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WASTEWATER DISINFECTION BY AOPs: EFFECT ON ANTIBIOTIC RESISTANCE AND CONTAMINANTS OF EMERGING CONCERN

DISINFEZIONE DELLE ACQUE REFLUE MEDIANTE AOPs: EFFETTO SULL'ANTIBIOTICO RESISTENZA E SUI CONTAMINANTI EMERGENTI

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To my family for their
daily emotional support

"The unexamined life is not worth living." (Socrates)

"Do not hurry; do not rest." (J.W. von Goethe)

"Today is only one day in all the days that will ever be. But what will happen in all the other days that ever come can depend on what you do today." (E. Hemingway)

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ABSTRACT

Urban wastewater treatment is called for facing different challenges worldwide, such as energy saving, resource recovery and reuse. Conventional wastewater treatments that are the state of art in most world regions, are not designed to remove the so-called contaminants of emerging concern (CECs) whose concentrations range from high ng L^{-1} to low μg L^{-1}. Among CECs antibiotics play an important role because they may promote the selection and the diffusion of antibiotic resistance patterns into the environment, such as the development of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs). Due to the incomplete removal by conventional treatments, CECs, ARB and ARGs occur in treated wastewater and may exert chronic toxic effects in aquatic and terrestrial organisms as well as they may pose environmental and public health risks. This scenario raises major concern in the light of wastewater reuse that is regarded as an interesting option for augmenting available water supplies in many Countries.

Advanced oxidation processes (AOPs) have been deeply investigating in the removal of such micropollutants accordingly. The main aim of this PhD thesis work was to investigate solar driven AOPs as wastewater disinfection methods to control or minimise the diffusion of antibiotic resistance into the environment especially in the light of reuse of treated wastewater for agricultural purposes. In particular, this thesis was focused on the following objectives: (i) the evaluation of different solar driven AOPs (H_2O_2/sunlight, H_2O_2/TiO_2/sunlight, TiO_2/sunlight, photo-Fenton) on the inactivation of ARB from urban wastewater; (ii) the investigation of the effect of UV/H_2O_2 process on the potential of antibiotic resistance transfer by means of molecular biology methods; (iii) the simultaneous removal of target ARB and CECs by H_2O_2/sunlight process and (iv) the subsequent assessment of chemical and microbial cross contamination on crops that have been irrigated with treated wastewater.

PhD thesis work was finalized with a multidisciplinary approach including environmental engineering, microbiology, molecular biology and analytical chemistry.

Cultivation methods were used to address the first relevant issue and an antibiotic resistant *E. coli* strain was selected from urban wastewater. When implemented at laboratory scale, H_2O_2/TiO_2/sunlight process allowed to achieve the detection limit (DL) with a cumulative energy per unit of volume (Q_{UV}) ranging between 3.3 and 4.2 kJ L^{-1}, depending on H_2O_2/TiO_2 ratio. Good performances were also obtained with photo-Fenton with Fe^{2+}/H_2O_2 ratio as low as 5/10 mg L^{-1}. Therefore, the energy was observed to be higher (Q_{UV}=14.9 kJ L^{-1}) than that required by H_2O_2/sunlight process (Q_{UV}=8.7 kJ L^{-1}, H_2O_2 dose of 50 mg L^{-1}). Subsequently disinfection experiments were carried out at pilot scale in compound parabolic collectors (CPCs) photo-reactors. The best disinfection efficiency was found for photo-Fenton at pH 4.0 (Fe^{2+}/H_2O_2 ratio of 5/10 mg L^{-1}), in terms of treatment time (20 min to reach DL) and required energy (Q_{UV}=0.98 kJ L^{-1}). All processes resulted in a complete inactivation (5 Log decrease) of antibiotic resistant *E. coli* strain until DL but, due to the expected higher costs related to both solar TiO_2 photocatalysis and solar photo-Fenton, H_2O_2/sunlight process at low hydrogen peroxide doses, namely 20 and 50 mg L^{-1}, may be regarded as the most feasible and cost-effective in small communities.

Since none of the implemented solar driven AOPs affected the antibiotic resistance of survived antibiotic resistant *E. coli* colonies, quantitative polymerase chain reaction (qPCR) based methods were investigated. bla_{TEM}, *qnr*S and *tet*W were selected as target ARGs and were quantified by qPCR in the survived colonies as well as in the whole suspension (total DNA). In spite of a bacterial inactivation both in tests on distilled water and on urban wastewater, UV/H_2O_2 process did not affect bla_{TEM} gene copies number per mL in total DNA. The presence of *qnr*S and *tet*W genes was not unequivocally proven in tests on distilled water, while no difference (p>0.05) was found for *qnr*S gene in urban wastewater between the initial (5.1×10^4 copies mL^{-1}) and the final (4.3×10^4 copies mL^{-1}) sample and a decrease (p<0.05) was observed for *tet*W gene. According to these results, H_2O_2/UV process may not be an effective tool to limit or minimise the potential spread of antibiotic resistance into the environment.

H_2O_2/sunlight process was also implemented as disinfection/oxidation treatment for urban wastewater to evaluate the simultaneous removal of ARB and CECs and the subsequent assessment of chemical and microbial cross contamination on crops irrigated with treated wastewater.

When the final bacterial density was below the DL in the treated wastewater, no microbial contamination of pathogens, such as antibiotic resistant *E. coli* and antibiotic resistant *E. faecalis*, was observed in lettuce leaves and soil samples. When H_2O_2/sunlight process resulted in a residual bacterial density, such as 5.6×10^2 CFU mL^{-1} for AR *E. faecalis*, the complete absence of microbial contamination in crops samples could not be guaranteed.

Carbamazepine (CBZ) and thiabendazole (TBZ) were partially degraded (36.9% and 68.2%, respectively), while flumequine (FLU) was totally removed (99.9%), after 300 min of solar exposure with the addition of 20 mg L^{-1} of H_2O_2. This led to a chemical contamination in lettuce and soil irrigated with treated urban wastewater. In fact after irrigation tests with 300 min treated wastewater, 48.0 ng g^{-1} of CBZ and 10.8 ng g^{-1} of TBZ were detected in lettuce samples, as well as 374.0 ng g^{-1} of CBZ and 120.8 ng g^{-1} of TBZ were measured in soil samples.

On the base of the obtained results, solar driven AOPs may be successful in the inactivation of ARB. H_2O_2/sunlight process may be used as advanced treatment of urban wastewater in small communities as a cost-effective technology but it must be properly designed and applied to effectively inactivate pathogens, such as ARB, as well as to maximise CECs degradation in order to reduce their subsequent accumulation/deposition/transfer/uptake on crops irrigated with the treated wastewater. However, this process may not entail the control of the potential spread of antibiotic resistance into the environment, at least at the applied oxidant dose and with respect to the investigated ARGs. Further research is then needed to deeply investigate how ARB and ARGs transfer antibiotic resistance, in order to minimise the spread of antibiotic resistance into the environment. An environmental and health risk assessment may be also a useful tool to evaluate other aspects in this research field.

SOMMARIO

Il risparmio energetico, il recupero ed il riutilizzo di materia, rappresentano *mission* strategiche anche nel trattamento delle acque reflue. Tuttavia, lo stato dell'arte mette in luce l'inefficacia o la scarsa efficacia dei processi di trattamento convenzionali, normalmente implementati negli impianti di depurazione, nella rimozione di contaminanti emergenti (CECs), presenti in concentrazioni dell'ordine dei ng L^{-1}/μg L^{-1}. Tra questi rientrano gli antibiotici che possono esercitare una pressione selettiva, facilitando la selezione di batteri antibiotico-resistenti (ARB) e la diffusione di geni che codificano la resistenza agli antibiotici (ARGs). Non rimossi dai processi di trattamento convenzionali, tali micro-contaminanti possono provocare effetti tossici cronici su organismi acquatici e terresti, nonché comportare un rischio per l'ambiente e la salute umana. In tale scenario desta maggiore preoccupazione il riutilizzo a scopi irrigui delle acque reflue trattate, opzione sempre più ambita in realtà connotate da una significativa scarsità della risorsa idrica.
Negli ultimi anni, i processi di ossidazione avanzata (AOPs) sono stati oggetto di investigazione scientifica per la rimozione dei contaminanti emergenti. Il principale obiettivo del presente lavoro di tesi concerne lo studio e la comparazione di diversi AOPs a radiazione solare come possibili processi di disinfezione al fine di controllare o minimizzare la diffusione di antibiotico-resistenza nell'ambiente, soprattutto nell'ottica del riutilizzo di acque reflue trattate per scopi irrigui. A tal fine, l'attività sperimentale è stata impostata al fine di perseguire i seguenti obiettivi specifici: (i) la valutazione di diversi AOPs a radiazione solare (UV/H_2O_2, UV/H_2O_2/TiO_2, fotocatalisi eterogenea con biossido di titanio, foto-Fenton) nell'inattivazione di ceppi di *E. coli* antibiotico-resistenti, selezionati da acque reflue urbane; (ii) lo studio dell'effetto del processo UV/H_2O_2 sul potenziale trasferimento di antibiotico-resistenza mediante metodi di biologia molecolare; (iii) l'investigazione della simultanea rimozione di ceppi batterici antibiotico-resistenti e contaminanti emergenti mediante il processo UV/H_2O_2 a radiazione

solare, al fine di valutare (iv) la successiva contaminazione chimica e microbica su colture target irrigate con acque reflue trattate.
Per conseguire gli obiettivi prefissati, l'attività sperimentale di tesi ha richiesto un approccio multidisciplinare che include l'ingegneria sanitaria ambientale, la microbiologia, la biologia molecolare e la chimica analitica, nonché la collaborazione con diversi gruppi di ricerca.
La selezione del ceppo di *E. coli* antibiotico-resistente è stata attuata con metodi di coltivazione su terreni selettivi. Quando implementato a scala di laboratorio, il processo $UV/H_2O_2/TiO_2$ ha consentito di raggiungere la completa inattivazione del ceppo batterico inoculato, con un'energia cumulata per unità di volume (Q_{UV}) tra 3.3 e 4.2 kJ L^{-1}, al variare del rapporto di concentrazione H_2O_2/TiO_2. Ottime performance sono state ottenute anche con il processo di foto-Fenton solare con un rapporto Fe^{2+}/H_2O_2 di 5/10 mg L^{-1}, sebbene tale processo abbia richiesto un'energia cumulata per unità di volume maggiore (Q_{UV}=14.9 kJ L^{-1}) rispetto al processo UV/H_2O_2 (Q_{UV}=8.7 kJ L^{-1}, con una dose di 50 mg L^{-1} di H_2O_2). Successivamente gli esperimenti sono stati implementati a scala pilota in foto-reattori, quali collettori parabolici composti (CPCs). In tal caso, le migliori rese sono state ottenute con il processo di foto-Fenton solare a pH 4.0 con un rapporto Fe^{2+}/H_2O_2 di 5/10 mg L^{-1}: il limite di detenzione (DL) è stato raggiunto in 20 minuti con un'energia cumulata per unità di volume di soli 0.98 kJ L^{-1}. Tutti i processi investigati hanno comportato la completa inattivazione del ceppo testato; tuttavia, alla luce dei costi maggiori attesi sia per la fotocatalisi eterogenea solare sia per il foto-Fenton solare, il processo solare UV/H_2O_2 a basse concentrazioni di perossido di idrogeno (20 e 50 mg L^{-1}), potrebbe risultare quello maggiormente implementabile in piccole comunità.
Nessun effetto è stato riscontrato sull'antibiotico-resistenza delle colonie batteriche sopravvissute al trattamento, pertanto, si è ricorso a metodi di biologia molecolare basati sulla reazione a catena della polimerasi (PCR). bla_{TEM}, *qnr*S e *tet*W sono stati identificati come geni target e quantificati mediante la PCR quantitativa (qPCR) sia nelle colonie batteriche sopravvissute al trattamento sia nella matrice acquosa (DNA totale). Dagli esperimenti condotti è emerso che l'inattivazione batterica non ha implicato la rimozione dei geni che codificano la resistenza agli antibiotici. Il processo UV/H_2O_2 non ha comportato alcun effetto sulla rimozione del gene bla_{TEM} dalla matrice acquosa (acqua distillata e, successivamente, acque reflue urbane). Negli esperimenti condotti su matrice reale (acque reflue urbane), nessuna differenza (p>0.05) è stata

riscontrata per il gene *qnr*S tra la concentrazione iniziale (5.1×10^4 copie mL^{-1}) e finale (4.3×10^4 copie mL^{-1}) dei campioni trattati, ed un effetto statisticamente significativo ($p<0.05$) è stato rilevato per il gene *tet*W a monte ed a valle del processo di disinfezione. Alla luce dei risultati ottenuti, il processo UV/H_2O_2 sembrerebbe inefficace nel controllo della diffusione dell'antibiotico-resistenza nell'ambiente, non impedendo il rilascio di ARGs.

Il processo UV/H_2O_2, tarato sull'inattivazione batterica, è stato ulteriormente investigato nella rimozione simultanea di ARB e CECs al fine di valutare la successiva contaminazione su lattuga irrigata con acque reflue trattate. Gli esperimenti condotti hanno evidenziato che la completa inattivazione dei ceppi patogeni non implica la contaminazione microbica di campioni di lattuga e suolo irrigati con acque reflue trattate, mentre una densità batterica residua (5.6×10^2 UFC mL^{-1} per *E. faecalis* antibiotico-resistenti) si può trasferire nelle colture. Il processo investigato ha comportato la parziale degradazione di carbamazepina (CBZ, 36.9%) e tiabendazolo (TBZ, 68.2%) e la totale rimozione della flumechina (FLU, 99.9%) dopo 300 minuti di trattamento. Ciò ha implicato una contaminazione chimica delle colture irrigate con acque reflue trattate: a valle degli esperimenti irrigui, 48.0 ng g^{-1} di CBZ e 10.8 ng g^{-1} di TBZ sono stati rilevati nei campioni di lattuga, 374.0 ng g^{-1} di CBZ e 120.8 ng g^{-1} di TBZ sono stati misurati nei campioni di suolo.

Alla luce dei risultati ottenuti, l'inattivazione di ceppi batterici antibiotico-resistenti può essere conseguita mediante gli AOPs a radiazione solare. Particolarmente efficace è risultata la disinfezione con UV/H_2O_2, tecnologia semplice da gestire ed economica da implementare in piccole comunità. Tuttavia un'adeguata progettazione e gestione sono imprescindibili per massimizzare la rimozione di CECs ed ARB al fine di evitarne il successivo accumulo su suolo ed assorbimento su colture. Inoltre, tale processo sembrerebbe non garantire il controllo della diffusione dell'antibiotico-resistenza nell'ambiente, almeno sulla base della dose di perossido di idrogeno utilizzata e con riferimento ai geni investigati. Ulteriore ricerca è auspicabile per comprendere come ARB e ARGs trasferiscono l'antibiotico resistenza al fine di individuare adeguate soluzioni per limitarne gli effetti di diffusione nell'ambiente e per valutarne i conseguenti rischi.

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ABOUT THE AUTHOR

Giovanna Ferro graduated with honours in Environmental Engineering in 2012 from University of Salerno discussing a thesis entitled "*Monitoring and removal of antibiotic resistant bacteria in urban wastewater treatment*", whose experimental work was carried out in microbiology laboratory at *Universidade Católica Portuguesa* of Oporto. She started her PhD research in 2013, after being awarded a 3 years PhD grant at Department of Civil Engineering of University of Salerno. Her research project was mostly focused on the investigation of advanced oxidation processes on antibiotic resistance. She spent six months as guest researcher in the *Plataforma Solar de Almería* in Spain, where she implemented solar driven advanced oxidation processes at pilot scale, in compound parabolic collectors photo-reactors and carried out irrigation tests. She had an oral presentation at *Society of Environmental Toxicology and Chemistry Europe 25th Annual Meeting* (Barcelona, 2015) and at *4th European Conference on Environmental Applications of Advanced Oxidation Processes* (Athens, 2015) about her research work. So far three papers about her research project have been published on international high impact factor journals and one paper is currently under review. Moreover she is co-author of three papers on international index journals. She was member of COST Action "*Detecting evolutionary hot spots of antibiotic resistances in Europe (DARE) TD0803*" and now is a member of COST Action "*New and emerging challenges and opportunities in wastewater reuse (NEREUS) ES1403*". She is also PhD Candidate of *European PhD School on Advanced Oxidation Processes*.

Giovanna Ferro ha conseguito la laurea specialistica con lode in Ingegneria per l'Ambiente ed il Territorio nel 2012 presso l'Università degli Studi di Salerno con una tesi dal titolo "*Monitoraggio e rimozione di batteri resistenti agli antibiotici nel trattamento dei reflui urbani*", la cui attività sperimentale è stata condotta presso l'*Universidade Católica Portuguesa* di Oporto. Nel 2013 è risultata vincitrice con borsa di studio del concorso per il XIV Ciclo del Dottorato di Ricerca in Ingegneria Civile per l'Ambiente ed il Territorio presso l'Università degli Studi di Salerno. La sua attività di ricerca è prevalentemente incentrata sullo studio dell'effetto indotto dai processi di ossidazione avanzata sull'antibiotico resistenza. Durante il periodo di dottorato ha trascorso sei mesi presso la *Plataforma Solar de Almería* in Spagna, principale centro europeo sulle tecnologie solari, dove ha implementato i processi di disinfezione a radiazione scolare a scala pilota ed ha condotto esperimenti irrigui. Ha presentato parte dei risultati conseguiti con la sua attività di ricerca al *Society of Environmental Toxicology and Chemistry Europe 25th Annual Meeting* (Barcellona, 2015) ed al *4th European Conference on Environmental Applications of Advanced Oxidation Processes* (Athene, 2015). È prima autrice di tre pubblicazioni scientifiche, inerenti alla sua attività di ricerca, su riviste internazionali ad alto impact factor, nonché co-autrice di tre articoli scientifici su riviste internazionali indicizzate. Un altro articolo è, attualmente, in fase di revisione. È stata membro dell'azione COST "*Detecting evolutionary hot spots of antibiotic resistances in Europe (DARE) TD0803*" ed è, attualmente, membro dell'azione COST "*New and emerging challenges and opportunities in wastewater reuse (NEREUS) ES1403*". È, infine, PhD Candidate della *Scuola Europea di Dottorato sui Processi di Ossidazione Avanzata*.

1 INTRODUCTION

1.1 NEW CHALLENGES IN URBAN WASTEWATER TREATMENT

According to an estimate by the United Nations, water use has been growing at more than twice the rate of population increase in the last century (WWAP 2015). By 2025, 1800 million people will be living in Countries or regions with absolute water scarcity, and two-thirds of the world population could be under stress conditions (FAO 2014, WWAP 2015). Facing demographic growth and economic development, it is increasingly hard to find new sources of water necessary to successfully satisfy growing water demand. This implies a significant pressure on renewable, but finite water resources, especially in arid and semiarid regions. The already complex relationship between world development and water demand is expected to be amplified by climate change and bio-energy demand (FAO 2014). Water scarcity has several dimensions, such as in availability, in access or due to the difficulties in finding a reliable source of safe water which is not time consuming and expensive, all characterizing developing Countries. All these issues make the wastewater reuse an interesting option for augmenting available water supplies (Scheierling et al. 2011). Wastewater reuse for agriculture irrigation is by far the most established application (Dreschel et al. 2010). Anyway in low-income countries, as well as in many middle-income countries, wastewater reuse involves the direct use of untreated wastewater or the indirect use of polluted waters from rivers that receive untreated urban discharges (World Bank 2010). The side effect of wastewater reuse for agricultural purposes is related to environmental and public health risks that are involved in a not planned or properly implemented integrated management of urban water resources (Dreschel et al. 2010, World Bank 2010). To address these risks, in particular microbial risks due to pathogens, the World Health Organization in 2006 issued new guidelines for the safe use of wastewater (WHO 2006) and the US Environmental Protection Agency updated the guidelines for water reuse in 2012 (US EPA 2012). Among possible routes of

transmission, the consumption of wastewater-irrigated food may be of public health importance.

Beyond regulatory aspects, it is important to achieve an integrated approach to wastewater reuse, including many economic/financial and social issues, but also technological ones (World Bank 2010). As wastewater reuse fits into the wider urban water cycle, one of the most significant challenges in urban wastewater treatment is related to the potential to reduce the amount of pathogens in order to meet the targets for different types of irrigation scenarios. It is important to point out that such health risks related to the presence of pathogenic microorganisms in wastewater characterise more lower-income and lower-middle income countries. As countries move to upper-middle income and higher-income levels, wastewater reuse for agricultural purposes relies on a more planned and regulated system. Moreover wastewater pollution tends to be related more to the presence of contaminants of emerging concern (CECs), such as disinfectants, endocrine disruptors, illicit drugs, personal care products, pesticides, pharmaceuticals and resistant microorganisms, among them antibiotic resistant bacteria (ARB), than to faecal contamination. Urban wastewater treatment plants (UWTPs), typically based on biological processes, may not be effective in removing CECs and in controlling ARB spread into the environment (Klamerth et al. 2010, Michael et al. 2013, Rizzo et al. 2013b). As reusable water must meet specific requirements (Miranda-García et al. 2010, US EPA 2012) including an absence of toxic compounds that generally escape conventional wastewater treatment (De Luca et al. 2013), the main challenge for urban wastewater treatment is removing many types of micropollutants from water.

If it is true that, in recent decades, water scarcity has led to reclaimed water use in different applications, it is also true that high costs are required to achieve proper levels of water quality. In this light, another important challenge lies in saving wastewater treatment costs, so making the wastewater reuse more feasible worldwide. This goal also fits into resources recovery and reuse. Some wastewater treatment processes may be driven by sunlight, so saving treatment costs. In this way solar driven processes may be an attractive option for wastewater treatment in small communities and in arid/semi-arid regions.

1.2 CONTAMINANTS OF EMERGING CONCERN

In upper-middle income and higher-income countries wastewater pollution is related to the presence of micropollutants known as CECs. This term is used to define different types of compounds, such as newly developed substances present in our daily lives, whose use cannot be controlled or eliminated (Klamerth et al. 2010) or compounds that have been lately discovered in the environment (Lapworth et al. 2012). Antibiotics, detergents, disinfectants, endocrine disruptors such as bisphenol A, fungicides, herbicides, pesticides, industrial additives, fragrances, personal care products, illicit drugs such as cocaine and THC, pharmaceuticals and other xenobiotic substances fall within CECs.
Due to their growing use, CECs have been detected in increasing quantities in surface water, wastewater and also in drinking water (Klamerth et al. 2010), in the range of high ng L^{-1} to low μg L^{-1} (Miralles-Cuevas et al. 2014). Many scientific papers critically review the fate of various CECs in wastewater treatment, focusing on different processes (*i.e.* biological processes and advanced treatment technologies) in view of the current concerns related to the induction of toxic effects in aquatic and terrestrial organisms (Fatta-Kassinos et al. 2011, Michael et al. 2013), and the occurrence of environmental and public health risks (Calderón-Preciado et al. 2012, Jiang et al. 2014, Wu et al. 2014).

1.2.1 Environmental and public health risks

The most important continuous pathways of such micropollutants into aquatic environment are UWTPs (De la Cruz et al. 2013). Due to the incomplete removal by conventional treatments, CECs occur in irrigation waters and may exert chronic toxic effects also at very low concentrations (Calderón-Preciado et al. 2012).
Anti-cancer drugs have been recognised, also at low concentrations, as potentially fetotoxic, genotoxic, mutagenic and teratogenic substances in non-target organisms (Lutterbeck et al. 2015). Nevertheless, there is still a lack of knowledge concerning the environmental fate of drugs and their metabolites after excretion and possible risks connected to their presence in the aquatic environment (Kümmerer et al. 2014).
Pharmaceutical residues, along with their metabolites and transformation products, are continuously released into the environment through irrigation or different discharge practices, becoming 'pseudo-persistent'.

Their long-term release may induce toxic effects towards aquatic or terrestrial organisms (Fatta-Kassinos et al. 2011). Some pharmaceuticals, such as carbamazepine (Shenker et al. 2011) and antibiotics (Jones-Lepp et al. 2010) can be also taken up by crops through irrigation waters. Clofibric acid, ibuprofen, naproxen, tonalide and triclosan can be accumulated in lettuce plant tissue (Calderón-Preciado et al. 2012). Caffeine, carbamazepine, DEET, dilantin, meprobamate, naproxen, primidone and triclosan can be easly taken up by plants irrigated with treated wastewater (Wu et al. 2014). Some personal care products and pharmaceuticals are persistent and bioaccumulative, potentially producing health and ecological impacts, such as adverse effects against algal, bacterial, aquatic vertebrates and invertebrate populations in the receiving waters of UWTPs effluents (Guerra et al. 2014). Malchi and co-workers assessed the health risk associated to the consumption of wastewater-irrigated root vegetables, namely carrots and sweet potatoes, through the threshold of toxicological concern approach. Their results highlighted that the threshold of toxicological concern value of lamotrigine can be achieved for a child at a daily consumption of half a carrot, namely almost 60 g (Malchi et al. 2014).

Among antibiotics, ampicillin and codeine have shown significant pollution risk quotients, indicating high risk to aquatic organisms in coastal waters (Jiang et al. 2014). Antibiotics play also an important role in the development and transfer of bacterial resistance (Michael et al. 2013, Xu et al. 2015). Moreover additive and synergistic effects represent another aspect of environmental exposure assessment that requires better understanding (Watkinson et al. 2007).

Among herbicides, atrazine has been detected in the atmosphere, in soil, in UWTPs effluents and also in groundwater and surface water because of agricultural runoff, although its use was banned from the EU in 2004. Atrazine is considered a potential contaminant that can cause cancer, harm the human endocrine system and lead to environmental problems (De Luca et al. 2013).

On the whole there is a general lack of knowledge in relation to environmental and public health risks derived from a lifetime exposure to so-called CECs (Calderón-Preciado et al. 2013, Shu et al. 2013, Wu et al. 2014). In this light CECs release into the environment should be restricted or at least optimised in order to limit risks to the environment and public health that are difficult to assess and manage.

1.2.2 Directive 2013/39/EU and Watch list

The Directive on Environmental Quality Standards (Directive 2008/105/EC), also known as the Priority Substances Directive, has set environmental quality standards for the substances in surface waters (river, lake, transitional and coastal) and has confirmed their designation as priority or priority hazardous substances, the latter being a subset of particular concern. The list was reviewed by the Commission and in 2012 a proposal for a Directive amending the Water Framework Directive and Directive 2008/105/EC as regards priority substances was put forward.
This proposal included a revised (second) list of priority substances; (i) 15 additional priority substances, six of them designated as priority hazardous substances; (ii) a provision for a watch-list mechanism designed to allow targeted EU-wide monitoring of substances of possible concern to support the prioritisation process in future reviews of the priority substances list, fall within main features.
Then Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amended Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. 45 priority substances are listed in Annex I to Directive 2013/39/EU as Annex X of the Water Framework Directive (Table 1.1), while environmental quality standards for priority substances and certain other pollutants are listed in Annex II to Directive 2013/39/EU.

Table 1.1 List of priority substances in the field of water policy (adapted from Annex I to Directive 2013/39/EU)

Number	Name of priority substance *	Identified as priority hazardous substance
(1)	Alachlor	
(2)	Anthracene	X
(3)	Atrazine	
(4)	Benzene	
(5)	Brominated diphenylether	X
(6)	Cadmium and its compounds	X
(7)	Chloroalkanes, C_{10-13}	X
(8)	Chlorfenvinphos	
(9)	Chlorpyrifos (Chlorpyrifos-ethyl)	
(10)	1,2-dichloroethane	
(11)	Dichloromethane	
(12)	Di(2-ethylhexyl)phthalate (DEHP)	X
(13)	Diuron	

Number	Name of priority substance *	Identified as priority hazardous substance
(14)	Endosulfan	X
(15)	Fluoranthene	
(16)	Hexachlorobenzene	X
(17)	Hexachlorobutadiene	X
(18)	Hexachlorocyclohexane	X
(19)	Isoproturon	
(20)	Lead and its compounds	
(21)	Mercury and its compounds	X
(22)	Naphthalene	
(23)	Nickel and its compounds	
(24)	Nonylphenol	X
(25)	Octylphenol	
(26)	Pentachlorobenzene	X
(27)	Pentachlorophenol	
(28)	Polyaromatic hydrocarbons (PAH)	X
(29)	Simazine	
(30)	Tributyltin compounds	X
(31)	Trichlorobenzenes	
(32)	Trichloromethane (chloroform)	
(33)	Trifluralin	X
(34)	Dicofol	X
(35)	Perfluorooctane sulfonic acid and its derivatives (PFOS)	X
(36)	Quinoxyfen	X
(37)	Dioxins and dioxin-like compounds	X
(38)	Aclonifen	
(39)	Bifenox	
(40)	Cybutryne	
(41)	Cypermethrin	
(42)	Dichlorvos	
(43)	Hexabromocyclododecanes (HBCDD)	X
(44)	Heptachlor and heptachlor epoxide	X
(45)	Terbutryn	

* Where groups of substances have been selected, unless explicitly noted, typical individual representatives are defined in the context of the setting of environmental quality standards.

The European Commission established a watch list of substances for Union-wide monitoring in the field of water policy (Table 1.2).

Table 1.2 Watch list of substances for Union-wide monitoring in the field of water policy (adapted from Commission Implementing Decision 2015/495)

Name of substance/group of substances	Maximum acceptable method detection limit (ng L^{-1})
17-Alpha-ethinylestradiol (EE2)	0.035
17-Beta-estradiol (E2), Estrone (E1)	0.4
Diclofenac	10
2,6-Ditert-butyl-4-methylphenol	3160
2-Ethylhexyl 4-methoxycinnamate	6000
Macrolide antibiotics	90
Methiocarb	10
Neonicotinoids	9
Oxadiazon	88
Tri-allate	670

Scientists at the European Commission's Joint Research Laboratory have developed a series of three water reference materials containing priority hazardous substances as defined under the EU Water Framework Directive. These three novel materials contain:

- eight polycyclic aromatic hydrocarbons (PAHs),
- six polybrominated diphenyl ethers (PBDEs),
- tributyltin.

By adding three pharmaceuticals on the 'watch list', the EU has set a first important step towards environmental pharmaceutical regulation (Van Doorslaer et al. 2014). Although pharmaceuticals have not been included yet in the priority substance list, this is a significant effort in taking note of environmental issues related to pharmaceutical pollution. On the other side, a major concern is the absence of limiting regulations for many CECs, typically detected in water and wastewater. Moreover the various existing national guidelines on wastewater reuse tend to focus mainly on microbiological risks and there is little mention of other trace pollutants (Fatta-Kassinos et al. 2011).

1.3 ANTIBIOTICS AND ANTIBIOTIC RESISTANCE

Antibiotics fall within CECs, whose increasing use for agricultural, human and veterinary purposes results in their continuous release into

the environment (Kümmerer 2009) due to incomplete metabolism or disposal of unused antibiotics. The occurrence of antibiotics may promote the selection and the diffusion of antibiotic resistance patterns into the environment (Davies and Davies 2010, Michael et al. 2013, Watkinson et al. 2007), such as the development of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) which reduce the therapeutic potential against animal and human pathogens (Rizzo et al. 2013b).

The report on global surveillance of antimicrobial resistance by the World Health Organization highlighted that the world is heading towards a post-antibiotic era, in which common infections and minor injuries may once again kill (WHO 2014). The continuous release of antibiotics into the environment entails serious consequences not just in terms of public health but also in terms of impacts onto the environment.

Among significant drivers for antibiotic resistance dissemination between the environment and humans and other animals, water plays an important role through urban water cycle (Vaz-Moreira et al. 2014). In fact potential paths of antibiotic resistance diffusion are related to water treatment, by means of the elimination/selection and release of clinically relevant ARB and ARGs (Manaia et al. 2015) that can reproduce and be spread across different hosts, respectively (Varela et al. 2015). Hospital effluents (Narciso-da-Rocha et al. 2014, Rodriguez-Mozaz et al. 2015) and UWTPs effluents (Rizzo et al. 2013a) have been identified as hotspots for antimicrobial resistance emergence and spread into the environment. In particular biological treatment processes may contribute to antibiotic resistance selection as well as transfer among bacteria which are continuously mixed with antibiotics at sub-inhibitory concentrations (Auerbach et al. 2007), although the knowledge regarding the effects of this aspect is contradictory (Kümmerer, 2009).

ARB and ARGs have been detected in different environmental compartments, although UWTPs effluents represent the most significant sources due to the absence of a final disinfection step or to the use of conventional disinfection systems that may be only partially effective in the inactivation of ARB (Munir et al. 2011, Rizzo et al. 2013b).

