Photocatalytic Disinfection of Antibiotic Resistant Bacteria and Resulting Antibiotic Resistance Gene Transfer in Urban Wastewater

Patrick SM Dunlop*, M Ciavola**, L Rizzo**, DA McDowell*, JA Byrne*

* NIBEC, Ulster University, Shore Road, Newtownabbey, County Antrim, BT37 0QB United Kingdom Email: psm.dunlop@ulster.ac.uk
** Department of Civil Engineering, University of Salerno, Via Giovanni Paolo II, 84084 Fisciano (SA), Italy Email: l.rizzo@unisa.it

INTRODUCTION

The excessive use of antibiotics has led to the development of antibiotic resistant bacteria (ARB) which have been isolated from both clinical and non-clinical facilities and throughout the environment [1]. ARB, and corresponding antibiotic resistance genes (ARGs), are not completely removed from water treated in traditional wastewater treatment plants (WWTP), indeed, the high bacterial load, sub-therapeutic concentration of antibiotics and the physical and mechanical processes used in WWTP can encourage the proliferation of ARB, with these selective pressures potentially promoting the evolution of antibiotic resistance and ARG transfer [2]. Given the potential for ARB and ARGs to persist and spread, the subsequent release of WWTP effluent and sludge could therefore increase the reservoir of ARB and ARGs in the environment contributing to the mobility of genetic elements and the development of antibiotic resistance in human pathogens. As such, new methods of water treatment/disinfection are required to target these emerging biological contaminants.

There is increasing interest in Advanced Oxidation Processes (AOPs) for both water and wastewater treatment and disinfection. UV-titanium dioxide (TiO_2) photocatalysis (PC) has been reported to inactivate a wide range of bacteria, viruses and protozoa commonly found in water and waste water via the production of biocidal reactive oxygen species [3] with few reports of the inactivation of ARB (Figure 1).

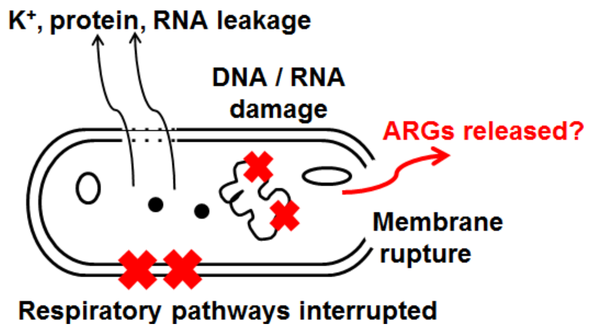

Figure 1 – Proposed mechanism/s of TiO_2 PC disinfection

METHODS

Photocatalytic disinfection experiments were conducted in a stirred tank reactor (STR) using immobilised titanium dioxide films as described by Alrousan et al [4]. *E. coli* K-12 (ATCC 23631) was cultured overnight in Luria-Bertani (LB) broth, prior to centrifugation, resuspension and dilution in distilled water or secondary effluent [4].

Two genetically modified strains of *E. coli* paired for conjugation, J-53R conferring resistance to rifampicin, and HT-99, harbouring a plasmid conferring resistance to chloramphenicol, were obtained from National Centre for Biotechnology Education at the University of Reading, UK. Individual liquid cultures of both organisms were prepared in LB broth as described by Dunlop et al [5]. The initial bacterial concentration in the photocatalytic experiments was approximately 3 $\log_{10}$ CFU mL^{-1}.

Photocatalytic treatment leading to sub-lethal stress and subsequent bacterial repair, was assessed by plating samples of treated cells onto both selective m-Endo agar (Fluka) and non-selective LB agar, as described in Dunlop et al [6]. To permit recovery & enumeration of injured cells, samples removed from the STR were incubated at 37°C for 24 hours, serially diluted in ¼ strength Ringer's solution and plated onto non selective LB agar.

