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The Ph. D. project herein deals with the application of a novel photocatalyst and its potential in wastewater treatment. Specifically, for wastewater intended for reuse in crop irrigation and with direct emphasis on antibiotic resistance dissemination. The project is divided in three work packages (WP). *WP A - Selection of a suitable photocatalyst* deals with the assessment of a number of photocatalyst that was carried out in order to identify a suitable catalyst showing higher efficiencies while having a low-cost synthetic route. The research carried out within this scope resulted in an optimised cerium doped ZnO catalyst which was identified as fitting the pre established requirements. This catalyst was subsequently studied in the immobilised form in *WP B - Catalyst immobilisation* here a procedure for coating stainless steel surfaces was set and the catalyst used for the removal of two model antibiotics trimethoprim and sulfamethoxazole, as well as testing the reusability of photocatalytic coatings. Additionally, the immobilised catalyst was used for the inactivation of bacteria in isotonic saline water and for the inactivation of autochthonous bacteria, including antibiotic resistant fractions in real wastewater. In the latter, Ce-ZnO was more efficient than the industry standard TiO_2-P25. This novel catalyst was also applied for controlled irrigation experiments. Lettuce plants (Lactuca sativa) were set up in four groups receiving either Ce-ZnO treated wastewater, chlorinated treated wastewater, fresh water or secondary wastewater in *WP C – HPC and chlorination for AR abetment in irrigation*. Both water treatments showed marginal differences in concentrations of the four selected genes (16S rRNA, bla_{OXA-10}, *qnrS* and *intI1*) in water concentrations. As for concentrations in soil after the irrigation campaign, both treatments showed lower levels than those from secondary wastewater but higher levels than fresh water with no major differences between treatments.

Antibiotic Resistance Mitigation Using Novel Heterogeneous Photocatalysts for Urban Wastewater Treatment and its Reuse in Agricultural Irrigation

Ian Zammit

Ph. D. Thesis
University of Salerno

Tesi di dottorato
Università degli Studi di Salerno

Ian Zammit

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Rischio e sostenibilità nei sistemi
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CICLO XXXII (2016-2019)

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Abstract

The Ph. D. project herein deals with the application of a novel photocatalyst and its potential in wastewater treatment. Specifically, for wastewater intended for reuse in crop irrigation and with direct emphasis on antibiotic resistance dissemination. The project is divided in three work packages (WP). *WP A - Selection of a suitable photocatalyst* deals with the assessment of a number of photocatalyst that was carried out in order to identify a suitable catalyst showing higher efficiencies while having a low-cost synthetic route. The research carried out within this scope resulted in an optimised cerium doped ZnO catalyst which was identified as fitting the pre-established requirements. This catalyst was subsequently studied in the immobilised form in *WP B - Catalyst immobilisation* here a procedure for coating stainless steel surfaces was set and the catalyst used for the removal of two model antibiotics trimethoprim and sulfamethoxazole, as well as testing the reusability of photocatalytic coatings. Additionally, the immobilised catalyst was used for the inactivation of bacteria in isotonic saline water and for the inactivation of autochthonous bacteria, including antibiotic resistant fractions in real wastewater. In the latter, Ce-ZnO was more efficient than the industry standard TiO_2-P25. This novel catalyst was also applied for controlled irrigation experiments. Lettuce plants (*Lactuca sativa*) were set up in four groups receiving either Ce-ZnO treated wastewater, chlorinated treated wastewater, fresh water or secondary wastewater in *WP C* –. Both water treatments showed marginal differences in concentrations of the four selected genes (16S rRNA, bla_{OXA-10}, *qnrS and intI1*) in water concentrations. As for concentrations in soil after the irrigation campaign, both treatments showed lower levels than those from secondary wastewater but higher levels than fresh water with no major differences between treatments.

Riassunto

La qui presente tesi di dottorato di ricerca concerne le applicazioni di un nuovo fotocatalizzatore per il trattamento di acque reflue. Specificamente, acqua reflua trattata a fine di un riutilizzo a scopo agricolo con enfasi alla mitigazione della propagazione di antibiotico resistenza nell'ambiente. Il suddetto progetto è diviso in tre work packages (WP). WP A - Selection of a suitable photocatalyst – Selezione di un Fotocatalizzatore Adeguato, si occupa di individuare un fotocatalizzatore con elevate prestazioni di inattivazione batterica pur rimanendo competitivo a livello di sintesi. Tramite WP-A un fotocatalizzatore a base di ossido di zinco dopato con 4% cerio è stato individuato è usato nei work packages successivi. WP B - Catalyst immobilisation – Immobilizzazione del Catalizzatore riguarda il processo di rivestimento di superfici dei dischi in acciaio al fine di ottenere un fotocatalizzatore attivo senza il bisogno di mantenere catalizzatori in polvere in sospensione. Il ZnO dopato con cerio è stato usato per la degradazione di 2 antibiotici – trimetoprim e sulfametossazolo, con prove di durabilità del disco sottoponendolo a 5 prove senza nessuno trattamento di rigenerazione del catalizzatore. Usando altri dischi, il fotocatalizzatore immobilizzato è stato anche usato per la disinfezione di Escherichia coli (DH5-α) in acqua salina isotonica e per la disinfezione di batteri autoctoni (*E. coli* e *Pseudomonas aeruginosa*) in acqua reflua secondaria, monitorando le frazioni resistenti a 2 antibiotici per ciascuna specie di batterio. Il catalizzatore ottimizzato in WP-A ha mostrato un livello di attività superiore a TiO2-P25 – un fotocatalizzatore commercialmente disponibile è usato come il benchmark nella letteratura. Il ZnO dopato con cerio, avendo dimostrato elevate prestazioni, è stato utilizzato per il trattamento di acqua reflua secondaria per irrigazione di lattuga (Lactuca sativa) in WP C – HPC and chlorination for AR abetment in irrigation – Fotocatalasi e Clorinazione per l'Abbattimento dell'Antibiotico Resistenza in Acqua di Irrigazione. Quattro serie da sei piante sono state irrigate con un tipo di acqua fra: acqua clorata, acqua trattata fotocataliticamente, acqua reflue secondaria non trattata e acqua potabile. Entrambi i processi di trattamento hanno registrato minuscole variazioni nelle concentrazioni in acqua dei quattro geni monitorati (16S rRNA, *bla*$_{OXA-10}$, *qnrS* and *intI1*). Le concentrazioni di questi geni nel suolo dopo la campagna di irrigazione mostrano una variazione più drastica. Nel suolo che ha ricevuto acqua non trattata è stata rilevata una concentrazione di geni molto più alta. Inoltre non è rilevata una differenza statisticamente significante fra i due trattamenti anche se questi livelli sono minori della serie che ha ricevuto acqua non trattata a più della serie che ha ricevuto acqua potabile.

About ANSWER-ITN

This Ph. D. project was developed within a European Union Horizon 2020 funded Marie Skłodowska-Curie Innovative Training Network (ITN) entitled *ANtibioticS and mobile resistance elements in WastEwater Reuse applications: risks and innovative solutions* (ANSWER) which ran from October 2015 to September 2019. It involved 15 early stage researchers (ESRs) at 10 beneficiaries in 9 different countries. The work carried out herein is directly linked to this project with the PhD candidate being one of the 15 ESRs hired within ANSWER-ITN. Prof. L. Rizzo was both the PhD supervisor and, as the UNISA grant holder, was also responsible for supervising the specific ESR project objectives.

ANSWER dealt with tackling gaps in knowledge pertaining to the distribution, proliferation and risk of antibiotics including transformation products, antibiotic-resistant bacteria and antibiotic resistance genes, in urban wastewater reuse applications. A major part of this was to better understand the steps necessary to mitigate the risk associated with the practice of wastewater reuse along the wastewater-soil-crop continuum.

Disclaimer:

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The content herein reflects only the author's views and the Research Executive Agency is not responsible for any use that may be made of the information it contains.

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List of Abbreviations

AOP(s)	Advanced Oxidation Process/-es
ARB	antibiotic resistant bacteria
ARG(s)	antibiotic resistance gene(s)
BET	Brunauer-Emmett-Teller
BOD_5	five-day biochemical oxygen demand
COD	chemical oxygen demand
DOC	dissolved organic carbon
DRS	UV–Vis diffuse reflectance spectroscopy
exDNA	extrachromosomal DNA
FT-IR	Fourier Transformed – Infrared Spectroscopy
gDNA	genomic DNA
IPCC	Intergovernmental Panel on Climate Change
LOQ	limit of quantification
ROS	reactive oxygen species
SMX	sulfamethoxazole
SS	stainless steel
SSA	specific surface area
TMP	Trimethoprim
TSS	total suspended solids
TWW	treated wastewater
UWTP(s)	urban wastewater treatment plant(s)
VSS	volatile suspended solids
WP(s)	work package(s)
WWTP(s)	wastewater treatment plant(s)
XRD	X-ray diffraction
XRF	X-ray fluorescence spectrometry

List of Papers Published

- Zammit, I.; Vaiano, V.; Iervolino, G.; Rizzo, L., Inactivation of an urban wastewater indigenous Escherichia coli strain by cerium doped zinc oxide photocatalysis. *RSC Advances* 2018, *8*, (46), 26124-26132.

- Manaia, C. M.; Rocha, J.; Scaccia, N.; Marano, R.; Radu, E.; Biancullo, F.; Cerqueira, F.; Fortunato, G.; Iakovides, I. C.; Zammit, I.; Kampouris, I.; Vaz-Moreira, I.; Nunes, O. C., Antibiotic resistance in wastewater treatment plants: Tackling the black box. Environment International 2018, 115, 312-324.

- Zammit, I.; Vaiano, V.; Ribeiro, A. R.; Silva, A. M. T.; Manaia, C. M.; Rizzo, L., Immobilised Cerium-Doped Zinc Oxide as a Photocatalyst for the Degradation of Antibiotics and the Inactivation of Antibiotic-Resistant Bacteria. Catalysts 2019, 9, (3), 222.

- Accepted for publication: Iervolino, G; Zammit, I.; Vaiano, V; Rizzo, L. Limitations and perspectives in wastewater treatment by UV and visible light active heterogeneous photocatalysis: a critical review

- In preparation: Zammit, I.; Marano R.; Vaiano V.; Cytryn E.; Rizzo L. Accumulation of antibiotic resistance genes in soil after irrigation with treated wastewater: a comparison between heterogeneous photocatalysis and chlorination

Last updated: 2019-11-26

1. Introduction

1.1. Water scarcity

For the majority of the population in developed countries, water scarcity is often thought as a remote issue confined to the middle east or undeveloped nations. The fact of the matter is that more than 3 billion people globally experience severe water scarcity for more than 3 months a year, amongst which a sizable population in industrialised nations in Europe, the US and Australia. Figure 1 shows the number of months a year where the blue water footprint is more than double the blue water availability.[1] In other terms, water scarcity here is defined as instances when freshwater sources (both ground and surfaces waters) are consumed at a rate that is at least twice larger than its availability, taking into account evaporative losses in agriculture, incorporation in produce/products or discharge to non-potable sources.

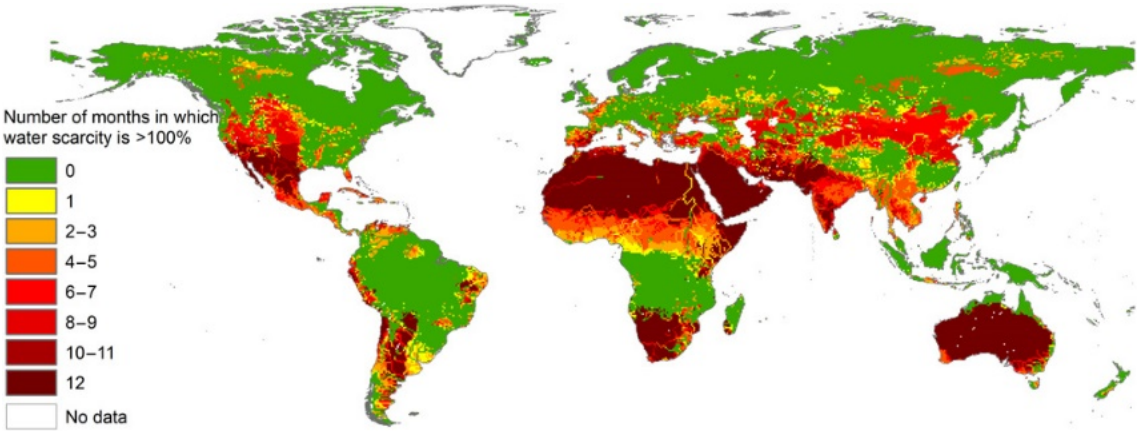


Figure 1 - Global annual water scarcity from Mekonnen and Hoekstra[1]

While the European Union, as a whole, fairs well in terms of renewable water availability, the distribution and consumption within the EU is highly variable. The Mediterranean European countries, which have warmer climates, lower rainfalls and strong agricultural sectors, experience elevated water stress and high water extraction volumes from river basis.[2] However European water stress is not limited to these countries, regions with high population densities in central European countries, such as Belgium, the Netherlands, Luxembourg. As well as the Greater London area, also suffer from elevated water stress. Thus, at a regional level within

the EU, water stress is a very real problem. While the issue of high water stress is by far not a new phenomenon nor is it one that is easily solved in the short term. Projections on the state of European water availability are cause of concern.

Figure 2 shows water stress, in terms of river basin water extraction, from the WaterGAP2 model [3] in Europe for the year 2000 as well as projections for the year 2030.

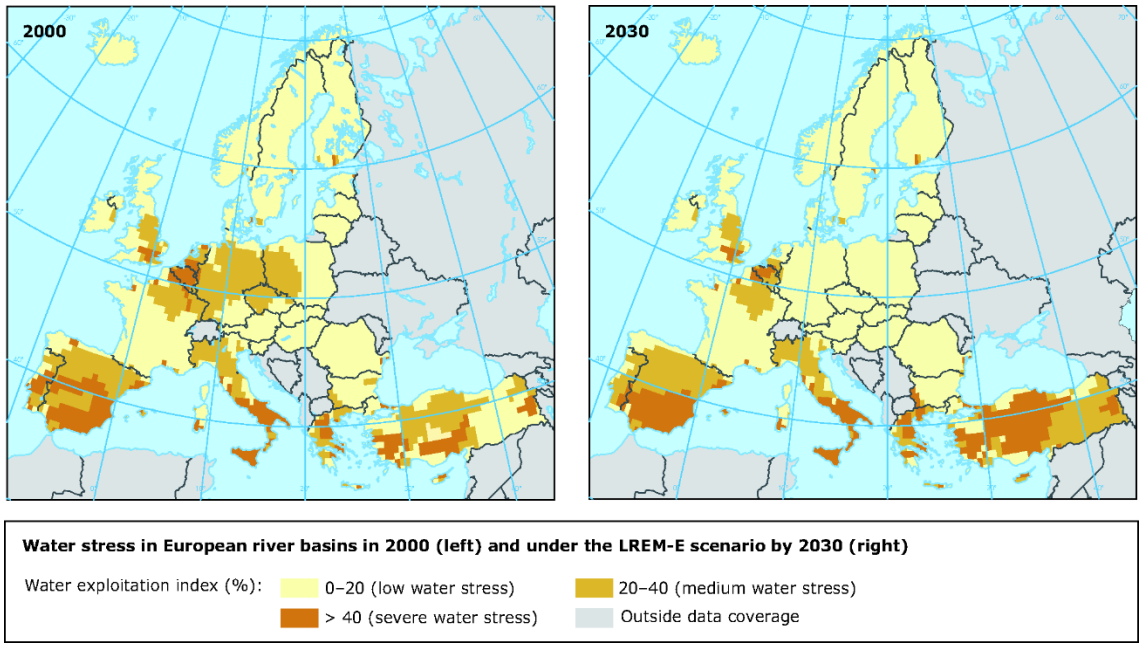


Figure 2 - Water stress in European river basins modelling[4] visualisation from [2]

Apart from the small island nations of Cyprus and Malta which have exceedingly high levels of water stress and as a consequence must rely heavily on desalination plants, Greece, Spain, Italy and Portugal experience elevated water stress. These countries are also the ones with the highest water percentage consumption for agricultural irrigation in the EU with more than 50% of extracted freshwater used in agriculture.[2] Under such heavy dependence of agriculture on irrigation (cf. rainfall irrigated), even temporary bouts of droughts can cause major economic damage in terms of agricultural productivity. In 2003, Europe experienced an extreme period of drought and heat with July temperatures being 6 °C above average and rainfalls at less than 50% of the annual averages.[5] This resulted in drastic economic and environmental effects with an estimated loss in agriculture and forestry sectors by more than 10 billion euros (4 billion euros in Italy alone)

and reversal of 4 years of carbon sequestration due to loss of plant growth.[5, 6] Similarly an intense drought that hit Italy in the summer of 2017 was estimated to have cost the Italian agricultural sector 2 billion euros (industry estimate by Coldiretti).[7]

The effects climate change has on water availability is also a cause of major concern. Apart from increases in frequency of abnormal events such as flooding, temperature extremes, temporal drying out of water streams, lower volumes of water recharging aquifers etc.[8] The Intergovernmental Panel on Climate Change (IPCC) paints a very bleak outlook for droughts in Europe, with Spain and Italy particularly impacted. Under the most extreme predictions, virtually all of Italy will experience what was previously the most drastic drought in a century, taking place every decade.[8]

Overall water availability or lack thereof is first and foremost an economic issue. Residential, industrial and agricultural sectors all, directly or indirectly depend heavily on the availability of affordable, clean water. Low water security or low water quality results in economic, health and productivity losses to societies.

1.2. Future water management and treated wastewater reuse

Future water management will include myriad approaches in both policy as well as infrastructure such as, water pricing, building new reservoirs, dams, desalination plants and water transfer networks, increasing rainwater harvesting, improving water utilisation efficiency, reducing freshwater losses, prospecting for unexploited sources of ground water and treated wastewater (TWW) reuse.[8, 9]

Wastewater reuse in particular stands out due to its large potential, in terms of volumes and lower costs. In developed countries with an arid climate, wastewater reuse is already implemented at a large scale. Notable examples are Australia, California, Israel and Singapore. Within the EU, very high proportions of wastewater reuse are reported in Cyprus (almost complete reuse) and Malta (≈60 %), while in absolute terms Spain (347 Mm^3/yr) and Italy (233 Mm^3/yr) dominate albeit it proportionally to their respective volumes of wastewater

generated only 12 % (Spain) and 5% (Italy) * are reused.[10] However the low relative reuse of wastewater (WW) in these two countries is also an opportunity for expansion. Political momentum to expand TWW reuse exists. At a European level, the European Commission recently published a proposal[11] to unify minimum water quality requirements for wastewater reuse, within this document they state:

> The general objective is to contribute to alleviating water scarcity across the EU, in the context of adaptation to climate change, notably by increasing the uptake of water reuse – *EC COM(2018) 337 final*

Wastewater reuse, is thus considered to be an integral part of the EU's action plan for the circular economy as outlined in COM(2015) 614 final [12]

1.3. Applications for treated wastewater

Wastewater that has been treated to suitable levels finds numerous uses, generally classified in two main types, potable and non-potable with a third category of unplanned reuse (Figure 3).

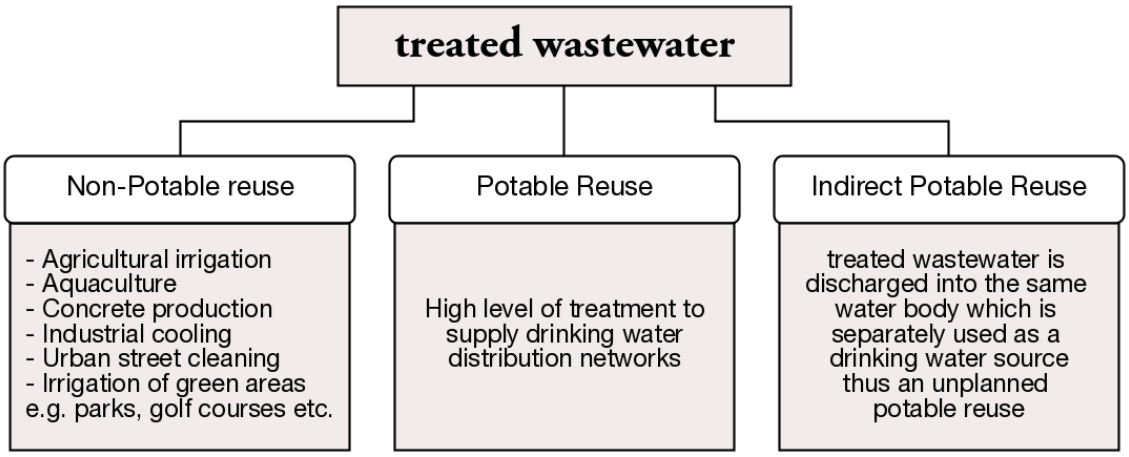


Figure 3 - Types of uses for reclaimed wastewater

Potable reuse is a minor application of treated wastewater. Windheok in Namibia has had a programme of recycle wastewater to potable water for over four decades however no known operations take place in Europe.[13, 14] The extremely stringent water quality requirements needed and the potential hazards outweigh the benefits

* All figures from 2006

especially when water desalination is an option. Thus, direct potable reuse is generally reserved as a last resort in areas which are far away from the sea.

Indirect potable reuse on the other hand is the discharge of treated wastewater in a waterbody that also acts as a source for drinking water treatment plants. The treated wastewater would not have been treated to a higher standard than normally required for discharge and thus wastewater and drinking water are linked in an unplanned manner. More recently, a middle ground between potable reuse and indirect potable reuse is becoming more commonly implemented. This involves the treating of wastewater to higher standards, followed by stabilisation in an environmental buffer such as is an aquifer, which is to be used as a drinking water source after a planned period of incubation.

By far the most extensive use of treated wastewater however is non potable reuse.[15] This includes treated wastewater for cleaning of roads, irrigation of parks, in aquaculture, cooling in industrial installations, and most significantly agricultural irrigation.[15]

1.4. Wastewater treatment plants

When talking about treated wastewater, it is essential to understand the role of wastewater treatment plants (WWTPs) as the sites of collection of raw sewage from sewage networks and processing prior to discharge or reuse. While EU member states, at a national level, can have more stringent regulations, at an EU level wastewater treatment is regulated under the Council Directive 91/271/EEC which was adopted in May 1991.[16] Amongst its articles, it stipulates the need to treat all wastewater from all agglomerates of more than 2000 population equivalents (i.e. 60 g of 5-day biological oxygen demand in the water per population equivalent) to at least secondary levels, to monitor water quality, and regulate discharge to sensitive areas and the disposal of sludge. While variations in the applied technologies in WWTPs exist, however they all rely on simple physical and biological sequential stages for removal of macro-contaminants and to reduce

biodegradable organic load to acceptable levels. A simplified schematic of an activated sludge urban wastewater treatment plant (UWTP) is shown in Figure 4.

