Antibiotic Resistance spread potential in urban wastewater effluents disinfected by UV/H_2O_2 process

Giovanna Ferro[1], Francesco Guarino[2], Stefano Castiglione[2], Luigi Rizzo[1]*.

[1]Department of Civil Engineering, University of Salerno, Via Giovanni Paolo II, 132, 84084 Fisciano (SA), Italy

[2]Department of Chemistry and Biology, University of Salerno, Via Giovanni Paolo II, 132, 84084 Fisciano (SA), Italy

*Corresponding author: Tel.: +39089969334; fax: +39089969620

E-mail address: l.rizzo@unisa.it

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Abstract

Urban wastewater treatment plants (UWTPs) are among the main hotspots of antibiotic resistance (AR) spread into the environment and the role of conventional and new disinfection processes as possible barrier to minimise the risk for AR transfer is presently under investigation. Accordingly, the aim of this work was to evaluate the effect of an advanced oxidation process (AOP) (specifically UV/H_2O_2) on AR transfer potential. UV/H_2O_2 disinfection experiments were carried out on real wastewater samples to evaluate the: i) inactivation of total coliforms, *E. coli* and antibiotic resistant *E. coli* as well as ii) possible removal of target antibiotic resistance genes (ARGs) (namely, *bla*$_{TEM}$, *qnr*S and *tet*W). In particular, DNA was extracted from both antibiotic resistant *E. coli* bacterial cells (intracellular DNA), grown on selective culture media, and the whole water suspension (total DNA) collected at different treatment times. Polymerase chain reaction (PCR) assay was performed to detect the absence/presence of the selected ARGs. Real Time quantitative Polymerase Chain Reaction (qPCR) was used to quantify the investigated ARGs in terms of copies mL^{-1}. In spite of the bacterial inactivation and a decrease of ARGs in intracellular DNA after 60 min treatment, UV/H_2O_2 process was not effective in ARGs removal from water suspension (total DNA). Particularly, an increase up to 3.7x10^3 copies mL^{-1} (p>0.05) of *bla*$_{TEM}$ gene was observed in total DNA after 240 min treatment, while no difference (p>0.05) was found for *qnr*S gene between the initial (5.1x10^4 copies mL^{-1}) and the final sample (4.3x10^4 copies mL^{-1}). On the base of the achieved results, the investigated disinfection process may not be effective in minimising AR spread potential into the environment. The death of bacterial cells, which results in DNA release in the treated water, may pose a risk for AR transfer to other bacteria present in the receiving water body.

Keywords: advanced oxidation processes, antibiotic resistant bacteria, antibiotic resistance genes, DNA, qPCR

1. Introduction

Antibiotic resistance (AR) has become an ongoing clinical and public health issue of concern worldwide (WHO 2014). Depending on the scenario, failing to tackle AR threat will mean that the world population by 2050 may decrease by 11 million (if resistance rates will be successfully kept at a relatively low rate) and 444 million (it reflects a scenario for a world with no effective antimicrobial drugs) than it would otherwise be in the absence of AR (Taylor et al., 2014). Moreover, the world economy would be smaller by between 0.06% and 3.10%, again depending on the scenario, and world Gross Domestic Product losses may result in a cumulative loss that ranges between $2.1 trillion and $124.5 trillion. AR threat can be successfully tackled only if the environmental issue, and not only the clinical one, is also addressed. The release of antibiotics into the environment through treated and untreated wastewater disposal contributes to the selection and development of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Rizzo et al., 2013). Hospital effluents (Varela et al., 2013; Rodriguez-Mozaz et al., 2015) and urban wastewater treatment plants (UWTPs) are among the most serious sources of AR spread into the environment due to several suitable conditions, such as a high cell density and a contamination with both antibiotics and bacteria (Rizzo et al., 2013). Screening campaigns have revealed the presence of ARGs in various environmental compartments. β-lactams (bla_{TEM}) and vancomycin (*van*A) resistance genes have been detected both in hospital effluents and in raw and treated effluent of UWTPs receiving hospital sewage (Narciso-da-Rocha et al., 2014). Tetracycline (*tet*O and *tet*W) and sulfonamide (*sul*-I) resistance genes have been found in the range of non-detectable–10^8 copies mL^{-1} in the final effluents of five UWTPs in Michigan and until 10^9 copies g^{-1} in biosolids (Munir et al., 2011). Significant ARGs concentrations (10^3–10^5 copies mL^{-1}) have been measured in the treated effluents

of two UWTPs in northern China (Mao et al., 2015). *Sul*-I, *sul*-II, tetO and *tet*W have been detected in dairy lagoon water, irrigation ditch water, urban/agriculturally impacted river sediments; *tet*O and *tet*W also in treated drinking water and recycled wastewater (Pruden et al., 2006). Another study showed how high concentrations of *bla*$_{TEM}$, *qnr*S, *erm*B, *sul*-I and *tet*W, still present in the treated UWTP effluent, have affected the receiving river (Rodriguez-Mozaz et al., 2015). The occurrence of ARGs in different environmental compartments (Schwartz et al., 2003) is a matter of concern being associated with mobile genetic elements which can promote horizontal gene transfer (Davies and Davies, 2010). Different mechanisms that take place in UWTPs and cause ARGs transfer potential are under investigation (Rizzo et al., 2013), although horizontal gene transfer is regarded as the most important one (McKinney and Pruden, 2012). Moreover a few works deal with the effect of disinfection processes in terms of potential spread of AR by means of molecular biology methods (Öncü et al., 2011). Just one paper reports the effect of photocatalysis on the potential to induce ARGs transfer within sub-lethally injured ARB, but by cultivation-based methods (Dunlop et al., 2015).