1.3.1 Detection of antibiotic resistant bacteria and antibiotic resistance genes

Methicillin resistant *Staphylococcus aureus* (MRSA) has been found in the effluent of four US UWTPs (Goldstein et al. 2012). Vancomycin resistant enterococci and β-lactam-hydrolysing Enterobacteriaceae have been identified in wastewater biofilms and less frequently in biofilms from the surface water environment (Schwartz et al. 2003). The occurrence of ciprofloxacin and vancomycin resistant enterococci has been observed in hospital effluents and UWTPs influent and effluent (Varela et al. 2013). Antibiotic resistant *Escherichia coli* have been detected in the surface water (Zhang et al. 2014), while antibiotic resistant enterococci in UWTPs effluents, and some resistance types, such as quinolone resistance in enterococci, tended to become more prevalent in the final effluent, after activated sludge process, than in the raw wastewater (Ferreira da Silva et al. 2006). In another study it has been shown that variations on the bacterial community structure of the final effluent were significantly correlated with the occurrence of penicillins, quinolones, sulfonamides, tetracyclines and triclosan in the raw inflow (Novo 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). ARGs and ARB have been quantified in final UWTP effluents in Michigan in the range of 9.1×10^7-1.1×10^8 copies mL^{-1} and 1.1×10^1-3.1×10^3 CFU mL^{-1}, respectively and significant correlations (R^2=0.75-0.83, p<0.05) between numbers of ARB and antibiotic concentrations were observed in raw influent and final effluent (Gao et al. 2012). 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). UWTPs have been confirmed to be important hotspots of tetracycline resistance determinants, namely *tet*A-E, *tet*G, *tet*M, *tet*O, *tet*Q and *tet*S, as compared to the lake water samples (Auerbach et al. 2007). Concentrations of doxycycline resistant bacteria harbouring the *tet*B gene have been observed to be higher in wastewater

and downstream river samples than in upstream water samples (Harnisz et al. 2015). In another work it has been shown that high concentrations of bla_{TEM}, *erm*B, *qnr*S, *sul*-I and *tet*W, still present in the treated UWTP effluent, may affect the receiving river (Rodriguez-Mozaz et al. 2015). Dairy lagoon water, irrigation ditch water, urban/agriculturally impacted river sediments and also treated drinking water, as well as recycled wastewater, can be regarded as important sources of *sul*-I, *sul*-II, *tet*O and *tet*W genes (Pruden et al. 2006). *van*A gene has been found not only in wastewater biofilms but also in drinking water biofilms in the absence of enterococci, indicating possible gene transfer to autochthonous drinking water bacteria, while *mec*A gene, encoding methicillin resistance in staphylococci, has been detected in hospital wastewater biofilms but not in any other compartment. Enterobacterial *amp*C resistance gene, encoding β-lactamase activities, have been detected in wastewater, surface water and drinking water biofilms (Schwartz et al. 2003). So drinking water is also regarded as a potential route of antibiotic resistance spread to humans: bla_{TEM}, *int*I-1 (encoding an integrase protein), *sul*-I, *tet*W, *van*A have been found in biofilm or planktonic DNA from tap water samples, although at low concentrations (Pruden et al. 2006, Schwartz et al. 2003, Xi et al. 2009).

In a review dealing with environmental pollution by antibiotics and antibiotic resistance determinants it has been highlighted that the same ARGs found at clinical settings are currently disseminated among pristine ecosystems without any record of antibiotic contamination (Martinez 2009). Moreover, unlike other contaminants, ARB can reproduce and ARGs can be spread across different hosts, having been detected in urban water streams, gulls or hospitalised patients (Varela et al. 2015). Anyway the current state of knowledge is not sufficient to assess the abundance and distribution of ARB and ARGs in the environment at regional, national or global levels and that represents a limit in assessing the risk of transmission of antibiotic resistance from the environment to human-associated bacteria (Berendonk et al. 2015). So global efforts are required to characterise and quantify antibiotic resistance in the environment.

1.3.2 Drawbacks of conventional technologies

The occurrence of both ARB carrying antibiotic resistance genetic material that can be spread into the environment (Łuczkiewicz et al. 2010) and ARGs in different environmental compartments 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. 2013b), although horizontal gene transfer is regarded as the most important one (McKinney and Pruden 2012).

In this scenario water and urban wastewater treatment play an important role. Moreover different screening campaigns have revealed, though the presence of ARB and ARGs in final effluents of UWTPs, that conventional treatment systems may be only partially effective in the inactivation of ARB and in the removal of ARGs (paragraph 1.3.1).

The relative abundance of different ARGs have shown significant variations among the wastewater treatment systems in rural and urban areas (Chen et al. 2013). The difference of ARGs levels in the raw influent and secondary effluent has suggested that UWTPs may induce to increase the abundance of resistance genes (Xu et al. 2015). Irrespective of any significant variation on the antibiotic resistance prevalence, a well functioning UWTP with secondary treatment will discharge continuously to the environment high doses of ARB and ARGs (Vaz-Moreira et al. 2014) and this load may be much higher than the receiving environment is able to eliminate (Manaia et al. 2015). This is because traditional UWTPs have not been designed for the removal of antibiotics or antibiotic resistance determinants (Pruden et al. 2013).

Disinfection processes can be regarded as possible barriers to control or minimise the diffusion of antibiotic resistance into the environment, especially in the light of reuse of treated wastewater for agricultural purposes (Fatta-Kassinos et al. 2011). Unfortunately, also even disinfection systems are implemented, they may not be effective in controlling antibiotic resistance spread into receiving water.

The proportion of several ARB was observed to increase after chlorination, especially for chloramphenicol-resistant bacteria exposed to 10 mg Cl_2 L^{-1} for 10 min (Huang et al. 2011). High doses (>1.0 mg Cl^2 L^{-1}) of chlorine with a 10 min contact time during chlorination were observed to increase the average tetracycline resistance of tetracycline-

resistant *E. coli* (Huang et al. 2013). In another study an increase of the proportion of bacterial isolates resistant to six types of antibiotics was registered from 3.8% in the influent to 6.9% in the chlorinated effluent (Al-Jassim et al. 2015). Chlorination process did not affect the antibiotic resistance of *E. coli* strains investigated in another work (Rizzo et al. 2013a).

A monitoring of the release of ARB and ARGs into the environment through the effluents of five UWTPs in Michigan has shown that target disinfection processes, namely chlorination and UV radiation, did not significantly contribute to the decrease of ARB and ARGs ($p>0.05$). ARB and ARGs concentrations (*sul*-I, *tet*O, *tet*W, 16S rRNA) in the final effluents were in the range of 5.0×10^{2}-6.1×10^{5} CFU mL^{-1} and non-detectable-2.3×10^{8} copies mL^{-1} respectively. Despite the unavailability of data about chlorine and UV doses as well as contact times, this result confirms that under realistic operative conditions, traditional disinfection processes may not be effective in controlling antibiotic resistance spread.

No apparent decrease has been observed in the concentrations of tetracycline resistance genes (*tet*O and *tet*W) and sulfonamide resistance gene (*sul*-I) by chlorine disinfection (Gao et al. 2012). Investigation of the effects of UV disinfection on wastewater effluents has shown no reduction in the number of detectable tetracycline resistance genes, such as *tet*A-E, *tet*G, *tet*M, *tet*O, *tet*Q and *tet*S (Auerbach et al. 2007).

Alternative disinfection processes have been starting to be investigated in order to control antibiotic resistance spread into the environment, overcoming drawbacks of traditional technologies.

1.4 ADVANCED OXIDATION PROCESSES

Conventional wastewater treatment systems have not been designed for the removal of CECs because of their high chemical stability and/or low biodegradability. In this context, non-conventional treatment technologies have been raising great interest for the degradation/removal of such micropollutants. In particular Advanced Oxidation Processes (AOPs) have been widely and successfully investigated for the removal of a wide range of inorganic and organic microcontaminants as well as for the improvement of the

biodegradability of industrial wastewater (Malato et al. 2009) as evidenced by several reviews recently published (Bacardit et al. 2006, De Luca et al. 2013, Klavarioti et al. 2009, Mantzavinos et al. 2009, Miralles-Cuevas et al. 2014).
AOPs are oxidation methods/technologies relying on the promotion of highly reactive species such as hydroxyl and other radicals which have very low selectivity and permit the degradation (partial or total) of a wide spectrum of both inorganic and organic pollutants. The generation of hydroxyl radicals can be achieved electrochemically, photochemically, sonochemically, by homogenous and heterogeneous catalysis.

The fundamentals of photochemistry and photophysics underlying the **heterogeneous photocatalysis** employing the semiconductor titanium dioxide (TiO_2) catalyst have been intensively described in the literature. Briefly the semiconductor TiO_2 has been widely utilised as a photocatalyst in order to induce a serious of oxidative and reductive reactions on its surface. When photon energy (*hν*) of greater than or equal to the bandgap energy of TiO_2 is illuminated onto its surface, usually 3.2 eV (anatase) or 3.0 eV (rutile), the lone electron will be photoexcited to the empty conduction band in femtoseconds. The light wavelength for such photon energy usually corresponds to $\lambda<400$ nm. The photonic excitation leaves behind an empty unfilled valence band, and thus creating the electron-hole pair (e^-–h^+). In the absence of electron scavengers, the photoexcited electron recombines with the valence band hole in nanoseconds with simultaneous dissipation of heat energy. Thus, the presence of electron scavengers is vital to prolong the recombination and successful functioning of photocatalysis. Without the presence of water molecules, the highly reactive hydroxyl radicals (HO•) could not be formed and impede the photodegradation of liquid phase organics. For heterogeneous photocatalysis, the liquid phase organic compounds are degraded to its corresponding intermediates and further mineralised to carbon dioxide and water, if the irradiation time is extended (Chong et al. 2010). TiO_2 is a catalyst which works at mild conditions with mild oxidants. However, as concentration and number of contaminants increase, the process becomes more complicated and challenging problems, such as catalyst deactivation, slow kinetics, low photoefficiencies and unpredictable mechanisms need to be addressed (Malato et al. 2009). This has led to the use of doped and modified TiO_2 (*i.e.* with noble metals), as well as to the enhancement of the

photocatalytic reaction rate by adding cheaper transition metals, such as Au, Mo, Ru or V on TiO_2 surface (Vaiano et al. 2014). The removal of catalyst after treatment represents another limit of this process that should be taken into count in a global assessment for wastewater treatment. In this light advancements in photocatalyst immobilization and supports are recently under investigation.

In aqueous solution the most abundant iron species have an oxidation number of +II (ferrous iron) and +III (ferric iron), the latter being the more critical iron species in the **photo-Fenton** process, because its hydroxides precipitate at lower pH than those of ferrous iron. In the presence of iron ions in aqueous solution, hydrogen peroxide is decomposed to oxygen and water in the **Fenton** reaction. Mixtures of ferrous iron and hydrogen peroxide are called Fenton reagent. If ferrous is replaced by ferric iron it is called Fenton-like reagent. The regeneration of ferrous iron from ferric iron is the rate limiting step in the catalytic iron cycle, if iron is added in small amounts (Malato et al. 2009).
The Fenton reaction does not involve any light irradiation: hydrogen peroxide is decomposed by ferrous ions present in the aqueous phase, resulting in the formation of hydroxyl radicals. When a light source is present, the rate of photo-Fenton was reported to be positively enhanced compared to the dark condition. This is mainly due to the regeneration of $Fe^{2+}(aq)$ from the photochemical effect of light and the concurrent generation of the HO• radicals in the system. Such a reversion cycle of $Fe^{2+}(aq)/Fe^{3+}(aq)/Fe^{2+}(aq)$ continuously generates HO•, provided that the concentration of H_2O_2 in the system is substantial (Chong et al. 2010).
Although the photo-Fenton has higher photoactivity than the heterogeneous photocatalysis and has been successfully applied in the field of wastewater decontamination characterised by total organic carbon as high as 10-25 g L^{-1} (Malato et al. 2009), its performances are strongly dependent upon several quality parameters of waters, namely pH and ions content. It is well known that the optimum pH for photo-Fenton reaction is 2.8 which does not allow the iron precipitation and promotes the presence of dominant iron species of $[Fe(OH)]^{2+}$ in water.
Then, in wastewater treatment, acidification before treatment and neutralization afterwards are required. Moreover the presence of different ions such as carbonate, chlorine, phosphate, sulphate also affects the iron equilibrium water. Due to this operative aspects

involving high chemical costs and due to the formation of precipitated iron to be subsequently removed, Fenton and photo-Fenton processes may not be really attractive on the economic point of view. In order to overcome these drawbacks, many recent studies are focused on enhancements of Fenton and photo-Fenton process by avoiding the initial acidification (De Luca et al. 2014, Papoutsakis et al. 2015a, Romero et al. 2016), by using different catalysts (Doumic et al. 2015, Ruales-Lonfat et al. 2015) or by combing different technologies (Miralles-Cuevas et al. 2014, Sánchez Peréz et al. 2014). Several authors have proposed the direct interaction of the oxidative mechanisms of photo-Fenton with other oxidative processes, *i.e.* the simultaneous combined action of photo-Fenton and ozone, TiO_2, ultrasound and also with biological processes (Malato et al. 2009). In general AOPs can be applied as pre- and/or post-treatment of biological processes in order to improve the biodegradability of wastewater and remove those micropollutants that are not completely degraded during the biological treatment respectively.

Other main processes to obtain hydroxyl radicals are described below (as adapted from Esplugas et al. 2002).

- O_3: In an ozonation process two possible ways of oxidising action may be considered: the direct way due to the reaction between the ozone and the dissolved compounds, and the radical way owing to the reactions between the generated radicals produced in the ozone decomposition (hydroxyl radicals) and the dissolved compounds.
- O_3/H_2O_2: Hydroxyl radicals are formed trough a radical-chain mechanism by interaction between the ozone and the hydrogen peroxide. The global reaction is:
  $H_2O_2 + 2O_3 \rightarrow 2HO\bullet + 3O_2$
  UV radiation may improve the efficiency of the process.
- UV: This method is based on providing energy to the chemical compounds as radiation, which is absorbed by reactant molecules. Those can pass to excited states and have sufficient time to promote reactions.
- UV/O_3: The energy provided by UV radiation interacts with ozone. The global reaction is as follows:

$O_3 + H_2O + h\nu \rightarrow 2HO\bullet + O_2$

- UV/H_2O_2: Radiation with a wavelength lower than 400 nm is able to photolize H_2O_2 molecule. The mechanism accepted for the photolysis of hydrogen peroxide is the cleavage of the molecule into hydroxyl radicals with a quantum yield of two HO• radicals formed per quantum of radiation absorbed according to the following reaction:

  $H_2O + h\nu \rightarrow 2HO\bullet$

  - UV/O_3/H_2O_2: This method allows the complete and fast mineralisation of pollutants.
  - UV/H_2O_2/TiO_2: The use of hydrogen peroxide together with TiO_2 photocatalyst improves the efficiency of the photocatalytic process since H_2O_2 reduces the recombination of hole–electron pairs on the catalyst surface and reacts with conduction band electrons and superoxide radical anions to produce additional hydroxyl radicals.

AOPs have been widely and successfully investigated in water and wastewater treatment for the removal of inorganic and organic contaminants as well as for the enhancement of the biodegradability of industrial wastewater. Unfortunately, the partial oxidation of organic contaminants may result in the formation of intermediates more toxic than parent compounds. In order to avoid this drawback, AOPs are expected to be carefully implemented, operated and monitored (Rizzo 2011).

1.5 SOLAR DRIVEN ADVANCED OXIDATION PROCESSES

Although different investigations have shown that AOPs may be effective in water and wastewater treatment due to their capacity to oxidise and mineralise almost any organic contaminants, technical applications are still scarce due to high operative costs. Significant efforts have been made to overcome those limits. One proposed cost-cutting measure deals with the proper combination of conventional biological processes, typically implemented in UWTPs, with AOPs. The aim is to

apply AOPs as pre-treatment of non-biodegradable and toxic wastewater. Once higher biodegradability has been achieved, the effluent is transferred to a cheaper biological treatment relying on a lower residence time and requiring less reagent consumption (Malato et al. 2009).

Another significant proposed cost-cutting measure is based on the use of renewable energy sources, *i.e.* sunlight, as irradiation source to run AOPs. In other words AOPs can take advantage of natural sunlight like source of photons, thereby saving treatment costs (Malato et al. 2009). In this way solar driven AOPs may be an attractive option for water and wastewater treatment in small communities.

Among solar driven AOPs, heterogeneous and homogeneous photocatalysis (*i.e.* TiO_2 and photo-Fenton) have received most research attention in recent decades for wastewater treatment purposes (Agulló-Barceló et al. 2013, Ortega-Gómez et al. 2014, Papoutsakis et al. 2015b, Polo-López et al. 2014, Rodríguez-Chueca et al. 2014, Soares et al. 2015, Velegraki and Mantzavinos 2015).

The heterogeneous solar photocatalytic detoxification process consists of making use of the near-ultraviolet (UV) band of the solar spectrum (wavelength shorter than 400 nm) to photo-excite a semiconductor catalyst in contact with water and in the presence of oxygen. Once hydroxyl radicals have been generated, they produce a progressive break-up of molecules until yielding CO_2, H_2O and diluted inorganic acids. The main benefits relying on solar heterogeneous photocatalysis applications are based on (i) the complete oxidation of pollutants into CO_2 and other inorganic species; (ii) the oxygen necessary for the reaction can be directly taken from atmosphere; (iii) the process is implemented at room temperature and without overpressure; (iv) the catalyst is cheap, innocuous and can be reused; (v) the catalyst can be attached to many types of inert matrices; (vi) the energy to photo-excite the catalyst is taken directly from the sun (Malato et al. 2009).

Different solar reactors for photocatalytic water treatment have been designed, such as concentrating and non-concentrating collectors, in order to ensure efficient conversion of incident photons to charge carriers, both in the detoxification and in the disinfection field.

1.6 ISSUES TO BE ADDRESSED

Although many efforts have been made in the field of wastewater detoxification, important gaps to be filled still exist in wastewater disinfection in respect of antibiotic resistance.
Current limitations are based on the lack of a proper definition of antibiotic resistance for environmental strains of bacteria as well as the numerous databases with scant information on antibiotic resistance in the environment (Berendonk et al. 2015). This is a limiting factor which needs to be dealt with in the light of further risk assessment and management of antibiotic resistance as well as of wastewater quality in respect to ARB and ARGs.
The above mentioned aspects are preliminary steps for the evaluation of the proper disinfection technology.
Solar driven AOPs have been successfully investigated for the removal of a wide range of CECs from water and wastewater. Unfortunately, so far a few and non-exhaustive works are available in the scientific literature about their effect on antibiotic resistance in terms of (i) ARB inactivation, (ii) ARGs removal, (iii) antibiotic resistance genetic elements release after treatment, (iv) simultaneous removal of ARB and CECs, (v) risk of antibiotic resistance transfer, as well as CECs diffusion, in receiving aquatic systems where treated wastewater are discharged or in crops irrigated with treated wastewater in the wider light of wastewater reuse. In particular, only a few works have addressed the effect of disinfection processes on the potential of antibiotic resistance transfer through molecular analysis (Guo et al. 2013, McKinney and Pruden 2012, Munir et al. 2011, Yuan et al. 2015) or cultivation methods (Dunlop et al. 2015).
This PhD thesis work aims at addressing the above quoted issues and lacks of knowledge trough a multidisciplinary approach including environmental engineering, microbiology, molecular biology and analytical chemistry.

2 OBJECTIVES AND RATIONALE

The aims of this PhD work were to investigate the main effects of AOPs on antibiotic resistance transfer and on CECs removal. In particular the effects on antibiotic resistance transfer were evaluated through (i) the inactivation of both antibiotic resistant bacterial strains selected from urban wastewater and indigenous ARB as well as through (ii) the assessment of variations of ARGs in DNA extracted from cell cultures and in total DNA after disinfection treatment. The second objective was evaluated trough (i) the removal of CECs and the simultaneous inactivation of ARB from urban wastewater as well as (ii) CECs accumulation/deposition/transfer/uptake by crops irrigated with treated wastewater in the light of wastewater reuse.

The above quoted objectives were addressed in the following way:

- The comparison among different AOPs, namely UV/H_2O_2, UV/TiO_2, UV/H_2O_2/TiO_2, photo-Fenton and the evaluation of their effect on the inactivation of target antibiotic resistant *E. coli* and *E. faecalis* strains selected from urban wastewater (chapters 3 and 4);
  Firstly experiments were carried out at laboratory scale (chapter 3 and sub-chapter 4), then at pilot scale (sub-chapter 4) in compound parabolic collectors photo-reactors;
- The evaluation of the effect of a selected AOP, namely UV/H_2O_2, on the potential of antibiotic resistance transfer by means of molecular biology analyses (chapter 5).
  Firstly experiments were run at laboratory scale working on DNA-free water spiked with an antibiotic resistant *E. coli* strain selected from urban wastewater. Then experiments were carried out on real urban wastewater to evaluate the effect of disinfection process on indigenous ARB;
- The evaluation of simultaneous removal of ARB, namely antibiotic resistant *E. coli* and *E. faecalis* strains selected from urban wastewater, and CECs, namely carbamazepine, flumequine

and thiabendazole, by a selected solar driven AOP, such as UV/H_2O_2 (chapter 6);

- The assessment of chemical and microbial cross contamination on lettuce crops irrigated with wastewater that have been treated by UV/H_2O_2 (chapter 6).

3 WASTEWATER DISINFECTION BY HETEROGENEOUS PHOTOCATALYSIS: EFFECT ON TETRACYCLINE RESISTANT *ENTEROCOCCUS* STRAIN

3.1 ABSTRACT

Solar simulated heterogeneous photocatalysis (SSHP) with suspended TiO_2 was investigated in the inactivation of tetracycline resistant/sensitive *Enterococcus* (TRE/TSE) strains in the effluent of an urban wastewater treatment plant (UWTP). The effect of solar simulated disinfection (SSD) on the inactivation of the same *Enterococcus* strains was investigated as control. SSHP process (0.05 g L^{-1} of TiO_2) was found to be effective in the inactivation of both *Enterococcus* strains; total inactivation (approximately 7 Log unit) was observed after 60 min of irradiation. On the contrary, SSD process did not show any significant inactivation after 90 min of irradiation. The effect of both processes on the antibiotic resistance phenotypes of the survived enterococci was also evaluated. TRE cells surviving the SSHP treatment have shown that disinfection process did not affect the antibiotic resistance pattern after 45 min irradiation. Accordingly, antibiotic resistance determinants can be spread into the receiving water body when antibiotic resistant strains survive to disinfection process.

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3.2 INTRODUCTION

Due to the ever-increasing use of antibiotics for agriculture, human (domestic and hospital use) and veterinary purposes, these compounds are continuously released into the environment from different anthropogenic sources (Brown et al. 2006, Grassi et al. 2013, Kümmerer 2009). In addition to the adverse effects as chemical pollutants of the environment, antibiotics are also associated with the spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs). The contamination of the environment and the related risk for human health through the food chain is presently considered a serious public health problem as confirmed by the World Health Organization which identified the development of antibiotic resistance as one of the major global threats to the human society (WHO 2013). UWTPs are suspected to be among the main anthropogenic sources for the development of antibiotic resistance because of the simultaneous occurrence of antibiotics, ARB and ARGs (Michael et al. 2013, Novo and Manaia 2010, Rizzo et al. 2013a). UWTPs are not designed to remove these contaminants and consequently they are released into the effluent almost unaffected, possibly promoting antibiotic resistance spread into the environment (Lupo et al. 2012). Moreover, wastewater reuse, especially for irrigation purposes in areas with limited rainfall, is a current practice, but the risk for human health that is related to the transfer of ARB is almost unknown (Fatta-Kassinos et al. 2011, Gatica and Cytryn 2013).
Disinfection process should be the ultimate barrier to control antibiotic resistance spread before UWTP effluent disposal or reuse, but conventional disinfection processes, such as chlorination and UV radiation, may not effectively control the release of ARB and ARGs (Rizzo et al. 2013b). This drawback calls for the study of alternative/new processes, such as AOPs, which have been successfully investigating in water/wastewater treatment (Lofrano et al. 2009, Rizzo et al. 2008, Rizzo et al. 2013c, Zapata et al. 2010). Among AOPs, heterogeneous photocatalysis with TiO_2 has recently emerged as an interesting water disinfection option (Dunlop et al. 2011, Robertson et al. 2012) that can be operated with solar radiation thus saving money for energy (Malato et al. 2009). Solar driven photocatalysis could be an interesting disinfection

option, alternative to conventional processes, in particular in arid and semi-arid areas, to improve the quality and safety of the effluent from small UWTPs (*i.e.*<2,000 population equivalent) before reuse.
The most studied bacteria for antibiotic resistance in wastewater belong to the common indicators of faecal contamination, namely coliforms and enterococci. Among coliforms, *Escherichia coli* have been recently used as indicators of antibiotic resistance to evaluate the effect of conventional (Huang et al. 2013, Rizzo et al. 2013b, Templeton et al. 2009) and new disinfection processes (Rizzo et al. 2014a). The effect on antibiotic resistant enterococci strains was investigated using TiO_2 photocatalysis (Tsai et al. 2010) and solar photo-Fenton (Michael et al. 2012).
Tetracyclines belong to a group of broad-spectrum antibiotics which are generally used in the treatment of infections of the gastrointestinal and urinary tracts. Accordingly, tetracycline is one of the most frequently detected antibiotics in wastewater (Michael et al. 2013, Watkinson et al. 2007) and different *Enterococcus* spp. (among which *Enterococcus faecalis* and *Enterococcus faecium*) were found to be capable of acquiring resistance to tetracycline (Cauwerts et al. 2007).
In the present study two *Enterococcus* spp. strains, one resistant to tetracycline (TRE) and one sensitive to tetracycline (TSE) were isolated from the effluent of a biologically treated urban wastewater and used as bio-indicators to test the disinfection efficiency of simulated solar driven TiO_2 photocatalysis process. The effect of disinfection process was evaluated on the base of *Enterococcus* survival and antibiotic resistance of survived *Enterococcus* colonies.

3.3 MATERIALS AND METHODS

3.3.1 Wastewater samples

Wastewater samples were collected from an UWTP placed in the province of Salerno (Italy), from the effluent of the biological process (activated sludge). The UWTP also receives sewage from a hospital. The samples were collected in sterilized 1 L amber glass bottles and analysed for conductivity, pH, BOD_5, COD and TSS. Wastewater were characterised as follows: conductivity=1,453.0 μS cm^{-1}, pH=7.9,

BOD_5=15.0 mg L^{-1}, COD=35.0 mg L^{-1}, TSS=28.0 mg L^{-1}. On the base of the measured parameters, wastewater met the Italian standards for wastewater disposal into surface water but did not meet the standard set by Italian legislator for TSS for wastewater reuse (10.0 mg L^{-1}).

3.3.2 Selection and identification of *Enterococcus* strains

Resistant/sensitive *Enterococcus* strains were isolated from UWTP effluent sample on Slanetz Bartley agar (SBA) culture medium (10.0 g L^{-1} bacteriological agar, 4.0 g L^{-1} dipotassium phosphate, 2.0 g L^{-1} glucose, 0.4 g L^{-1} sodium azide, 20.0 g L^{-1} tryptose, 0.1 g L^{-1} TTC, 5.0 g L^{-1} yeast extract), using the membrane filtration method. A resistant *Enterococcus* strain was selected on SBA culture medium supplemented with 16 mg L^{-1} of TET. Briefly, 5 mL of wastewater or its serial dilutions were added to 45 mL of saline solution and filtered through membranes; membranes were subsequently transferred onto SBA culture medium and incubated at 37 °C for 48 h. A confirmation test was conducted by transferring the membranes with typical red colonies onto Bile Esculin Azide (BEA) agar (13.00 g L^{-1} agar, 1.00 g L^{-1} esculin, 0.50 g L^{-1} iron ammonium citrate, 10.00 g L^{-1} ox bile, 3.00 g L^{-1} peptone, 0.15 g L^{-1} sodium azide, 5.00 g L^{-1} sodium chlorine, 17.00 g L^{-1} tryptose, 5.00 g L^{-1} yeast extract). Some colonies were randomly picked up and frozen in 15% glycerol Triptone Soy Broth at -20 °C.
Colonies that were able to grow on SBA supplemented with 16 mg L^{-1} of TET were regarded as TRE.

3.3.3 Inoculum and sample preparation

Autoclaved (15 min at 121 °C) wastewater samples were inoculated with TRE and TSE strains separately. For disinfection experiments, 500 mL autoclaved wastewater samples were spiked with fresh cell cultures of the selected strains until obtaining an initial bacterial density as high as 10^5 colony forming units (CFU) mL^{-1}. A standard plate counting method on SBA was used to evaluate bacterial inactivation. Culture plates with 20-200 colonies were the basis for bacterial counts.

3.3.4 Photocatalytic tests

Photocatalytic experiments were carried out in 2.2 L cylindrical glass batch reactor (13.0 cm in diameter) filled with 500 mL wastewater sample (5.0 cm water height). The reactor was placed in a water bath to keep constant temperature (roughly 25 °C) during the experimental procedure. The wastewater solution was kept stirred during the experiments. Simulated solar radiation was provided by a wide spectrum 250 W lamp equipped with a UV filter (Procomat, Italy), fixed at 40 cm from the upper water level in the reactor. 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 (emission spectrum 340-460 nm; average intensity: 53.46 μW cm^{-2}; max intensity (375 nm): 104.0 μW cm^{-2}). 0.05 g TiO_2 L^{-1} was used in photocatalytic experiments, according to previous results (Rizzo et al. 2014b). The autoclaved wastewater sample with TiO_2 powder (Degussa P25) was sonicated for five min before inoculum addition. Control tests without any photocatalyst addition (solar simulated photolysis tests) were also performed.

3.3.5 Antibiotic resistance assays

The antibiotic resistance patterns of the bacterial isolates prior to and after the photocatalytic treatment was tested by Kirby–Bauer method. Briefly, the colonies that survived to photocatalytic treatment and grew on SBA medium were selected (4-5 colonies randomly picked up from each one agar/irradiation time) and transferred to 10 mL physiological solution up to achieve 10^8 CFU mL^{-1} (0.5 McFarland). Bacterial suspensions were spread onto Mueller Hinton agar (Biolife, Italy) using a sterile cotton swab. Antibiotic-impregnated discs of ampicillin (AMP) (10 μg), ciprofloxacin (CIP) (5 μg), tetracycline (TET) (30 μg) and vancomycin (VAN) (30 μg) (all from Biolife) were placed onto the surface of each inoculated plate. After 24 h of incubation at 37 °C, the diameters of each antibiotic inhibition zone were measured. The procedure was duplicated and the average values were plotted. The results were compared with antibiotic resistance and inhibition zone diameters (mm) of *Enterococcus faecalis* for Kirby-Bauer method available in EUCAST (2013) database and summarised in Table 3.1.

Table 3.1 Summary of antibiotic resistance and inhibition zone diameter values (mm) of *Enterococcus faecalis* for AMP, CIP, TET and VAN (Kirby-Bauer method) available in EUCAST database (2013)

	AMP10	CIP5	TET30	VAN30
Resistant (R)	≤ 16	≤ 15	≤ 14	< 16
Intermediate (I)	–	16 – 20	15 – 18	–
Sensitive (S)	≥ 17	≥ 21	≥ 19	> 16

3.4 RESULTS AND DISCUSSION

3.4.1 *Enterococcus* strains inactivation by solar simulated photocatalysis

Solar simulated heterogeneous photocatalysis and solar simulated photolysis were simultaneously investigated in the inactivation of TRE and TSE strains. The photocatalytic process allowed both the tested strains to achieve the detection limit (DL) within 60 min of exposure (Figure 3.1). In particular, an inactivation percentage of 98.63% was observed after 30 min irradiation for TRE strain (average initial concentration as high as 1.8×10^5 CFU mL^{-1}). A similar trend was observed for the sensitive strain (average initial density 1.3×10^5 CFU mL^{-1}): an inactivation of 95.11% was reached after 30 min treatment.

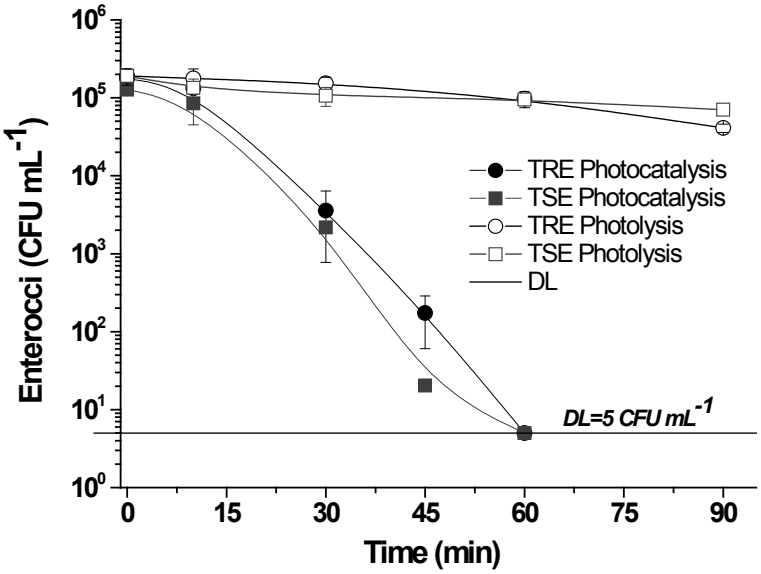

Figure 3.1 Inactivation of TRE and TSE strains by solar simulated photolysis and solar simulated photocatalysis (0.05 g TiO_2 L^{-1})

In parallel, solar simulated photolysis tests did not show any inactivation of both strains working on the same operative conditions of photocatalytic assays.

These results seem to support the idea that *Enterococcus* may be used as indicator of the occurrence of antibiotic resistant *Enterococcus* after disinfection process. Specifically, if the disinfection process effectively inactivates *Enterococcus* indicators, the simultaneous inactivation of the resistant strains may be expected.