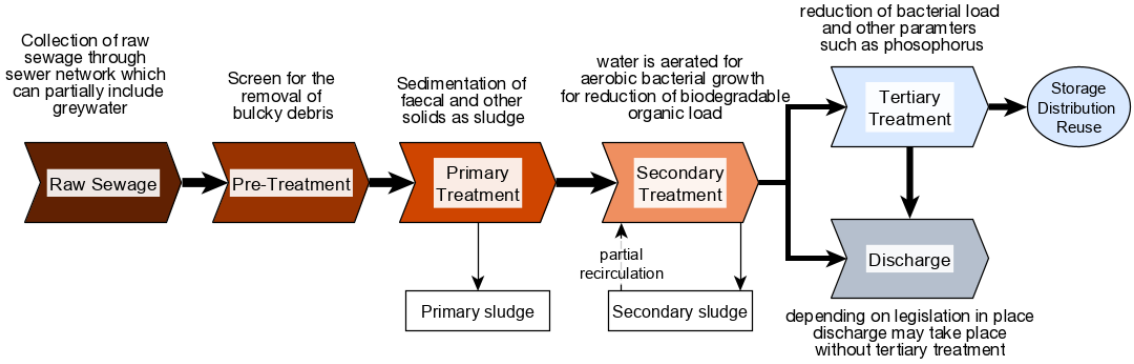


Figure 4 - Simplified schematic of an activated sludge WWTP

Raw sewage that is agglomerated from different non-industrial units are first pre-screened to remove large debris that may obstruct operation of the plant. This stage is followed by a primary sedimentation stage where solids are precipitated from wastewater under gravity and grease and oils are skimmed off the surfaces if present. The core of a WWTP is the secondary stage, also known as the biological stage. Here the WW is aerated to allow the rapid growth of aerobic bacteria which consume biodegradable organic matter in WW and incorporate it as biomass which is then removed as sludge during the secondary sedimentation stage.

Figure 5 - Secondary treatment tanks at the Prat de Llobregat WWTP, Barcelona during and ANSWER-ITN visit. June 2016

Depending on – the regulation in place, site of discharge and efficiency of the WWTP – treated wastewater may be discharged to receiving water bodies or wetlands as is, or if required, it is treated at a tertiary level and then discharged or reused. Tertiary treatment technology, such as would be the application of heterogeneous photocatalysis in WW treatment, will be discussed in following sections.

1.5. Tertiary wastewater treatment

Tertiary treatment is an additional water polishing step that – may be needed prior to discharge and that is virtually always applied prior to wastewater reuse – in order to satisfy discharge/reuse regulation in its relevant jurisdiction. The definition is rather arbitrary and may include any additional treatment after the biological (secondary) treatment (Figure 4). It is, however, often understood, to include additional nitrogen and phosphorus removal which cause eutrophication of receiving water bodies, filtrations steps and disinfection. Disinfection is the pertinent process herein, – it is the goal in the application of heterogeneous photocatalysis and is the process relevant to antibiotic resistance mitigation.

While regulations were established in order to protect public health and environmental concerns, the parameters to monitor and the thresholds, are a matter of debate amongst scientists and policymakers. It is clear however that WWTPs are major point sources with environmental consequences; the identification of a specific issue and how this issue can be targeted during wastewater treatment results in major benefits for the environment.

1.5.1. Heterogeneous Photocatalysis as a Tertiary wastewater treatment

Heterogeneous photocatalysis (HPC) is a type of Advanced Oxidation Process (AOP). AOPs are a group of water treatment technologies that are based off the generation of reactive oxygen species (ROS). HPC involves a solid semiconductor that acts as a photoactivated catalyst that upon irradiation and in the presence of water, it is able to generate ROS. Figure 6 shows a schematic of a semiconductor acting as a photocatalyst. These materials possess a valance band, where most

electrons are found, that is separated from the conduction band by forbidden energy states, this separation is termed the band gap and has a specific energy for a material at a set temperature.[17] At absolute zero (0 K) the valance band is fully occupied by electrons while the conduction band is devoid of electrons. In photocatalysis, a phenomenon called photoconductivity of semiconductors is exploited. This phenomenon is the promotion of electrons from the valence band to the higher energy level i.e. the conduction band, by absorption of photons which have an energy level equal or higher to that of the band gap as per the Planck Einstein relation (Eq. 1).[17]

$$E = \frac{hc}{\lambda} \qquad \textit{Eq. 1}$$

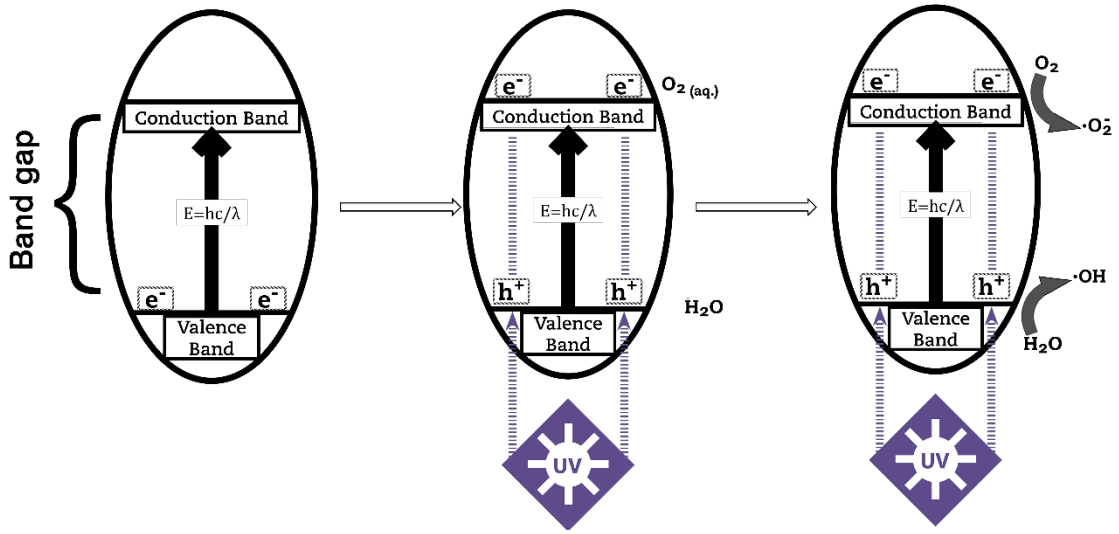


Figure 6 - Semiconductor photocatalysis and generation of ROS

In terms of environmental photocatalysis, the two most commonly employed semiconductor photocatalysts are TiO_2 and ZnO. Both have different crystal lattice forms which have different band gaps. For TiO_2, at room temperatures, these are 3.00 eV for rutile, and 3.21 eV for anatase, [18] while for ZnO in its main form (wurtzite) the band gap is 3.20 eV.[19] Using the Planck Einstein relation, the longest wavelength that can induce promotion to the conduction band is 413 nm for bandgaps of 3.0 eV and 386 nm for bandgaps of 3.21 eV, i.e. at the UV-A fringe of the electromagnetic spectrum.

On irradiation of a photocatalyst with wavelengths of suitable energy and in the presence of water, results in electrons from the valence band being promoted to the conduction band, leaving behind and electron hole (h^+) (Figure 6). The electron hole is a state in which an electron would be energetically stable and regaining an electron at the electron hole is thus favourable. There are multiple ways in which the absorbed energy is dissipated to reform the lower energy states, as far as HPC for water treatment is concerned semiconductors oxidise water by a transfer of electron from the water molecule to the electron hole to produce hydroxyl radicals ($OH^{\bullet}$) and a proton. The electron promoted to the conduction band is of higher energy and thus prone to reaction, dissolved oxygen acts as an electron acceptor to form superoxide radicals ($O_2^{\bullet-}$). Hydroxyl radicals and to a lower extent other ROS, are the driving force of HPC for water treatment. The high reactivity of these radicals and their non-selectivity results in HPC being active on virtually all organic water pollutants, including bacteria and other pathogens in wastewater disinfection.

Heterogeneous photocatalysis for water treatment has major advantages over other wastewater treatment technologies, such as: there is no need to continuously add an oxidant/disinfectant since the oxidant is generated *in situ* by the absorption of energy in the form of electromagnetic radiation. Solar photocatalysts, that is, materials that are active under solar radiation have also been developed through semiconductor doping,[20-23] and thus it is possible to use solar energy and thus reduce costs.

Despite the potential and intense research carried out on HPC in the past decades, it has failed to meet its expectations and become established outside of the lab.[24] HPC was first used for the oxidation of environmental contaminants more than 40 years ago[25] and for bacterial inactivation more than 25 years ago.[26] The most limiting issue with HPC is its high cost relative to competing water treatment technologies. With a lack of need for higher water quality HPC cannot realistically compete with the alternatives such as chlorination or UVC disinfection. It is

estimated that HPC needs to become one order of magnitude more efficient in order to compete head on with other treatment technologies.[24, 27]

In the short term, it is improbable that HPC will replace conventional technologies in fulfilling current water quality targets. However, HPC can find specific uses as an alternative technology and/or niche applications in order to overcome the shortcomings and/or deleterious side-effects (such as formation of toxic by-products) conventional technologies are known to have.[28]

HPC would become feasible with stricter wastewater discharge requirements. An example of a shift to more intensive and costly treatments becoming feasible is provided by a change in law in Switzerland. The new Swiss Water Protection Ordinance required the removal, at high percentages, of selected organic micropollutants and this resulted in the adoption of ozonation systems at mass scale in UWTPs.[29, 30] Another promising field in which HPC may offer considerable improvements over conventional disinfection is its application for the mitigation of antibiotic resistance in the environment. It is not yet clear if HPC sufferance from the same limitations as other wastewater treatments process, specifically if AR prevalence increases after treatment.

1.6. Antibiotic resistance and the environmental dimension

Antibiotic resistance in bacteria is a natural biological process in which bacteria respond to a deleterious stimulus, such as the presence of antibiotics, and evolve defensive mechanism to counteract the bioactivity of these antibiotics. This process is by no means new, Alexander Fleming, the discoverer of the first therapeutic antibiotic (Penicillin) way back in his 1945 Nobel Prize lecture predicted that:

> The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the [uninformed person] may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant
>
> A. Fleming, December 11, 1945, Stockholm, Sweden

The mechanisms of AR resistance have been reviewed in detail by Blair*, et al.*[31] In summary the mechanisms used by bacteria to combat the effect of antibiotics fall into one of three categories. (a) the use of efflux pumps that transport antibiotic molecules, that have bypassed the cell membrane, back to the extracellular matrix. (b) bacterial alternations to the target site of the antibiotic or synthesis of competitive binders, both of which prevent binding of the antibiotic to its target site. (c) direct action on antibiotic molecule such as synthesis of enzymes that degrade the antibiotic. All of these defensive mechanisms involved changes to the bacteria at a genetic level which, in suitable conditions, are propagated vertically by naturally selection and, additionally in bacteria, also horizontally via transduction, conjugation and transformation.[32]

While the nosocomial acquisition of antibiotic resistance is by far the most direct and significant dimension for pathogenic bacteria to acquire resistance, the environmental dimension should not be ignored and is part of the action plan of the Food and Agriculture Organization of the United Nations.[33] The concern is that resistance that develops in environmental bacteria, for example due to improper management of wastewater, can transfer resistance genes to pathogenic bacteria. In other words, that the environmental resistome may act as a source to the clinical resistome.[34]

While it is not clear how probable AR acquisition is within an UWTP , it is well established that UWTPs are point sources for the release of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs) into the evniornment.[35] There is also a large body of evidence showing that the disinfection processes applied at UWTPs, while they succeed at reducing the absolute number of ARB and ARGs, are more effective on non-resistant bacteria rather than the resistant ones. The differential mortality of non-resistant bacteria cf. ARB results in an increase in prevalence of resistance as defined by Eq. 2

$$\text{AR prevalance} = \frac{\text{number of ARB}}{\text{total number of bacteria}} \qquad \textit{Eq. 2}$$

ARB and ARGs are not like chemical pollutants, they have a major difference, that is the ability to replicate after being released into the environment. Replication is dictated by population dynamics such as the available environmental resources and inter- and intra- species competition. Thus, the prevalence of AR discharged is as relevant as the absolute quantity of ARB and ARGs released to the environment and any treatment aimed to mitigate AR effect should target both.

Disinfection treatments however have been developed to reduce the quantity of indicator bacteria without taking into account antibiotic resistance. Research on AR prevalence after chlorination,[36, 37] UVC,[38, 39] and ozonation[38-40] all have shown an increase in prevalence of resistance. At the start of this Ph. D. project, it was not clear if HPC will also result in increasing AR prevalence on treatment and research on this topic is one of the projects strongest novelty factors. The goal here is to anticipate future regulatory demands in which AR mitigation might be put as a specific treatment goal. Thus, while HPC is not currently economically feasible, the development of a treatment process involving HPC that at the very least doesn't increase AR prevalence and at best reduces it, coupled with specific AR mitigation targets would justify the higher cost of this treatment.

1.7. Mechanisms of reactivity of HPC

To better understand the effects of HPC as a water treatment technology, one has to understand the chemical mechanisms of action. While HPC shares common mechanisms with the other AOPs, it diverges substantially in that it is a heterogeneous process and that the reactive radicals are generated *in situ* in a highly endothermic process (the O-H bond dissociation energy of water is 491.9 kJ/mol).[41]

As relevant to bacterial degradation and DNA damage (for ARG degradation), two main categories exist - oxidation reactions via valance band holes and reactions via ROS as are illustrated in Figure 7. Direct oxidation proceeds by an electron from e.g. bacterial cell walls or an electron rich moiety is transferred to the valance bond. This leaves behind a radical that is unstable and goes on to react further or

rearrange. Reactions with ROS especially hydroxyl radicals are however the major driving force of HPC treatments.

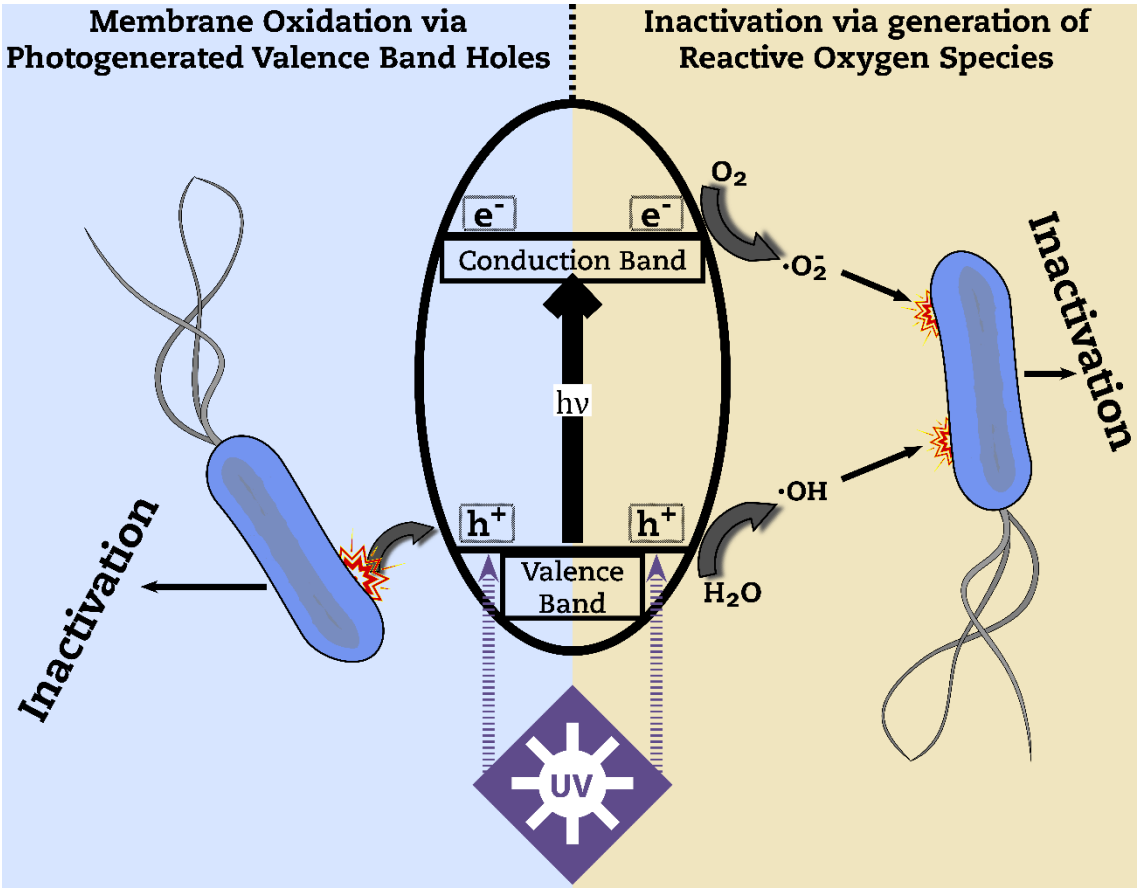


Figure 7 - HPC mediated reactions in water treatment

ROS - as detailed in section 1.5.1 are generated on photo-activation of the catalyst in the presence of water. The main ROS generated in HPC are hydroxyl radicals and superoxide radicals. The former however is the most dominant since its reactions with many organic compounds, approach the theoretical diffusion-controlled limit and as confirmed by quencher studies, OH• have reaction rate constants and order of magnitude higher than other ROS. [42-45]

Hydroxyl radicals react in two main ways. An electrophilic attack on a carbon hydrogen bond, where a hydrogen and one electron is used to form a water molecule and an aliphatic radical (Figure 8(i)). Hydroxyl radicals also reacted with via radical addition (Figure 8(ii)) at a higher rate than hydrogen abstraction. The hydroxyl radical here adds to the electron rich unsaturated bond to form a hydroxyl

adduct. Both these types of reactions are electrophilic attacks and hence are dependent on the electron density present at the bond of reaction.

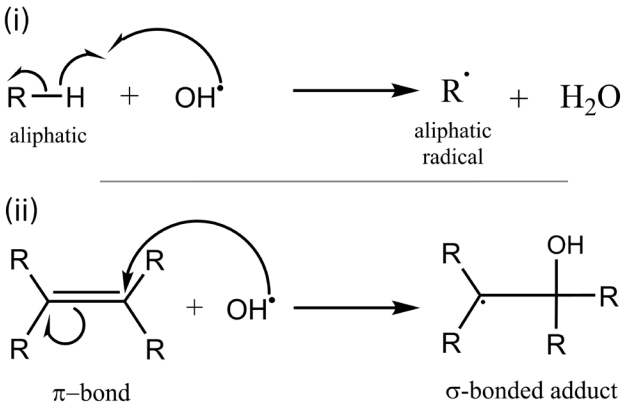


Figure 8 - (i) hydrogen abstraction by hydroxyl radicals and (ii) radical addition of hydroxyl radicals to aliphatic compounds

In a real wastewater matrix electron density of the myriad chemical components is a crucial factor in treatment. This is since the efficacy of treatment for a target pollutant, be it bacteria or ARGs, depends on the mixture of other components and how electron rich they are since these would compete with the target pollutants in reacting with hydroxyl radicals. Such considerations have also been discussed in more detail in a review paper in which the undersigned is a co-author.[46]

These two types of reactions are also the mechanisms that result in bacteria inactivation and DNA degradation. Cho*, et al.*[47] demonstrated that a linear relationship between concentration of hydroxyl radicals ([OH•]) and rate of bacterial inactivation exists. Concordantly, Kiwi and Nadtochenko[48] looked at FT-IR bands of biochemical components of bacteria cell wells, specifically polysaccharide bands, acyl bands, methyl C=C bands all of which form components in the cell walls. They demonstrated that with reaction time during photocatalysis, the intensity of these bands was diminishing due to the reaction of the compounds that produce them with hydroxyl radicals. Additionally, new bands (i.e. those from aldehydes, ketones, and carboxylic acid bands) that correspond to the expected products of the reactions of hydroxyl radicals with the aforementioned components arose. Other studies dealing with identifying reaction components in bacterial peroxidation follow the generation of a marker of oxidative stress (malondialdehyde) as a proxy for general lipid peroxidation.[49, 50]

Bacterial DNA exists in two main forms, genomic (gDNA) and extrachromosomal (exDNA). In a prokaryote, such as are all bacteria, gDNA is circular, not bound to proteins, smaller in size, and is not housed within a nuclear envelope. Moreover, Bacteria possess exDNA in the form of plasmids. These are smaller (fewer base pairs) circular DNA units that are of high relevance to AR transfer since they often house ARGs and are more easily exchanged horizontally amongst bacteria. DNA itself, is a biopolymer formed of nucleobases (Figure 9) linked on a phosphate deoxyribose backbone containing numerous bonds highly susceptible to hydroxyl radical attack. However direct attack on DNA inside a living bacteria cell during HPC is extremely improbable. This is because DNA is found intracellularly, and ROS in HPC are generated extracellularly. For the ROS to react with DNA inside a cell it would need to bypass the bacteria's cell wall, membrane and other components, while also being reactive to many constituents of these components. Thus, DNA degradation during treatment in real terms takes place after the treatment reaches a certain threshold of reactions on the bacterial cell external components that result in cell lysis, and hence release of DNA into the matrix. A very comprehensive review of the reactions of radicals with DNA is provided by von Sonntag[45] These four constituent nucleobases of DNA have the same moieties that are susceptible to oxidative attack by ROS. Intracellularly, ROS are generated naturally during aerobic metabolism. Bacteria have evolved mechanisms to decrease the activity of superoxide and hydrogen peroxide in producing hydroxyl radicals, however there are no known defence mechanisms directly acting on hydroxyl radicals.[51, 52] In artificial matrices to study the effect AOP chemistry has directly on DNA, the expected reaction pathways are observed. Wamer, Yin and Wei[53] and later confirmed by Hirakawa*, et al.*[54] identified a hydroxylation product (8-Oxo-2'-deoxyguanosine) of guanine deoxyribose upon photocatalytic treatment, i.e. an electrophilic attack of the

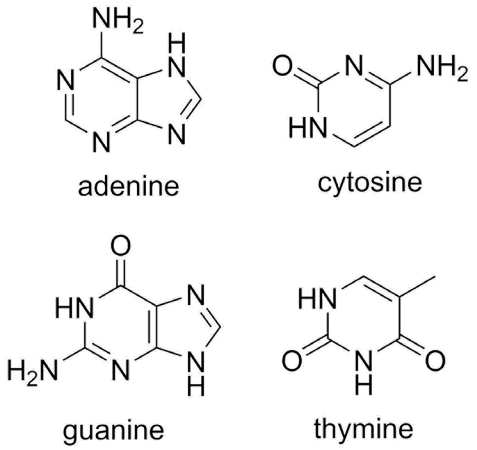


Figure 9 - the four constituent nucleobases of all DNA molecules

electron dense imine bond in guanine. The quantity of this hydroxylation product was dependent on irradiation time and quantity of photocatalyst, linking the reaction directly to the product. In summary, reaction between ROS and DNA is now quite well understood, however in complicated matrices such as WW it is not given that DNA and thus any ARGs are sufficiently inactivated to halt AR spread.

1.8. Project-Wide Objectives and their Divisions into Work packages

With the overall objective being the development of an HPC based process with increased efficiency of disinfection, reduction of treatment costs and to investigate changes in AR prevalence on reusing treated wastewater in crop irrigation, the Ph. D. project was divided into three Work packages (WP).

A) Selection of a suitable photocatalyst.

   The goal was to identify a novel catalyst that shows higher efficiency than the industry standards while also establishing a synthesis protocol that is easy to upscale and uses inexpensive materials that are commercially available.

B) Reduction in operating costs via catalyst immobilisation

   HPC treatments involving powdered catalysts in suspension have major drawbacks pertaining to post-treatment catalyst recovery such as high decantation times or filtration. Additionally, treatment using a powdered catalyst also involves a high energy expenditure in and maintaining the catalyst in suspension i.e. for maintaining the turbulent flow by agitation of air bubbling for mixing.

C) Investigating changes in AR prevalence in soil microbiota after agricultural irrigation

   Directly investigating changes in antibiotic resistant genes and associated genes in soil after an irrigation campaign of lettuce plants using different water regimes. Specifically, HPC treated wastewater, a conventional

treatment (chlorination) and non-disinfected secondary wastewater and fresh water.