The above mentioned studies point out the importance to find effective solutions for minimising AR spread. In this scenario, disinfection process can be considered as possible barrier to control the diffusion of AR into the environment, especially in the light of reuse of treated wastewater for agricultural purposes (Fatta-Kassinos et al., 2011; Ferro et al., 2015). In spite of successful ARB inactivation by Advanced Oxidation Processes (AOPs) (Fiorentino et al., 2015; Karaolia et al., 2014; Rizzo et al., 2014a; Tsai et al., 2010), there are still crucial gaps to be filled about the potential spread of AR. Some questions to be addressed concern the fate of ARGs and extracellular materials during and after the disinfection process. To date a few works are available about the effect of disinfection in terms of ARGs removal or their fate when all pathogens have been inactivated (Guo et al., 2013; McKinney

and Pruden, 2012; Munir et al., 2011; Yuan et al., 2015; Zhuang et al., 2015). The above mentioned studies have been mainly focused on conventional disinfection processes, namely chlorination and UV radiation; they may not be successful in ARGs removal, at least with realistic doses of disinfectants or UV radiation. Moreover target ARGs were mostly quantified regardless of DNA belonged to survived bacterial cells (namely intracellular DNA) or released from inactivated bacterial cells. In our recently published paper, the effect of UV/H_2O_2 process on an antibiotic resistant *E. coli* strain selected from urban wastewater and inoculated in deionized water was evaluated through cultivation methods Vs PCR based methods (Ferro et al., 2016). In spite of total inactivation of antibiotic resistant *E. coli* strain, target ARG (*bla*$_{TEM}$) was still present in water suspension after 300 min treatment. According to the results achieved and considering possible effects of real aqueous matrices and indigenous bacterial population, the effect of UV/H_2O_2 process on indigenous ARB in real wastewater is worthy of investigation. In this context, the aim of the present work was to investigate the effect of UV/H_2O_2 process in the simultaneous (i) inactivation of indigenous ARB and (ii) the removal of target ARGs, namely *bla*$_{TEM}$, *qnr*S and *tet*W. Particularly, the effect of the disinfection process on intracellular and total DNA was investigated. Experiments were carried out at laboratory scale, working on real UWTP effluents collected downstream of the biological process.

2. Material and methods

2.1 Experimental design

Real wastewater samples were freshly collected from the effluent of the biological process (activated sludge) and undergone UV/H_2O_2 process until to 240 min. Both bacterial inactivation and ARGs fate were monitored during the treatment. In

particular, aliquots of treated wastewater were spread onto selective culture media in order to monitor bacterial growth, and DNA (from here on “intracellular”) was extracted from antibiotic resistant *E. coli* after 24 h growth. Simultaneously, almost 450 mL of treated wastewater were filtered onto polycarbonate membranes (0.22 μm) in order to extract DNA (genetic material of live bacteria and/or released from inactivated bacteria) from wastewater (from here on “total”).

2.2 Wastewater samples

Wastewater samples were freshly collected from a large UWTP (700,000 population equivalent) placed in Salerno (Italy), from the effluent of the biological process (activated sludge) and upstream of disinfection unit. In order to reduce fluctuations and to get a more representative sample, 24 h composite samples were taken using an automatic sampling equipment (ISCO 2009 Sampler) programmed to collect 500 mL aliquots at each hour. UWTP samples were collected in sterilized glass bottles, transported refrigerated to the laboratory and analysed within 4 h. Three wastewater samples were collected in three consecutive days, characterized and the average values were the following: pH 7.6, 20.0 mg BOD_5 L^{-1}, 48.0 mg COD L^{-1}, 18.0 mg TSS L^{-1}, 7.9 mg Total N L^{-1}, 1.8 mg Total P L^{-1}. Initial bacterial densities were $3.6x10^4$ CFU mL^{-1} of total coliforms and $7.8x10^2$ CFU mL^{-1} of *E. coli* on average.

2.3 Disinfection experiments

UV/H_2O_2 experiments were carried out in a 2.2 L cylindrical glass reactor (13.0 cm in diameter) filled with 500 mL wastewater sample (5.0 cm water height), according to optimized working conditions in a previous study (Rizzo et al., 2014b). The reactor was placed in a water bath in order to keep a constant temperature of 25 °C and it was magnetically stirred during the experiments. A wide spectrum 250 W lamp equipped with a UV filter (Procomat, Italy) (main radiation emission in the

range 320-450 nm) fixed at 40 cm from the upper water level in the reactor was used as UV source. A spectrometer model HR-2000 from Ocean Optics (Florida, USA), equipped with cosine corrector with Spectralon diffusing material, was used to measure irradiance spectra of UV lamp. Wastewater samples were exposed to a range of UV doses (0-2.5x10^5 μW s cm^{-2}) by varying the exposure time from 0 to 240 min.

Prior to radiation exposure, one sample was collected and analysed (from here on T0). Then H_2O_2 at 30 wt% (Titolchimica, Italy) was used as received and diluted into the reactor; the working solution was stirred for three minutes in the dark to ensure homogenization. Initial dose of hydrogen peroxide was set to 20 mg L^{-1}, according to a previous work (Fiorentino et al., 2015) and the residual concentration was monitored by a colorimetric method based on the use of titanium (IV) oxysulfate (Sigma-Aldrich), which forms a stable yellow complex with H_2O_2 detected by absorbance measurements at 410 nm. Absorbance was measured using a spectrophotometer (PerkinElmer, USA) and was linearly correlated with H_2O_2 concentration in the range 0.1-100.0 mg L^{-1}. When decreases of hydrogen peroxide were measured, a suitable volume of the oxidant solution was added to the solution to keep constant the concentration. Catalase was added to water samples collected at different treatment times in order to eliminate residual H_2O_2: 1 mL sample was mixed with 20 μL of 2300 U mg^{-1} bovine liver catalase at 0.1 g L^{-1} (Sigma-Aldrich). H_2O_2 and catalase have been demonstrated to have no detrimental effects on bacterial viability at these concentrations (García-Fernández et al., 2012). Serial UV/H_2O_2 experiments were carried out (one for each treatment time) in order to perform molecular analyses with representative wastewater volume (450 mL).

