**DOTTORATO DI RICERCA IN INGEGNERIA CIVILE PER L'AMBIENTE ED IL TERRITORIO**
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# ANTIBIOTIC RESISTANCE IN STREAM: MONITORING, MODELING AND EFFLUENT CONTROL BY PHOTOCATALYTIC DISINFECTION

## ANTIBIOTICO RESISTENZA NEI CORSI D'ACQUA: MONITORAGGIO, MODELLAZIONE E CONTROLLO DEGLI EFFLUENTI MEDIANTE DISINFEZIONE FOTOCATALITICA

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Ai miei nonni

In finale mi confinarono in ottava corsia, non ero contento, non potevo controllare gli avversari. All'uscita della curva ero penultimo, Wells indemoniato era tre metri avanti. Penso: non avrò altre occasioni. Dodici anni di lavoro e di dolore per niente. Allora riparto, risento tutto, rientro in gara, recupero, vinco, alzo le braccia e il ditino. (Pietro Mennea, Olimpiade di Mosca 1980)

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# ABSTRACT

Since the 1940s, the ever-increasing use of antibiotics for human, veterinary and agricultural purposes, contributes to their continuous release into the environment due to incomplete metabolism or due to disposal of unused antibiotics. The concern for the release of antibiotics into the environment isrelated to the development of antibiotic resistance genes (ARGs) and bacteria (ARB), which reduce the therapeutic potential against human and animal pathogens. Urban wastewater treatment plant (UWWTP) effluents, hospital discharges, livestock farms represent today the major contamination sources of surface water from antibiotics and ARB. The consequence is that antibiotics, exerting selective pressure, may facilitate the selection of ARB or the acquisition of resistance genes by horizontal transfer.

The aim of this work was to investigate the spread of ARB in the environment, particularly in water system, as well as to minimize the related risk through the investigation of effective wastewater disinfection methods. Accordingly, experimental activity was addressed to (i) the monitoring of ARB in river, (ii) modelling ARB fate in river and (iii) minimize ARB release in river through effective wastewater disinfection.

The monitoring of ARB fate in a water body was performed by analysing samples along Tusciano river (Salerno province, Italy), upstream and downstream of UWWTPs and a Hospital discharges. The proportion of Escherichia coli and Enterococcus strains resistant to three antibiotics (ampicillin (AMP), ciprofloxacin (CPX) and tetracycline (TET)) and their mixture , was evaluated. Three monitoring campaigns in winter (C1) spring (C2) and autumn (C3) 2014 were performed in order to assess seasonal variability. A drastic increase in the proportion of E.coli strains resistant to AMP (from 28 to 49%, in C1) and to CPX (from 7 to 17% and from 3 to 20%, in C2 and C3 respectively) downstream of hospital effluent discharge was detected. A drastic increase in Enterococcus resistant to TET (from 9 to 41% in C3) downstream of hospital discharge was also observed.

The effect on ARB fate of natural attenuation (dark condition), solar radiation, sediment adsorption and multiple UWWTP effluents discharges was evaluated as well as the corresponding inactivation/adsorption kinetics in a lab scale hydraulic channel built to this end. The rate of average inactivation for E.coli and Enterococcus resistant bacteria was 2 log units after 6 hours exposure to sunlight. In the presence of the sediment, the rate of inactivation was close to 3 log units, and 1 log unit was adsorbed to the sediment. In the presence of simulated discharges an increase of the portion adsorbed to the sediment was observed. The inactivation/adsorption of target ARB was found to obey to a first order kinetic. Half-live time (t1/2) between 1.3 h and 2.26 h were observed in the water under the effect of solar radiation, while a t1/2 values between 3.71 and 7.92 h in sediments were observed.

Finally, to minimize the release of ARB in the environment alternative disinfection processes were investigated also by a comparison with conventional disinfection processes. In particular:

(i) UV-C radiation and non-conventional disinfection by $TiO_2$ photocatalysis were investigated to evaluate their effect on the transformation (mutants formation) of antibiotic resistant microorganism (Salmonella typhimurium). In UV-C experiments for the tested strain a total inactivation of 9 units log was reached after about 45 min and after 15 and 10 min with $10^8$ and $10^7$ CFU $mL^{-1}$ of initial concentration. In photocatalytic tests the complete Salmonella inactivation was achieved in 60, 30 and 15 min of irradiation as initial bacterial density was decreased respectively. In UV-C and $TiO_2$ photocatalysis tests an increase in formation of mutants was detected. The formation of mutants increased as UV dose increased up to a maximum of $1.1 \times 10^3$, $1.9 \times 10^3$ and $3.9 \times 10^3$ CFU $mL^{-1}$ respectively and the same trend was observed in the $TiO_2$ photocatalytic tests with a maximum of $9.5 \times 10^2$, $1.1 \times 10^3$ and $1.4 \times 10^3$ CFU $mL^{-1}$ mutants formation respectively.

(ii) $TiO_2$ photocatalytic process by using different light sources was evaluated on the inactivation of antibiotic resistant E. coli strains (selected from real wastewater) as well as on antibiotic resistance of colonies survived to disinfection process. The higher efficiency was

observed in the absence of $TiO_2$ when the wastewater was irradiated using 250W lamp. In the presence of $TiO_2$ a decreasing inactivation trend was observed (99,76% and 72,22% inactivation after 10 min irradiation at 0.10 and 2.00 g $TiO_2$ $L^{-1}$ respectively). Under solar simulated conditions the highest inactivation efficiency (93.17%) after 10 min of irradiation was achieved at the lower photocatalyst loading (0.05 g $TiO_2$ $L^{-1}$).

(iii) solar driven AOPs and conventional disinfection by chlorine were investigated in the inactivation of indigenous E. coli strain selected from real wastewater according to its resistance to three antibiotics (ampicillin (AMP), ciprofloxacin (CIP) and tetracycline (TET)). 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. 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 Cl2 $L^{-1}$) 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).

According to these results, the suggested strategy to control antibiotic resistance spread into the environment includes two approaches: (i) implementation of effective policies for controlling the use of antibiotics in order to avoid any misuse or unnecessary use; (ii) an effective control of antibiotic resistance contamination point sources through the application of highly effective AOPs based disinfection technologies, by selecting case by case either solar driven or artificial light based technologies.

# SOMMARIO

Dal 1940 il crescente impiego di antibiotici per uso umano, agricolo e veterinario ha contribuito ad un loro continuo rilascio nell'ambiente in quanto queste sostanze sono solo parzialmente metabolizzate dagli organismi. La preoccupazione per il rilascio di antibiotici nell'ambiente è correlata allo sviluppo di geni di resistenza agli antibiotici (ARGs) e batteri resistenti (ARB), che riducono il potenziale terapeutico contro patogeni umani ed animali. Gli effluenti degli impianti di trattamento delle acque reflue (UWWTP), degli ospedali, e degli allevamenti rappresentano oggi la principale fonte di contaminazione di ARB nelle acque superficiali. La conseguenza è che gli antibiotici, esercitando una pressione selettiva, possono facilitare la selezione di ARB o l'acquisizione di geni di resistenza

Lo scopo del presente lavoro è stato quello di studiare la diffusione ARB nell'ambiente, in particolare nei corpi idrici, così da minimizzare ogni rischio ambientale, tramite la ricerca di metodi efficaci di disinfezione delle acque reflue. Di conseguenza, l'attività sperimentale è stata indirizzata a (i) il controllo di ARB nei fiumi, (ii) il destino e la modellazione degli ARB nei fiumi e (iii) minimizzare il rilascio ARB nei copri idrici attraverso una efficace disinfezione delle acque reflue.

E' stato effettuato il monitoraggio di ARB in un corpo idrico analizzando campioni prelevati lungo il fiume Tusciano (Provincia di Salerno, Italia), a monte e valle degli scarichi di UWWTPs e di un ospedale. E' stata valutata la proporzione di ceppi di Escherichia coli ed Enterococcus resistenti a tre antibiotici (ampicillina (AMP), ciprofloxacina (CPX) e la tetraciclina (TET)) singolarmente ed insieme. Le analisi sono state condotte in tre campagne di monitoraggio ovvero in inverno (C1) estate (C2) e in autunno (C3) nel 2014 al fine di valutare la variabilità stagionale. E' stato rilevato un drastico aumento della proporzione di ceppi di E.coli resistenti ad AMP (da 28 a 49%, in C1) e CPX (da 7 a 17% e da 3 a 20%, in C2 e C3 rispettivamente) a valle dell'effluente ospedaliero. Inoltre un drastico aumento di Enterococcus

resistenti a TET (9-41% in C3) è stato riscontrato a valle dell'immissione nel fiume del refluo ospedaliero.

E' stato realizzato un canale idraulico a scala di laboratorio al fine di simulare l'effetto di attenuazione naturale degli ARB nei corpi idrici, per mezzo della radiazione solare, e dell'adsorbimento ai sedimenti fluviali, anche in presenza di scarichi di UWWTP simulati, nonché le corrispondenti cinetiche di inattivazione/adsorbimento. Il tasso di inattivazione medio di ceppi di E.coli e Enterococcus resistenti è stato di circa 2 unità log dopo 6 ore di esposizione alla luce solare. In presenza del sedimento, il tasso di inattivazione era di circa 3 unità log, mentre 1 unità log è stata adsorbita dal sedimento. In presenza di scarichi simulati è stato osservato un aumento della porzione adsorbita al sedimento. L'inattivazione/adsorbimento degli ARB erano conformi ad una cinetica di primo ordine. Il tempo di dimezzamento ($t_{1/2}$) variava tra 1,3 e 2,26 h in acqua per effetto della radiazione solare, mentre sono stati osservati $t_{1/2}$ compresi tra 3,71 e 7,92 h nei sedimenti.

Infine, per ridurre al minimo il rilascio di ARB nell'ambiente sono stati studiati processi di disinfezione alternativi e confrontati con i processi di disinfezione convenzionali. In particolare:

(i) è stato confrontato il trattamento di disinfezione con radiazione UV-C ed il trattamento non convenzionale di fotocatalisi con $TiO_2$ al fine di valutare l'effetto sulla trasformazione (formazione mutanti) di microrganismi antibiotico resistenti (Salmonella typhimurium resistente alla tetracyclina). Negli esperimenti con UV-C è stata raggiunta un'inattivazione totale di 9 unità log dopo circa 45 minuti e di 8 e 7 unità log dopo 15 e 10 minuti rispettivamente. Nei test fotocatalitici l'inattivazione Salmonella completa è stata ottenuta in 60, 30 e 15 min di irradiazione rispettivamente. Sia negli esperimenti con UV-C che nei test fotocatalitici con $TiO_2$ è stato rilevato un aumento della formazione di mutanti. La formazione di mutanti è aumentata all'aumentare del dosaggio UV fino a un massimo di $1.1 \times 10^3$, $1.9 \times 10^3$ e $3.9 \times 10^3$ CFU $mL^{-1}$ rispettivamente al diminuire della carica batterica iniziale, e la stessa tendenza è stata osservata nelle prove di fotocatalisi con un massimo di $9.5 \times 10^2$, $1.1 \times 10^3$ e $1.4 \times 10^3$ di formazione di mutanti rispettivamente.

(ii) è stato analizzato il processo fotocatalitico con $TiO_2$ utilizzando diverse sorgenti luminose, studiando l'inattivazione di ceppi di E.coli resistenti agli antibiotici (selezionati da un refluo reale) ed è stata valutata la variazione di resistenza agli antibiotici nelle colonie sopravvissute al processo di disinfezione. La maggiore efficienza è stata osservata in assenza di $TiO_2$ quando l'acqua reflua è stata irradiata con una lampada di 250W. In presenza di $TiO_2$ è stata osservata una tendenza inattivazione decrescente (99.76% e 72.22% di inattivazione dopo 10 min di irradiazione a 0.10 e 2.00 g $TiO_2$ $L^{-1}$, rispettivamente). Con la radiazione solare simulata invece la massima efficienza di inattivazione (93.17%) è stata raggiunta dopo 10 min di irradiazione con la minore concentrazione di catalizzatore (0.05 g $TiO_2$ $L^{-1}$).

(iii) sono stati esaminati processi di ossidazione avanzata (AOPs) con radiazione solare e processi di disinfezione convenzionale con cloro nell'inattivazione di ceppi di E. coli selezionati da acque reflue in base resistenti a tre antibiotici (ampicillina (AMP), ciprofloxacina (CIP) e tetraciclina (TET)). I tassi di inattivazione maggiori sono stati ottenuti con $H_2O_2/TiO_2$/radiazione solare (l'energia cumulata per unità di volume ($Q_{UV}$) era compresa nell'intervallo 3-5 KJ $L^{-1}$, al variare del rapporto di concentrazione $H_2O_2/TiO_2$) e $H_2O_2$/radiazione solare (la $Q_{UV}$ era pari a 8 KJ $L^{-1}$). Inoltre il processo $H_2O_2$/radiazione solare è stato confrontato con il processo di disinfezione convenzionale con cloro al fine di valutare il potenziale di ricrescita batterica e in particolare la percentuale di E. coli MDR rispetto al totale della popolazione di E. coli. Il processo con Clorazione (1.0 mg $Cl_2$ $L^{-1}$) è stato più efficace del $H_2O_2$/radiazione solare (50 mg $H_2O_2$ $L^{-1}$) nella completa inattivazione degli MDR E. coli (15 min Vs 90 min), ma meno efficace nel fenomeno di ricrescita batterica (24 h Vs 48 h).

In base a questi risultati, la strategia suggerita per controllare la diffusione di batteri resistenti agli antibiotici nell'ambiente comprende due approcci: (i) l'attuazione di politiche efficaci per controllare l'uso di antibiotici, per evitare qualsiasi abuso o uso non necessario; (ii) un efficace controllo delle fonti di contaminazione dei batteri resistenti agli antiobitici attraverso l'applicazione di tecnologie di disinfezione AOPs altamente efficaci, selezionando a seconda del caso processi solari guidati o tecnologie basate su luce artificiale.

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Antonio della Sala, amico leale con il quale ho condiviso gran parte delle idee e dei progetti durate questo percorso.

# ABOUT THE AUTHOR

**Antonino Fiorentino** ha conseguito la laurea specialistica in Ingegneria per l'Ambiente e il Territorio nel 2011 presso l'Università degli Studi di Salerno con una tesi dal titolo "*Trattamento avanzato di acque reflue urbane con radiazione UV e successiva fotolisi: effetto sui batteri resistenti agli antibiotici*". Da tale attività sono stati tratti due lavori pubblicati su riviste internazionali. Nello stesso anno è risultato vincitore del XIII ciclo del Dottorato di ricerca in Ingegneria Civile per l'Ambiente e il Territorio presso l'Università di Salerno. La sua attività di ricerca si è incentrata sullo studio dell'antibiotico resistenza dei microrganismi, relativamente alla presenza nei corpi idrici e alle cinetiche di inattivazione naturali. Inoltre ha studiato l'applicazione di processi di disinfezione alternativi nell'inattivazione degli ARB. Durante il dottorato è stato visiting researcher presso la *Plataforma Solar de Almeria* in Spagna e la *Univeriteè de Loreienne de Nancy* in Francia, dove ha svolto attività di ricerca sugli effetti della disinfezione sulla mutagenecità dei microrganismi e ha studiato processi di ossidazione avanzata per l'inattivazione degli ARB. E' stato membro della azione COST "*Detecting evolutionary hot spots of antibiotic resistances in Europe (DARE) TD0803"* ed è ora membro dell'azione COST *"New and emerging challenges and opportunities in wastewater reuse (NEREUS) ES1403"*. È inoltre co-autore di diverse pubblicazioni scientifiche.

**Antonino Fiorentino** graduated in Environmental Engineering in 2011 discussing a thesis entitled *"Advanced urban wastewater treatment plants with radiation UV and next photolysis: effect on antibiotic resistant bacteria"*. Two publications on international journals have been published from this research activity. He began the Ph.D. programme in the same year at the Department of Civil Engineering of University of Salerno. His research activity has been focused on the evaluation of the antibiotic resistance bacteria and modelling of their presence in water stream. During the Ph.D. he was a visiting research at the *Plataforma Solar de Almeria* in Spain and at the *Univeriteè de Loreienne de Nancy* in France, where he studied the effect of the disinfection on the mutagenicity of the antibiotic resistance bacteria and the application of the advanced oxidation process for their inactivation. He was also member of the action COST "*Detecting evolutionary hot spots of antibiotic resistances in Europe (DARE) TD0803"* and he now is a member of the *"New and emerging challenges and opportunities in wastewater reuse (NEREUS) ES1403"*. He is co-autor of several scientific papers on international journals.

# 1 INTRODUCTION

The 2014 World Health Organization (WHO) report (WHO, 2014) focused on antibiotic resistance bacteria (ARB) as one of the most 39 critical human health challenges of the next century and heralded the need for "a global strategy to contain resistance." According to the report, counted more than two 25.000 deaths per year in Europe, more than 25.000 deaths in USA.

Antibiotics are used for improving human health. Besides this fundamental application, antibiotics (antimicrobials at large) have also been used for preventing and treating animals and plants infections as well as for promoting growth in animal farming (McManus, 2002; Smith et al., 2002; Singer et al., 2003; Cabello, 2006). All these applications made antibiotics to be released in large amounts in natural ecosystems.

The main concern for the release of antibiotics into the environment is related to the development of antibiotic resistance genes (ARGs) and bacteria , which reduce the therapeutic potential against human and animal pathogens (Kemper, 2008; Zhang et al., 2009).

Effluents from wastewater treatment plants (WWTPs) are suspected to be among the main anthropogenic sources for antibiotics (no maximum contaminant levels have been set by EU and other international institutions/organizations), ARGs and ARB spread into the environment (Ferreira da Silva et al., 2006; Figueira et al., 2011; Kümmerer, 2009; Lupo et al., 2012). The biological treatment process creates an environment potentially suitable for resistance development and spread because bacteria are continuously mixed with antibiotics at sub-inhibitory concentrations (Auerbach et al., 2007; Davies et al., 2006).

The release of ARB into the environment from WWTPs effluents may be due to either: the absence of a final disinfection step or the use of conventional disinfection systems, which have been proven only partially effective in the inactivation of ARB (Munir et al. 2011, Rizzo et al. 2013).

Accordingly, a continuous release of ARB, including faecal microorganisms, has been observed in different aquatic environments, including rivers, lakes and the sea (Edge 2005; Watkinson et al., 2007; Ferreira da Silva 2007; Reinthaler et al., 2010; Tao et al., 2010).

Several authors detected the presence of ARB in the rivers, especially downstream of hospital structure or WWTP (Li et al. 2014, Zhang et al. 2014, Sidrach-Cardona et al. 2014). However, there are still gaps in knowledge about the seasonal effect relative to the consuming specific antibiotics depending on the season.

Moreover, it has not yet well understood which mechanisms significantly affect the fate of pathogens in rivers and not specific information is available about ARB. Solar radiation, temperature, salinity, pH and turbidity (Yukselen et al., 2003; Kay et al., 2005) as well as adsorption of bacteria to the sediments can affect pathogens fate in river. To better address the contribution of different mechanisms to the fate of bacteria in river, some investigators simulated the process by artificial system (Walters et al. 2014).

Finally, in order to limit the release of ARB in water bodies, new disinfection methods should be investigated to minimize the presence of ARB in WWTPs discharges. Advanced oxidation processes (AOPs) (e.g., Fenton, photo- Fenton, TiO2 photocatalysis, UV/O3, UV/H2O2 etc.) have been successfully investigated for the removal of a wide range of contaminants (Zapata et al., 2010; Rizzo, 2011; Sannino et al., 2013; Murcia et al., 2013). Although several studies on the inactivation of microorganisms by AOPs are available in scientific literature (Malato et al., 2009; Dunlop et al., 2011), only a few works addressed inactivation of ARB (Tsai et al., 2010; Dunlop et al., 2014) and still less focused on indigenous ARB (Rizzo et al., 2014). Moreover, disinfection processes, such as chlorination and UV radiation, can result in the formation of mutagens (Glaze et al., 1993; DeMarini et al., 1995, Monarca et al. 2000), but no information is available in scientific literature on the formation of mutants of ARB following AOPs treatment.

# 2 OBJECTIVE AND RATIONALITY

The aim of this work was to investigate the spread of ARB in the environment, particularly in water system, as well as to minimize the related risk through the investigation of effective wastewater disinfection methods. Accordingly, experimental activity was addressed to (i) monitor ARB fate in river, (ii) modelling ARB fate in river and (iii) minimize ARB release in river through effective wastewater disinfection (Figure 2.1).

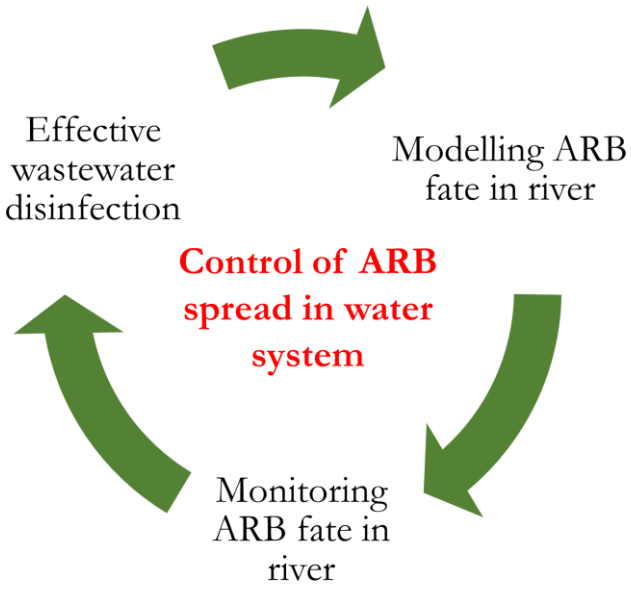


**Figure 2-1 Scheme of the objectives of the thesis**

In particular, the following specific objectives were pursued:

- Monitoring the prevalence of ARB (namely, E.coli and Enteroccus strains resistant to three antibiotics ampicillin (AMP), ciprofloxacin (CPX),and tetracycline (TET) and a mixture of these) in

different seasons (January-February, May-June, September-October 2014) and different sections of a river located in Southern Italy and affected by municipal WWTPs and, Hospital wastewater discharges (Chapter 3); ;

- Investigation and kinetic modelling of different mechanisms and conditions on the fate of indigenous E. coli and Enterococcus antibiotic resistant strains in simulated river. In particular, the effect of natural attenuation (dark condition), solar radiation, sediment adsorption and multiple UWWTP effluents discharges was evaluated through outdoor experiments by using a purposely built lab scale hydraulic channel, located outside the Laboratory of Sanitary and Environmental Engineering, at University of Salerno, Fisciano (Italy) (Chapter 4);
- Controlling the release of ARB from urban WWTPs through effective disinfection processes (Chapter 5), and particularly:
  - o By comparatively investigating the effect of conventional (UV-C radiation) and non-conventional ($TiO_2$ photocatalysis) disinfection processes on the transformation (mutants formation) of antibiotic resistant microorganism (Salmonella typhimurium) (Sub-chapter 5.1);
  - o By evaluating the effect of $TiO_2$ photocatalytic process and different light sources on the inactivation of antibiotic resistant E. coli strains (selected from real wastewater) as well as on antibiotic resistance of colonies survived to disinfection process (Sub-chapter 5.2);
  - o By investigating solar driven AOPs and conventional disinfection by chlorine in the inactivation of indigenous E. coli strain selected from real wastewater according to its resistance to three antibiotics (ampicillin (AMP), ciprofloxacin (CIP) and tetracycline (TET)). Finally, $H_2O_2$/sunlight process was compared with chlorination in the inactivation of indigenous antibiotic resistant E.coli as well as to investigate post-treatment bacterial regrowth (Sub-chapter 5.3).

# 3 MONITORING OF ARB IN WASTEWATER POLLUTED STREAM

## 3.1 ABSTRACT

The study of antibiotic resistance spread into the environment started with the monitoring of the prevalence and the investigation of spatial dynamics of antibiotic-resistant bacteria (Escherichia coli, and Enterococcus) in a river impacted by Hospital and urban wastewater treatment plant (WWTP) discharges. The resistance to three antimicrobial agents and their mixture, ampicillin (AMP), ciprofloxacin (CPX) and tetracycline (TET), was evaluated using the agar dilution method for both bacteria,. Three monitoring campaigns in winter (C1) spring (C2) and autumn (C3) were performed in order to assess the seasonal variability. A drastic increase in E.coli resistant to AMP (from 28 to 49%, in C1) and in E.coli resistant to CPX (from 7 to 17% and from 3 to 20%, in C2 and C3 respectively) downstream of hospital effluent discharge was detected. A drastic increase in Enterococcus resistant to TET (from 9 to 41% in C3) downstream of hospital dischrarge was also detected. In general after WWTPs discharges a percentage increase of the resistance to antibiotics was detected. These results clearly demonstrate that both the Hospital effluent and the WWTPs contribute to the emergence and spread of antibiotic resistance in the environment.

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The present chapter, after an adaptation in terms of formatting style, will be submitted for possible publication in a peer reviewed indexed journal.

## 3.2 INTRODUCTION

Antibiotics are widely used to contrast different types of microbial infections, in men, animals and plants (Chagas et al. 2011, Galvin et al. 2010). The ever increasing use of antibiotics into medical practice, and the abuse of these compounds in human and veterinary medicine, animal husbandry, agriculture, aquaculture and food technology have resulted in an increase in antibiotic resistance and multidrug-resistant bacteria (Baquero et al. 2008).

Large amounts of antibiotics are released into municipal wastewater due to incomplete metabolism in humans or due to disposal of unused antibiotics (Nagulapally et al., 2009). Wastewater get wastewater treatment plants (WWTPs) through the sewage and conditions favorable to the development of resistance may occur (Ferreira da Silva et al. 2006; Rizzo et al. 2013; Manaia et al., 2012). WWTPs effluents are typically released into surface waters which are ideal habitat for the acquisition and spread of antibiotic resistance, (Koczura et al., 2012).