1.9. Project Novelty

The project explores multiple new research perspectives. While HPC has been studied extensively in the past, very few of this research deals with applying HPC for the abetment of antibiotic resistance. Prior to the start of the Ph. D. project, only 35 publications dealt with antibiotic resistance and photocatalysis[†] as opposed to 144 at the time of writing. The recent identification of the negative issues that chlorination, ozonation and other disinfection methods have, most importantly the increase of AR prevalence after treatment, makes the search for an alternative treatment ever more pressing. While HPC could be this treatment, prior to the start of the project, very little was known on the effects HPC has on AR prevalence. Another strong point of the Ph. D. project is the multidisciplinarity, both at a local level and at an ANSWER project-wide level. With the help of colleagues with a very vast set of expertise, the Ph. D. project delivered a novel photocatalyst, synthesised from scratch, tested in various matrices and against different pollutants as well as ultimately being used for a practical end goal. Thus having multidisciplinarity ingrained into the project. Likewise, at an ANSWER project level, the work carried out herein, was designed in order to be relevant to the bigger picture. Though extensive coordination between the ANSWER researchers working on different aspects (e.g. geneticists, environmental microbiologists, analytical chemists etc.) of the same issue.

[†]Scopus search for: TITLE-ABS-KEY (antibiotic AND resistance AND photocatalysis) AND (EXCLUDE (PUBYEAR , 2020) OR EXCLUDE (PUBYEAR , 2019) OR EXCLUDE (PUBYEAR , 2018) OR EXCLUDE (PUBYEAR , 2017) OR EXCLUDE (PUBYEAR , 2016))

2. WP A – Selection of a suitable photocatalyst

Part of the work detailed in this section was published under an open access Creative Commons Attribution-Non Commercial 3.0 licence as *Zammit, et al.*[55]:

> Zammit, I.; Vaiano, V.; Iervolino, G.; Rizzo, L., Inactivation of an urban wastewater indigenous Escherichia coli strain by cerium doped zinc oxide photocatalysis. *RSC Advances* 2018, *8*, (46), 26124-26132.

and can be found as Annex 1

2.1. Rationale

The application of HPC in wastewater treatment is severely limited by its cost relative to conventional treatments. It is estimated that HPC needs to be at least 1 order of magnitude more efficient for it to be considered feasible in wastewater treatment.[24, 27] The properties of semiconductor materials can be tweaked using two main methods:

- Doping – Defects i.e. a third ion, are deliberately introduced into the semiconductor crystal lattice. Both anions and cations are used. Doping is subdivided into n-type where the dopant contributes electrons to the lattice and p-type where the dopant having less electrons creates electron holes in the lattice. E.g. of an n-type doped semiconductor is Al^{3+} doped $Ti(IV)O_2$ while Ta^{5+} doped $Ti(IV)O_2$ is a p-type doped semiconductor.[56]
- Coupling – involves using two different semiconductors which have different energy levels and can transfer charges between each other. This can result both in an extended absorption spectrum but also changes in electron-hole recombination. Numerous coupled catalysts have been synthesised in the past two decades such as graphene and graphene oxide coupled with TiO_2.

In order to identify a novel photocatalyst that satisfies the objectives in §1.8A a total of 21 catalysts have been synthesised and pre-screened. Of these, 14 catalysts were

studied to identify their efficiency in inactivating *Escherichia coli* - a common indicator bacteria in wastewater treatment.

Both doped (cerium doped ZnO) photocatalyst and coupled photocatalysts (graphene oxide - ZnO) were studied. Cerium doped ZnO was chosen after promising results in the literature for the removal of chemical pollutants.[57-59] The application of this catalyst for the inactivation of bacteria had not been studied prior to the research detailed herein. Additionally, the quantity of cerium doping, ranging from 0.25% at./at. to 10% at./at., in the ZnO lattice was studied in order to optimise the inactivation rate.

2.2. Methods and Observations

2.2.1. Synthesis of ZnO and cerium doped ZnO

The hydroxide induced hydrolysis of zinc nitrate was used to synthesise the ZnO based catalysts. The method was adapted in collaboration with colleagues at the Department of Industrial Engineering of the University of Salerno (Led by Prof. V. Vaiano) from McBride, Kelly and McCormack[60] 5.0 g (16.8 mmol) of zinc nitrate hexahydrate (Sigma-Aldrich: Purum, >99.0%) were dissolved in 75 mL of MilliQ water, separately 2.2g (55 mmol) of sodium hydroxide (Carlo Erba, RPE) was dissolved in 25 mL of MilliQ water with caution due to the exothermic nature of the dissolution. Once all the NaOH was dissolved it was added dropwise to the Zn^{2+} solution under vigorous stirring at such a rate that it took ≈5 min to add the 25 mL base solution. A white precipitate of zinc(II) hydroxide starts forming after the addition of the first drops of sodium hydroxide as per Eq. 3

$$Zn(NO_3)_2.6H_2O_{(aq.)} + 2OH^-_{(aq.)} \rightarrow Zn(OH)_{2(s)} + 2NO_3^- \qquad Eq.\ 3$$

After the addition of all of the base, the precipitate was left to stir for an additional hour after which it was separated by centrifugation at 1200 ×*g* for 5 min. The water was decanted and 100 mL of water added for washing by resuspending the precipitate, mixing and centrifuging again with the same parameters. Washing water was again removed and the white paste collected and spread on a ceramic container. Water was removed by evaporation at 110°C for 30 min inside an oven

after which the zinc hydroxide was thermally decomposed to the final photocatalyst, zinc oxide, by calcination at 300°C for 1 h as per Eq. 4

$$Zn(OH)_{2(s)} + \text{heat} \rightarrow ZnO_{(s)} + H_2O_{(g)} \qquad \textit{Eq. 4}$$

Cerium doped ZnO was synthesised using an identical method, with the addition of a Ce(III) salt prior to the addition of the base to form the precipitate. A specific amount of cerium(III) nitrate hexahydrate (Sigma-Aldrich, >99%) to obtain the final desired atom ratio of Ce^{3+} and Zn^{2+} was dissolved with the zinc nitrate hexahydrate in the same volume of water. After 5 min of stirring, the NaOH was added drop wise in the same way undoped ZnO was prepared. Seven atomic ratios of Ce : Zn prepared specifically, 0.0025, 0.0050, 0.0100, 0.0150, 0.0400, 0.0700, 0.1000 per atom of zinc.

A difference source of Zn^{2+} was also tested. Zinc acetate dihydrate (Sigma-Aldrich: ACS reagent, ≥98% was used to investigate any difference in the resulting surface area and other physico-chemical properties. The recovery of ZnO in the synthesis ranged from 62-78% in molarity terms.

2.2.2. Synthesis of graphene oxide and coupling with ZnO

A coupled photocatalyst was also synthesised in collaboration with the Department of Chemistry and Biology of the University of Salerno (Prof. A. Proto's Lab). Graphene oxide was produced via the ubiquitous Hummers' method starting from graphite powder as per the method outlined by Chen*, et al.*[61]. The product was filtered overnight, rinsed with water and dried at 60°C for 24 h. The zinc oxide used for the coupling was produced in the same batch as detailed in §2.2.1.

Figure 10 - Autoclave for hydrothermal synthesis of GO-ZnO

The coupling with ZnO was done following the hydrothermal method published by Wu*, et al.*[62] and modified as follows. 10 mL of 0.2% GO in water and the desired quantity of ZnO in 35 mL of water were mixed and sonicated for 10 min. The mixture was transferred to a stainless-steel autoclave with an internal Teflon recipient containing a magnetic follower (Figure 10). The autoclave was placed in an oil bath at 150-160°C for 12 h with the internal airtight components constantly magnetically stirred. After 12 h the heating element was turned off and the autoclave allowed to cool overnight. The contents were then separated by centrifugation at 3400 ×*g* for 10 min, washed with 25 mL of water and centrifuged again under the same conditions. Three loadings of GO were synthesised, i.e. 0.5%, 1.0% and 2.0% w/w relative to ZnO.

2.2.3. Characterisation of catalysts

All synthesised catalysts were fully characterised. This included specific surface area (SSA) via Brunauer-Emmett-Teller (BET) analysis for surface area, X-ray diffraction (XRD) pattern, Raman Spectroscopy and UV–Vis diffuse reflectance spectroscopy (DRS) with Kubelka-Munk analysis for band gap estimation. Additionally for GO, Fourier Transformed-Infrared Spectroscopy (FT-IR) was carried out. In order to confirm the level of incorporation of Ce^{3+} inside the ZnO crystal lattice, x-ray fluorescence spectrometry was used on these samples.

Table 1 - catalyst characterisation results from XRF, SSA and Kubelka-Munk band gap (E_{bg}) estimation

ID	Cerium content (atomic ratio of Ce:Zn)		SSA (m^2g^{-1})	E_{bg} (eV)
	As prepared (precursor ratio)	As measured by XRF		
ZnO	Not Doped	Not Doped	14	3.21
Ce-25	0.0025:1	0.002:1	19	3.19
Ce-50	0.0050:1	0.005:1	23	3.18
Ce-100	0.0100:1	0.011:1	19	3.17
Ce-150	0.0150:1	0.017:1	23	3.16
Ce-400	0.0400:1	0.038:1	22	3.15
Ce-700	0.0700:1	0.074:1	27	3.00
Ce-1000	0.1000:1	0.140:1	28	2.97
TiO_2-P25	N/A	N/A	47	3.30
0.5% GO-ZnO	N/A	N/A	11	3.08
1.0% GO-ZnO	N/A	N/A	13	3.11
2.0% GO-ZnO	N/A	N/A	14	3.10

Table 1 shows XRF, SSA and band gap measurements of the synthesised catalysts. The cerium doping preparation used, successfully incorporated the Ce^{3+} ion in the ZnO crystal lattice. The atomic ratio of Ce:Zn as measured by XRF was also within the expected range from the molar ratio of the cerium and zinc salts added during the preparation. Cerium doping also produced an increase in SSA, undoped ZnO had a relatively low SSA of 14 m^2g^{-1} while Ce-1000 showed the highest SSA of the ZnO based catalysts at 28 m^2g^{-1}. A very clear trend with Ce doping is the shifting of the band gap. The measured band gap of undoped ZnO was 3.21 eV which gradually decreased in energy with quantity of Ce to 2.97 eV in the highest level of Ce doping. In terms of wavelength this means that the undoped ZnO can produce electron hole pairs at wavelengths that are ≤386 nm while the threshold wavelength for Ce-1000 is 417 nm.

The commercial TiO_2-P25 had the highest SSA, this is probably due to the particular method of synthesis that is used which involves gas-phase flame spraying.[63] The measured band gap is concordant with literature since commercial TiO_2-P25 is mainly composed of anatase.[63]

The 3 levels of GO-ZnO synthesised had the lowest SSA of all catalysts and GO at this level did not affect the band gap substantially.

The XRD patterns are shown stacked in Figure 11. XRD of undoped ZnO (red) shows 5 patterns at 2θ 32.06°, 34.74°, 36.53°, 47.85° and 56.90°. In respective order these are associated with the (1,0,0), (0,0,2), (1,0,1), (1,0,2) and (1,1,0,) planes of hexagonal wurtzite ZnO crystal structure [64] confirming the successful synthesis. Since doping was a minor component it did not affect much the diffraction pattern of the crystal lattice, but higher Ce loads did produce weaker peaks. With higher loads of Ce the wide band peak at 28.5° starts to become visible and is attributed to cerium oxide impurities.

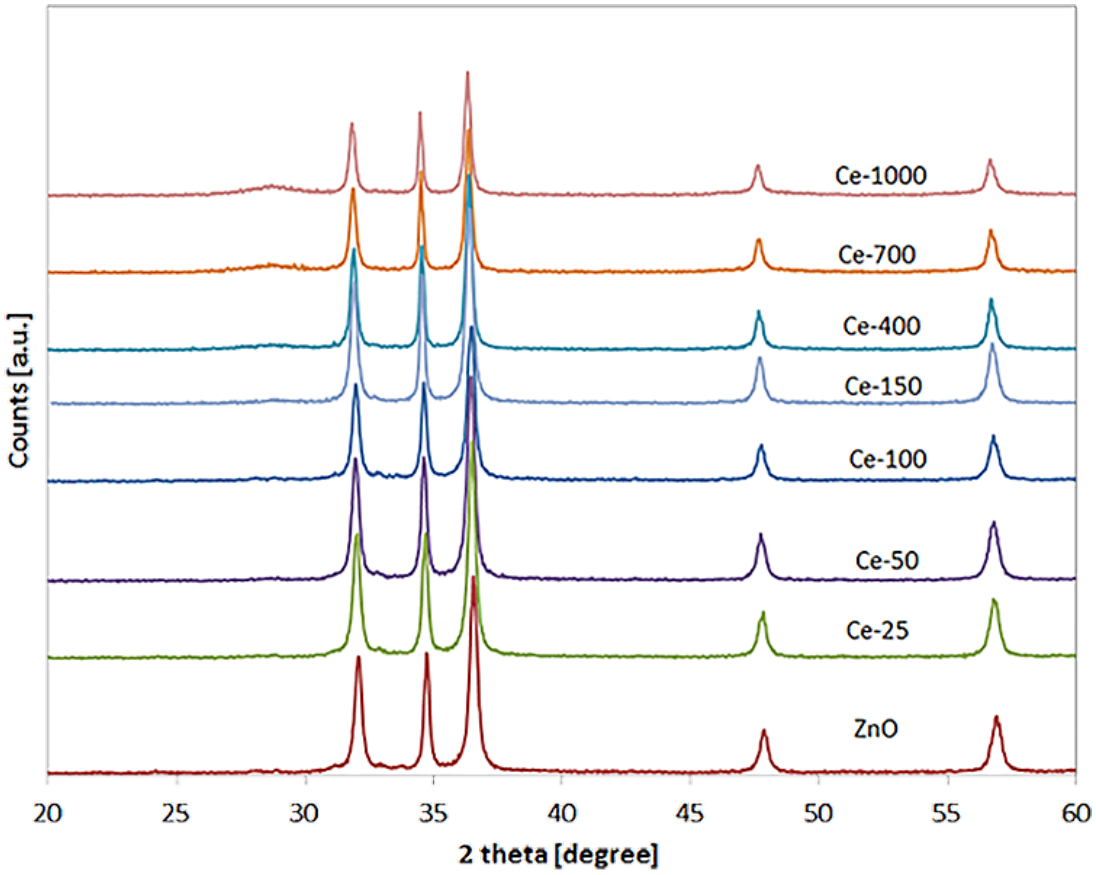


Figure 11 - XRD patterns of the different loadings of Ce as a dopant in ZnO

On increasing quantities of Ce as a dopant a pattern arising from plane (1,0,0) (2θ = 32.06°) is clearly observed when measuring the XRD diffraction pattern at higher resolution (Figure 12). This pattern is attributed to the differences in the ion radii of Ce^{3+} (102 pm) and Zn^{2+} (74 pm) with which partial substitution of the cerium ions in Zn^{2+} sites are inducing a shift and thus confirming that doping is actually taking place not simply the conformation of cerium (IV) and zinc (II) oxides.[65]

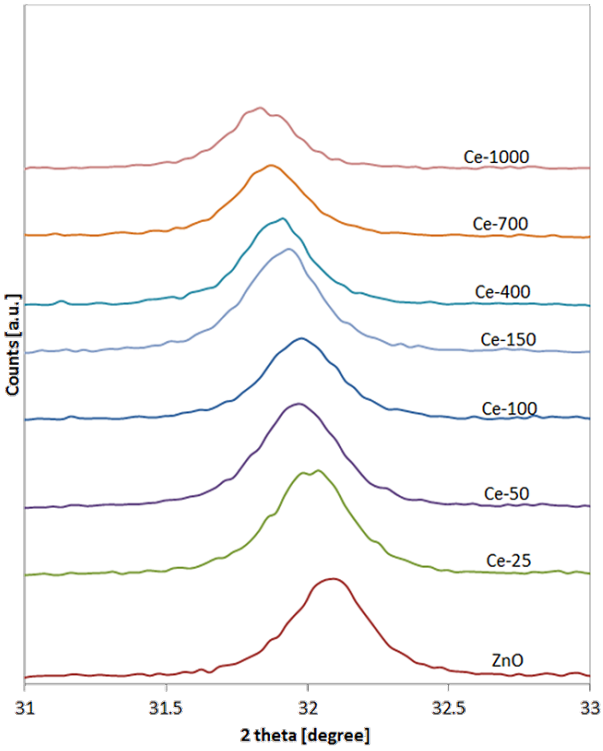


Figure 12 - high resolution XRD pattern (2 θ 31°-33°) of Ce doped ZnO

Raman spectroscopy (Figure 13) also confirmed the successful synthesis of cerium doped ZnO and the impurities of cerium oxide at the highest doping levels. The peaks at 332 cm^{-1}, 379 cm^{-1}, and 438 cm^{-1} are known wurtzite vibration modes.[66] The widening of the peak at higher Ce loads is due to a CeO_2 peak at 456 cm^{-1} merging with the 437 cm^{-1} peak of wurtzite ZnO. Thus also confirming that higher loads of Ce used in preparation also induce higher cerium (IV) oxide impurities.[67] As for the GO-ZnO composite photocatalysts, the successful formation of this catalyst was confirmed by Raman spectroscopy (Figure 14). While also other characterisation techniques were carried out such as XRD, the low quantity of GO and its amorphous nature make this characterisation method less useful.

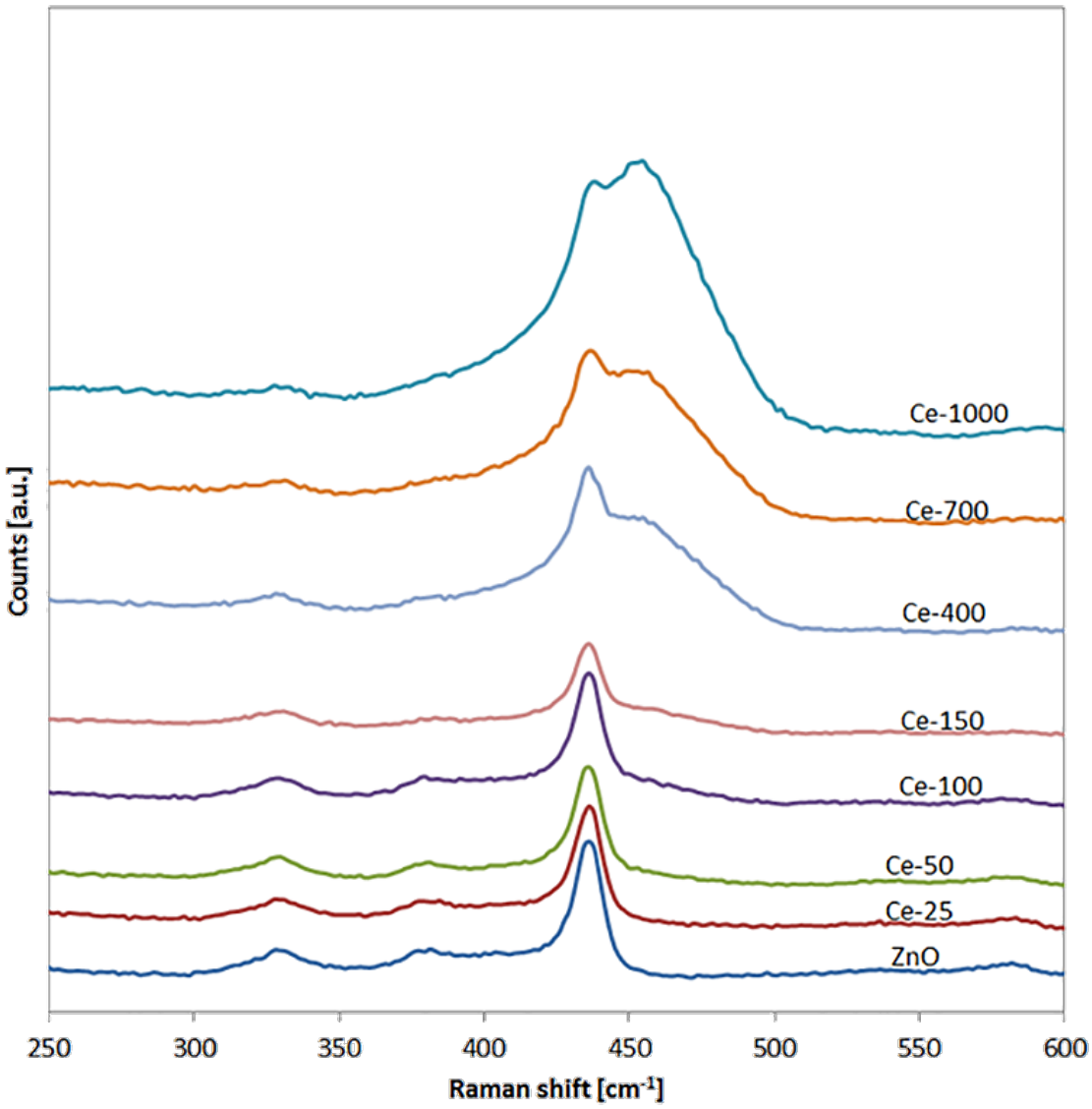


Figure 13 - Raman spectroscopy of Ce-ZnO photocatalysts

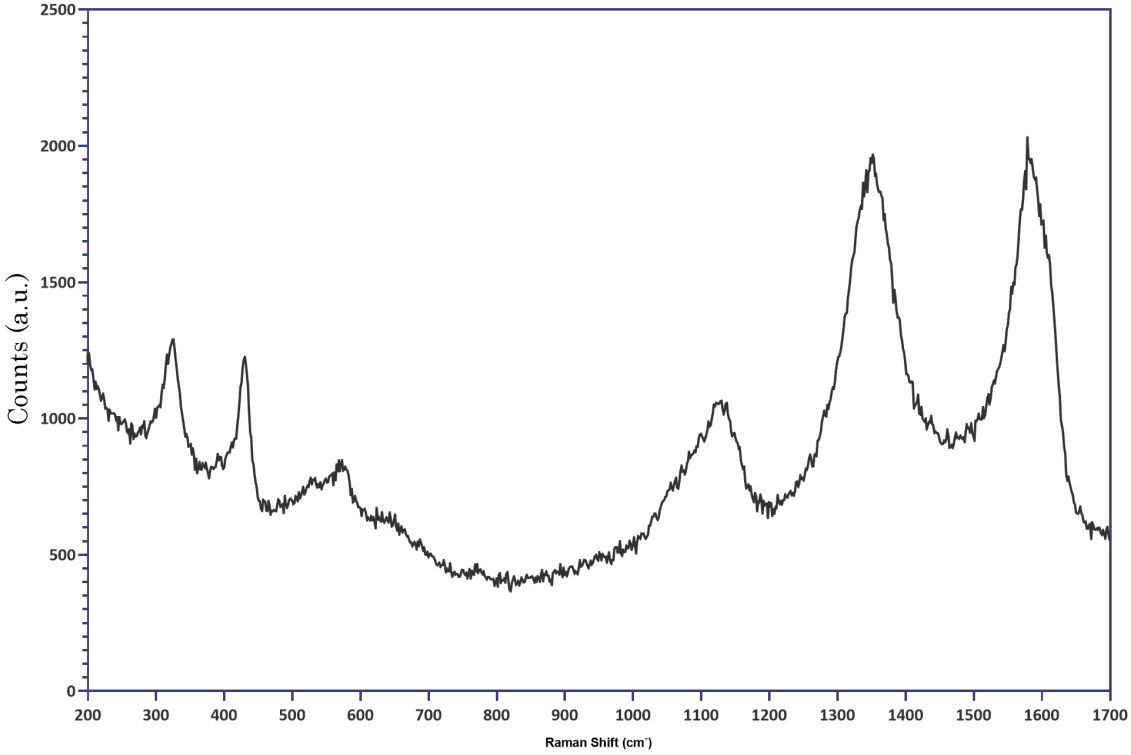


Figure 14 - Raman spectrum of 1% GO-ZnO

Again, in GO-ZnO the wurtzite peak at 438 cm^{-1} is observed which arises from E_2 vibrational modes.[68] The characteristic bands for GO are observed at 1600 cm^{-1} which corresponds to the E_{2g} symmetric stretching of an sp^2 hybridised carbon-carbon bond and at 1355 cm^{-1} showing the D-band that originates from disordered single layer graphene. The broad band peak at ≈1100 cm^{-1} arises from a combination of various types of carbon-carbon bonds and of multiphonon processes in ZnO and are not indicative of a specific structure.[69]

2.2.4. Isolation of *Escherichia coli* from urban wastewater

Secondary effluent wastewater was sampled from a UWTP in Salerno (40.6305700°, 014.8448900°). WW was serial diluted, and three levels of dilutions plated on a selective chromogenic agar (TBX) and incubated at 37°C for 24 h. *E. coli* form blue colonies on TBX by the action of the enzyme group β-glucuronidase which breaks down a proto-dye. An isolated colony was selected and grown in LB broth overnight for stock preservation. The overnight grown broth was then used to produce glycerol stocks kept at -80°C and agar stabs for routine use in disinfection experiments using the same isolate.