2.4 Bacterial detection and count

A standard plate counting method was used through 10-fold serial dilutions in phosphate buffer saline (PBS) after an incubation period at 37 °C for 24 h. 50-100-200 μL samples or their serial dilutions were spread onto Tryptone Bile X–glucuronide Agar (TBX, Sigma-Aldrich). Particularly, Total Coliforms, *E. coli* and antibiotic resistant *E. coli* were counted. In order to count antibiotic resistant *E. coli*, TBX was supplemented with a mixture of three antibiotics (8 mg L^{-1} of ampicillin (AMP), 0.064 mg L^{-1} of ciprofloxacin (CIP), 8 mg L^{-1} of tetracycline (TET)), typically occurring in wastewater (Michael et al., 2013) and among the most used antibiotics in the geographical are where the UWTP is located (Assessorato alla Sanità della Regione Campania, 2014). Taking into account the current limitations about phenotypic resistance in environmental settings (Berendonk et al., 2015), antibiotic concentrations were selected on the base of the respective epidemiological cut-off values available in EUCAST database (EUCAST 2015). Antibiotics, all high purity grade (>99%), were purchased from Sigma-Aldrich. *E. coli* colonies that were able to grow on TBX agar supplemented with the mixture of antibiotics were regarded as resistant to antibiotics. The detection limit (DL) of this experimental method was 5 CFU mL^{-1}. Antibiotic resistant *E. coli* colonies were then used to extract intracellular DNA.

2.5 DNA extraction

DNA was extracted using the DNeasy® Blood & Tissue Kit (Qiagen; Milano, Italy) following the manufacturer's instructions. In particular DNA was extracted from bacterial cells at the following treatment times: 30 min (T30), 60 min (T60) and 90 min (T90). At 120 min it was not possible to harvest cells because no colonies grew on the plates. In particular, the intracellular DNA was extracted from all bacterial colonies grown on plates after each treatment time and resuspended in 1.0 mL of sterile water up to obtain 10^9 harvest cells useful for DNA extraction. Cellular density of water bacterial suspensions was spectroscopically measured (three

199 replicates) by optical density at 600 nm. Total DNA (e.g. genetic material of live
200 bacteria and/or released from inactivated bacteria) was extracted from UV/H_2O_2
201 treated wastewater samples collected at each treatment time.
202 450 mL of wastewater samples were filtered through membranes (polycarbonate,
203 0.22 μm pore size, 47 mm diameter, GE Healthcare). The filters were stored at -20
204 °C until DNA extraction. The filters were put in tubes and centrifuged for 10 min at
205 14,000 rpm; the resulting pellet was resuspended in 180.0 μL of extraction buffer
206 (ATL) and used for DNA extraction.
207 The nucleic acid quality and quantity were estimated on agarose gels and
208 spectrophotometrically at 260 nm (NanoDrop 2000C, NanDrop Technologies,
209 Willmington, DE). All DNA samples were stored at -20 °C.

210 ***2.6 Qualitative PCR analysis***

211 Specific primers were designed on the basis of literature (Rodriguez-Mozaz et al.,
212 2015) and used both for qualitative and quantitative PCR analyses. Qualitative PCR
213 reactions were performed to check the presence or absence of ARGs in UV/H_2O_2
214 treated and untreated wastewater samples. PCR reactions were performed in a
215 25.0 uL reaction mixture containing 10 ng of DNA and 0.5 U of Taq polymerase
216 (AmpliTaq® DNA polymerase, Applied Biosystems), according to the
217 manufacturer's instructions. PCR reaction cycling parameters were as follows: 5
218 min of initial denaturation at 94 °C, followed by 35 cycles at 94 °C for 1 min, 60 °C
219 for 1 min and 72 °C for 1 min. The final extension was set at 72 °C for 7 min. PCR
220 products were separated by electrophoresis on 1% agarose gels.

221 ***2.7 Quantification of ARGs***

222 DNA was amplified with specific primers (*bla*$_{TEM}$, *qnr*S, *tet*W, 16S, whose efficiency
223 was >99%) and iQSYBR Green Super Mix (Bio-Rad Laboratories, Milano, Italy) in
224 quantitative qPCR assays. qPCR assays were carried out in triplicate for each

sample and performed twice, using the iQ5 (Biorad-Laboratories, Milano, Italy). A negative (no-template) control was used to test for false-positive results and/or contaminations. Moreover, DNA of *E. coli* strain was used as positive control to check PCR conditions. PCR reactions cycling parameters were as follows: an initial denaturing step at 95 °C for 3 min followed by 45 cycles, with one cycle consisting of denaturation at 95 °C for 15 s, annealing temperature was 60 °C for 30 s. Data collection and analysis were performed using the Optical System Software (iQ5 version 2.0). To quantify ARGs copies number, standard curves were created, using the 16S rRNA as standard, and the following steps were performed: i) estimation of the genome size (the *E. coli* genome size was around 4.6 Kbp) (Blattner et al., 1997; Grenier et al., 2014); ii) estimation of the genome mass; iii) calculation of the mass of DNA containing the copy of interest; iv) calculation of the concentration of DNA needed to achieve the copy of interest; v) preparation of serial DNA dilutions. Diluted DNA was used for qPCR assays. Since the detection limit for 16S rRNA was at 10^{-4} ng, the standard curves were generated using a 3-fold dilution series to produce four quantities of *E. coli* DNA ranging from 1 ng ($1.8x10^{-5}$ copies of 16S rRNA) to 10^{-3} ng ($1.8x10^{-2}$ copies). The threshold cycle (Ct) values of ARGs were correlated with the standard curves in order to estimate the copies number.

