater (e.g., Aeromonads, Pseudomonads), surviving ozonation, have contributed to the increase of the abundance of the examined genes. A recovery on the examined genes was also observed for the more severe experimental conditions of HRT and ozone dose. These results could be explained through the assumption made by Alexander et al. (2016), who suggested that bacteria may develop different levels of robustness or resistance against oxidative stress. Additionally, the high organic content of the wastewater (Table S2) may react with the majority of the produced oxidizing species, resulting in a very small fraction able to cause lethal damages to the live cells existing in the medium.

Alexander et al. (2016) investigated the efficiency of ozonation treatment to inactivate selected ARGs in a pilot-scale system (18 ± 2 min of HRT and ozone dose up to 0.9 ± 0.1 gO_3 $gDOC^{-1}$) and the obtained results showed variations of the examined genes removed through ozonation, with ARGs of *vanA*, bla_{VIM}, *ampC* and *ermB* being reduced by 49.9%, 18.7%, 69.8% and 99.3%, respectively. Zhuang et al. (2015) reported a slight removal of the examined genes at low ozone doses using a bench-scale system with ozone dose varying between 27 and 128 mg L^{-1} a fact that may be attributed to the rapid consumption of ozone by the organic matter present in wastewater (e.g. COD: 13–29 mgO_2 L^{-1}). Also, the inactivation of the selected genes observed by Zhuang et al. (2015) was found to be enhanced by increasing ozone doses, and this is in line with the results presented in this study. Continuous mode ozonation experiments performed in synthetic and real wastewater samples with constant ozone dose for different contact times (e.g. 15, 30 and 60 min) indicated that increasing contact time led to the inactivation of the selected genes (e.g. *16S rRNA gene, intI1*, bla_{TEM}, *qnrS* and *sul1*) up to 99% (in relation to the initial load before treatment) after 30 min of exposure (Sousa et al., 2017). In addition, Sousa et al. (2017) showed that the regrowth after 72 h of incubation occurred for samples treated for 15 and 30 min, whereas no reactivation was observed after 60 min, suggesting that this period of contact time was adequate to kill the majority of the cells. However, in the real wastewater samples, which are characterized by higher microbial diversity and chemical complexity, compared to synthetic wastewater, the inactivation efficiency and the regrowth potential in the examined samples were lower and more intense, respectively. This may be ascribed to the fact that the structural organization of the bacterial cells is different, with real wastewater containing a high number of aggregated cells forming flocs of different dimensions.

It is already known that the suspended solids, the dE_fOM and the complexity of the microbiota in the wastewater may be the major factors explaining the variations of the efficiency of ozonation treatment (Zhuang et al., 2015; Sousa et al., 2017). The fluctuation of the wastewater qualitative characteristics can influence the reaction mechanism of ozone affecting thus the overall disinfection efficiency towards the living cells in UWTPs. Although not covered in this study, according to Pak et al. (2016) damage of the surface of the cells by ozonation may lead to the release of DNA. If relatively intact, ARGs containing free fragments may be transferred to other microorganisms, through horizontal mechanisms, and contribute to the spread of antibiotic resistance. All those factors seemed to have influence on the ozonation efficiency and more detailed studies are needed to identify the proper conditions required to achieve the optimum performance of the ozonation treatment in terms of genes and ARGs inactivation.

3.4. Mineralization and phytotoxicity assessment

Fig. 7a shows the results for the mineralization observed in the treated effluents, in terms of DOC removal. It must be pointed out that the contribution of the antibiotics to the DOC of the spiked wastewater effluent, was negligible since the DOC of the samples