2.2.5. Photocatalytic disinfection

The same standardised method was used for disinfection experiments. 0.1g/L of catalysts was used in 500 mL of 0.85% saline solution. The light source was an Osaka 125 W mercury UVA lamp producing 1.26mW/cm^2 at 365 nm peak, (full emissions spectrum is available as supplementary information in [55]) placed 32 cm from the base of an open cylindrical reactor. Catalysts were initially sonicated for 15 min prior to use. An initial bacterial load of 1x10$^{6-7}$ CFU/mL was introduced by growing the stocks overnight, separating the bacterial mass by centrifugation at 1200 $\times g$ for 5 min and spiking a suitable quantity to reach the desired initial bacterial load.

Prior to starting the experiment, the initial bacterial load was enumerated via the plate count method on TBX agar. After which the matrix was kept under stirring in the dark for 60 min for equilibration of physical effects. Only after 60 min was UV light introduced (the lamp was warmed up before) and aliquots taken at regular times to follow the rate of bacterial inactivation. GO-ZnO catalysts were also used in ¼ ringer solution instead of 0.85% saline.

2.3. Results and Discussion

A small decrease in viable counts are observed at time -60 min and 0 min i.e. after 60 min of dark incubation. This is attributed to physical effects such as agglomeration of photocatalyst particles around the bacteria that prevent culturability. The different doping levels of Cerium showed a clear trend (Figure 15). Doped ZnO performed increasingly better with increasing cerium load, up to 4% cerium (Ce-400) after which the higher activity is maintained but not improved. Ce-400 also performed better than the industry standard TiO_2-P25. The starkest difference is between undoped ZnO and the doped ZnO which show a very substantial improvement by the simple modification of Ce doping. After 60min of irradiation Ce-400 inactivated up to 4 orders of magnitude more bacteria than the undoped form Figure 16 - normalised inactivation after 60 min for Ce-ZnO catalysts.

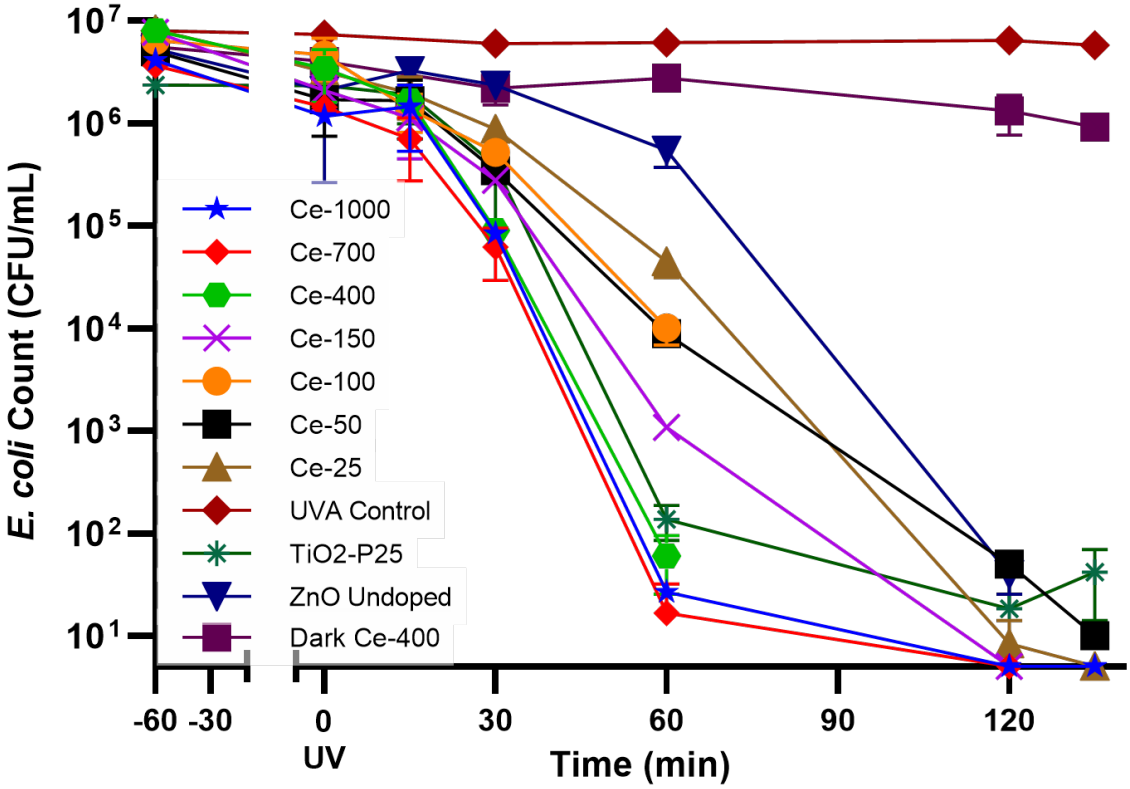


Figure 15 - Ce doping level and bacterial inactivation rates. Err. Bar - SD

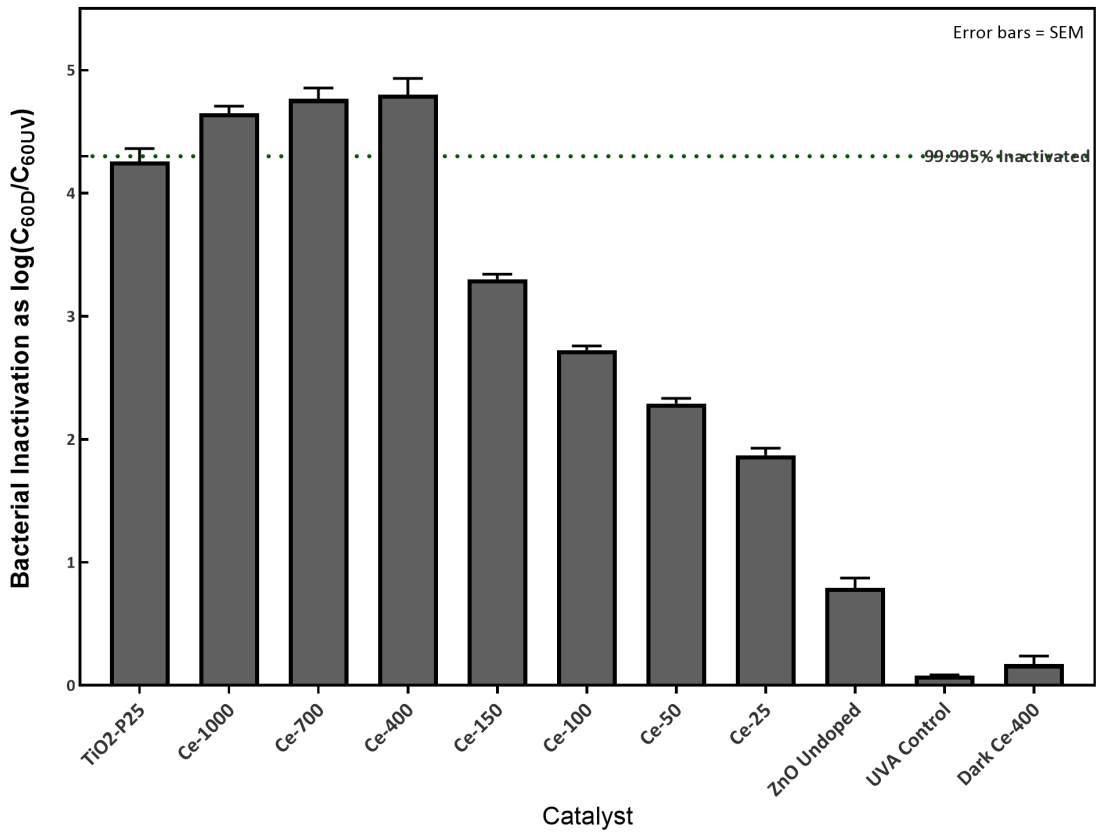


Figure 16 - normalised inactivation after 60 min for Ce-ZnO catalysts

Control experiments were also carried out. The affect of UVA on bacterial mortality was measured by exposing the *E. coli* at the same exact conditions which showed no decrease in viability after 135 min (Figure 15) proving that a photocatalytic process in giving the inactivation and being concordant with literature.[70] Likewise the best performing Ce doped ZnO was used in the dark to confirm that it is not due to any toxic or physical effects that are resulting in bacterial mortality. With a 0.6 $\log_{10}$ difference in viable bacterial loads this can also be excluded (Figure 15).

The loading of GO in the GO-ZnO composite catalyst on the other hand maxed out at 1% GO in ¼ Ringer's solution (Figure 17) but again 1% GO-ZnO exceeded the performance of TiO_2-P25.

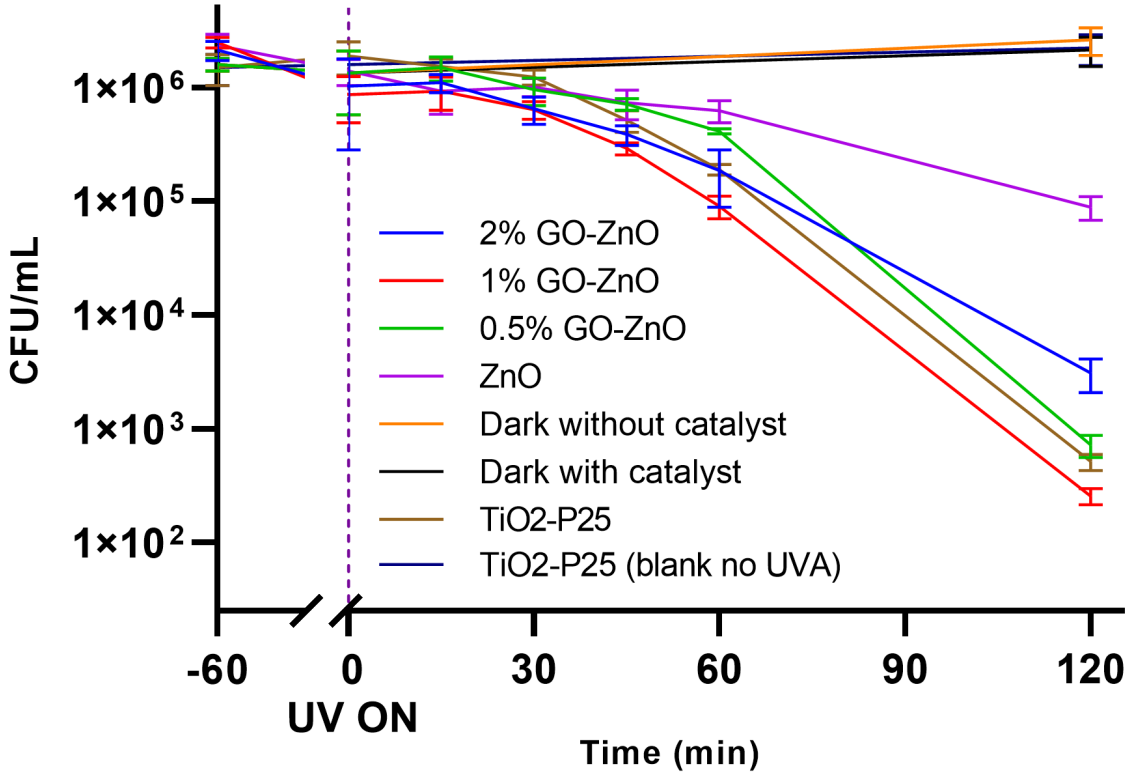


Figure 17 - GO-ZnO composite catalysts inactivation rates with GO load

A direct comparison between the two best performing catalysts, that is Ce-400 and 1% GO-ZnO, in exactly the same conditions showed negligible differences between their performances and the other criteria in §1.8A are more pertinent to selection.

Putting the results in a broader perspective, the modifications provided further evidence that cerium is a viable dopant in ZnO and the results by Calza*, et al.*[59] on a chemical pollutant are also vindicated for bacterial inactivation. The higher percentages of cerium studied herein (0.25%-10% vs. up to 1% therein) also indicate that there might be a potential to further improve the performance of Calza*, et al.*[59] by testing higher cerium loads. While a large amount of different catalysts are available in literature, the current trend is to improve the efficacy of the catalysts at the expense of synthesis simplicity. Zhu*, et al.*[71] for example synthesised a five-component composite photocatalyst showing higher activity than the industry standard TiO_2P-25. While this catalyst might find uses in specialised reactors where efficiency is of the utmost importance, it will not be suitable for wastewater treatment where cost of treatment is crucial. For future photocatalytic systems for wastewater another potentially suitable catalyst might be that synthesised by Venieri*, et al.*[22] which modified TiO_2 to give complete deactivation after 15 min of treatment, albeit at a light intensity that was more than 1 order of magnitude higher than that used herein.

2.4. Conclusions

Overall both 4%Ce-ZnO and 1%GO-ZnO were major improvements to commercially available catalyst under the criteria set out in §1.8A. 4% Ce-ZnO however has two major advantages over the graphene oxide composite:

- Synthesis cost and ease
  the preparation of Ce-ZnO involves a one pot synthesis with a total preparation time under 4 h. The preparation for GO-ZnO involves the separate preparation of GO via the Hummer's method. This uses environmentally problematic compounds and the two steps of synthesis combined take more than 24 h

- Robustness

  Graphene oxide is not thermally stable. This becomes an issue for the criteria of §1.8B where the goal is catalyst immobilisation. Ce-ZnO is compatible with calcination of the precursor directly on a macroscopic support while for GO-ZnO using high temperatures will result in degradation of the graphene oxide composite compromising the efficacy of the catalyst.

For these two main reasons it was decided that the catalyst of choice would be 4% Ce-ZnO and catalyst immobilisation (§1.8B) and use in crop irrigation (§1.8C) were carried out using this catalyst.

3. WP B – Catalyst immobilisation

Most of the work detailed in this section was published under an open access Creative Commons Attribution-Non Commercial 4.0 licence as Zammit*, et al.*[72]

Zammit, I.; Vaiano, V.; Ribeiro, A. R.; Silva, A. M. T.; Manaia, C. M.; Rizzo, L., Immobilised Cerium-Doped Zinc Oxide as a Photocatalyst for the Degradation of Antibiotics and the Inactivation of Antibiotic-Resistant Bacteria. *Catalysts* 2019, *9*, (3), 222.

and can be found as Annex 2.

3.1. Rationale

Photocatalysis, being a heterogeneous process, has substantial limitations for its application at full scale in WWTPs. For environmental water treatment, the cost of such a treatment is crucial in determining if it is feasible. Apart from process efficiency, the two main issues limiting HPC are:

- The high energy cost of keeping a catalyst in suspension
  If a powdered photocatalyst is allowed to precipitate to the bottom of the reactor, this would be highly detrimental for the efficiency of the process. The absorption of electromagnetic radiation would be at its lowest since the agglomeration of particles would shadow each other and the distance from the light source would be at its maximum. Thus through Beer-Lambert's law the transmittance through the water would be at the lowest (i.e. the path length would be at its highest).
- Catalyst recovery
  Once the desired contact time is reached, the powdered catalyst needs to be separated from the water phase. This is needed both because the photocatalyst may have detrimental environmental effects on discharge and also because the catalyst is easily reusable and it makes economic sense to recover it.

In this regard, many studies have addressed the use of photocatalysis in the immobilised form.[73-76] Immobilisation is the fixing/coating of a photocatalyst on to a macroscopic support which can easily be physically separated from the water phase. Compared to a suspended powder, a fixed photocatalytic process does not involve post treatment filtration or settling to remove the photocatalysts.

Many substrates have been used in literature as support material, including but not limited to, metallic surfaces, glass, plastics, fibres, alumina and silica.[77] Novel membrane reactors are also incorporating photocatalytic coatings onto the membranes in an effort to reduce fouling during filtration.[78] While immobilisation makes the process more convenient to operate, especially when it is at a large scale, it not only does not enhance the activity of the catalyst but most probably also reduces it. In powder form, the surface area of a catalyst in contact with the aqueous phase that is illuminated is higher. This is because it is not partially bound to the support which cuts off light and does not allow electron-hole pairs to produce ROS since water is not in close proximity. As regards to bacterial inactivation, it is not so straightforward. In order to induce inactivation, multiple reactions in spatial proximity need to take place to reach a threshold of inactivation. This is contrasted with a chemical pollutant where a stable reaction at 1 site is enough to produce a transformation product. Some researchers have put forward a hypothesis[79, 80] that since an immobilised catalyst results in a more spatially concentrated reaction, this is enough to overcome for the drawbacks that an immobilisation catalyst has towards bacterial inactivation.

The aim of this work package was to validate a cost-effective method to immobilise the catalysts optimised in the previous section and to test its efficacy for the inactivation of bacteria and antibiotic resistant bacteria in real wastewater. After some preliminary trials with polymethyl methacrylate, polystyrene and stainless steel (SS) support materials SS was used.

3.2. Methods and Observations

3.2.1. Catalyst immobilisation

Catalyst immobilisation was carried out with the help of the group of Prof. A.M.T. Silva at the Laboratory of Catalysis and Materials of the University of Porto and with equipment provided by Adventech Lda (São João da Madeira, Portugal). Discs ANSI 304 grade stainless steel were obtained from commercial suppliers. These discs were then subject to mechanical roughing of their surface by scraping with aluminium oxide P60 sandpaper. This was followed by sonication in acetone for 15 min to remove any fine powders and surface lubricants from the SS cutting process. After cleaning and drying the discs were heated in a furnace to 400°C for 1 h in order to introduce a slight surface oxidation.

These discs were then initially coated with a silica interlayer. The two-step hydrolysis process by Schaefer and Keefer[81] was used. Tetraethyl orthosilicate (Sigma Aldrich, 98 %), propan-2-ol (Merck, >99.0 %) and water were mixed together in a 1:3:1 mole ratio and heated to 60°C. To this 0.7millimoles of hydrochloric acid per mole of water used were added and the first hydrolysis step was left under stirring for 1.5 h at 60°C. The second hydrolysis step was initiated by adding 11 mole parts of water and 0.0043 mole parts of HCl, to give a pH between pH 2-3. This mixture was left overnight under stirring.

The interlayer coating was achieved by direct spraying of the hydrolysed tetraethyl orthosilicate on to the heated individual SS discs. Each disc was coated multiple times with gaps to allow for evaporation in between. After complete drying, the discs were calcined at 400°C for 1 h with a heating ramp of 2°C/min and passive cooling. The result was a light scattering layer coating the metal disc (Figure 18A). The photocatalysts was produced as previously optimised and detailed in §2.2.1. The only difference being that the zinc hydroxide precursor was first sprayed onto a metal disc and then calcined at 300°C for 1 h with a heating ramp of 2°C/min and passive cooling producing a white coating (Figure 18B).

As comparisons, undoped ZnO coated discs and TiO_2-P25 coated discs, were also produced. The undoped ZnO disc was produced in exactly the same method but without adding cerium nitrate (i.e. the same method used in §2.2.1). The TiO_2-P25 on the other hand was produced directly from a 1% w/w suspension of the photocatalyst in water followed by the same thermal treatment.