Moreover, despite each bacterium strain responds to stress conditions in different ways, the obtained results are in agreement with those plotted in a previous work where the photocatalytic process was investigated in the inactivation of an antibiotic resistant *E. coli* strain (Rizzo et al. 2014b). In particular, photocatalysis tests run in the same operative conditions, showed a total inactivation, as high as 5 Log removal, after 60 min irradiation. Xiong and Xu investigated the effect of a UV-A/LED/TiO_2 system (TiO_2 particles coated reactor, 8,000 μW cm^{-2} light intensity) on the inactivation of an antibiotic resistant *E. coli* strain (ATCC 700891) containing ampicillin and streptomycin resistance markers. In particular 4.5 Log inactivation (initial bacterial density as

high as 6×10^4 CFU mL^{-1}) was observed in 90 min of irradiation time approximately (Xiong and Xu 2013). Tsai and co-workers investigated the effect of TiO_2 photocatalytic process on three clinical isolates antibiotic resistant bacteria, among them vancomycin-resistant *Enterococcus* faecalis (VRE). Unlike the present work, photocatalytic experiments were carried out relying on higher intensity irradiation (400-800 μW cm^{-2}) and photocatalyst loads (0.0625-0.1250 g L^{-1}) (Tsai et al. 2010). According to results that have been obtained in the present study, no significant effect on VRE inactivation under light irradiation was observed. The addition of catalyst (0.0625 g L^{-1}, 400 μW cm^{-2}) involved better performances in terms of inactivation (10^3-10^5 CFU mL^{-1} initial bacterial density), but total inactivation was not observed during the experiment (max irradiation time 90 min). When light irradiation intensity and photocatalyst load were increased up to 800 μW cm^{-2} and 0.1250 g L^{-1} respectively, total inactivation was achieved in 20-25 min (Tsai et al. 2010). In another study, the effect of solar-photo-Fenton on enterococci (initial density ≈$2.5\text{-}4.0\times10^2$ CFU mL^{-1}) was investigated in terms of both inactivation rate and resistance percentage (ratio between resistant and total enterococci). DL was reached after 180 min treatment under sunlight radiation (Michael et al. 2012).

3.4.2 Effect of solar simulated heterogeneous photocatalysis on antibiotic resistance

The average values of each inhibition zone diameter for AMP (10 μg), CIP (5 μg), TET (30 μg) and VAN (30 μg), before disinfection (T0), were compared with the corresponding clinical breakpoints values for *Enterococcus* from EUCAST database (Table 3.1). TRE strain was found to be sensitive (S) to AMP, CIP and VAN but resistant (R) to TET.
As shown by Kirby-Bauer test results, disinfection process did not affect TRE strain resistance to each tested antibiotic (Table 3.2).

Table 3.2 Antibiogram results on TRE strain before (t=0) and after photocatalysis: inhibition zone diameters (mm)

time (min)	AMP10	CIP5	TET30	VAN30
0	24.3	24.6	10.0	20.0
10	23.8	24.5	10.0	20.0
30	24.0	24.8	10.0	20.0
45	31.5	26.5	10.0	20.0

In parallel solar simulated photolysis process did not show any effect on the inhibition zone diameters (no trend was observed) for all investigated antibiotics.
The effect of photocatalytic process on an antibiotic resistant *E. coli* strain was investigated in a previous work (Rizzo et al. 2014b). The resistance of the survived colonies to CIP and VAN was found to decrease (the diameters of inhibition zone increased from 12.8 to 13.8 mm and from 10.2 to 10.9 mm, respectively) as irradiation time increased (from 0 to 30 min), respectively. In another work, the effect of solar photo-Fenton process on antibiotic resistance of enterococci was investigated in terms of resistance percentage (Michael et al. 2012). After 180 min treatment under sunlight radiation, a decrease of resistance occurred for ofloxacin and trimethoprim resistant enterococci (initial resistance percentages ≈20% and ≈10% respectively).

3.5 CONCLUSIONS

TiO_2 solar simulated photocatalysis was found to be effective in the inactivation of TRE and TSE strains selected from UWTP biological effluent. Remarkably, photocatalytic disinfection tests showed comparable inactivation rates for both strains. On the base of the achieved results, the inactivation of cultivable enterococci may also involve the inactivation of cultivable antibiotic resistant enterococci. Accordingly, *Enterococcus* may be used as indicator of photocatalytic process efficiency.
Moreover the photocatalytic process did not affect antibiotic resistance of the survived TRE colonies. In this light the investigated disinfection process may only minimise the spread of antibiotic resistance into the receiving water bodies in which the treated effluent is disposed off.

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3.7 APPENDIX

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WASTEWATER DISINFECTION BY SOLAR HETEROGENEOUS PHOTOCATALYSIS: EFFECT ON TETRACYCLINE RESISTANT/SENSITIVE *Enterococcus* strains

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ABSTRACT

Solar simulated heterogeneous photocatalysis (SSHP) with suspended TiO_2 was investigated in the inactivation of tetracycline resistant/sensitive *Enterococcus* (TRE/TSE) strains in the effluent of an urban wastewater treatment plant (UWTP). The effect of solar simulated disinfection (SSD) on the inactivation of the same *Enterococcus* strains was investigated as control. SSHP process (0.05 g l^{-1} of TiO_2) was found to be effective in the inactivation of both *Enterococcus* strains with total inactivation (~7 log unit) observed after 60 min of irradiation. On the contrary, SSD process did not show any significant inactivation after 90 min of irradiation. The effect of both processes on the antibiotic resistance phenotypes of the surviving enterococci was also evaluated. TRE cells surviving the SSHP treatment showed that disinfection process did not affect the antibiotic resistance pattern after 45 min irradiation. The same was observed for the TSE strain. Accordingly, antibiotic resistance can spread into the receiving water body when antibiotic resistant strains survive to disinfection process.

Keywords: advanced oxidation processes, antibiotic resistant bacteria, bioindicator, urban wastewater, wastewater reuse.

4 EFFECT OF SOLAR DRIVEN AOPS ON THE INACTIVATION OF ANTIBIOTIC RESISTANT *E. COLI*

4.1 ABSTRACT

Solar driven advanced oxidation processes (AOPs), namely H_2O_2/sunlight, TiO_2/sunlight, H_2O_2/TiO_2/sunlight, solar photo-Fenton, were compared and evaluated in the inactivation of antibiotic resistant (AR) bacteria in real urban wastewater. An AR *Escherichia coli* strain isolated from the effluent of the biological process of an urban wastewater treatment plant was chosen as target AR bacterium. Experiments were firstly carried out at laboratory scale, then at pilot scale in compound parabolic collectors photo-reactors. In the first case, H_2O_2/TiO_2/sunlight process allowed to achieve the highest inactivation rates relying on a cumulative energy per unit of volume (Q_{UV}) in the range 3-5 kJ L^{-1}, on the base of H_2O_2/TiO_2 ratio. H_2O_2/sunlight process required 8 kJ L^{-1} on average to reach the detection limit (DL). When implemented at pilot scale, the best disinfection efficiency in terms of treatment time (20 min to reach the DL) and required energy (Q_{UV}=1 kJ L^{-1}) was observed for photo-Fenton at pH 4 (Fe^{2+}/H_2O_2:5/10 mg L^{-1}). All investigated processes resulted in a complete inactivation (5 Log decrease) of AR *Escherichia coli* until DL.
Antimicrobial susceptibility was tested by Kirby-Bauer disk diffusion method. Ampicillin and ciprofloxacin (to which the selected strain was resistant), cefuroxime and nitrofurantoin were chosen as tested antibiotics. None of the investigated processes affected antibiotic resistance of survived colonies.

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4.2 INTRODUCTION

Due to the release of antibiotic resistant (AR) bacteria through urban wastewater treatment plants (UWTPs) effluents, disinfection processes represent an important tool to control/minimise the risk of antibiotic resistance spread into the environment. Although several studies deal with the effects of AOPs on the inactivation of microorganisms (Agulló-Barceló et al. 2013, Bichai et al. 2012, Dunlop et al. 2011, Malato et al. 2009), only a few works address AR bacteria fate within and after disinfection processes (Dunlop et al. 2015, Tsai et al. 2010) and still less focus on indigenous AR bacteria (Rizzo et al. 2014b). It is well known that AOPs can be driven by sunlight thus saving energy costs (Malato et al. 2009) and becoming competitive to conventional technologies (Bichai et al. 2012), particularly for wastewater treatment in small communities. Moreover they may decrease health risk for consumers of wastewater-irrigated crops in developing countries (Bichai et al. 2012).
Among solar driven AOPs, heterogeneous and homogeneous photocatalysis have received most research attention in recent decades for wastewater treatment purposes (Agulló-Barceló et al. 2013, Ortega-Gómez et al. 2014, Rodríguez-Chueca et al. 2014).
Aim of this work was to evaluate the effect of solar disinfection (SODIS) and solar driven AOPs on the inactivation of an indigenous *E. coli* strain selected from real urban wastewater as resistant to three antibiotics, namely ampicillin (AMP), ciprofloxacin (CIP) and tetracycline (TET).

Specifically, H_2O_2/sunlight, TiO_2/sunlight, H_2O_2/TiO_2/sunlight, solar photo-Fenton processes (at natural pH) were implemented at laboratory scale testing different catalyst doses in order to (i) assess and compare their effect on the target AR *E. coli* strain inactivation and (ii) investigate the effect of disinfection processes on the antibiotic resistance of survived colonies.
On the base of the achieved results, solar driven AOPs were subsequently implemented at pilot scale, in compound parabolic collectors (CPCs), in order to assess their potential to decrease the microbial risk of treated and reclaimed UWTPs effluents. Also in this case different catalyst doses were tested and compared. This work is the first dealing with the investigation and comparison among different solar driven AOPs in the inactivation of an indigenous AR *E. coli* strain in real UWTP effluent, at pilot scale.

4.3 LABORATORY SCALE EXPERIMENTS

4.3.1 Materials and methods

4.3.1.1 Selection of antibiotic resistant *E. coli* strain

An AR *E. coli* strain was selected from a large UWTP (700,000 population equivalent) placed in southern Italy. Particularly the target strain was isolated from the biological effluent of an activated sludge process through membrane filtration and subsequent cultivation on selective medium (at 37 °C for 24 h), namely Tryptone Bile X-glucuronide agar (TBX, Sigma-Aldrich) supplemented with a mixture of three antibiotics (16 mg L^{-1} of AMP, 2 mg L^{-1} of CIP, 8 mg L^{-1} of TET). AMP and CIP concentrations were selected as the double values of the respective minimum inhibitory concentration (MIC) values available in EUCAST database (2015). Colonies that were able to grow in these stress conditions were regarded as antibiotic resistant. Some colonies were randomly picked up and frozen in 15% glycerol Triptone Soy Broth at -20 °C.

4.3.1.2 Inoculum and sample preparation

Antibiotic resistant *E. coli* colonies were unfrozen and reactivated by streaking onto ChromoCult® Coliform agar (Merck KGaA, Darmstadt, Germany) and incubating at 37 °C for 18-24 h. Three colonies from the plate were inoculated into 14 mL sterile Luria Bertani broth (LB, Sigma-Aldrich, USA) and incubated at 37 °C for 18 h by constant agitation in a rotator shaker to obtain a stationary phase culture. Bacterial cells were harvested by centrifugation at 3,000 rpm for 10 min and the pellet was resuspended in 14 mL phosphate buffer saline (PBS, Oxoid), yielding a final density of 10^9 CFU mL^{-1} approximately.

Wastewater samples were freshly collected from the UWTP of Almería, El Bobar (Spain), from the effluent of the biological process (activated sludge), on the morning of each disinfection experiment. Wastewater samples were subsequently filtered through 0.22 μm sterile Millipore filters in order to remove all indigenous bacteria and then evaluate the effect of each disinfection process on the inactivation of the selected AR *E. coli* strain.

Filtered wastewater samples were spiked with the selected AR *E. coli* strain until reaching an initial density as high as 10^{5-6} CFU mL^{-1} approximately.

All the filtered samples were characterized using a conductivity sensor (GLP31, CRISON, Spain), a pH-meter (multi720, WTW, Germany), a thermometer (Checktemp, Hanna instruments, Spain), a Total Organic Carbon analyzer (Shimadzu TOC-5050, Kyoto, Japan). The average values were the following: conductivity: 1,020-1,510 μS cm^{-1}, pH: 7.00-7.92, TOC: 18.20-27.80 mg L^{-1}.

4.3.1.3 Bacterial count

A standard plate counting method through a serial 10-fold dilutions in PBS after an incubation period at 37 °C for 24 h was used to count bacterial colonies. When the bacterial density was expected to be high, volumes of 20 μL were plated in triplicate onto Endo agar (Fluka, Sigma-Aldrich, USA). When the bacterial load was expected to be low, 250 or 500 mL samples were spread onto ChromoCult® Coliform agar.

The detection limit (DL) of this experimental method was found to be 2 CFU mL^{-1}.

4.3.1.4 Catalysts and oxidants doses: analytical measurements

Hydrogen peroxide

Different H_2O_2 (Riedel-de Haën, Germany) concentrations were used:

- 50, 75 and 100 mg L^{-1} in H_2O_2 dark assays;
- 10, 20 and 50 mg L^{-1} in H_2O_2/sunlight tests;
- 10 and 50 mg L^{-1} in H_2O_2/TiO_2/sunlight tests;
- 10, 20 and 40 mg L^{-1} in solar photo-Fenton experiments.

H_2O_2 at 30 wt% (Merck, Germany) was used as received and diluted into bottles filled with filtered wastewater samples. H_2O_2 concentration in wastewater was measured by means of a colorimetric method based on the use of titanium (IV) oxysulfate (Riedel-de Haën, Germany), which forms a stable yellow complex with H_2O_2 detected by absorbance measurements at 410 nm (PG Instruments Ltd T-60-U). The signal was read with reference to a H_2O_2 standard in distilled water. Absorbance measurements were linearly correlated with H_2O_2 concentrations in the range 0.1–100.0 mg L^{-1}.
A freshly prepared solution of bovine liver catalase (0.1 g L^{-1}, Sigma-Aldrich, USA) was added to wastewater samples to eliminate residual H_2O_2 and avoid Fenton reactions after samples collection. Particularly 1 mL wastewater samples were mixed with 20 µL of 2,300 U mg^{-1} catalase. Hydrogen peroxide and catalase have been demonstrated to have no detrimental effects on *E. coli* viability (García-Fernández et al. 2012).

Titanium dioxide

Aeroxide P25 (Evonik Corporation, Germany) TiO_2 was used as received from the manufacturer as slurry to perform heterogeneous photocatalytic experiments.
The concentration of titanium dioxide was 100 mg L^{-1} in TiO_2/sunlight and in H_2O_2/TiO_2/sunlight assays.

Iron
Ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$, PANREAC, Spain) was used as Fe^{2+} source for homogeneous photo-Fenton reaction at concentrations of 5, 10 and 20 mg L^{-1}. Fe^{2+} concentrations were

measured according to ISO 6332. All samples were filtered through 0.20 μm CHROMAFIL® XtraPET-20/25 (PANREAC, Spain) and measured by means of a spectrophotometer (PG Instruments Ltd. T-60-U) at 510 nm. The concentration ratio of iron and hydrogen peroxide was 1:2. For photo-Fenton tests, a freshly prepared solution of bovine liver catalase (0.1 g L^{-1}, Sigma-Aldrich, USA) was added to wastewater samples in a ratio 0.1/5 (v/v) to eliminate residual hydrogen peroxide and avoid Fenton reactions after samples collection.

4.3.1.5 Solar experiments

All solar experiments were carried out in duplicate under natural solar irradiation on clear sunny days at Plataforma Solar de Almería, Spain (37° 84' N and 2° 34' W) from September 2013 to May 2014.

Borosilicate glass (DURAN, Schott, Germany) bottles were used as reactors. Glass covers (Schott, Germany) were used in order to allow the entrance of solar radiation from all directions. They were filled with 200 mL filtered wastewater samples, exposed to sunlight and kept stirring during each experiment performed at laboratory scale. Prior to sunlight exposure, bottles with 200 mL of real filtered wastewater were spiked with 20 μL of AR *E. coli* inoculum solution until reaching an initial bacterial density as high as 10^{5-6} CFU mL^{-1}. Then reagents (catalysts/oxidants) were added to the sample. Bottles were kept stirring in the dark for a few minutes in order to make the solution homogeneous and then exposed to natural solar irradiation. Reagents were also added during the experiment to keep constant concentration of the catalysts/oxidants.

Treated wastewater samples were collected at regular intervals: sampling frequency varied on the base of treatment. Water temperature was measured hourly in each reactor by a thermometer (Checktemp, Hanna instruments, Spain): it ranged from 21.2 °C to 44.0 °C. pH (multi720, WTW, Germany), Fe^{2+} and H_2O_2 were also measured in the reactor during the assays. A water sample was taken as control for each test, kept in the dark at laboratory temperature and plated at the end of the assay. Inactivation values are plotted as average of at least two replicates for each solar driven experiment.

4.3.1.6 Solar UV-A radiation measurement

Solar UV-A radiation was measured with a global UV-A pyranometer (300-400 nm, Model CUV4, Kipp & Zonen, Netherlands) tilted 37°, the same angle as the local latitude. This instrument provides data in terms of incident UV-A (in W m^{-2}), which is the solar radiant UV-A energy rate incident on a surface per unit area. The inactivation rate is plotted as function of both experimental time (t) and cumulative energy per unit of volume (Q_{UV}) received in the photo-reactor, and calculated by Eq. (1):

$$Q_{UV,n} = Q_{UV,n-1} + \frac{\Delta t_n \overline{UV}_{G,n} A_r}{V_t} \qquad \Delta t_n = t_n - t_{n-1}$$

where $Q_{UV,n}$ and $Q_{UV,n-1}$ are the UV energy accumulated per liter (kJ L^{-1}) at times n and n-1, $UV_{G,n}$ is the average incident radiation on the irradiated area, Δt_n is the experimental time of sample, A_r is the illuminated area of collector (m^2), V_t is the total volume of treated water (L). Q_{UV} is commonly used to compare results under different operative conditions (Malato et al. 2009).

4.3.1.7 Antibiotic resistance assays

Antibiotic resistance phenotypes were tested by Kirby-Bauer disk diffusion method according to standard recommendations (EUCAST, 2015). Briefly, *E. coli* colonies, prior to and after treatment, were randomly collected from some agar/irradiation time and transferred onto a physiological solution up to achieve a suspension of 0.5 McFarland (standard turbidity), approximately corresponding to 1-2×10^8 CFU mL^{-1} suspension. Then it was spread onto Mueller Hinton agar II (Fluka, Sigma-Aldrich, USA) using a sterile cotton swab. Antibiotic discs (Biolife, Italy) of ampicillin (AMP, 10 μg), ciprofloxacin (CIPR, 5 μg), cefuroxime (CXM, 30 μg) and nitrofurantoin (NI, 100 μg) were placed onto the surface of each inoculated plate. After 18 h of incubation at 35 °C, the diameters of each inhibition zone were measured and recorded as susceptible (S), intermediary (I) or resistant (R) to respective antibiotics. The criteria used for these interpretations, based on the inhibition zone diameters, were as follows (mm): AMP (10 μg): S≥14, R<14; CIPR (5 μg): S≥22, 19≤I<22, R<19; CXM (30 μg): S≥18, R<18; NI (100 μg):

S≥11, R<11. These values are available on EUCAST (2015) database. The procedure was carried out in duplicate.

4.3.2 Results and discussion

4.3.2.1 Thermal effect on antibiotic resistant *E. coli* strain

Some tests were performed in order to evaluate the thermal effect of temperature on AR *E. coli* inactivation rate. The bottles were covered by aluminum sheets, placed into stoves and exposed to three different temperatures, such as 40, 45 and 50 °C. In parallel a sample was kept as control at room temperature (≈25 °C) in the dark.
AR *E. coli* density did not show any significant variation, except a mild decrease (0.5 Log) after 240 min in the test which was carried out at 50 °C (Figure 4.1).

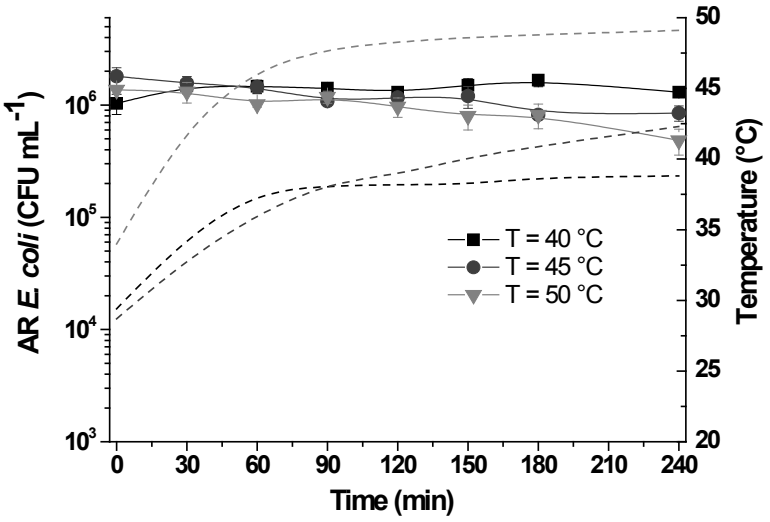

Figure 4.1 Temperature effect on AR *E. coli* strain

The effect of temperature on *E. coli* inactivation during SODIS experiments has been previously investigated and the obtained results are in agreement with those available in scientific literature (Dunlop et al. 2011, McGuigan et al. 2012). McGuigan et al. (2012) investigated the roles of optical and thermal inactivation mechanisms by simulating

conditions of optical irradiance and temperature. They found out that the thermal inactivation of *E. coli* was negligible at temperatures below 40-45 °C and sufficiently important only at water temperatures higher than 45 °C.

4.3.2.2 SODIS

The effect of solar radiation on the inactivation of AR *E. coli* was investigated and results are plotted in Figure 4.2.

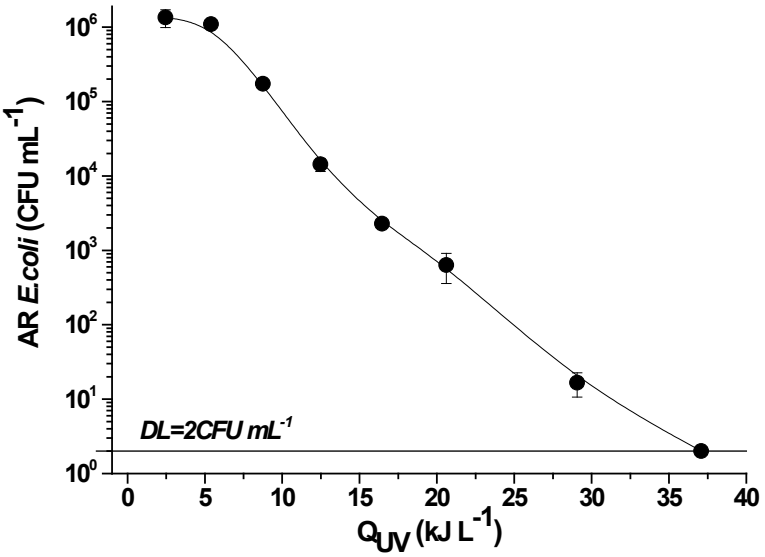

Figure 4.2 AR *E. coli* inactivation by SODIS

AR *E. coli* inactivation rates enhanced under solar exposure as UV dose increased. A 6-Log decrease was observed for the tested strain and a total inactivation (residual bacterial density below the DL, 2 CFU mL^{-1}) was reached after about 4 h of sunlight exposure. In terms of Q_{UV}, SODIS required 37.00 kJ L^{-1} to get the DL. The inactivation of AR *E. coli* is due to the effect of solar radiation as it has been demonstrated that the synergistic effect of UV-A photons and mild thermal heating mechanisms takes place when temperature is above 45 °C (McGuigan et al. 1998). Temperature varied in the range 27.0-35.0 °C, therefore sufficiently lower than 45 °C to observe any significant temperature-related synergistic effect.

SODIS has been deeply investigated for disinfection of contaminated water in terms of indigenous pathogens inactivation and in particular conditions (up to 2.0-2.5 L in bottles and static conditions) (McGuigan et al. 1998). In a study dealing with the evaluation of SODIS on *E. coli* inactivation, DL was achieved between 180 and 300 min and with a Q_{UV} energy in the range 12-30 kJ L^{-1} (Helali et al. 2014). The higher energy demand may be due to the higher resistance of AR *E. coli* and the different water matrix (saline solution Vs real filtered urban wastewater). UV-A and early visible wavelength (320-450 nm) of sunlight spectrum have been reported to be able to generate reactive oxygen species that cause strand break-age and changes in DNA (Acra et al. 1989). These toxic reactive oxygen species can also disrupt protein synthesis. Moreover, UV-B is known to induce direct damage to DNA via formation of the cyclobutane pyrimidine dimer photoproduct (Lyons et al. 1998). The formation of these dimers alters gene expression, inhibits DNA replication and causes genetic mutations (Britt 1996).

4.3.2.3 Solar photo-Fenton

Solar photo-Fenton experiments were carried out at different Fe^{2+}:H_2O_2 ratios (5:10, 10:20 and 20:40 mg L^{-1}) without modifying pH to evaluate possible improvement compared to H_2O_2/sunlight experiments. The complete inactivation was achieved under all investigated conditions, with a Q_{UV} request between 14.9 and 23.1 kJ L^{-1} (Figure 4.3).

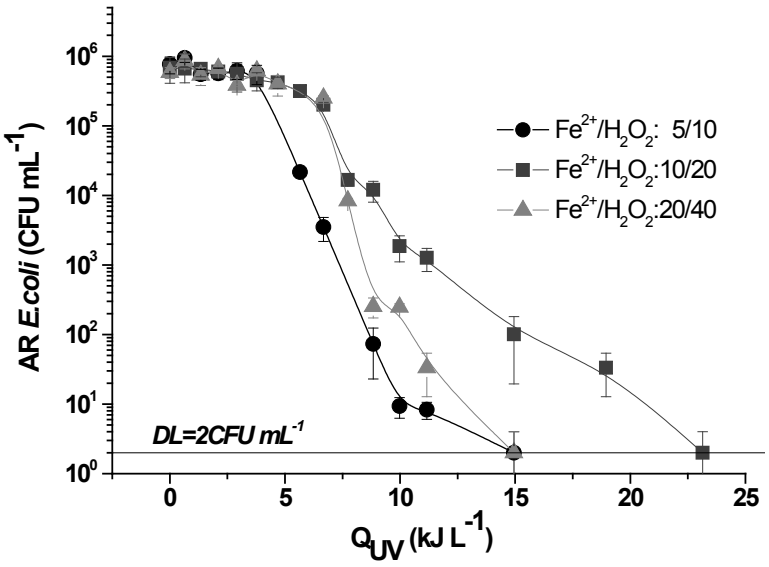

Figure 4.3 AR *E. coli* inactivation by solar photo-Fenton at laboratory scale

The best performance was observed with 5:10 of Fe^{2+}:H_2O_2 ratio. Temperature varied between 19.1 and 40.2 °C and pH between 7.13 and 8.32.

Spuhler et al. observed a total inactivation of *E. coli* strain K12 (10^6-10^7 CFU mL^{-1} initial bacterial density) in mineral water (pH 7.0-7.5) during solar simulated photo-Fenton process at low Fe^{2+} concentration (0.6/10.0 mg L^{-1} of Fe^{2+}:H_2O_2 ratio), in a shorter treatment time (approximately 95 min) (Spuhler et al. 2010). Possibly, the lower Fe^{2+} initial concentration and pH resulted in a decreased iron precipitation and then in a faster inactivation efficiency. The more complex water matrix (wastewater Vs mineral water), the different light source (natural sunlight Vs solar simulator) as well as the target bacteria (AR *E. coli* Vs *E. coli* K12) are also expected to affect process efficiency.

4.3.2.4 H_2O_2/sunlight process

H_2O_2/sunlight process was investigated in detail at different hydrogen peroxide concentrations: 10, 20 and 50 mg L^{-1} (Figure 4.4). DL was reached for all tests with a Q_{UV} of 30.7 kJ L^{-1} (H_2O_2 dose of 10 mg L^{-1}), 18.1 kJ L^{-1} (H_2O_2 dose of 20 mg L^{-1}), 8.7 kJ L^{-1} (H_2O_2 dose of 50 mg L^{-1}). Water temperature was measured during each solar assay and varied

from 27.0 °C to 35.2 °C. H_2O_2 concentration was also monitored throughout all tests; when it decreased, a small volume was added in order to keep constant it.

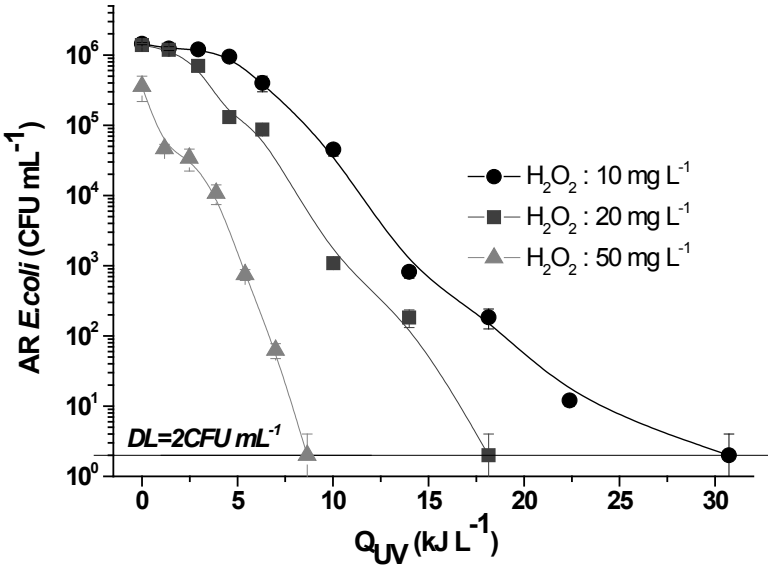

Figure 4.4 AR *E. coli* inactivation by H_2O_2/sunlight process at laboratory scale

4.3.2.5 TiO_2/sunlight and H_2O_2/TiO_2/sunlight processes

The inactivation of AR *E. coli* by heterogeneous photocatalysis with suspended TiO_2 is shown in Figure 4.5. DL was achieved in 150 min of solar treatment with 100 mg L^{-1} of TiO_2 and a Q_{UV} of 20.6 kJ L^{-1}. Inactivation rate was faster than SODIS experiment where DL was reached after 300 min treatment with a higher Q_{UV} (38.0 kJ L^{-1}).

The effect of H_2O_2 and TiO_2 doses was also investigated. The complete inactivation was faster (60 min) than heterogeneous photocatalysis (150 min) and was achieved with both investigated H_2O_2/TiO_2 ratios (10:100 mg L^{-1} and 50:100 mg L^{-1}) (Figure 4.5) with a significantly lower cumulative energy (4.2 and 3.3 kJ L^{-1}, respectively).

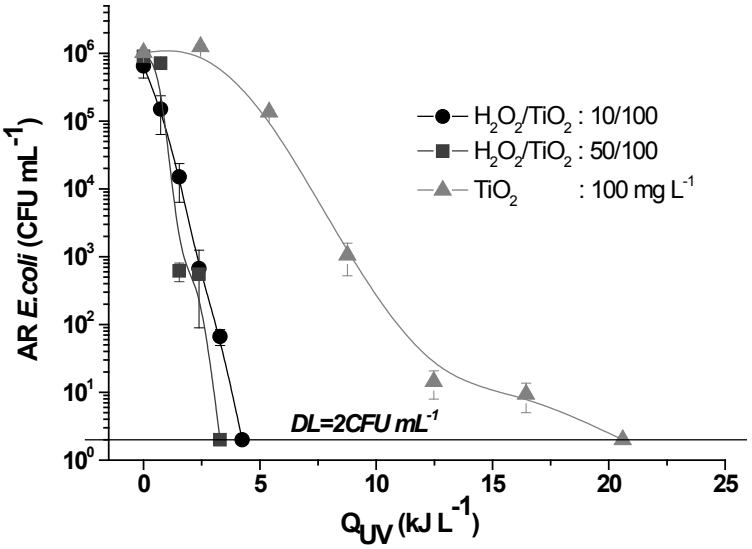

Figure 4.5 AR *E. coli* inactivation by H_2O_2/TiO_2/sunlight and TiO_2/sunlight processes at laboratory scale

The addition of H_2O_2 in presence of TiO_2 promotes the formation of hydroperoxyl radicals ($HO_2^{\bullet}$), and low values of hydroxyl radicals (HO•) (Lousada et al. 2013). This was explained by the change in the extent of decomposition of H_2O_2, comparing effective area/volume ratios, and it may be caused by different factors such as the presence of rutile in P25. However, referring to the finding by Du and Rabani, it is possible to speculate whether a loss of effective surface area due to an agglomeration of particles took place (Du and Rabani 2006). This would result in a reduced production of hydroxyl radicals. On the other hand, agglomeration is possibly due to interactions among $HO_2^{\bullet}$ to the surface of TiO_2. For this reason, the presence of $HO_2^{\bullet}$ is stabilised at high concentrations, with a best performance of inactivation.

The achieved results are in a quite good agreement with those that have been described and discussed in the previous chapter.

Another comparative study at laboratory scale has shown that photocatalytic oxidation by TiO_2 did not affect significantly the inactivation of both methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* ($p>0.05$), whereas the inactivation rate was two times higher for multi-drug resistant *Acinetobacter baumanni* than for multi-drug sensitive *Acinetobacter baumanni* ($p<0.05$) and 2.4 times higher for

vancomycin-sensitive *Enterococcus faecalis* than for vancomycin-resistant *Enterococcus faecalis* (p<0.05) (Tsai et al. 2010). According to these results, the bacterial strain may play an important role on the performance of the photocatalytic process.

4.4 PILOT SCALE EXPERIMENTS

4.4.1 Materials and methods

4.4.1.1 Experimental design

On the base of results that have been achieved in previous tests implemented at laboratory scale, disinfection experiments were run at pilot scale in CPCs photo-reactors at Plataforma Solar de Almería (Spain) on the same AR *E. coli* strain.