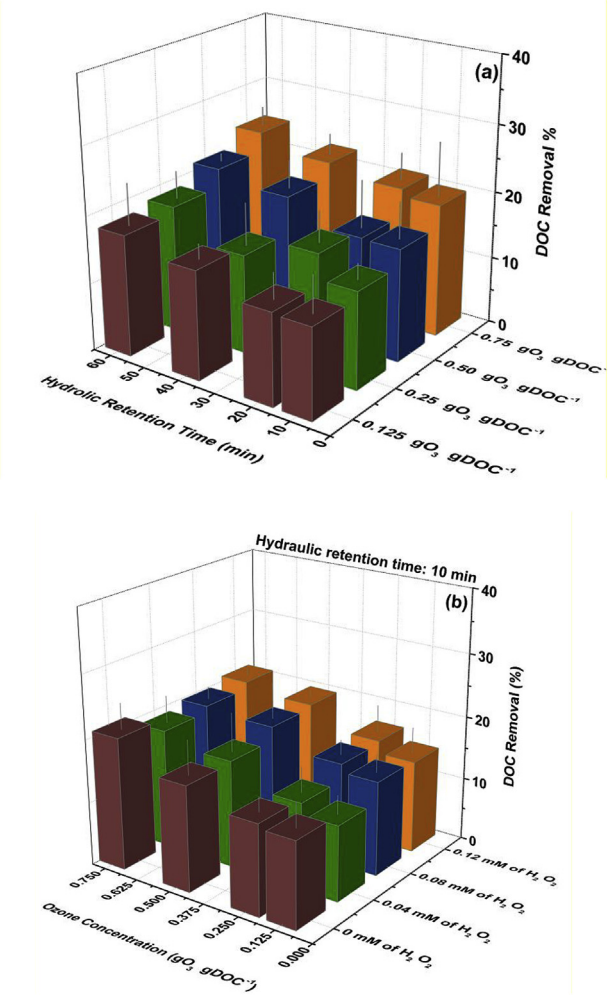

Fig. 7. DOC removal percentages by continuous mode ozonation: (a) varying the O_3 doses (0.125, 0.25, 0.5 and 0.75 gO_3 $gDOC^{-1}$) and HRTs (10, 20, 40, 60 min); and (b) using an HRT of 10 min and varying the O_3 doses and H_2O_2 concentrations (0, 0.04 mM, 0.08 mM, 0.12 mM of H_2O_2). Experimental conditions: $[A_0] = 100$ μg L^{-1}; matrix: secondary treated effluents; pH 7.3–7.8; $T = 24 \pm 1$ °C.

varied between 18.2 and 21.2 mg L^{-1} (Table S2), whereas the theoretical DOC corresponding to the 8 spiked antibiotics was 0.45 mg L^{-1} (<3%). A significant improvement was observed when increasing both the ozone dose and HRT. Specifically, when applying an ozone dose of 0.25 gO_3 gDOC $^{-1}$ and HRT of 10 min, DOC removal was found to be 10–12%, whereas an ozone dose of 0.75 gO_3 gDOC $^{-1}$ and HRT of 60 min led to DOC removal up to 25%. Several studies are in line with the results presented herein and many of them attributed the low yield of mineralization to the formation of recalcitrant organic intermediates deriving from the oxidation of dE_fOM originally present in wastewater (Von Sonntag and Von Gunten, 2012; Margot et al., 2013; Prieto-Rodríguez et al., 2013; Rodríguez-Chueca et al., 2015; Marce et al., 2016; Michael-Kordatou et al., 2017). In a pilot-scale study dealing with ozonation of secondary-treated effluents, Liu et al. (2014) reported a DOC removal up to 28%, after 30 min of contact time, using an ozone concentration of 4 mg L^{-1}. Carbajo et al. (2015) observed 35% of DOC removal even using 130 mg L^{-1} of ozone and a contact time of 20 min. No remarkable differences were found for DOC removal by adding H_2O_2 (Fig. 7b), and this is in accordance to the results observed for the antibiotics in Section 3.1, suggesting that H_2O_2 did not improve the removal of the oxidation products formed during ozonation.

In this work, it was clearly shown that the degradation of the parent antibiotic compounds (down to their limit of detection) required mild ozone doses, while under these conditions the reduction of the organic matter (in terms of COD and DOC) was low (15–25%). dE_fOM contains a variety of organic compounds that during their reaction with ozone and hydroxyl radicals, may lead to the formation of persistent oxidation products. These products may be not susceptible to ozone and can be even more toxic than the initial compounds.

Phytotoxicity tests using three plants species (*Lepidium sativum, Sinapis alba and Sorghum saccharatum*) were considered suitable to evaluate the toxicity of the treated wastewater before its use for agriculture irrigation (Rizzo, 2011). To evaluate the phytotoxicity of the treated samples and assess the influence of the ozone dose and HRT, three experimental conditions reported in Section 3.3 were selected. The phytotoxicity of the treated samples towards *L. sativum, S. alba* and *S. saccharatum* was expressed as percentage of inhibition in the seed germination (GI), shoot germination (SI) and root growth (RI). The experiments were conducted using wastewater effluent samples as collected (Fig. 8a and b) and the same samples spiked with the target antibiotics (Fig. 8c and d), in order to elucidate the role of the oxidation products formed during ozonation in the phytotoxicity against the three plants species. The raw wastewater samples did not cause any GI, whereas an inhibition effect was observed in the ozonated samples, with *S. saccharatum* being the only seed showing a GI in the three sets of experiments (Fig. S5).