Previous reports have shown the occurrence of antimicrobial-resistant strains of bacteria in different aquatic environments (Edge 2005; Watkinson et al., 2007; Ferreira da Silva 2007; Reinthaler et al., 2010; Tao et al., 2010). However Chagas et al. (2011) and Korzeniewska et al. (2013) claim that in existing literature there is a lack of information on the emission of bacteria and ARB from effluent of hospitals and their transfer in to the water reservoirs. Mohanta et Goel (2014) studied the prevalence of antibiotic-resistant bacteria in three different water sources: the River Hooghly in Kolkata, River Kangsabati and groundwater from Kharagpur, West Bengal over three seasons: autumn,

winter and summer. The percentages of multidrug antibiotic-resistant (MDR) bacteria the percentage of MDR was always above 10%, and was highest in winter. Wen Yang et al. (2014) compared the prevalence of ARB in two different stream, Huanghsi river which presented a natural acidic (pH 4) and Waishuanghsi river with a neutral (pH 7) water environment. In both water bodies the prevalce of carbenicillin and vancomycin resistant bacteria were dedected without substantial differences compared to the change in pH. Zhang at al. (2014) detected the prevalence of E.coli MDR in a river which was exposed to the discharge of a livestock, and high rates of resistance were found against all the eight antibiotics tested.

An comparation between the effect of discharges WWTPs and discarge of plant for the production of antibiotic in river was studied by Sidrach-Cardona et al. (2014). A sensible higher percentage of E.coli MDR was detected after point of discharge of production of antibiotic demonstrating that the presence of antibiotics in the environment can contribute to an increase of resistant. However, there are still gaps in knowledge about the extent that the presence of antibiotics may provide in the increased resistance, and on seasonal effect relative to the consuming specific antibiotics depending on the season.

In the 2013 report on antibiotic resistance and use of antibiotics in Campania, Italy (area where was carried out monitoring activities), in the 15 major public hospitals have been isolated resistant and multiresistant bacteria approximately in 40.000 patients (the region has about 6.000.000 inhabitants). Among the antibiotics used in hospitals structure, most administered were ampicillin (1.8%) and ciprofloxacin (9.7%) respectively, the consumption of these antibiotics was of 2.14% and 6.9% in Italy and 2.8% and 7.9% in Europe. Among the isolates most common belonged to the family of E.coli, about 7.000 patients (20%), also resistant Enterococcus were widespread with approximately 2.500 infected patients (6.8%).

In this study, the prevalence of antibiotic resistant E.coli and Enteroccus in different sections of a stream in Southern Italy was evaluated during different seasons (January-February, May-June, September-October 2014). Sampling points were located (i) in the upper stream section, (ii) just downstream of two municipal WWTP effluents, (iii) just

downstream of Hospital effluent and (iv) close to the estuary. E. coli and Enterococcus were plated in agar without and with addition of three antibiotics ampicillin (AMP), ciprofloxacin (CPX),and tetracycline (TET) and a mixture of these.

## 3.3 MATERIAL AND METHODS

### 3.3.1 Study Area

Tusciano river is located in Campania region, southern Italy. The source (Lat. 40°45' Long. 15°03') is between Monte Polveracchio and Monte Cervialto, in the Monti Picentini range of the southern Apennines, the mouth is in the locality Spineta in the municipality of Battipaglia (Lat. 40°37' Long. 14°55'). It passes through the three municipalites that count together about 60.000 inhabitants. Having passed several small countries crosses Battipaglia, to flow into the Tyrrhenian Sea in the Gulf of Salerno. The Tusciano has a length of 37 km, an average flow rate of 300 L $s^{-1}$ and receives effluents from three municipal wastewater treatment plants (WWTPs) and a hospital.

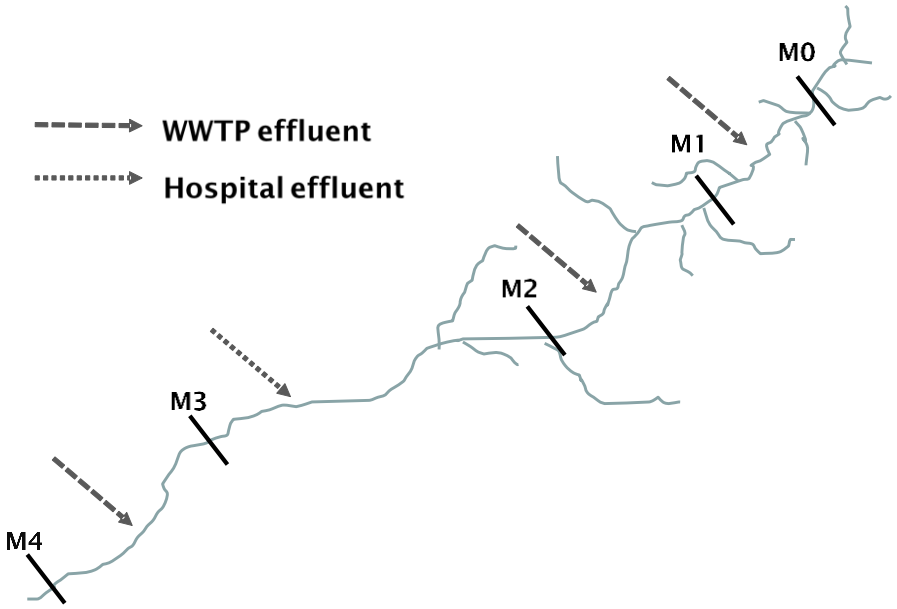


**Figure 3-1 Schematization of Tusciano river with related discharge points**

### 3.3.2 Sample collection

Water samples were taken in January-February (winter time), June-July (spring-summer time) and September-October (summer-autumn) 2014, in five different points: (i) close to the river source (M0), (ii) just downstream of two municipal WWTP effluents, (M1) and (M2), (iii) just downstream Hospital effluent (M3) and (iv) after Battipaglia municipality WWTP effluent and close to the estuary (M4). WWTPs are activated sludge plants with final disinfection process with chlorine to the service respectively of 3.000 PE (M1), 10.000 PE (M2) and 50.000 PE (M4). The Battipaglia Hospital has approximately 200 beds.
The WWTPs which discharge into Tusciano river receive mainly civil wastewater and uses chlorination disinfection as tertiary treatment.
Two replicate 500 mL water samples were collected at each site from the middle of stream in flowing water in the morning in sterile 500 mL bottles.

### 3.3.3 Enumeration of total and resistant E.coli and Enterococcus strains

Total, antibiotic resistant and multidrug-resistant E.coli and Enterococcus were determined in all water samples collected. Water sample were undiluited and undiluited in a final volume of 50 mL in sterile Falcon containing sterilized phosphate buffered saline (PBS, pH 7.4) and flitered trough a 0.45 μm membrane (Millimore; Darmsadt, Germany). Total Enterococcus were enumerated on Slanetz Bartley Agar (Biolife, Milano, IT) a selective medium for Enterococcus including tryptone (20.0 g $L^{-1}$), yeast extract (5.0 g $L^{-1}$), glucose (2.0 g $L^{-1}$), potassium phosphate dibasic (4.0 g $L^{-1}$), sodium azide (0.4 g L-1), TTC 0.1 (g $L^{-1}$), Agar (10.0 g $L^{-1}$). Total E.coli were enumerated on intryptone bile X-glucur onide (TBX) agar medium (Biolife, Milano, IT), a selective chromogenic medium for the detection and enumeration of E. coli, including tryptone (20.0 g $L^{-1}$), bile salts (1.5 g $L^{-1}$), agar (15.0 g $L^{-1}$), X-glucuronide (0.075 g $L^{-1}$).

For the enumeration of antibiotic resistant bacteria, antibiotics AMP, CIP and TET (Sigma Aldrich, St. Louis, MO, USA) were added to the culture media. Specifically, antibiotics stock solutions were prepared in sterile water (Milli-Q water system, Millipore, Billerica, MA,USA), subsequently diluted in the culture media to get a final concentration of 8 mg $L^{-1}$ for AMP, 1 mg $L^{-1}$ for CIP and 16 mg $L^{-1}$ for TET, separately and as a mixture for the detection of antibiotic resistant E. coli, and 16 mg $L^{-1}$ for AMP, 4 mg $L^{-1}$ for CIP and 16 mg $L^{-1}$ for TET, for Enterococcus. Antibiotic concentrations were chosen according to the respective MICs listed in EUCAST "The European Committee on Antimicrobial Susceptibility Testing" database (2014).

## 3.4 RESULT AND DISCUSSION

### 3.4.1 Chemical properties of stream

Chemical proprieties were measured to determine as the concentration varies from upstream to downstream, afterward the discharge of effluents. No significant temporal variations were observed. The Table below shows an average of the measured values.

**Table 3.1 Chemicals properties of the Tusciano stream**

| | M0 | M1 | M2 | M3 | M4 |
|---|---|---|---|---|---|
| **pH** | 7.47±0.2 | 7.10±0.3 | 7.21±0.4 | 7.23±0.3 | 6.95±0.5 |
| **Temperature °C** | 9.88±0.5 | 11.03±0.4 | 11.78±0.3 | 13.07±0.8 | 14.21±1.2 |
| **DO mg $L^{-1}$** | 9.35±0.2 | 9.50±0.2 | 8.60±0.5 | 8.19±0.7 | 6.84±0.8 |
| **COD mg $L^{-1}$** | <DL | 5±0.0 | 5±0.0 | 10±0.0 | 15±0.5 |
| **$BOD_5$ mg $L^{-1}$** | <DL | <DL | <DL | <DL | 5±0.0 |
| **TSS mg $L^{-1}$** | <DL | <DL | <DL | 57.0±1.5 | 59.0±2.5 |

Biochemical oxygen demand ($BOD_5$) was detected for all campaigns, only at the last sampling point, as well as to the total suspended solids (TSS), detected only in the last two sampling points. For chemical oxygen demand (COD) a slight increase from upstream to downstream was detected.

### 3.4.2 Behavior of total E. coli and Enterococcus

Total E. coli and Enterococcus in stream were determined in three monitoring campaigns: January-February (C1), May-June (C2) and September-October (C3) as shown in Fig. 3.2 and 3.3. The relationship between the number of E.coli and Enterococci does not vary significantly in the three campaigns for ever section. However the number of Enterococci was slightly lower than that of E.coli. Moreover, the number of total bacteria increased slightly from the sampling point $M_1$ to $M_3$, in the range log 3.2-3.7 CFU $mL^{-1}$ for E.coli and log 3.0-3.5 CFU $mL^{-1}$ for Enteroccoccus. An increase of bacterial count was observed in the point $M_4$, near the mouth of the river, about log 4.9 CFU $mL^{-1}$ and log 4.6 CFU $mL^{-1}$ were detected for E.coli and Enterococcus, respectively. No significant seasonal differences in the total number of fecal bacteria were found, however, a slight increase in bacterial concentration was detected in May-June campaigns. The average increase compared to the other two campaigns was only 0.2 log units. However, the bacterial load for both bacteria monitored is increased gradually from upstream to downstream, and this, as lawful wait, was closely related to discharges of WWTPs and Hospital discharge.

### 3.4.3 Prevalence of antibiotic resistant E. coli along Tusciano river

In all of the monitoring campaigns, and in all the sampling points but M0 (which is located upstream of any WWTP effluent discharge) antibiotic and multi-drug resistant E.coli resistant were found, as shown in Fig. 3.2.

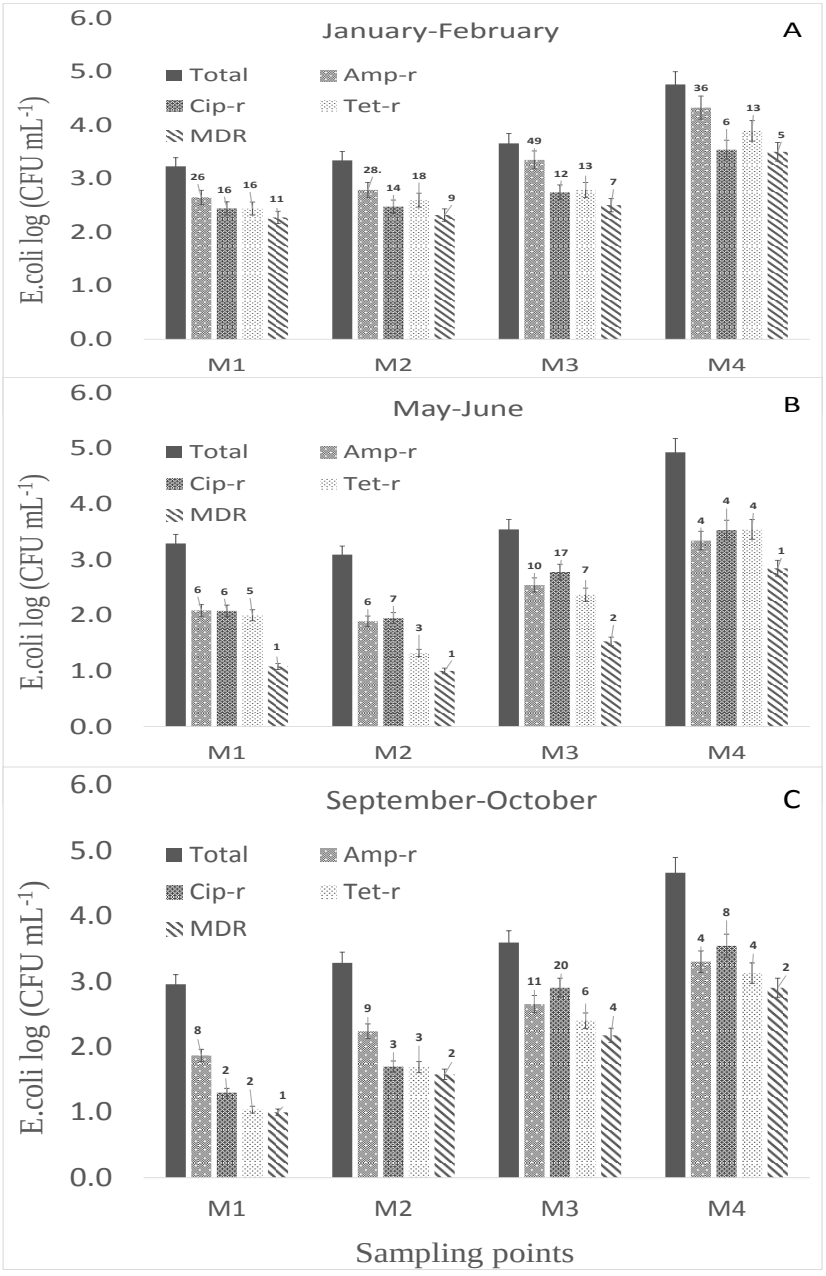


**Figure 3-2 Prevalence of E. coli resistant and multi-resistant to antibiotics in the Tusciano river**

In the winter campaign (C1), AMP-r E.coli was found to be the most prevalent antibiotic resistant E. coli among single antibiotic and multidrug resistant E. coli investigated. Just downstream of the discharge of WWTPs (M1 and M2), the prevalence of AMP-r E. coli were found to be really similar (as high as 27%), however, downstream of Battipaglia WWTP and Hospital effluents discharges (M3) the prevalence drastically increased to 49%. Then finally decreased to 39% in the last section (M4). Contrary to AMP-r E.coli, CIP-r and TET-r prevalence did not significantly change from upstream to downstream but for CIP-r in the last sampling location (6%). Finally, MDR E. coli prevalence decreased from 11% in M1 to 5% in M4. In C2 campaign (May-June), the percentages of resistance was lower than the $C_1$ campaign, however, the proportion of resistant and multi-resistant E. coli, increased dramatically just downstream of hospital wastewater disposal (M3). E. coli prevalence from S2 to S3 increased from 6% to 10%, 7% to 17%, 3% to 7% and from 1% to 2% for AMP-r, CIP-r, TET-r and MDR, respectively. In M4 point the percentage decreased to the levels of the point M2 and M1. A similar trend, was found in C3 campaign. The percentage of resistant E.coli between M1 and M2 was quite similar, but from M2 and M3 the increase of resistance was from 9% to 11%, 3% to 20%, 3% to 6% and from 2% to 4% for AMP-r, CIP-r, TET-r and MDR, respectively; in S4 point the percentage of resistant E. coli decreased to 4%, 4%, 8% and 2%, respectively. In all campaigns an increase of E.coli resistant downstream of the Battipaglia WWTP and Hospital was found. However in C1 campaign a drastic increase in the AMP-r was found (from 28 to 49%), in C2 and C3 campaign a drastic increase in the CIP-r was found (from 7 to 17% and from 3 to 20% respectively).

In the annual (2013) report on antibiotic resistance and use of antibiotics in Campania of 8.000 E. coli isolates in hospitals about 68% were resistant to AMP while 41% were resistant to CPX, which suggests a correlation between the presence of resistant organisms in hospitals and in the water body.

Sidrach-Cardona et al. (2014) monitored the Bernesga River downstream of the city of León (Northwest Spain), and they detected an high level (35%) of AMP-r E. coli which remained almost constant along the river. However a drastical increase (from 40% to 83%) in ampicillin resistance

was observed downstream of an antibiotic production plant (APP) discharge; a moderate decrease was observed along the river, even after receiving the WWTP effluent. The resistance to other antibiotics was also investigated and a decrease in TET-r E.coli (from 20% to 16%) downstream of APP discharges was found. In general a low rate of resistance was observed against azithromycin, with maximum values of 31.6% in water samples.

Proportions of E. coli resistant to ampicillin, tetracycline, and ofloxacin were determined in June, September and December 2006, and March 2007 by Akiyama and Savin (2010) in Mud Creek, a tributary of the Illinois River, located in Fayetteville, AR, USA, receiving discharge from a municipal WWTP. Temporal variations were observed in antibiotic-resistant E. coli with higher percentage in December (36%) and lower percentages in June and September (less than 10%). Despite temporal variability, antibiotic-resistant E. coli were highest in effluent across sampling times and progressively decreased in the two downstream sites but remained higher than upstream. According to our work, Savin and Aykama (2010) also an higher presence of AMP-r in the winter months.

Korzeniewska et al. (2013) investigated the contamination degree in terms of antibiotic resistance from hospital effluents and municipal sewage (inflow, sewage in aeration tank, outflow) and observed that the strains of E. coli isolated from hospital effluents are characterized by higher resistance rates than those from municipal sewage. With WWTP effluent input, levels of antibiotic resistance among bacteria in an effluent-receiving stream may be altered, but antibiotic resistance may also be related to temporal dynamics of physicochemical conditions of the effluent and stream water (Graves et al., 2007).

### 3.4.4 Prevalence of antibiotic resistant Enterococcus along Tusciano river

In the winter campaign (C1), CIP-r Enteroccoccus, were found to be the most common among the investigated strains. In M1 and M2 point,

downstream of WWTPs discharge in river, CIP-r Enteroccoccus prevalence was as high as 40% and 25%, respectively;
However, after the discharge of the Hospital wastewater effluent, the percentage increases up to 54% (M3). Finally, the percentage of CIP-r Enteroccoccus decreased to 35% in the last sampling section (M4) as shown in Fig 3.3.

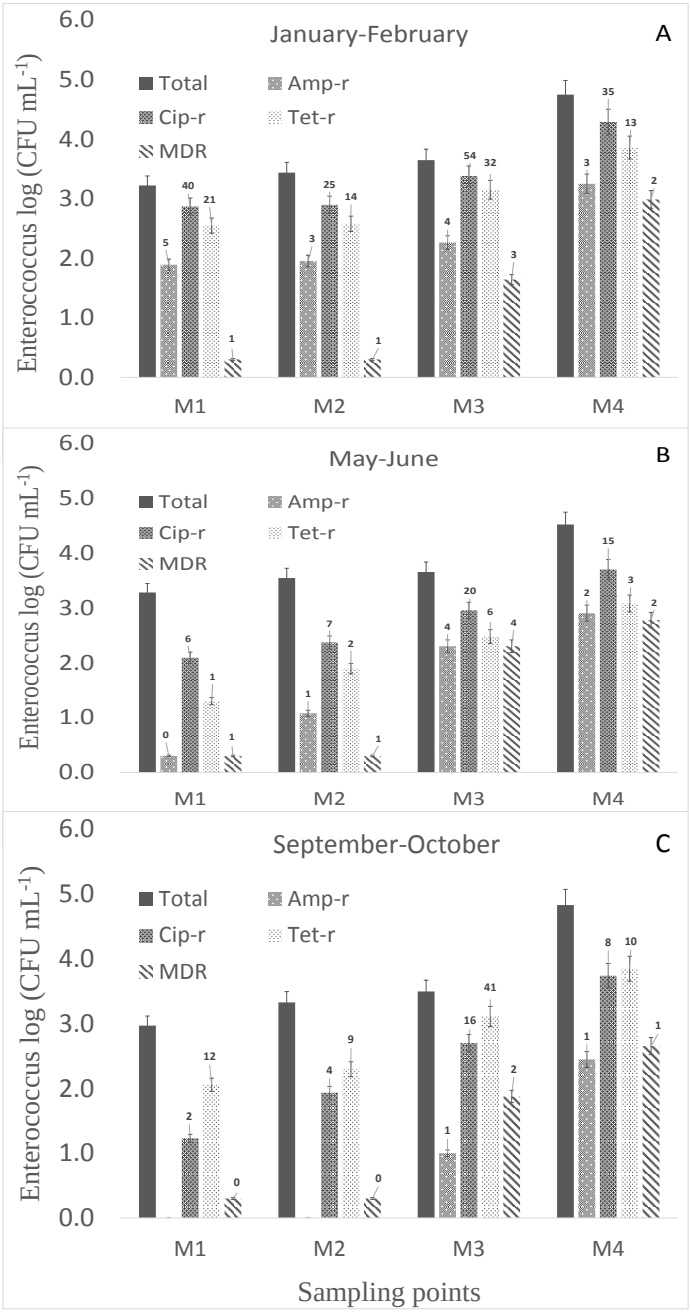


**Figure 3-3 Prevalence of Enterococcus resistant and multi-resistant to antibiotics in the Tusciano river**

A similar trend was observed for Tet-r Enteroccoccus where the percentage increased after the discharge of hospital effluent, although to a lesser extent than the CPX. In C2 and C3 campaigns an increase of Enteroccoccus resistant to both single and the mixture of the three antibiotics was detected in M3, with the highest increase, for CIP and TET resistant Enteroccoccus. In C3 campaign a drastic increase in TET-r from 9% in S2 to 41% in S3 was also observed. In all three campaigns, however, a low percentage of AMP-r, and MDR was detected.

In the annual (2013) report on antibiotic resistance and use of antibiotics in Campania the percentage of resistant Enterococcus was high, about 6% (it is the fifth micro-organism in terms of percentages of resistance), however lower than the percentage of E. coli (about 20%). Even in the water body, average a level of less resistance in Enterococcus was detected compared with E.coli. About resistance data than individual antibiotics, have been analysed only the Enterococcus resistant to AMP, and not to CIP and TET. Of 2.700 Enterococcus isolates, approximately only 5% were resistant to AMP.

Sidrach-Cardona et al. (2014) monitored, in addition to E.coli resitant, also the Enterococcus resistant in Bernesga River. They found out a high resistance to group of cephalexin, which reached 60% immediately after receiving the APP discharge, but it decreased along the river. However, partially in according to our study where no AMP-r in C2 (M1) and C2 (M1 and M2) were detected, they did not find any AMP-r Enterococcus isolate in water samples collected in the river or at the APP discharge point, whereas the rate of resistant isolates was about 10% at the WWTP discharge location. In all cases (except for AMP) there were resistant isolates in the environment before receiving the treatment plant discharges.

The presence in environment of Enterococcus, in coast of northern Pahang, Malaysia, near the mouth of the river (affected by WWTPs discharges) was studied by Ahmad et al. (2014). The differences in the levels of antibiotic resistant isolates recovered from the beach area and the river area were investigated. The river area isolates generally presented higher resistance proportions; in particular, the level of antibiotic resistance was highest for kanamycin, about 90.2%, while TET

and chloramphenicol resistance levels were at 44.79% and 37.59% respectively. A similar percentage of resistance for the TET was detected, in our work, in C1 and C2 after the discharge of the hospital effluent.

The increase of resistance in CIP and TET in S3, found in our work, after discharge in river of hospital effluent, finds confirmation in the work of Varela et al. (2013). They claim that ciprofloxacin and vancomycin resistant Enterococci were higher in the hospital effluent than in the raw urban wastewater, and that the percentage of bacteria resistant to both antibiotics was significantly higher in the hospital effluent than in the (raw and treated) urban effluent. This because the hospital effluent the average concentration of antibiotics was higher than in the raw wastewater, as to be expected. Moreover they also claim that in the hospital effluent may exist a higher percentage of culturable persisters (bacteria that can grow in the presence of antibiotic, although do not possess specific resistance determinants).

Chong et al. (1994) detected a prevalence of TET-r Enterococcus of 60.6% downstream of a hospital effluent disposal. Differently from our work, Dada et al. (2013) detected a quite high prevalence of AMP-r Enterococcus (25%) after effluent hospital discharge.

## 3.5 CONCLUSION

This work demonstrated that both Hospital effluent and WWTP discharges significantly contribute to the spread of antibiotic resistance in river. The higher abundance of the target ARB was observed downstream of Hospital effluent. The relative abundance of the target ARB was not only affected by wastewater discharges but also by the season. An increasing trend in the relative abundance of ARB from upstream to downstream observed during all seasons. These results call for a better understanding of both the fate of ARB in rivers and inactivation mechanisms, as well as for the investigation of effective disinfection processes for controlling antibiotic resistance release from

both Hospital effluent and WWTP discharges. Both issues were respectively addressed in the subsequent chapters.