Wastewater samples were freshly collected from the UWTP of Almería, El Bobar (Spain), from the effluent of the biological process (activated sludge) and autoclaved (15 min at 121 °C) to inactivate all indigenous bacteria. Autoclaved wastewater samples were spiked with the selected AR *E. coli* strain until reaching an initial density as high as 10^{5-6} CFU mL^{-1} approximately.

All the autoclaved samples were characterised using a conductivity sensor (GLP31, CRISON, Spain), a pH-meter (multi720, WTW, Germany), a thermometer (Checktemp, Hanna instruments, Spain), a Total Organic Carbon analyser (Shimadzu TOC-5050, Kyoto, Japan).

Autoclaved wastewater characterisation is reported in Table 4.1.

Table 4.1 Chemical characterization of the secondary UWTP effluent (El Bobar, Almería, Spain) after autoclaving process

Parameter	Average value	Standard deviation	Unit
Conductivity	1,504.00	154.00	$\mu S\ cm^{-1}$
pH	9.05	0.12	-
Turbidity	50.00	16.00	NTU
TC	70.21	9.90	$mg\ L^{-1}$
IC	48.85	7.94	$mg\ L^{-1}$
TOC	21.35	4.80	$mg\ L^{-1}$

Bacterial count was performed as previously described (see paragraph 4.3.1.3) as well as analytical parameters were measured as reported in paragraph 4.3.1.4. In this case catalysts and oxidants doses were set as follows:

Hydrogen peroxide

- 40 and 75 mg L^{-1} in H_2O_2 dark assays;
- 20, 50 and 75 mg L^{-1} in H_2O_2/sunlight experiments;
- 20 and 50 mg L^{-1} in H_2O_2/TiO_2/sunlight experiments;
- 10, 20 and 40 mg L^{-1} in solar photo-Fenton experiments.

Titanium dioxide

50 and 100 mg L^{-1} were tested as different concentrations in H_2O_2/TiO_2/sunlight experiments.

Iron

Ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$, PANREAC, Spain) was used as Fe^{2+} source for homogeneous photo-Fenton reaction at concentrations of 5, 10 and 20 mg L^{-1}.

4.4.1.2 Solar photo-reactors

Pilot-scale experiments were carried out in a CPCs plant. This system is made up of tube modules placed on a tilted platform connected to a recirculation tank and a centrifugal pump. They are cylindrical prototypes made of borosilicate glass of 2.5 mm thickness that allows a 90% transmission of UV-A in the natural solar spectrum. The photo-reactor is inclined at 37 °C with respect to the horizontal to maximise solar radiation collection and is equipped with static CPC (Navntoft et al. 2008) whose concentration factor is equal to 1.

The solar photo-reactor (Figure 4.6) volume is 8.5 L, the illuminated volume is 4.7 L, the irradiated collector surface is 0.4 m^2. Water flow rate was set as 16 L min^{-1} in order to guarantee a turbulent regime (Re=8,600) which results in a proper homogenisation of water samples. Heterogeneous photocatalysis also requires to maintain titanium dioxide nanoparticles perfectly suspended and homogeneously distributed. The set flow regime also ensures the best conditions to achieve a good

contact between bacteria and catalyst nanoparticles during photocatalytic disinfection; any bacterial removal associated with particles sedimentation can be discarded.
The experimental setup allowed two experiments to be run simultaneously in two identical solar CPC photo-reactors.

Figure 4.6 Photo of solar CPC photo-reactors

A diagram of one of the two identical solar CPC photo-reactors is depicted in Figure 4.7.

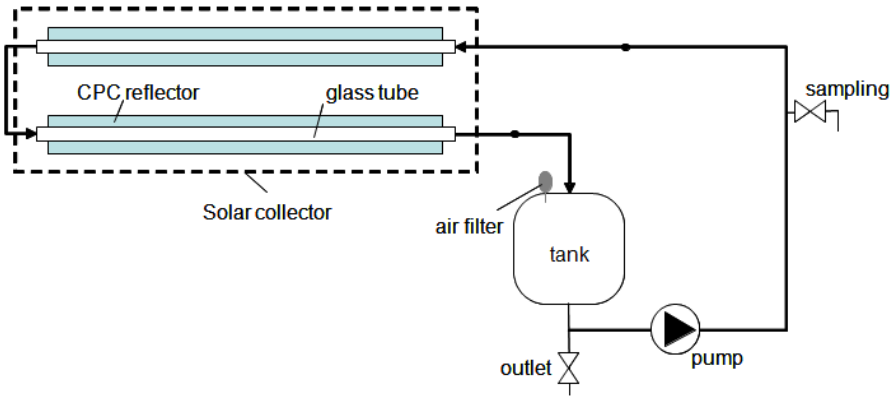

Figure 4.7 Diagram of one solar CPC photo-reactor

4.4.1.3 Solar experiments

Solar photo-reactor was filled with 8.5 L of autoclaved real wastewater and spiked with the selected strain until obtaining an initial bacterial density as high as 10^{5-6} CFU mL^{-1} approximately. The suspension was homogenised while the solar photo-reactor was still covered. Reagents were added to each reactor tank and wastewater were re-circulated for some minutes to ensure homogenisation. Then the first sample was collected and the cover was removed. Treated wastewater samples were collected at regular intervals: sampling frequency varied on the base of treatment.

Water temperature was measured hourly in each photo-reactor by a thermometer (Checktemp, Hannain struments, Spain): it ranged from 21.2 to 44.0 ◦C. pH (multi720, WTW, Germany) and H_2O_2 were also measured in each reactor during the experiments. For each assay, a water sample was taken and kept in the dark at laboratory temperature as control and was plated at the end of the experiment. Inactivation results are plotted as the average of at least two replicates for each solar driven experiment.

Solar UV-A radiation was measured as described in paragraph 4.3.1.6. The average solar UV-A irradiance for all tests was 37.3±4.30 W m^{-2} within the period 10:00–16:00 local time, with maximum values of 44.4 W m^{-2}.

4.4.1.4 Antibiotic resistance assays

Antibiotic resistance phenotypes were tested by Kirby-Bauer disk diffusion method as described in paragraph 4.3.1.7.

4.4.1.5 Kinetics evaluation

The inactivation kinetics of different AOPs were calculated as kinetic disinfection rates against the energy parameter (Q_{UV}, in kJ L^{-1}) instead of real time, as the solar flux integrated with time per unit of volume is the driving parameter (Sichel et al. 2007). The statistical analysis of experimental data resulted in the kinetic constants is shown in Table 4.2.

Table 4.2 AR *E. coli* inactivation kinetics

Process	Fe^{2+} mg L^{-1}	H_2O_2 mg L^{-1}	TiO_2 mg L^{-1}	k L kJ^{-1}	R^2	Model*
Solar disinfection				0.36±0.08	0.91±0.86	2
H_2O_2/dark		40		0.26±0.02	0.99±0.31	1
		75		0.34±0.04	0.97±0.52	1
Solar photo-Fenton	5	10		0.35±0.04	0.95±0.56	2
	10	20		0.34±0.05	0.93±0.60	3[a]
	20	40		0.29±0.03	0.93±0.63	2
Solar photo-Fenton (pH4)	5	10		5.12±0.48	0.97±0.42	1
H_2O_2/sunlight		20		0.66±0.06	0.97±0.48	1
		50		0.80±0.17	0.89±1.01	1
		75		0.88±0.14	0.93±0.74	1
TiO_2/sunlight			50	0.59±0.11	0.87±0.96	1
			100	0.64±0.09	0.93±0.79	1
H_2O_2/TiO_2/sunlight		5	50	0.86±0.12	0.92±0.92	1
		20	100	1.46±0.13	0.98±0.46	1

* Model 1: Log-linear; 2: shoulder + Log linear; 3: shoulder + Log linear + tail
a: Tail is the N_{res}=0.47 Log

These kinetic models are very similar to those reported elsewhere (García-Fernández et al. 2015):

i) Log-linear decay of the density of bacteria (N) from an initial value (N_0), with a kinetic rate (k) according to the Chick' law [Eq. (2)];
ii) A 'shoulder phase' given by constant density of bacteria (N_0) (or very smooth decay), attributed to loss of cells viability

after the accumulation of oxidative damages during the process, followed by a Log-linear decay [Eq. (3)];

iii) A 'shoulder phase' followed by a Log-linear decay and a 'tail phase' at the end of the process [Eq. (4)]. The 'tail' shape of this kinetics represents the residual density (N_{res}) of bacteria remaining at the end of the experiment due to a strong reduction on the photocatalytic activity of the process and/or the presence of a population of cells that are resistant to the treatment.

$$Log\left(\frac{N}{N_0}\right) = a - k \cdot Q_{UV} \qquad \textit{Eq. (2)}$$

$$Log\left(\frac{N}{N_0}\right) = \begin{cases} 0, & N \geq N_0 \\ a - k \cdot Q_{UV}, & N < N_0 \end{cases} \qquad \textit{Eq. (3)}$$

$$Log\left(\frac{N}{N_0}\right) = \begin{cases} 0, & N \geq N_0 \\ a - k \cdot Q_{UV}, & N_{res} < N < N_0 \\ b, & N \geq N_{res} \end{cases} \qquad \textit{Eq. (4)}$$

4.4.2 Results and discussion

4.4.2.1 Solar photo-inactivation

The effect of solar radiation on the inactivation of AR *E. coli* strain is shown in Figure 4.8.

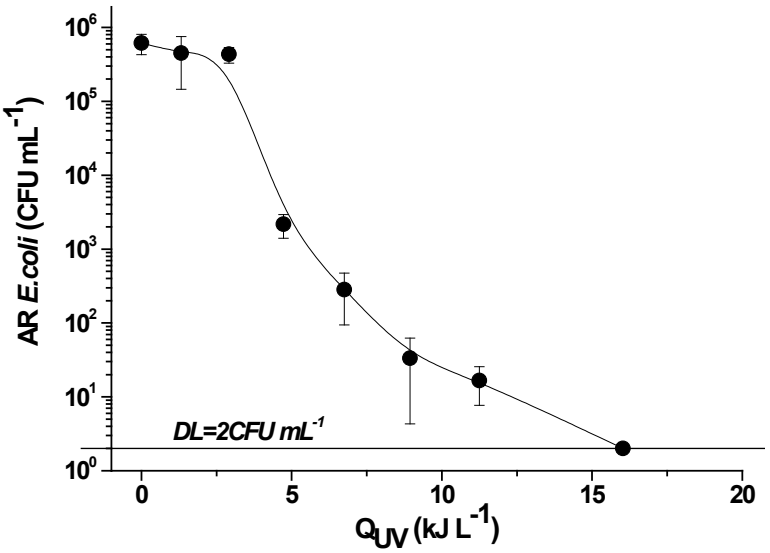

Figure 4.8 AR *E. coli* inactivation by solar radiation

Solar-photo inactivation required 16.03 kJ L^{-1} in terms of Q_{UV} and about 4 h to get the DL.
Temperature varied in the range 26.3-41.0 °C, therefore sufficiently lower than 45 °C to observe any significant temperature-related synergistic effect.
The negative influence of flow rate and intermittent delivery of solar radiation have been reported to limit moderately the disinfection efficiency (McGuigan et al. 1998).
However solar photo-inactivation of AR *E. coli* in real wastewater has never been investigated before at pilot scale, even under continuous flow conditions. Agulló-Barceló and co-workers investigated the effect of solar photo-inactivation on naturally occurring *E. coli* in real wastewater, in CPC reactors. This process allowed to reach the DL for indigenous *E. coli*, but the treatment time was 60 min longer on average and the trend of the inactivation curve is quite constant with treatment time (Agulló-Barceló et al. 2013). Differently, in the present work, during the first hour of solar exposure, zero decrease in AR *E. coli* was observed (60 min duration shoulder), whereas a faster kinetic occurred later, with a clear liner tendency until nearly the end of the process. Moreover, the Q_{UV} energy required to achieve a 4-Log decrease was much higher (Q_{UV}≈35

kJ L^{-1}) than that required in this study (Q_{UV}≈16 kJ L^{-1} as shown in Figure 4.8) for 5-Log abatement.
The comparison with laboratory scale results has shown that DL was achieved within the same treatment time (as high as about 4 h) but with a much lower energy (Q_{UV}≈16 kJ L^{-1} on average in pilot scale experiments Vs Q_{UV}≈37 kJ L^{-1} on average in laboratory scale tests). This may be due to the total volume of treated water that may play a major role than the irradiated area.

4.4.2.2 Solar photo-Fenton

Solar photo-Fenton process was investigated both at natural pH (8.72±0.15), at different Fe^{2+}/H_2O_2 ratios, and at pH 4.00 and results are plotted in Figure 4.9.

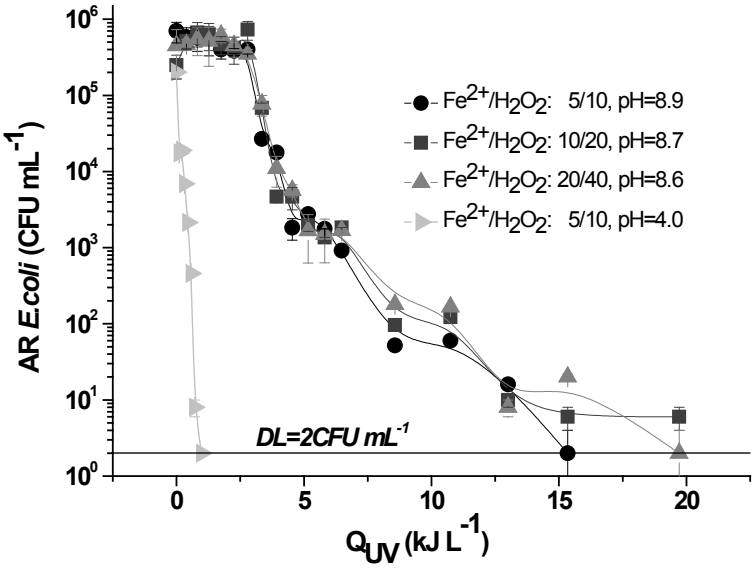

Figure 4.9 AR *E. coli* inactivation by solar photo-Fenton at pilot scale

The inactivation kinetics were found to be slow at all the tested conditions and DL was not achieved with a Fe^{2+}/H_2O_2 ratio of 10/20 within 300 min of solar exposure (differently from laboratory scale result under the same operative conditions). The best disinfection performance was obtained with 5/10 mg L^{-1} of Fe^{2+}/H_2O_2: DL was reached within 240 min of solar treatment and with 15.34 kJ L^{-1} of Q_{UV}. The DL was

also got in the case of 20/40 mg L^{-1} of Fe^{2+}/H_2O_2 during 300 min of solar experiment with a higher Q_{UV}, namely 19.71 kJ L^{-1}. The average temperatures were 35.0±5.3 °C, 35.2±5.5 °C and 36.7±4.9 °C respectively. pH remained almost constant during all treatments ($pH_{initial}/pH_{final}$ were 8.9/8.6, 8.7/8.4, 8.6/8.4, respectively). The lower inactivation rates may be due to the precipitated iron at near natural pH of wastewater that could negatively affect the efficiency of the process because of a lack of hydroxyl radicals as well as the screening effect of precipitated iron (Rodríguez-Chueca et al. 2014). This hypothesis is supported by the measurements of dissolved iron which were zero or below the DL of the quantification method for all near natural pH photo-Fenton experiments. If the dissolved iron is zero, the investigated process may be regarded as a H_2O_2/sunlight one.

The same DL for naturally occurring *E. coli* in a real secondary UWTP effluent was reached at 10/20 mg L^{-1} of Fe^{2+}/H_2O_2 with 13.1 kJ L^{-1} of Q_{UV} within 240 min of solar photo-Fenton treatment at pH 5 (Rodríguez-Chueca et al. 2014). According to the mentioned work, the complete inactivation may be due to the limited oxidation action of the process that still exists and causes lethal damages in *E. coli* cells, although the generation of hydroxyl radicals could be limited by the precipitated iron. Agulló-Barceló et al. showed that different microorganisms may have different responses to the same treatment: 10 mg L^{-1} of Fe^{2+} and 20 mg L^{-1} of H_2O_2 at natural pH were enough to inactivate indigenous *E. coli* and F-specific RNA bacteriophages, but not for somatic coliphages and sulfite reducing clostridia (Agulló-Barceló et al. 2013). In this perspective, the incomplete inactivation at the same concentration of iron and hydrogen peroxide (Figure 4.9), may be due to the different sensitivity of the tested AR *E. coli*.

Much better performances were obtained in experiments at pH 4.00 with a Fe^{2+}/H_2O_2 ratio of 5/10 (Figure 4.9). In this case, after 20 min of treatment, DL was reached with 0.98 kJ L^{-1} of Q_{UV}. Although 10 mg L^{-1} of iron was added, the measured dissolved iron at pH 4.00 was between 0.1 and 3.4 mg L^{-1}, not as high as the initially added but enough to promote photo oxidative damages in *E. coli* cells, due to the hydroxyl radicals that are produced during this process in agreement with other publications on photo-Fenton for *E. coli* and *Fusarium* (García-Fernández et al. 2012).

The effect of acid conditions (at pH 4.00) on AR *E. coli* survival was evaluated in dark under similar operative conditions, *i.e.* water matrix and

initial bacterial density without the addition of any reagent. The density of AR *E. coli* remained constant for 300 min.
The inactivation rate fits quite well with the results that have been obtained by Agulló-Barceló et al. for the inactivation of naturally occurring *E. coli* in a UWTP secondary effluent treated by photo-Fenton at pH 3.00 (Agulló-Barceló et al. 2013). On the contrary, inactivation rate does not fit with the results by Karaolia et al. dealing with the inactivation of enterococci in real UWTP effluent by solar photo-Fenton at pH 4.00, possibly due to the different target bacteria (Karaolia et al. 2014). The only work available in the scientific literature about the inactivation of AR bacteria in real UWTP effluents by solar AOPs at pilot scale is carried out by Karaolia et al. The authors investigated the effect of solar photo-Fenton at pH 4.00 on a mixture of antibiotics as well as the disinfection effect on enterococci and on their resistance to clarithromycin and sulfamethoxazole; complete removal, as high as 5 Log reduction, was achieved within 140 min in the presence of 5 mg L^{-1} of Fe^{2+} and 50 mg L^{-1} of H_2O_2 (Karaolia et al. 2014).

4.4.2.3 H_2O_2/sunlight process

H_2O_2/sunlight process was investigated at different hydrogen peroxide doses, such as 20, 50 and 75 mg L^{-1} and results are plotted in Figure 4.10 as average values.

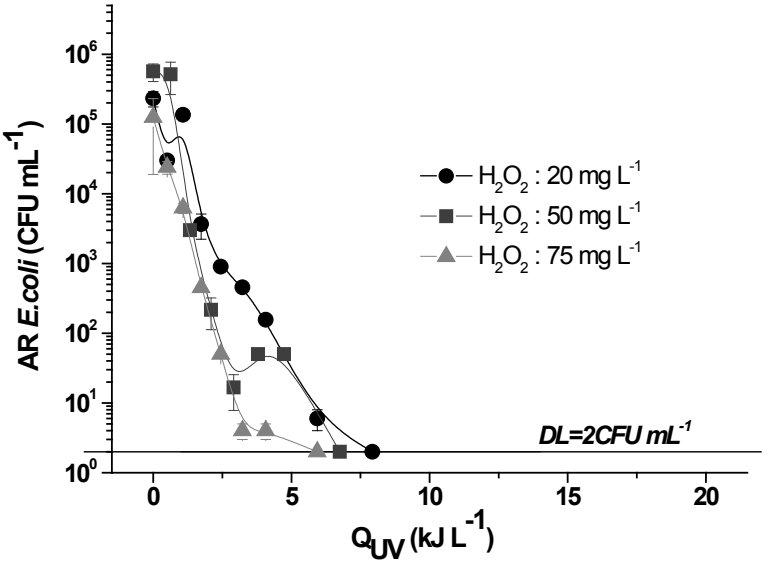

Figure 4.10 AR *E. coli* inactivation by H_2O_2/sunlight process at pilot scale

The synergistic effect of H_2O_2 and solar radiation contributed to the best performances among all evaluated solar driven AOPs, after solar photo-Fenton at pH 4.00. DL was got in 150 min in the presence of 20 mg L^{-1} of H_2O_2 (Q_{UV}=7.93 kJ L^{-1}), in 120 min in the presence of 50 mg L^{-1} of H_2O_2 (Q_{UV}=6.75 kJ L^{-1}), in 120 min in the presence of 75 mg L^{-1} of H_2O_2 (Q_{UV}=5.93 kJ L^{-1}). Water temperature increased from 23.8 °C to 40.7 °C, and also in this case, temperature effect on bacterial inactivation may be excluded. H_2O_2 concentration was also monitored throughout each experiment; when it decreased, adequate amounts were added so that the concentration was kept almost constant during the test.

In another work the same hydrogen peroxide doses (20 and 50 mg L^{-1}) allowed to obtain a similar inactivation of indigenous *E. coli* (DL was achieved within 180 min of solar treatment) (Agulló-Barceló et al. 2013).

Dark control tests were performed in CPCs reactors, under the same operative conditions, except that the reactors were covered, in order to assess the influence of H_2O_2. As shown in Figure 4.11, hydrogen peroxide resulted in a total inactivation of the tested strain: DL was achieved within 240 min in the presence of 40 mg L^{-1}, while after 210 min in the presence of 75 mg L^{-1}. The average temperature that has been registered was lower than 45 °C (31.3±1.5), so that a thermal inactivation mechanism may be excluded.

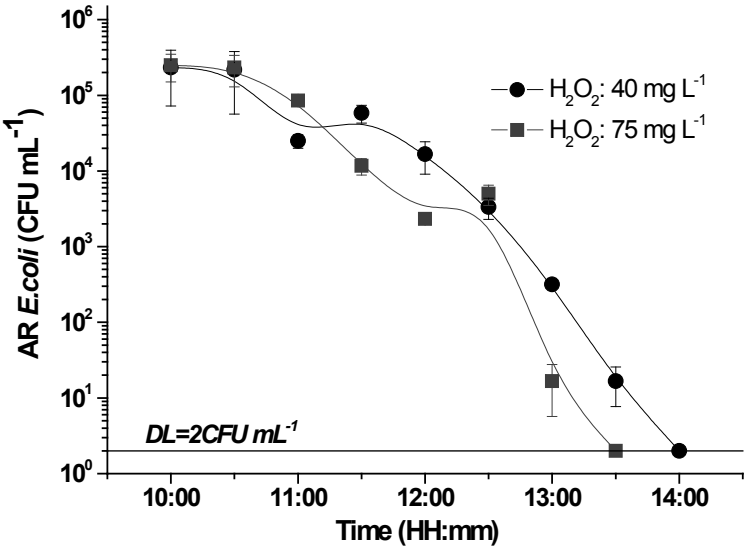

Figure 4.11 H_2O_2 dark control

A similar inactivation curve in the presence of 50 mg L^{-1} of H_2O_2, in dark conditions, was observed by Rodríguez-Chueca et al. (2014). Although these authors observed a ≈6 Log units decrease (the initial density was ≈10^6 CFU mL^{-1}) in a simulated UWTP secondary effluent, DL was not reached. They also pointed out that the direct oxidative effect of hydrogen peroxide on bacteria viability was very low compared with the synergistic effect of H_2O_2 and solar radiation. Although a much better inactivation is reached when H_2O_2 and sunlight are applied simultaneously, as can be seen through the different shapes of inactivation curves, an important direct oxidation effect of only hydrogen peroxide at these concentrations (up to 75 mg L^{-1}) may not be ruled out on the base of results that have been obtained in this work.

By comparison between near-neutral pH solar photo-Fenton (Figure 4.9) and H_2O_2/sunlight process (Figure 4.10), it is possible to note that almost similar H_2O_2 concentrations (40 and 50 mg L^{-1} respectively) have led to very different disinfection results, being solar photo-Fenton much slower than H_2O_2/sunlight. It is important to remark that the total amount of dissolved iron at near-natural pH was zero or below DL, so that solar photo-Fenton process at near-neutral pH may be regarded as H_2O_2/sunlight process occurring in the presence of precipitated iron.

According to some authors, this precipitated and partially suspended in water samples iron results in disinfection efficiency losses (García-Fernández et al. 2012, Rodríguez-Chueca et al. 2014). Therefore, if not all added iron is dissolved, its presence may negatively affect the bactericidal effect of H_2O_2/sunlight process. The chemical quality of wastewater also plays a role: in this study, pH and turbidity average values were higher than those reported in the above-mentioned work (pH=9.04 Vs pH=7.31 and turbidity=53 NTU Vs turbidity=8 NTU) (Agulló-Barceló et al. 2013). This may have resulted in a less efficiency of disinfection process.

The obtained results are also in agreement with those that have been achieved in another study, where a complete inactivation of *E. coli* took place with 12.0 kJ L^{-1} of Q_{UV} in the presence of 20 mg L^{-1} of H_2O_2 and with 7.4 kJ L^{-1} of Q_{UV} in the presence of 50 mg L^{-1} of H_2O_2 (Rodríguez-Chueca et al. 2014).

All the tested hydrogen peroxide doses allowed to reach a complete inactivation of the target AR *E. coli* strain. Anyway, in some cases, limits into the discharge of treated effluents for crop irrigation require a H_2O_2 concentration lower than 50 mg L^{-1} (Coosemans 1995). A decrease of H_2O_2 concentrations was observed downstream of the treatment; this is possibly due to the reactions with organic matter present in wastewater and auto-decomposition of hydrogen peroxide into oxygen and water, which is favored at higher temperatures. In all cases, except for doses of 75 mg L^{-1} of H_2O_2, the residual hydrogen peroxide concentrations were below the guide value for crop irrigation. Although the energy required for bacterial inactivation was lower in the presence of higher doses of H_2O_2 (75 mg L^{-1}), this may not fit with guide values for treated wastewater for crop irrigation to avoid toxic effects.

4.4.2.4 TiO_2/sunlight and H_2O_2/TiO_2/sunlight processes

The inactivation of AR *E. coli* by heterogeneous photocatalysis with suspended TiO_2 is shown in Figure 4.12. DL was reached in 150 min of solar treatment with 50 mg L^{-1} of TiO_2 (Q_{UV}=7.9 kJ L^{-1}) and in 180 min of solar exposure with 100 mg L^{-1} of TiO_2 (Q_{UV}=9.9 kJ L^{-1}). The higher concentration of catalyst did not improve the performance of disinfection efficiency and required both more cumulative energy per unit of volume and treatment time. This may be due to the increase of the turbidity of wastewater that affects negatively the penetration of solar

UV-A. This behaviour is in agreement with results by Benabbou et al. that observed a total inactivation of *E. coli* after 180 min of treatment with 250 mg L^{-1} of TiO_2, and just a 4 Log units decrease after the same exposure time to irradiation with a 10 times higher TiO_2 concentration, namely 2.5 g L^{-1} (Benabbou et al. 2007).
When a catalyst load of 100 mg L^{-1} has been used, during the first 40 min of solar exposure, inactivation kinetics were slow, and, in general, much slower than for 50 mg L^{-1}. This initial trend is similar to that reported by Agulló-Barceló et al. (2013). This work should be the first one on the inactivation of AR bacteria by TiO_2/sunlight process at pilot scale.

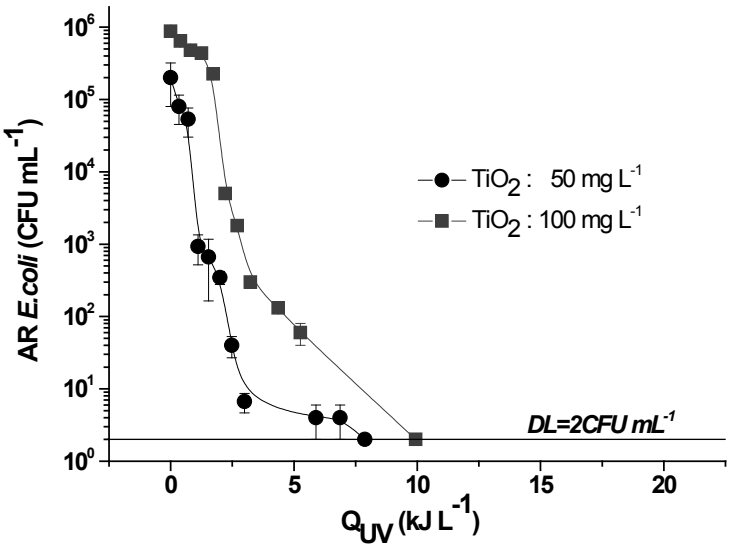

Figure 4.12 AR *E. coli* inactivation by TiO_2/sunlight process at pilot scale

The trend that was observed at laboratory scale is almost similar: with a dose of titanium dioxide as high as 100 mg L^{-1}, DL was achieved in 150 min (Vs 180 min) on average and with a cumulative energy per unit of volume of 20.6 kJ L^{-1} (Vs 9.9 kJ L^{-1}).
Finally the effect of hydrogen peroxide and titanium dioxide was simultaneously investigated in order to test if small doses of H_2O_2 (5 mg L^{-1} in the presence of 50 mg L^{-1} of TiO_2, and 20 mg L^{-1} in the presence of 100 mg L^{-1} of TiO_2) may positively affect the disinfection efficiency (Figure 4.13).

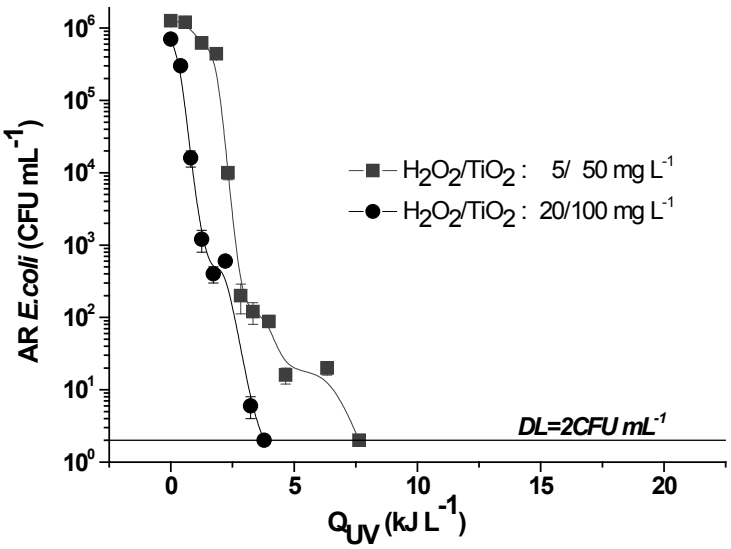

Figure 4.13 AR *E. coli* inactivation by H_2O_2/TiO_2/sunlight process at pilot scale

DL was achieved in 180 min with 5 mg L^{-1} of H_2O_2, 50 mg L^{-1} of TiO_2 and a Q_{UV} of 7.6 kJ L^{-1}; in 80 min with 20 mg L^{-1} of H_2O_2, 100 mg L^{-1} of TiO_2 and a Q_{UV} of 3.8 kJ L^{-1}. In the first case the small amount of the added hydrogen peroxide did not improve the process efficiency, whilst a significant increase in disinfection performance was observed with the addition of 20 mg L^{-1} of H_2O_2 (55.6% time-saving and 61.9% energy-saving). If this enhancement is compared with H_2O_2/sunlight process using the same dose of hydrogen peroxide, namely 20 mg L^{-1}, the percentages decrease: 46.7% time-saving and 52.1% energy-saving.

4.4.2.5 Inactivation mechanisms description

The mechanism of action of microorganism inactivation in water by solar photo-Fenton and solar heterogeneous photocatalysis has been widely recognised to be due to the oxidative attack of reactive oxygen species (ROS), mainly hydroxyl radicals (HO•), which are generated during these processes (Malato et al. 2009). In the case of solar heterogeneous photocatalysis, a semiconductor particle is photo-excited by UV-A photons and eventually can generate hydroxyl radicals in the presence of water. For solar photo-Fenton process the dissolved photo-active iron species react with hydrogen peroxide and generate hydroxyl

radicals as well as oxidised iron species through the action of photons of wavelengths below 550 nm approximately. Moreover, the mere action of solar photons has detrimental effect over bacterial cell viability (Figure 4.2) that has to be considered also when the photocatalytic processes are occurring.
The inactivation mechanisms that take place during solar driven processes can be summarised as follows:

i) In the case of photo-Fenton, microorganism inactivation is believed to be achieved through the action of species, such as the hydroxyl radicals that have been generated by means of the catalytic cycle of photo-Fenton described in equations (5) and (6), which can indistinctly oxidise several parts of the cell walls as these are external reactions. Moreover, species like Fe^{2+} or H_2O_2 may diffuse inside cells, which, under solar radiation, induce an increase of the inactivation efficiency by internal generation of ROS, mainly HO•, through internal photo-Fenton reactions (García-Fernández et al. 2012, Polo-López et al. 2011, Rodríguez-Chueca et al. 2014).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\bullet \qquad \textit{Eq. (5)}$$

$$Fe(OH)^{2+} + h\nu \rightarrow Fe^{2+} + OH\bullet \qquad \textit{Eq. (6)}$$

ii) In the case of heterogeneous photocatalysis, it has been proven that the photo-excitation of titanium dioxide particles generates hydroxyl radicals (Cho et al. 2004). Bacterial cells in TiO_2 aqueous suspensions are surrounded by TiO_2 nanoparticles and aggregates (Gumy et al. 2006) which allow a very close and fast attack of hydroxyl radicals to the components of the outer layer of the cell wall (Polo-López et al. 2010, Sichel et al. 2007). This mechanism involves the first recognised oxidative damage of photocatalysis on bacteria, *i.e.* loss of cell wall permeability that results in cell death. The majority of photocatalytic studies recognises hydroxyl radicals as the main ROS responsible for microorganism inactivation, although other ROS, such as hydrogen peroxide and the superoxide anion radical $(O_2^{\bullet-})$ have also been reported to be involved in the process. Other proposed

mechanisms of cell death include DNA/RNA damages, increased ion permeability, membrane disruption or respiratory chain damages (Keane et al. 2014).

iii) The use of both H_2O_2 and TiO_2 improves the efficiency of the photocatalytic process since H_2O_2 reduces the recombination of hole-electron pairs on the catalyst surface and reacts with conduction band electrons (Legrini et al. 1993) and superoxide radical anions to produce additional hydroxyl radicals (Tuhkanen 2004). Therefore, H_2O_2/TiO_2 photocatalysis acts against bacteria in a similar way as TiO_2 alone, via hydroxyl radicals direct attack. Neverthless, when H_2O_2 concentrations are enough, the process is not enhanced, but delayed or disfavored because of the oxidation of H_2O_2 by the photo-generated holes, which also lead to a decrease in HO• (Pablos et al. 2013, Wang and Hong 1999).

iv) The clear synergistic killing of microorganisms by hydrogen peroxide and sunlight in water has been reported for bacteria and fungi. The mechanism of action of H_2O_2/sunlight process was firstly attributed to the direct oxidative action of H_2O_2 over bacterial cells making them more sensitive to solar radiation. Then it has been recognised the capability of H_2O_2 molecules to diffuse inside cells, reacting with the free iron and generating internal HO• by photo-Fenton or Fenton-like reactions, causing internal damages inside cells and eventually causing cell death (Feuerstein et al. 2006, Polo-López et al. 2011, Sichel et al. 2009, Spuhler et al. 2010).