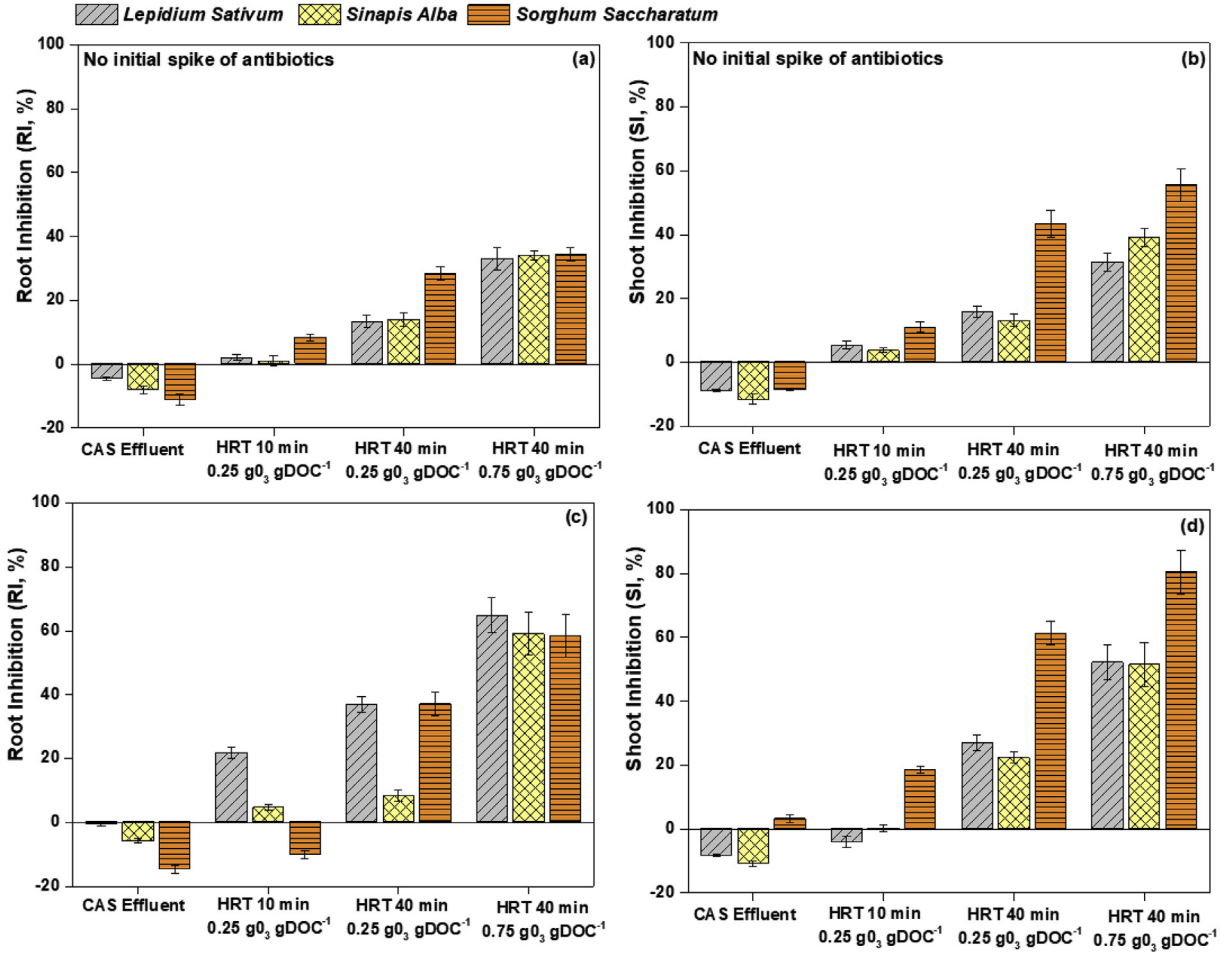

Fig. 8. Root growth inhibition (RI) and shoot growth inhibition (SI) of *Lepidium sativum*, *Sinapis alba* and *Sorghum saccharatum*, before and after continuous mode ozonation of wastewater samples, as collected or spiked at 100 μg L^{-1}, using the selected experimental conditions: 0.25 gO_3 gDOC $^{-1}$ and HRT of 10 min; 0.25 gO_3 gDOC $^{-1}$ and HRT of 40 min; and 0.75 gO_3 gDOC $^{-1}$ and HRT of 40 min. Experimental conditions: Matrix: secondary treated effluents; pH 7.3–7.8; T = 24 ± 1 °C.

The main finding of the phytotoxicity assessment (Fig. 8) was that the higher ozone doses or contact times increased the phytotoxicity levels of the treated samples. The inhibition expressed as SI and RI percentages in the spiked experiments were higher than those observed in the non-spiked experiments. According to Fig. 8, the highest SI and RI were observed in the experiment applying ozone dose of 0.75 gO_3 $gDOC^{-1}$ with HRT of 40 min. These results are in agreement with those obtained by Michael-Kordatou et al. (2017), showing that ozonation resulted in the augmentation of the phytotoxicity (i.e. root and shoot inhibition) even after 15 min of treatment, indicating that dE_fOM oxidation products were potentially more toxic than the original matrix. This behaviour might be due to direct oxidation of various organic constituents present in the dE_fOM of the wastewater samples or to their oxidation via a hydroxyl radical-mediated mechanism. The high values of DOC and COD of secondary treated effluent used for the implementation of the experiments are an indication of the existence of those organic constituents. It is well known that a number of oxidation products can be formed during ozonation from the oxidation of both the examined antibiotics and the dE_fOM present in the secondary-treated wastewater effluents (Von Sonntag and Von Gunten, 2012; Lee and Von Gunten, 2016; Michael-Kordatou et al., 2017). In fact, the low DOC removal could be correlated with the existence of ozonation products in the treated samples, which could contribute to the increased phytotoxicity of the samples. Also, results obtained from other studies using a variety of toxicological tests indicated that ozonation may cause other biological effects, e.g. toxicity and genotoxicity (Stalter et al., 2010).