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# 4 MODELLING THE FATE PF ARB IN RIVERS

## 4.1 ABSTRACT

The results from previous chapter 3 call for a better understanding of the fate of ARB in rivers as well as of the possible inactivation mechanisms. Accordingly, the purpose of this work was the investigation of different mechanisms and conditions on the fate of E. coli and Enterococcus antibiotic resistant strains in simulated river. In particular, the effect of natural attenuation (dark condition), solar radiation, sediment adsorption and multiple UWWTP effluents discharges was evaluated as well as the corresponding inactivation/adsorption kinetics. To this end a lab scale hydraulic channel to simulate a real river was built. E. coli and Enteroccoccus multidrug resistant strains were selected from a large urban WWTP upstream the disinfection unit. Bacterial inactivation was mainly dependent on sunlight intensity. The rate of average inactivation for both bacteria was 2 log units after 6 hours exposure to sunlight. In the presence of the sediment, the rate of inactivation was close to 3 log units, and 1 log unit was adsorbed to the sediment. In the presence of discharges an increase of the portion adsorbed to the sediment was observed. The inactivation/adsorption of target ARB was found to obey to a first order kinetic. Half-live time ($t_{1/2}$) between 1.3 h and 2.26 h were observed in the water under the effect of solar radiation, while a $t_{1/2}$ values between 3.71 and 7.92 h in sediments were observed. In spite of solar inactivation contribution significantly affected ARB strains in simulated river, under the investigated conditions (weak slope and slow current), inactivation efficiency is expected to drastically decrease as water height and turbidity increase. A significant effect on ARB fate is expected also from adsorption to sediments, but this mechanism may contribute to the spread of resistance in the environment through flora and fauna river and consequently through food chain.

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Giacomo Viccione, Professor, University of Salerno and Nicola Immediata, doctor, University of Salerno, for help and advice to designing of the artificial stream.

The present chapter, after an adaptation in terms of formatting style, will be submitted for possible publication in a peer reviewed indexed journal.

## 4.2 INTRODUCTION

Urban wastewater treatment plant (UWWTP) effluents, hospital discharges, livestock farms are among the major contamination sources of surface water from antibiotic resistant bacteria (ARB). Accordingly, several authors have detected ARB, particularly E.coli and Entorococchi strains, in different aquatic ecosystems, (Edge 2005; Watkinson et al., 2007; Tao et al., 2010; Araújo et al., 2010; Korzeniewska et al. 2010), . Therefore, it is really important to understand the mechanisms and fate of ARB in rivers, in order to better address possible solutions to minimize the spread of antibiotic resistance in the environment.

It is usually assumed that bacteria are free floating microorganisms when dispersed/suspended in river. However researchers have found that a significant amount of faecal bacteria may attach to sediment particles (Bai et Lang 2005). Different mechanisms drive bacterial fate into stream water, but they can be lumped in to physical transport (downstream or to bed sediments) and inactivation.

The fate and transport of enteric microorganisms in a waterbody are typically modelled with a form of the advection-dispersion-reaction equation (Cho et al., 2010; Gao et al., 2011). Several studies have investigated the sources, fate and transport of faecal indicator bacteria in waterbodies, and revealed that the levels of faecal indicator bacteria are significantly influenced by meteorological conditions (Marsalek and

Rochfort, 2004; Kim et al., 2007). In particular, the fate of microorganisms in aquatic systems has been significantly associated with solar intensity (Brookes et al., 2004). In recent years research focused on determining the bacterial inactivation rate under several environmental factors such as light intensity, radiance, temperature, salinity, pH and turbidity (Yukselen et al., 2003; Kay et al., 2005). Several authors have tried to shape the fate of microorganisms in water bodies by analysing water samples taken at different points of the water body (Sperling von and de Lemos Chernicharo, 2005 Chapra and Pelletier, 2003, Kashefipour et al.,2006). Moreover, some works studied the inactivation kinetics by artificial system able to simulate a water body (Schultz-Fademrecht et al. 2008, Walters et al. 2014). But, to our knowledge, no work is available in scientific literature deals with the simulation of ARB fate in rivers.

Accordingly, the purpose of this work was the investigation of different mechanisms and conditions on the fate of E. coli and Enterococcus antibiotic resistant strains in simulated river. In particular, the effect of natural attenuation (dark condition), solar radiation, sediment adsorption and multiple UWWTP effluents discharges was evaluated. To this end a lab scale hydraulic channel to simulate a real river was built. The system was located outside the Laboratory of Sanitary and Environmental Engineering at University of Salerno, Fisciano (Italy). E. coli and Enteroccoccus multidrug resistant strains were selected from a large urban WWTP upstream the disinfection unit.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Artificial stream

An artificial stream system (Fig. 4.1) was constructed to simulate the fate of ARB in a real river

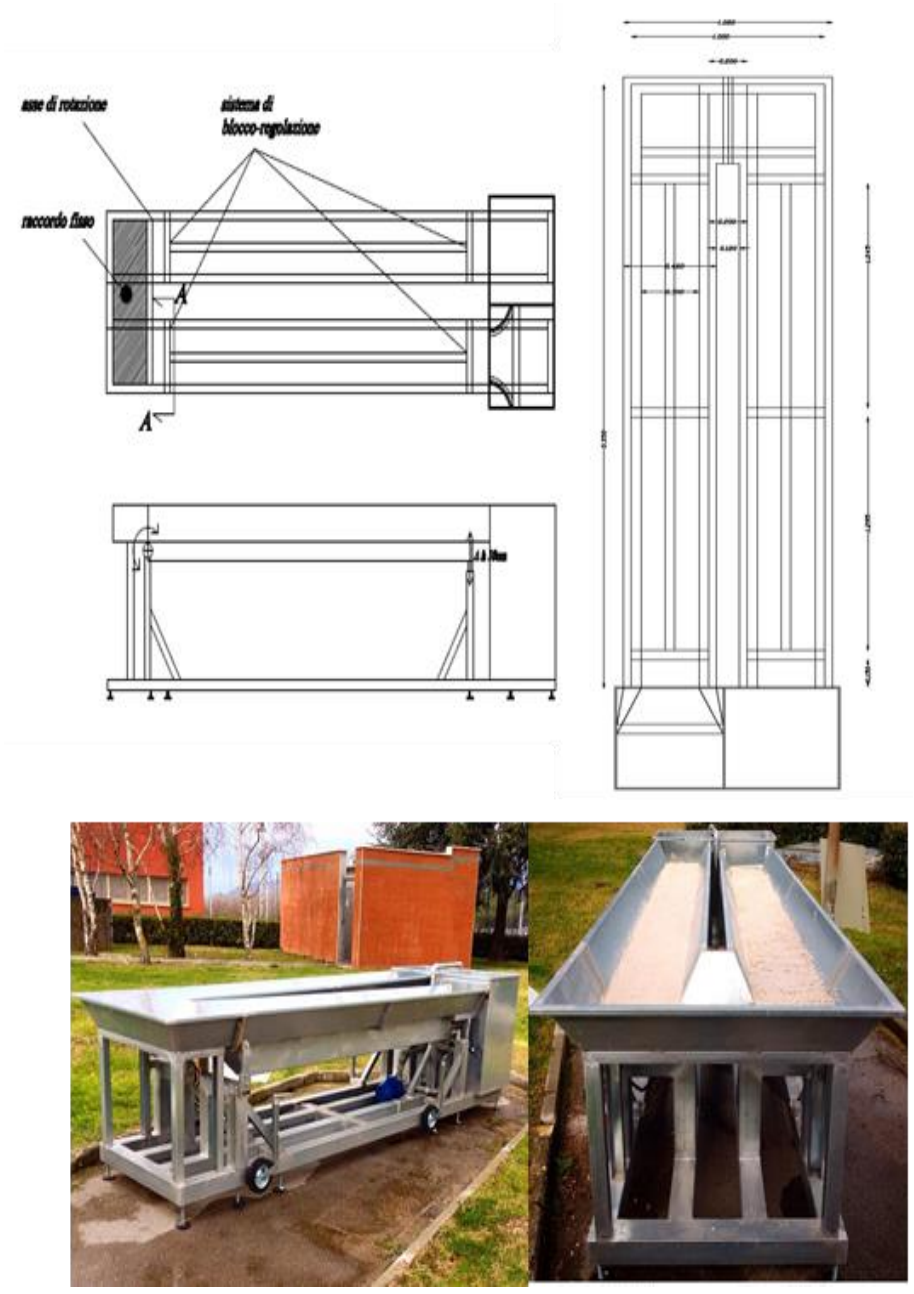


**Figure 4-1 Drawings and photos of the artificial stream designed**

The shape is designed as a "U" in order to limit the points of curvature, and simulate, as closely as possible, a river. The flume, constructed of aluminum, has a trapezoidal section ($b_1$=30cm, $b_2$=50cm, h=18cm) to minimize the hampering to solar radiation during the experiment. The flume was equipped with a removable covering, in order to operate control tests under dark conditions. The volumetric capacity of the flume was about 120 L. The flow velocity averaged 0.2 m $s^{-1}$, the average water height was 2.8 cm. To evaluate bacterial adsorption to sediment some experiments were carried out by placing sand (0.8 cm depth) on the bottom of the flume. The sand (grain sizes in the range of 0.39-0.45 mm) was taken from Sele river (Salerno province, Italy) and sterilized before use in autoclave at 120 °C for 15 min.

The detection of antibiotic resistant E.coli and Enterococci strains in water and in the sediments was carried out by taking samples before, during and at the end of the experiments. The experiments were carried out outside of the laboratory of Sanitary and Environmental Engineering (Lat. 44°77'61"N Long. 14°78'70"), Department of Civil Engineering, University of Salerno. Four different type of experiments were carried out:

i) dark (control) test to assess the natural decay of bacteria;
ii) sunlight experiment to simulate solar inactivation;
iii) sunlight experiments in presence of sand in the flume, to assess bacterial inactivation by solar radiation as well as to assess the contribution of sand adsorption to bacteria fate;
iv) sunlight with sand in the flume simulating WWTPs' discharges;

Surface water was freshly taken for each experiment from upstream location (free of fecal contamination) of river in Salerno province (Italy). The water was checked before each experiment in order to determine the complete absence of fecal microorganisms. Prior to start each experiment, the water was spiked with 2 mL of E. coli or Enterococcus ($10^9$ CFU $mL^{-1}$), to obtain an initial bacterial concentration of $10^5$ CFU $mL^{-1}$.

### 4.3.2 Selection of multidrug resistant E. coli and Enteroccosus strain for inactivation experiments

E. coli and Enteroccoccus multidrug resistant strains were respectively selected from a river located in southern Italy, just downstream of a WWTP effluent. The samples were filtered (membrane filtration) and subsequently cultivated on selective medium (24 h incubation time at 44 °C), according to the procedure described by Rizzo et al. (2014b). Culture medium for the selection of resistant E.coli strain was prepared with bile X-glucur onide (TBX) agar medium (Biolife, Milano, IT), a selective chromogenic medium for the detection and enumeration of E. coli , including tryptone (20.0 g L-1), bile salts (1.5 g L-1), agar (15.0 g L-1), X-glucuronide (0.075 g L-1). with Tryptone, Bile salts, X-glucuronide (TBX, Oxoid), and supplemented with a mixture of three antibiotics (16 mg L-1 of AMP, 2 mg L-1 of CIP and 8 mg L-1 of TET).

Culture medium for the selection of resistant Enetococcus strain was prepared with Slanetz Bartley medium (Biolife, Milano, IT) a selective medium including tryptone (20.0 g $L^{-1}$), yeast extract (5.0 g $L^{-1}$), glucose (2.0 g $L^{-1}$), potassium phosphate dibasic (4.0 g $L^{-1}$), sodium azide (0.4 g $L^{-1}$), TTC 0.1 (g $L^{-1}$), Agar (10.0 g $L^{-1}$) and supplemented with a mixture of three antibiotics (16 mg $L^{-1}$ of AMP, 4 mg $L^{-1}$ of CIP and 16 mg $L^{-1}$ of TET). Antibiotic concentrations were selected according to the results from a previous study (Rizzo et al, 2013). Some colonies were randomly picked up and frozen in 15% glycerol Triptone Soy Broth (TSB) at -20 °C for the subsequent experiments. The full antibiotic concentrations were chosen according to the respective MICs listed in EUCAST "The European Committee on Antimicrobial Susceptibility Testing" according to data provided in 2014.

### 4.3.3 Enumeration of E.coli and Enterococcus

Water samples were collected from the flume at different time to enumerate E.coli and Enterococcus colonies, respectively. Water samples, depending on the bacterial load hypothesized, were diluited and undiluited in a final volume of 50 mL in sterile Falcon containing sterilized phosphate buffered saline (PBS, pH 7.4) and filtered through

0.45 μm membrane (Millimore; Darmsadt, Germany). Total Enterococcus were enumerated on Slanetz Bartley Agar (Biolife, Milano, IT) and total E.coli were enumerated on tryptone bile X-glucur onide (TBX) agar medium (Biolife, Milano, IT). For the enumeration of E.coli and Enteroccoccus in sediment, 5 g of sediment were taken and centrifuged for 10 minutes at 4.000 rpm. The supernatant was filtered on the respective culture media.

### 4.3.4 Analytical instrument

The BLACK-Comet Stellar Net UV-VIS (StellarNet, Florida, USA) spectrometer was used for solar irradiance monitoring during the experiments. The instrument was kept switched on during the entire duration of the experiments. All chemical parameters (temperature, conductivity, pH etc.) were monitored by the multi-parameter meter, model HI 9828 (Hanna Instruments USA). The turbidity was measured by 2100N Laboratory Turbidimeter, EPA 115 Vac (Hach, USA)

## 4.4 RESULT AND DISCUSSION

### 4.4.1 Physical-chemical properties of water

The experiments were carried out in the period between November 2014 and January 2015. The water temperature in all experiments was measured and resulted in the range of 10.0-12.0 °C. A so low temperature does not result in any effect on bacteria inactivation, because temperature was found to affect the fate of bacteria in water suspension for values higher than 40°C (Dunlop et al., 2011). pH (7.5) and conductivity (410 μS cm-1) were also measured and they did not change significantly. The turbidity was approximately as low as 0.1 NTU in water, which is not expected to significantly affect bacterial inactivation rate (Dunlop et al., 2011). The flow rate of the flume was kept low (0.1 m $s^{-1}$) to simulate a river in weak slope in slow current.

### 4.4.2 E. coli and Enterococcus inactivation rates by sunlight

The flume was initially coated (dark experiments) with a suitable system in order to assess the natural bacterial decay of the bacterial. The decay rate for E.coli and Enterococci (Fig 4.2 A-B, respectively) in 6 h experiment, was not significant (0.2 log units).

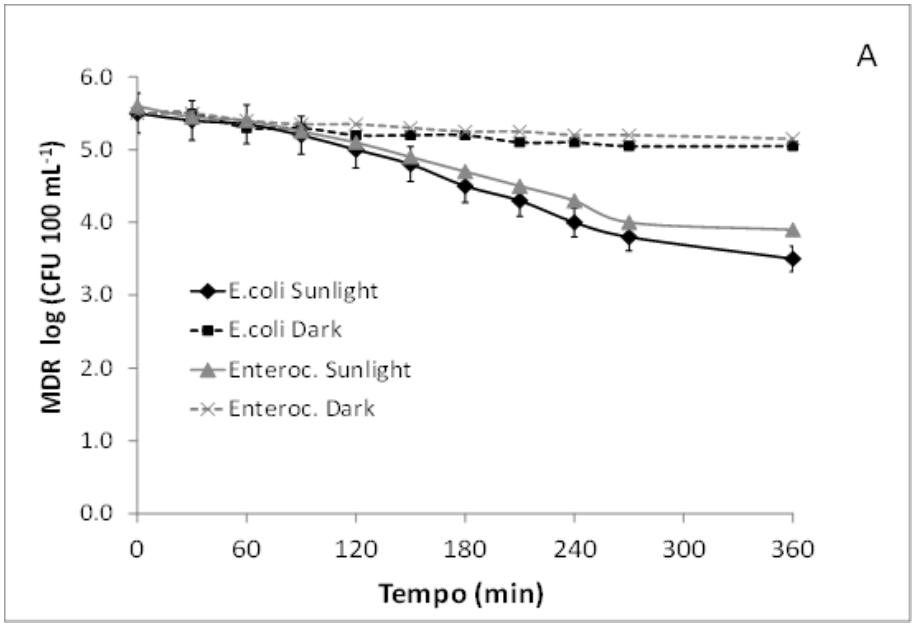


**Figure 4-2 Natural decay and inactivation of E.coli and Enterococcus by solar radiation**

In the experiments with sunlight (from 9.30 am to 3.30 pm in Fisciano, Italy Lat 40°46'25"68 N Long 14°48'1"08 E) the inactivation rate for both microorganisms was comparable (about 2 log units) after 6 h of exposure (fig 4.2 A). However, a slightly higher inactivation in E.coli (2.0 log units, 99.00% inactivation) compared to Enterococcus (1.7 log units, 97.90% inactivation) was observed.

### 4.4.3 E. coli and Enterococcus inactivation rates in the presence of sediments

The contribution of sediment adsorption mechanism to the fate of bacteria in the flume was assessed by putting a layer of sterile sand on the bottom of the plume (height in cross section of about 0.8 cm). The inactivation rate after 6 h of exposure to sunlight which corresponded approximately to 50 W s $cm^{-2}$ of UV dose, in the presence of sand was about 2.8 and 2.6 log units for E.coli and Enterococcus, respectively (Figure 4.3 A-B).

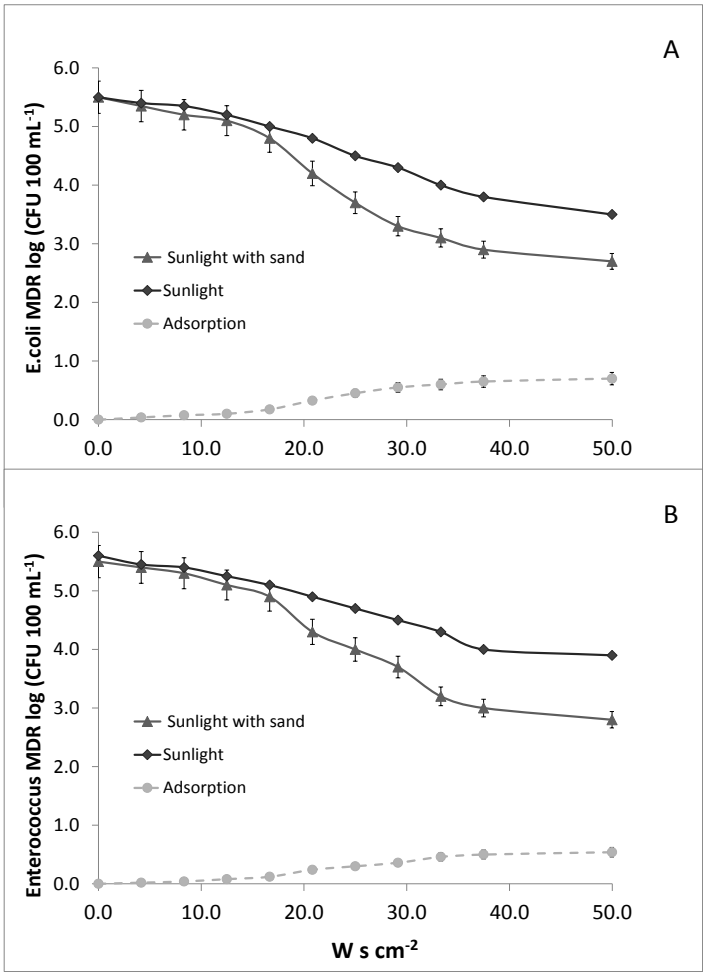


**Figure 4-3 Inactivation rate of E.coli (A) and Enterococcus (B) in presence of sediments**

The inactivation rate compared to the experiment without sediment in which the rate of bacterial inactivation was only 2 unit logs, was higher. In the experiment with sediment, the water turbidity did not significantly affect bacterial inactivation by sunlight and the total inactivation was higher because of the additional contribution of sediment adsorption mechanism. After 6 h of experiment the contribution of sand adsorption to bacterial removal was estimated as high as 0.7 and 0.5 unit logs for E.coli and Enterococcus, respectively.

### 4.4.4 Simulation of WWTPs' discharges

To simulate WWTPs' discharges, E. coli and Enterococcus strains were respectively inoculated at the beginning of the experiment, after 1.5 h and after 3 h. as shown in Fig. 4.4 A-B.

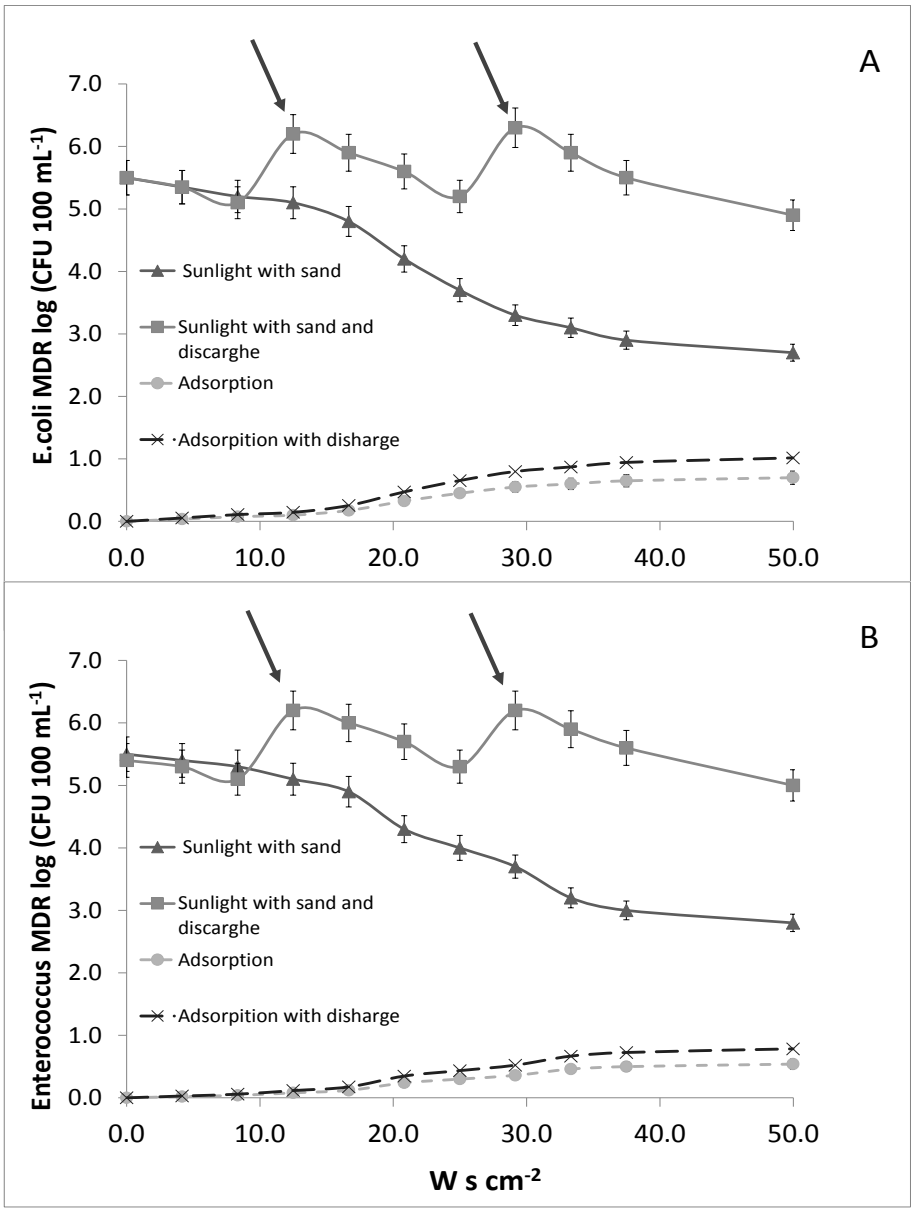


**Figure 4-4 Inactivation rate of E.coli (A) and Enterococcus (B) in presence of sediments and discharge simultaed**

When both strains were inoculated an increase in the respective bacterial loads was observed. The inactivation rate between two consecutive

inoculations is similar, but at the end of the experiment a slight difference between the two strains was observed (0.5 and 0.4 log units inactivation for E.coli and Enterococcus, respectively). As expected, total inactivation rate at the end of the respective experiments was lower compared to the experiment with single (initial) inoculum, because total cumulative bacterial density affected inactivation rate as well as the same irradiation time was not sufficient to reach a comparable inactivation. Moreover, the bacterial load adsorbed to the sand was higher compared to single inoculum experiment too. An increase of about 0.3 unit logs for both bacteria was detected at the end of experiments after 6 h.

### 4.4.5 Inactivation kinetics

The fate of ARB in the flume exposed to sunlight radiation and adsorption to sediment was found to fit quite well a first-order kinetic. Table 4.1 shows the values of kinetic parameters (constant k and half-life time $t_{1/2}$) and correlation coefficient ($R^2$) for both the investigated strains under different experimental conditions.

**Table 4.1 kinetic parameters (k and t1/2) and correlation coefficient R2 (between brackets)**

| | | **K [$d^{-1}$]** | | **$T_{1/2}$ [h]** | |
|---|---|---|---|---|---|
| | | E.coli | Ent. | E.coli | Ent. |
| **sunlight experiment** | water | 20.2 (0.960) | 18.1 (0.967) | 2.06 | 2.26 |
| **sunlight experiments with sand** | water | 31.6 (0.931) | 29.52 (0.938) | 1.31 | 1.40 |
| | sediments | 5.25 (0.932) | 5.76 (0.938) | 7.92 | 7.22 |
| **sunlight with sand and discharges simulated** | water | 29.88 (0.921) | 29.16 (0.911) | 1.39 | 1.42 |
| | sediments | 11.52 (0.931) | 8.64 (0.938) | 3.61 | 4.81 |

$T_{1/2}$ values are quite similar between the investigated ARB strains, and Enterococcus inactivation/adsorption time was slower compared to E. coli .
In sunlight experiment, kinetic constant varied between 18 and 31 $d^{-1}$ in water with a $t_{1/2}$ between 1.31 and 2.26 h. In the experiment with sediment the half-life time was lower because part of the bacterial load was adsorbed by sediment. Kinetic constant related to adsorption of ARB to sediment varied between 5.25 and 11.52 $d^{-1}$ with half-life time between 3.61 and 7.92 h. In this case, a considerably greater adsorption capacity was detected with increasing bacterial load suspended in water.