4.4.2.6 Effect of solar driven AOPs on antibiotic resistance

The average values of inhibition zone diameters for AMP, CIPR, CXM, NI for the selected AR *E. coli* strain, before each disinfection process (T0), in the middle of each experiment and at the end (Table 4.3), were compared with the corresponding clinical breakpoint values for *E. coli* from EUCAST database (EUCAST, 2015). Inhibition zone diameters were also monitored for tetracycline (TET, 30 μg) and vancomycin (VAN, 30 μg), although the corresponding clinical breakpoint values are not reported in EUCAST online database. The tested strain was resistant (R) to AMP, CIPR and TET, as expected, but also to VAN. It was sensitive (S) to both CXM and NI. The results of resistance assays on the

colonies that have been survived to the disinfection processes have shown that none of the investigated solar driven AOPs affected the antibiotic resistance. This was observed both in the middle of each experiment and at the end, when still at least one cultivable and detectable colony grew on plates and allowed to apply the antibiogram protocol. The tested strain did not lose its resistance to AMP, CIPR, TET and VAN during the process because no variation was observed in the inhibition zone diameters.

Table 4.3 Antibiogram results on AR *E. coli* at the end of each disinfection process at pilot scale: inhibition zone diameters (mm)

Process	AMP10	CIPR5	CXM30	NI100
Solar disinfection	10	10	21	23
H_2O_2/sunlight 20 mg L^{-1}	10	10	18	25
H_2O_2/sunlight 50 mg L^{-1}	10	10	20	23
H_2O_2/sunlight 75 mg L^{-1}	10	10	22	24
Solar photo-Fenton (pH4)	10	10	21	23
Solar photo-Fenton Fe^{2+}/H_2O_2: 5/10 mg L^{-1}	10	10	18	23
Solar photo-Fenton Fe^{2+}/H_2O_2: 10/20 mg L^{-1}	10	10	22	26
Solar photo-Fenton Fe^{2+}/H_2O_2: 20/40 mg L^{-1}	10	10	22	22
TiO_2/sunlight 50 mg L^{-1}	10	10	21	23
TiO_2/sunlight 100 mg L^{-1}	10	10	21	23
H_2O_2/TiO_2/sunlight 20:100 mg L^{-1}	10	10	21	27

Although antibiogram is a qualitative proof that does not allow to deeply investigate changes in antibiotic resistance from a genetical point of view, it showed that antibiotic resistance was not affected. In the literature, only a few works are available about the investigation of solar photo-Fenton process on antibiotic resistance of enterococci but in terms of resistance percentage (Karaolia et al. 2014, Michael et al. 2012). The profile of antibiotic resistance percentage, calculated by comparing bacterial colonies on culture media supplemented with antibiotics with the corresponding bacterial colonies on culture media without any antibiotics, plotted as function of treatment time, showed a decrease in ofloxacin and trimethoprim resistance percentage (Michael et al. 2012). According to these results, solar photo-Fenton process at pilot scale (Fe^{2+}=5 mg L^{-1}, H_2O_2=75 mg L^{-1}, pH=2.8-2.9) affected antibiotic resistance, but in percentage terms. The same approach was followed by Karaolia et al.; also in this case a decrease of clarithromycin and sulfamethoxazole resistant *Enterococcus* in real UWTP effluent was observed as treatment time increased (solar photo-Fenton process at

pilot scale, Fe^{2+}=5 mg L^{-1}, H_2O_2=50 mg L^{-1}, pH=4.0, in the presence of 100 μg L^{-1} of clarithromycin and sulfamethoxazole) (Karaolia et al. 2014). Some changes in antibiotic resistance have been observed in some studies where MIC-based method (Rizzo et al. 2013) and Kirby-Bauer disk diffusion method (Rizzo et al. 2014a) were used in order to characterise antibiotic resistance of *E. coli* strains downstream of UV radiation and TiO_2 photocatalysis, respectively. An AR *E. coli* strain after UV radiation tests (UV dose=1.25×10^4 μW s cm^{-2}), was observed to change its resistance to ciprofloxacin (MIC=12 mg L^{-1}), but not to amoxicillin (MIC>256 mg L^{-1}) and sulfamethoxazole (MIC>1024 mg L^{-1}) (Rizzo et al. 2013). In another study, the effect of solar simulated TiO_2 photocatalysis on AR *E. coli* was investigated (Rizzo et al. 2014a). While no detectable change in resistance levels was found for cefuroxime, ciprofloxacin and vancomycin, a significant increasing trend (p=0.03<α=0.05) was observed for tetracycline. As expected, the same strain may have different responses to different antibiotics. Moreover, although no change in antibiotic resistance was observed in the present work, it does not necessarily mean that no change in antibiotic resistance occurred at all, but only that no change was observed in bacterial cells randomly selected among those survived to disinfection process at the given sampling times.

4.5 CONCLUSIONS

Different solar driven AOPs were evaluated and compared to disinfect real UWTP effluents contaminated by AR *E. coli*.

When implemented at laboratory scale, H_2O_2/TiO_2/sunlight process allowed to achieve DL with Q_{UV} ranging between 3.3 and 4.2 kJ L^{-1}, depending on H_2O_2/TiO_2 ratio. The best performance of photo-Fenton process was observed for Fe^{2+}/H_2O_2 ratio as low as 5/10 mg L^{-1} (Q_{UV}=14.9 kJ L^{-1}). Therefore, the energy was observed to be higher than that required by H_2O_2/sunlight process (Q_{UV}=8.7 kJ L^{-1}, H_2O_2 dose of 50 mg L^{-1}). Considering the expected higher cost related to both TiO_2 photocatalysis (due to additional treatment for catalyst removal downstream of treatment) and solar photo-Fenton, H_2O_2/sunlight process may be regarded as the most attractive for wastewater disinfection in small communities.

On the base of the achieved results at laboratory scale, solar driven AOPs were also implemented in a pilot scale CPCs plant. In this case the best disinfection efficiency was found for photo-Fenton at pH 4.0 (Fe^{2+}/H_2O_2 ratio of 5/10 mg L^{-1}), in terms of treatment time (20 min to reach DL) and required energy (Q_{UV}=0.98 kJ L^{-1}). This high efficacy is due to photo-Fenton reaction between solar photons, added hydrogen peroxide and dissolved iron in the wastewater sample. Anyway the treatment of UWTP effluents by photo-Fenton would require acidification upstream of treatment and neutralisation afterwards with the formation of precipitated iron that should be subsequently removed, making this process not really attractive from the economic point of view. When this process is operated at near-neutral pH, iron precipitates and the process can be regarded as a H_2O_2/sunlight process. The efficiency that has been obtained for H_2O_2/sunlight process was very similar for the three tested H_2O_2 doses, such as 20, 50 and 75 mg L^{-1}. Solar TiO_2 photocatalysis was also very promising, but the removal of catalyst after treatment should be taken into account in a global assessment for wastewater reuse application.

In the light of urban wastewater reuse for crop irrigation each of all investigated solar driven AOPs may be promising, except solar photo-Fenton at near-natural pH with Fe^{2+}/H_2O_2 ratio of 10/20 mg L^{-1}. On the base of the above explained drawbacks related to both solar photo-Fenton and H_2O_2/TiO_2/sunlight process, H_2O_2/sunlight process at low hydrogen peroxide doses, namely 20 and 50 mg L^{-1}, may be regarded as the most feasible. Moreover, at low doses, H_2O_2/sunlight process may also meet the guide values for H_2O_2 residual concentration in wastewater reuse for crops irrigation.

None of the implemented solar driven AOPs affected the antibiotic resistance of survived AR *E. coli* colonies. In this perspective genetic analysis may be required to deeply investigate some aspects related to antibiotic resistance.

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4.7 APPENDIX

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Inactivation and regrowth of multidrug resistant bacteria in urban wastewater after disinfection by solar-driven and chlorination processes

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ABSTRACT

Solar disinfection and solar-driven advanced oxidation processes (AOPs) (namely H_2O_2/sunlight, TiO_2/sunlight, H_2O_2/TiO_2/sunlight, solar photo-Fenton) were evaluated in the inactivation of indigenous antibiotic-resistant bacteria (ARB) in real urban wastewater. A multidrug resistant (MDR) *Escherichia coli* strain isolated from the effluent of the biological process of an urban wastewater treatment plant was the target ARB. The higher inactivation rates (residual density under detection limit, 2 CFU mL^{-1}) were achieved with H_2O_2/TiO_2/sunlight (cumulative energy per unit of volume (Q_{UV}) in the range 3–5 kJ L^{-1}, depending on H_2O_2/TiO_2 ratio) and H_2O_2/sunlight (Q_{UV} of 8 kJ L^{-1}) processes. All investigated processes did not affect antibiotic resistance of survived colonies. Moreover, H_2O_2/sunlight was compared with conventional chlorination process to evaluate bacterial regrowth potential and particularly the proportion of indigenous MDR *E. coli* with respect to total indigenous *E. coli* population. Chlorination (1.0 mg Cl_2 L^{-1}) was more effective than H_2O_2/sunlight (50 mg H_2O_2 L^{-1}) to achieve total inactivation of MDR *E. coli* (15 min Vs 90 min) but less effective in controlling their regrowth (24 h Vs 48 h). Interestingly, the percentage of MDR *E. coli* in H_2O_2/sunlight treated samples decreased as incubation time increased; the opposite was observed for chlorinated samples.

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Urban wastewater disinfection for agricultural reuse: effect of solar driven AOPs in the inactivation of a multidrug resistant *E. coli* strain

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ABSTRACT

The occurrence of antibiotics in urban wastewater treatment plants (UWTPs) may result in the development of antibiotic resistance and subsequently in the release of multidrug resistant bacteria (MDR) and genes into the effluent. Conventional disinfection processes are only partially effective in controlling ARB spread, so advanced oxidation processes (AOPs) have been investigated as alternative option in this work. In particular, the aim of this work was to comparatively assess the efficiency of solar disinfection and solar driven AOPs (namely H_2O_2/sunlight, TiO_2/sunlight, H_2O_2/TiO_2/sunlight, natural photo-Fenton) for the inactivation of a multidrug (namely ampicillin, ciprofloxacin and tetracycline) resistant *E. coli* strain isolated from the effluent of the biological process of an UWTP. Different concentrations of H_2O_2 (0.588–1.470–2.205 mM), TiO_2 (50–100 mg L^{-1}), H_2O_2/TiO_2 (0.147 mM/50 mg L^{-1}, 0.588 mM/100 mg L^{-1}) and Fe^{2+}/H_2O_2 (0.090/0.294, 0.179/0.588, 0.358/1.176 mM) were evaluated at pilot-scale (in compound parabolic collector reactor) in real biologically treated wastewater. All investigated processes resulted in a complete inactivation (5-log decrease) of bacteria until detection limit, but the best disinfection efficiency in terms of treatment time (20 min to reach the detection limit) and required energy (0.98 kJ L^{-1}) was observed for photo-Fenton at pH 4 (Fe^{2+}/H_2O_2:0.090/0.294 mM). Antimicrobial susceptibility was tested by Kirby-Bauer disk diffusion method. Ampicillin and ciprofloxacin (to which the selected strain is resistant), cefuroxime and nitrofurantoin were chosen as tested antibiotics. None of the investigated processes affected antibiotic resistance of survived colonies.

5 ANTIBIOTIC RESISTANCE SPREAD *POTENTIAL* IN WATER AND WASTEWATER DISINFECTED BY UV/H_2O_2 PROCESS

5.1 ABSTRACT

Water is one of the most important habitats and routes of antibiotic resistance dissemination between the environment and humans. Particularly, urban wastewater treatment plants (UWTPs) are among the main hotspots of antibiotic resistance spread into the environment and the role of conventional and new disinfection processes as possible barriers to minimise the risk for antibiotic resistance transfer is presently under investigation.

Aim of this work was the evaluation of the effect of an advanced oxidation process (AOP), such as UV/H_2O_2 on the base of the previous results, on antibiotic resistance transfer *potential* trough cultivation methods Vs polymerase chain reaction (PCR) based methods. bla_{TEM}, *qnr*S and *tet*W were selected as target antibiotic resistance genes (ARGs) and were quantified by quantitative PCR (qPCR) in the survived colonies as well as in the whole suspension (total DNA).

UV/H_2O_2 disinfection experiments were firstly carried out on (i) DNA-free water spiked with a selected antibiotic resistant (AR) *E. coli* strain, in order to work on controlled operative conditions on a selected strain, then on (ii) urban wastewater samples collected from the effluent of an UWTP biological process in order to evaluate the effect on indigenous bacterial population.

In DNA-free water tests, the detection limit (DL) of residual AR *E. coli* colonies (5 CFU mL^{-1}) was reached after 240 min treatment, but bla_{TEM} gene was still present in total DNA after 300 min (2.8×10^6 copies mL^{-1}), and no effect was observed in DNA extracted from cell cultures (3.3×10^2 copies CFU^{-1} after 90 min). Moreover, qPCR assay revealed only few or undetectable copies of *qnr*S and *tet*W genes at each treatment time in DNA extracted from cell cultures and in total DNA.

In disinfection tests that have been carried out on urban wastewater, despite 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, the copies number of *bla*$_{TEM}$ gene was mostly unchanged after 240 min treatment, while no difference (p>0.05) was found for *qnr*S gene between the initial (5.1×10^4 copies mL^{-1}) and the final sample (4.3×10^4 copies mL^{-1}). The less abundant gene in total DNA was *tet*W (6.0×10^1 copies mL^{-1}) which decreased (p<0.05) up to 1.1×10^1 copies mL^{-1} after 240 min of treatment.
On the base of the achieved results, the investigated disinfection process may not be effective at minimising antibiotic resistance spread *potential* into the environment. The inactivation of bacterial cells, which results in DNA release in the treated water, may pose a risk for antibiotic resistance transfer to other bacteria present in the receiving water body.

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5.2 INTRODUCTION

The main aspects and consequences that are related to antibiotic resistance have been deeply discussed in paragraph 1.3. It is important to remark here that this issue of concern has serious consequences not just in terms of public health but also in terms of impacts onto the

environment as the occurrence of antibiotics may promote the selection and the diffusion of antibiotic resistance patterns into the environment (Rizzo et al. 2013). Potential paths of antibiotic resistance dissemination from the environment to humans are related to water treatment, through the elimination/selection and release of clinically relevant antibiotic resistant (AR) bacteria and antibiotic resistance genes (ARGs) (Manaia et al. 2015). Unlike other contaminants, AR bacteria can reproduce and ARGs can be spread across different hosts detected in urban water streams, gulls or hospitalised patients (Varela et al. 2015). Hospital effluents (Narciso-da-Rocha et al. 2014, Rodriguez-Mozaz et al. 2015) and urban wastewater treatment plants (UWTPs) (Kümmerer 2009, Watkinson et al. 2007) have been identified as main environmental hotspots for antimicrobial resistance emergence and spread, 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. bla_{TEM} (encoding resistance to β-lactams), *int*I1 (encoding an integrase protein), *sul*-1 (encoding resistance to sulfonamide), *tet*W (encoding resistance to tetracyclines), *van*A (vancomycin resistance gene that is mainly associated with *Enterococcus* and is uncommon in the environment) have been detected in hospital effluents (Narciso-da-Rocha et al. 2014, Rodriguez-Mozaz et al. 2015), in biofilm or planktonic DNA from tap water samples, although at low concentrations (Pruden et al. 2006, Schwartz et al. 2003, Xi et al. 2009). Tetracycline (*tet*O and *tet*W) and sulfonamide (*sul*-I) resistance genes have been found in UWTPs effluents (Munir et al. 2011), as well as in dairy lagoon water, irrigation ditch water, urban/agriculturally impacted river sediments, in treated drinking water and recycled wastewater (Pruden et al. 2006).

The 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 (McKinney and Pruden 2012). So far only a few works deal with the effect of disinfection processes in terms of *potential* spread of antibiotic resistance by means of molecular biology methods (Öncü et al. 2011). One paper reports the effect of photocatalysis on the potential to induce ARGs transfer within sub-lethally injured AR bacteria, but by cultivation-based methods (Dunlop et al. 2015).

In this scenario disinfection processes play an important role in controlling antibiotic resistance diffusion, especially in the light of reuse of treated wastewater for agricultural purposes. Unfortunately conventional disinfection processes may not be effective (Auerbach et al. 2007, Huang et al. 2013, Munir et al. 2011). Advanced oxidation processes (AOPs) have been successfully investigated in the inactivation of AR bacteria (Karaolia et al. 2014, Tsai et al. 2010); anyway, there are still crucial gaps to be filled about the *potential* spread of antibiotic resistance. Some questions to be addressed concern the fate of ARGs and extracellular materials during and after disinfection process. To date a few works are available in the scientific literature 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 which may not be successful in ARGs removal, at least with realistic doses of disinfectants or UV radiation. Moreover target ARGs have been mostly quantified in the water suspension regardless the genetic material was released by inactivated bacterial cells or belonged to survived bacterial cells (intracellular DNA). McKinney and Pruden investigated the effect of UV radiation on intracellular and extracellular DNA, observing a higher (p<0.05) removal of extracellular ARGs than intracellular ARGs at dose as high as 400 mJ cm^{-2} (McKinney and Pruden 2012). Further research is therefore needed to evaluate ARGs fate after disinfection/oxidation processes in order to minimise the *potential* for subsequent antibiotic resistance spread into the environment.

In this context, aim of the present work was to investigate the effect of UV/H_2O_2 process (selected on the base of results that have been described in the previous chapter) in the simultaneous (i) inactivation of AR *E. coli* and (ii) the variation of bla_{TEM} (conferring resistance to β-lactams, penicillins and extended-spectrum cephalosporins), *qnr*S (encoding reduced susceptibility to fluoroquinolones) and *tet*W (encoding resistance to tetracyclines) genes. These ARGs have been normally detected in environmental settings and suggested as possible indicators to assess the antibiotic resistance status (Berendonk et al. 2015).

Particularly, disinfection processes were firstly carried out on (i) DNA-free water spiked with an AR *E. coli* strain selected from urban wastewater, in order to work under controlled operative conditions and

on a selected strain, then on (ii) UWTP effluents collected downstream of the biological process in order to evaluate the effect of the disinfection process on indigenous bacterial population. Moreover the effect on total DNA and DNA extracted from cell cultures was simultaneously investigated.

5.3 EFFECTS OF UV/H_2O_2 PROCESS ON SELECTED ANTIBIOTIC RESISTANT STRAIN

5.3.1 Materials and methods

5.3.1.1 Experimental design

A schematic view of experimental design that is related to DNA-free water tests is shown in Figure 5.1.

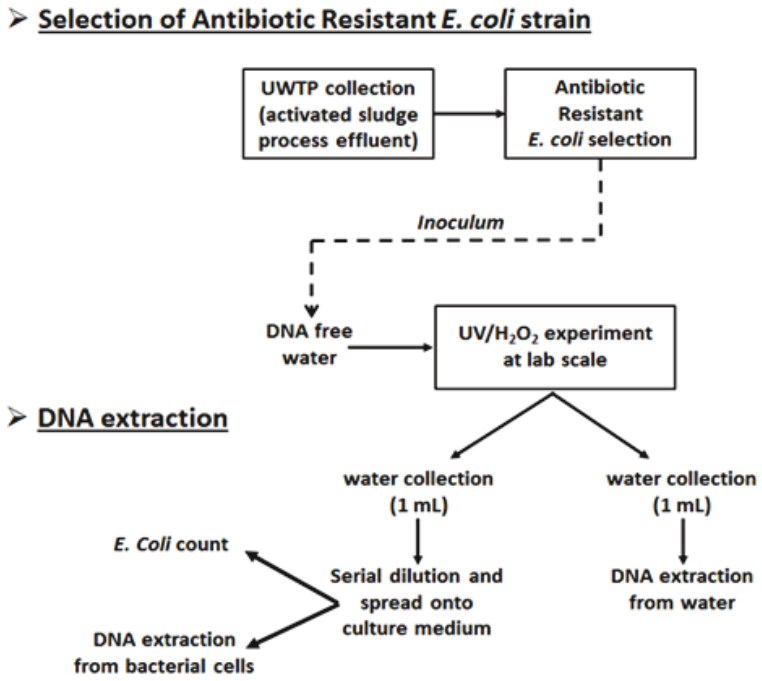


Figure 5.1 Schematic view of experimental design for DNA-free water tests

An AR *E. coli* strain, previously isolated, was inoculated in sterile DNA-free water (10^5 CFU mL^{-1}). The water spiked with AR *E. coli* strain was exposed to UV/H_2O_2 process up to 300 min. Water samples were collected during the process at different times: T30 (30 min), T60 (60 min), T90 (90 min), T120 (120 min), T240 (240 min), T300 (300 min). During the treatment, bacterial inactivation and ARGs variations were monitored. In particular, aliquots of treated water were spread onto selective culture media in order to monitor bacterial growth, and DNA was extracted from AR *E. coli* cells after 24 h growth until 10^9 harvest cells useful for DNA extraction. Simultaneously, DNA (genetic material from live bacteria and/or released from inactivated bacteria) was extracted from water samples collected at the same time (from here on "total").

5.3.1.2 Selection of antibiotic resistant *E. coli* strain

The selection of AR *E. coli* strain was the first step to perform DNA-free water tests. The AR *E. coli* strain was isolated from the effluent of the activated sludge process of the UWTP of Salerno by membrane filtration and subsequent cultivation on selective culture medium. Briefly 50 mL of wastewater and its serial dilutions were filtered through membranes (cellulose nitrate, 0.45 μm pore size, 47 mm diameter, Millipore) that were incubated at 37 °C for 24 h on Tryptone Bile X–glucuronide agar (TBX, Sigma-Aldrich) 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)). Antibiotics, all high purity grade (>99%), were purchased from Sigma-Aldrich. On the base of current limitations about phenotypic resistance in environmental settings (Berendonk et al. 2015), antibiotic concentrations were selected according to the respective epidemiological cutoff values available in EUCAST database (EUCAST, 2015). Colonies that were able to grow on selective culture medium supplemented with the mixture of antibiotics were regarded as resistant to respective antibiotics. Some colonies were randomly picked up and frozen in 15% glycerol Triptone Soy Broth at -80 °C and reactivated before disinfection experiments. AR *E. coli* were also spread onto TBX supplemented with AMP (8 mg L^{-1}), CIP (0.064 mg L^{-1}), TET (8 mg L^{-1}) separately, in order to monitor a potential loss of antibiotic resistance and check weak antagonistic effects among antibiotics.

5.3.1.3 DNA-free water inoculation

DNA-free water (UltraPure™ DNase/RNase-Free Distilled Water – ThermoFischer MI-ITALIA) was used in disinfection tests in order to work under controlled operative conditions. In this way the detected ARGs were only due to the inoculated strain, being experiments carried out in sterile conditions. DNA-free water was spiked with the selected AR *E. coli* strain, according to the protocol described elsewhere (Bichai et al. 2012). Briefly, bacterial strain was cultivated on TBX agar plates and incubated at 37 °C for 24 h. Some colonies were grown into 14 mL sterile Luria-Bertani liquid medium (LB, Sigma-Aldrich) at 37 °C for 18 h. Pellets of AR *E. coli* strain were collected by centrifugation at 3,000 rpm for 10 min and resuspended in 14 mL phosphate buffer saline (PBS) solution, up to reach a final density of 10^5 CFU mL^{-1}, approximately. DNA-free water was spiked with 0.5 mL of AR *E. coli* inoculum.

5.3.1.4 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 of DNA-free water, according to working conditions optimized in a previous study (Rizzo et al. 2014). The reactor was placed in a water bath in order to keep constant temperature at 25 °C and 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. The water samples were exposed to a range of UV doses (0.0-2.5 10^4 μW s cm^{-2}) by varying the exposure time from 0 to 300 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 homogenisation. Initial dose of hydrogen peroxide was set at 20 mg L^{-1}, according to the results that have been described in the previous chapter, 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}.
Catalase was added to water samples collected at different treatment times in order to eliminate residual H_2O_2: 1 mL samples were mixed with 20 µL of 2,300 U mg^{-1} bovine liver catalase at 0.1 g L^{-1} (Sigma-Aldrich). Catalase and H_2O_2 have been demonstrated to have no detrimental effects on bacterial viability at these concentrations (García-Fernández et al. 2012). Aliquots of UV/H_2O_2 treated water were plated onto TBX agar culture media in order to monitor bacterial growth.
Aliquots of water samples were also used to obtain total DNA. Control tests using inoculated DNA-free water under UV radiation were also carried out in parallel to UV/H_2O_2 tests and no significant variation compared to initial bacterial density was detected.

5.3.1.5 Bacterial count

Samples of water treated by UV/H_2O_2 were collected at regular time for bacterial count. Aliquots of starting solution and of serial dilutions were plated onto TBX agar culture media and incubated at 37 °C for 24 h.
Bacterial growth and colony count were followed through visual inspection of the Petri dishes over a time period of 24 h. The detection limit (DL) of this experimental method was 5 CFU mL^{-1}. Some *E. coli* colonies were picked up from plates and resuspended in 1.0 mL of sterile water up to obtain 10^9 harvest cells useful for DNA extraction.

5.3.1.6 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: 0, 30, 60 and 90 min. At 120 min it was not possible to harvest cells because of the low number of colonies in the plates. In particular, DNA was extracted from bacterial colonies picked up from plates, 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 replicates) by optical density at 600 nm. Total DNA (*i.e.* genetic material from live bacteria

and/or released from inactivated bacteria) was extracted from UV/H_2O_2 treated water samples collected at each treatment time.
Aliquots (1.0 mL) of each water sample were centrifuged at 14,000 rpm for 10 min in order to collect and concentrate genetic material up 0.25 OD (600 nm). Pellets were resuspended in 180.0 µL of extraction buffer (ATL) and used for DNA extraction.
The nucleic acid quality and quantity were estimated on agarose gels and spectrophotometrically at 260 nm (NanoDrop 2000C, NanDrop Technologies, Willmington, DE). All DNA samples were stored at -20 °C.

5.3.1.7 Qualitative PCR analysis

Specific primers were designed on the base of literature (Rodriguez-Mozaz et al. 2015, as reported in Table 5.1) and used both for qualitative and quantitative (qPCR) PCR analyses.
Qualitative PCR reactions were performed to check the presence or absence of ARGs in UV/H_2O_2 treated water samples. PCR reactions were performed in a 25.0 uL reaction mixture containing 10 ng of DNA and 0.5 U of Taq polymerase (AmpliTaq® DNA polymerase, Applied Biosystems), according to the manufacturer's instructions. PCR reaction cycling parameters were as follows: 5 min of initial denaturation at 94 °C, followed by 35 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. The final extension was set at 72 °C for 7 min. PCR products were separated by electrophoresis on 1% agarose gels.

Table 5.1 qPCR primer sequences and reaction conditions

Target	Primer	Sequence	Annealing T (°C)	Product (bp)
16S rRNA	16S rRNA F 16S rRNA R	GTGSTGCAYGGYTGTCGTCA ACGTCRTCCMCACCTTCCTC	60	146
bla$_{TEM}$	RTblaTEMFX RTblaTEMFR	GCKGCCAACTTACTTCTGACAACG CTTTATCCGCCTCCATCCAGTCTA	60	247
*qnr*S	qnrSrtF11 qnrSrtR11	GACGTGCTAACTTGCGTGAT TGGCATTGTTGGAAACTTG	62	240
*tet*W	tet(W)-FX tet(W)-FV	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	60	168

5.3.1.8 Quantitative PCR analysis

DNA was amplified with specific primers (*bla*$_{TEM}$, *qnr*S, *tet*W, 16S; primers efficiency was >99% for each primer) and iQSYBR Green Super Mix (Bio-Rad Laboratories, Milano, Italy) in triplicate for each sample and performed twice, using the iQ5 (Bio-Rad Laboratories, Milano, Italy). The main qPCR parameters according to reference (Bustin et al. 2009) were evaluated and reported in Table 5.2.

Table 5.2 qPCR parameters

Target	Efficiency (mean value ± standard deviation)	R^2	Slope
16S rRNA	110±10	0.994 ± 0.004	-2.923
bla$_{TEM}$	106 ± 5	0.998 ± 0.002	-2.776
*qnr*S	115 ± 4	0.997 ± 0.002	-2.913
*tet*W	101 ± 8	0.995 ± 0.004	-2.678

A negative (no-template) control was used to test for false-positive results and/or contaminations. However, 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). 16S rRNA gene was used as normalising gene for all experiments. To quantify ARGs copies number, standard curves were created, using the 16S rRNA as standard and following the steps here reported: (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 DNA mass containing the copy of interest; (iv) calculation of DNA concentration 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, from 1 ng (1.8×10^{-5} copies of 16S rRNA) to 10^{-3} ng (1.8×10^{-2} copies). The threshold cycle (Ct) values of ARGs were correlated with the standard curves in order to estimate the copies number.

5.3.1.9 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 process on ARGs copies number.

5.3.2 Results

5.3.2.1 Bacterial inactivation

The effect of UV/H_2O_2 process on the inactivation of the AR *E. coli* strain inoculated in DNA-free water is plotted in Figure 5.2.

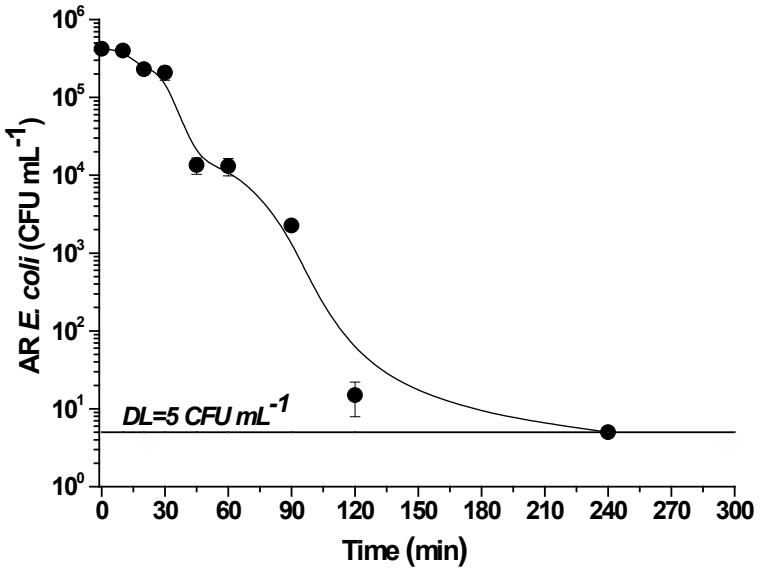


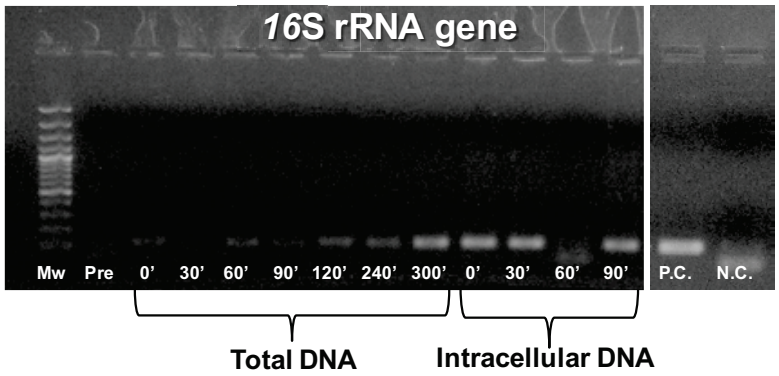
Figure 5.2 AR *E. coli* inactivation by UV/H_2O_2 process in DNA-free water tests

A 4-Log decrease was observed after 120 min UV exposure (density as low as 1.5×10^1 CFU mL^{-1}) and the DL (5 CFU mL^{-1}) was achieved after 240 min treatment. Hydrogen peroxide concentration was monitored throughout the experiment; no addition of oxidant was required to keep almost constant concentration. The low H_2O_2 consumption (1 mg L^{-1} after 300 min treatment) may be due to the water matrix, such as distilled

water which does not include compounds (*i.e.* nitrogen and organic matter) that react with oxidants, thus reducing hydrogen peroxide concentration. A continuous regeneration of H_2O_2 may not be also excluded.
Despite DL was achieved in 240 min, disinfection process was extended up to 300 min to simultaneously evaluate the variations of target ARGs which may be released after bacterial cells inactivation.