2.8 Statistical analysis

One-way analysis of variance (ANOVA) was performed using SigmaPlot 12.0 software package followed by a Holm-Sidak post-hoc test, with $p < 0.05$ as the significance cut-off, in order to evaluate the effect of UV/H_2O_2 on ARGs copies number.

3. Results

3.1 Bacterial inactivation

Antibiotic resistant *E. coli* inactivation by UV/H_2O_2 disinfection is plotted in Figure 1. The DL (5 CFU mL^{-1}) was achieved after 90 min treatment. Within 30 min of disinfection, a high consumption of hydrogen peroxide was observed and no Log reduction was observed. The consumption of hydrogen peroxide (4.22 mg L^{-1}) is possibly related to the oxidation of organic matter, nitrogen and other oxidable compounds typically occurring in wastewater. A suitable volume of H_2O_2 solution was added to keep constant the concentration. After the addition of the oxidant, a higher inactivation was observed.

Figure 1.

During treatment antibiotic resistant *E. coli* population (initial density of $7.0x10^1$ CFU mL^{-1}) decreased at a higher rate compared to *E. coli* and total coliforms (Figure 2). Particularly, the percentage of antibiotic resistant *E. coli* with respect to total coliforms and *E. coli* populations, respectively 0.2% and 9.9% increased within the first 10 min of treatment. This percentage with respect to total coliforms increased at T20, then decreased at T30 and T45 and increased at T60 (Figure 2). The percentage of antibiotic resistant *E. coli* increased throughout the treatment (from 0.2% to 2.3%), and the DL was achieved at T90 when a fraction of *E. coli* and total coliforms still survived the disinfection process. Therefore *E. coli* may be a suitable indicator of antibiotic resistant *E. coli* fraction occurrence.

Figure 2.

3.2 Occurrence of ARGs

Qualitative PCR assays were performed on total and intracellular DNA in order to detect the presence or absence of target ARGs in wastewater samples exposed to UV/H_2O_2 disinfection process. Total DNA was extracted from wastewater samples collected at different times and supposedly containing genetic material of living residual bacterial cells and/or released from inactivated bacterial cells due to oxidation/disinfection process. Results of electrophoretic run of PCR products on gel agarose are shown in Figure 3.

Figure 3.

PCR highlighted the presence of 16S rRNA gene both as total and intracellular. The intensity of the band of the 16S rRNA gene (total DNA) did not show any variation as treatment time increased (only a slight increase was observed after 240 min). The *bla*$_{TEM}$ gene, initially present in intracellular DNA, seem to be removed from UV/H_2O_2 process, while it may be absent in total DNA or present at not-detectable concentration. As the *bla*$_{TEM}$ gene, also the *qnr*S gene was detected in intracellular DNA and the intensity of the band decreased as treatment time increased. Also in this case an effect of disinfection process on the removal of the *qnr*S gene in intracellular DNA may not be excluded. In the case of the *qnr*S gene in total DNA, a slight presence was observed at T0 and T30, then an absence at T60 and T90, and a new mild presence at T120 and T240. The *tet*W gene was detected in intracellular DNA and the band is still visible after 90 min treatment.. As for the *bla*$_{TEM}$ gene, the *tet*W gene is likely to be absent or present in total DNA at very low concentration that would make it not detectable through a PCR assay.

3.3 ARGs quantification

qPCR analyses were carried out in order to quantify the ARGs copies number, using the 16S rRNA gene as standard. The Ct values of ARGs were correlated with

297 the 16S rRNA standard curves in order to estimate the copies number (R^2 value =
298 0.992).

299 *3.3.1 Effect of UV/H_2O_2 process on intracellular DNA*

300 The effect of UV/H_2O_2 process on antibiotic resistant *E. coli* was also monitored
301 through the evaluation of intracellular DNA. Figure 4 shows ARGs copies number
302 normalized with respect to the corresponding residual antibiotic resistant *E. coli*
303 colonies where DNA was extracted from.

304 **Figure 4.**

305 While *bla*$_{TEM}$ gene copies number slightly decreased from T0 to T30, *qnr*S gene
306 copies number did not show any variation during the same treatment time. However
307 bacterial inactivation was higher than ARGs removal, so that a significant apparent
308 increase (p<0.05) was observed when data are plotted as copies number per
309 CFUs. Disinfection process affected *bla*$_{TEM}$ gene in agreement with the results from
310 Figure 3: $2.8x10^0$ copies CFU^{-1} remained after 90 min treatment. UV/H_2O_2 process
311 also affected the removal of *qnr*S gene, which was not detected at T60, in
312 agreement with PCR results (Figure 3). Unlike the other two genes, *tet*W gene in
313 intracellular DNA was not detected by means of qPCR.

314 *3.3.2 Effect of UV/H_2O_2 process on total DNA*

315 Total DNA was also monitored during UV/H_2O_2 process in order to evaluate the
316 possible release of ARGs following bacterial cells inactivation. All ARGs in total
317 DNA were detected at each treatment time until to the end of the experiment (240
318 min) (Figure 5).

319 **Figure 5.**

Unlike intracellular DNA results, the higher relative abundance in the water solution was observed for the *qnr*S gene. This result can be explained by the possible occurrence of both: other bacteria encoding *qnr*S gene and suspended DNA. By comparing the initial total DNA ($5.1x10^4$ copies mL^{-1}) with the final one ($4.3x10^4$ copies mL^{-1}), no statistically significant difference was found ($p>0.05$). Possibly, the slight increases/decreases observed during treatment may be regarded as an overall mild effect of UV/H_2O_2 on *qnr*S gene removal.