## 4.5 DISCUSSION

Inactivation rate of the target ARB strains in a flume were investigated under different conditions, to evaluate the inactivation related to the effect of solar radiation, the adsorption to the sediment and the effect of continuous discharges of WWTPs along the river channel.

The rate of average inactivation for both strains was 2 log units after 6 hours exposure to sunlight. In the presence of the sediment, the rate of inactivation was close to 3 log units, and 1 log unit in terms of adsorption to the sediment. In the presence of discharges an increase of the portion adsorbed to the sediment was observed.

Similar experiments in a flume were carried out by Schultz-Fademrecht et al. (2008). They simulated a river with a flume with a sand bed by using artificial lamp to simulate sunlight. Three different light intensity I=40.0, I=8.0 and I=0.08 W $m^{-2}$ were investigated to simulate midday sun in June, a radiation intensity conform with the annual mean radiation in Germany and the minimum of ultraviolet radiance, respectively. According to our work, they did not observe a significant difference between the inactivation of E.coli and Enterococci. However, differently from our work (6 h treatment, 50.0 W s $cm^{-2}$ light intensity), a total inactivation of 6 log units was achieved after 6 and 10h for I=40.0 I=8.0 W $m^{-2}$ respectively (86.4 W s $cm^{-2}$ and 28.8 W s $cm^{-2}$ respectively). After

24 h they observed that about 2 log units were adsorbed to sediment (the grain size was of about 30-40 mm).

If we compare the inactivation kinetics, in the work of Schultz-Fademrecht et al. (2008) the kinetic constant related to bacteria suspended in water, varied between 10 and 22 $d^{-1}$ ($t_{1/2}$ between 0.8 and 1.7 h), compared to our work where the values varied between 18 and 31 d-1 ($t_{1/2}$ included between 1.31 and 2.26h). In sediments the kinetic constant related to adsorption mechanism varied between 21.4 and 13 $d^{-1}$ in work of Schultz-Fademrecht et al. (2008) while in our work the lower values were observed (5.25 and 11.52 $d^{-1}$, respectively). The differences can be explained by the different working conditions. In our work, indigenous ARB selected from an urban WWTP were inoculated and investigated; moreover, experiments were carried out outside under natural sunlight (differently, in the quoted work the experiments were carried out inside the laboratory with artificial light sources, were very limited). Furthermore, the different results observed in the adsorption of target bacteria to the sediment, can be explained by the different sand particle size (significantly smaller in our work).

Menon et al. (2003) and Servais et al. (2007) investigated the fate of fecal bacteria in a river, downstream of a discharge of WWTP. In their studies a complete inactivation (5 log units) was observed between 24 and 48 h of sunlight exposure.

Balzer et al. (2007) analyzed samples of water and biofilms of different rivers and they found that the amounts of E. coli and Enterococci was approximately from one to three log units higher in sediments than in the water phase.

Aquatic sediments has been described as reservoirs for pathogens and fecal indicator microorganisms (Alm et al., 2003). However, there is still lack of knowledge on the role of benthic biofilms as reservoir for pathogens in rivers. Sediments can be a sort of substrate, nutrients source and a protection against ultraviolet radiation which might allow pathogens to survive or even to grow.

## 4.6 CONCLUSION

Different mechanisms and conditions can affect the fate of bacteria in rivers. In this work, the fate of E. coli and Enterococcus antibiotic resistant strains in simulated river was investigated to evaluate the effects of solar radiation, adsorption to sediments and multiple WWTPs discharges. The results from kinetic modeling showed that target ARB can be inactivated in weak slope and slow current (simulated) streams in a few hours, but solar inactivation efficiency is expected to drastically decrease as water height and turbidity increase. A significant effect on ARB fate is expected also from adsorption to sediments, but this mechanism may contribute to the spread of resistance in the environment through flora and fauna river and consequently through food chain.

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# 5 WASTEWATR DISINFECTION: EFFECT OF PHOTOCATALYS AND CONVENTIONAL DISINFECTION PROCESSES ON ANTIBIOTIC RESISTANCE

The results from previous chapters showed that ARB can occur in rivers because of WWTP and Hospital discharges. Accordingly, conventional disinfection processes, if any, are not effective in controlling the release of ARB and they do not look to be an effective barrier to the spread of antibiotic resistance in the environment. ARB released in the river can be only partially affected by natural phenomena such solar radiation. The ARB absorbed to river sediments may accumulate in aquatic flora and fauna, with a subsequent risk of antibiotic resistance transfer through food chain.. In order to minimize this risk as well as to elucidate the effect of conventional and new disinfection processes on the fate and inactivation mechanisms of ARB in wastewater, the following issues were addressed in the subsequent sub-chapters (5.1, 5.2, 5.3):

- UV-C radiation and non-conventional disinfection by $TiO_2$ photocatalysis were investigated to evaluate their effect on the transformation (mutants formation) of antibiotic resistant microorganism (Salmonella typhimurium) (Sub-chapter 5.1);

- $TiO_2$ photocatalytic process by using different light sources was evaluated on the inactivation of antibiotic resistant E. coli strains (selected from real wastewater) as well as on antibiotic resistance of colonies survived to disinfection process (Sub-chapter 5.2);

- Solar driven AOPs and conventional disinfection by chlorine were investigated in the inactivation of indigenous E. coli strain selected from real wastewater according to its resistance to three antibiotics (ampicillin (AMP), ciprofloxacin (CIP) and

tetracycline (TET)). Finally, $H_2O_2$/sunlight process was compared with chlorination in the inactivation of indigenous antibiotic resistant E.coli as well as to investigate post-treatment bacterial regrowth (Sub-chapter 5.3).

## 5.1 COMPARISON BETWEEN UV-C RADIATION AND TIO2 PHOTOCATALYSIS IN THE INACTIVATION AND MUTAGENICITY OF AR SALMONELLA TYPHIMURIUM

### 5.1.1 Abstract

The efficiency of UV-C radiation and $TiO_2$ Photocatalysis in the inactivation of Salmonella Typhimorium antibiotic resistant bacteria (ARB) in water was evaluated. Three different initial concentrations of Salmonella, $10^9$, $10^8$ and $10^7$ CFU $mL^{-1}$, were investigate for both disinfection treatments. In UV-C experiments for the tested strain a total inactivation of 9 units log was reached after about 45 min (UV-C dose 30 mWs $cm^{-2}$). As initial bacterial density was decreased to $10^8$ and $10^7$ CFU $mL^{-1}$ total Salmonella inactivation was achieved in 15 and 10 min, respectively while the corresponding UV-C dose was approximately the same (6 mWs $cm^{-2}$). In photocatalytic tests the complete Salmonella inactivation was achieved in 60, 30 and 15 min of irradiation as initial bacterial density was decreased from $10^9$ to $10^8$ and to $10^7$ CFU $mL^{-1}$ respectively (the energy required to achieve total Salmonella was in the range 2 - 20 mWs $cm^{-2}$). In UV-C and $TiO_2$ Potocatalysis tests an increase in formation of mutants was detected. The formation of mutants increased as UV dose increased up to a maximum of 1.1 x $10^3$, 1.9 x $10^3$ and 3.9 x $10^3$ for $10^9$, $10^8$ and $10^7$ CFU $mL^{-1}$ initial bacterial density, respectively. The corresponding percentage increase with respect to the initial density of mutants was 58, 210 and 358%, respectively The same trend was observed in the $TiO_2$ photocatalytic tests (a maximum of 9.5 x $10^2$, 1.1 x $10^3$ and 1.4 x $10^3$ mutants formation for $10^9$, $10^8$ and $10^7$ CFU $mL^{-1}$ initial bacterial density, respectively). The percentage of mutants was significantly lower (35, 77 and 120%, respectively) compared to UV-C disinfection experiments.

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The present chapter, after an adaptation in terms of formatting style, will be submitted for possible publication in a peer reviewed indexed journal

### 5.1.2 Introduction

Salmonella represent today a major public health problem worldwide. Salmonella serovars are increasing in importance as significant pathogens to both humans and animals (Turki et al. 2012). Food and water still play the main roles in the transmission of Salmonella. Salmonellae are excreted through animal and human feces into the environment (Baudart et al 2000). They remain viable outside the host environment for longer time periods and can persist for months in soil, faeces and water (Hussong, Burge & Enkiri, 1985).

Salmonellae are frequently found in environmental samples. They are usually present in large numbers in raw sewage and can still persist in wastewater effluent also after disinfection process (Wéry et al. 2008). A study indicated that 80% of wastewater samples used as an irrigation source was positive for Salmonella (Nutt et al., 2003). Moreover, it has been detected in various types of natural waters such as rivers, lakes, coastal waters, as well as contaminated ground water (Haley et al. 2009; Levantesi et al., 2010; Martinez-Urtaza et al., 2004; Polo et al., 1999; Wilkes et al., 2009). The growth of Salmonella in non-host environments such as wastewater sludge has also been reported (Zaleski et al 2005) and

the growth of Salmonella in water supplies is also considered possible, due to its ability to colonize surfaces and replicate in biofilms of distribution system pipes (Jones et Bradshaw, 1996).

The genus Salmonella comprises two species S. bongori and S. enterica. The species S. enterica, as Salmonella Typhimurium, is mainly associated to human and other warm blooded vertebrates (Popoff 2001). On the basis of the caused clinical syndromes Salmonella are divided in to two distinct groups namely the typhoidal and non typhoidal one (Pond, 2005). In the last years the emergence of multidrug resistant (MDR) strains of Salmonella Typhimurium was associated to the observed incidence of invasive salmonellosis (Gordon et Graham, 2008). The increased frequency of MDR Salmonella strains in human infections is an emerging issue of major health concern (Lightfoot, 2004; Lynch et al., 2009). As a consequence, the possible role of fecally contaminated waters in the spread of MDR Salmonella, both typhoidal and non-typhoidal, as well as antibiotic resistance genes though horizontal gene transfer is of great interest (Levantesi et al. 2012).

Different studies have investigated the inactivation of Salmonella strains by disinfection processes in water and wastewater. The effect of different disinfection processes on Salmonella strains in synthetic wastewater effluent was studied by Koivunen and Heinonen-Tanski (2005). In particular, the treatment with paracetic acid and UV radiation, has been shown to be faster than UV radiation alone and chlorine processes. Moreover, Salmonella has been found to be more resistant to chlorine than others indicator bacteria, including total coliforms and Enterococcus, (Li et al. 2012). Moreover, it is also more resistant to UV radiation than several indicator strains such as E.coli and Enterococcus, (Berney et al., 2006; Smith et al., 2002; Rincon and Pulgarin, 2003, Rincon and Pulgarin 2007, Sciacca et al. 2011).

However conventional disinfection by either chlorination or UV radiation may be not effective in controlling antibiotic resistance spread (Rizzo et al., 2013), therefore new disinfection methods should be investigated. Advanced oxidation processes (AOPs) (e.g., Fenton, photo-Fenton, TiO2 photocatalysis, UV/$O_3$, UV/$H_2O_2$ etc.) have been found effective in the removal of a wide range of contaminants (Zapata et al., 2010; Rizzo, 2011; Sannino et al., 2013; Murcia et al., 2013). Among

AOPs, $TiO_2$ photocatalysis is an effective water disinfection option, due to its capacity to inactivate a wide range of pathogens in water; however, the effectiveness of $TiO_2$ photocatalysis on the inactivation of selected Salmonella strain and mutagenic effect has not been investigated to authors' knowledge.

The objective of the present work was the investigation of $TiO_2$ photocatalysis process on the inactivation and mutants formation of a model antibiotic resistant microorganism. Salmonella was chosen as target/model organism (its genus belongs to the same family as Escherichia, which includes the species E.coli) because of (i) its spread in aqueous matrices (particularly in natural water and wastewater), (ii) the related concern for human health (it can cause illnesses such as typhoid fever and paratyphoid fever), (iii) its role in antibiotic resistance spread and (iv) the availability of a well-established test (Ames) to evaluate the formation of mutants. Moreover, the effect of $TiO_2$ photocatalysis on the inactivation and mutants formation of Salmonella was compared with a conventional disinfection process such as UV-C disinfection

### 5.1.3 Material and method

#### 5.1.3.1 Salmonella Typhimurium strain

Salmonella Typhimurium strains TA102 were maintained in frozen stoks and grown as described in Ames et al. (1973). The Salmonella Typhimurim TA102 have a hisG type of mutation in the histidine operon, a rfa mutation (that causes partial loss of the membrane integrity) concern lypoplysacharide membrane (LPS), pAQ1 (in tetracycline resistance) plasmid that contains an ochre mutation at the his G428 wich increases the sensitivity of the strains towards radical, a wild type gene in bacterial acetyltransferase (OAT) and a base pari substitution in term of reversion event. The strain was tested on the basis of associated genetic markers raising it from a single colony from the master plate as described by Maron and Ames (1983).

#### 5.1.3.2 Sample preparation and control tests

Briefly, TA102 colonies were unfrozen and reactivated by streaking on minimal glucose agar and incubated at 37 °C for 48 h. A single colony from the plate was inoculated into 10 mL sterile Luria Bertani broth (LB,

Sigma-Aldrich, USA) and incubated at 30◦C for 18 h by constant agitation (160 rpm) in a rotator shaker to obtain a stationary phase culture. Cells were harvested by centrifugation at 5000 rpm for 2 min twice, and the pellet was re-suspended in 10 mM $MgSO_4$ in different volume to obtain a final concentration of $10^9$, $10^8$ and $10^7$ CFU $mL^{-1}$; these suspensions were then used in disinfection experiments. The pre-incubation test was performed as described by Maron and Ames (1983). Seven doses of Mytomicine C (5, 2.5, 0.5, 0.25, 0.05, 0.025 and 0.005 μl) diluted in ethanol and 10 mM MgSO4 to control ethanol toxicity, were incorporated in the minimal glucose agar containing traces of histidine and biotine. 0.1 mL Salmonella bacterial culture was plated in triplicates and incubated at 37C for 48-72h

#### 5.1.3.3 UV-C disinfection tests

UV-C experiments were carried out in 40 mL glass Petri dishes (diameter: 90 mm) filled in with 25 mL of sample. Petri dish was put under a collimating tube, and gently stirred throughout the exposure time. The irradiance at the center of the beam at the water surface was about 0.008 mW $cm^{-2}$. The lamp was located horizontally 20 cm above the surface of the water.

#### 5.1.3.4 TiO2 photocatalysis tests

Photocatalytic tests were carried out in a 2.2 L cylindrical glass reactor (13.0 cm in diameter) filled in with the 400 mL of sample. The thickness of liquid was constant and equal to the liquid depth (3 cm). The reactor was placed in a water bath to control the temperature at 30 °C during the experiments. The solution in the reactor was stirred continuously. A wide spectrum 250 W lamp (Procomat, Italy) was used as light source.. The lamp was placed horizontally 40 cm above the surface of the water. A spectrometer model HR-2000 from Ocean Optics (Florida, USA) was used to measure light intensity. Degussa P25 $TiO_2$ was used as received from the manufacturer as slurry to perform heterogeneous photocatalytic experiments. They were carried out at concentration of 100 mg $L^{-1}$ according to previously set conditions (Rizzo et al., 2014)

#### 5.1.3.5 Bacterial count and mutagenic index

In UV-C and $TiO_2$ photocatalysis experiments, serially dilution of sample in 10 Mm $MgSO_4$, from $10^0$ to $10^7$ CFU $mL^{-1}$ were prepared and 100 μL spread in triplicate on minimal glucose agar with (MGA+H) and without (MGA) histidine. Plates were incubated at 37°C for 48-72h. Each experiment was performed in triplicate. The detection limit of this experimental method was found to be 2 CFU $mL^{-1}$. Agar plate composition included distillated water, agar, VB salt solution (50X) and glucose solution (40%) with L-Histidine 20 mM and biotin 0.5 mM in MGA+H and only Biotine 0.5 mM for MGA. Mutagenix index = number of his- revertants induced in the sample for each time/number of his- before the process. Where his- was the number of colonies, for each time, on the agar.

### 5.1.4 Result and discussion

#### 5.1.4.1 Salmonella resistant strain inactivation tests

**UV-C disinfection tests**

Experiments using UV-C lamp were performed with different initial bacterial densities: $10^9$, $10^8$ and $10^7$ CFU $mL^{-1}$. The experiments with Salmonella were carried out in Milli-Q water (pH 7.7) inoculated with Salmonella strain, and represented as a function of UV energy (Fig. 5.1 A-B-C). Initial bacterial density did not change during control experiment in dark (sample stored at room temperature for 4 hours, results not shown).

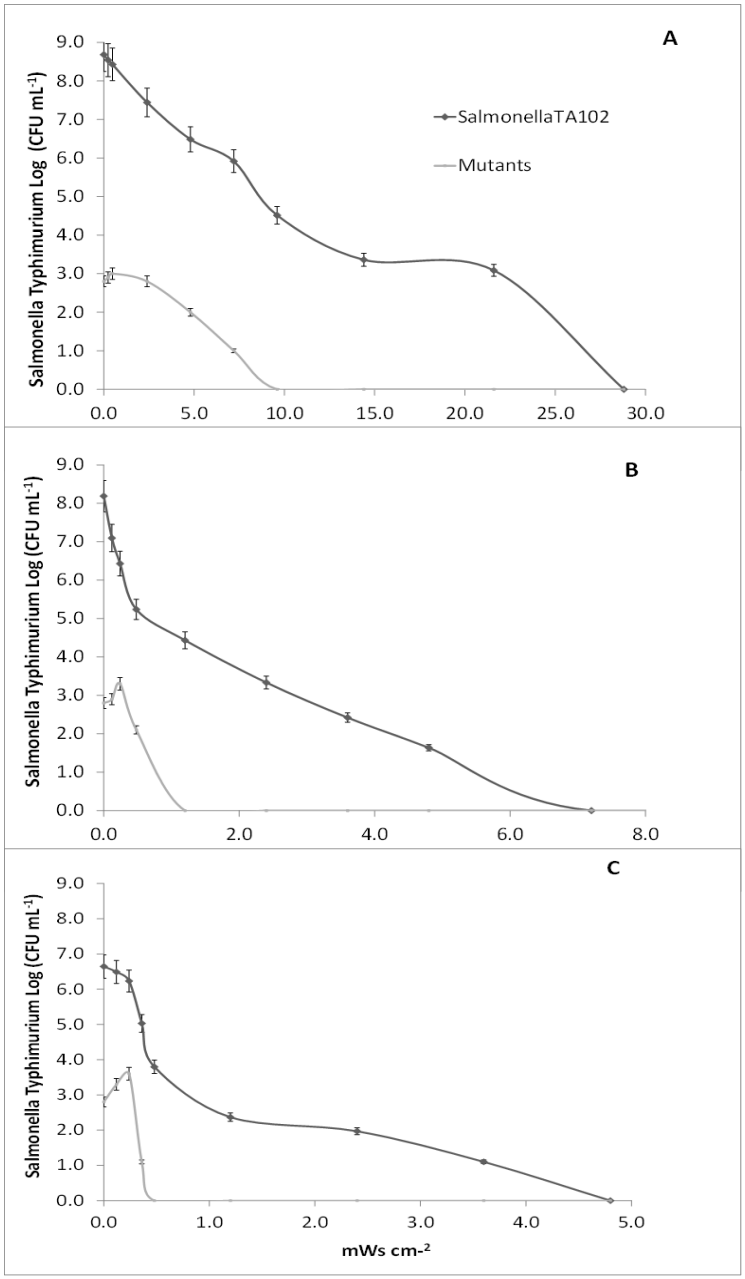


**Figure 5-1 Inactivation of Salmonella strain by UV-C radiation and mutants formation**

Salmonella inactivation increased as UV-dose increased. Roughly 9 log units decrease was observed for the tested strain and total inactivation was reached after about 45 min of UV exposure (Fig. 5.1A). In terms of UV-C dose 30 mWs $cm^{-2}$ (45 min irradition) was necessary to get the total inactivation.

As initial bacterial density was decreased to $10^8$ (Fig. 5.1B) and $10^7$ (Fig. 5.1C) CFU $mL^{-1}$ total Salmonella inactivation was achieved in 15 and 10 min, respectively while the corresponding UV-C dose was approximately the same (6 mWs $cm^{-2}$ in both experiments). A direct dependence of initial concentration of microorganism and UV dose was expected, as its estimation was based on the averaged incident radiation energy over the reactor volume that is a dynamic function of the absorption coefficient, which itself depends dynamically on the concentration of active cells (Silva et al. 2013). UV-C disinfection relies on the sensitivity of the microorganism to UV radiation. This is unique to each microrganism and is determined by its ability to absorb at 200–280 nm (germicidal wavelength range), so inactivating their active cells through UV-induced damages such as the formation of pyrimidine dimers in their DNA.

UV-induced damages disrupt the DNA structure, so that, if a critical number of dimers is formed, the DNA cannot replicate (Hignen et al 2006). The effect of UV-C radiation on Salmonella has been previously investigated. Koivunen et al. (2005) observed inactivation as high as 0.9 and 2.6 log10 in Salmonella Enteredis aqueous suspensions ($10^7$ CFU $mL^{-1}$ initial density) after 10 min irradiation with two different dose (6.0 and 10 mWs $cm^{-2}$, respectively). Graca et al. (2013) observed a complete inactivation (7.0 log CFU $mL^{-1}$) after 4 h treatment and two different UV-C doses (1.0 and 0.5 kJ $m^{-2}$).

**$TiO_2$ photocatalysis tests**

The bacterial inactivation mechanism during disinfection by $TiO_2$ photocatalysis is related to the formation of radicals (e.g., hydroxyl radical) with no significant contribution of UV dose. Accordingly, the results of Salmonella inactivation by photocatalysis were plotted as function of irradiation time (Fig.5.2 A-B-C).

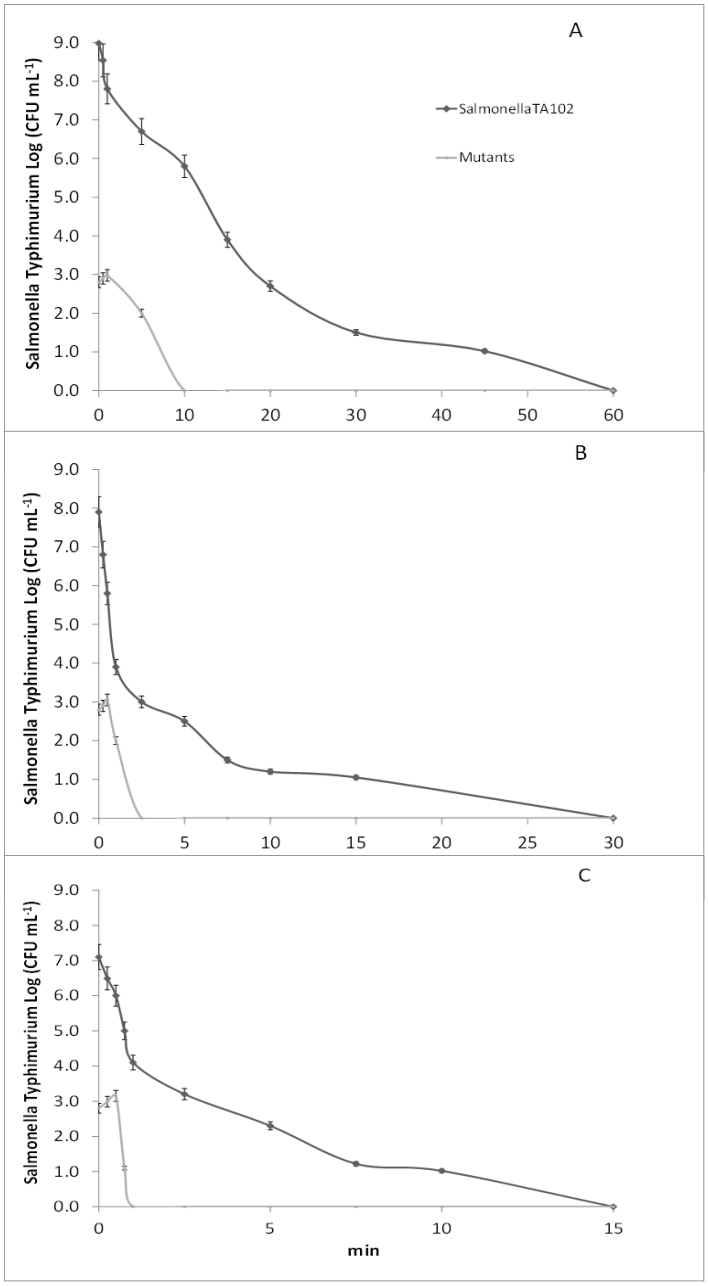


**Figure 5-2 Inactivation of Salmonella strain by $TiO_2$/UV radiation and mutants formation**

Initial bacterial density strongly affected inactivation rate by $TiO_2$ photocatalysis (Figure 5.2). The complete Salmonella inactivation was achieved in 60, 30 and 15 min of irradiation as initial bacterial density was decreased from $10^9$ to $10^8$ and to $10^7$ CFU $mL^{-1}$ respectively. The complete inactivation was faster in the UV-C disinfection experiments compared to photocatalytic disinfection experiments for all initial bacterial densities investigated. However, photocatalytic process was more energy efficient than UV-C process because a lower energy was required to achieve total Salmonella inactivation (in the range 2 - 20 mWs $cm^{-2}$). The inactivation of Salmomella Typhimurium by photocatalysis with UV-A lamp and $TiO_2$ at concentration of 0.5 g $L^{-1}$ was studied by Long et al. (2014).

Three different initial load concentration were investigated: $10^7$, $10^6$ and $10^5$ CFU $mL^{-1}$, and a complete inactivation was reached after 180, 70 and 60 min respectively. Slower inactivation rates, as might be expected, were obtained with only the UV-A lamp.

A similar study, with inactivation of Salmonella Enteretidis ($10^8$ CFU $mL^{-1}$ initial bacterial density) by UV-A lamp and $TiO_2$ at concentration of 1.0 g $L^{-1}$ was carried out by Robertson et al. (2005). A decrease of 5 log units was reached after 120 min of treatment, however, quite surprisingly, little difference was observed in experiments without catalyst and only with UV-A lamp. In contrast to the work of Robertson et al. (2005), photocatalysis has proved much more effective than UV treatment in our experiments. The faster photocatalytic inactivation compared to UV-C can be explained by the different mechanism of action of the two processes. The mechanism for bacterial destruction by TiO2 has been proposed to occur via attack by hydroxyl radicals generated on the photocatalyst surface and the mode of microbial destruction suggest that initial target for photocatalytic attack is the bacterial cell wall (Matsuanga et al 1985; Sunada et al. 2003).