5.3.2.2 Occurrence of ARGs

Qualitative PCR assays were performed on total DNA and on DNA extracted from cell cultures in order to detect the presence or absence of target ARGs in water samples spiked with AR *E. coli* exposed to UV/H_2O_2 disinfection process. Total DNA was extracted from water samples collected at different times and containing genetic material of living residual bacterial cells and/or released from inactivated bacterial cells due to disinfection/oxidation process.
PCR revealed the presence of 16S rRNA and bla_{TEM} genes both in total DNA and in DNA extracted from cell cultures (Figure 5.3). The intensity of 16S rRNA and bla_{TEM} PCR product increased, in the case of total DNA, as treatment time increased. bla_{TEM} gene was detected both in total DNA and in DNA extracted from cell cultures. On the contrary, *qnr*S and *tet*W genes were likely to be absent in total DNA and in DNA extracted from cell cultures, or present at very low concentrations that made them not detectable through the qualitative PCR assay.



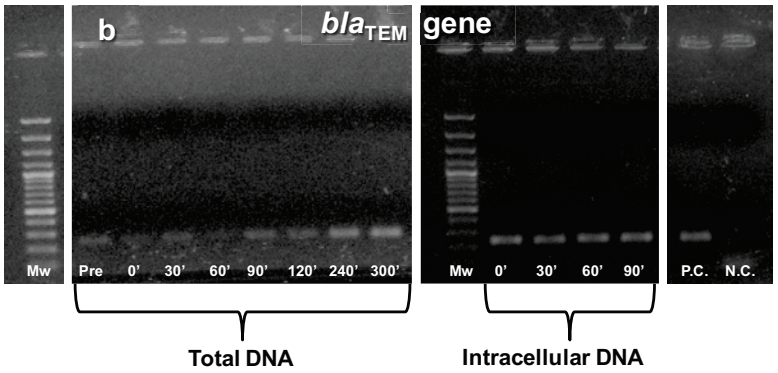


Figure 5.3 Results of electrophoretic run of PCR product on gel agarose: 16S rRNA gene (a), *bla*$_{TEM}$ gene (b), in DNA-free water assays

Pre = water samples collected before the inoculum. P.C. = positive control; N.C. = negative control.

5.3.2.3 Quantification of ARGs

qPCR analyses were carried out in order to quantify ARGs copies number, using 16S rRNA gene as standard. The Ct values of ARGs were correlated with the 16S rRNA standard curves in order to estimate the copies number (R^2 value=0.992).

qPCR assays revealed only few or undetectable copies number of *qnr*S and *tet*W genes, at each treatment time, in both total DNA and in DNA extracted from cell cultures (data not shown), according to PCR analysis.

5.3.2.4 Effect of UV/H_2O_2 process on DNA extracted from cell cultures

DNA concentration was monitored during treatment in order to understand if the disinfection process may affect to some extent the antibiotic resistance of the survived bacterial cells.

bla$_{TEM}$ gene was detected in DNA samples up to T90, when it was still possible to harvest cells (Figure 5.4). As it can be observed, UV/H_2O_2 process did not result in any relevant effect on the removal of DNA extracted from cell cultures. From T0 to T60 a slight decrease was obtained that is not statistically significant (p>0.05); the copies number per mL was found to decrease from 2.7×10^8 (T0) to 2.4×10^8 (T30) to 1.9×10^8 (T60). The mild increase that is observed at T90 is not

statistically significant (p>0.05) and entails that *bla*$_{TEM}$ gene copies number was mostly unchanged within the treatment.

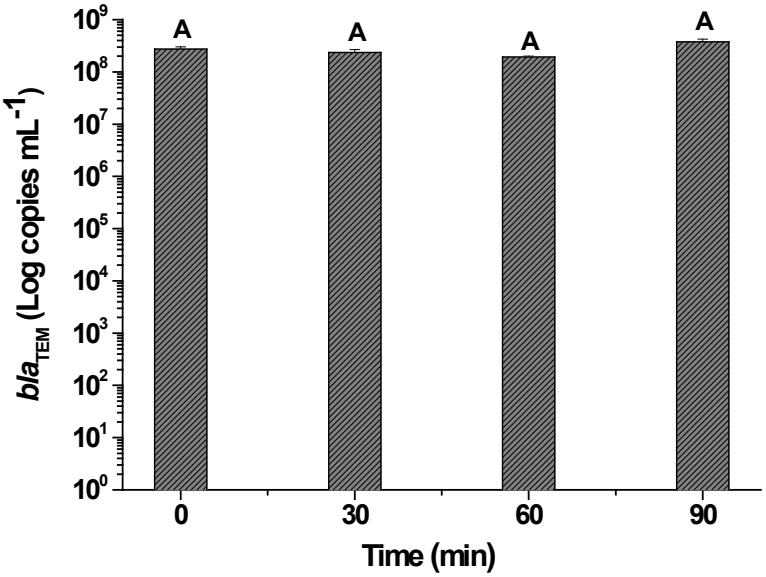


Figure 5.4 Concentrations of *bla*$_{TEM}$ gene in DNA extracted from cell cultures as function of treatment times of UV/H_2O_2 process when carried out on DNA-free water

A indicates significantly (p<0.05) different groups of *bla*$_{TEM}$ gene (Log copies mL^{-1}) among the four investigated treatment times.

5.3.2.5 Effect of UV/H_2O_2 process on total DNA

Total DNA was also monitored during UV/H_2O_2 process in order to evaluate the possible release of ARGs following bacterial cells inactivation as well as their variations during disinfection process.

bla$_{TEM}$ gene was detected at each treatment time until to the end of the experiment (300 min), as it is shown in Figure 5.5. In particular, *bla*$_{TEM}$ gene copies number slightly decreased from T0 to T30 (p<0.05), then it remained constant up to T90. From T90 to T240 a new decrease of *bla*$_{TEM}$ gene copies number was observed (p<0.05), while the copies number increased at T300 reaching the initial value. Therefore, UV/H_2O_2 process did not significantly affect ARGs removal. Despite the total inactivation of AR *E. Coli* within 240 min, *bla*$_{TEM}$ gene was detected at T300.

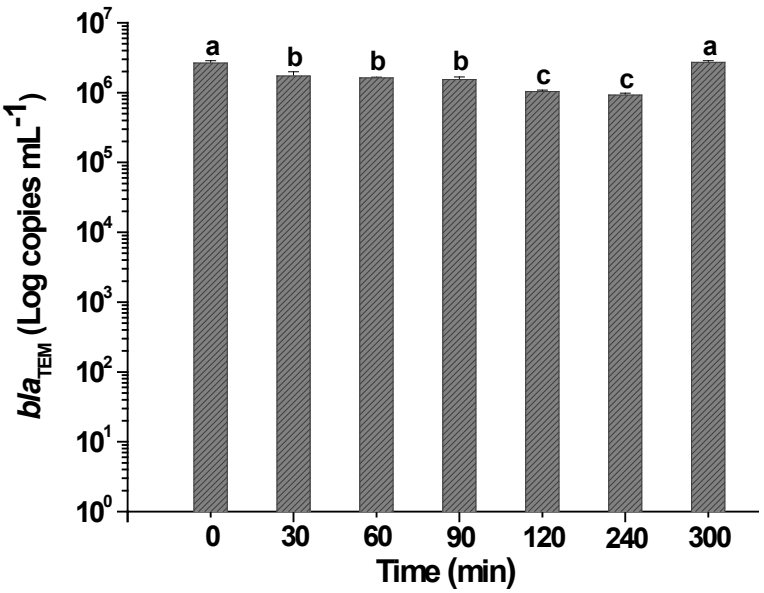


Figure 5.5 Concentrations of *bla*$_{TEM}$ gene in total DNA as function of treatment times of UV/H_2O_2 process when carried out on DNA-free water

a, b, c, indicate significantly (p<0.05) different groups of *bla*$_{TEM}$ (Log copies mL^{-1}) among the investigated treatment times.

The developed method allowed the quantification of *bla*$_{TEM}$ gene at each treatment time: 3.8×10^8 copies mL^{-1} (T90) and 2.7×10^6 copies mL^{-1} (T300) were detected in DNA extracted from cell cultures and in total DNA, respectively. The differences of ARGs copies number between DNA extracted from cell cultures and total DNA may be explained on the base of the developed/adopted method. Briefly, total DNA was extracted from bacterial cells present in 1 mL of treated water. The copies numbers that are plotted in Figure 5.4 were estimated starting from DNA extracted from bacterial cells present in treated water and grown exponentially on selective culture medium, namely TBX agar plates. Consequently, in last case, ARGs copies number was higher than that present in 1 mL of water because the number of AR *E. coli* was higher.

5.3.3 Discussion

The simultaneous effect of UV/H_2O_2 process on the inactivation of a selected AR *E. coli* strain and the variation of target ARGs was investigated in the present study.

The main inactivation mechanism of bacteria by AOPs is due to HO• radicals attack to cell wall. Although the UV-A wavelengths bordering on visible light are not sufficiently energetic to directly modify DNA, they play an important role in the formation of radical oxidising species in water, such as singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl radical, which can damage DNA (McGuigan et al. 2012). However, control tests with UV lamp as standalone process did not show any effect on the inactivation of the spiked strain.

UV/H_2O_2 process was evaluated as possible option to control the *potential* spread of antibiotic resistance into the environment. As shown in chapter 4, it allowed to reach the DL to AR *E. coli* strain that has been inoculated in filtered wastewater, after 150 min of solar exposure and with 18.0 kJ L^{-1} of cumulative energy per unit of volume, using the same dose of oxidant. In the present work, cell density was 1.5×10^{1} CFU mL^{-1} at T120; no realistic treatment times up to 240 min was investigated because the main aim of the present work was the evaluation of the variations of ARGs due to treatment. Some treatment times were chosen to monitor the bacterial inactivation that took place at 240 min; moreover, the disinfection process was extended up to 300 min to be sure that all cultivable bacteria could be inactivated.

In no work data are plotted as copies number per mL, as in the present study. The mild increase observed at T300 for bla_{TEM} gene in total DNA may mean that the copies number remained mostly unchanged, therefore the investigated process did not have any effect on this ARG. *qnr*S and *tet*W genes were investigated because the *E. coli* strain was selected from wastewater according to its resistance to ampicillin, ciprofloxacin and tetracycline; *qnr*S is a gene encoding resistance to quinolones whose class ciprofloxacin belongs to, as well as *tet*W is a gene encoding resistance to tetracyclines. Unfortunately, qPCR assays revealed only few or undetectable copies of *qnr*S and *tet*W genes both in DNA extracted from cell cultures and total DNA; accordingly their presence was not unequivocally proven.

In the present work the attempt to characterise the contribution of both total DNA and DNA extracted from cell cultures was made to better

understand the *potential* of antibiotic resistance spread after disinfection process. As it has been recognised the capability of hydrogen peroxide molecules to diffuse inside bacterial cells, reacting with the free iron, generating internal HO• by photo Fenton or Fenton-like reactions and causing cells inactivation (Polo-López et al. 2011, Sichel et al. 2009), the effects on DNA are still unclear. On the base of the obtained results, in a wider scenario, ARGs that are not removed by UV/H_2O_2 process may spread into the environment.
To date there is a main gap to fill related to design/operative parameters that may influence the mechanisms through which ARGs are removed, survive or multiply during the application of advanced treatment (Manaia et al. 2015). Moreover due to the scarcity of data on the occurrence of antibiotic resistance and horizontal gene transfer in the environment, which is regarded as one of the main transfer mechanisms (Rizzo et al. 2013), it is currently difficult to develop validate models that can be applied in the framework of environmental risk assessment (Berendonk et al. 2015). Despite extracellular DNA cannot be evaluated, in the present work, as difference between total DNA and DNA extracted from cell cultures due to the employed methods (last one is an "amplification" of the real intracellular DNA in live bacterial cells), the above discussed results have been a useful first step in the characterisation of the *potential* for antibiotic resistance transfer after disinfection process.

5.4 EFFECTS OF UV/H_2O_2 PROCESS ON INDIGENOUS BACTERIAL POPULATION

Disinfection experiments were subsequently carried out on real urban wastewater in order to evaluate the effect of UV/H_2O_2 process on antibiotic resistance transfer *potential* among indigenous bacterial population.

5.4.1 Materials and methods

5.4.1.1 Experimental design

The previous experimental design was simplified to carry out tests on UWTP effluents (Figure 5.6).

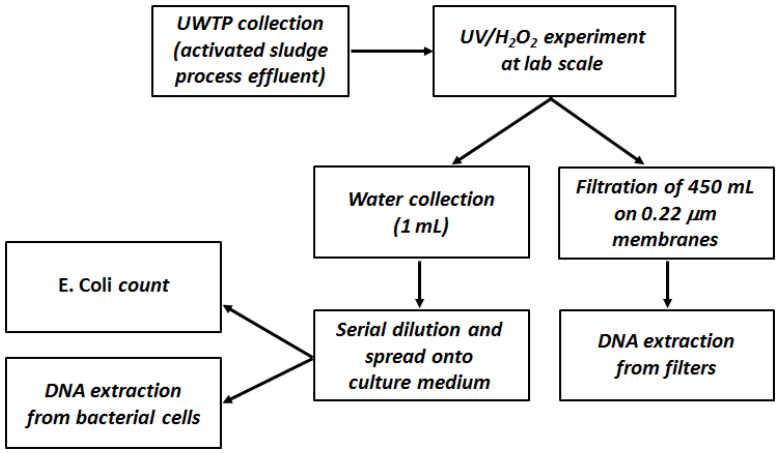


Figure 5.6 Schematic view of experimental design for urban wastewater assays

Urban wastewater samples were freshly collected from the effluent of the biological process (activated sludge) and exposed to 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 AR *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 from live bacteria and/or released from inactivated bacteria) from wastewater (from here on "total").

5.4.1.2 Wastewater samples

Urban wastewater samples were used to carry out tests on real water matrices. They 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 the disinfection unit. 24 h composite samples were taken using an automatic sampling equipment (ISCO 2009 Sampler) programmed to collect 500 mL aliquots

at each hour in order to reduce fluctuations and to get a more representative sample. UWTP samples were collected in sterilised glass bottles, transported refrigerated to the laboratory and analysed within 4 h. Wastewater samples were chemically characterised and the average values were the following: BOD_5: 20.0 mg L^{-1}, COD: 48.0 mg L^{-1}, TSS: 18.0 mg L^{-1}, Total N: 7.9 mg L^{-1}, Total P: 1.8 mg L^{-1}, pH: 7.6.

5.4.1.3 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 of urban wastewater (5.0 cm water height), according to working conditions that have been described in paragraph 5.3.1.4.
When decreases of hydrogen peroxide were measured, a suitable volume of the oxidant was added to wastewater samples to keep constant concentration.
In this case, serial UV/H_2O_2 experiments were carried out (one for each treatment time) in order to perform molecular biology analyses on a representative wastewater volume (≈450 mL).

5.4.1.4 Bacterial count

Samples of untreated and treated wastewater were collected at regular times for bacterial count that was performed through the standard plate count method. Aliquots of starting solution and of serial dilutions were plated onto TBX agar culture media and incubated at 37 °C for 24 h.
In particular Total Coliforms, *E. coli* and AR *E. coli* were counted. TBX was supplemented with a mixture of three antibiotics (8 mg L^{-1} of AMP, 0.064 mg L^{-1} of CIP, 8 mg L^{-1} of TET) in order to count AR *E. coli*. Taking into account the current limits 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). *E. coli* colonies that were able to grow on TBX agar supplemented with the mixture of antibiotics were regarded as resistant to antibiotics. AR *E. coli* colonies were then used to extract intracellular DNA.
The detection limit (DL) of this experimental method was 5 CFU mL^{-1}.

5.4.1.5 DNA extraction

DNA was extracted from bacterial cells as described in paragraph 5.3.1.6. At 120 min it was not possible to harvest cells because no AR *E. coli* colony grew on the plates.

Total DNA (*i.e.* genetic material from live bacteria and/or released from inactivated bacteria) was extracted from UV/H_2O_2 treated wastewater samples collected at each treatment time. In this case, 450 mL of wastewater samples were filtered through membranes (polycarbonate, 0.22 μm pore size, 47 mm diameter, GE Healthcare). The filters were stored at -20 °C until DNA extraction. Then they were put in tubes and centrifuged at 14,000 rpm for 10 min; the resulting pellet was resuspended in 180.0 μL of extraction buffer (ATL) and used for DNA extraction.

The nucleic acid quality and quantity were estimated on agarose gels and spectrophotometrically at 260 nm (NanoDrop 2000C, NanDrop Technologies, Willmington, DE). All DNA samples were stored at -20 °C.

5.4.1.6 PCR analysis

Qualitative and quantitative PCR analyses were performed as described in paragraphs 5.3.1.7 and 5.3.1.8, respectively.

5.4.1.7 Statistical analysis

Statistical analysis was performed as described in paragraph 5.3.1.9

5.4.2 Results

5.4.2.1 Bacterial inactivation

Bacterial inactivation by UV/H_2O_2 disinfection in urban wastewater tests is plotted in Figure 5.7.

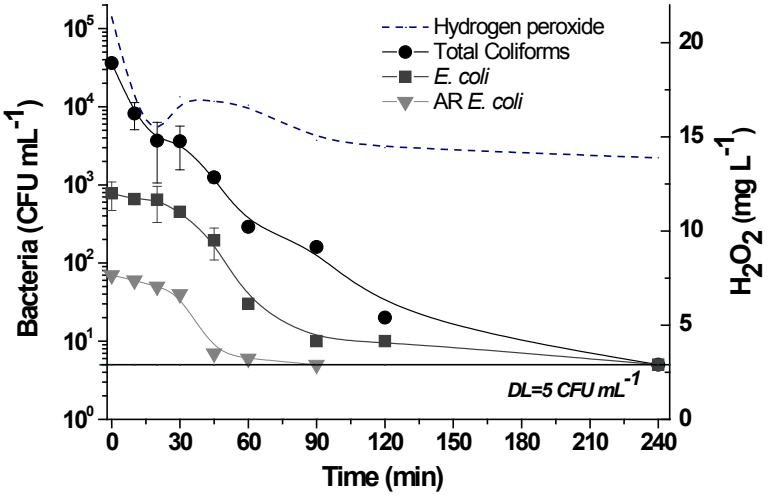


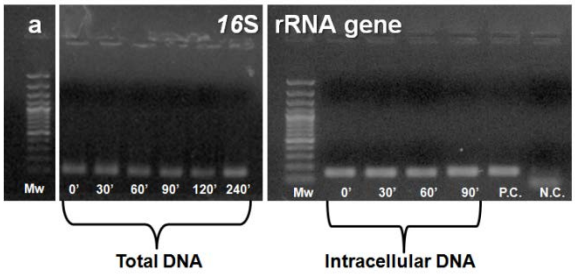
Figure 5.7 Bacterial inactivation by UV/H_2O_2 process in urban wastewater experiments

The inactivation curves for Total Coliforms and *E. coli* have a similar trend: DL (5 CFU mL^{-1}) was achieved at 240 min of treatment.
Within 30 min of treatment, a high consumption of hydrogen peroxide was observed and 1-Log reduction was reached by Total Coliforms. The consumption of hydrogen peroxide (4.2 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 in wastewater samples in order to keep constant concentration of H_2O_2. After the addition of oxidant, a higher inactivation was observed.
AR *E. coli* inactivation was also monitored; their initial density was as low as 7.0×10^1 CFU mL^{-1} and the DL was reached after 90 min of treatment (compared to 240 min for both Total Coliforms and *E. coli*). Therefore *E. coli* may be a suitable indicator of AR *E. coli* fraction occurrence. The percentages of AR *E. coli* were calculated to be 0.2% and 9.9% with respect to Total Coliforms and *E. coli* population, respectively.

5.4.2.2 Occurrence of ARGs

Qualitative PCR assays were performed on total DNA and on DNA extracted from cell cultures in order to detect the presence or absence of target ARGs in urban wastewater samples exposed to UV/H_2O_2

disinfection process. In particular, total DNA was extracted from untreated wastewater sample (T0) and from wastewater samples collected at different times and containing genetic material of living residual bacterial cells and/or released from inactivated bacterial cells due to disinfection/oxidation process.
In urban wastewater tests, PCR pointed out the presence of 16S rRNA gene both in intracellular and total DNA (Figure 5.8a). The intensity of the band of 16S rRNA gene (in total DNA) did not show any variation as treatment time increased. bla_{TEM} gene, initially present in intracellular DNA, seems to be removed by UV/H_2O_2 process, whilst it may be absent in total DNA or present at not-detectable concentrations (Figure 5.8b). As bla_{TEM} gene, *qnr*S gene was also detected in intracellular DNA and the intensity of the band decreased as treatment time increased (Figure 5.8c). A mild effect of disinfection process on the removal of *qnr*S gene in intracellular DNA may not be excluded. In the case of *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. *tet*W gene was detected in intracellular DNA and the intensity of the band slightly decreased as treatment time increased (Figure 5.8d). As for bla_{TEM} gene, *tet*W gene is likely to be absent or present in total DNA at very low concentrations that would make it not detectable through a PCR assay.



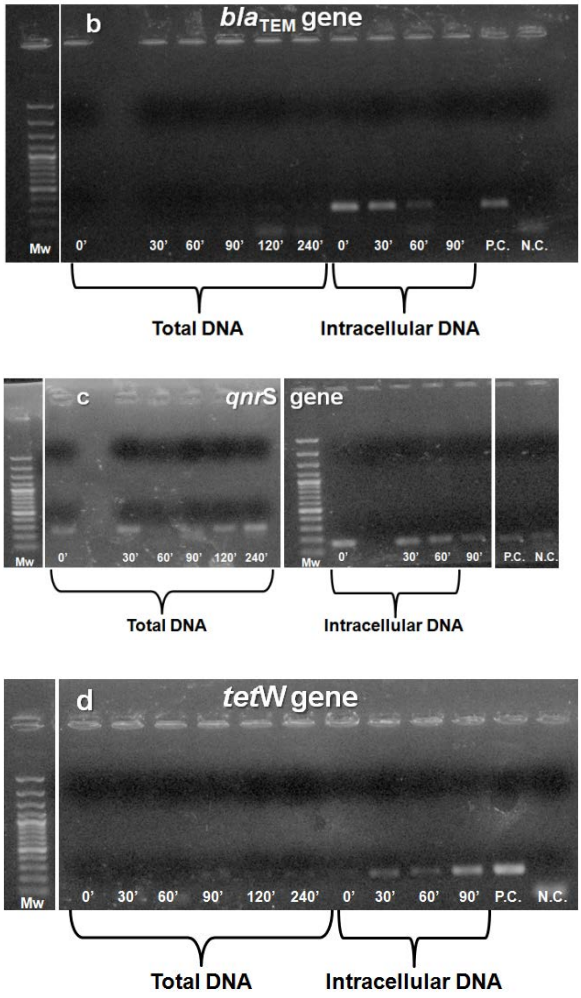


Figure 5.8 Results of electrophoretic run of PCR product on gel agarose: 16S rRNA gene (a), *bla*$_{TEM}$ gene (b), *qnr*S gene (c), *tet*W gene (d) in urban wastewater tests

P.C. = positive control; N.C. = negative control.

5.4.2.3 Effect of UV/H_2O_2 process on DNA extracted from cell cultures

In urban wastewater experiments, copies number was normalised with respect to the corresponding residual AR *E. coli* colonies from which DNA has been extracted (Figure 5.9). While *bla*$_{TEM}$ gene copies number

slightly decreased from T0 (7.8×10^5) to T30 (5.3×10^5), *qnr*S gene copies number did not show any variation during the same treatment time ($p>0.05$). However, bacterial inactivation was higher than ARGs removal, so that a significant apparent increase ($p<0.05$) was observed when data are plotted as copies number per CFUs. Disinfection process affected bla_{TEM} gene removal in agreement with results that have been obtained through PCR assays: 2.8×10^0 copies CFU^{-1} remained after 90 min treatment. UV/H_2O_2 process also affected the removal of *qnr*S gene, which was not detected at T60 in agreement with PCR results. Unlike the other two genes, *tet*W gene was not detected in intracellular DNA by means of qPCR.

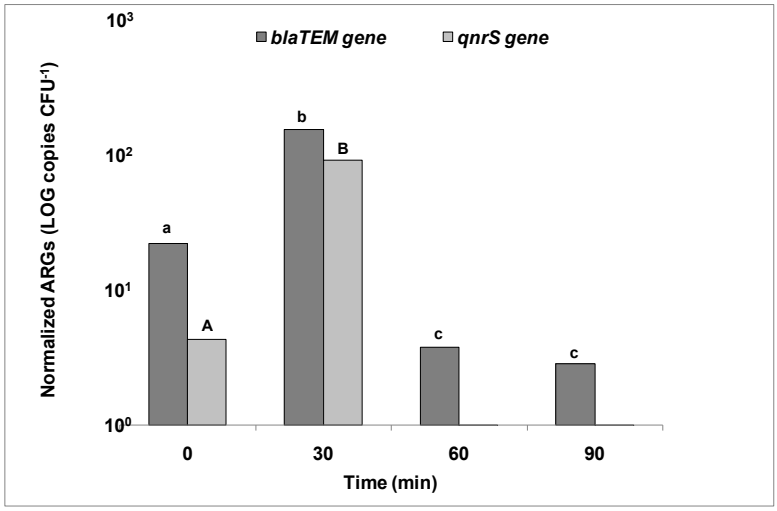


Figure 5.9 Concentrations of ARGs in DNA extracted from cell cultures as function of treatment times of UV/H_2O_2 process when carried out on urban wastewater

A, B, a, b, c indicate significantly ($p<0.05$) different groups of normalised ARGs (Log copies CFU^{-1}) among the four investigated treatment times.

5.4.2.4 Effect of UV/H_2O_2 process on total DNA

Differently, in urban wastewater assays, all target ARGs were detected in total DNA at each treatment time until to the end of the experiment (240 min), as shown in Figure 5.10.

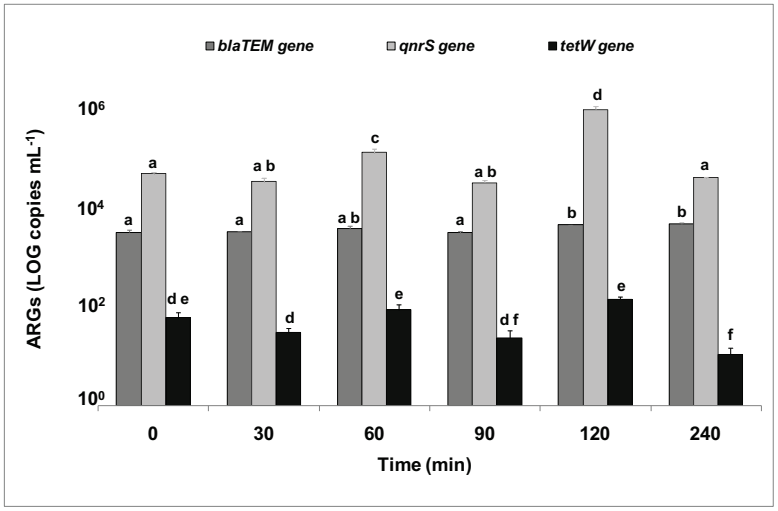


Figure 5.10 Concentrations of ARGs in total DNA as function of treatment times of UV/H_2O_2 process when carried out on urban wastewater

a, b, c, d, e, f, indicate significantly (p<0.05) different groups of ARGs (Log copies mL^{-1}) among the investigated treatment times.

Unlike intracellular DNA results, the higher relative abundance in wastewater was observed for *qnr*S gene. This result may be explained through the possible occurrence of both: other bacteria encoding *qnr*S gene and suspended DNA. By comparing the initial total DNA (5.1×10^4 copies mL^{-1}) with the final one (4.3×10^4 copies mL^{-1}), no difference was found (p>0.05). Possibly, the slight increases/decreases that are observed during treatment, may be regarded as an overall mild effect of UV/H_2O_2 process on *qnr*S gene removal.

Differently from PCR results, qPCR revealed the presence of 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 AR bacteria, the disinfection process did not significantly affect the occurrence of bla_{TEM} gene in total DNA.

The mild variations that are observed during treatment may suggest that UV/H_2O_2 process would have affected the removal of tet_W gene to some extent, but a clear trend was not observed. However, by comparing the initial concentration (6.0×10^1 copies mL^{-1}) with the final one (1.1×10^1 copies mL^{-1}), a significant decrease (p<0.05) was noted.

5.4.3 Discussion

Only a few works have addressed the effect of water/wastewater disinfection processes on the *potential* of antibiotic resistance transfer and mostly by conventional disinfection processes. Munir et al. monitored the release of ARB and ARGs into the environment through the effluents of different UWTPs. Despite no availability of data about chlorine and UV doses, as well as contact times, their results have shown that the target disinfection processes (namely chlorination and UV radiation) did not significantly contribute to the decrease of ARB and ARGs (p>0.05). In particular *sul*-I, *tet*O and *tet*W concentrations in the final effluents were in the range of non-detectable-2.3×10^{8} copies mL^{-1}. Higher removal (1-3 Log units) of *tet*W gene (from not detectable to 10^{4} copies mL^{-1}) was observed in samples after MBR and UV radiation processes (Munir et al. 2011). Rodriguez-Mozaz and co-workers monitored the occurrence of ARGs in a UWTP and observed that *erm*B and *tet*W genes (in terms of copies number normalised to the 16S rRNA gene copies number) decreased (p<0.05) as a result of wastewater treatment, while bla_{TEM}, *qnr*S and *sul*I genes increased (Rodriguez-Mozaz et al. 2015). In another study dealing with the monitoring of different ARGs in a UWTP, it was observed that the relative abundance of bla_{TEM} (ratio bla_{TEM} per 16S rRNA) decreased of 1 Log unit (p<0.05) (Narciso-da-Rocha et al. 2014). The quoted studies pointed out (i) a lower resistance of *tet*W gene to the treatment compared with the other investigated ARGs and (ii) that the effect of disinfection process depended on the target ARG.

Zhuang et al. compared chlorination, UV radiation and ozonation in the removal of target ARGs, namely *int*I1, *tet*G, *sul*1, from a urban wastewater. The most effective process 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 by UV radiation (UV dose of 12.5 mJ cm^{-2} for 2.5-2.7 Log reductions) and by ozonation (ozone dose of 177.6 mg L^{-1} for 1.7-2.6 Log reductions). *tet*G gene (from 10^{5} to 10^{6} copies mL^{-1}) was more abundant than *tet*W gene in the present study (6.0×10^{1} copies mL^{-1}) and was removed more easily than other ARGs by ozonation. However, just 2.6 Log reductions were observed with an 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 the results that have been achieved in the present study. 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 a not realistic dose. Another study on plasmid DNA isolated from a multi-resistant *E. coli* HB101 and subsequently treated by different disinfection processes by Öncü et al. (2011) has shown that increasing ozone doses (from 1.00 to 4.42 mg L^{-1}) resulted in a gradual decrease in the intensity of bands of plasmid DNA, until bands disappeared. A similar trend was observed for the bands of plasmid DNA treated by TiO_2 photocatalysis that were totally removed within 75 min treatment (Öncü et al. 2011). On the base of their results, ozone and titanium dioxide induced a damage in the plasmid DNA structure, caused strand breaks resulting in conformational change and decreases of the cell transformability.
The effect of Fenton (without acidification) and ozonation processes on *tet*M gene in total DNA, in parallel to its host, namely multi-resistant *E. coli* HB101 inoculated (10^7 CFU mL^{-1}) in a synthetically contaminated cow manure, was monitored by Cengiz and co-workers. Increased exposure to oxidants (up to 40 mM H_2O_2/4 mM Fe^{2+} and up to 7.5 mg ozone) resulted in reduced band intensity (Cengiz et al. 2010). Although the matrix was different from that analysed in the present study (synthetic cow manure Vs urban wastewater), high doses of oxidant (1,360.6 mg L^{-1} Vs 20.0 mg L^{-1}) did not result in ARB inactivation as well as ARGs removal. In disagreement with that, a work by Oh et al. (2014) has shown that ozonation reduced *E. coli* DH5α and its multi-resistant gene pB10 from a synthetic wastewater more than 90% with an ozone dose of 3 mg L^{-1} within 15 min: the addition of some catalysts, such as peroxymonosulfate, persulfate, hydrogen peroxide (concentration of 1 mg L^{-1}), resulted in better performances (Oh et al. 2014). Anyway no information is available about the working volume, as well as the initial concentrations of ARB and ARGs.
In the literature a work is available about the effect of photocatalysis (TiO_2 immobilised onto borosilicate glass plates: 0.5 mg cm^{-2}) on the transfer of ARGs in urban wastewater by means of a cultivation method: it pointed out that ARB, once sub-lethally injured, upon recovery, may spread antibiotic resistance into the environment (Dunlop et al. 2015).
McKinney and Pruden showed that UV doses ranging from 200 to 400 mJ cm^{-2} (at least one order of magnitude higher than those for the inactivation of host bacterial cells) were required to remove from 3- to 4-Log units of ARGs, such as *amp*C, *mec*A, *tet*A and *van*A (McKinney and Pruden 2012). Despite the effect of UV-C on damage to ARGs was

successful, two drawbacks were emphasised by authors: (i) the required doses of UV radiation to achieve from 3 to 4 Log reduction in ARGs would be impractical for water utilities and (ii) the potential of re-growth and photo-reactivation cannot be excluded. On the contrary UV treatment, implemented at laboratory scale at doses of 5 and 10 mJ cm^{-2} on UWTP effluents, was effective in the removal of *ere*A, *ere*B, *erm*A, *erm*B, *tet*A, *tet*O (Guo et al. 2013). Particularly, the concentrations of four erythromycin genes were under the DL with a dose of 10 mJ cm^{-2}. However, in this work, initial concentrations were lower (from 1.8×10^{1} to 2.4×10^{3} copies mL^{-1}) than those detected in other studies (*i.e.* McKinney and Pruden 2012, Munir et al. 2011).