To assess the potential for ARG transfer resulting from photocatalytic treatment, experiments using a 9:1 mixture of J-53R (recipient) to HT-99 (donor) were undertaken (i.e. the ARG recipient within the conjugated pair was present in ten-fold excess). The initial bacterial concentration used was approximately 5 $\log_{10}$ CFU mL^{-1}. Samples of bacterial suspension were removed from the STR as a function of treatment time and pre-incubated overnight at 37°C in 10 mL of LB broth. Following serial dilution in ¼ strength Ringer's solution aliquots (100 μL) were plated in triplicate on LB agar containing 100 μL mL^{-1} rifampicin and 25 μL mL^{-1} chloramphenicol (i.e. only organisms which contained both resistance genes via conjugative exchange were permitted to grow for enumeration).

The number of gene conjugate colonies enumerated following photocatalytic treatments was expressed as a percentage against that observed in the no treatment controls.

RESULTS

Inactivation of J-53R (Figure 2) and HR-99 was observed, but the rate of inactivation was slower than that for K12 [5]. Comparison of the number of colonies observed on selective and non-selective agars suggested sub-lethal injury of the ARB - bacterial regrowth was observed following treatment of J-53R and HR-99, but not with K12.

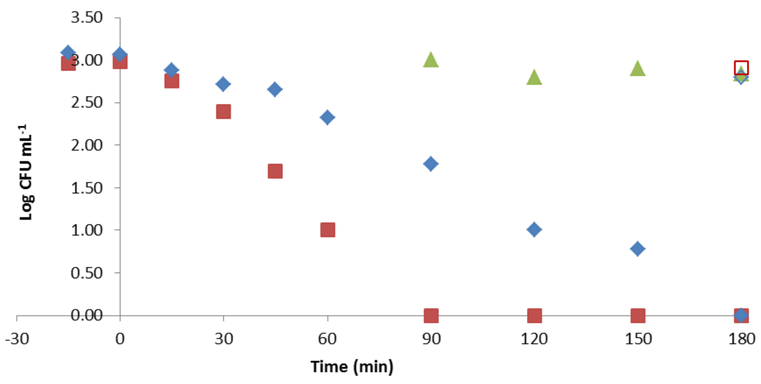

Figure 2 – PC treatment of J-53R, rifampicin resistant.

An increase in the number of gene pair conjugates, as a function of photocatalytic treatment of ARB in distilled water and secondary effluent was observed (Figure 3).

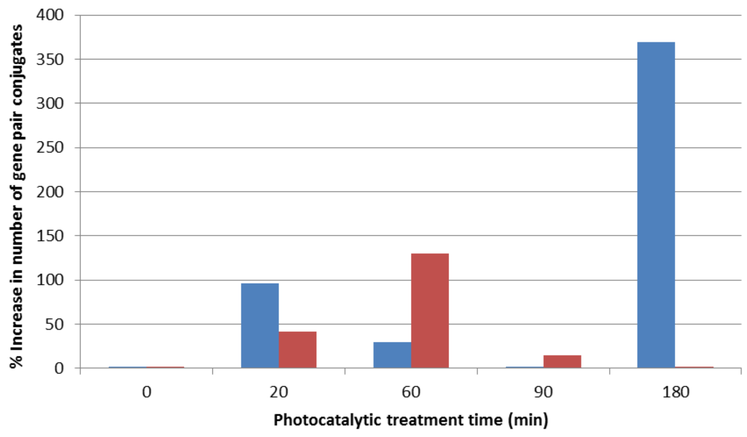

Figure 3 – Percentage increase in gene pair conjugate numbers during photocatalysis.

DISCUSSION

Photocatalytic experiments using individual cultures of K12 and ARB showed a reduction in counts to the detection limit. Re-growth was evident for both J-53R and HT-99, but not for K12. This suggests that ARB are more resistant to the biocidal radicals produced by photocatalytic treatment than antibiotic sensitive *E. coli* and highlights the need for caution in projecting results from laboratory based experiments using attenuated strain of bacteria to "real world" situations.

Horizontal gene transfer (HGT) occurred in the conjugated pair, as shown in Figure 4, and enumeration of gene conjugate colonies on LB agar containing both rifampicin and chloramphenicol was possible (Figure 5).