Wang et al. (2013) also reported that the rate of inactivating bacteria or actual antimicrobial activity by TiO2 depends on the initial bacterial concentration as well as bacteria population during the inactivation. Possibly, when the initial bacteria population is too high, there are many dead cells and mineralization product in the reaction process; these intermediate products of photocatalysis have the function of protecting

live bacteria, and the factor likely to be contributing to the loss of antibacterial activity is competition between these intermediate and bacteria for OH radicals.

Rizzo et al. (2014) investigated the effect of UV radiation on antibiotic resistant E.coli strains at laboratory scale (500 mL), and a total inactivation (initial bacterial load was $10^6$ CFU $mL^{-1}$) was reached after 10 min of treatment. The total inactivation in $TiO_2$/Sunlight experiment was achieved after 60 min, with 100 mg $L^{-1}$ of $TiO_2$. Berney et al. (2006) claim that inactivation mechanisms can be different according to the enteric pathogen considered. Salmonella strain inactivation under sunlight irradiation has been shown to be one of the most resistant strains to disinfection (Evison, 1988). Sciacca et al. (2011) claim that in disinfection context, a pathogen bacterium such as Salmonella should often be considered as an indicator of the disinfection effectiveness.

#### 5.1.4.2 Reversion of Salmonella strain in disinfection treatment

**UV-C disinfection tests**

Disinfection experiments on Salmonella aqueous suspensions resulted in the formation of mutants (Fig. 5.1). Salmonella mutants should be considered as potentially carcinogenic for human and animals (Zeiger, 1998).

The formation of mutants increased as UV dose increased up to a maximum of 1.1 x $10^3$, 1.9 x $10^3$ and 3.9 x $10^3$ for $10^9$, $10^8$ and $10^7$ CFU $mL^{-1}$ initial bacterial density respectively. The corresponding percentage increase with respect to the initial density of mutants was, 58, 210 and 358%, respectively. UV doses higher than 6.0, 1.0 and 0.5 mWs $cm^{-2}$ respectively resulted in a progressive decrease of mutants and although Salmonella still persist, no further formation of mutants was detected (Fig. 5.1).

The drastic increase of mutants with decreasing initial bacterial load, is possibly due to the corresponding lower turbidity of the solution which results in a higher UV radiation absorption in microorganisms. Higher absorbed radiation means higher stress to bacteria which possibly resulted in a higher formation rate of mutants.

Although several studies reported the relationship between genotoxicity of waters and the intensity of human activities (Vargas et al., 2001; Tagliari et al., 2004) only a few studies have been focused on mutagenic activity of conventional water/wastewater disinfection processes, such as chlorination or UV-C radiation. Basically these studies investigated the effect of the treatment process on target contaminants or real water/wastewater matrix to evaluate its effect on mutagenicity; accordingly, Ames test has been used as a tool to evaluate whether disinfection/oxidation process affected mutagenicity. For example, Monarca et al. (2000) observed that, unlike biological process, disinfection affected mutagenic activity in urban wastewater treatment plants. In particular, unlike UV disinfection, chlorination process (about 110 mutants per liter were detected after treatment with 1.5 mg $L^{-1}$ of chlorine) resulted in an increased mutagenicity. The genotoxic activity of water samples before and after treatment by UV radiation was investigated by Haider et al. (2002). Water samples from different geographic areas in Austria showed a weak genotoxic activities after treatment by low pressure UV-C irradiation, compared to drinking water samples from other countries under similar experimental conditions. . To authors' knowledge, no study investigated the effect of disinfection process on the formation of mutants after Salmonella suspensions treatment. But Tate et al. (2006) characterized the damage of UV-C radiation in Salmonella Typhimurim; with the exception of TA98 strain, the damage has been found primarily localized in the nucleic acids and results from direct absorption of photons by the target molecules. Moreover, they claim that the damage produced by UV-C is predominately in the form of pyrimidine dimers and 6-4 photoproducts resulting from direct photon absorption by DNA.

**$TiO_2$ photocatalysis tests**

The same trend in the formation of Salmonella mutants was confirmed in the $TiO_2$ photocatalytic tests, where an increase up to a maximum of 9.5 x $10^2$, 1.1 x $10^3$ and 1.4 x $10^3$, for $10^9$, $10^8$ and $10^7$ CFU $mL^{-1}$ initial bacterial density, respectively was observed. The percentage of mutants was significantly lower (35, 77 and 120% for $10^9$, $10^8$ and $10^7$ CFU $mL^{-1}$ initial bacterial density, respectively) compared to UV-C disinfection experiments (Fig. 5.2). The different mechanism of bacterial inactivation, physical (UV radiation) and chemical (radicals formation in $TiO_2$

photocatalytic process), possibly affected mutants' formation in different ways.

The effect of different oxidants/disinfectants (namely, chlorine, chlorine dioxide and ozone) on genotoxicity has been studied by different authors (Monarca et al., 2000; Mišík et al., 2011; Magdeburg et al., 2014), but no information is available in scientific literature (to authors' knowledge) on their and photocatalytic process effect on the formation of mutants in Salmonella strains after treatment. For example, Magdeburg et al. (2014) studied the effect of ozone, as tertiary treatment, in a pilot scale wastewater treatment. They observed genotoxic effects were reduced by both tertiary treatments with better performance of ozonation leading to a >70% elimination of genotoxic activity at the lowest ozone dose tested. However, the Ames assay using the YG7108 strain revealed an ozone-dose dependent mutagenicity increase after WW ozonation, indicating the formation of alkylating mutagenic oxidation byproducts. The impact of ozone treatment on genotoxic and acute toxic effects of tertiary treated municipal wastewater was investigated by Mišík et al. (2011). After ozone treatment they observed a decrease of the mutagenic activity of the samples and the bactericidal effects were reduced by ozonation. The influence of disinfectants as chlorine dioxide, ozone, peracetic acid and UV radiation, on the formation of mutagenic has been evaluated by Monarca et al. (2000). They tested mutagenicity using the Ames test and all disinfectant treatments investigated, produced bacterial mutagenicity, particularly chemical treatment with $ClO_2$ or ozone, in contrast with physical treatment with UV-C lamp. $ClO_2^-$ treated water seems to promote cellular divisions in order to favor the formation of mutants. In addition to wastewater before biological treatment and wastewater disinfected with peracetic acid showed a significant increase in chromosomal anaphase aberrations, revealing the presence of unknown genotoxic compounds. Unknown direct mutagenic compounds caused mainly frame-shift point mutations in the Ames test.

### 5.1.5 Conclusion

This work shows that both UV-C disinfection and $TiO_2$ photocatalytic disinfection can effectively inactivate Salmonella but, in the same time they can result in the formation of mutants. UV-C radiation process was slightly faster in the inactivation of Salmonella but photocatalytic process

required lower UV dose . The formation of mutants increased as UV dose increased. UV doses higher than 6.0, 1.0 and 0.5 mWs $cm^{-2}$ in the experiments with $10^9$, $10^8$ and $10^7$ CFU $mL^{-1}$ initial bacterial density, respectively, resulted in a progressive decrease of mutants and although Salmonella still persist, no further formation of mutants was detected. The same trend in the formation of Salmonella mutants was confirmed in the $TiO_2$ photocatalytic tests, but the percentage of mutants was significantly lower compared to UV-C disinfection experiments.

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## 5.2 EFFECT OF DIFFERENT LIGHT SOURCES ON THE PHOTOCATALYTIC INACTIVATION OF AR E.COLI STRAIN

### 5.2.1 Abstract

The effect of $TiO_2$ photocatalysis on the inactivation of an antibiotic resistant Escherichia coli strain selected from an urban wastewater treatment plant (UWWTP) effluent was investigated. Different light sources including a 250 W wide spectrum lamp, a 125 W UV-A lamp and solar radiation, as well as, photocatalysts loadings ($TiO_2$ Degussa P25) in the range from 0.05 to 2.00 g $TiO_2$ $L^{-1}$ were evaluated. The higher efficiency (total bacterial inactivation after 10 min of irradiation) was observed in the absence of $TiO_2$ when the wastewater was irradiated using the 250W lamp. In the presence of $TiO_2$ a decreasing inactivation trend was observed (99.76% and 72,22% inactivation after 10 min irradiation at 0.10 and 2.00 g $TiO_2$ $L^{-1}$ respectively). Under solar simulated conditions the highest inactivation efficiency (93.17%) after 10 min of irradiation was achieved at the lower photocatalyst loading (0.05 g $TiO_2$ $L^{-1}$). The concept of "reactor optical thickness" was introduced to explain the rates of disinfection observed. The optimum photocatalyst loading estimated by radiation absorption-scattering modeling was found to be 0.1 g $TiO_2$ $L^{-1}$ for all lamps. The difference between experimental tests and modeling may be due to $TiO_2$ particles aggregation. Comparative kinetic tests between solar and solar simulated photocatalytic (SSP) processes using 0.05 g $TiO_2$ $L^{-1}$ in suspension showed a quite similar inactivation behavior up to 30 min of irradiation, but only the SSP process resulted in a total inactivation of bacteria after 60 min of exposure. Antibiotic resistant test (Kirby-Bauer) on survived colonies showed that the SSP and SP processes affected in different ways the resistance of E. coli strain to the target antibiotics.

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Antonio Della Sala, former MSc student at University of Salerno, Italy;

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### 5.2.2 Introdution

The widespread use of antibiotics for human, veterinary and aquaculture purposes results in their continuous release into the environment (Díaz-Cruz et al., 2003; Batt et al., 2006; Watkinson et al., 2009; Fram and Belitz, 2011) since these species are only partially metabolized by organisms. Their occurrence into the environment is of particular concern because of the development of antibiotic resistance in bacterial populations (ARB), which results in the loss of antibiotics effectiveness for the treatment of several diseases (Schwartz et al., 2003). There are four main genetic reactors in which antibiotic resistance evolves (Baquero et al., 2008): (i) human and animal microbiota, (ii) hospitals, long-term care facilities, farms, or any other place in which susceptible individuals are crowded and exposed to bacterial exchange, (iii) wastewater facilities and (iv) soil, surface and ground water, where the bacterial organisms originated in the previous reactors, mix and counteract with environmental organisms.

Among wastewater facilities, conventional urban wastewater treatment plants (UWWTPs) typically operated by biological processes, are suspected to contribute to ARB selection, as well as, resistance transfer among bacteria (Rizzo et al., 2013a). Therefore, the release of ARB into the receiving water should be controlled by an effective disinfection treatment since conventional disinfection processes (e.g., chlorination and UV radiation) are partially effective in controlling ARB spread (Munir et al., 2011; Rizzo et al., 2013b). Consequently, alternative/new disinfection processes should be investigated to reduce the formation of ARB and antibiotic resistance of survived colonies.

Advanced oxidation processes (AOPs) (e.g., Fenton, photo-Fenton, $TiO_2$ photocatalysis, $UV/O_3$, $UV/H_2O_2$ etc.) have been successfully investigated for the removal of a wide range of contaminants (Zapata et

al., 2010; Rizzo, 2011; Sannino et al., 2013; Murcia et al., 2013). However, despite of the extensive literature on the inactivation of microorganisms by AOPs (Malato et al., 2009; Dunlop et al., 2011), only a few studies have focused on the effect of AOPs on ARB (Tsai et al., 2010; Öncü et al., 2011) and particularly on indigenous ARB (Rizzo et al., 2014). Among AOPs, $TiO_2$ photocatalysis has recently emerged as an effective water disinfection option, an alternative to disinfection with chemicals which results in the formation of harmful and toxic disinfection by-products (Richardson et al., 1996; Fernández et al., 2005; Rizzo, 2009a). Despite an increasing number of scientific studies in photocatalysis has demonstrated its efficacy in water and wastewater treatment, industrial applications are still limited possibly because of some technical/economical limitations. Among these, the removal of the catalyst after treatment is a challenge not yet successfully addressed (Rizzo, 2009b).

The $TiO_2$ photocatalytic process has been found to be effective in the inactivation of a wide range of pathogens in water, including indicator bacteria, e.g. Escherichia coli (Bekbölet, 1997), bacterial spores (Dunlop et al., 2008) and protozoa (Sunnotel et al., 2010). However, the effectiveness of TiO2 photocatalysis on the inactivation of ARB and their resistance in wastewater effluents has not been investigated. E. coli are typically detected in UWWTP effluents, and E. coli strains resistant to antibiotics have been isolated (Reinthaler et al., 2003; Ferreira da Silva et al., 2007; Rizzo et al., 2012). Therefore, the treatment of UWWTP effluent by new/alternative disinfection processes to effectively control the extent of ARB release in wastewater-polluted stream is in need of investigation.

In the present study, the effect of $TiO_2$ photocatalysis process on a multi drug resistant E. coli strain selected from the effluent of an UWWTP was investigated under different irradiation conditions (using artificial and solar light) and photocatalysts loadings. Antibiotic resistance of E. coli strain to ciprofloxacin (CIP), cefuroxime (CEF), tetracycline (TET) and vancomycin (VAN), before and after photocatalytic treatment, was evaluated by Kirby-Bauer method. In contrast to previous studies on water disinfection, in this study we introduce the concept of reactor optical thickness to explain the rates of disinfection observed

### 5.2.3 Material and Methods

#### 5.2.3.1 Wastewater samples

Wastewater samples were collected from a large UWWTP (250,000 equivalent inhabitants) located in southern Italy, from the effluent of the biological treatment process (activated sludge) just upstream of the disinfection unit currently employing chlorination. Samples were collected in sterilized 1 L amber glass bottles. The wastewater was characterized as follows: pH 7.9, $BOD_5$ 10.0 mg $L^{-1}$, COD 23.3 mg $L^{-1}$, TSS 32.5 mg $L^{-1}$, redox potential 63.6 mV, conductivity 1105 μS $cm^{-1}$.

#### 5.2.3.2 Inoculum and sample preparation

Multi drug resistant E. coli strain was selected according to the methodology published in Rizzo et al., (2012). Briefly, 50 mL of wastewater sample were filtered through 0.45 μm membrane filters (Millipore, Billerica, MA, USA) and then cultivated (24 h incubation time at 44 °C) in tryptone bile X-glucuronide (TBX) agar medium (Oxoid, Basingstoke, UK), a selective, chromogenic medium for the detection and enumeration of E. coli. Ten colonies were randomly collected from TBX agar medium after the incubation period and used in the subsequent step for the selection of the resistant strains. Each colony was cultivated (24 h incubation time at 37 °C) in four different tryptone soya agar (TSA) media (Oxoid, Basingstoke, UK) prepared with a mixture of three antibiotics (amoxicillin, ciprofloxacin, sulphametoxazole). The antibiotics concentrations were chosen according to the respective minimum inhibiting concentrations for E. coli listed in "Clinical and Laboratory Standards Institute" documentation (CLSI, 2011). The E. coli strains were taken from the Petri dish, transferred in 15% glycerol tryptic soya broth (TSB) (Oxoid, Basingstoke, UK) and frozen at - 20 °C. The selected strains were identified by the Rapid One System method (Remel, Lenexa, KS, USA). In particular, among the selected colonies, the E. coli strain that showed the higher resistance, which was growth on the medium enriched with 1 mg $L^{-1}$ of ciprofloxacin, 8 mg $L^{-1}$ of amoxicillin, 32 mg $L^{-1}$ of sulphametoxazole (Rizzo et al., 2013b) was used for the photocatalytic tests.

Wastewater samples were first autoclaved (15 min at 121°C) to remove indigenous bacteria and then inoculated with the selected E. coli strain. The E. coli strain was unfrozen and transferred into 10 mL physiological solution to achieve $10^7$ CFU 100 $mL^{-1}$ (0.5 McFarland). The physiological solution was then added to 500 mL wastewater sample.

#### 5.2.3.3 $TiO_2$ photocatalysis tests

Photocatalytic experiments were carried out in a 2.2 L cylindrical glass reactor (13.0 cm in diameter) filled in with the 500 mL wastewater sample. The thickness of liquid traversed by photon beams was constant and equal to the liquid depth (5 cm). The reactor was placed in a water bath to control the temperature at 30°C during the experiments. The solution in the reactor was stirred continuously. Three different artificial light sources were used to irradiate the wastewater: (i) a wide spectrum 250 W lamp (Procomat, Italy), (ii) the same lamp equipped with a filter to simulate solar radiation, and (iii) a 125 W black light fluorescent lamp. The lamps were located horizontally 40 cm (250 W lamp) and 15 cm (125 W lamp) above the surface of the water. 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 lamps and solar light (Fig. 5.3).

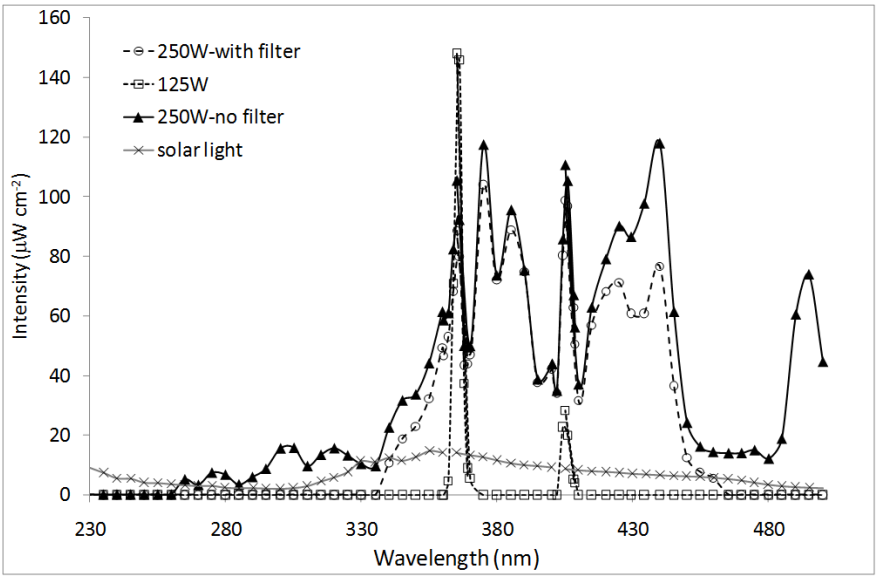


**Figure 5-3 Irradiance spectra of UV lamps and solar light**

In photocatalytic experiments, a suitable amount of $TiO_2$ powder (Degussa P25) was added to the autoclaved wastewater sample and sonicated for 5 min. The inoculum was subsequently added to the wastewater. Preliminary E. coli inactivation experiments under irradiation lasting 10 min were carried out in the absence of catalyst and varying the concentration of $TiO_2$ in the range 0.05-2.00 g $L^{-1}$. Further experiments (60 min long) were performed under irradiation with solar light and simulated solar light, using an optimized catalyst concentration related to the irradiation conditions and reactor geometry used.

#### 5.2.3.4 Bacterial count

Bacterial count was performed by the spread plate method. Briefly, small amounts of wastewater samples were diluted according to the expected number of colonies. 100 μL were spread on TBX agar medium (Sigma Aldrich) and incubated at 44 °C for 24 h. Measurements were carried out in triplicates and average values and standard deviation were plotted as CFU 100$mL^{-1}$.

#### 5.2.3.5 Antibiotic resistance assay

The antibiotic resistance of bacterial colonies, before and after the photocatalytic treatment, was tested by Kirby–Bauer method according to standard recommendations (Clinical Laboratory Standards, 2006). Briefly, the colonies survived to the photocatalytic treatment were collected from the TBX agar medium and transferred into a 10 mL physiological solution to achieve 107 CFU 100 $mL^{-1}$ (0.5 McFarland). Then bacterial suspensions were spread on Mueller Hinton agar (Biolife, Italy) using a sterile cotton swab. Antibiotic discs of CIP (5 mg), CEF (30 mg), TET (30 mg) and VAN (30 mg) (all from Biolife) were placed on the surface of each inoculated plate. After 24 h of incubation at 37°C, the diameters of antibiotic inhibition of growth were measured and recorded as susceptible (S), intermediary (I) or resistant (R) according to the corresponding values set by EUCAST (2012) where available (Table 5.1).

**Table 5.1 Inhibition and resistance diameter values (mm) according to EUCAST database**

| CIP | CEF | TET | VAN |
|---|---|---|---|
| R ≤ 19 | R ≤ 18 | R ≤ 11 | - |
| 19 < I ≤ 22 | - | 11 < I ≤ 15 | - |
| S > 22 | S > 18 | S > 15 | - |

### 5.2.3.6 Radiation absorption–scattering modeling

The effect of optical thickness τλ on the rate of E. coli inactivation was analysed using the model proposed by Li Puma and Brucato (2007).
The optical thickness is a function of the thickness of wastewater penetrated by photon beams δ, the specific extinction coefficient (βλ, scattering and absorption) of the aqueous suspension of photocatalyst and the photocatalyst concentration, Ccat (Eq. 1).

$$\tau\lambda = \beta\lambda C_{cat}\delta \qquad \text{(Eq. 1)}$$

Since $\beta_\lambda$ is a function of wavelength of the incident radiation, $\lambda$, a spectral averaged value of the extinction coefficient, $\beta$, can be calculated as a weighted function of the spectral intensity of the incident radiation $I_\lambda$ (Eq. 2).

$$\beta = \frac{\int_{\lambda_{min}}^{\lambda_{max}} \beta_\lambda I_\lambda \, d\lambda}{\int_{\lambda_{min}}^{\lambda_{max}} I_\lambda \, d\lambda} \qquad \text{(Eq. 2)}$$

where $\lambda_{min}$ and $\lambda_{max}$ are the lowest and highest wavelengths that can be absorbed by the photocatatalyst. In such way the emission spectra of each different radiation source (artificial light with and without filter or solar radiation) are considered in the evaluation of a spectral averaged value of the optical thickness (Eq. 3).

$$\tau = \beta C_{cat} \delta \qquad \text{(Eq. 3)}$$

Eq. 3 allows a comprehensive investigation of the combined effect of catalyst concentration and light sources on the rate of inactivation of a multi drug resistant *E. coli* strain selected from the effluent of an UWWTP

### 5.2.4 Result and Discussion

#### 5.2.4.1 Radiation absorption–scattering modeling

The efficiency of $TiO_2$ photocatalysis in wastewater disinfection depends on the wastewater characteristics, photocatalyst loading, the radiation field and light source. In water disinfection processes in the absence of photocatalyst, the liquid is often transparent to light (very low extinction coefficient). In consequence, the irradiance gradient across the thickness of the liquid is often neglected and the disinfection rate becomes a function of the UV dose received by the water. Therefore, the rate of disinfection depends solely on the intensity of the incident radiation and the exposure time. In contrast, in $TiO_2$ photocatalysis there are strong gradients of the irradiance across the liquid depth penetrated by light due to the high extinction coefficients of $TiO_2$ suspensions, therefore, the efficiency of the disinfection process is also governed by the photocatalyst loading, the light source and the reactor geometry. The optical thickness of the aqueous suspension in the reactor is the fundamental dimensionless parameter that interrelates the above factors, that determines the optimal use of the photon energy and that affects the rate of disinfection.

Figure 5.4 shows the extinction coefficient of Degussa P25 as a function of photon wavelength in the range 250- 384 nm measured with a UV-vis spectrophotometer.

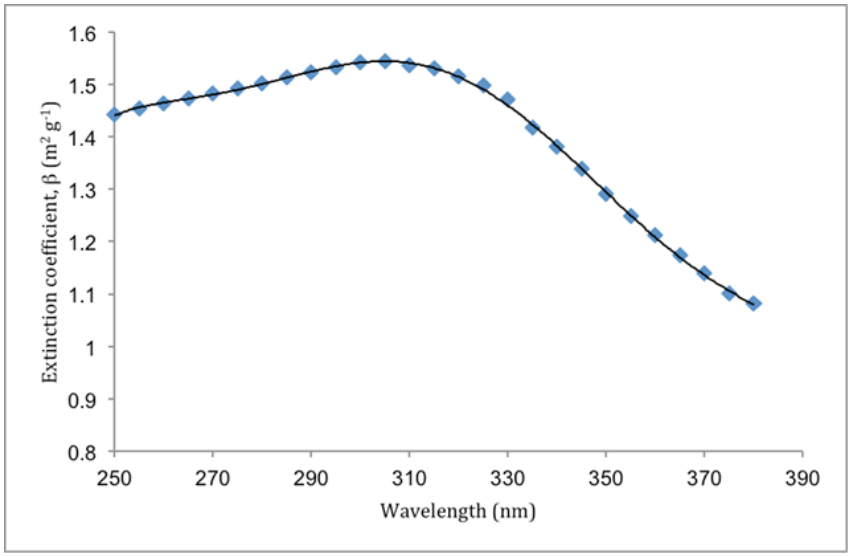


**Figure 5-4 Extinction coefficient of Degussa P25 as a function of photon wavelength in the range 250- 384 nm**

Above 384 nm $TiO_2$ does not absorbs radiation and all incident radiation is scattered. From these data and from the lamps emission spectra (Fig. 5.1) Eq. (3) can be evaluated to calculate the spectral averaged extinction coefficients. The results in Table 5.2 show the spectral averaged extinction coefficients for each irradiation arrangement of the suspension and the optical thicknesses calculated at different catalyst concentrations.