Yuan and co-workers investigated the effect of chlorination (15 mg Cl_2 $min\ L^{-1}$) on ARGs removal from a UWTP effluent collected after the biological process, and observed that *ere*A and *erm*B genes (initial concentration of 3.2×10^{4} and 2.1×10^{4} copies mL^{-1}, respectively) persisted in chlorinated samples (6.6×10^{3} and 1.3×10^{4} copies mL^{-1}, respectively) (Yuan et al. 2015). *tet*A gene (1.3×10^{4} copies mL^{-1}) also underwent a slight decrease (9.9×10^{3} copies mL^{-1}). In agreement with results that have been achieved in the present work, ARGs may survive as dissociative DNA although their bacterial hosts have been inactivated. Moreover the trend of each ARG removal was not constant; slight increases, followed by decreases, were observed during the treatment.

Another study by Shi and co-workers also pointed out ARB and ARGs prevalence in drinking water after chlorination (residual chlorine from 0.6 to 0.8 mg L^{-1}): ARGs, such as *amp*C, *aph*A2, bla_{TEM-1}, *erm*A, *erm*B, *tet*A and *tet*G were found to be enriched by chlorination ($p<0.05$) (Shi et al. 2013). According to their results, chlorination (2-4 h as treatment time) may promote antibiotic resistance in drinking water.

In the above mentioned works no information was provided about the nature of DNA. Only one work available in the scientific literature dealt with the effect of UV disinfection both on extracellular and intracellular DNA: a higher removal ($p<0.05$) of extracellular ARGs than intracellular ARGs was observed but only at a dose as high as 400 mJ cm^{-2} (McKinney and Pruden 2012).

It is worth mentioning a recent published work by Zhang and co-workers (2016) that is focused on the effect of Fenton oxidation and UV/H_2O_2 process on the removal of *intI*1, *sul*1, *tet*G, *tet*X and 16S rRNA genes from urban wastewater whose concentrations ranged from 10^{5} to 10^{9} copies mL^{-1}. It is highlighted that in UV/H_2O_2 experiments (30 min

irradiation time, 0.01 mol L^{-1} H_2O_2 concentration and pH 3.5), all ARGs were reduced of 2.8-3.5 Log units. This significant reduction is due to different operative conditions compared with those investigated in the present work. First of all, the reactor was equipped with a low-pressure 254 nm (UV-C) mercury vapour lamp, while a wide spectrum lamp, whose main radiation emission ranges from 320 to 450 nm, was used in the present study. This entails different treatment times as well as mechanisms of action: it is known that UV-C light can penetrate the UV-transparent structures in the cell and primarily be absorbed by the nucleobases comprising DNA and RNA (Dodd 2012). Differently, the effect on DNA in the present work is expected to be mainly due to hydroxyl radicals that are produced during oxidation process. Another difference is due to the pH of wastewater: 3.5 Vs natural pH (7.6); the authors evaluated the effect at pH 3.5 because of the lower removal (1.5-2.3 Log decrease) achieved at pH 7.0 (Zhang et al. 2016). Finally the dose of hydrogen peroxide was set at 0.01 mol L^{-1} (340 mg L^{-1}) which is much higher than the dose that has been applied in the present study, namely 20 mg L^{-1}. A so high concentration is not realistic because does not make the investigated technology cost-effective and would be toxic to plants in agricultural wastewater reuse practise (Sichel et al. 2009). A dose as low as 20 mg L^{-1} was chosen according to previous results (chapter 4), but also in the light of a possible wastewater reuse as a cost-effective technology.

In urban wastewater experiments, despite the successful inactivation of Total Coliforms, *E. coli* and AR *E. coli*, bla_{TEM}, *qnr*S and *tet*W genes were still present in wastewater after 240 min treatment. 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 that may promote antibiotic resistance spread into the environment.

5.5 CONCLUSIONS

The effect of UV/H_2O_2 process on the simultaneous inactivation of bacteria, among them AR *E. coli*, and the variations of genes that confer antibiotic resistance, was investigated firstly in DNA-free water, then in real urban wastewater.

In disinfection tests that have been run on DNA-free water spiked with a selected AR *E. coli* strain, despite a total inactivation (the DL was reached within 240 min of treatment), no removal was obtained for bla_{TEM} gene in total DNA. *qnr*S and *tet*W genes were also investigated but their presence was not unequivocally proven.
DNA analysis was a useful tool to better understand the *potential* of antibiotic resistance spread after disinfection process. Once developed, the protocol was subsequently applied to work on a more complex environmental matrix, such as real urban wastewater collected downstream of the biological process. In this way the attention was focused on indigenous bacteria and genetic materials.
Also in this case, despite the DL of AR *E. coli* and other investigated cultivable bacteria was reached at 90 and 240 min, respectively, ARGs were still present in the wastewater sample at the end of 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 (4.3×10^4 copies mL^{-1} after 240 min treatment). However, the investigated disinfection process allowed to get a significant decrease ($p<0.05$) of the copies number of *tet*W gene (1.1×10^1 copies mL^{-1}) after 240 min treatment.
According to these results, UV/H_2O_2 process may not be an effective disinfection process to limit or minimise the *potential* spread of antibiotic resistance under realistic conditions, at least at the applied oxidant dose, such as 20 mg L^{-1}, and regarding the investigated ARGs. The survived ARB and/or ARGs that are released from inactivated bacteria and/or naturally present in wastewater may contribute to antibiotic resistance diffusion/transfer into the environment (namely, aquatic ecosystems in which the treated wastewater is discharged or irrigated crops/soils in the case of wastewater reuse).

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5.7 APPENDIX

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β-lactams resistance gene quantification in an antibiotic resistant *Escherichia coli* water suspension treated by advanced oxidation with UV/H_2O_2

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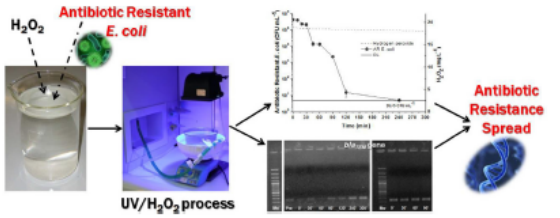
HIGHLIGHTS

- An antibiotic resistant *E. coli* strain has been effectively inactivated by UV/H_2O_2 process.
- Bacterial cells released antibiotic resistant genes (ARGs) during UV/H_2O_2 treatment.
- UV/H_2O_2 process did not affect antibiotic resistance of survived colonies.
- UV/H_2O_2 process did not significantly change the copy number per mL of *bla*TEM gene.

GRAPHICAL ABSTRACT



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Water disinfection

ABSTRACT

Water is one of the most important habitats and route for the spread of antibiotic resistance (AR) in the environment and disinfection processes can be a potential barrier to minimise this risk. In this study the effect of UV/H_2O_2 process on the potential of AR transfer was investigated through cultivation methods vs (polymerase chain reaction) PCR based methods. bla_{TEM} was selected as target antibiotic resistance gene (ARG) and was quantified by qPCR in the survived colonies and the whole suspension (total DNA). The detection limit of residual antibiotic resistant *Escherichia coli* (*E. coli*) colonies (5 CFU mL^{-1}) was reached after 240 min treatment, but bla_{TEM} gene was still present in total DNA after 300 min (2.8×10^6 copies mL^{-1}), and no effect was observed in DNA extracted from cell cultures (3.8×10^8 copies mL^{-1} after 90 min). Accordingly, the investigated disinfection process may select for unaffected ARGs, therefore contributing to the potential transfer of AR in the environment.

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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/H2O2) on AR transfer potential. UV/H2O2 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, blaTEM, qnrS and tetW). 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/H2O2 process was not effective in ARGs removal from water suspension (total DNA). Particularly, an increase up to 2.8x106 copies mL-1 ($p>0.05$) of blaTEM gene was observed in total DNA after 240 min treatment, while no difference ($p>0.05$) was found for qnrS gene between the initial (5.1x104 copies mL-1) and the final sample (4.3x104 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.

6 CHEMICAL AND MICROBIAL CONTAMINATION OF LETTUCE CROPS IRRIGATED WITH H_2O_2/SUNLIGH TREATED WASTEWATER

6.1 ABSTRACT

H_2O_2/sunlight process has recently been regarded as a sustainable alternative option in advanced treatment of urban wastewater to be reused for crops irrigation. On the base of previous results, H_2O_2/sunlight was implemented as disinfection/oxidation treatment for urban wastewater treatment plant (UWTP) effluents in compound parabolic collectors (CPCs) photo-reactors to evaluate subsequent cross-contamination of lettuce leaves and top soil by contaminants of emerging concern (CECs), among which antibiotic resistant (AR) bacteria, after irrigation with treated UWTP effluents. CECs were quantified by means of quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction method and LCQqLIT-MS/MS analysis. Carbamazepine (CBZ), flumequine (FLU) and thiabendazole (TBZ) were selected as target CECs and spiked in real autoclaved UWTP effluents up to reach the concentration of 100 μg L^{-1}. Simultaneously two AR strains, namely *Escherichia coli* (*E. coli*) and *Enterococcus faecalis* (*E. faecalis*), selected as resistant to a mixture of target antibiotics, were inoculated in autoclaved UWTP effluents. The detection limit (DL) of 2 CFU mL^{-1} was achieved after 120 min of solar exposure with 20 mg L^{-1} of hydrogen peroxide for AR *E. coli* and after 240 min for AR *E. faecalis* on average. Both CBZ and TBZ were poorly removed after 90 min (12% and 50%, respectively) compared with FLU (94%) on average. Irrigation assays were carried out for five weeks. CBZ and TBZ were accumulated in soil up to 472 ng g^{-1} and 256 ng g^{-1}, respectively and up-taken by lettuce up to 109 and 18 ng g^{-1}, respectively, when wastewater treated for 90 min were used for irrigation purposes. No bacterial contamination was observed when the AR density in treated wastewater was below the DL. A proper treatment time should be guaranteed in order to avoid the transfer of pathogens from disinfected wastewater to irrigated crops and soil.

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6.2 INTRODUCTION

A crucial challenge of urban wastewater treatment is based on avoiding serious risks to human health and environment when treated wastewater are reused for irrigation purposes. The ever increasing use of treated wastewater calls for more effective advanced treatment before wastewater reuse. The main risks arise from the occurrence of pathogenic microorganisms and a wide variety of chemical pollutants in wastewater. Guidelines on treated wastewater reuse are mainly focused on microbiological risks and there is little mention of other trace pollutants (Fatta-Kassinos et al. 2011). When irrigated with reclaimed wastewater, crops are exposed to micropollutants or so-called contaminants of emerging concern (CECs) that have been detected in urban wastewater in the range of high ng L^{-1} to low μg L^{-1} (Klamerth et al. 2010, Miralles-Cuevas et al. 2014). Conventional treatments that are the state of the art in most world regions rely on well-established biological and physical methodologies (Manaia et al. 2015), but unfortunately are not intended for the removal of CECs (Klamerth et al.

2010). So they occur in irrigation waters and may exert chronic toxic effects also at very low concentrations (Calderón-Preciado et al. 2012). Specifically CECs may enter the plants through different pathways, among which root uptake, and may result in potentially deleterious effects on environment and human health by entering the food chain if a proper treatment is not foreseen and applied before reuse (Calderón-Preciado et al. 2011). It has been reported that some pharmaceuticals, such as carbamazepine (Shenker et al. 2011), and antibiotics (Jones-Lepp et al. 2010), can be up-taken by crops through irrigation waters. Long-term release of pharmaceutical residues may induce toxic or other effects toward aquatic and terrestrial organisms. So far there is general lack of knowledge about the related environmental and human risks (Fatta-Kassinos et al. 2011, Jiang et al. 2014, Wu et al. 2014).

The use of domestic wastewater effluents for irrigation purposes raises public health concerns, especially when vegetables are eaten raw or undercooked, such as green leaves, if a proper disinfection process has not removed pathogens (Beuchat 2002, Bichai et al. 2012). In fact wastewater effluents are regarded as a vehicle for conventional pathogens, such as bacteria and fungi, which generate human and plant diseases (García-Fernández et al. 2012), but also for emerging resistant microorganisms, such as antibiotic resistant (AR) bacteria (Rizzo et al. 2013).

As highlighted in introduction chapter, conventional wastewater treatment processes may not be effective in controlling AR bacteria and CECs that are so released into water bodies in which the treated wastewater is discharged (Calderón-Preciado et al. 2012, Guo et al. 2013, Munir et al. 2011). On the contrary, advanced oxidation processes (AOPs) may be an effective and environmental friendly option for advanced wastewater treatment in small communities and areas, especially if they are solar driven (Malato et al. 2009). On the base of results that have been obtained in chapter 4, H_2O_2/sunlight process may be a sustainable alternative compared with other solar driven AOPs, in particular if residual hydrogen peroxide concentration is lower than 50 mg L^{-1} in order to avoid any phytotoxic effect on irrigated crops (Sichel et al. 2009). Unfortunately, scientific knowledge is currently lacking in some aspects related to the effects of AOPs, among which solar driven AOPs, on (i) the simultaneous removal of CECs and AR bacteria, as well as on (ii) the subsequent uptake of the respective residuals by crops when irrigated with treated wastewater (Bichai et al. 2012, Calderón-

Preciado et al. 2011). Many gaps to be filled concern the safety of water/wastewater treated by AOPs and subsequently reused for crops irrigation as well as the assessment and management of related risks for consumers (Christou et al. 2014).
In this light the present work aims at assessing chemical and microbial cross contamination both on lettuce leaves and top soil when irrigated with real urban wastewater treatment plant (UWTP) effluents treated by H_2O_2/sunlight process implemented at pilot scale in a solar compound parabolic collector (CPC) system. In particular (i) the chosen dose of hydrogen peroxide was as low as 20 mg L^{-1}; (ii) carbamazepine (CBZ), flumequine (FLU), and thiabendazole (TBZ) were selected as target CECs; (iii) AR *E. coli* and AR *E. faecalis* were singled out as target AR bacteria; (iv) lettuce was chosen as target crop. Real autoclaved UWTP samples were spiked with CBZ, FLU and TBZ and simultaneously with AR bacterial strains. Residual concentrations of AR bacteria and CECs in wastewater after photocatalytic treatment were evaluated. Irrigation assays were carried out. Finally residual concentrations of AR bacteria and CECs both in lettuce leaves and top soil irrigated with treated wastewater were evaluated.

6.3 MATERIALS AND METHODS

6.3.1 Experimental design

A schematic view of experimental design is depicted in Figure 6.1. Briefly UWTP effluents were freshly collected, sterilised in autoclave and spiked with selected AR strains ($\approx 10^5$ CFU mL^{-1}) as well as with three target CECs ($\approx$100 μL^{-1} each one). Then H_2O_2/sunlight experiments were carried out. Two experimental conditions were investigated on the base of different treatment times, namely 300 min (R1) and 90 min (R2), in order to evaluate bacteria transfer from urban UWTP samples treated by H_2O_2/sunlight process at pilot plant in CPCs photo-reactors (from here on "treated wastewater") to lettuce leaves and top soil. On the base of previous experiences, 300 min of solar exposure were chosen in order to be sure that all selected AR bacteria have been inactivated. Differently, 90 min exposure experiments were carried out to obtain partially treated urban wastewater with residual pathogen density ($>10^2$ CFU mL^{-1} for

AR *E. faecalis*, and <DL for AR *E. coli*, on average) that might be transferred to the tested crops through irrigation waters. Cultivated lettuce and top soil were irrigated with both R1 and R2 disinfected/oxidized wastewater. Irrigation experiments were performed for five weeks. Seven days after irrigation, leaves and top soil samples were aseptically collected and analysed for the evaluation of AR *E. coli* and AR *E. faecalis* transfer, as well as for CECs uptake. A presence/absence detection method of AR bacteria on lettuce leaves and top soil samples was adapted from the literature (Bichai et al. 2012). Simultaneously the accumulation/deposition of CECs on lettuce and soil was measured as described below.

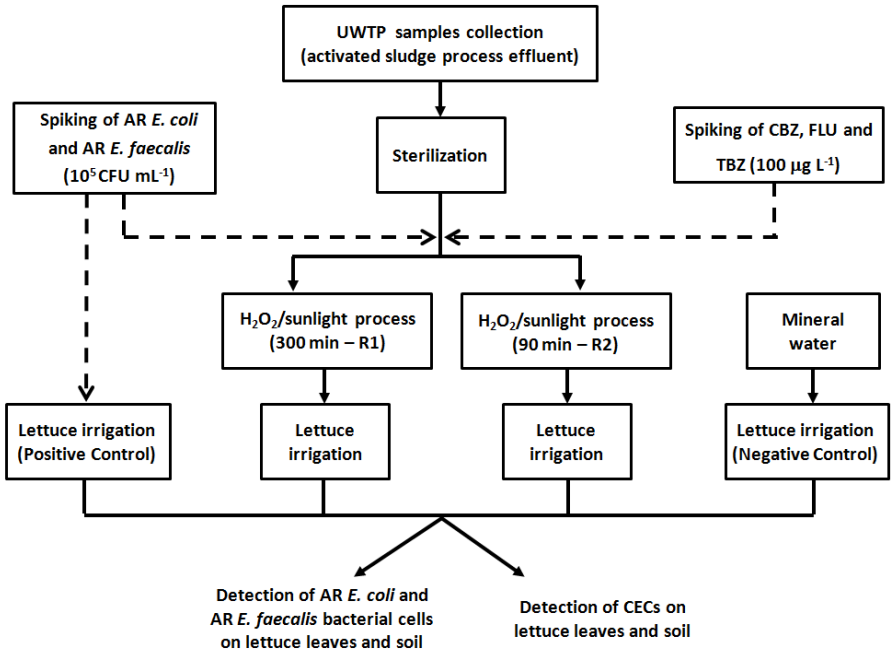

Figure 6.1 Schematic view of experimental design

6.3.2 Chemicals

CBZ (Ahmed and Chiron 2014), FLU (Van Doorslaer et al. 2014) and TBZ (Guerra et al. 2014) were selected as target CECs because normally detected in urban wastewater. CBZ, FLU, TBZ, ampicillin (AMP), ciprofloxacin (CIPR), ofloxacin (OFL), and tetracycline (TET), all high

purity grade (>99%), were purchased from Sigma-Aldrich. CBZ and TBZ were dissolved in MeOH at 2.5 g L^{-1} and stored at -20 °C, whereas FLU was dissolved in demineralised water with NaOH at 2.5 g L^{-1} and stored at 4 °C. Urban wastewater samples were spiked with the selected CECs (initial concentrations of 100 μg L^{-1}) before bacteria inoculum. H_2O_2 (Riedel-de Haën, Germany) at 30 wt% was used as received and diluted into the photo-reactor filled with autoclaved urban wastewater until obtaining an initial concentration of 20 mg L^{-1}. HPLC-grade acetonitrile (ACN) and formic acid (98% purity) were supplied by Fluka (Buchs, Germany). Water used for LC-MS analysis was generated from a Direct-Q 5 Ultrapure Water System from Millipore (Bedford, MA, USA) with a specific resistance of 18.2 MΩ cm. Anhydrous $MgSO_4$ and NaOAc were supplied by Panreac (Barcelona, Spain). Primary-secondary amine was provided by Supelco (Bellefonte, PA, USA), and C18 sorbent was purchased from Agilent Technologies (Santa Clara, CA).

6.3.3 Antibiotic resistant bacteria selection

Antibiotic resistant *E. coli* and *E. faecalis* strains were selected from the UWTP of Almería (Spain), from the effluent of the activated sludge process, by membrane filtration and subsequent cultivation on selective medium. Briefly, for *E. coli* isolation, 50 mL of wastewater and its serial dilutions were filtered through sterile membranes (cellulose nitrate, 0.45 μm pore size, 47 mm diameter, Millipore) which were incubated at 37 °C for 24 h on ChromoCult® Coliform agar (Merck KGaA, Germany), supplemented with a mixture of three antibiotics (8 mg L^{-1} of AMP, 0.064 mg L^{-1} of CIPR, and 0.25 mg L^{-1} of OFL). For *E. faecalis* isolation, membranes were incubated 37 °C for 48 h on Slanetz & Bartley agar (Scharlau, Spain), supplemented with a mixture of three antibiotics (4 mg L^{-1} of AMP, 4 mg L^{-1} of CIP, and 4 mg L^{-1} of TET). Antibiotic concentrations were selected according to the respective epidemiological cutoff values available in EUCAST database (EUCAST, 2015). Some colonies were randomly picked up and frozen in 15% glycerol Triptone Soy Broth at -20 °C.

The cultivation method allowed to select and isolate bacterial strains that are able to grow in the presence of mixtures of antibiotics, typically detected in urban wastewater effluents and among which weak antagonistic effects can be expected (Ocampo et al. 2014).

6.3.4 Inoculum and sample preparation

Wastewater samples were freshly collected (20 L) from the UWTP of Almería, El Bobar (Spain), from the effluent of the activated sludge process, on the morning of each disinfection experiment. Wastewater samples were autoclaved at 121 °C for 15 min in order to remove indigenous bacteria (control tests after autoclaving did not show any colonies growth), and to avoid any potential interference with the selected AR bacterial strains. Once autoclaved, wastewater was spiked with the selected AR bacterial strains. Briefly, some colonies were unfrozen and reactivated by streaking onto selective culture media (Chromo-Cult® Coliform agar for AR *E. coli* and Slanetz & Bartley agar for AR *E. faecalis*) and, then, incubated at 37 °C (AR *E. coli*, 24 h; AR *E. faecalis*, 48 h). Two or three colonies from each plate were inoculated into 14 mL sterile Luria-Bertani broth (Sigma-Aldrich) and incubated at 37 °C for 18 h by constant agitation in a rotator shaker to obtain a stationary phase culture. Cells were harvested by centrifugation at 3,000 rpm for 10 min and the pellet was resuspended in 14 mL of phosphate buffer saline (PBS, Oxoid), reaching a final density of 10^5 CFU mL^{-1} approximately. Urban wastewater characterisation is reported in Table 6.1. Total carbon (TC), inorganic carbon (IC) and total organic carbon (TOC) were analysed by Shimadzu TOC-5050 (Shimadzu Corporation, Kyoto, Japan). Anions were quantified using Metrohm 872 Extension Modules 1 and 2 configured for gradient analysis. Cations were determined using a Metrohm 850 Professional IC configured for isocratic analysis.

Table 6.1 Chemical characterization of the secondary UWTP effluent (El Bobar, Almería, Spain) after autoclaving process

Parameter	Average value	Standard deviation	Unit
Conductivity	1,411.00	169.00	$\mu S\ cm^{-1}$
pH	8.99	0.10	-
Turbidity	59.60	8.10	NTU
TC	51.60	9.25	$mg\ L^{-1}$
IC	37.64	10.25	$mg\ L^{-1}$
TOC	13.96	1.16	$mg\ L^{-1}$
Cl^-	315.50	30.60	$mg\ L^{-1}$
NO_2^-	1.20	0.50	$mg\ L^{-1}$
NO_3^-	29.00	15.30	$mg\ L^{-1}$
PO_4^{3-}	2.90	0.04	$mg\ L^{-1}$
SO_4^{2-}	82.30	6.40	$mg\ L^{-1}$
NH_4^+	20.30	12.70	$mg\ L^{-1}$

6.3.5 Solar photo-reactor and disinfection/oxidation tests

H_2O_2/sunlight experiments were run in a pilot scale CPC plant with 20 mg L^{-1} of H_2O_2. This system, described elsewhere (Fernández-Ibáñez et al. 2009), consists of tube modules connected to a recirculation tank and a centrifugal pump. The tube modules are cylindrical prototypes made of borosilicate glass (2.5 mm thickness) with 90% transmission in the solar UV-A spectrum. The photo-reactor is inclined at 37° with respect to the horizontal to maximise solar radiation collection and is equipped with static CPC with concentration factor 1. Total photo-reactor volume is 8.5 L, with an illuminated volume of 4.7 L and irradiated CPC surface of 0.4 m^2; the water flow rate was set at 16 L min^{-1}. Experiments were carried out simultaneously using two identical CPC photo-reactors. Disinfection/oxidation experiments were run during 300 (R1) and 90 min (R2) of solar exposure on clear sunny days at Plataforma Solar de Almería (South of Spain, latitude 37° 84' N and longitude 2° 34' W) from June 2014 to July 2014. The solar photo-reactor was filled with 8.5 L of real autoclaved urban wastewater spiked with CECs (100 μg L^{-1}). Before testing the mix of CECs, a single test with each CEC was performed at laboratory scale in order to assess possible negative effects over bacterial viability. The results (data not shown) revealed the absence of any interference among bacteria and CECs. Therefore, wastewater samples were also spiked with the selected AR bacteria (initial density as high as 10^5 CFU mL^{-1} approximately) and homogenised while the photo-reactors were still covered. Hydrogen peroxide was added to each photo-reactor and re-circulated for three min to ensure homogenization. Then the first sample was taken and the cover was removed. Samples were collected at regular treatment times in order to monitor the process within 300 min. Water temperature (T) was measured in each reactor (checktemp, Hanna, Spain) and it ranged from 24.0 °C to 44.3 °C. pH (multi720, WTW, Germany) and H_2O_2 concentration were also measured in the photo-reactors during the assays. Solar UV-A radiation was measured with a global UV-A pyranometer (300−400 nm, CUV5, Kipp&Zonen, The Netherlands) tilted at 37°. It provided UV-A incident data (in W m^{-2}). The average solar UV-A irradiance for assays conducted for 90 min was 38±4 W m^{-2}, while for 300 min tests the average value was 47±4 W m^{-2}. Bacterial inactivation curves are plotted as function of both experimental time (t) and cumulative energy per unit of volume (Q_{UV}) received in the photo-reactor:

$$Q_{UV,n} = Q_{UV,n-1} + \frac{\Delta t_n \overline{UV}_{G,n} A_r}{V_t} \qquad \Delta t_n = t_n - t_{n-1}$$

where $Q_{UV,n}$ and $Q_{UV,n-1}$ are the UV energy accumulated per liter (kJ L^{-1}) at times n and n-1, $UV_{G,n}$ is the average incident radiation on the irradiated area (W m^{-2}), Δt_n is the experimental time of sample (s), A_r is the illuminated area of collector (m^2), V_t is the total volume of treated water (L). Q_{UV} is commonly used to compare results under different operative conditions (Malato et al. 2002).
Eight experiments with real urban wastewater were run within 90 min (R2) and 300 min (R1) in order to collect enough volume of treated wastewater for subsequent irrigation tests. Disinfection assays were carried out once a week in order to avoid the stagnation of treated wastewater. The treated wastewater was stored at 4 °C; chemical and microbiological parameters were monitored before using for lettuce irrigation daily.

6.3.6 Irrigation assays

Lettuce plants (≈10-15 cm height) were transplanted in 20 rectangular pots (three plants for each pot) and kept outside. Pots were filled with autoclaved commercial peat containing NPK in a ratio (w/v) of 13-14-13 g L^{-1}, respectively, pH 7, and conductivity of 120 mS m^{-1} according to manufacturer. A 50 mL sample of H_2O_2/sunlight treated wastewater was used to irrigate each lettuce plant daily according to a protocol (Bichai et al. 2012) that has been adapted for the present research work. The first two weeks drip irrigation was simulated in order to assess the transfer of bacteria mainly on the top soil. During the subsequent weeks, sprinkle irrigation (50 mL) was simulated by using a Pasteur pipet to carefully cover the entire surface of the lettuce in order to study the transfer of bacteria, especially on lettuce leaves. These two irrigation systems were chosen because they are the most applied methods in many geographical areas.
The following protocol was arranged for each irrigation assay: (i) at the end of each H_2O_2/sunlight experiment, treated wastewater samples (8.5 L) were collected (R1 and R2, respectively) and used for lettuce irrigation daily (the not-used water was stored at 4 °C for five days); (ii) one pot was irrigated with free-bacteria mineral (potable) water as negative

control; (iii) another pot was irrigated with mineral water spiked with AR *E. coli* and AR *E. faecalis* (10^5 CFU mL^{-1} approximately) as positive control. The microbial quality of mineral water and treated wastewater (R1 and R2) in the stored samples was monitored, as described before, to evaluate whether AR *E. coli* and AR *E. faecalis* were absent or present. Particularly, bacterial presence/absence was monitored during the storage period and the corresponding count was performed at 24 and 48 h using the same bacterial monitoring protocol; no regrowth was observed in R1, whist in R2 residual bacteria were still present.
Irrigation assays were carried out for five weeks (June 30 to August 1, 2014). Each Friday lettuce leaves and top soil samples were collected using alcohol sanitised gloves and disinfected spoons and tweezers. For each test-crop, the inner and outer lettuce leaves were aseptically cut in small pieces (5 g each); each 5 g sample was mixed with 45 mL of saline solution in a sterile Stomacher bag and homogenised in a Stomacher 400 (Seward, UK) set at 260 rpm for five min. At the same time, 10 g of top soil (7 cm depth) around each plant were aseptically collected in a sterile plastic container and mixed with 90 mL of saline solution in a sterile polypropylene specimen container and homogenised by shaking at 150 rpm for 10 min (Oliveira et 2012). Triplicate samples were taken from the same pot.

6.3.7 Antibiotic resistant bacteria detection

A standard plate counting method was used through 10-fold serial dilutions in PBS after an incubation period at 37 °C (for 24 h for AR *E. coli* and for 48 h for AR *E. faecalis*). Volumes of 20 µL were spread onto Endo agar and Slanetz & Bartley agar (Fluka, Sigma-Aldrich) for AR *E. coli* and AR *E. faecalis* count, respectively. When very low density of bacteria was expected, 200 or 500 µL samples were spread onto ChromoCult® Coliform agar and Slanetz & Bartley agar for AR *E. coli* and AR *E. faecalis*, respectively. The DL of this experimental method was 2 CFU mL^{-1}. In the bacterial count of soil samples, moisture content of soil was determined by drying 3 g of soil at 105±1 °C for 24 h in a drying oven and weighing the residual. CFUs in the soil were calculated per dry weight (dw).

6.3.8 Analytic determination

The dose of hydrogen peroxide used in solar disinfection/oxidation experiments was set at 20 mg L^{-1} on the base of results that have been achieved previously (see chapter 4). H_2O_2 concentration was measured with a colorimetric method based on the use of titanium (IV) oxysulfate (Riedel-de Haën, Germany) which forms a stable yellow complex with H_2O_2 detected by means of absorbance measurements at 410 nm. Absorbance was measured through a spectrophotometer (PG Instruments Ltd. T-60-U) and linearly correlated with H_2O_2 concentration in the range 0.1−100.0 mg L^{-1}. Catalase was added to wastewater samples in order to eliminate residual hydrogen peroxide; particularly, 1 mL sample was mixed with 20 μL of 2,300 U mg^{-1} bovine liver catalase at 0.1 g L^{-1} (Sigma-Aldrich). It has been demonstrated that catalase and H_2O_2 at these concentrations have no detrimental effects on bacterial viability (García-Fernández et al. 2012).

The concentrations of CBZ, FLU and TBZ in wastewater samples, before and after treatment by H_2O_2/sunlight tests, were measured through ultraperformance liquid chromatography (UPLC, Agilent Technologies, series 1200) with a UV-DAD detector and a C-18 analytical column. The initial conditions were 95% water with 25 mM formic acid (A) and 5% ACN (B). A linear gradient progressed from 10% to 85% B in 13 min. Re-equilibration time was three min with a flow rate of 1 mL min^{-1}. The injection volume was 100 μL. A 10 mL sample was filtered through a 0.2 μm syringe-driven filter, and the filter was washed with 1 mL of ACN mixed with the filtered water sample (to remove any adsorbed compounds). Detection wavelengths were 286 nm for CBZ, 248 nm for FLU and 300 nm for TBZ.

Extraction of CECs in both lettuce leaves and top soil was performed by the application of the quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction method (Lehotay et al. 2005); only one extraction was applied since the precision of the method was within acceptable limits (<20%). Briefly, a representative aliquot of previously homogenised sample (10 g of lettuce and 2 g of soil) was weighed in a 50 mL PTFE centrifuge tube. Then 10 mL of ACN (and 10 mL of Milli-Q water in the case of soil samples) were added together with 6 g of $MgSO_4$ and 1.5 g of NaOAc. The tube was vigorously shaken for one min to prevent coagulation of $MgSO_4$ and then centrifuged (3,500 rpm for five min). A 5 mL aliquot of the supernatant was transferred to a 15 mL

centrifuge tube containing 125 mg of primary-secondary amine (PSA), 150 mg of C18 and 750 mg of $MgSO_4$. Then the tube was shaken for 30 s in a Vortex and centrifuged again (at 3,500 rpm for 5 min). A 4 mL sample of the extract was filtered through a 0.45 μm PTFE filter (Millex FG, Millipore, Milford, MA, USA) and transferred onto a vial in which 50 μL of ACN with 1% of formic acid was added. Finally, 100 μL of the extract was evaporated under gentle nitrogen stream and reconstituted with 100 μL of ACN:H_2O (10:90) prior to the LC−MS injection. Recoveries that have been obtained under these conditions were 109% CBZ, 72% FLU, 78% TBZ in lettuce, and 77% CBZ, 74% FLU, 70% TBZ in soil.