Differently from PCR results, qPCR revealed the presence of the *bla*$_{TEM}$ gene in total DNA. Overall no Log removal was obtained after 240 min of treatment. In spite of an inactivation of indigenous bacteria and among them ARB, the disinfection process did not significantly affect the occurrence of *bla*$_{TEM}$ gene in total DNA. The slight variations observed at each treatment time may suggest that the investigated process would have affected the removal of *tetW* gene to some extent but a clear trend was not observed. However, by comparing the initial concentration ($6.0x10^1$ copies mL^{-1}) with the final one ($1.1x10^1$ copies mL^{-1}), a statistically significant decrease ($p<0.05$) was obtained.

4. Discussion

UV/H_2O_2 process was investigated in the present study as possible option to control the potential spread of AR into the environment. In spite of a successful inactivation of total coliforms, *E. coli* and antibiotic resistant *E. coli* (Figure 2), *bla*$_{TEM}$, *qnr*S, and *tet*W genes were still present in wastewater after 240 min of treatment (Figure 5). While UV/H_2O_2 process showed an effect on the removal of ARGs in intracellular DNA, the side effect of the treatment may be the release of genetic material, and specifically ARGs in water solution which may promote AR transfer in the environment.

345 Similar inactivation trends of *E. coli* by UV/H_2O_2 have been observed in scientific
346 literature. Argulló-Barceló and co-workers (2013) investigated solar driven UV/H_2O_2
347 process (same H_2O_2 dose) and DL was achieved after 180 min treatment; in that
348 case, experiments were carried out in compound parabolic collector reactors filled
349 with 10 L of wastewater. Compared to our previous work dealing with the
350 investigation of the effect of solar driven AOPs on real wastewater spiked with an
351 antibiotic resistant *E. coli* strain, 150 min of solar radiation were needed to get the
352 DL (Fiorentino et al., 2015). In that case, experiments were performed with the
353 same dose of hydrogen peroxide (20 mg L^{-1}), but borosilicate glass bottles with a
354 total volume of 250 mL were used and the initial antibiotic resistant *E. coli*
355 concentration was 10^6 CFU mL^{-1}.

356 Only a few works in scientific literature have addressed the effect of
357 water/wastewater disinfection processes on the potential of AR transfer and most of
358 them deal by conventional disinfection processes (namely, chlorination, UV
359 radiation and ozonation). Munir and co-workers (2011) monitored the release of
360 ARGs, among them *tet*W, in full scale UWTP effluents and observed no significant
361 removal (p>0.05) between pre-disinfected and post disinfected samples when
362 chlorination and UV radiation were implemented as disinfection processes. Higher
363 removal (1-3 Log units) of *tet*W gene (from not detectable to 10^4 copies mL^{-1}) was
364 observed in samples after MBR and UV radiation processes, while other ARGs
365 were still present in the final effluents (Munir et al., 2011). Rodriguez-Mozaz et al.
366 (2015) monitored the occurrence of ARGs in UWTP and observed that *erm*B and
367 *tet*W genes (in terms of copies number normalized to the 16S rRNA gene copies
368 number) decreased (p<0.05) as a result of wastewater treatment, while *bla*$_{TEM}$, *qnr*S
369 and *sul*I genes increased. Both quoted studies point out (i) a lower resistance of
370 *tet*W gene to the treatment compared to the other investigated ARGs and (ii) that
371 the effect of disinfection process depends on the target ARG.

Zhuang et al. (2015) compared chlorination, UV radiation and ozonation in the removal of target ARGs, namely *intI*1*, tet*G*, sul*1, from a real urban wastewater. The most effective was chlorination (chlorine dose of 160 mg L^{-1} and contact time of 120 min for 3.0-3.2 Log reductions of ARGs). Lower removals were achieved with UV radiation (UV dose of 12.5 mJ cm^{-2} for 2.5-2.7 Log reductions) and with ozonation (ozone dose of 177.6 mg L^{-1} for 1.7-2.6 Log reductions). The *tet*G gene (from 10^5 to 10^6 copies mL^{-1}) was less abundant than the *tet*W gene in our study ($6.0x10^1$ copies mL^{-1}) and was removed more easily than other ARGs by ozonation. However, just 2.6 Log reductions were observed with ozone dose as high as 177.6 mg L^{-1}. In spite of no-realistic disinfectants doses, ARGs were still present in the treated wastewater, in agreement with our results. The higher effect of chlorine in terms of ARGs removal was linked to its ability to penetrate the cell envelope (Zhuang et al., 2015), but at not realistic dose. Another study on plasmid DNA isolated from a multi-resistant *E. coli* HB101 and treated by three disinfection processes showed that chlorine (0.5 - 5.0 mg L^{-1}) did not affect the cell transformability, while titanium dioxide (0.17 mg L^{-1}) and ozone (1.0 - 4.2 mg L^{-1}) induced damage in the plasmid DNA structure and then decreased the cell transformability (Öncü et al., 2011). This study also revealed that the removal of all plasmid DNA required an ozone dose as high as 4.2 mg L^{-1} and a photocatalytic treatment time as long as 75 min. Multi-resistant *E. coli* HB101 was spiked (10^7 CFU mL^{-1}) in a synthetically contaminated cow manure treated by both Fenton (without acidification) and ozonation processes (Cengiz et al., 2010). In spite of very high doses of H_2O_2 and Fe^{2+}, such as 50 and 5 mM respectively, just 0.56 Log reduction was obtained within 24 h of treatment; 1.83 Log reduction was achieved with doses of ozone as high as 3.12 mg per gram of manure slurry. Also the effect on the *tet*M gene was monitored during Fenton and ozonation processes: increased exposure to oxidants resulted in reduced band intensity (Cengiz et al., 2010). Although the matrix was different from that analysed