**Table 5.2 Spectral averaged extinction coefficients for each irradiation arrangement of the suspension and optical thickness as a function at catalyst concentration. Liquid depth is 0.05 m**

| | *Wide spectrum 250W lamp – no filter* | *Wide spectrum 250W lamp – with UVC/B filter* | *125 W blacklight lamp* |
|---|---|---|---|
| Spectral averaged extinction coefficient $\beta$ ($m^2$ $Kg^{-1}$) | 1252 | 1160 | 1172 |
| Catalyst concentration | Optical thickness | Optical thickness | Optical thickness |

| ($Kg\ m^{-3}$) | | | |
|---|---|---|---|
| 0 | 0 | 0 | 0 |
| 0.05 | 3.13 | 2.90 | 2.93 |
| 0.1 | 6.26 | 5.80 | 5.86 |
| 0.2 | 12.5 | 11.6 | 11.7 |
| 0.3 | 18.8 | 17.4 | 17.6 |
| 0.4 | 25.0 | 23.2 | 23.4 |
| 0.5 | 31.3 | 29.0 | 29.3 |
| 1 | 62.6 | 58.0 | 58.6 |
| 1.5 | 93.9 | 87.0 | 87.9 |
| 2.0 | 125 | 116 | 117 |

For the conditions of the experiments, the optical thicknesses varied from 0 to 125. For the scattering albedo of Degussa P25 equal to 0.7396 the optical thickness that optimizes the absorption of radiation should be about the value of 6 (Li Puma and Brucato, 2007). This corresponds to the condition of irradiation of the entire suspension volume which also yields the highest rate of photon absorption of the incident radiation. Such situation should yield the highest rate of photocatalytic activity and E. coli inactivation, which is realized with all lamps when 0.1 g $L^{-1}$ of $TiO_2$ was used. Above or below such optimum catalyst concentration, lower photocatalytic inactivation rates are expected due to the darkening of the farther region of the catalyst suspensions at higher catalyst concentrations, or to excessive losses of radiation by transmission through the suspension at lower catalyst concentrations.

#### 5.2.4.2 Effect of light source and photocatalyst loading

Figure 5.5 shows the E. coli inactivation achieved under irradiation with the 250 W lamp, without filter, and varying the photocatalyst loading in the range 0.00-2.00 gr $TiO_2$ $L^{-1}$. As shown, total bacterial inactivation (100%) was achieved in the absence of photocatalyst, due to the UV-C germicidal effect exhibited by this lamp (Fig. 5.3). In the presence of TiO2, bacterial inactivation results from the combined effect of UV-C radiation and hydroxyl radicals formed by UV activation of $TiO_2$. The highest rate of deactivation was achieved at 0.1 g $L^{-1}$, which

corresponded to the optimal optical thickness for the suspension in the reactor.

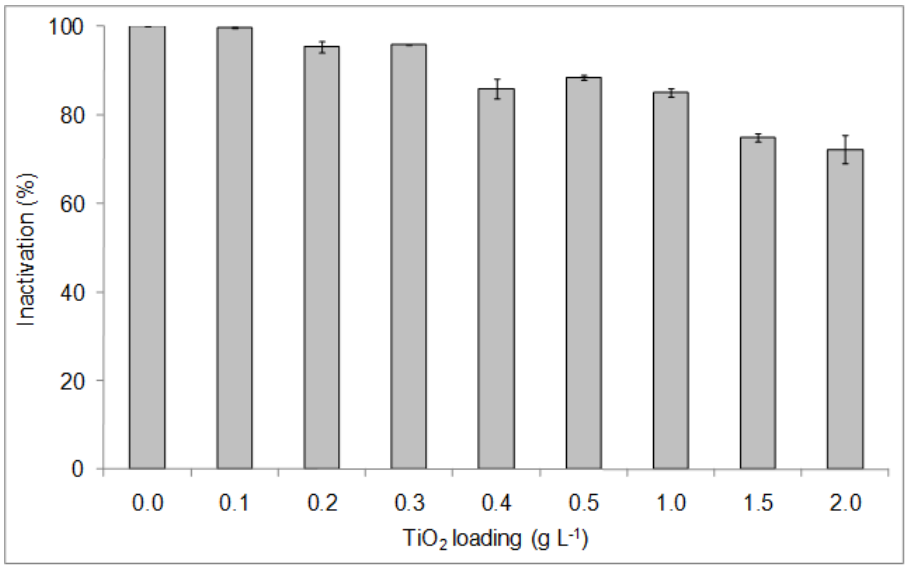


**Figure 5-5 Inactivation of multi drug resistant E. coli strain using 250 W lamp without filter and varying the photocatalyst loading in the range 0.00-2.00 gr TiO2 L-1**

At higher $TiO_2$ concentrations ranging from 0.2 to 2.0 g $L^{-1}$ a progressively larger fraction of the reactor nearer the back wall of the reactor was being obscured and, as a result, the bacterial inactivation decreased. The rate of inactivation at 2.0 g $L^{-1}$ $TiO_2$ (72.22%) was relatively high in comparison to what could be expected in a flow-through reactor at such large values of the optical thickness of the suspension. This relatively high rate of deactivation was realized since the solution was continuously stirred during the irradiation experiments which resulted in the transport of fresh bacteria from the darker region of the reactor volume towards the illuminated region.

When the reactor was irradiated with the 250 W lamp through a UVA-B filter to simulate solar radiation, the bacterial inactivation varied significantly (Fig. 5.6). In particular, in the absence of $TiO_2$, a mild rate

of bacteria deactivation (23.86%) was observed, since the energy of the photons was insufficient to penetrate the cell wall.

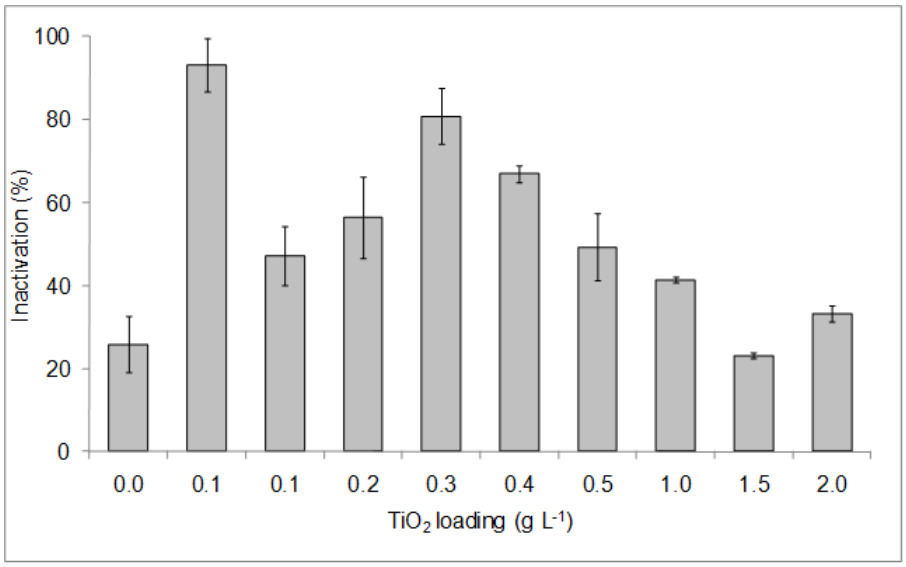


**Figure 5-6 Inactivation of multi drug resistant E. coli strain using simulated solar radiation (250 W lamp with filter) and varying the photocatalyst loading in the range 0.00-2.00 gr TiO2 L-1**

The lower bacterial inactivation rate can be easily explained according to the emission spectra of both lamps (Fig. 5.3). In particular, the intensity peak at 254 nm (bactericidal wavelength) of solar simulated lamp was much lower compared to the lamp without the filter. In the solar simulated lamp the contribution to bacteria inactivation by hydroxyl radicals alone was significantly higher compared to inactivation by direct UV-C irradiation. When the effect of photocatalyst loading was investigated in solar simulated photocatalytic tests, the higher inactivation efficiency was observed at the lowest $TiO_2$ loading (0.05 g $L^{-1}$). This result was also observed in our previous study, in which we determined that particle size and particle size distribution varied at different catalyst loadings due to catalyst aggregation, which affected the disinfection efficiency (Rizzo et al., 2014). Moreover, these results are in agreement with previous studies of inactivation of pure culture of E. coli in a solar reactor with suspended $TiO_2$ P25 particles (Fernández et al.,

2005). The behaviour of photocatalytic inactivation under different photocatalysts loadings in our study matches the typical behaviour observed in slurry photocatalytic reactors (Malato et al., 2009). Specifically, the initial inactivation rate increases as photocatalyst loading increases, up to reach a certain value. Then, further increases in the photocatalyst loading result in a decrease of the disinfection efficiency due to the light screening effect that outer layers of photocatalysts particles offer to the inner layers in the photoreactor.

E. coli inactivation was also performed using a typical light source used in photocatalytic experiments (UV-A black light lamp with an emission peak typically < 380 nm). In the absence of $TiO_2$ the inactivation rate was very low (2.87%, Fig. 5.7) since this lamp did not emit in the germicidal region (UV-C and UV-B, Fig. 5.3). In contrast, in the presence of TiO2 the higher inactivation efficiencies were observed for the lower catalyst loadings with a peak at 0.1 gr $TiO_2$ $L^{-1}$, in agreement with the optimal optical thickness for this reactor. The E. coli inactivation mechanism is due to the attack by the hydroxyl radicals formed by photocatalysis.

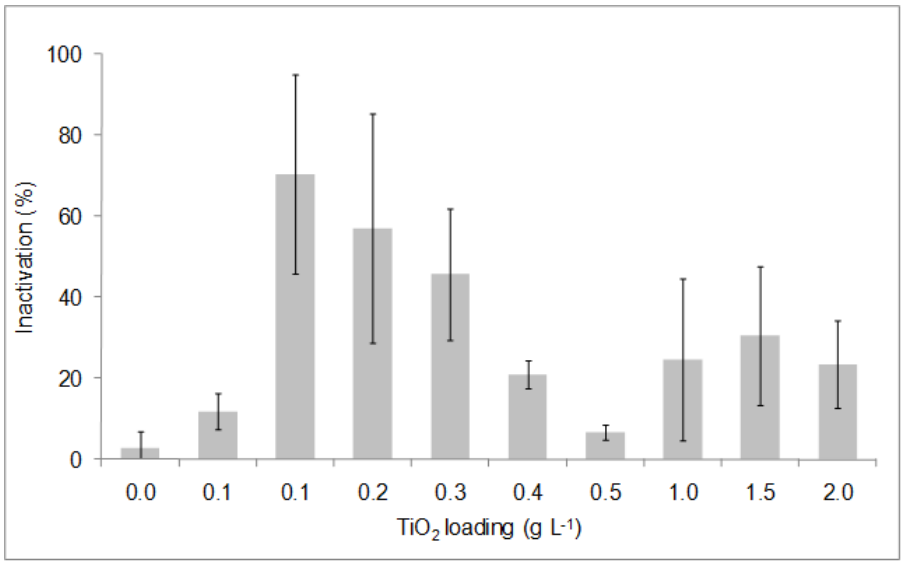


**Figure 5-7 Inactivation of multi drug resistant E. coli strain using 125 W UV-A lamp and varying the photocatalyst loading in the range 0.00-2.00 gr TiO2 L-1**

Tsai et al. (2010) also used an UV-A light source for the $TiO_2$ photocatalytic inactivation of clinical ARB isolates (namely, methicillin-resistant Staphylococcus aureus, multidrug-resistant Acinetobacter baumannii and vancomycin-resistant Enterococcus faecalis). The lower inactivation efficiency at 10 min irradiation time observable in most of these experiments were due to the differences in the (i) aqueous matrix, (ii) type and (iii) initial density of model ARB.

#### 5.2.4.3 Inactivation kinetics of multi drug resistant E. coli strain: comparison between solar and solar simulated TiO2 photocatalytic processes

Although the higher inactivation efficiencies of the multi drug E. coli strain colonies were observed with the 250 W lamp, the high energy consumption compared to conventional UV lamps used in wastewater disinfection does not make this option economically attractive. In contrast, in the last years solar driven photocatalytic treatment has been increasingly investigated because of the possibility to be economical on energy consumption, thus making this technology particularly attractive (Malato et al., 2009). Accordingly, we evaluated the inactivation kinetics of the multi drug resistant E. coli strain by applying solar simulated $TiO_2$ photocatalysis (SSP) and natural solar driven photocatalytic (SP) process. Both processes were operated with 0.05 gr $TiO_2$ $L^{-1}$, approximately $10^7$ CFU 100 $mL^{-1}$ initial bacterial density and 60 min total irradiation time. The inactivation of multi drug resistant E. coli strain colonies was quite similar (90% inactivation) in the early 30 min of irradiation in both processes (Fig. 5.8). However, total inactivation was observed after 60 min irradiation time only for SSP. The SP process did not result in total bacteria inactivation, but 99% removal of initial bacterial density (16000 CFU 100 mL-1 residual density) was observed after 60 min irradiation time.

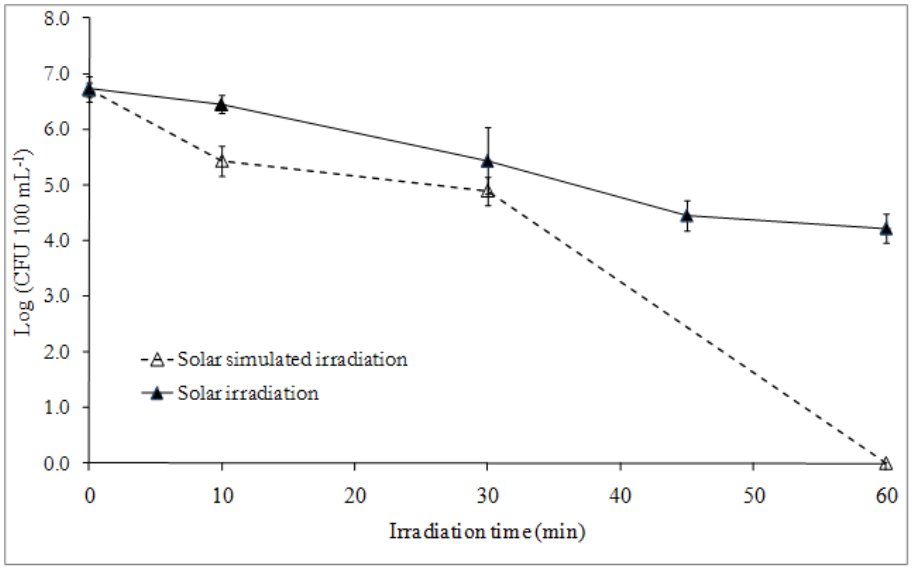


**Figure 5-8 inactivation kinetic of multi drug resistant E. coli strain by solar simulated (250 W lamp with filter) photocatalysis and natural solar driven photocatalytic process (0.05 gr TiO2 $L^{-1}$, approximately $10^7$ CFU/100 mL initial bacterial density).**

The lower inactivation rate in the SP process can be explained by the appearance of a cloud coverage of the sky in the last 15 min of the experiment combined with the significantly lower solar irradiance compared to the irradiance with the simulated solar light (Fig. 5.3). The effect of photocatalytic processes on bacterial inactivation has been widely investigated on pure culture of microorganisms suspended in purified or deionized water, but only a few studies have addressed the combination of actual wastewater and natural bacteria (Lydakis-Simantiris et al., 2010; Rizzo et al., 2014). In our previous study we have investigated the effect of N-doped $TiO_2$ photocatalyst to improve solar radiation absorption and consequently the photocatalytic disinfection efficiency, which resulted in the total bacteria inactivation, was achieved after 60 min of irradiation (Rizzo et al., 2014). The present results are also in a quite good agreement with Liu et al. (2006) work, where a total inactivation occurred within 120 min of irradiation (average light intensity 10 mW $cm^{-2}$), using an N-doped $TiO_2$ photocatalyst, but starting with a higher E. coli density ($10^9$ CFU $mL^{-1}$) compared to our work.

#### 5.2.4.4 Effect on antibiotic resistance: comparison between solar and solar simulated TiO2 photocatalytic processes

The effect of SSP and SP processes on the resistance of the selected E. coli strain to the target antibiotics (CIP, CEF, TET and VAN) was investigated by the Kirby-Bauer test. According to this method, the resistance of bacterial colonies to the target antibiotic is evaluated in terms of inhibition diameter, which is the diameter of the area around the antibiotic disc on the culture media, where bacterial colonies are incapable of growing. The larger the inhibition diameter, the lower the bacterial resistance to the antibiotic.
The average value of inhibition diameter for CIP, CEF, VAN and TET before SSP treatment (t = 0) was found to be as high as 12.8, 25.8, 10.2, and 10.1 mm, respectively. Compared to the corresponding clinical breakpoints values for E. Coli from EUCAST database (Table 1), the E. coli strain selected from the UWWTP effluent is resistant to CIP and TET, but susceptible to CEF (no data available for VAN in EUCAST database) (t = 0, Fig. 5.9).

As the E. coli suspension was disinfected by the SSP process the resistance of the survived colonies to CIP, CEF and VAN decreased (inhibition diameter increased from 12.8 to 13.8 mm, from 25.8 to 27.3 mm and from 10.2 to 10.9 mm, respectively) as irradiation time was increased (from 0 to 30 min, respectively, Fig. 5.7), but these trends were not found to be statistically significant.

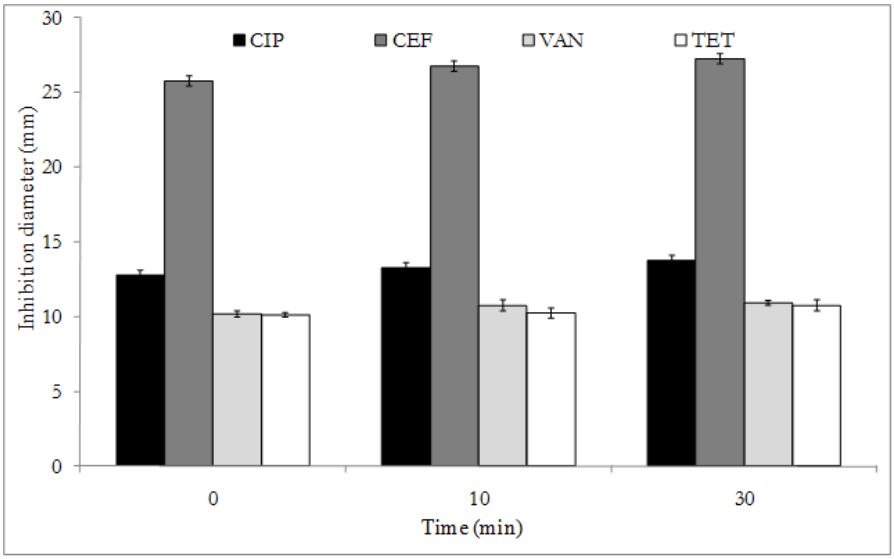


**Figure 5-9 Average value and standard deviation of inhibition diameter for CIP, CEF, VAN and TET before and after solar simulated (250 W lamp with filter) photocatalysis treatment**

Conversely, a significant statistically increasing trend ($p = 0.033 < \alpha < = 0.05$) was observed for the E. coli resistance to TET (inhibition diameter increased from 10.1 to 10.8 mm).

Figure 5.10 shows the effect of the SP process on antibiotic resistance of the survived colonies, at different irradiation times. The resistance of survived colonies to CIP and CEF decreased (inhibition diameter increased from 13.0 to 14.0 mm, and from 25.5 to 28.8 mm, respectively) as irradiation time was increased (from 0 to 60 min, respectively). These trends were found to be statistically significant ($p = 0.019$ and 0.0008, respectively) for both antibiotics. No statistically significant trends were observed for resistance to TET and VAN.

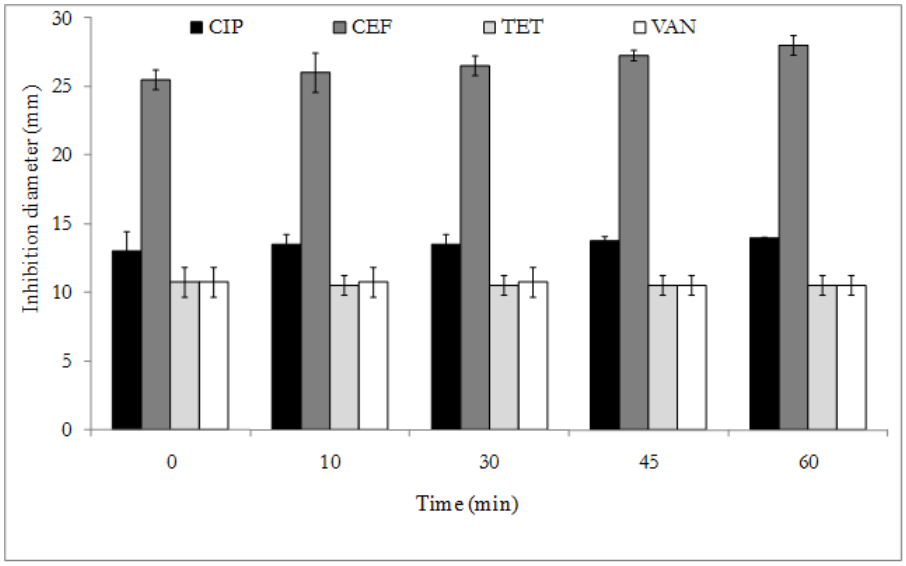


**Figure 5-10 average value and standard deviation of inhibition diameter for CIP, CEF, VAN and TET before and after natural solar photocatalysis treatment**

These results are in good agreement with our previous work where N-doped $TiO_2$ was used as photocatalyst under similar operating conditions (Rizzo et al., 2014). The differences in terms of resistance between SSP and SP processes may be due to the different light sources and intensities (lamp and sun light respectively) which resulted in different inactivation efficiencies (Fig. 5.8) as well as different DNA damage of survived colonies from UV-C component of light source.

### 5.2.5 Conclusion

The effect of $TiO_2$ photocatalysis on the inactivation of an antibiotic resistant E. coli strain selected from an UWWTP effluent was investigated. SP and SSP processes (0.05 g $TiO_2$ $L^{-1}$ in suspension) showed similar inactivation behavior up to 30 min of irradiation, but only the SSP process resulted in the total inactivation of bacteria after 60 min of exposure. SSP and SP processes affected in different ways the resistance of E. coli strain to the target antibiotics. Accordingly, $TiO_2$ photocatalysis can be effective in controlling ARB release from UWWTP effluents, but suitable operating conditions should be set out to optimize

the inactivation efficiency. Optimizing the reactor optical thickness may provide the most efficient rate of disinfection. However, no conclusive results on the effect of the investigated photocatalytic processes on antibiotic resistance of survived colonies show that the risk of antibiotic resistance spread exists and the potential for resistance transfer among bacteria in UWWTP effluent receiving stream is worth of further investigation.

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## 5.2.7 Appendix

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# Disinfection of urban wastewater by solar driven and UV lamp – $TiO_2$ photocatalysis: Effect on a multi drug resistant *Escherichia coli* strain

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ABSTRACT

The effect of $TiO_2$ photocatalysis on the inactivation of an antibiotic resistant *Escherichia coli* strain selected from an urban wastewater treatment plant (UWWTP) effluent was investigated. Different light sources including a 250 W wide spectrum lamp, a 125 W UV-A lamp and solar radiation, as well as, photocatalysts loadings ($TiO_2$ Degussa P25) in the range from 0.05 to 2.00 g $TiO_2$ $L^{-1}$ were evaluated. The higher efficiency (total bacterial inactivation after 30 min of irradiation) was observed in the absence of $TiO_2$ when the wastewater was irradiated using the 250 W lamp. In the presence of $TiO_2$ a decreasing inactivation trend was observed (99.76% and 72.22% inactivation after 10 min irradiation at 0.10 and 2.00 g $TiO_2$ $L^{-1}$ respectively). Under solar simulated conditions the highest inactivation efficiency (93.17%) after 30 min of irradiation was achieved at the lower photocatalyst loading (0.05 g $TiO_2$ $L^{-1}$). The concept of "reactor optical thickness" was introduced to explain the rates of disinfection observed. The optimum photocatalyst loading estimated by radiation absorption-scattering modeling was found to be 0.1 g $TiO_2$ $L^{-1}$ for all lamps. The difference between experimental tests and modeling may be due to $TiO_2$ particles aggregation. Comparative kinetic tests between solar and solar simulated photocatalytic (SSP) processes using 0.05 g $TiO_2$ $L^{-1}$ in suspension showed a quite similar inactivation behavior up to 30 min of irradiation, but only the SSP process resulted in a total inactivation of bacteria after 60 min of exposure. Antibiotic resistant test (Kirby–Bauer) on survived colonies showed that the SSP and SP processes affected in different ways the resistance of *E. coli* strain to the target antibiotics.



## 1. Introduction

The widespread use of antibiotics for human, veterinary and aquaculture purposes results in their continuous release into the environment (Díaz-Cruz et al., 2003; Batt et al., 2006; Watkinson et al., 2009; Fram and Belitz, 2011) since these species are only partially metabolized by organisms. Their occurrence into the environment is of particular concern because of the development of antibiotic resistance in

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## 5.3 INACTIVATION AND REGROWTH OF ARB: COMPARISON BETWEEN SOLAR AOPS AND CHLORINATION

### 5.3.1 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) E. 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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### 5.3.2 Introduction

The broad use of antibiotics for human, veterinary and aquaculture purposes, only partially metabolized by organisms, results in their continuous release into the environment (Batt et al. 2006, Watkinson et al. 2009, Fram and Belitz 2011). Un-metabolized antibiotics can reach urban wastewater treatment plants (WTPs) through the sewage, where they are typically detected at low concentrations (from fractions to some hundreds ng $L^{-1}$). Unfortunately, such low concentrations are suspected to promote the development of antibiotic resistance among bacteria and WTPs effluents can contribute to the spread of antibiotic resistance into the environment (Rizzo et al. 2013a). The release of antibiotic resistant bacteria (ARB) into the environment from WTPs effluents may be due to either: the absence of a final disinfection step or the use of conventional disinfection systems, which have been proven only partially effective in the inactivation of ARB (Munir et al. 2011, Rizzo et al. 2013b). Therefore new disinfection methods should be investigated to minimize the risk of antibiotic resistance spread from WTPs.
Advanced oxidation processes (AOPs) (e.g., Fenton, photo- Fenton, TiO2 photocatalysis, $UV/O_3$, $UV/H_2O_2$ etc.) have been successfully investigated for the removal of a wide range of contaminants (Zapata et al. 2010, Rizzo 2011, Sannino et al. 2013, Murcia et al. 2013). Although several studies on the inactivation of microorganisms by AOPs are

available in scientific literature (Malato et al. 2009, Dunlop et al. 2011), only a few works addressed inactivation of ARB (Tsai et al. 2010, Dunlop et al. 2014) and still less focused on indigenous ARB (Rizzo et al. 2014a). UV driven AOPs can be operated by sunlight thus saving energy costs (Malato et al. 2009) and they could be a viable alternative to conventional treatments (Bichai et al. 2012), particularly for wastewater treatment in small communities.