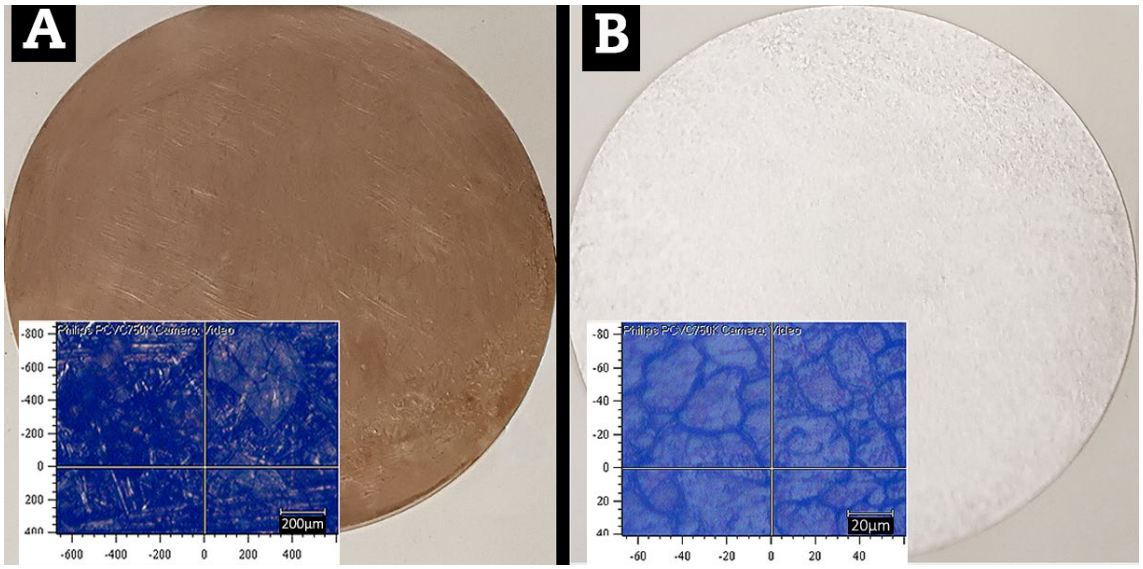


Figure 18 - (A) SS disc coated with a silica interlayer; (B) Ce-ZnO coating on the same disc. Light microscope inserts showing the coating is reproduced in the lower left corner of each segment

3.2.2. Coating characterisation

The various coatings and bare stainless steel were analysed by X-ray diffraction using a Brucker D8 diffractometer, with a scan rate equal to 0.05°/s

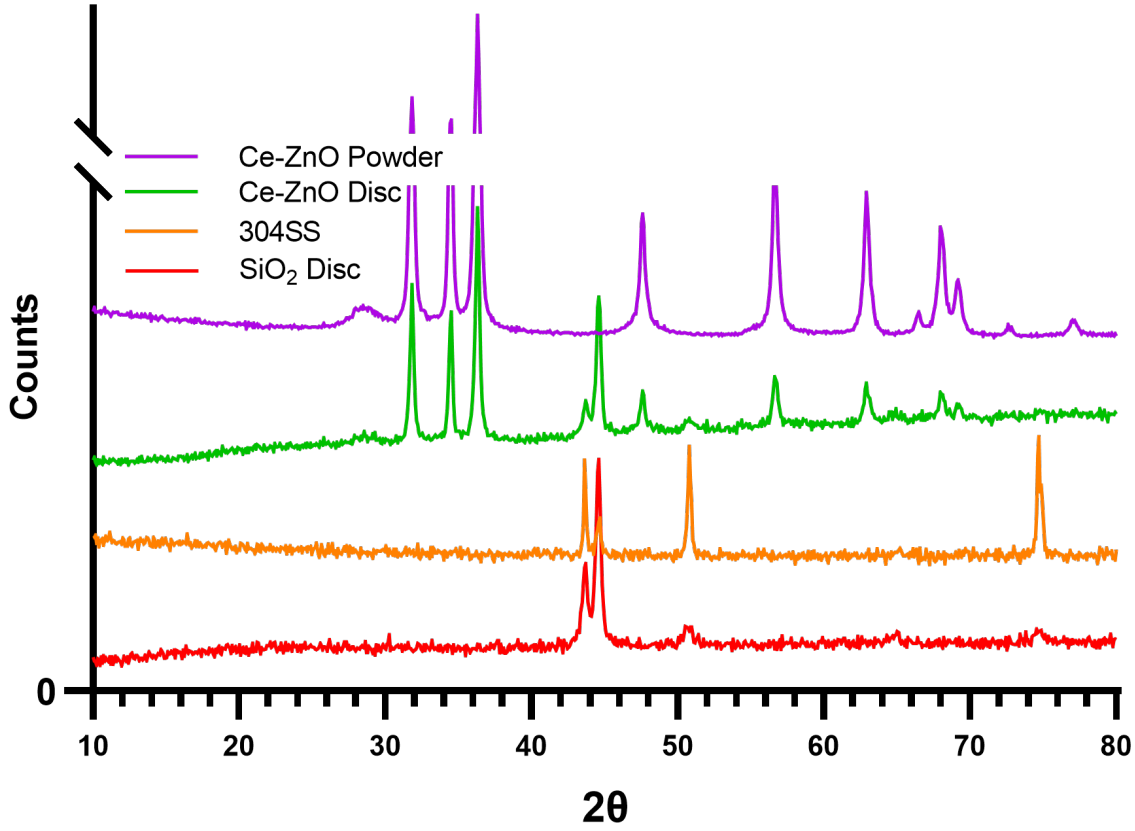


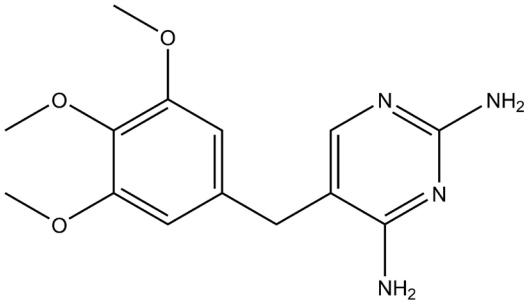
Figure 19 - Stacked XRD pattern of SS disc with the different coatings and Ce-ZnO in powder form

The bare stainless steel disk shows diffraction patterns (Figure 19 orange line) that are congruent with literature, specifically 2θ = 43° attributed to γ(1,1,1), 45° to α(1,1,0), 51° to γ(2,0,0), 75° to γ(2,2,0).[82] On coating the SS disc with the silica interlayer the 51° to γ(2,0,0) and 75° to γ(2,2,0) peaks were severely reduced in intensity. The amorphous nature of silica does not produce specific X-ray diffraction peaks but rather increases non-specific background scattering that can mask other peaks. The Ce-ZnO disc on the other hand shows well the combined peaks of the powdered Ce-ZnO (Figure 19 purple line) and that of the interlayer coated disc. The XRD pattern also matches that of the Ce-ZnO previously synthesised (Figure 11) which has been already described in §2.2.3.

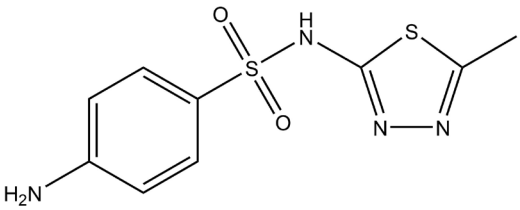
3.2.3. Immobilised photocatalytic experiments – Antibiotic degradation

The coated discs were used, separately, for (i) the degradation of two antibiotics (Figure 20) in pure water, (ii) for the inactivation of a strain of *E. coli* in isotonic saline solution and (iii) for the inactivation of autochthonous bacteria in real wastewater and simultaneously following specific antibiotic resistant fractions of their respective populations.

For the degradation of antibiotics, three different coatings, i.e. Ce-ZnO, undoped ZnO and TiO_2-P25 were prepared on 11.5 cm diameter (area = 104 cm^2) discs and used for the simultaneous degradation of sulfamethoxazole (SMX) and trimethoprim (TMP). The antibiotics were chosen since they were previously prioritised based on their crop uptake potential, widespread use and environmental persistence within the ANSWER project (H2020-MSCA-ITN-2015 number 675530) and NEREUS COST Action (Cost Action ES1403).[83]



Trimethoprim
Chemical Formula: $C_{14}H_{18}N_4O_3$
Molecular Weight: 290.32



Sulfamethazole
Chemical Formula: $C_9H_{10}N_4O_2S_2$
Molecular Weight: 270.33

Figure 20 - the two antibiotics used for photocatalytic experiments

A stock solution in methanol of 0.25 mM of each SMX and TMP (both Sigma-Aldrich, >98%) was prepared and stored in the dark at 4°C. Prior to each experiment, the stock solution was brought to room temperature and well homogenised, making sure there are no precipitated particles. 1 mL of stock solution was accurately pipetted to a 1 L volumetric flask and a gentle flow of nitrogen introduced to evaporate off the methanol. The volumetric flask was then filled up with 1 L of MilliQ water and briefly sonicated to dissolve the antibiotics. Thus, the final concentration of each antibiotic was 0.25 µM i.e. SMX = 6.332 µg/L and TMP = 7.258 µg/L giving a pH of 6.22. The aqueous solution was then transferred to a cylindrical borosilicate glass container of 18 cm diameter to give a water column height of 4 cm. This container was magnetically stirred and placed inside a circulating water bath for thermoregulation. The disc, which was previously rinsed with water to remove any poorly adhered coating, was placed at the bottom of the container. After 60 min of dark phase stirring to reach homeostasis, two Osram Dulux®L BL UVA 18W/78 (spectrum in the supplementary information of annex 2) coupled with an Osram Quicktronic Professional®Optimal QTP-OPTIMAL 2X18-40 ballast were

used as a UVA light source. For the Ce-ZnO disc, the stability of the coating and its efficacy on reuse was also tested by reusing the same disc up to a maximum of 5 times with only a drying phase at 80°C for 0.5 h in between reuses. The degradation of SMX and TMP was followed by UHPLC-MS/MS from Shimadzu Corporation (Kyoto, Japan) consisting of a Nexera UHPLC coupled to a triple quadrupole mass spectrometer (Ultra Fast Mass Spectrometry series LCMS-8040), with an ESI source operating in both positive and negative ionisation modes. A Kinetex™ 1.7 µm XB-C18 10 nm pore size column (Phenomenex, Inc., Torrance, CA, USA) was employed for chromatographic separation under isocratic mode, using a mixture (1:4, v/v) of an aqueous solution of 0.1% formic acid (Fisher Scientific) and a solution of methanol and acetonitrile (1:1, v/v) (both MS grade VWR International), at a flow rate of 0.20 mL/min. Column oven was set at 35°C while the autosampler at 4°C. The injection volume was 10 µL. Selected reaction monitoring (SRM) was performed for analysis, evaluating the two SRM transitions between the precursor ion and the two most abundant fragment ions for each compound, with a scan time of 100 ms per transition. The most abundant fragment ion used for quantification and the second most abundant for analyte confirmation and other analytical parameters are shown in Table 2.

Table 2 - UHPLC-MS/MS parameters for the analysis of TMP and SMX

Analyte	M_r	pKa	SRM1	SRM2
trimethoprim	290.32	7.12	252.10 > 156.00	252.10 > 92.10
sulfamethoxazole	253.28	pKa1 = 1.6 pKa2 = 5.7	290.80 > 230.00	290.80 > 123.05

Argon at 230 kPa was the CID gas. Drying gas flow = 15 L/min; nebulizing gas flow = 3.0 L/min; capillary voltage = 4.5 kV; desolvation temperature = 400°C; source Temperature = 250°C

3.2.4. Immobilised photocatalytic experiments – Bacterial inactivation

In collaboration with the School of Biotechnology of the Catholic University of Portugal (Prof. C. Manaia's Lab), the applicability of the immobilised catalysts in inactivating bacteria were tested. For these experiments, 1 L of saline water or 750 mL of secondary wastewater and larger 22 cm diameter (area = 380 cm^2) discs coated with either Ce-ZnO or TiO_2-P25 were used while maintaining the same

irradiative setup. Bacterial enumeration was performed by the membrane filtration method, using 0.45 µm membranes after incubating at 37°C for 24 h. For the experiments with secondary wastewater, this was collected from the secondary clarifier of a UTWP in northern Portugal. Dissolved organic carbon (DOC) measurements were conducted using a Shimadzu TOC-L analyser, after filtering the samples and are reported in Table 3 along with other data supplied by the UWTP. The wastewater was stored at 4°C and used for experiments within 3 days of sampling. The UV-VIS transmittance spectrum of the WW is available as part of Annex 2.

Table 3 - wastewater parameters measured inhouse or provided by the UWTP

Monthly:	Secondary Clarifier					
	pH	Conductivity (µS/Cm)	BOD_5 (mg/L)	COD (mg/L)	TSS (mg/L)	VSS (mg/L)
Average	7.2	707	31.3	90.3	33.5	31.4
Min	7.0	590	15.6	16.0	23.0	22.0
Max	7.5	859	95.3	204.0	76.0	76.0

Measured parameters: DOC WW1 = 20.6 mg/L; WW2 = 34.6 mg/L

Initially the rate of inactivation of an *Escherichia coli* strain (DH5-Alpha kindly supplied by C. Merlin LCPM/Université de Lorraine CNRS) was tested using Ce-ZnO coated discs. To avoid additional stress to the bacteria not due to the photocatalytic process, the measurements were carried out in isotonic saline solution (0.85% NaCl). In order to test reproducibility of the efficacy of different coating runs, two separate discs were used under identical conditions with starting bacterial loads of ≈1x10^5 CFU/mL and a third measurement was carried out to test the capacity to inactivate higher bacterial loads with 1 order of magnitude higher initial bacterial density.

The use of Ce-ZnO and TiO_2-P25 coated discs was also used in real wastewater for the inactivation of autochthonous bacteria. Real wastewater has numerous properties that affect the efficiency of a photocatalytic process. This includes reduced transmission of UVA, presence of organic compounds that compete for the

generated ROS and presence of ions (such as carbonates) that interfere with the photocatalytic process. Thus, it was important to test the HPC process in a real matrix. Additionally, the effect HPC has on antibiotic resistant fractions of bacteria was tested. A treatment process that aims to reduce the proliferation of antibiotic resistance in the environment must take into account the prevalence of resistance. This is defined as the ratio of a specific bacterium that shows resistance to a specific antibiotic over the total number of this species present in any given matrix. In environmental matrices, it is possible to estimate this prevalence using standard plate counts in conjunction with plate counts having the addition of relevant levels of antibiotics, as has been reported in literature (see [84-88]).

$$\text{prevalence of resistance} = \frac{\text{colonies on antibiotic spiked agar}}{\text{colonies on agar without antibiotic}} \qquad \textit{Eq. 5}$$

Thus, the prevalence of resistance would be the enumeration of bacteria on the antibiotic enriched medium over the number of bacteria on a medium without any antibiotic as per Eq. 5. Herein two selective media were used to assess the change in AR prevalence after treatment for *E. coli*, other coliforms and *Pseudomonas aeruginosa* against two antibiotics of each category. Chromogenic Coliform Agar (Merck Millipore), referred to as CCA, was used to putatively identify *E. coli* and other coliforms based on chromogenic colony differentiation as per manufacturers specifications (*E. coli* - blue and other coliforms - pink). The prevalence of ofloxacin and azithromycin resistance for CCA cultivable bacteria was assessed using the aforementioned method and CLSI guidelines[89] for antibiotic concentrations that determine the cut-off between resistance and susceptible. These concentrations are 8 µg/mL for ofloxacin and 32 µg/mL for azithromycin. *P. aeruginosa* on the other hand was enumerated on 0.3% cetrimide agar (referred to as CET) with 15 µg/mL of nalidixic acid as per the method reported by Goto and Enomoto[90]. The AR prevalence of *P. aeruginosa* against ofloxacin (8 µg/mL) or ciprofloxacin (1 µg/mL) was assessed. The CLSI cut-off concentration for ofloxacin was used while for ciprofloxacin a more realistic (1 µg/mL) environmental concentration from literature (see [91, 92]) was used.

3.3. Results and Discussion

3.3.1. Antibiotic Degradation

The three different photocatalytic coatings used were effective at degrading the model pollutants at varying degrees (Figure 21). UVA only controls showed little to no degradation, resulting from photolysis from 365 nm and thus demonstrating that the removal process is photocatalytic based.

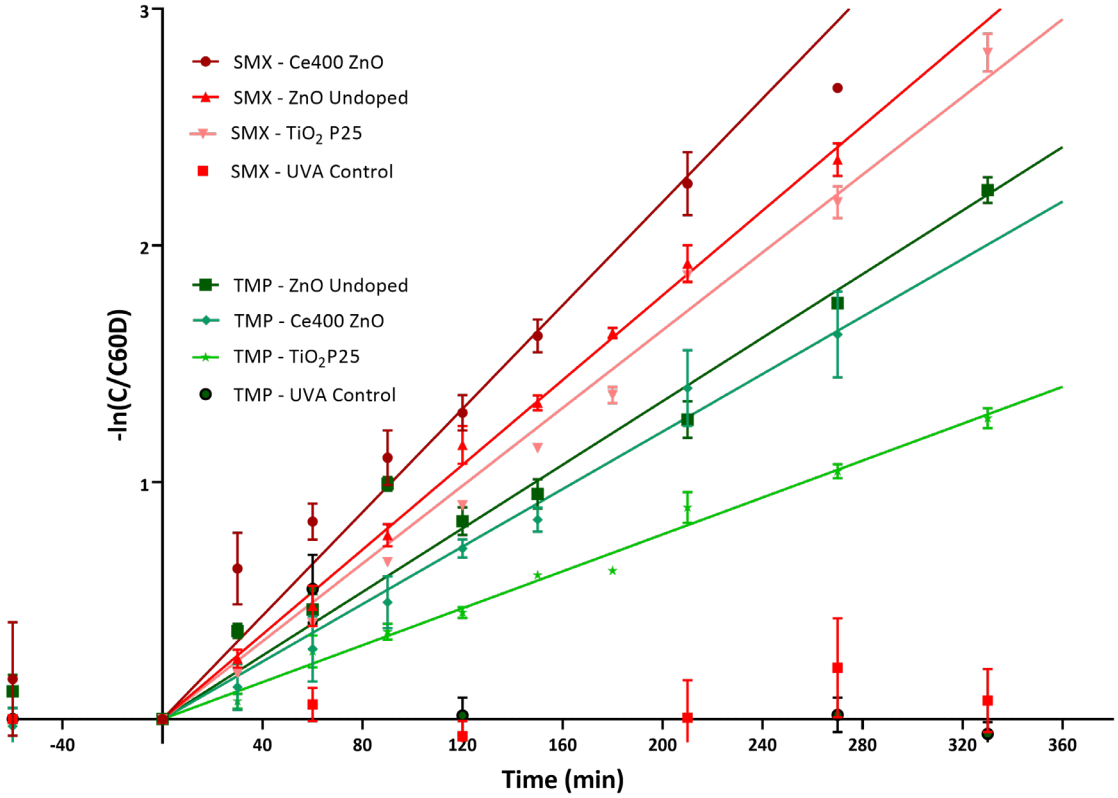


Figure 21 - natural log of the normalised concentrations of SMX and TMP with treatment time. Err. Bars = SD

TiO_2-P25 was the catalyst that showed the weakest activity for both SMX and TMP. In the case of the ZnO based catalysts, the cerium doped ZnO showed the highest activity against SMX while the undoped ZnO was more active against TMP. In all cases TMP was more recalcitrant and degraded slower. The pseudo first order rate constants as well as the half-life for each removal were obtained from the linear regression of the data in Figure 21 and are presented in Table 4. It should also be pointed out during the initial dark phase (i.e. time -60 min to 0 min) less than 10% of the antibiotics were removed from the aqueous phase, indicating adsorption is

not very strong as is expected from an immobilised catalyst which has a lower surface area than its suspended form.

Table 4 – linear regression parameters of the pseudo first order plot presented as Figure 21

	SMX				TMP			
	Ce400 ZnO	ZnO Undoped	TiO_2-P25	UVA Control	Ce400 ZnO	ZnO Undoped	TiO_2-P25	UVA Control
Std. Err ($x10^{-4}$)	2.63	0.88	0.99	1.52	1.44	1.79	0.55	3.32
R^2	0.943	0.993	0.988	0.083	0.956	0.941	0.983	-0.248
k (min^{-1}) ($x10^{-4}$)	109	89.5	82.1	3.28	60.7	67.1	39.0	0.945
$t_{1/2}$ (min)	63.6	77.4	84.4	2113.3	114.2	103.3	177.7	7334.9

The fact that SMX was less persistent than TMP is not explained solely by their respective reactivities with free form hydroxyl radicals. The hydroxyl radical rate constant (k_{OH}) of both of these antibiotics has been experimentally determined. SMX has a log(k_{OH}) of 9.76 which is lower than that for TMP at (k_{OH}) of 9.84. [93, 94] This difference in rate constants in a competitive kinetics scenario, would result in SMX being degraded slower, specifically at a rate which is 20% slower than that for TMP. Thus, the fact that TMP degraded slower in the photocatalytic setup used herein indicates that other reactions are taking place. These reactions could be both a substantial contribution of other ROS, such as super oxide radicals showing higher reactivities with SMX over TMP, or SMX showing higher adoption to the coated surface and thus allowing a substantial contribution from surface generated hydroxyl radicals.[95] The contribution from surface adsorbed reactions would also explain one catalyst is not the most effective at degrading both antibiotics. The different chemical structures result in different affinities of adsorption to a specific photocatalyst. Thus, a higher adsorption of one catalyst to TMP over SMX would produce a higher removal rate of TMP since more reactions can take place in the adsorbed form. These results show that while ROS generation rates are very important in deterring rate of reaction, it is not the sole substantial contributor.

The stability of the photocatalytic coating over different reuses was probed by utilising the same disc multiple times without any chemical cleaning in between. The pseudo first order rate constants were determined from the linear regression of each experimental run and plotted in Figure 22.

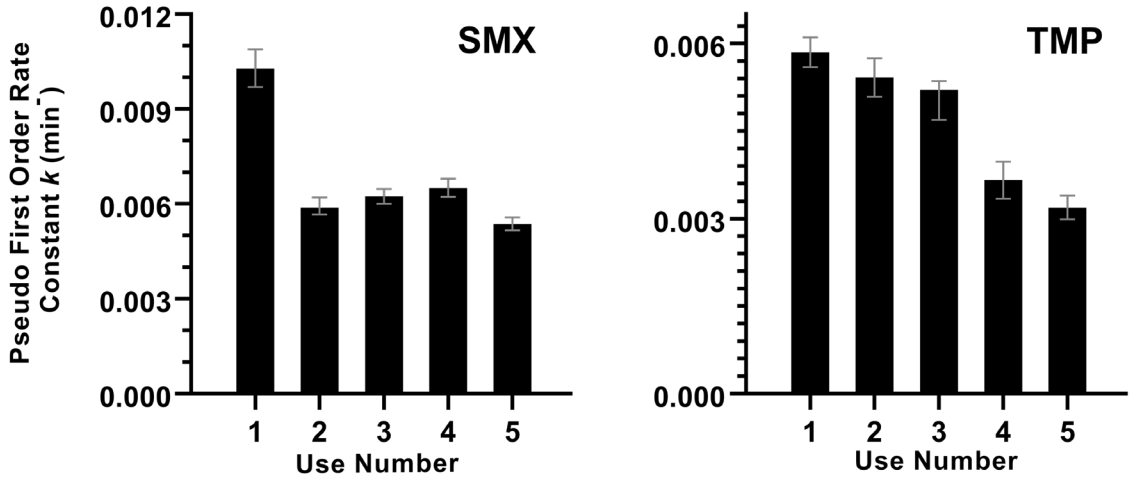


Figure 22 - Pseudo first order rate constants with number of reuses. Err. Bars = SD

For SMX, straight from the second use, there was a substantial drop in removal rates with approximately 40% slower removal kinetics. For TMP, on the other hand the same discs during the same experimental runs showed a more gradual decrease in removal rates. In all cases for both antibiotics the removal rate was nonetheless more than 50% of the initial rate even after 5 reuses. The differences in decreases of removal rates might again be tied to the different adsorptivities of the antibiotics. The adsorption of SMX was saturated after 1 use and further uses was not able to exploit adsorption for higher reactivities. The overall reduction in rates is however expected as the catalyst can be poisoned by the antibiotics or their transformation products and/or these adsorbed compounds could also have a higher absorbance of UV light at the wavelength of interest (365 nm in this case).[96] As regard to the robustness of the coating and the discs, no flaking off of the catalysts was observed with the naked eye and no photochemical corrosion was observed at the circumneutral pH.

While this is the first instance were the reusability of cerium doped ZnO was investigated, other authors did study the reuse of other photocatalysts as a major

strongpoint of HPC over the other AOPs. Titania was reused by Pekakis, Xekoukoulotakis and Mantzavinos[97] for the photocatalytic degradation of industrial dyes. While did not report a decrease in reactivity, they operated a catalyst load which was much higher than the lowest load that showed optimal removal rates. Similarly, Nageswara Rao, Sivasankar and Sadasivam[98], report no decrease in activity for up to 5 uses when 2 g/L of ZnO were used for the degradation of salicylic acid. After more than 5 uses however a decrease was reported. The high catalyst use and the fact that after additional reuses a decrease was nonetheless observed suggest that catalyst poisoning is taking place at every use, but since these studies used catalysts loads that were much higher than the catalyst load that saturated the reaction rate, then any poisoned catalysts was being replaced by fresh catalyst and overall the reaction rate didn't suffer. Fernández-Ibáñez*, et al.*[23] reinforce this point since in their study they showed an immediate decrease in reactivity of two titania based catalysts under solar illumination at a catalyst load of 0.2 g/L. The much lower catalyst load used here resulted in any poisoning of catalysts not being able to be replaced by fresh catalyst since it is operating below the load which would be considered optimal. Additionally, they observed that one of the two titania catalysts was more susceptible to poisoning since it showed a faster decline in reactivity. The use of immobilised photocatalysts in literature is far less common. The studies that do utilise immobilised photocatalysts often do not report data on their reuse (e.g. see [76, 99-102]) which is a missed opportunity since the reuse of an immobilised photocatalyst is one of its major advantages. An example of immobilised titania being reused and showing encouraging results is provided by Lalhriatpuia*, et al.*[103]. They reused the catalysts five times for the degradation of tetracycline and diclofenac which showed low reductions in removal rates with the sequential uses. When it comes to catalysts regeneration, ZnO has been used as a catalyst in organic synthesis, albeit this use is very much less common than environmental catalysis.[104] Here it was reported that any reduction in reaction rate is easily reverted by a simple wash in dichloromethane.[105] A non-halogenated and thus greener polar solvent would probably also work while satisfying environmental concerns. The washing of a photocatalyst coating metallic surfaces

is also very straightforward and feasible in mass scale, as opposite to plastic based supports which might not be compatible with most solvents.