The above findings clearly indicate that the ozonation treatment is insufficient to completely mineralize the wastewater and poses risks of producing oxidation products with potentially phytotoxic effects, in particular when the most severe operating conditions are tested. Considering that nowadays ozonation finds an increasing application in full-scale UWTPs, more research should be performed on this issue in order to identify the specific oxidation products that may be responsible for the observed phytotoxic effects.

4. Conclusions

The findings of this study demonstrated that ozonation of wastewater operated at continuous mode constitutes a promising treatment technology for the elimination of the selected A&ARB&ARGs. The results revealed that low ozone doses accompanied by prolonged treatment time was needed to eliminate the parent compounds of the examined antibiotics to concentration below their LOD in the spiked experiments, whereas the mildest conditions (i.e. lower HRT of 10 min for the same ozone dose of 0.125 gO_3 $gDOC^{-1}$) were sufficient to degrade the parent compounds in the non-spiked experiments. No substantial mineralization was observed at the end of the treatment time at all experimental conditions examined, a fact that may explain the enhanced phytotoxicity of the ozonated samples towards the tested plant species. Ozonation operated at high HRT and ozone doses (HRT 40 min; 0.25 gO_3 $gDOC^{-1}$) was capable of inactivating total as well as TMP- or SMX-resistant *E. coli*, with the simultaneous reduction of the abundance of the examined genes. However, regrowth cannot be avoided after ozonation, as it was demonstrated that living cells might still exist in the treated samples even after prolonged treatment. Overall, it was shown that ozonation upon its proper optimisation constitutes an efficient treatment option for the removal of antibiotic-related microcontaminants from wastewater effluents. Nevertheless, it is suggested that after ozonation, post-treatment by biological filtration or activated carbon adsorption, may overcome the main limitations of this process in relation to phytotoxicity. Also, more studies are needed to establish conditions capable of limiting regrowth of ARB after ozonation. These efforts will contribute to improving the quality of the treated wastewater, while minimising the potential risks of spread of antibiotic resistance in the receiving environment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.05.025.

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