An important issue related with water disinfection and sanitation is the residual persistence of disinfection effect after the treatment because injured bacteria injured can regrow under suitable conditions in water (Rizzo et al. 2004). Thus, bacterial regrowth may be a problem when treated wastewater is reused for different end uses, like for instance crops irrigation. The use of AOPs for water/wastewater disinfection has been reported to produce a lower persistence of residual disinfecting agent (HO•) compared to chlorination (Rincón and Pulgarin 2005). Residual disinfecting quality of a process is also a factor that should be considered to assess its efficiency. This is limited for AOPs, by the nature of the highly reactive HO• radicals, which usually have a short half-life in the water environment. Wist et al. (2002) observed a drastic increase in E.coli density in river water samples after 24 h of UV irradiation. Similar bacterial regrowth problem was observed by Rincón and Pulgarin (2007) after 48 h in samples disinfected by UV radiation (30-300 min treatment). Reactivation and regrowth of indigenous bacteria exposed to low chlorine doses, after 24 h storage, was also observed by Li et al. (2013). Differently, when solar AOPs were investigated, no bacterial regrowth was observed during 24 h after sunlight exposure (Rincón and Pulgarin 2007).

In the present study the performance of solar disinfection (SODIS) and solar driven AOPs on the inactivation of an indigenous E. coli strain selected from real wastewater according to its resistance to three antibiotics (ampicillin (AMP), ciprofloxacin (CIP) and tetracycline (TET)), was investigated. More specifically, $H_2O_2$/Sunlight, $TiO_2$/sunlight, $H_2O_2$/$TiO_2$/sunlight, solar photo-Fenton (at natural pH), were operated under different temperatures and catalyst doses to (i) evaluate and compare their effect on the target E. coli strain inactivation and (ii) investigate the effect of disinfection processes on antibiotic resistance of surviving colonies. Additionally, and according to the

results achieved in the comparative solar AOPs experiments, $H_2O_2$/sunlight process was subsequently compared with conventional disinfection by chlorine in the inactivation of indigenous antibiotic resistant E.coli as well as to investigate post-treatment bacterial regrowth.

### 5.3.3 Material and Methods

#### 5.3.3.1 Chemicals

Aeroxide P25 (Evonik Corporation, Germany) $TiO_2$ was used as received from the manufacturer as slurry to perform heterogeneous photocatalytic experiments. Ferrous sulphate heptahydrate (FeSO4·7H2O, PANREAC, Spain) was used as $Fe^{2+}$ source for homogeneous photo-Fenton reaction. $H_2O_2$ at 35% w/v (Merck, Germany) was used in photo-Fenton and $H_2O_2$/Solar treatments. Sodium hypochlorite solution at 10% (Sigma-Aldrich, St. Louis, MO) was used in chlorination tests.

#### 5.3.3.2 Selection of multidrug resistant E. coli strain

E. coli multidrug resistant strain was selected from a large urban WTP (250,000 equivalent inhabitants) located in southern Italy. It was isolated from the effluent of the biological process (activated sludge) upstream of the disinfection unit. The sample was filtered (membrane filtration) and subsequently cultivated on selective medium (24 h incubation time at 44 °C), according to the procedure described by Rizzo et al. (2014b). Culture medium for the selection of resistant strain was prepared with Tryptone, Bile salts, X-glucuronide (TBX, Oxoid), and supplemented with a mixture of three antibiotics (16 mg L-1 of AMP, 2 mg $L^{-1}$ of CIP and 8 mg $L^{-1}$ of TET). Antibiotic concentrations were selected according to the results from a previous study (Rizzo et al. 2013b). Some colonies were randomly picked up and frozen in 15% glycerol Triptone Soy Broth (TSB) at -20 °C for the subsequent disinfection experiments.

#### 5.3.3.3 Inoculum and sample preparation

Disinfection experiments were carried out at Plataforma Solar de Almería (Spain) and wastewater samples were freshly collected from the

WTP of Almería, from the effluent of the biological process (activated sludge). All the samples were first autoclaved (15 min at 121 °C) with the purpose to remove all indigenous bacteria included in the samples and then inoculated with the selected multidrug resistant (MDR) E. coli strain. The reactivation of MDR E. coli was performed by streaking on ChromoCult® Coliform Agar (Merck KGaA, Darmstadt, Germany) and incubating at 37 °C for 18 h. 14 ml of sterile Luria Bertani broth (LB, Sigma-Aldrich, USA) was used to inoculate a single colony. Then, it was incubated at 37 °C for 18 h under continuously agitation in a rotator shaker to reach stationary phase culture. Cells were centrifuged at 3000 rpm for 10 min.
All the autoclaved samples were characterized using a Total Organic Carbon analyzer (Shimadzu TOC-5050, Kyoto, Japan), pH-meter (multi720, WTW, Germany), thermometer (Checktemp, Hanna instruments, Spain) and conductivity sensor (GLP31, CRISON, Spain). The values for each parameter ranged between 18.20-27.80 mg L-1 for TOC, pH from 8.00 to 8.92 and 1020-1510 μS cm-1 of conductivity.

#### 5.3.3.4 Bacterial count

Bacterial count was performed by standard plated counting method through a serial 10-fold dilutions in Phosphate Buffer Saline (PBS, Oxoid) after an incubation period of 24 h at 37 ºC. When the bacterial load was expected to be high, volumes of 20 μL were plated on Endo agar (Fluka, Sigma–Aldrich, USA). When the bacteria load was expected to be low, 250 or 500 μL samples were spread onto ChromoCult® Coliform Agar plates. Measurements were carried out in triplicates and average values and standard deviation were plotted as CFU 100 $mL^{-1}$. The detection limit of this experimental method was found to be 2 CFU $mL^{-1}$.

#### 5.3.3.5 Solar experiments

All experiments were carried out under natural solar irradiation at the Plataforma Solar of Almeria, Spain, located at 37° 84' N and 2°34' W, in clear sunny days. Solar stirred tank reactors exposed to sunlight were borosilicate glass (DURAN, Schott, Germany) bottles magnetically stirred during all experiment with a total volume of 250 mL. Glass

covers (Schott) permitting the entrance of the solar radiation from all directions were used. Prior to solar exposure, bottles with 200 mL of real wastewater, previously autoclaved, were spiked with 80 μL of E. coli ($10^9$ CFU $mL^{-1}$) solution to obtain an initial bacterial concentration of $10^6$ CFU $mL^{-1}$. The reagents (catalyst/oxidants) were added to the sample, and after a few minutes of stirring in the dark for homogenization, the reactors were exposed to natural solar irradiation. Reagents were added to Duran bottles at the beginning and during the experiment to ensure a constant concentration of the catalysts/oxidants. In $TiO_2/H_2O_2$/Sunlight experiments two different ratios $TiO_2$:$H_2O_2$ were investigated (10:100 and 50:100 mg $L^{-1}$). In Fenton and photo-Fenton processes, $Fe^{2+}$ and $H_2O_2$ were combined in three different ratios (5:10, 10:20 and 20:40 mg $L^{-1}$) in dark and one ratio (20:40 mg $L^{-1}$) with sunlight at natural pH. Different $H_2O_2$ concentrations (50, 75 and 100 mg $L^{-1}$) were investigated in $H_2O_2$ dark experiments, and in $H_2O_2$/sunlight experiments (10, 20 and 50 mg L-1). Sampling frequency varied depending on the 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) and $H_2O_2$ were also measured in the reactor during the experiments. For each experiment test, a water sample was taken and kept in the dark at laboratory temperature as a control which was plated at the end of the assay. Inactivation results are plotted as the average of at least two replicates for each solar driven experiments.

Solar UVA radiation was measured with a global UVA 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 UVA (in W $m^{-2}$), which is the solar radiant UVA energy rate incident on a surface per unit area. The average solar UVA irradiance for all tests was 37.34 4.30 W $m^{-2}$ within the period 10:00–16:00 local time, with maximum values of 44.38 W $m^{-2}$. In this study, the inactivation rate is plotted as function of both experimental time (t) and cumulative energy per unit of volume ($Q_{UV}$) received in the photoreactor, and calculated by Eq. (1):

$$Q_{UV,n} = Q_{UV,n-1} + \Delta t_n UV_{G,n} A_r / V_t \qquad \Delta t_n = t_n - t_{n-1}$$

where $Q_{UV}$,n, $Q_{UV}$,n-1 is the UV energy accumulated per litre (KJ $L^{-1}$) at times n and n-1, UVG,n is the average incident radiation on the irradiated area, Δtn is the experimental time of sample, Ar is the illuminated area of collector ($m^2$), Vt is the total volume of water treated (L). $Q_{UV}$ is commonly used to compare results under different conditions (Malato et al., 2009).

#### 5.3.3.6 Chlorination test

Sodium hypochlorite solution was added to wastewater sample (500 mL) to simulate the chlorination process. Different chlorine doses (0.5, 1.0 and 2.0 mg $L^{-1}$) were tested in order to find the best compromise between total bacterial inactivation and residual chlorine at the end of experiment (roughly 0.2 mg $L^{-1}$ meets the Italian standard for residual disinfectant in WTP effluent). After sampling, 0.1 mL of sodium thiosulfate solution (10%) was added to each 100 mL sample to remove residual chlorine before bacterial count. Chlorination experiments were done in duplicate

#### 5.3.3.7 Antibiotic resistance assay

Antibiotic resistance phenotypes were tested by Kirby–Bauer method according to standard recommendations (EUCAST, 2014). Briefly, colonies, prior to and after the AOPs treatment, were randomly selected from some agar/irradiation time and transferred into physiological solution to achieve a suspension of 0.5 McFarland (standard turbidity), approximately corresponding to 1–2x$10^8$ CFU $mL^{-1}$ suspension. Then the colonies weres spread onto Mueller Hinton agar II (Fluka, Sigma–Aldrich, USA) using a sterile cotton swab. Antibiotic resistance versus AMP (10 μg), CIP (5 μg), cefuroxime (CXM, 30 μg), nitrofurantoin (NI, 100 μg) was tested by placing the corresponding antibiotic disc (Biolife, Italy) on the surface of each inoculated plate. After 18 h of incubation at 35 °C, the diameters of antibiotic inhibition of growth were measured and recorded as susceptible (S), intermediary (I) or resistant (R). The criteria used for these interpretations, based on inhibition zone diameters, were as follows (mm): AMP 10: S≥14, R<14; CIP 5: S≥22, 19≤I<22, R<19; CXM 30: S≥18, R<18; NI 100: S≥11, R<11. These values are available in EUCAST (2014) database. The procedure was duplicated.

#### 5.3.3.8 Bacterial regrowth and comparative chlorination and H2O2/sunlight experiments

Wastewater samples for comparative inactivation and regrowth experiments with chlorine and $H_2O_2$/sunlight were freshly collected from the effluent of the biological process (activated sludge) of Salerno WTP (southern Italy), on the morning of each experiment. In order to evaluate the regrowth of indigenous antibiotic resistant E.coli population after disinfection experiments, wastewater samples were used as received. Untreated (as control), an treated WTP samples with chlorination (after treatment, chlorine neutralization was done by sodium thiosulfate) and $H_2O_2$/sunlight were incubated at 20°C for 6, 12, 24 and 48 h. The samples for regrowth experiments were taken before, in the middle (according to the inactivation rate) and at the end (total bacterial inactivation) of disinfection experiments. $H_2O_2$/sunlight experiments were performed using the most effective $H_2O_2$ dose (50 mg L-1). Chlorine dose was set at 1 mg $L^{-1}$, a compromise between bacterial inactivation efficiency and residual chlorine at the end of the experiment. The samples were plated on TBX agar prepared with and without a mixture of three antibiotics (16 mg $L^{-1}$ of AMP, 2 mg $L^{-1}$ of CIP and 8 mg $L^{-1}$ of TET). The antibiotic concentrations were chosen according to the respective minimum inhibiting concentrations for E. coli according to "Clinical and Laboratory Standards Institute" database (CLSI, 2011). E. coli colonies able to grow on the antibiotic added agar were referred to as multidrug resistant (MDR) E. coli. The percentage of MDR E. coli colonies inactivated by disinfection processes or regrowth after treatment was calculated as follow: MDR-E. coli colonies × 100/total E. coli colonies

#### 5.3.3.9 Analytical measurements

$H_2O_2$ at 30 wt% was used as received and diluted into the reactor filled with wastewater sample. $H_2O_2$ concentration in wastewater was determined by a colorimetric method based on the use of Titanium 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 measurement was linearly correlated with $H_2O2$ concentration in the range 0.1–100 mg $L^{-1}$.
$Fe^{2+}$ concentrations were measured according to ISO 6332. All samples were filtered with 0.20 μm CHROMAFIL® XtraPET-20/25 (PANREAC, Spain) and measured with spectrophotometer (PG Instruments Ltd. T-60-U) at 510 nm. For photo-Fenton tests, a freshly prepared solution of bovine liver catalase (0.1 g $L^{-1}$, Sigma–Aldrich, USA) was added to samples in a ratio 0.1/5 (v/v) to eliminate residual $H_2O_2$ and avoid Fenton reactions after samples collection. Free residual chlorine was measured by a portable spectrophotometer (Pocket colorimeter Chlorine, Hach, Loveland CO, USA).

### 5.3.4 Result and Discussion

#### 5.3.4.1 Solar heating effect on E.coli multidrug strain

The heating due to the IR spectrum may affect E. coli inactivation rate, therefore tests were carried out to separate the effect of AOPs from temperature. The bottles were covered by aluminum paper and exposed inside stoves at three different temperatures: 40, 45 and 50 °C. Moreover, a control sample was taken and kept at room temperature (24-26◦C) in the dark. The E. coli density during experiments is showed in Table 5.3.

**Table 5.3 Temperature effect on E. coli strain density (CFU $mL^{-1}$)**

| Temperature (°C) | 0 | 30 min | 60 min | 120 min | 180 min | 240 min |
|---|---|---|---|---|---|---|
| 40 | $1.0*10^6$ | $1.0*10^6$ | $1.0*10^6$ | $1.1*10^6$ | $1.2*10^6$ | $1.2*10^6$ |
| 45 | $1.2*10^6$ | $1.2*10^6$ | $1.2*10^6$ | $1.2*10^6$ | $1.1*10^6$ | $1.1*10^6$ |
| 50 | $1.2*10^6$ | $1.1*10^6$ | $1.0*10^6$ | $1.0*10^6$ | $1.0*0^6$ | $0.7*10^6$ |

E. coli concentration remained almost unchanged during temperature experiments. Only a slight decrease (0.5 log) was observed in the 50°C temperature experiment after 180min.
The effect of temperature on E. coli inactivation during solar disinfection experiments has been previously investigated and our results are in agreement with those available in scientific literature (McGuigan et al. 2012; Dunlop et al., 2011). 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, being sufficiently important only at water temperatures higher than 45◦C.

#### 5.3.4.2 Sodis

Experiments using natural solar irradiation were performed at different days in September 2013. These experiments were run from 10:45 a.m. to 15:45 p.m., when the solar irradiance reaches the highest levels. The results of the effect of sunlight on E. coli inactivation were represented as a function of $Q_{UV}$ (Fig. 5.11). Temperature (◦C) was also monitored during experiments.

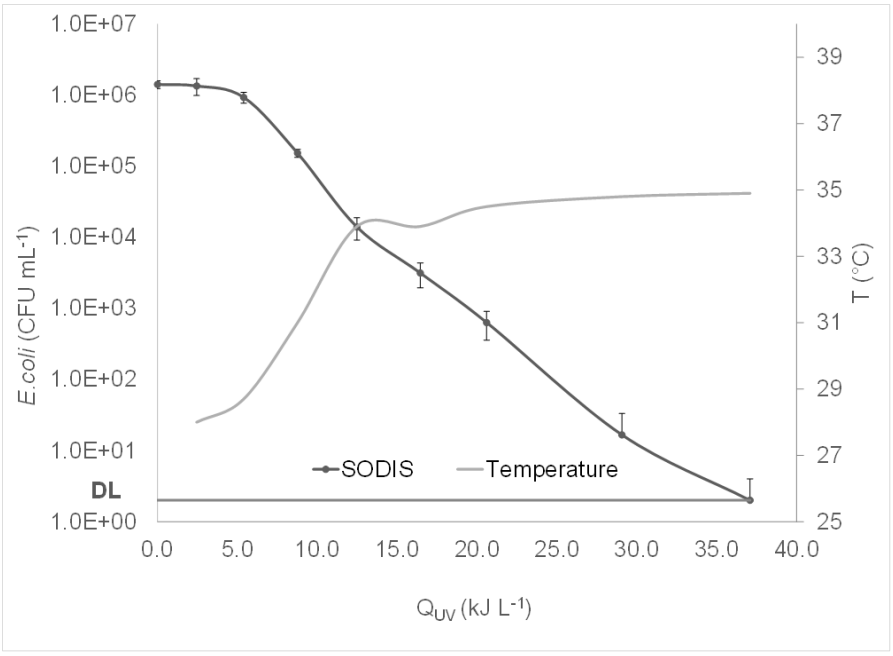


**Figure 5-11 Inactivation of MDR E. coli by SODIS and temperature profile**

Figure 5.11 shows that E. coli inactivation rates enhanced in the presence of solar radiation and that the inactivation increased as UV-dose increased. Roughly 6 log units decrease was observed for the tested strain and a total inactivation (residual bacterial density under detection limit, 2 CFU $mL^{-1}$) was reached after about 4 h of solar exposure. In terms of $Q_{UV}$, SODIS has required 37.00 KJ $L^{-1}$ to get the detection limit. In these experimental tests temperature varied in the range 27.0-35.0 °C, so that comparing these results with table 5.3 bacteria inactivation can be clearly attributed to the effect of UV-A radiation with no or only marginal temperature contribution. The two inactivation curves present a shoulder, with a very slow E. coli inactivation during the first hour and half of irradiation, followed by a faster linear-phase of the inactivation rate.

The effect of SODIS on E. coli in saline solution, at lab-scale, has been investigated by Helali et al. (2014). They observed a total inactivation of E.coli between 3 and 5 h and with a $Q_{UV}$ energy in the range 12-30 KJ $L^{-1}$ with the same initial bacterial concentration. The higher energy demand to achieve total inactivation in our experiments may also be explained by the higher resistance of the investigated MDR E. coli. Acra et al. (1989)

have reported that UV-A and early visible wavelength (320–450 nm) of the sunlight spectrum were able to generate reactive oxygen species which cause strand break-age and changes in DNA. These toxic reactive oxygen species can also disrupt protein synthesis.
Moreover, UV-B (290–320 nm) 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, inhibit DNA replication, and causes genetic mutation (Britt et al 1996).

### 5.3.4.3 $TiO_2$/Sunlight and $H_2O_2$/$TiO_2$/Sunlight

The inactivation of MDR E. coli by heterogeneous photocatalysis with suspended $TiO_2$ is shown in Figure 5.12.

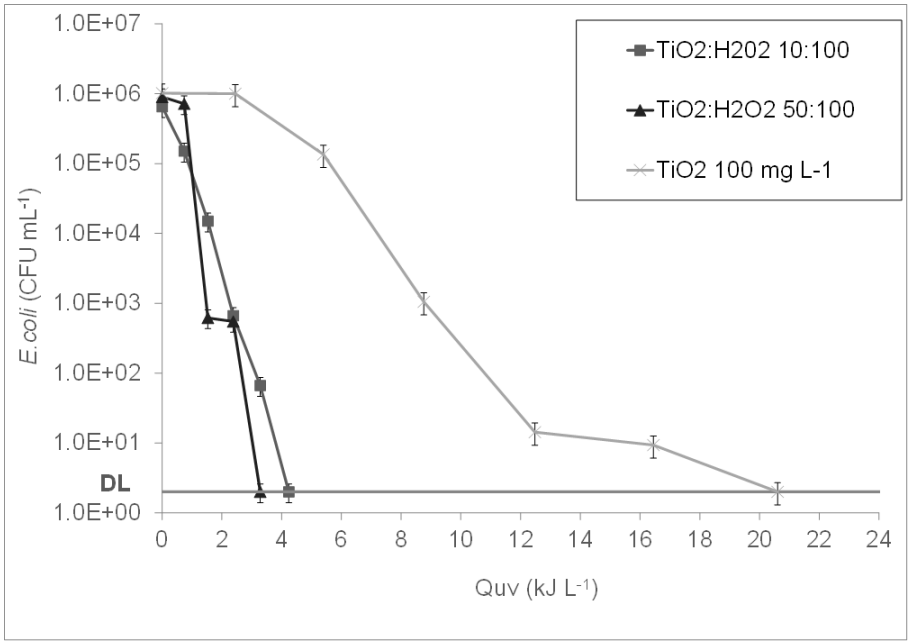


**Figure 5-12 Inactivation of MDR E. coli by TiO2 and TiO2/H2O2 photocatalytic processes**

The complete inactivation was achieved in 150 min of solar treatment with 100 mg $L^{-1}$ of $TiO_2$ a $Q_{UV}$ = 20 kJ $L^{-1}$. Inactivation rate was faster compared to SODIS experiment where total inactivation was achieved after 5 hours treatment with an higher energy requirement (38 kJ $L^{-1}$).

The effect of $TiO_2$ and $H_2O_2$ doses has been investigated too. The complete inactivation was faster (60 min) compared to $TiO_2$ experiment (150 min) and it was achieved with both $H_2O_2$:$TiO_2$ ratios investigated (10:100 and 50:100) (Fig. 5.12) with a significantly lower cumulative energy (3 and 4 kJ $L^{-1}$, respectively).

The addition of $H_2O_2$ in presence of $TiO_2$ promote the formation of hydroperoxyl ($HO_2$•) radicals, and low values of hydroxyl (HO•) radicals (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 et al. (2006), it is possible to speculate whether a loss of effective surface area due to an agglomeration of particles takes place. This would result in a reduced production of hydroxyl radicals. On the other hand, agglomeration is possibly due to interactions among $HO_2$• to the surface of $TiO_2$. For this reason, the presence of $HO_2$• is stabilized at high concentrations, with a best performance of inactivation. The results achieved are in a quite good agreement with two just published works where $TiO_2$ photocatalytic process was investigated in the inactivation of multidrug resistant E. coli and Enterococcus strains in wastewater (Rizzo et al. 2014a, Rizzo et al. 2014b). E.coli multidrug resistant strain were halved (initial density $10^5$ CFU 100 $mL^{-1}$) after about 60 min of treatment with solar radiation (50 mg $L^{-1}$ of $TiO_2$). Enterococcus multidrug resistant strain (initial density $10^5$ CFU 100 $mL^{-1}$), was completely inactivated in 60 min treatment (50 mg $L^{-1}$ of $TiO_2$).

#### 5.3.4.4 $H_2O_2$/sunlight and solar photo-Fenton

The $H_2O_2$/sunlight process has been investigated in detail with different $H_2O_2$ concentrations: 10, 20, and 50 mg $L^{-1}$ (Fig. 5.13).

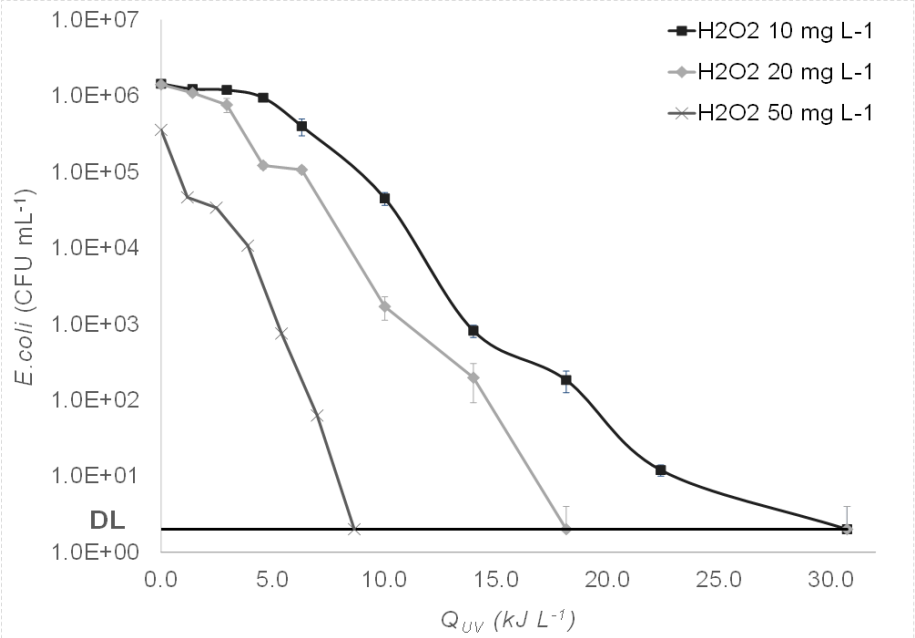


**Figure 5-13 Inactivation of MDR E. coli by H2O2/UV process**

The detection limit was reached for all experiments, with a complete inactivation at 10 mg $L^{-1}$ and $Q_{UV}$= 30 kJ $L^{-1}$, at 20 mg $L^{-1}$ and $Q_{UV}$= 18 kJ $L^{-1}$, and at 50 mg $L^{-1}$ and $Q_{UV}$ =8 kJ $L^{-1}$. Water temperature was measured during the solar tests and it was observed to increase from 28 °C to 36 °C. $H_2O_2$ concentration was also monitored throughout the tests; when it decreased, a small volume was added in order to keep it constant.

Photo-Fenton experiments were carried out at different $Fe^{2+}$:$H_2O_2$ ratios (5:10, 10:20 and 20:40 mg $L^{-1}$), under natural pH conditions 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 15 and 23 kJ $L^{-1}$ (Fig 5.14.).