3.3.2. Bacterial inactivation

The immobilised Ce-ZnO catalyst was also effective in the inactivation of *E. coli DH5-Alpha* in isotonic saline water. The measurements carried out with similar initial bacterial loads (i.e. trendlines B1 and B2 in Figure 23), showed a very similar inactivation rate obtained using different discs coated in the same manner. This indicates that the coating method is able to produce coatings that give reproducible and thus predictable results. This is needed to be able to estimate treatment times in photocatalytic reactors when treating water to a specific threshold level.

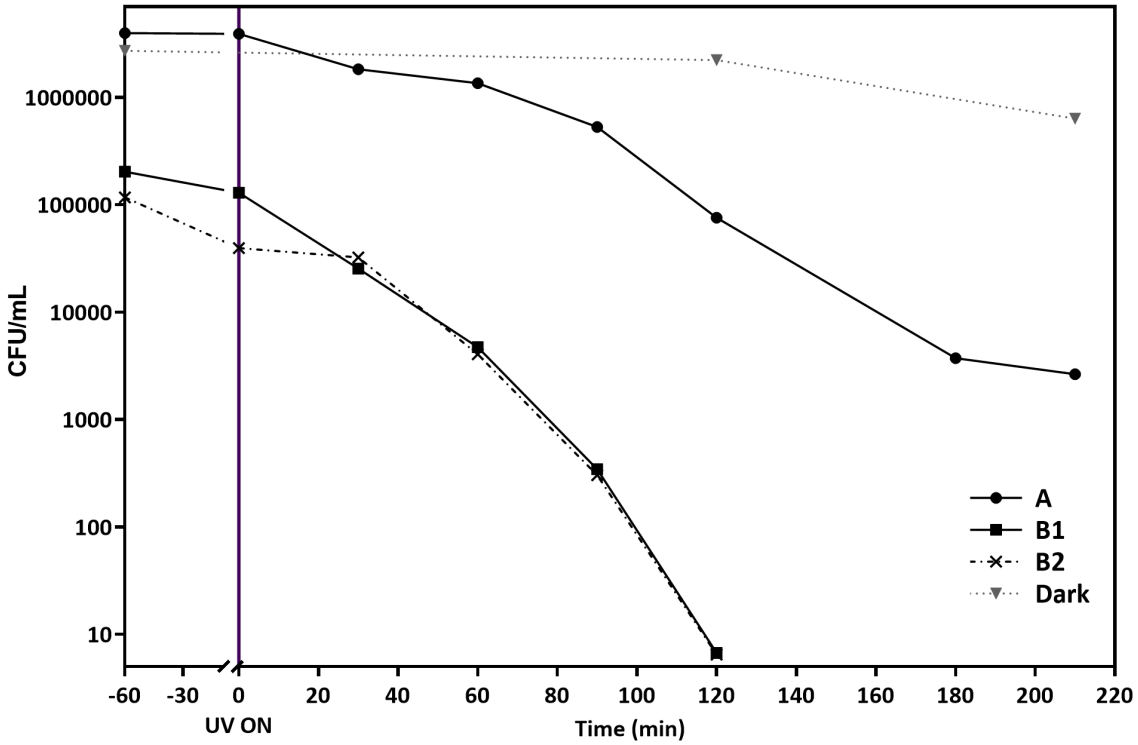


Figure 23 - Inactivation of *E. coli* starting from 10^6 CFU/mL (A) and 105 CFU/mL (B1 and B2) and dark control

The higher initial load of bacteria (trendline A Figure 23) as expected requires longer treatment times to reduce bacterial levels. As already stated, bacterial inactivation takes place when multiple oxidation reactions occur on a single bacterium cell until a threshold level is reached which results in inactivation.[48] Thus with a high bacterial density the fixed number of reactants would be distributed more sparsely and this threshold of inactivation for individual cells

would take place at longer reaction times. A dark control, shown in Figure 23, proves that the inactivation due to any toxicity effects, or passive inactivation as less than $1\log_{10}$ after 210 min and relatively negligible to that during the photocatalytic treatment.

Ce-ZnO and TiO2-P25 coated discs were also used for the inactivation of bacteria in real secondary wastewater sampled from a secondary clarifier (before any disinfection step) in a UWTP in northern Portugal. No spiking of bacteria was carried out and all disinfection experiments were on autochthonous bacteria. Two replicates of Ce-ZnO in real wasted water sampled on different weeks (identified are WW1 and WW2) were carried out.

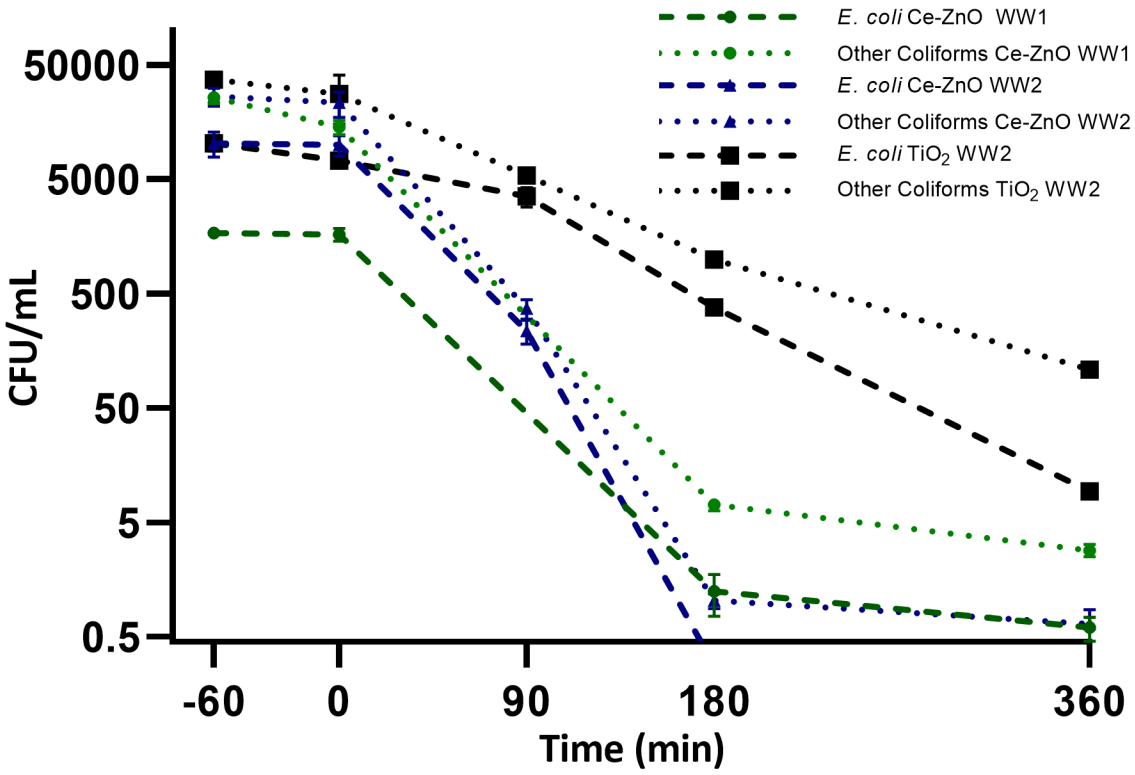


Figure 24 - inactivation of coliforms in real secondary WW. Err. Bars = SD.

A direct comparison between Ce-ZnO and the industry standard TiO_2-P25 was also carried out by using exactly the same wastewater (identified as WW2). The initial bacterial loads were found to be rather high, with an average initial *E. coli* load of 6900 CFU/mL. In real wastewater the dark phase (from time -60 to 0 min) resulted in virtually no bacterial inactivation (Figure 24). This is most definitely because the

bacteria have been well acclimatised to the matrix in the UWTP before sampling and the WW itself contains all the nutrients needed to sustain them.

E. coli are were slightly more susceptible to treatment, while other coliforms (i.e. all CCA cultivable non-*E. coli* coliforms) are more abundant the rate of inactivation for *E. coli* are marginally higher. For experiments using Ce-ZnO, differences in the kinetics between the two wastewaters are observed (i.e. WW1 and WW2). A major contributor here is probably the fact that WW2 has a higher initial load of bacteria and hence longer treatment times are needed to reach the same levels, as was the case in Figure 23. However, this is not the only factor, WW2 was identified as having a higher DOC load (WW1 = 20.6 mg/L; WW2 = 34.6 mg/L), thus the extra organic matter is competing for ROS with the bacteria lowering the total inactivation capacity. This is also observed in literature were immobilised titania used in distilled water for the inactivation of *E. coli* and an amoebae species showed higher rates than the same catalyst in synthetic wastewater.[80] In the best-case scenario, the Ce-ZnO coated disc was able to reduce the level of *E. coli* to below the LOQ of 0.5 CFU/mL. In the direct comparison between the two catalysts, Ce-ZnO was orders of magnitude more effective than the titania coated disc. For example, after 180 min using the TiO_2-P25 coated disc, there were 3 orders of magnitude higher levels of other coliforms when compared to Ce-ZnO in the same identical wastewater. Giving the simplicity of the coating with Ce-ZnO this result is highly encouraging. These results are also consistent with those obtained for the removal of antibiotics in §3.3.1. Therein, titania was the least efficient of the three catalysts tested for antibiotic degradation. The linear relationship that exist between concentration of ROS and bacterial inactivation rates[47] indicates that the titania coating is less efficient at generating ROS and thus exhibiting lower degradation rate against antibiotics and lower inactivation rates against bacteria. While this is true, there is discrepancy observed between degradation rates (antibiotics) and disinfection rates (bacteria). That is, for degradation, titania, while less efficient was on the same order of magnitude, however for disinfection, Ce-ZnO was orders of magnitude more efficient. This discrepancy could potentially be explained by the surface charge of the photocatalyst. By doping ZnO with Ce, the semiconductor is

shifted towards a positive charge since Ce^{3+} is substituting Zn^{2+}. This, coupled with the fact that Gram-negative bacterial cell walls are circumneutral pH are negatively charged[106] means that there is a much higher attractive force between the photocatalytic surface and bacteria than there is between the photocatalytic surface and the studied antibiotics.

The variability of *P. aeruginosa* in the two wastewaters samples was much lower, with both showing approximately the same initial bacterial loads of 50000 CFU/mL which are very high levels.[107] Titania again showed orders of magnitude slower inactivation kinetics than Ce-ZnO in the same wastewater (Figure 25) The inactivation of *P. aeruginosa* between WW1 and WW2 was less predictable and the higher DOC water (WW2) gave a higher inactivation rate.

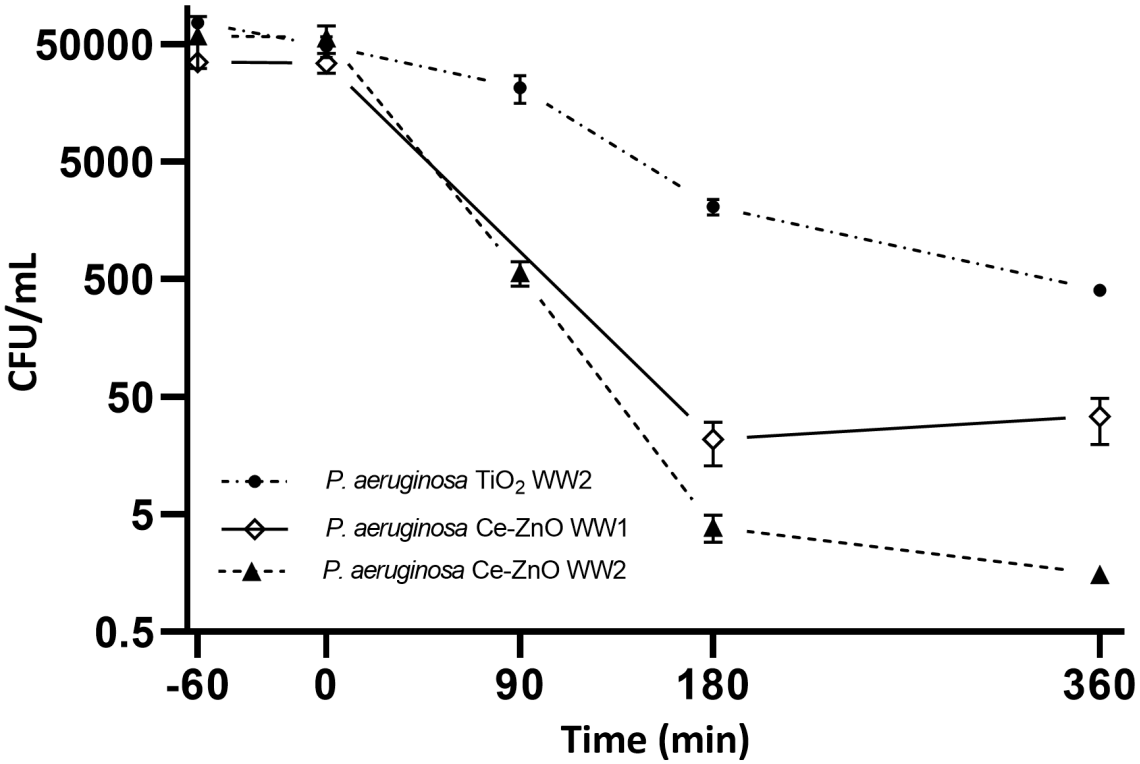


Figure 25 - inactivation of *P. aeruginosa* in real secondary WW. Err. Bars = SD

Overall *P. aeruginosa* is more resilient to treatment and even after 360 min of photocatalytic treatment levels of a few CFU/mL persisted. This bacterium is not currently monitored for regulatory purposes, however it was followed in order to investigate the antibiotic resistance differential mortality on treatment.

In parallel to the bacterial inactivation measurements carried out and presented in Figure 24 and Figure 25, the antibiotic fractions of these populations were also assessed through cultivation methods and inactivation rates amongst these different sub-populations are presented in Figure 26.

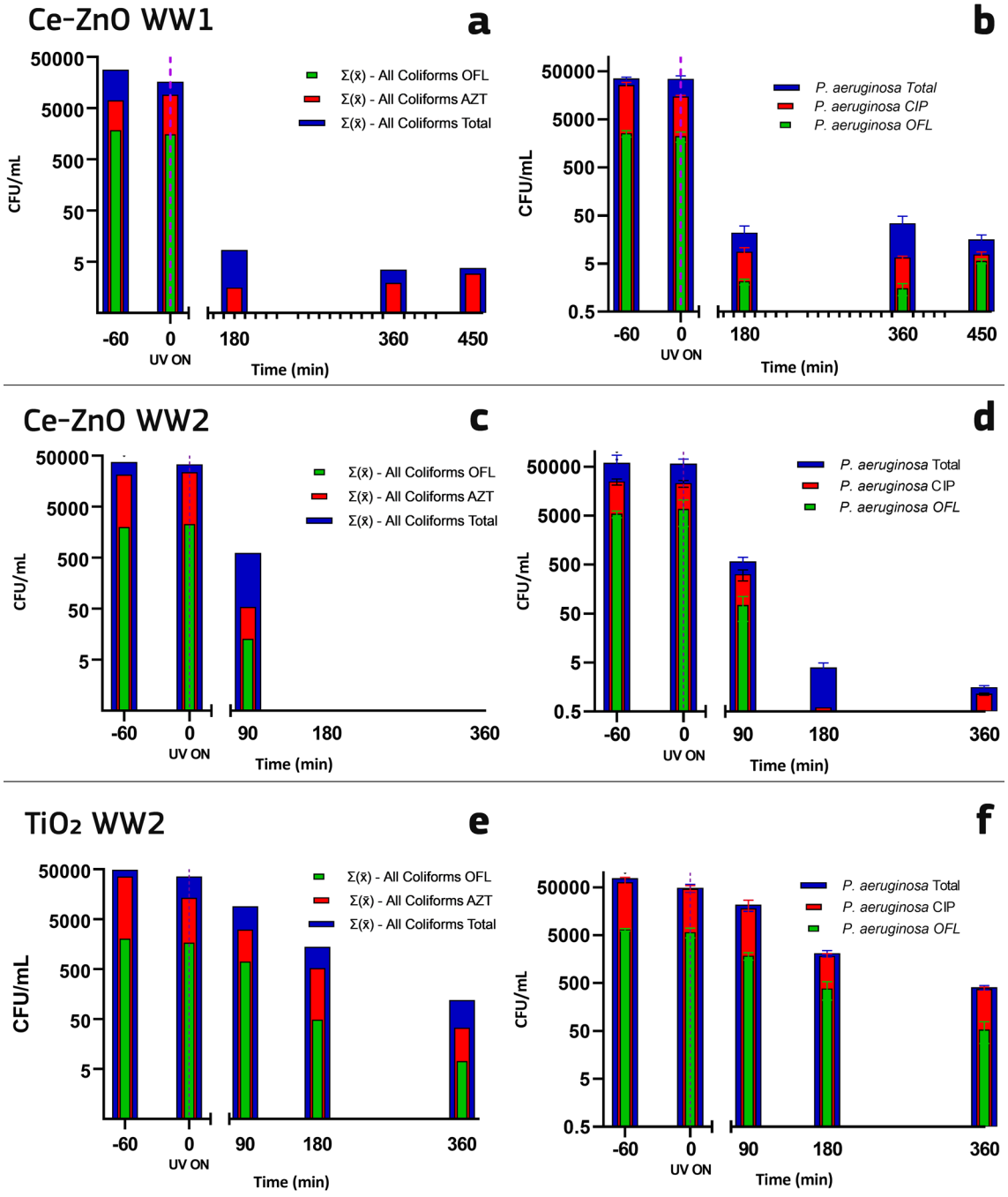


Figure 26 - inactivation of antibiotic resistant sup-populations of autochthonous bacteria
The rows represent the different experiments carried out while the left column (i.e. a,c,e) shows results for coliforms and the right column (i.e. b.d.f) those for *P. aeruginosa*. Err. Bars = SD

In all cases, and for both coliforms and *P. aeruginosa*, ofloxacin resistance was the least common, being present at approximately 5 %-10 % (Figure 26 a-f). Azithromycin resistance amongst *E. coli* (Figure 26 a,c,e) and especially ciprofloxacin resistance amongst *P. aeruginosa* (Figure 26 b,d,f) were very high, reaching almost unity in some cases. The photocatalytic treatment did not result in major changes in the prevalence of resistance and was effective on all sub populations. OFL resistant coliforms were below the limits of detection already after 180 min of treatment (Figure 26 a) while the remaining low levels of coliforms were not further reduced. For *P. aeruginosa* with Ce-ZnO in WW1 (Figure 26 b) inactivation was much slower and both resistant fractions were still quantifiable even after 450 min of treatment, indicating that an additional oxidant or higher UV intensity is needed to reach these lower levels in real wastewater. As already discussed, in Figure 24 the inactivation of coliforms in WW2 ((Figure 26 c) was higher than that in WW1 (Figure 26 a) and no coliforms could be quantified after 90 min of treatment. This trend was also observed, albeit, to a lower extent for *P. aeruginosa* in the same run (Figure 26 d) where the lower resistant sub-population, i.e. OFL resistant *P. aeruginosa* were not quantifiable after 90 min. Immobilised TiO_2-P25 showed a similar consistent trend of inactivating all sub-populations with approximately equal intensity and the order of magnitude differences in AR prevalence were also maintained here. Looking more into detail at the change of AR prevalence after 90 min (Table 5) shows somewhat lower prevalence for both coliform groups studied with titania and even lower with Ce-ZnO. *P. aeruginosa*, when disinfected using either catalyst, is more resilient and no changes were observed. While these changes might be too minor to discern with 1 round of disinfection, in a wastewater recycling situation were the effluent will eventually influence the influent, these small changes may build up to a substantial change in prevalence.

Table 5 - change in AR prevalence after 90 min of treatment as range percentages

		Resistant % at start (t=-60)			Resistant % at 90 min Treatment		
		OFL	AZT	CIP	OFL	AZT	CIP
Ce ZnO	*E. coli*	7.0-11.5	56.0-67.1	N/A	2.1-2.8	2.9-8.0	N/A
	Other Coliforms	3.6-4.6	53.4-56.7	N/A	1.5-2.2	9.8-10.9	N/A
	P. aeruginosa	7.2-14.6	N/A	32.7-67.9	7.9-15.9	N/A	52.8-55.4
TiO_2	*E. coli*	8.7-8.8	55.7-74.2	N/A	8.6-9.7	5.6-15.7	N/A
	Other Coliforms	1.6-4.0	75.0-78.0	N/A	4.0-9.1	43.7-53.1	N/A
	P. aeruginosa	8.5-8.6	N/A	62.8-99.5	8.0-9.6	N/A	56.3-99.5

Overall the changes are too small to ascertain increase or decrease in prevalence in either direction.

3.4. Conclusions

Positive results were achieved for the main goals of this work package (§3.1). A cost-effective immobilisation method was established. The method is in accordance with our requirements of having an easy to upscale method. This requirement is satisfied by the fact that the precursor can by synthesised in large quantities and sprayed under a flow of air onto metallic surfaces, followed by heating to relatively low temperatures to produce a coating of the active photocatalyst.

Catalyst reusability was also sufficiently positive. Reusing the same discs up to five times in pure water gave removal kinetics upward of 60% of the initial rate. As for bacterial disinfection, the coated discs were effective for disinfection in isotonic saline solution, giving very similar removal kinetics and hence confirming the robustness of the coating method in giving predictable outcomes. In real wastewater, the efficacy of the discs was much lower than in saline due to the numerous competing reactions happening in real wastewater, a ubiquitous observation in literature. Overall Ce-ZnO was much more effective than TiO_2-P25, albeit it for trimethoprim, the undoped ZnO was even faster than the doped counterpart. As for changes in prevalence of antibiotic resistance in the studied

WW microbiota, the changes are too minimal to be able to discern any specific changes. What can be concluded however is that there is no evidence for increases in AR prevalence. With this regard, it was decided to investigate the AR prevalence changes directly in soil as it is the receiving environment for wastewater reuse for agricultural irrigation.

4. WP C – HPC and chlorination for AR abetment in irrigation

At time of writing, a manuscript detailing the work conducted within this scope, is being prepared. The tentative title is "Accumulation of antibiotic resistance genes in soil after irrigation with treated wastewater: a comparison between heterogeneous photocatalysis and chlorination" and will include the following authors:
Ian Zammit, Roberto B. M. Marano, Vincenzo Vaiano, Eddie Cytryn, Luigi Rizzo

4.1. Rationale

For numerous reasons, the presence of ARGs in treated wastewater does not automatically translate to changes in the microbiome of the receiving environment. Urban wastewater — agricultural soil easily fulfils the definition of an ecological boundary by Cadenasso*, et al.*[108]. A high genetic fitness in one environment is unlikely to correspond to a high genetic fitness past the ecological boundary since the requirements are very different and many other factors, which as yet we often cannot model, are highly influential in determining the outcome.

In the context of AR resistance in urban WW and irrigation, resistant bacteria that show higher fitness throughout the urban water cycle will face a completely different environment once used for irrigation. The factors that potentially impart selective advantages for AR resistance to thrive, such as co-selection due to the presence of heavy metals[109-111] or presence of sublethal levels of antibiotics[112-114], might not be present in soil. In such a case both retaining AR resistance and transferring AR resistance would not confer any evolutionary advantages and thus it is expected that they would be outcompeted in a few generations. Another potential obstacle for AR uptake by the soil microbial communities is the fact that post-treatment, while ARGs are often detected at substantial levels,[35, 115-120] these genes could be both from viable and non-viable bacterial cells. Non-viable bacterial are not able to reproduce and thus cannot transfer ARGs vertically. Additionally, non-viable bacteria cannot partake in bacterial conjugation and horizontal gene transfer of ARGs to the autochthonous soil bacteria can only take place via

transformation or via bacteriophages which were not inactivated during WWTP treatment.