399 in our study, high doses of oxidants did not result in ARB inactivation and ARGs
400 removal. In disagreement with that, a study by Oh et al. (2014) shows that
401 ozonation may reduce *E. coli* DH5α and its multi-resistant gene pB10 from a
402 synthetic wastewater more than 90% with an ozone dose of 3 mg L^{-1} within 15 min:
403 the addition of some catalysts, such as peroxymonosulfate, persulfate, hydrogen
404 peroxide (concentration of 1 mg L^{-1}), resulted in better performances. Anyway no
405 information are available about the working volume, the initial concentrations of
406 ARB and ARGs. Finally, the effect of photocatalysis (TiO_2 immobilised onto
407 borosilicate glass plates: 0.5 mg cm^{-2}) on the transfer of ARGs in urban wastewater
408 by means of a cultivation method was investigated: the authors highlighted the
409 importance of applying photocatalytic treatment “long enough” to avoid post
410 treatment recovery from sub-lethal injury and the highly undesirable transfer of
411 ARGs amongst bacteria during wastewater treatment (Dunlop et al., 2015).

412 In our recently published paper, UV/H_2O_2 process did not result in any relevant
413 effect on the removal of DNA extracted from cell cultures of an antibiotic resistant *E.*
414 *coli* strain (Ferro et al., 2016). Differently, with respect to the same target gene, in
415 the present study, disinfection process affected bla_{TEM} gene in intracellular DNA
416 ($2.8x10^0$ copies CFU^{-1} after 90 min treatment). This different effect may be due to
417 the fact that, in the former work, DNA was extracted from cell cultures of an
418 inoculated strain that has been previously selected as resistant to a mixture of
419 antibiotics, among which ampicillin. Differently, in the present study, DNA was
420 extracted from cell cultures of all indigenous antibiotic resistant *E. coli* cells.
421 Moreover, in agreement with the present work and in spite of the inactivation of the
422 spiked strain, bla_{TEM} gene was still present after 300 min treatment ($2.8x10^6$ copies
423 mL^{-1}). Although the different water matrix (distilled water Vs real wastewater),
424 UV/H_2O_2 process was not effective on bla_{TEM} gene removal because (i) the oxidant
425 dose may be low (20 mg L^{-1}); (ii) the mechanism of action of hydroxyl radicals on

426 DNA may be mild. A recent work by Zhang et al. (2016) focused on the effect of
427 Fenton oxidation and UV/H_2O_2 process on the removal of *intI*1, *sul*1, *tet*G, *tet*X and
428 16S rRNA genes from UWTP effluent, whose concentrations ranged from 10^5 to 10^9
429 copies mL^{-1}. The authors pointed out that in UV/H_2O_2 experiments (30 min
430 irradiation time, 0.01 mol L^{-1} hydrogen peroxide concentration and pH 3.5), all
431 ARGs were reduced of 2.8-3.5 Log units. This significant reduction is due to
432 different operative conditions compared to those investigated in our study. First the
433 reactor was equipped with a low-pressure 254 nm (UV-C) mercury vapour lamp,
434 while a wide spectrum lamp, (main radiation emission range from 320 to 450 nm
435 (UV-A)), was used in our work. This entails different treatment times as well as
436 mechanisms of action: UV-C light can penetrate the UV-transparent structures in
437 the cell and primarily be absorbed by the nucleobases comprising DNA and RNA
438 (Dodd, 2012). Differently, the effect on DNA in the present work is expected to be
439 mainly due to hydroxyl radicals produced during UV/H_2O_2 process. Another
440 difference is due to the pH of wastewater investigated (3.5 Vs natural pH (7.6) in
441 our study); the authors evaluated the effect at pH 3.5 because of the lower removal
442 (1.5-2.3 Log) achieved at pH 7 (Zhang et al., 2016). Finally the dose of H_2O_2 was
443 set at 0.01 mol L^{-1} (340 mg L^{-1}) which is much higher than the dose applied in our
444 study (20 mg L^{-1}). A so high concentration is not realistic because does not make
445 the investigated technology cost-effective and would be toxic to plants (either, in the
446 receiving water body or eventually irrigated with the effluent in agricultural
447 wastewater reuse practice) (Sichel et al., 2009). A concentration as low as 20 mg L^{-1}
448 was chosen in our work according to previous studies (Agulló-Barceló et al., 2013;
449 Bichai et al., 2012; Fiorentino et al., 2015), but also in the light of a possible
450 wastewater reuse as a cost-effective technology.

5. Conclusions

The effect of UV/H_2O_2 process on the inactivation of indigenous bacteria (among them antibiotic resistant *E. coli*) and ARGs was investigated in real urban wastewater. In spite of the DL of antibiotic resistant *E. coli* and the other investigated cultivable bacteria was reached after 90 and 240 min treatment, respectively, ARGs were still present in the wastewater sample at the end of the treatment. In particular, UV/H_2O_2 process did not affect the copies number of bla_{TEM} gene and poorly affected (p>0.05) the copies number of *qnr*S gene ($4.3x10^4$ copies mL^{-1} after 240 min treatment). However, the investigated disinfection process allowed to obtain a significant decrease (p<0.05) of the copies number of the tet_W gene ($1.1x10^1$ copies mL^{-1}) after 240 min treatment. Simultaneously UV/H_2O_2 process showed a successful effect on the removal of ARGs in intracellular DNA.

According to these results, UV/H_2O_2 process may not be an effective disinfection process to limit or minimise the potential spread of AR under realistic conditions. The survived ARB and/or free ARGs released from inactivated bacteria may contribute to AR transfer into the environment (namely, aquatic ecosystems in which the treated wastewater is disposed off or irrigated soils/crops in case of wastewater reuse).

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594

595 **Figures captions**

596 **Figure 1.** Antibiotic resistant *E. coli* inactivation by UV/H_2O_2 process.