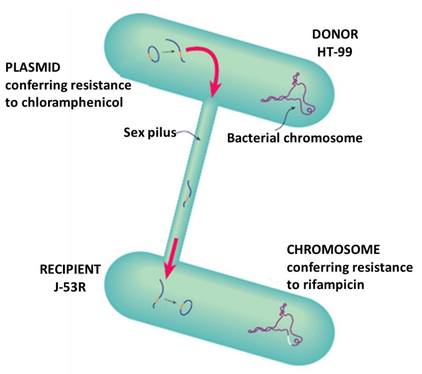

Figure 4 – HGT in the J-53R HT-99 pair

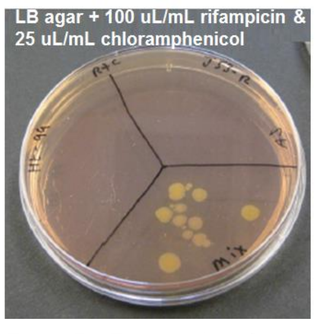

Figure 5 – Enumeration of gene conjugate colonies

Figure 3 shows the percentage increase in the number of colonies detected on LB agar containing both antibiotics (gene pair conjugates) as a function of photocatalytic treatment of ARB in distilled water and secondary effluent, in comparison to the (no treatment) control where a baseline of natural HGT was determined.

Within the initial 30 min of treatment in distilled water, photocatalysis induced sub-lethal stress/damage and caused an increase in ARG transfer between the conjugated *E. coli* pair. At the end of the three hour treatment the percentage of gene pair conjugates increased by approximately four-fold from that observed in the (no treatment) control.

A similar increase in the percentage of gene pair conjugates was also observed in experiments conducted in (filter sterilised) secondary wastewater effluent, however to a lesser extent. We hypothesise that this is due to a reactive oxygen species scavenging effect by the organic and inorganic content of the waste water - essentially "protecting" the bacteria from the biocidal ROS such as hydroxyl radicals, superoxide radical anion and peroxide species. Coupled with the lower number of culturable cells in the reactor (a 95% reduction from the initial cell density was detected on LB agar) this clearly demonstrates a significant increase in ARG transfer following 60 min treatment of the mixture of both ARB.

Given the complexity of natural systems and the variability in water quality, additional research is required to further investigate ARG transfer using organisms sourced from WWTPs. ROS generation via photocatalysis is relatively slow and therefore AOP generating larger concentrations of ROS should also be assessed for their potential to cause sub-lethal stress induced HGT, and in cases where membrane rupture is observed, an assessment of the potential for natural transformation of ARGs should be considered.

CONCLUSIONS

A high level of sub-lethal environmental stress, approaching that with the potential to completely inactivate a population of organisms, can increase the rate of genetic transfer amongst pathogens.

- Photocatalysis can inactivate antibiotic sensitive and resistant organisms - if treatment times are sufficiently long.
- The production of sub-lethal concentrations of ROS resulted in subsequent bacterial regrowth - observed only if bacteria are cultured in non-selective media (permitting repair).
- Sub-lethal stress resulted in increased horizontal gene transfer in the J-53R HT-99 pair.
- Care must be taken when extrapolating laboratory based disinfection kinetics of antibiotic sensitive bacteria for "real world" applications.
- Should antibiotic resistant genes be considered as pollutants of concern?

References:
[1] Berendonk et al. (2015), Nat. Rev. Microbiol., 13, 310-317. [2] Rizzo et al. (2013), Sci. Total Environ. 447, 345. [3] Byrne et al. (2015), Molecules, 20(4), 5574. [4] Alrousan et al. (2009), Water Res., 43, 47. [5] Dunlop et al (2015), Catal. Today, 240, 55-60. [6] Dunlop et al. (2011), Chemosphere, 85, 1160.

Acknowledgement:
The funding awarded to Marco Ciavola by the Faculty of Engineering at the University of Salerno is gratefully acknowledged (Rep. no 1363, Prot. no. 20378). The authors also wish to thank EU COST Actions "DARE": Detecting Evolutionary Hotspots of Antibiotic Resistances in Europe (TD0803) and "NEREUS": New and Emerging Challenges and Opportunities in Wastewater Reuse (ES1403) for supporting our collaboration.