While acknowledging that a cause and effect relationship between ARGs in water used for irrigation and receiving soil is not straightforward, the fact of the matter is that it is not known if our current water reuse standards are sufficient to hinder AR spread along the wastewater — soil — crop continuum. Regulations and guidelines for TWW reuse are often based on indicator parameters that are easy to measure, such as bacterial loads of faecal coliforms, *Escherichia coli* or other bacteria. For example, the Italian regulation[121] for TWW intended for unrestricted crop irrigation, which is one of the more restrictive laws, set a maximum *Escherichia coli* load of 10 CFU/100 mL. Ideally regulations, need to be based on analysis that are straightforward, low cost and implementable with rudimentary training of personnel; which is why bacterial enumeration by plate counts is ubiquitously found in wastewater reuse standards. One of the most common treatments to achieve the required bacterial load threshold as per any applicable regulations is the use of chlorination,[122] i.e. the addition of hypochlorite (OCl^-) salts or bubbling chlorine gas. While a number of issues have been identified with chlorination, such as the formation of toxic disinfection by-products, it is by far cheaper than alternatives such as ozonation and does not require UV irradiation such as UV-C disinfection or UV-C/peroxide AOPs which require more complex reaction tanks.

In an effort to shed more light on the relationship between the type of water used for irrigation and the effect it has on the loads of ARGs quantifiable in soils, a comparative experiment was conceptualised. In a controlled environment, lettuce plants (*Lactuca sativa*) were irrigated with one of four water types, untreated secondary wastewater, fresh water and two TWW by chlorination and TWW by heterogeneous photocatalysis. In both treatment cases, the wastewater was treated inhouse to the Italian regulatory level of < 10 CFU/100 mL of *E. coli*. The main goals here are:

- to establish if irrigating with untreated wastewater over a short time gives significant increases of ARGs and associated genes in the soil cf. fresh water,
- to investigate if reaching the indicator level of <10 CFU/100 mL of *E. coli* is sufficient to limit AR spread in the short term.
- To investigate if there are any differences in AR spread amongst treatments at the same indicator level. i.e. if the mechanisms of inactivation of one treatment results in more effective hinderance of AR spread.

With this regard, two ARGs (*bla*$_{OXA-10}$, *qnrS*) and an ARG associated gene (*intI1)* were chosen. *intI1* was chosen as it is an abundant TWW-associated gene and indicative of anthropogenic pollution.[123] qnrS was chosen since, unlike the other genes, it is a plasmid associated ARG,[124, 125] while *bla*$_{OXA-10}$ was chosen of the basis that it is strongly associated with wastewater but not commonly found in soil[126] and thus a good indicator that any increase is due to the irrigation.

4.2. Methods and Observations

All the work detailed in this section, apart from catalyst preparation and synthesis, was carried out in Dr. Eddie Cytryn's Lab at the Agricultural Research Organization, (Volcani Center) of the State of Israel.

4.2.1. Photocatalyst synthesis and characterisation

The photocatalyst used was the one optimised in §2 and used in real wastewater in the immobilised form in §3. Synthesis was carried out in the exact same way as already described and characterisation carried out prior to starting the irrigation campaign. The synthesis of the catalyst was upscaled by one order of magnitude with ease in order to have sufficient catalyst for the entire irrigation period. The methods for synthesis and characterisation have already been explained in detail in the aforementioned section. XRD and Raman analysis of the product matched those of previously synthesised Ce-ZnO 4% Ce:Zn.

4.2.2. Crop Setup

Well characterised[127] sandy soil from Rehovot (Israel) which had never received wastewater was collected and sieved through a 1 mm mesh. 90 kg of this soil was

well homogenised in large tanks and twenty-four 3 L (15cm base circumference) plastic pots were filled with approximately 3.3 kg of soil. To each pot, one lettuce seedling was transplanted and labelled according to the water type it would receive so as to create 4 series of 6 pots each as shown in Figure 27.

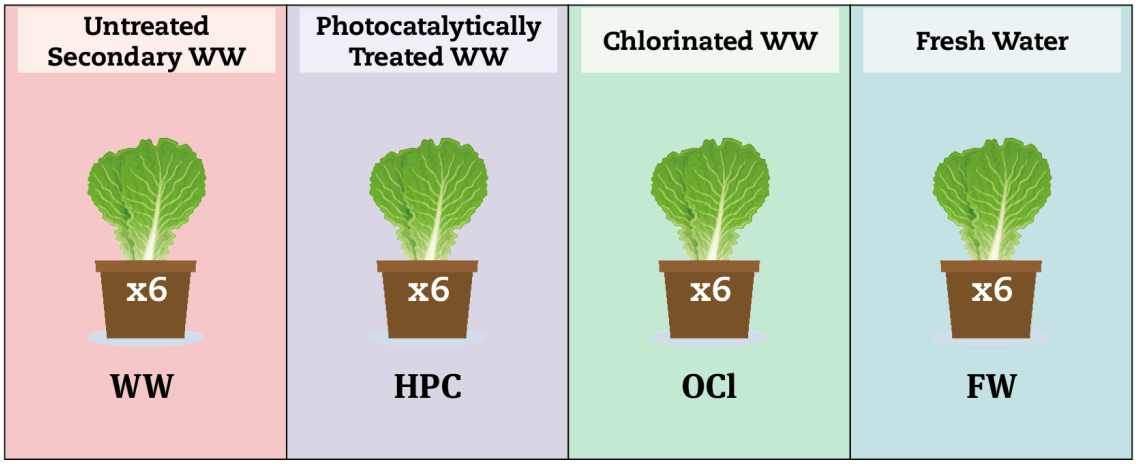


Figure 27 - 6 plants were set up in 4 series identified by their corresponding water type: WW, HPC, OCl & FW

The pots were placed inside a green house and distributed randomly over a table in order to reduce the effect microclimates inside the greenhouse, might have on results. The transplantation was carried on the 18th of October 2018 (Day 0) in Rishon LeTsiyon (Israel). A plastic mesh canopy was erected above the plants to reduce insolation and temperature which were considered excessive. Following transplantation all pots were irrigated with the same quantity of fresh municipal tap water (FW) for 18 days in order to stabilise the bacterial communities in the soil. After which each of the 4 series received exclusively its respective water type for the entire growth period of the lettuce, i.e. till day 54 after transplanting. DNA sampling from soil was carried out twice from each pot, the pre-irrigation regime samples at day 18 and the post-irrigation regime samples at day 54 (Figure 28).

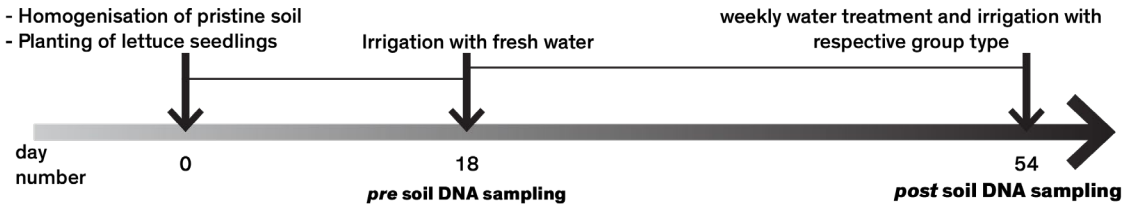


Figure 28 - timeline of the irrigation campaign

Every plant was given the same total amount of water (2.1 L of freshwater up to day 18 and 3.73 L of their respective water type over day 18-54). This was supplemented with NPK fertiliser at 27.8 mg of total N per pot spread over the growing period. The temperature for the entire growing period ranged from 25°C-16°C.

4.2.3. Wastewater Sampling

Secondary treated wastewater was obtained from the Dan Region Wastewater Treatment Plant (Shafdan) in Rishon LeTsiyon, Israel. Sampling was split in two sessions, WW1 was sampled on the 2018-11-04 and 75 L were collected to be used for the following 3 weeks of irrigation. WW2 was sampled on the 2018-11-25 to be used for the last 3 weeks of irrigation. Wastewater characterisation data was partially measured in house and partially provided by Shafdan.

Table 6 - WW parameters of both sampling sessions

Parameter	WW1	WW2
Chemical oxygen demand *	40 mg/L	34 mg/L
Biological oxygen demand *	6 mg/L	7 mg/L
Dissolved organic carbon †	9.2 mg/L (unspiked) 11.9 mg/L (spiked)	8.9 mg/L (unspiked) 10.7 mg/L (spiked)
Dissolved total carbon †	51.0 mg/L (unspiked) 55.0 mg/L (spiked)	43.1 mg/L (unspiked) 44.9 mg/L (spiked)
Total nitrogen †	16.2 mg/L	14.4 mg/L
Total suspended solids †	6.1 mg/L	7.0 mg/L
Absorbance at 365 nm †	0.0634 A	0.0698 A
Turbidity (NTU) *	2.2	2.7
pH*	7.4	7.5
Unspiked *E. coli* load †	667 CFU/mL	467 CFU/mL
Unspiked other coliforms load †	3300 CFU/mL	2567 CFU/mL

* provided by Shafdan WWTP, † self-measured

Wastewater was kept at 4°C in the dark and subsamples taken to be disinfected weekly as needed. The WW was not used for more than 3 weeks after sampling.

4.2.4. Preparation of the bacterial stock

A stock of bacteria was produced by mixing 1 part of WW from freshly sampled WW1 to 19 parts of sterile LB broth in culture tubes. These tubes were incubated overnight at 30°C under constant shaking at 140 RPM and the following morning centrifuged at 1000 × *g* for 5 min. The liquid was decanted, and the pellets combined in sterile 0.85% NaCl as to concentrate the growth by a factor of 8 relative to the original LB broth growth. The combined bacterial growth was again centrifuged at 1000 × *g* for 5 min to remove traces of LB broth and finally resuspended in sterile glycerol/water 1:1 v/v and stored at -80°C to be used as weekly spiking mother suspensions in order to reduce the fluctuation of viable bacterial densities between different weeks.

4.2.5. Bacterial Enumeration

Bacteria were enumerated on Chromocult Coliform Agar (Merck Millipore) after appropriate dilution in sterile 0.85% NaCl and filtration on a 0.45 µm cellulose nitrate membrane (Sartorius Stedim). This agar is used (e.g. in ISO 9308-1:2014) to simultaneously enumerate *E. coli* and other coliforms based on selective enzymatic cleaving of chromogenic compounds. Bacteria were enumerated each week before each spiking with the bacterial stock and before each disinfection (Figure 29). Bacterial spiking approximately doubled the bacterial load from the unspiked WW and was carried out in order to have WW that is less subject to drastic variations.

For bacterial enumeration post treatment, 100 mL of water was filtered in order to be able to have a quantification limit lower than 10 CFU/100mL. The stored disinfected WW at 4°C was checked for regrowth at 1, 2 and 4 days after disinfection using the same method. All measurements were conducted in triplicate.

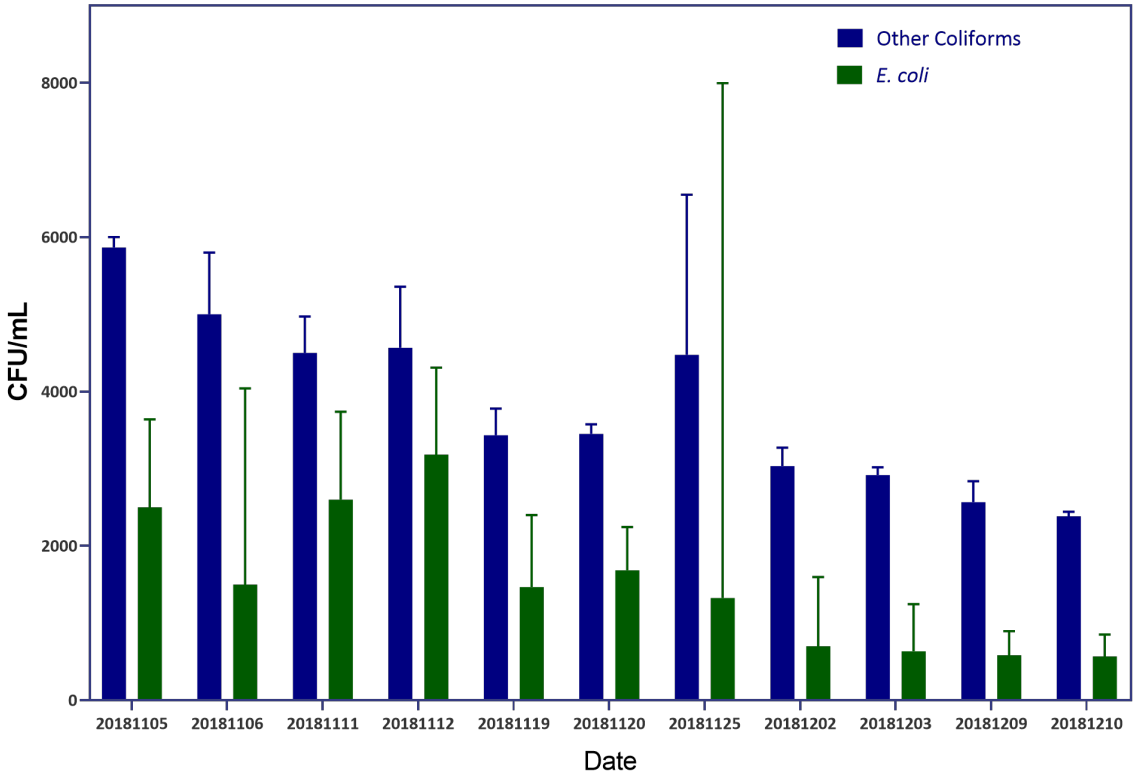


Figure 29 - bacterial loads prior to each weekly disinfection with HPC and chlorination. Err. Bars = SEM

Table 7 - figure 29 summarised data

	(CFU/mL)				
	Mean	SD	Max	Min	n
Other Coliforms	3777	1317	6550	2200	31
E. coli	1529	954	3600	350	31

4.2.6. Protocols for Disinfection by HPC and Chlorination

At the start of each week a subsample (6.5-7.5L depending on the type and number or analysis to be carried out) of WW was taken and brought to room temperature. In a sanitised container under stirring, 10 µL of bacterial stock per litre of WW was added and allowed to mix. Aliquots of WW were taken for bacterial enumeration, both before and after spiking.

For HPC disinfection, the spiked WW was placed in a rectangular PET tank of 54 cm by 21 cm with three magnetic stirrers at the bottom. Separately 0.1g of Ce-ZnO per litre of WW used was weighed, suspended in a minimal amount of sterile water and sonicated using a QSonica Q125(CT, USA) probe sonicator at an amplitude of 70% of the maximum for 5 min. The Ce-ZnO was then added to the WW in the reaction

tank and left to equilibrate in the dark for 30 min. After which, two pre-warmed *Osram Dulux L BL UVA 55W/78* coupled to an *Osram Quicktronic Professional Optimal* ballast were placed 35 cm from the base of the rectangular tank. The reaction time was set at 3 h after which the treated WW was sampled for bacterial enumeration and as needed for other tests such as dissolved organic carbon measurements. A portion of this TWW was used for irrigation of the 6 pots in the series on the same day, and the rest was stored at 4°C to be used for irrigation the following days up to a maximum of 4 days.

Disinfection by chlorination was carried out using commercial 6% sodium hypochlorite which was diluted ten-fold and its concentration verified using MQuant active chlorine test strips (Merck Millipore #117924). A suitable quantity of this diluted hypochlorite solution in order to achieve an initial concentration of 2 mg/L of active chlorine was added to 6.5-7.5 L of WW under constant stirring as required for that week. The concentration of active chlorine added to the WW was tested on addition and 90 min after addition using MQuant active chlorine DPD kit (Merck Millipore #114434). The active chlorine concentration measured initially was approximately 1.8-2.2 mg/L and that after 90 min was < 0.2 mg/L. This residual active chlorine, after 90 min contact time, was not quenched since they are allowed by Italian regulation and is even lower than WHO recommendations for drinking water disinfection.[128, 129] As was the case for HPC TWW, it was used for irrigation on the same day and the rest was stored at 4°C to be used in the 4 days that followed disinfection.

4.2.7. Water sampling for DNA Extraction

Water samples were filtered through a 0.45 µm membrane (Sartorius; Göttingen, Germany to be processed for DNA extraction and subsequent qPCR analysis. Five different water samples were taken, and their corresponding volumes (later to be used for normalisation) are presented in Table 8 below. All samples were taken in triplicate.

Table 8 - water samples and their respective volumes for DNA extraction

Type	Volume (mL)
Fresh water	500
Unspiked wastewater	250
Spiked wastewater	250
Post HPC treated wastewater	300
Post chlorination wastewater	300

In order to decrease as much as possible the detection limit post-treatment, the maximum quantity of water before clogging of the membrane, was taken. The membranes used for filtering each sample were stored at -80°C until processed for DNA extraction using DNeasy PowerWater Kit (Qiagen; Hilden, Germany). DNA extraction was carried out as per manufacturer's instructions, the final elution volume was 100 µL which was split into two subsamples and stored at -80°C.

4.2.8. Soil sampling for DNA Extraction

A total of 48 soil samples were taken, i.e. two soil samples from each pot, specifically the pre-irrigation regime samples at day 18 (Figure 28) i.e. after irrigating with freshwater for 18 days to stabilise the bacterial community and the post-irrigation regime at the end of the growing cycle 54 days after transplantation. Post-irrigation soil sampling was carried out 24 h after the last irrigation in order to avoid sampling water directly. Sampling was carried out by thoroughly mixing the top layer (up to a depth of 3-5 cm) of soil of each pot and transferring ≈15 g of soil into a sterile Falcon tube which were stored at -80°C until DNA extraction and other analysis were carried out.

Qiagen's DNeasy PowerSoil Kit (Hilden, Germany) was used for DNA extraction as per manufacturer's instructions. 250 mg of soil was subsampled and homogenised using a MP Biomedicals FastPrep-24™ Classic (CA, USA) (5 m/s for 23 s with a gap of 5 min between cycles to avoid overheating. The final elution volume was 100 µL which was split into two subsamples and stored at -80°C.

4.2.9. Auxiliary Measurements

Other complimentary analysis were carried out of the various samples. Namely, dissolved organic carbon and total nitrogen were measured using a Shimadzu

TOC-V Analyser (Kyoto, Japan). Total suspended solids were measured gravimetrically by filtering a 300 mL aliquot of water and drying at 105°C taking into account changes in the mass of blank filters. Soil dry mass was measured according to ASTM D2216-10[130] but using 5 g of soil until there was not further reduction in mass at 110°C which took 24-36 h. Plant aerial height was measured as the part of the plant from the soil to the upper most part extended perpendicularly upwards. The dry weight of the plants was measured by cutting the whole aerial part and drying at 80°C for 36-48 h until no further loss in mass was measured.

4.2.10. Quantitative real time PCR analysis

A total of four genes in both water and soil samples were analysed by qPCR, namely 16S rRNA, *intI1*, *qnrS* and *bla*$_{OXA-10}$ using a method previously employed by Marano*, et al.*[131]. Two plasmids were used as templates for standard curve calibration, the pMARPAT for blaOXA-10 [131]and The pNORM1[132] for the remaining genes. The plasmids were extracted from fresh bacterial cultures using QIAprep Spin Miniprep Kit (Qiagen; Hilden, Germany) and enzymatically linearised with EcoRI (ThermoScientific; MA, USA) prior to use. The concentration of the plasmid extract was determined using a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific; MA, USA) and the dsDNA BR Assay Kit (Thermo Fisher Scientific; MA, USA).

Copy number quantifications were carried out in duplicate and PCR grade water was used as a negative control. A StepOnePlus real-time PCR running StepOne software v2.3 (Applied Biosystems, CA, USA) with a 96-well plate tray was used. FAST SYBR® Green MasterMix (Thermo Scientific; MA, USA) was used to amplify the 16S rRNA gene, whereas POWER SYBR® Green MasterMix (Thermo Scientific; MA, USA) was used for the other genes. Each well contained 10 µL of the respective Mastermix, 1 µL of sample extract and 0.5 µM of both the reverse and forward primer to making up a total well volume of 20 µL. The primers used and the cycling conditions for each gene are reported in Table 9. Only results that had an efficiency of 100 ±10% and R^2 values greater than 0.99 are reported. After accounting for elution volumes, sample volumes and other parameters such as dilutions and dry

weight, results are presented as normalised values per unit volume (water samples) or per unit dry mass (soil).

Table 9 - qPCR primers and cycling parameters adapted with permission from [131]

Gene	Primers	Primer sequence	Amplicon size (bp)	Cycling conditions	
16S rRNA	331F 518R	TCCTACGGGAGGCAGCAGT ATTACCGCGGCTGCTGG	195	1x [95°C - 5 min] 40x [95°C - 5 s; 60°C - 30 s]	[133, 134]
intI1	intILC5_fw intILC1_rv	GATCGGTCGAATGCGTGT GCCTTGATGTTACCCGAGAG	196	1x [95°C - 10 min] 40x [95°C - 15 s; 60°C - 1 min]	[135]
bla$_{OXA10}$	OXA10-fw OXA10-rv	AGAGGCTTTGGTAACGGAGG TGGATTTTCTTAGCGGCAAC	191		[136]
*qnr*S	qnrSrtF11 qnrSrtR11	GACGTGCTAACTTGCGTG TGGCATTGTTGGAAACTT	118		[137]

4.3. Results and Discussion

4.3.1. Bacterial Inactivation and Regrowth

The conditions used for disinfection was sufficient to reach the established target of <10 CFU/100mL of *E. coli* albeit a few colonies were still detected in most samples. As for other coliforms both disinfection products were unable to reduce their level to less than <10 CFU/100mL. This is not an experimental limitation in any way, the standard is set specifically for *E. coli* and it is expected that more resilient bacteria such as faecal coliforms or *Pseudomonas aeruginosa*, after treatment, can be present at higher levels than *E. coli* since different organisms have different inactivation kinetics.[138-140]Bacterial regrowth of treated wastewater that was stored at 4°C for up to 5 days was not significant and *E. coli* levels remained below <10 CFU/100mL. When TWW is used in real agricultural irrigation it is almost always stored for multiple days at ambient temperatures and bacterial regrowth is known to occur within a few days irrespective of treatment applied (e.g. see [115, 119, 141]), albeit it depending on the intensity it is applied for.

4.3.2. Plant Growth

Growth metric of the 24 lettuce plants were also collected to investigate any signs of detrimental growth effect due to the wastewater or treatment by-products that can induce phytotoxicity. As for aerial height (Figure 30), the only significance (α=0.05) in height was observed between plants receiving FW and those receiving chlorinated wastewater (one-way ANOVA: mean FW=26.17 cm, mean OCl=23.00 cm, p=0.0365). Collectively the means are not significantly different from one another (p=0.0592).