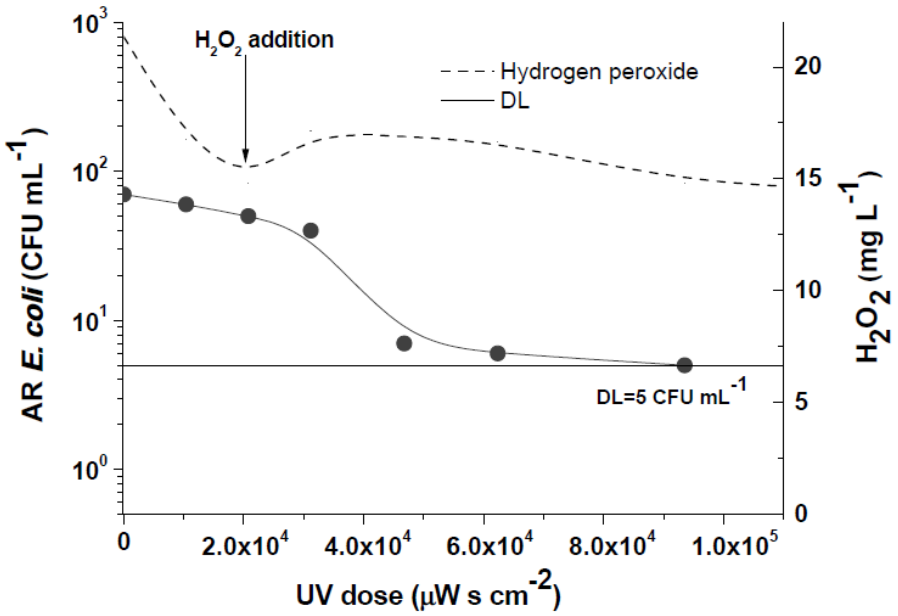
597 **Figure 2.** Relative abundance of bacterial population during UV/H_2O_2 treatment and
598 Antibiotic Resistant *E. coli* percentages calculated with respect to total coliforms
599 (upper placed values) and *E. coli*.

600 **Figure 3.** Results of electrophoretic run of PCR product on gel agarose: 16S rRNA
601 gene (a), *bla*$_{TEM}$ gene (b), *qnr*S gene (c), *tet*W gene (d). P.C. = positive control; N.C.
602 = negative control.

603 **Figure 4.** Concentrations of ARGs in intracellular DNA as a function of treatment
604 times of UV/H_2O_2 process. A, B, C, a, b, indicate significantly (p<0.05) different
605 groups of normalized ARGs (Log copies CFU^{-1}) among the four investigated
606 treatment times.

607 **Figure 5.** Concentrations of ARGs in total DNA as a function of treatment times of
608 UV/H_2O_2 process; a, b, c, d, e, f indicate significantly (p<0.05) different groups of
609 ARGs (Log copies mL^{-1}) among the six investigated treatment times.

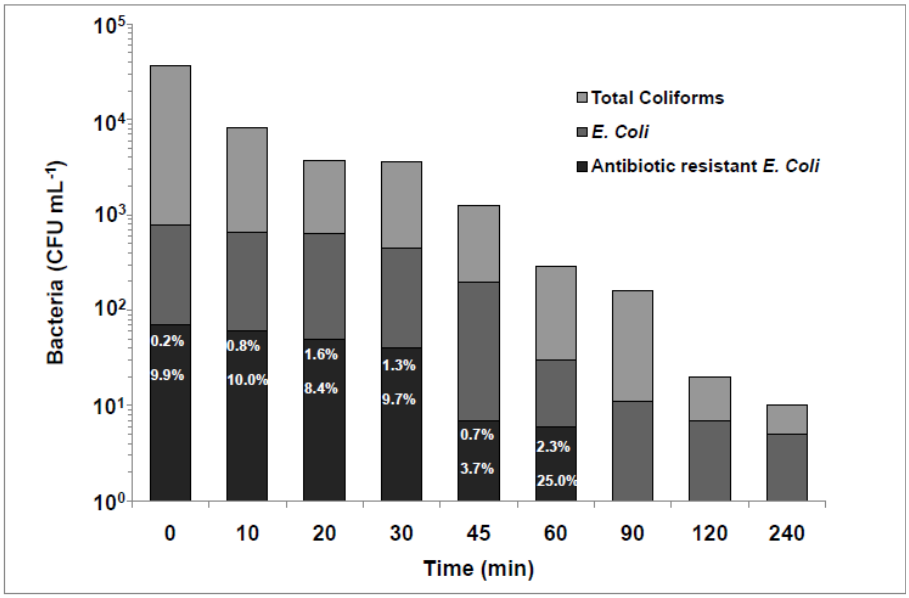
610



611

612 **Figure 1.** Antibiotic resistant *E. coli* inactivation by UV/H_2O_2 process.