A hybrid triple quadrupole-linear ion trap-mass spectrometer (QqQLIT-MS) (5500 QTRAP LC/MS/MS system, AB Sciex Instruments, Foster city, CA) was used for the analysis of the target compounds. Chromatographic separation was carried out using an HPLC system (Agilent series 1200) provided with a Zorbax Eclipse Plus C18 column (150 mm×4.6 mm, 5 μm; Agilent Technologies). The mobile phases were ACN (A) and 0.1% formic acid in water (B) with the following gradient: 10% (A) for two min, increased to 100% in 40 min, kept constant for 10 min, and reduced to 10% in 0.1 min. The injection volume was 5 μL, and the flow rate was 0.4 mL min^{-1}.

The HPLC system was connected to a QqQLIT-MS/MS with an electrospray interface operated in positive ionization mode. The operating source settings were ionspray voltage: 5500 V; source temperature: 500 °C; curtain gas: 20 (arbitrary units); GS1: 50 psi; and GS2: 40 psi. Nitrogen was used as nebulizer gas, curtain gas, and collision gas. The MS was operated in SRM mode with a resolution set to low and unit for Q1 and Q3, respectively. The SRM transitions and MS parameters were optimised for maximum response. AB SCIEX Analyst software 1.5.1 was used for data acquisition and processing. LODs/LOQs obtained with the proposed method were as follows: 0.01/0.05 ng g^{-1} for CBZ, 0.02/0.05 ng g^{-1} for FLU and 0.005/0.005 ng g^{-1} for TBZ in lettuce, and 0.18/0.18 ng g^{-1} for CBZ, 1.3/2.0 ng g^{-1} for FLU and 0.02/0.04 ng g^{-1} for TBZ in soil.

6.4 RESULTS

6.4.1 Antibiotic resistant bacteria inactivation

The inactivation of bacterial strains selected as antibiotic resistant is depicted in Figure 6.2. Particularly, the average values and relative standard deviations of experiments that have been run within five weeks are plotted.
AR *E. coli* required an average Q_{UV} of 6.3 kJ L^{-1} and 120 min of solar exposure to achieve the DL, whilst AR *E. faecalis* was found to be more resistant to treatment as the DL was reached at higher Q_{UV}, namely 14.9 kJ L^{-1}, and treatment time (240 min).

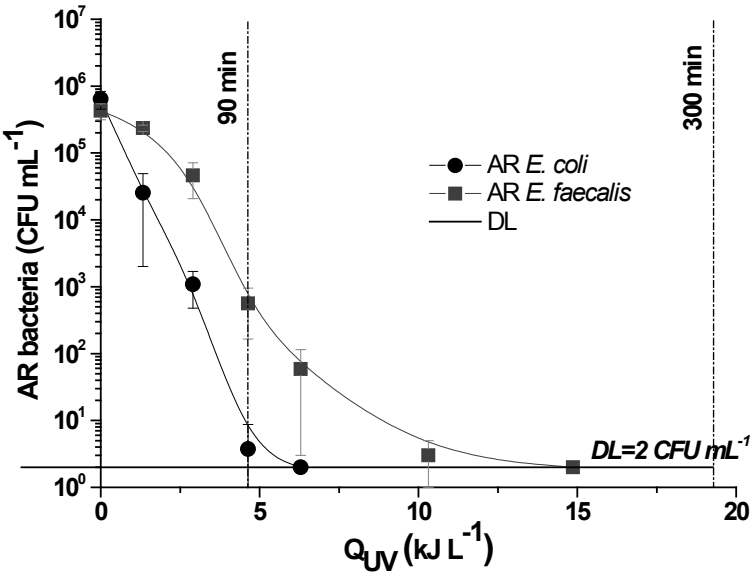

Figure 6.2 AR bacteria inactivation by H_2O_2/sunlight process

H_2O_2 concentration was measured within treatment; at 90 min it was 17 mg L^{-1} (as average value) and at 300 min it was 14 mg L^{-1} (as average value). In each experiment water temperature was also monitored: it increased from 16.5 °C to 39.3 °C.

6.4.2 Degradation of CECs

The average degradation of target CECs by H_2O_2/sunlight process is plotted in Figure 6.3. As it is shown, the average degradation percentages for CBZ were 12.3% after 90 min (Q_{UV}=4.6 kJ L^{-1}) and 36.9% after 300 min (Q_{UV}=19.3 kJ L^{-1}). This means that the average final concentrations in treated wastewater were 84.8 μg L^{-1} after 90 min and 61.0 μg L^{-1} after 300 min, respectively. A higher average degradation was observed for TBZ: 49.5% after 90 min and 68.2% after 300 min. This means that the average final concentrations in treated wastewater were 48.0 μg L^{-1} after 90 min and 30.2 μg L^{-1} after 300 min, respectively. The best performances were obtained for FLU removal that were as high as 94.0% after 90 min and 99.9% after 120 min (Q_{UV}=6.3 kJ L^{-1}). This means that the average final concentrations in treated wastewater were 5.6 μg L^{-1} after 90 min and below the DL after 300 min, respectively.

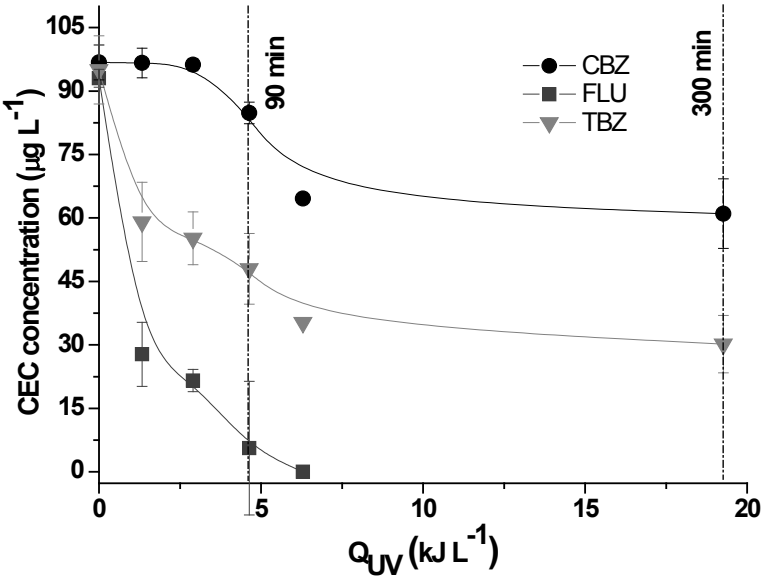

Figure 6.3 CECs degradation by H_2O_2/sunlight process

Solar experiments (without the addition of hydrogen peroxide) were also performed for each CEC separately: a no-significant degradation of each compound was observed (data not shown). When 20 mg L^{-1} of H_2O_2 were added, an enhancement of photodegradation of CECs was obtained.

The detection and measurement of metabolites and transformation byproducts of the target CECs were not investigated in this study because the main aim was to evaluate their fate when, still present in the treated water/wastewater, may be up-taken by crops.

6.4.3 Antibiotic resistant bacteria transfer to lettuce

The adequate implementation of the protocol that has been developed in this study in order to evaluate the microbial contamination of crops when irrigated with disinfected wastewater, was confirmed by the absence of AR bacteria in all negative control samples, as shown in Table 6.2.

Table 6.2 Antibiotic resistant bacteria absence/presence on lettuce leaves and top soil irrigated with UWTP effluents treated by H_2O_2/sunlight process

Irrigation week	Irrigation water		AR *E. coli*		AR *E. faecalis*	
			Lettuce leaves*	Top soil**	Lettuce leaves*	Top soil**
1st	R1	1	–	–	–	–
		2	–	–	–	–
		3	–	–	–	–
	R2	1	–	–	–	–
		2	–	–	–	–
		3	–	–	–	–
	NC	1	–	–	–	–
		2	–	–	–	–
		3	–	–	–	–
	PC	1	–	1.7×10^3	–	9.7×10^3
		2	–	1.7×10^3	–	5.6×10^3
		3	–	1.7×10^3	1.2×10^3	7.9×10^3
2nd	R1	1	–	–	–	–
		2	–	–	–	–
		3	–	–	–	–
	R2	1	–	–	–	–
		2	–	–	–	–
		3	–	–	–	–
	NC	1	–	–	–	–
		2	–	–	–	–
		3	–	–	–	–
	PC	1	–	2.2×10^2	1.7×10^3	7.4×10^2
		2	–	1.3×10^2	5.0×10^4	2.5×10^3

Irrigation week	Irrigation water		AR *E. coli*		AR *E. faecalis*	
			Lettuce leaves*	Top soil**	Lettuce leaves*	Top soil**
		3	–	3.4×10^{2}	1.0×10^{4}	1.1×10^{3}
		1	–	–	–	–
	R1	2	–	–	–	–
		3	–	–	–	–
		1	–	–	–	–
	R2	2	–	–	–	–
3rd		3	–	–	–	–
		1	–	–	–	–
	NC	2	–	–	–	–
		3	–	–	–	–
		1	2.5×10^{4}	5.4×10^{4}	1.7×10^{6}	4.5×10^{3}
	PC	2	6.3×10^{4}	8.4×10^{4}	6.3×10^{4}	4.5×10^{3}
		3	3.5×10^{4}	5.0×10^{4}	2.5×10^{6}	7.3×10^{3}
		1	–	–	–	–
	R1	2	–	–	–	–
		3	–	–	–	–
		1	–	–	–	–
	R2	2	–	–	–	–
4th		3	–	–	–	–
		1	–	–	–	–
	NC	2	–	–	–	–
		3	–	–	–	–
		1	4.3×10^{4}	1.3×10^{4}	3.1×10^{4}	1.9×10^{3}
	PC	2	8.5×10^{5}	2.3×10^{5}	6.8×10^{5}	2.0×10^{4}
		3	2.7×10^{5}	9.5×10^{2}	4.0×10^{5}	1.0×10^{4}
		1	–	–	–	–
	R1	2	–	–	–	–
		3	–	–	–	–
		1	–	2.5×10^{1}	6.4×10^{3}	–
	R2	2	–	–	–	–
5th		3	–	–	–	–
		1	–	–	–	–
	NC	2	–	–	–	–
		3	–	–	–	–
		1	1.1×10^{7}	2.0×10^{4}	9.5×10^{5}	4.5×10^{3}
	PC	2	4.7×10^{6}	1.3×10^{6}	4.7×10^{5}	2.5×10^{4}
		3	4.5×10^{6}	7.2×10^{4}	1.9×10^{4}	1.1×10^{4}

- indicates the absence of AR *E. coli* or AR *E. faecalis* in lettuce leaves samples or top soil samples;

R1 indicates UWTP effluent disinfected by H_2O_2/sunlight process for 300 min;
R2 indicates UWTP effluent disinfected by H_2O_2/sunlight process for 90 min;
Negative control (NC) refers to mineral (potable) water, while positive control (PC) refers to mineral (potable) water where both AR strains were inoculated (≈10^5 CFU mL^{-1}).
* in lettuce leaves samples AR strains are expressed as CFU $100mL^{-1}$;
** in top soil samples AR strains are expressed as CFU $100g^{-1}$ per dry weight.

During the first two weeks, when a drip irrigation system was simulated, positive control did not result in any growth of AR *E. coli* in lettuce leaves samples. By contrast, positive control was detected as positive for AR *E. faecalis*. Moreover, the positive control was detected as positive for both strains in top soil samples. When the irrigation system was modified with sprinkling, all positive control samples for both strains were detected as positive in lettuce leaves and in top soil samples.
Through all irrigation tests using treated wastewater by H_2O_2/sunlight process for 300 min in which the final concentrations of AR bacteria were below DL, all lettuce leaves and top soil samples were detected as negative for the presence of both AR *E. coli* and AR *E. faecalis*. No regrowth (24 h and 48 h post treatment) was observed in treated wastewater that have been stored at 4 °C and used for crop irrigation daily.
Wastewater that have been treated within 90 min were characterised by average final concentrations lower than DL for AR *E. coli* (expect in one assay) and 5.6×10^2 CFU mL^{-1} for AR *E. faecalis*. These values remained mostly stable within storage at 4 °C until the irrigation experiments. After irrigation tests, 14 out of 15 lettuce and top soil samples were detected as negative for both AR bacteria. In the fifth irrigation week, one lettuce sample was detected as positive for AR *E. faecalis* (6.4×10^1 CFU mL^{-1}) and one top soil sample was detective as positive for AR *E. coli* (0.25×10^0 CFU g_{dw}^{-1}).

6.4.4 CECs uptake by lettuce and accumulation on soil

The results of irrigation tests regarding CECs uptake by lettuce leaves and accumulation/deposition on top soil are reported in Table 6.3 and in Table 6.4 respectively.

Table 6.3 CBZ and TBZ uptake by lettuce leaves

Irrigation week	Carbamazepine (ng g^{-1})		Thiabendazole (ng g^{-1})	
	R1	R2	R1	R2
I	7.23	17.56	0.00	3.38
III	11.16	71.30	1.01	2.05
V	47.96	109.35	10.83	18.46

R1 refers to treated wastewater within 300 min of H_2O_2/sunlight process and then used as irrigation water;
R2 refers to treated wastewater within 90 min of H_2O_2/sunlight process and then used as irrigation water.

FLU was detected neither in lettuce leaves nor in top soil samples. This result was expected because of high degradation of FLU whose average residual concentration was 5.5 μg L^{-1} after 90 min of H_2O_2/sunlight treatment. On the contrary, an accumulation/deposition/uptake of CBZ and TBZ was observed in both investigated matrices. In particular, after five weeks of irrigation with 300 min treated wastewater (R1), 48.0 ng g^{-1} and 374.0 ng g^{-1} of CBZ were detected in lettuce leaves and top soil samples, respectively. These concentrations were higher (109.3 ng g^{-1} and 472.4 ng g^{-1}, respectively) when 90 min treated wastewater (R2) have been used for irrigation. This is due to the higher concentration of CBZ (84.8 μg L^{-1} as average value) in treated wastewater.

Table 6.4 CBZ and TBZ accumulation/deposition on top soil

Irrigation week	Carbamazepine (ng g^{-1})		Thiabendazole (ng g^{-1})	
	R1	R2	R1	R2
I	104.84	303.09	24.21	106.15
II	222.85	488.91	62.11	239.03
III	252.81	469.10	62.11	191.45
IV	260.30	525.59	95.17	294.24
V	374.01	472.44	120.82	256.36

R1 refers to treated wastewater within 300 min of H_2O_2/sunlight process and then used as irrigation water;
R2 refers to treated wastewater within 90 min of H_2O_2/sunlight process and then used as irrigation water.

After five weeks of irrigation with 300 min treated wastewater (R1), 10.8 ng g^{-1} and 120.8 ng g^{-1} of TBZ were detected in lettuce leaves and top soil samples, respectively. Higher values (18.5 ng g^{-1} in lettuce leaves and 256.4 ng g^{-1} in top soil samples) were measured when 90 min treated wastewater (R2) have been used.

6.5 DISCUSSION

H_2O_2/sunlight process was selected among solar driven AOPs according to results that have been obtained in previous experiments and discussed in chapter 4. In particular this process was chosen on the base of its disinfection performances.

H_2O_2 dose was set as low as 20 mg L^{-1} on the base of results that have been achieved in previous experiments as well as to avoid any phytotoxic effect on irrigated crops. In fact in hydroponic cultures a phytotoxic effect was found at 50 mg L^{-1} of hydrogen peroxide (Sichel et al. 2009). In this light hydrogen peroxide concentration was monitored and was observed to decrease because of chemical reactions with organic matter naturally present in wastewater as well as self-decomposition of H_2O_2.

Water temperature was also monitored during each test in order to exclude any thermal effect on bacterial inactivation. The maximum average value was recorded at 39.3 °C as plotted in Figure 6.2. As the synergistic effect of UV radiation and temperature on bacterial inactivation rises at water temperature higher than 45 °C (García-Fernández et al. 2012), this effect can be excluded in the present study.

As H_2O_2/sunlight was mostly chosen as disinfection process, good performances in terms of bacterial inactivation were expected because of the synergistic effect of hydrogen peroxide and solar radiation. The mechanisms of action are due to (i) the direct oxidation over bacterial cells that makes them more sensitive to solar radiation (Spuhler et al. 2010); (ii) the reaction of hydrogen peroxide with free iron inside bacterial cells that leads to the formation of internal HO•, subsequent internal cell injuries and cell inactivation (Feuerstein et al. 2006, Polo-López et al. 2011, Sichel et al. 2009). The enhancements due to the addition in water of low doses of H_2O_2 (<50 mg L^{-1}) to natural sunlight have been reported for several microorganisms (Agulló-Barceló et al. 2013, García-Fernández et al. 2012, Rodríguez-Chueca et al. 2014).

The results in terms of bacterial inactivation that have been obtained in the present work are in agreement with those reported in scientific literature. In a study by Rodríguez-Chueca and co-workers dealing with the treatment of real UWTP effluents by H_2O_2/sunlight process at pilot scale, in the presence of 20 mg L^{-1} of H_2O_2 (pH 5.0), it was observed that the cumulative energy per unit of volume required to achieve the DL was higher for *E. faecalis* (Q_{UV}=25.9 kJ L^{-1}) than for *E. coli* (Q_{UV}=12.0 kJ L^{-1}) (Rodríguez-Chueca et al. 2014). As reported in the above mentioned

study, in the present work AR *E. faecalis* were also more resistant to treatment than AR *E. coli*: in particular, within 90 min of solar exposure, the average residual bacterial density was 3.8×10^{0} CFU mL^{-1} for AR *E. coli* and 5.6×10^{2} CFU mL^{-1} for AR . *faecalis*, respectively.

The obtained bacterial inactivation results are also in agreement with those achieved by Agulló-Barceló et al. who investigated the effect of H_2O_2/sunlight process on indigenous *E. coli* (initial bacterial density as high as 10^5 CFU mL^{-1}) at the same dose of hydrogen peroxide. In the above quoted study, the DL was got within 180 min of solar exposure, although the shape of inactivation curve is quite different (Agulló-Barceló et al. 2013).

As previously highlighted, H_2O_2/sunlight was chosen mostly as disinfection process. In literature several works deal with the degradation of CECs by AOPs, such as electrochemical oxidation, ozonation, photo-Fenton, ultrasound, etc (Miralles-Cuevas et al. 2014). H_2O_2/sunlight process has been investigated for CECs removal by means of solar simulator systems and UV lamps. This should be the first attempt to apply a solar driven UV/H_2O_2 to degrade/remove micropollutants. De La Cruz et al. investigated the oxidation of a wide range of micropollutants using UV-C, UV-C+H_2O_2, UV-C+H_2O_2+Fe^{3+} in a pilot scale (37 L) treatment plant equipped with five low pressure mercury lamps (De La Cruz et al. 2013). An average degradation of 92.0% and 97.0% was found for CBZ (initial concentration of 332.7 ng L^{-1}) after an UV-C+H_2O_2 treatment with 20 and 30 mg L^{-1} of hydrogen peroxide, respectively. Wavelength ultraviolet (254 nm) photolysis resulted in a removal percentage of 55% for CBZ (De La Cruz et al. 2013).

UV-C and UV-C/H_2O_2 (initial concentrations of hydrogen peroxide as low as 1.5 and 3.0 mg L^{-1}) were also investigated at pilot scale for the removal of 16 CECs, among which CBZ, from a secondary UWTP effluent and ranging from 1.0 to 1,000.0 ng L^{-1}) (Merel et al. 2015). The removal rate ranged from 10% to >90% on the base of the target micropollutant. CBZ was poorly degraded by photolysis (<30% with all investigated UV doses: 0.4, 0.9 and 1.3 J cm^{-2}); it was found that the addition of 3.0 mg L^{-1} of hydrogen peroxide increased the removal up to 30-70% with medium (0.5-1.0 J cm^{-2}) and high (1.0 J cm^{-2}) UV fluencies (Merel et al. 2015).

In another study aimed at evaluating the effect of UV-C photolysis on the degradation of CECs, among which CBZ, in Milli-Q water, an enhancement of the oxidation rate was observed when 25 mg L^{-1} of

H_2O_2 have been added (Shu et al. 2013). It is important to underline that in the above mentioned work, the initial concentrations of the target contaminants were not realistic, being as high as 10-20 mg L^{-1}, and light was provided by a medium pressure lamp which emitted in the 200-300 nm range. Moreover, 0.68 J cm^{-2} and 2.25 J cm^{-2} were required to obtain a removal of 50% and 90% for CBZ, respectively, in the presence of 25 mg L^{-1} of H_2O_2 (Shu et al. 2013).

Another comparison can be made with a work dealing with the simultaneous inactivation of *E. coli*, whose initial density was 10^3 CFU mL^{-1}, and removal of four pharmaceuticals (20 μg L^{-1}) from synthetic UWTP effluents (Pablos et al. 2013). A complete bacterial inactivation was achieved within 15 min of UV-C treatment, but bacterial reproduction was affected only when a dose as high as 100 mg L^{-1} of H_2O_2 has been added (Pablos et al. 2013). On the base of kinetic constants, the authors have observed that the photocatalytic degradation of CECs was really slow compared with bacterial inactivation, also when hydrogen peroxide has been added. Pharmaceuticals were completely removed by UV-A/TiO_2 process, although a longer irradiation time was required to inactivate bacteria (Pablos et al. 2013).

In the present study a key aspect is the initial concentration of target CECs that have been investigated. It is known that, in this kind of experiments, a compromise between realistic conditions and experimental needs should be found. Accordingly the initial concentration was set as low as possible in order to make the investigated conditions as close as possible to real ones, but sufficiently high to evaluate their photocatalytic degradation as well as to detect their subsequent deposition on soil and uptake by crops. Moreover, H_2O_2/sunlight process did not result in a complete degradation of CBZ and TBZ. This allowed to investigate the fate of residual micropollutants in treated wastewater that have been reused for lettuce irrigation. The final concentrations of CBZ and TBZ in treated wastewater can be compared with those present in final UWTP effluents treated by conventional processes that are not designed to remove such emerging contaminants. In this way the simulated irrigation scenario can be regarded as realistic.

Irrigation tests have shown that the sprinkling system is more likely to transfer AR bacteria from water to lettuce leaves than the drip system. This is in agreement with a previous study dealing with the evaluation of

transfer of *E. coli* O157:H7 (10^7 CFU g_{dw}^{-1} approximately) from soil to lettuce leaves by means of both irrigation systems (Oliveira et al. 2012).
The microbial contamination of lettuce crops by *E. coli* after irrigation with wastewater that have been treated by H_2O_2/sunlight process was also investigated by Bichai et al. In this case solar experiments were carried out in 20 L CPCs photo-reactors evaluating two different doses of oxidant, namely 5 and 10 mg L^{-1} (Bichai et al. 2012). Differently from this work, lettuce was irrigated at the end of each disinfection process and microbial contamination was assessed after 24 h irrigation in terms of presence/absence of *E. coli*. No *E. coli* were detected on lettuce leaves that have been irrigated with treated wastewater (Bichai et al. 2012).
Another contribution focusing on the reuse of treated UWTP effluents for tomato crops irrigation by means of a drip system pointed out the absence of microbial contamination with respect to Total Coliforms, faecal Coliforms, *E. coli*, *Listeria* and *Salmonella* (Christou et al. 2014). In particular, tomato fruit flesh, peel and homogenate were examined after a period of irrigation of 150 days with two tertiary (namely MBR/UV radiation and slow sand filtration/chlorination, respectively) UWTP effluents and no *E. coli* were detected (Christou et al. 2014).
The results obtained in the present work also showed that residual chemical micropollutants that have not been removed by advanced/oxidation treatment may be up-taken by crops and accumulated on soil. In the literature it has been reported that CBZ can be regarded as a slow-mobile compound in organic matter-rich soil layers because it was observed to be retarded in the 0-5 cm soil samples rich in organic matter (Chefetz et al. 2008). This moderate mobility in soil may explain the high accumulation/deposition of CBZ on top soil that was observed in the present work. In fact the peat that has been used had a high content in organic matter (90% before being autoclaved).
In another study CBZ was detected in cucumber plants at a concentration of 39.1 ng g^{-1} in leaves, 4.5 ng g^{-1} in roots and 1.9 ng g^{-1} in stems (Shenker et al. 2011). In this case the cucumber plants were grown in sandy soil and then irrigated with reclaimed wastewater that have been spiked with 1.0 μg L^{-1} of CBZ (Shenker et al. 2011).
Finally, in a recent study, CBZ was measured in apple tree leaves and in alfalfa at concentrations as low as 0.043 ng g^{-1} and 0.024 ng g^{-1} respectively, after an irrigation with water containing 0.13 μg L^{-1} of CBZ (Calderón-Preciado et al. 2011).

The presence of CECs in different crops, such as carrot, green bean and lettuce that have been irrigated with groundwater and reclaimed water (namely secondary UWTP treated effluent, UWTP effluent disinfected by chlorine and UWTP disinfected by photocatalysis-UV radiation) was assessed by Calderón-Preciado et al. that carried out greenhouse experiments for three months (Calderón-Preciado et al. 2013). CECs concentrations in crops were observed to range from less than the DL to 571 ng g^{-1}; the highest values were measured in lettuce that has been irrigated with secondary UWTP effluents with no additional treatment (Calderón-Preciado et al. 2013).

Drip and sprinkling irrigation systems were simulated in the present study because (i) typically used worldwide and (ii) also in order to depict a scenario which might be as close as possible to the real one. Anyway this choice resulted in the impossibility of identification of the main route by which CECs were introduced to lettuce plants. On the base of high concentrations of CECs that have been detected both in lettuce leaves and in top soil samples, neither mechanism, such as accumulation/deposition/transfer or uptake, may be excluded. CBZ is a non-ionic organic molecule that has a high potential to be up-taken by roots and accumulated on leaves because of the water flow (Malchi et al. 2014). In the light of this property, a synergic effect of uptake by roots and deposition on leaves may explain the high concentrations of CBZ that have been measured on lettuce samples.

Although the extent of accumulation/deposition/transfer/uptake of CECs is due to many factors, such as (i) the chemical and physical properties of each pollutant, (ii) the physiological properties of crops and (iii) the characteristics of soil (Malchi et al. 2014), consequences should be taken into account in terms of human health risk and/or toxicological impact. In fact the chemical risk due to the uptake of pharmaceuticals in edible portions of crops may not be neglected on the base of the allergenic potential and long-term effect of some CECs (Fatta-Kassinos et al. 2011). The possible toxic effects of four anticancer drugs on lettuce have been investigated by Lutterbeck and co-workers, but no significant effect was observed on the seed germination index (Lutterbeck et al. 2015).

6.6 CONCLUSIONS

H_2O_2/sunlight process was chosen as possible advanced treatment in the light of wastewater reuse for crops irrigation. On the base of the developed protocol and the obtained results, when the final bacterial density was below the DL in the treated wastewater, no microbial contamination of pathogens, such as AR *E. coli* and AR *E. faecalis*, was observed in lettuce leaves and top soil samples. When H_2O_2/sunlight process resulted in a residual bacterial density, such as 5.6×10^2 CFU mL^{-1} for AR *E. faecalis* on average, the complete absence of microbial contamination in both lettuce leaves and top soil samples could not be guaranteed.

The analytic method that has been applied, namely QuEChERS extraction followed by LC-QqLIT-MS/MS analysis, was a useful tool to extract, detect and quantify CECs at ng g^{-1} levels both in lettuce leaves and top soil samples. CBZ and TBZ were found to be partially degraded (36.9% and 68.2%, respectively), while FLU was found to be totally removed (99.9%), after 300 min of solar exposure with the addition of 20 mg L^{-1} of H_2O_2. Consequently, this led to the following order of chemical contamination in lettuce and soil samples: FLU<<TBZ<<CBZ. Therefore, on the base of the obtained results, H_2O_2/sunlight process may be used as advanced treatment of urban wastewater in small communities but it must be properly designed and applied to effectively inactivate pathogens, namely AR bacteria, as well as to maximise CECs degradation in order to reduce their subsequent accumulation/deposition/transfer/uptake on crops irrigated with the treated wastewater.

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6.8 APPENDIX

ENVIRONMENTAL Science & Technology

Article

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Cross-Contamination of Residual Emerging Contaminants and Antibiotic Resistant Bacteria in Lettuce Crops and Soil Irrigated with Wastewater Treated by Sunlight/H_2O_2

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Supporting Information

ABSTRACT: The sunlight/H_2O_2 process has recently been considered as a sustainable alternative option compared to other solar driven advanced oxidation processes (AOPs) in advanced treatment of municipal wastewater (WW) to be reused for crop irrigation. Accordingly, in this study sunlight/H_2O_2 was used as disinfection/oxidation treatment for urban WW treatment plant effluent in a compound parabolic collector photoreactor to assess subsequent cross-contamination of lettuce and soil by contaminants of emerging concern (CECs) (determined by QuEChERS extraction and LC-QqLIT-MS/MS analysis) and antibiotic resistant (AR) bacteria after irrigation with treated WW. Three CECs (carbamazepine (CBZ), flumequine (FLU), and thiabendazole (TBZ) at 100 $\mu g\ L^{-1}$) and two AR bacterial strains (*E. coli* and *E. faecalis*, at 10^5 CFU mL^{-1}) were spiked in real WW. A detection limit (DL) of 2 CFU mL^{-1} was reached after 120 min of solar exposure for AR E. *coli*, while AR *E. faecalis* was more resistant to the disinfection process (240 min to reach DL). CBZ and TBZ were poorly removed after 90 min (12% and 50%, respectively) compared to FLU (94%). Lettuce was irrigated with treated WW for 5 weeks. CBZ and TBZ were accumulated in soil up to 472 ng g^{-1} and 256 ng g^{-1} and up-taken by lettuce up to 109 and 18 ng g^{-1}, respectively, when 90 min treated WW was used for irrigation; whereas no bacteria contamination was observed when the bacterial density in treated WW was below the DL. A proper treatment time (>90 min) should be guaranteed in order to avoid the transfer of pathogens from disinfected WW to irrigated crops and soil.

7 CONCLUSIONS

Energy saving, resource recovery and reuse can be regarded as the greatest challenges to be faced by urban wastewater treatment in Europe and worldwide. Moreover several limits of conventional treatment processes on the removal of so-called contaminants of emerging concern (CECs), among which antibiotics, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs), call for advanced and more effective technologies. In particular disinfection processes can be possible barriers to control or minimise the diffusion of antibiotic resistance into the environment, especially in the light of wastewater reuse for agricultural purposes. In this light different advanced oxidation processes (AOPs) were compared and investigated as possible solutions. On the base of the main results that have been plotted and discussed in the previous chapters, the following conclusions may be drawn.

TiO_2 solar simulated photocatalysis was found to be effective in the inactivation of an antibiotic resistant *Enterococcus* strain selected from urban wastewater treatment plant biological effluent.
Solar driven AOPs may be effective in the inactivation of antibiotic resistant *Escherichia coli*, overcoming the drawbacks related to conventional disinfection systems. Among them, H_2O_2/TiO_2/sunlight process, TiO_2/sunlight and solar photo-Fenton are less feasible than H_2O_2/sunlight process at low hydrogen peroxide doses, namely 20 and 50 mg L^{-1} that allow to meet the guide values for H_2O_2 residual concentration in wastewater reuse for crops irrigation.

The implementation at pilot scale of such solar driven AOPs in compound parabolic collectors photo-reactors has shown the feasibility of these technologies as cost-cutting measures. If H_2O_2/sunlight process may be used as advanced treatment of urban wastewater in small communities, it must be properly designed and operated to effectively inactivate ARB as well as to maximise CECs degradation in order to reduce their subsequent accumulation/deposition/transfer/uptake on crops irrigated with the treated wastewater.

The occurrence of CECs in irrigation waters may exert chronic toxic effects also at very low concentrations. This aspect must be taken into account when a disinfection/oxidation process is identified, designed and implemented. So part of this research work was mostly focused on the evaluation of the fate of ARB and CECs after water treatment when, still present at residual concentrations in treated water, may be accumulated and transferred onto lettuce leaves and top soil samples. H_2O_2/sunlight process was partially effective in the removal of target CECs, namely carbamazepine and thiabendazole, whose residual concentrations in treated wastewater were accumulated on soil and up-taken by lettuce.

Moreover, the application of quantitative polymerase chain reaction-based methods has pointed out that the inactivation of indigenous bacteria and, among which ARB, did not result in target ARGs removal that are so spread into the environment contributing to the dissemination of antibiotic resistance patterns. UV/H_2O_2 process may not be effective to limit or minimise the *potential* spread of antibiotic resistance under realistic operative conditions, at least at the applied oxidant dose, such as 20 mg L^{-1}, and regarding the investigated ARGs, namely bla_{TEM}, *qnr*S and *tet*W. Therefore ARGs that have been released from inactivated bacteria and/or are naturally present in wastewater may contribute to antibiotic resistance diffusion/transfer into the environment (namely, aquatic ecosystems in which the treated wastewater are discharged or irrigated crops/soils in the case of wastewater reuse).

In the light of the achieved results, future perspectives may be focused on the deepest investigation aiming at assessing the effective transfer of antibiotic resistance. Experimental works should be intended to understand how ARB and ARGs can effectively spread antibiotic resistance. Finally other gaps to be filled are based on the environmental and human health risk assessment and management.

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