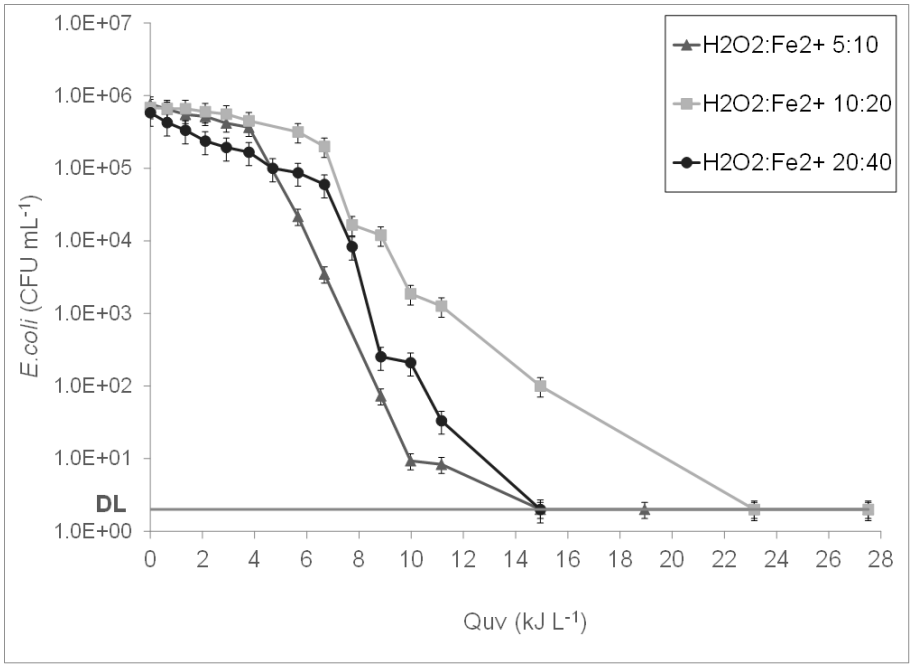


**Figure 5-14 Inactivation of MDR E. coli by solar photo-Fenton**

The best performance was observed with 5:10 of $Fe^{2+}$:$H_2O_2$ ratio. Temperature varied between 28 and 35°C and, and pH between 8.20 and 7.80. The inactivation rates were comparable with those observed in $H_2O_2$/sunlight process. The low levels of dissolved iron measured confirmed iron precipitation at natural pH of wastewater, which did not limit too much process efficiency. With a dissolved iron concentration approaching zero, the process can be considered as a $H_2O_2$/sunlight one. According to our results, Rodríguez-Chueca et al. (2014) observed that solar photo-Fenton process got the same detection limit for naturally occurring E. coli in a real secondary wastewater effluent at 10:20 mg $L^{-1}$ of $Fe^{2+}$:$H_2O_2$ with 13.1 kJ L-1 of $Q_{UV}$ within 4 h treatment at pH 5. However, Spuhler et al (2010) observed a total inactivation of E. coli strain K12 ($10^6$-$10^7$ CFU $mL^{-1}$ initial bacterial population) in mineral water (pH 7.0-7.5) during solar simulated photo-Fenton process at low $Fe^{2+}$ concentration (0.6:10 mg $L^{-1}$ $Fe^{2+}$:$H_2O_2$ ratio), in a shorter treatment time (approximately 95 min). Possibly, the lower $Fe^{2+}$ initial concentration and pH resulted in a decreased iron precipitation

compared to our work with a consequent faster inactivation efficiency. The more complex water matrix (wastewater Vs mineral water), the different light source (natural sunlight Vs solar simulator) and target bacteria (MDR E.coli Vs E. coli K12) are also expected to affect process efficiency.

#### 5.3.4.5 Effect of solar driven AOPs on antibiotic resistance

The average values of inhibition diameters for AMP, CIP, CXM, NI before each disinfection process (t=0) for the selected MDR E. coli were compared with the corresponding clinical breakpoint values for E. coli from EUCAST database (table 2). The tested strain was resistant (R) to AMP, CIP, TET, as expected, but also to VAN. It was sensitive (S) to CXM and NI. The colonies survived to the disinfection process did not show any change in the original clinical breakpoint. In particular, the tested strain did not lose its resistance to AMP, CIPR, TET and VAN during the process because no variations in the inhibition zone diameters were observed.

**Table 5.4 Inhibition zone diameter values (mm) of E. coli for AMP, CIP, CXM and NI (Kirby-Bauer method) available in EUCAST database (2014) and average values measured before each disinfection process (R: Resistant; I: Intermediary; S:Susceptible).**

| Disinfection process/EUCAST database | AMP10 | CIP5 | CXM30 | NI100 | TET30 | VAN30 |
|---|---|---|---|---|---|---|
| EUCAST database | R<14 | R<19 | R<18 | R<11 | - | - |
| | - | 19≤I<22 | - | - | - | - |
| | S≥14 | S≥22 | S≥18 | S≥11 | - | - |
| SODIS | 10 | 10 | 19 | 22 | 9 | 8 |

| | | | | | | |
|---|---|---|---|---|---|---|
| photo-Fenton $Fe^{2+}$:$H_2O_2$ 5:10 mg $L^{-1}$ | 10 | 10 | 22 | 28 | 10 | 10 |
| photo-Fenton $Fe^{2+}$:$H_2O_2$ 10:20 mg $L^{-1}$ | 10 | 10 | 22 | 24 | 10 | 10 |
| photo-Fenton $Fe^{2+}$:$H_2O_2$ 20:40 mg $L^{-1}$ | 10 | 10 | 22 | 22 | 10 | 10 |
| $H_2O_2$/sunlight 10 mg $L^{-1}$ | 10 | 10 | 17 | 25 | 10 | 10 |
| $H_2O_2$/sunlight 20 mg $L^{-1}$ | 10 | 10 | 19 | 26 | 10 | 10 |
| $H_2O_2$/sunlight 50 mg $L^{-1}$ | 10 | 10 | 22 | 27 | 10 | 10 |
| $TiO_2$/sunlight 100 mg $L^{-1}$ | 10 | 10 | 19 | 21 | 10 | 10 |
| $H_2O_2$/$TiO_2$/sunlight 10:100 mg $L^{-1}$ | 10 | 10 | 20 | 25 | 10 | 10 |
| $H_2O_2$/$TiO_2$/sunlight 50:100 mg $L^{-1}$ | 10 | 10 | 21 | 27 | 10 | 10 |

In the literature, the effect of AOPs on antibiotic resistance by cultivation methods has been investigated only recently. Michael et al. (2012) and Karaolia et al. (2014) investigated the effect of solar photo-Fenton process on antibiotic resistance of Enterococchi but in terms of resistance percentage (bacterial count on the culture media supplemented with antibiotics divided by the corresponding counts on the culture media without antibiotics). Michael et al. (2012) observed a decrease in ofloxacin and trimethoprim resistance percentage after solar photo-Fenton treatment, at pilot scale, ($[Fe^{2+}]$ = 0.090 mM; ($[H_2O_2]$ = 2.205; pH = 2.8-2.9). The same approach has been followed by Karaolia et al.

(2014), also in this case a decrease of clarithromycin and sulfamethoxazole resistant Enterococcus in real WTP effluent as treatment time increased was observed (solar photo-Fenton process at pilot scale, [$Fe^{2+}$] = 0.090 mM; ([$H_2O_2$] = 1.470 mM; pH = 4, in the presence of 0.1 mg L-1 of clarithromycin and sulfamethoxazole). The effect of solar and solar simulated $TiO_2$ photocatalytic processes on the resistance of an indigenous E. coli strain to four antibiotics (CIP, CEF, TET and VAN) was also investigated (Rizzo et al. 2014b); a statistically significant change in antibiotic resistance ($p = 0.033 < \alpha = 0.05$) was observed for TET (inhibition diameter increased from 10.1 to 10.8 mm).

#### 5.3.4.6 Inactivation of total and indigenous antibiotic resistant E.coli after chlorination and H2O2/Sunlight

Considering the good results achieved with $H_2O_2$/sunlight process too, and the expected higher cost related to $TiO_2$ photocatalysis (due to additional treatment for catalyst removal after treatment or $TiO_2$ supported/film reactors) and solar photo-Fenton ($Fe^{2+}$ dose optimization can decrease precipitation and sludge production), $H_2O_2$/sunlight process look to be a more attractive option for wastewater disinfection in small communities. Therefore this process was used for the subsequent comparative experiments with conventional chlorination process. The inactivation of total and MDR indigenous E.coli for the two disinfection processes is showed in Fig.5.15 A-B.

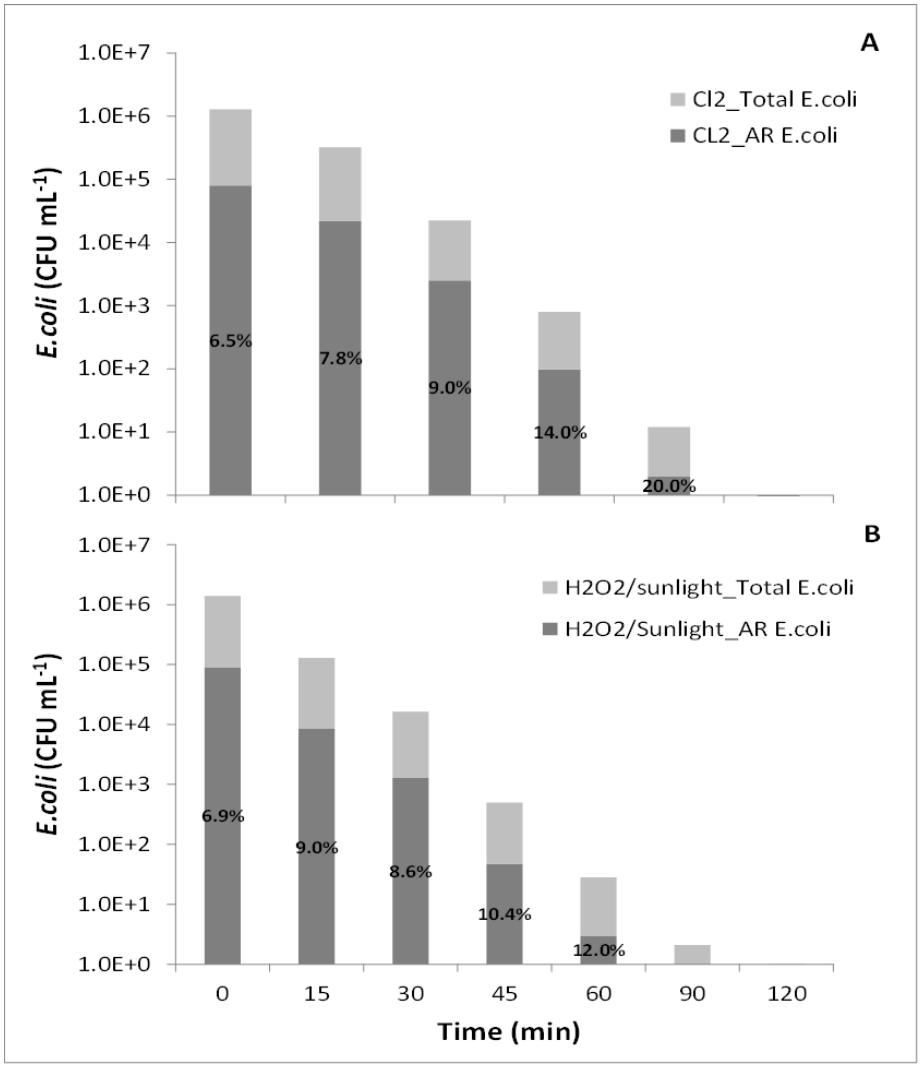


**Figure 5-15 A-B Inactivation of total and MDR indigenous E.coli by chlorination (A) and H2O2/Sunlight (B) processes**

Complete inactivation of total and MDR E.coli by chlorination was achieved in 15 min (Fig. 5.15A), i.e. until the experimental detection limit. However during treatment, the MDR E. coli population decreased at a slower rate compared to the total E. coli population (accordingly the percentage of MDR E. coli increased from 6.0% to 20% after 10 min). Differently, in $H_2O_2$/Sunlight process, total inactivation was observed

after 90 min of treatment, and the percentage of MDR E. coli increased at a lower rate compared to chlorination process (from 6.9% to 12.0% after 60 min treatment). According to these results, MDR E. coli showed much higher resistance to disinfection processes compared to a significant fraction of the total E. coli population. But finally, a total inactivation of MDR E. coli was observed when a fraction of total E. coli population still survived the disinfection process, thus making E. coli population a good indicator of MDR fraction occurrence.

Treatment by $H_2O_2$/Sunlight, although requires longer inactivation times compared to chlorination process, it seems to produce a higher proportional impact on MDR E.coli inactivation than chlorination at the evaluated dose. Just before complete inactivation, the percentage of resistant in chlorination treatment is almost double compared to $H_2O_2$/Sunlight process. Huang et al. (2013) studied the inactivation by chlorination of tetracycline-resistant E.coli strains (antibiotic sensitive strain E. coli CGMCC 1.3373, and tetracycline resistant strain 1.1595 harbouring the plasmid pBR322). In their work the inactivation of antibiotic sensitive E.coli, at 1.0 mg $Cl_2$ $L^{-1}$ (the same chorine concentration used in our work) did not change significantly from that of tetracycline resistant strain. Templeton et al. (2009) studied the inactivation of ampicillin-resistant E. coli 145, trimethoprim-resistant E. coli 018 and a wild strain of E. coli isolated from sewage sludge. The initial concentration of chlorine was 1.4 mg $Cl_2$ $L^{-1}$. The trimethoprim-resistant strain of E. coli 018 was more resistant to chlorine than the antibiotic-sensitive E. coli isolate, and the ampicillin-resistant E. coli 145 was less resistant to chlorine than the E. coli isolate. However, previous studies have shown different results after the chlorination process. Murray et al. also showed a small increase in the percentage of tetracycline-resistant bacteria in sewage after chlorination. Staley et al. (1988) showed an increase percentage of tetracycline-resistant total coliforms and faecal coliforms in secondary effluents after both low and high doses of chlorination.

The differences in term of inactivation rate of MDR E. coli and total E. coli in the two disinfection processes investigated in our work may be explained by the different inactivation mechanisms of disinfection processes. Opposite to chlorine, which does not affect plasmid DNA structure at the investigated doses, AOPs such as $H_2O_2$/sunlight, can

result in conformational change and the damage can increase as oxidant doses increases. In particular, $H_2O_2$/sunlight brings about oxidation by the generation of the highly reactive •OH radical. Hydroxyl radicals are able to kill bacteria mainly by destroying their cell membrane or walls (Watts et al. 1995). Bianchini et al. (2002) claimed formation of intermediate free radicals (secondary radicals) during oxidation of wastewater such as hydroxyl radicals and carbon centred radicals. Kikuchi et al. (1997) suggested that possibly superoxide radicals and hydrogen peroxide can diffuse into microorganisms cell through the membrane, and produce hydroxyl radicals by the Haber–Weiss reaction. UVB induces formation of the superoxide species that can react to form hydrogen peroxide, and if not scavenged can enhance the production of intracellular hydroxyl radical through Fenton or Haber–Weiss reaction. Moreover, a contribution to the inactivation by UV radiation in UV driven AOPs is possible too. Rusin et al. (2002) found out a similar inactivation rate of antibiotic-resistant E. coli and antibiotic-sensitive E. coli by UV irradiation. UV irradiation causes dimerization of bacterial DNA pyrimidine bases preventing molecular processes such as replication and transcription, leading to inactivation of the bacteria (Rusin et al. 2002).

#### 5.3.4.7 Regrowth of total and indigenous antibiotic resistant E.coli after chlorination and H2O2/Sunlight

The regrowth of total and MDR E.coli after chlorination and $H_2O_2$/Sunlight disinfection was investigated at different retention times (6, 12, 24 and 48 h) in dark conditions. A slight regrowth of MDR E.coli, in the sample taken in mid-process (at 2.5 min and 30 min for chlorination and $H_2O_2$/Sunlight, being the corresponding bacterial load as high as $2.0\times10^4$ and $1.5\times10^4$ CFU 100 $mL^{-1}$, respectively), was observed in both disinfection processes (Fig. 5.16 A-B). However MDR indigenous E.coli in the sample after chlorination increased in percentage from 12% to about 16% (Fig. 5.16 A), while in the sample after $H_2O_2$/Sunlight disinfection the percentage of MDR indigenous E.coli decreased from 8% to about 4% (Fig. 5.16 B). In wastewater samples taken at the end of disinfection experiments (total bacterial inactivation), a similar trend was observed. Reactivation of indigenous MDR E.coli took place after 12 h in chlorination process ($1.2\times10^2$ CFU $mL^{-1}$) (Fig. 5.16 A) and after 24 h in $H_2O_2$/Sunlight process ($0.3\times10^2$ CFU $mL^{-1}$)

(Fig. 5.16 B). The percentage of resistant was 5.6 and 6.7% in the chlorinated sample after 24 and 48 h, respectively (after 12 h only total E.coli was detected). In the sample treated by $H_2O_2$/Sunlight the MDR E.coli were detected only at 48h and the percentage on the total was of 3%.

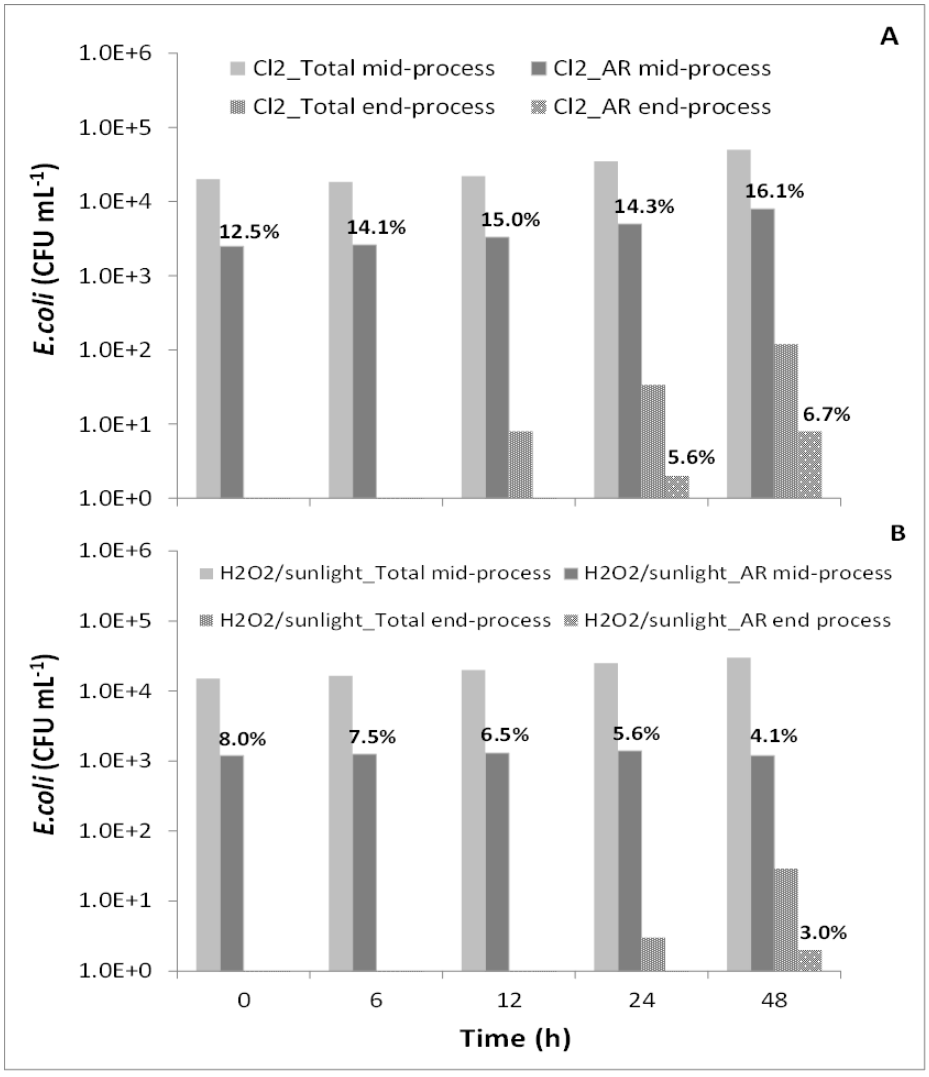


**Figure 5-16 A-B Regrowth of total and MDR indigenous E.coli after chlorination (A) and H2O2/Sunlight (B) treatment**

Inactivation and regrowth results from chlorination experiments are in quite good agreement with results from Huang et al. work. They investigated the inactivation and reactivation of antibiotic-resistant heterotrophic bacteria by chlorination in secondary effluents of a municipal wastewater treatment plant. They observed that the reactivation and decay of total heterotrophic bacteria and antibiotic-resistant bacteria occurred when the dosage of chlorine is lower than 2.0 mg $Cl_2$ L-1 for 10 min. In addition the reactivation and decay of total heterotrophic bacteria and antibiotic-resistant bacteria decreased gradually when the dosage of chlorination increased and the proportion of antibiotic-resistant bacteria in the secondary effluents had a significant increase after standing for 22 h.

The regrowth in photocatalytic processes: sunlight/$TiO_2$, sunlight/$TiO_2$/$Fe^{3+}$, sunlight/$Fe^{3+}$/$H_2O_2$, using a CPC reactor and natural water spiked with E.coli K 12 was analyzed by Rincon and Pulgarin (2005). In their work a bacterial regrowth by bare sunlight was reached after 24 h but no bacterial regrowth was observed during 24 h after stopping sunlight exposure. Though they did not investigate $H_2O_2$/Sunlight process, a comparison can be tried considering that all these processes result in the formation of hydroxyl radicals which are expected to significantly contribute to bacterial inactivation

### 5.3.5 Conclusion

Different photo-driven AOPs using natural sunlight ($H_2O_2$/sunlight, $TiO_2$/sunlight, $H_2O_2$/$TiO_2$/sunlight and photo-Fenton) were evaluated in the inactivation of an indigenous MDR E. coli strain in real urban wastewater. The best disinfection efficiency was observed for $H_2O_2$/$TiO_2$/sunlight process with total inactivation achieved for 3-5 KJ $L^{-1}$ ($Q_{UV}$), depending on $H_2O_2$/$TiO_2$ ratio. The best performance of Photo-Fenton process was observed for $Fe^{2+}$:$H_2O_2$=5:10 ratio at 15 KJ $L^{-1}$. Therefore, the required energy to decrease MDR E. coli under DL is higher than that required by $H_2O_2$/sunlight process ($Q_{UV}$=8 KJ $L^{-1}$, 50 mg $L^{-1}$ of $H_2O_2$). All investigated processes did not affect antibiotic resistance of survived colonies.

Considering the expected higher cost related to $TiO_2$ photocatalysis (due to additional treatment for catalyst removal after treatment or $TiO_2$ supported/film reactors), solar photo-Fenton ($Fe^{2+}$ dose optimization can decrease precipitation and sludge production) and $H_2O_2$/sunlight processes look to be a more attractive option for wastewater disinfection in small communities.

In spite of the slower inactivation rate, $H_2O_2$/sunlight process was more effective than chlorination process in controlling MDR E. coli regrowth, thus making this process possibly an alternative and effective solution for the disinfection of wastewater to be reused in crops' irrigation.

### 5.3.6 Reference

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### 5.3.7 Appendix

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Manuscript Draft

Manuscript Number:

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

Article Type: Full Length Article

Keywords: advanced oxidation processes; antibiotic resistance; bacterial regrowth; Escherichia coli; photo-Fenton; TiO2 photocatalysis

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Abstract: Solar disinfection and solar driven advanced oxidation processes (AOPs) (namely H2O2/Sunlight, TiO2/sunlight, H2O2/TiO2/sunlight, solar photo-Fenton) were evaluated in the inactivation of indigenous antibiotic resistant bacteria (ARB) in real urban wastewater. A multidrug resistant (MDR) E. 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 H2O2/TiO2/sunlight (cumulative energy per unit of volume (QUV) in the range 3-5 KJ L-1, depending on H2O2/TiO2 ratio) and H2O2/sunlight (QUV of 8 KJ L-1) processes. All investigated processes did not affect antibiotic resistance of survived colonies. Moreover, H2O2/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 Cl2 L-1) was more effective than H2O2/sunlight (50 mg H2O2 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 H2O2/sunlight treated samples decreased as incubation time increased; the opposite was observed for chlorinated samples.

# 6 CONCLUSION

The problem of the ARB is increasingly important in view of increase number of deaths that occur worldwide due to infections from ARB. In this thesis work different experimental works have been carried out in order to investigate the spread and fate of ARB into the environment as well as technological solutions to control the spread of antibiotic resistance. According to the results explained and discussed in the previous chapters, the following conclusions were achieved.

The monitoring of Tusciano rever, in Salerno province (Italy), showed that Hospital and UWWTP discharges can significantly contribute to the spread of antibiotic resistance into the environment. The higher abundance of the target ARB was observed downstream of Hospital effluent. The relative abundance of the target ARB was not only affected by wastewater discharges but also by the season.

Different mechanisms and conditions can affect the fate of ARB in rivers. The results from kinetic modelling achieved by simulating ARB fate in a purposely built channel showed that the target ARB can be inactivated in weak slope and slow current (simulated) streams in a few hours, but solar inactivation efficiency is expected to drastically decrease as water height and turbidity increase. A significant effect on ARB fate is expected also from adsorption to sediments, but this mechanism may contribute to the spread of resistance in the environment through flora and fauna river and consequently through food chain.

According to these results, the suggested strategy to control antibiotic resistance spread into the environment includes two approaches: (i) implementation of effective policies for controlling the use of antibiotics in order to avoid any misuse or unnecessary use; (ii) an effective control of antibiotic resistance contamination point sources through the application of highly effective disinfection technologies. AOPs investigated in this work showed to be more effective than conventional disinfection processes in controlling antibiotic resistance spread. In

particular, solar driven AOPs (namely, sunlight/$H_2O_2$) look to be a more attractive option for wastewater disinfection in small communities. Where solar technology is not suitable, artificial light lamp can effectively be used.

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