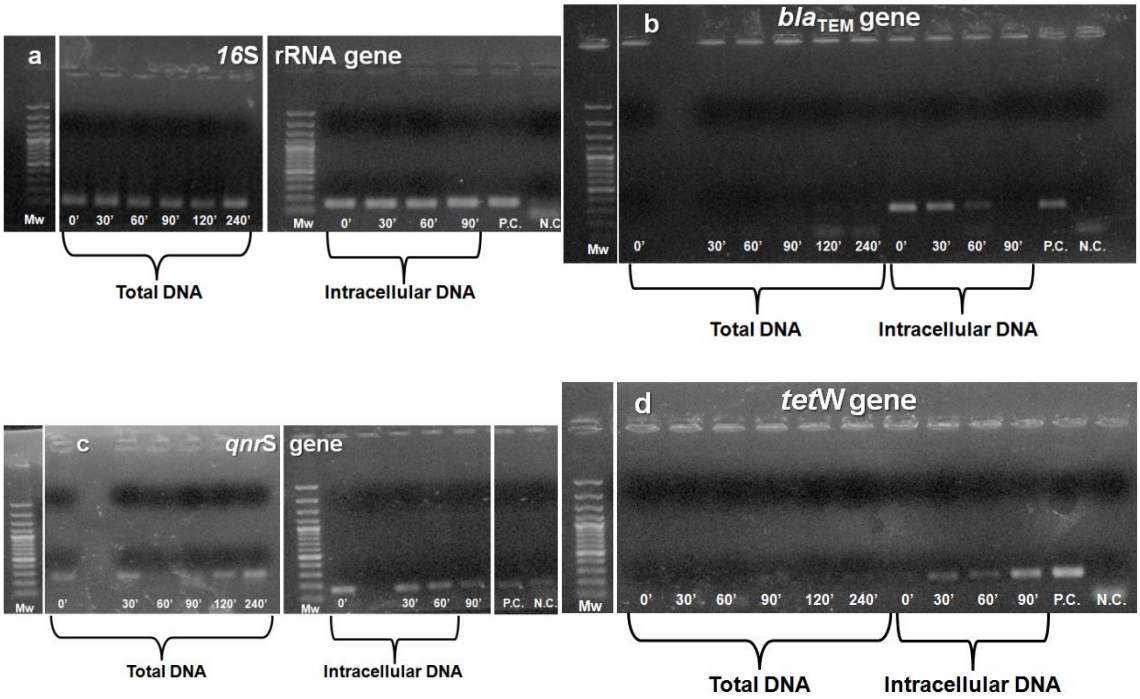
613



614

615 **Figure 2.** Relative abundance of bacterial population during UV/H_2O_2 treatment and Antibiotic
616 Resistant *E. coli* percentages calculated with respect to total coliforms (upper placed values) and *E.*
617 *coli*.

618



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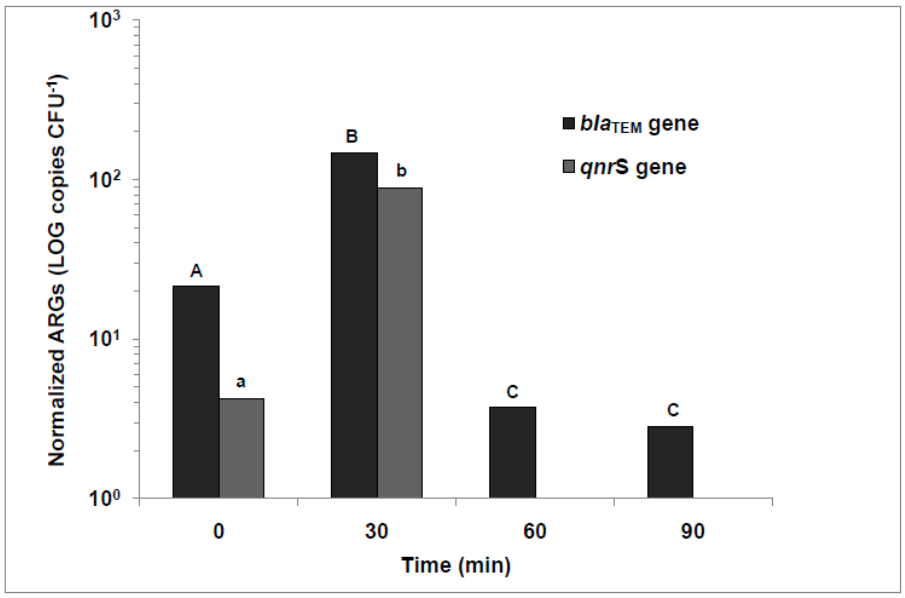

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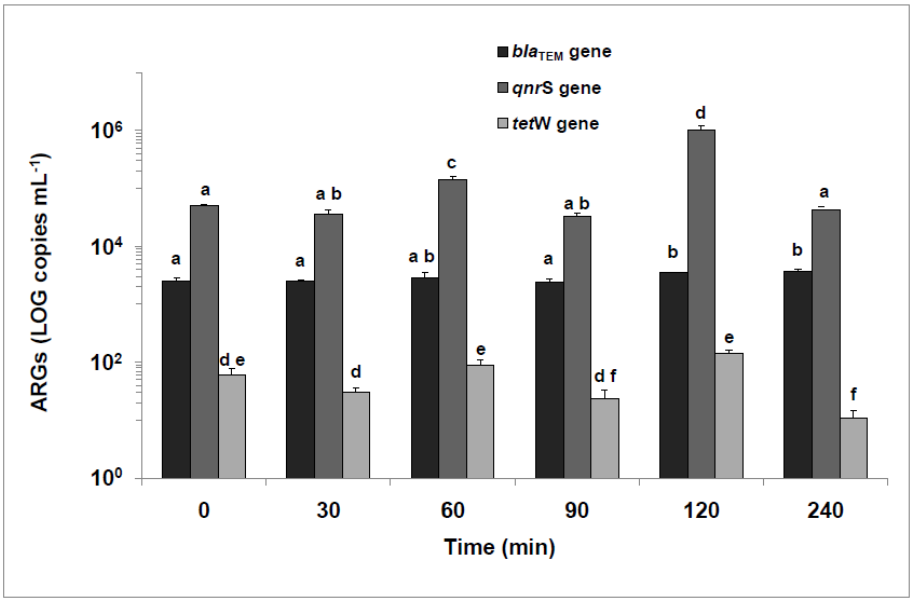

628

629 **Figure 5.** Concentrations of ARGs in total DNA as a function of treatment times of UV/H_2O_2 process.
630 a, b, c, d, e, f indicate significantly (p<0.05) different groups of ARGs (Log copies mL^{-1}) among the
631 investigated treatment times.

632