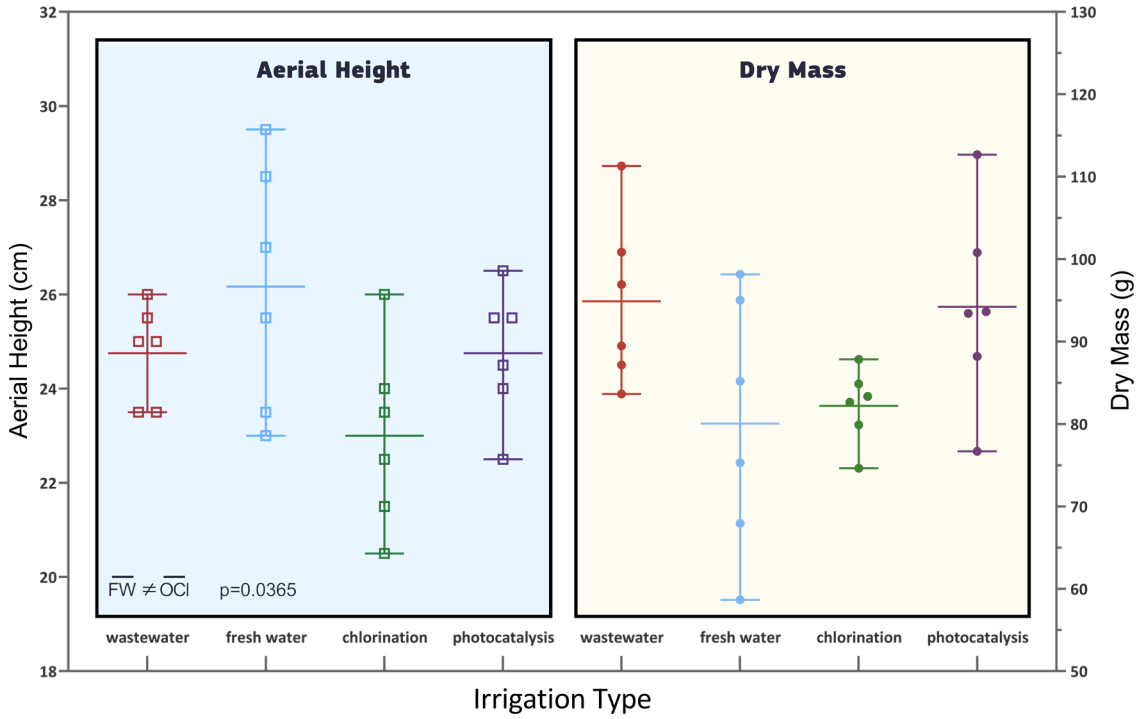


Figure 30 - plant growth metrics with water type. Err. Bars = range

Another growth metric that was measured was dry mass (Figure 30). No significance (α=0.05) was observed both individually and collectively between groups. It can be concluded that, within 1 growth cycle, the use of any of the four water types does not influence growth. Similar results are noted in literature, Manios, *et al.*[142] irrigated cucumber and tomato plants with various types of wastewater including fresh and chlorinated secondary wastewater. Tomato plants irrigated with secondary wastewater had a mean height of 70.9 cm while chlorinated and fresh water irrigated plants had means of 60.6 cm and 62.0 cm respectively. Cucumber plants showed and even more drastic difference between

water types. Secondary WW plants on average had a height of 155.8 cm while chlorinated WW (131.5 cm) and fresh water (79.0 cm) were substantially shorter. As for dry mass secondary WW tomato plants had a larger dry mass at (46.3 g) cf. chlorinated WW at 27.8 g and 32.0 g for fresh water. Cucumber plant mass was also substantially higher in the secondary WW irrigated series. Fresh water irrigation therein was augmented with fertiliser, yet the plants still seemed to benefit from the higher nutrient content in the WW series. The authors also note that the difference in the growth parameters amongst the different water types for cucumber is probably due to the fact that cucumber plants start to flower earlier and thus have a use for higher nutrient load at an earlier stage. Any of this difference was not observed in the lettuce plants grown in our study. The most plausible explanation for this is that lettuce plants are artificially selected and grown for their vegetative parts as opposed to cumber and tomatoes which are artificially selected to produce large amount of fruits. Thus, these plants have a high demand for nutrients to produce fruits. Lettuce plants being mostly leafy crops did not benefit from the additional nutrients in WW to give higher growth values. While there is no significance in the dry mass between any of the plants, the effect that chlorinated by-products have on plant growth cannot be discounted. Physiological effects on lettuce even at low levels such as was the residual chlorine concentration of <0.2 mg/L are known to occur.[143]

4.3.3. Water – qPCR Results

As for molecular results, 16S rRNA gene copy number, an indicator of total bacterial density,[134] showed little change amongst samples (Table 10). Spiking increased slightly the total load of 16S rRNA, the aim here was to achieve a bacteria load in the same order of magnitude as unspiked wastewater while reducing the variability of the samples over the weeks. The post spike had a higher mean ($p=0.0478$) and a lower standard deviation (as planned for). Comparing the means of the 2 TWW with the spiked mean i.e. the water before treatment shows that only chlorination was significant in reducing the total 16S rRNA copy number (OCl $p=0.0005$; HPC $p=0.7255$). Fresh water, as expected, had a much lower 16S rRNA gene count with 3 orders of magnitude less than spiked WW.

Table 10 - 16S rRNA copy number mean values in water samples

	16S rRNA copies per mL of water in:				
	Unspiked WW	Spiked WW	After OCl	After HPC	Fresh Water
Mean	1.44E+06	2.02E+06	1.17E+06	1.74E+06	2.05E+03
SD	6.33E+05	4.88E+05	3.40E+05	4.43E+05	1.84E+03

Overall, both treatments were relatively ineffective in reducing the levels of 16S rRNA gene indicating that while the treatment is effective at reducing viable bacterial loads as measured by plate counts, their reactivities are not enough to oxidise all biological components of these bacteria. Another possibility is that most of the 16S rRNA genes are present in bacteria which are not cultivable on the agar in use (CCA). On average bacteria have 4.2 copies of the 16S rRNA gene per genome (equivalent to per cell which in theory results in 1 colony); the average for Enterobacteriaceae is 7.[144]. Using the average combined values of E. *coli* and other coliforms in the spiked wastewater (5306 CFU/mL), we can assume that this contributes to approximately 3.71E+04 copies per mL of 16S rRNA of the total quantified 2.02E+06 copies per mL, i.e. less than 2%. Thus, is it possible that the 16S rRNA genes housed within the genome of the plate enumerated Enterobacteriaceae are in fact deactivated but more resilient bacteria are the major contributor to the measured quantity of 16S rRNA in water samples.

As for the measured ARGs and associated genes, the treatment again was not very effective (Figure 31). Pre and post treatment values are within the same order of magnitude. In FW all 3 genes were found to be below the quantification limit. As for treatments, chlorination was found to significantly (α=0.05) reduce the quantity of all 3 studied genes while one-way ANOVA tests of HPC and spiked WW showed no significance in the differences in means. There were also no statistical differences in the means of gene copy numbers for spiked and unspiked samples. Thus while E. *coli* densities were approximately doubled by spiking, the number of these genes conferring resistance to antibiotics did not increase. This enrichment of bacteria, having been conducted in the absence of selective pressures, has resulted in these genes not being transferred vertically on bacterial reproduction and thus did not increase in number after spiking.

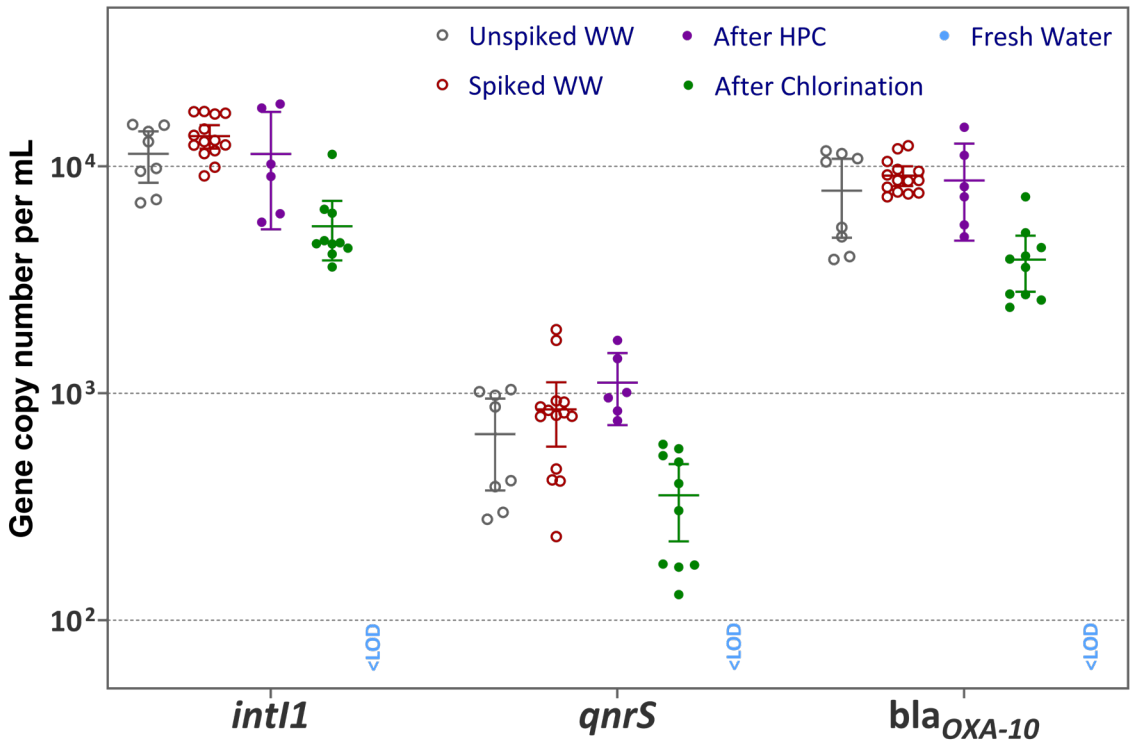


Figure 31 - *intI1, qnrS, bla*$_{OXA-10}$ concentrations in water samples including after treatment. Err. Bars = 95% C.I.

The absolute level of these genes corresponds well with literature values. Both *IntI1* and bla$_{OXA-10}$ are genes that are strongly associated with wastewater and were found in relatively high levels. Marano, *et al.*[131] also report concentrations of *intI1* and *bla*$_{OXA-10}$ in Israeli secondary WW both at levels ranging from ≈10^4 to 10^7 copies per mL, while *qnrS* was measured at lower levels ≈10^4 copies per mL.

Table 11 - gene copy number mean values in water samples

		Gene copies per mL of water:				
		Unspiked WW	Spiked WW	After OCl	After HPC	Fresh Water
intI1	Mean	1.14E+04	1.36E+04	5.44E+03	1.13E+04	<LOQ
	SD	3.46E+03	2.78E+03	2.23E+03	5.76E+03	<LOQ
qnrS	Mean	6.60E+02	8.49E+02	3.55E+02	1.11E+03	<LOQ
	SD	3.44E+02	4.63E+02	3.55E+02	3.72E+02	<LOQ
bla$_{OXA-10}$	Mean	7.81E+03	9.10E+03	3.87E+03	8.65E+03	<LOQ
	SD	3.55E+03	1.57E+03	1.50E+03	3.76E+03	<LOQ

4.3.4. Soil – qPCR Results

Soil samples taken before and after the irrigation campaign from each pot were analysed for the same genes in order to assess if these genes become more

abundant after a specific water regime is applied. As for water samples, 16S rRNA results indicated the total number of bacteria present in the sample.

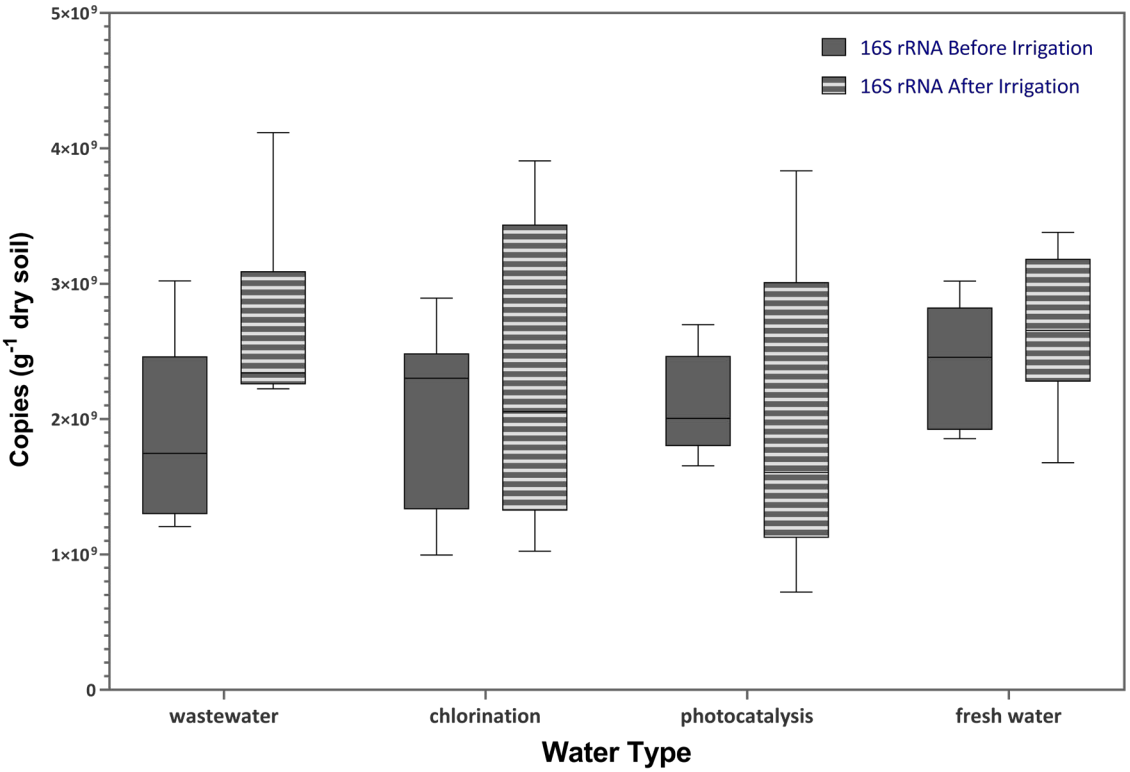


Figure 32 - changes in 16S rRNA gene copy number by irrigation regime.

The absolute abundance of 16S rRNA (Figure 32 and Table 12) was lower than that found in natural non-agricultural soils.[145] This is probably due to the fact that the soil utilised was almost entirely sandy with very little organic matter. Thus these results can be explained by the fact that 16S rRNA is present in all bacteria, and is thus used as an indicator of quantity of bacteria present.[144] and since soil type is the major determinant[146] for bacterial activity in soil.

No statistical significance was noted between all *before irrigation* samples indicating that the soil mixing and 17 days of equilibration were suitable. As for differences between the before and after irrigation of the same water type, only wastewater irrigation resulted in a significant increase (two-tailed t-Test, $p = 0.0066$) indicating that the much higher bacterial load present in this water type resulted in increases in bacterial loads present in the soil.

Table 12 - 16S rRNA soil copy numbers before and after irrigation

	16S rRNA copy number (g^{-} dry soil)			
	Before Irrigation		After Irrigation	
	Mean	SD	Mean	SD
WW	1.87E+09	6.30E+08	2.70E+09	6.47E+08
OCl	2.02E+09	6.36E+08	2.27E+09	1.07E+09
HPC	2.11E+09	3.69E+08	1.97E+09	1.11E+09
FW	2.42E+09	4.31E+08	2.64E+09	5.84E+08

It should be noted that this increase is not merely due to the addition of water with higher organic content. Both chlorination and heterogeneous photocatalysts resulted in slightly higher dissolved organic carbon content while dissolved inorganic carbon was reduced after treatment; HPC (17.3 mg/L) and chlorination (14.6 mg/L) from WW initially at 11.9 mg/L DOC. The higher mass of DOC on treatment could be the product of DOC being oxidised by the addition of hydroxyl and carbonyl groups and thus increasing in mass while maintaining or reducing their molarity. The high level of DOC in all WW samples however indicates that the viable bacteria are responsible for the increase in 16S rRNA gene copy number and it is not merely the more rapid multiplication of bacteria in soil by supplying nutrients in the form of dissolved organic carbon.

As for ARGs *intI1* (Table 12), a very interesting result was obtained (Figure 33 and Table 13). The quantity of all studied genes in the wastewater pots after irrigation increased significantly. Most notably, the gene that was found at highest quantities in WW (*intI1*) increased by more than 1 order of magnitude from the pre irrigation campaign levels. There was also a drastic change in $bla_{\text{OXA-10}}$ levels. Before the irrigation campaign was started, this gene was not present at levels higher than the detection limit in all samples, while after treatment it was found at relatively high levels in WW irrigated soil. As for *qnrS*, which is not generally associated with wastewater and was in fact quantified at the lowest level in the water samples, while showing an increase, it was much lower than the other two more abundant genes. *qnrS* is also associated with plasmids and these results may indicate that plasmid associated ARGs may be less prone to AR dissemination after wastewater

treatment. In summary for wastewater samples, there is a clear link that irrigating with a high bacteria load wastewater which contains the studied ARGs will result in higher ARG load also in the soil bacterial communities.

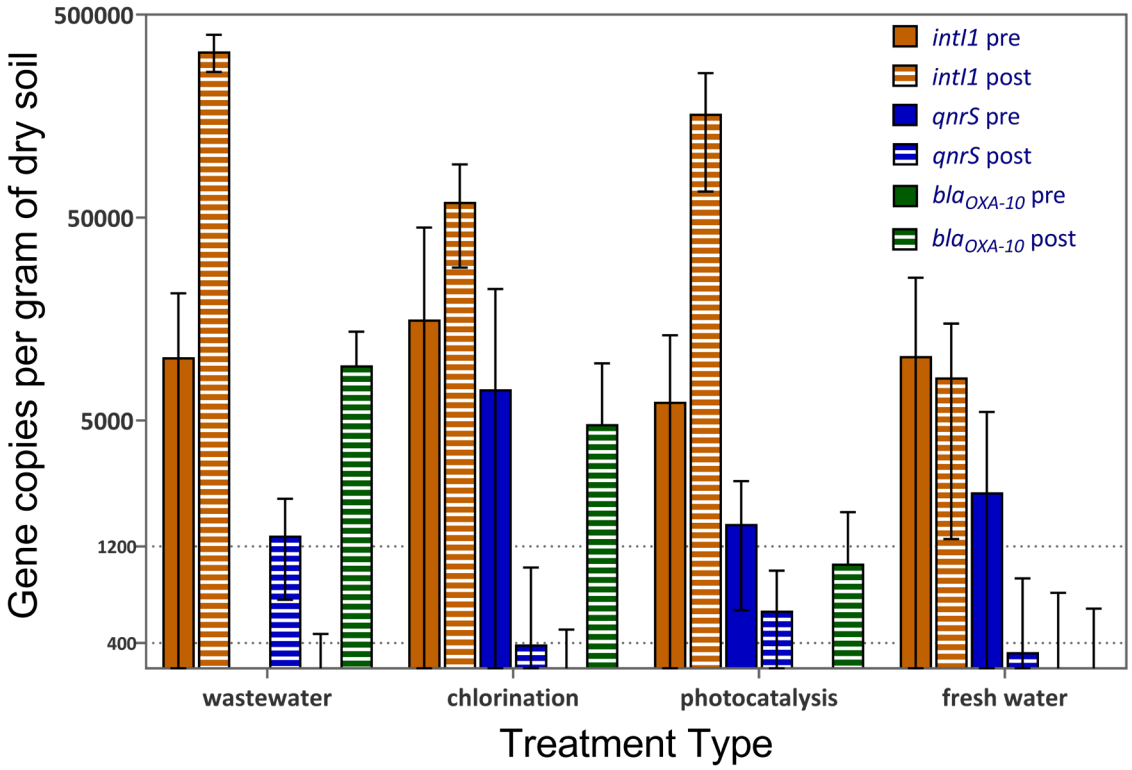


Figure 33 - gene copies of the studied genes in soil before and after irrigation. Err. Bars = 95% C.I.

As for FW samples, there was no increase in any gene, indicating that any increase in the WW samples was not due to multiplication of soil bacteria which housed the genes in question. Notably also is the fact that *qnrS* decreased significantly after irrigation with freshwater (which did not contain this gene) for 37 days. Thus, indicating that accumulation of ARGs could potentially be possible to reverse and offers opportunities in the realm of agricultural water management practices in order to control ARG levels in soil. For e.g. by alternating reused wastewater and other water sources every growing season to allow the soil to revert to previous levels. This would also have positive effects on other wastewater irrigated problems such as soil hydrophobicity and salinity.[147]

Table 13 - *gene* soil copy numbers before and after irrigation

	Before Irrigation		After Irrigation	
	***intI1* copy number (g^{-} dry soil)**			
	Mean	SD	Mean	SD
WW	1.03E+04	1.09E+04	2.70E+09	6.47E+08
OCl	1.57E+04	2.89E+04	2.27E+09	1.07E+09
HPC	6.19E+03	6.95E+03	1.97E+09	1.11E+09
FW	1.04E+04	1.49E+04	2.64E+09	5.84E+08
	***qnrS* copy number (g^{-} dry soil)**			
WW	1.30E+01	7.60E+00	1.36E+03	7.03E+02
OCl	7.14E+03	1.51E+04	3.94E+02	5.50E+02
HPC	1.55E+03	9.67E+02	5.78E+02	3.32E+02
FW	2.22E+03	3.30E+03	3.61E+02	4.71E+02
	***bla*$_{OXA-10}$ copy number (g^{-} dry soil)**			
WW	1.62E+02	2.82E+02	9.35E+03	4.35E+03
OCl	1.37E+02	3.29E+02	4.80E+03	4.78E+03
HPC	1.31E+02	1.34E+02	9.86E+02	7.82E+02
FW	2.46E+02	4.62E+02	2.82E+02	3.09E+02

While it is clear that untreated secondary WW increases the copy number of all the studied genes, the differences between both types of treatment is not so clear cut. *qnrS* values are significantly less after chlorination irrigation (p=0.0105) while no change is reported in HPC (p=0.7237). Thus, chlorination for *qnrS* approaches more the situation with fresh water irrigation, however HPC does not result in a significant differences in means, indicated that it is nonetheless more effective than untreated WW For *bla*$_{OXA-10}$ there is a significant (p= 0.0001) increase for chlorinated wastewater irrigation with a magnitude comparable to that of untreated wastewater, the differences in this gene for HPC treated wastewater is not reported as significant (p= 0.4411).

4.4. Conclusions

The data from the untreated WW irrigation water samples can be used as part of methodologies to construct emission limit values, that is values that when exceeded, will result in deleterious effects – in this case being increases in hazardous genes in the soil communities. The establishment of these values are at a rather rudimentary stage, and more data is needed for a more comprehensive evaluation.

Over an irrigation period of 37 days, each pot containing 3.3kg of dry soil received a total of 3730 mL of WW. Taking each pot as a homogeneous unit as an approximation, this results in the gene concentrations showed in Table 14

Table 14 - estimation of number of gene copies needed in irrigation water for increases in soil copy number

	intI1	*qnrS*	bla_{OXA-10}
mean copy number in WW (mL^{-})	1.36E+04	8.49E+02	9.10E+03
total copy number received over 37 days	5.06E+07	3.17E+06	3.39E+07
total copy number per g of soil	1.53E+04	9.60E+02	1.03E+04

Thus an irrigation unit receiving wastewater which contains ≈1530 copies of *intI1* per dry gram of soil, over a relative short period of time, is expected to result in an increase in that gene in soil and likewise for the other genes in Table 14. Having said that, the quantity of genes in water is by far not the only determining factor. Both WW treatments had very similar copy number values to that of untreated wastewater, however the magnitude of increase of the copy number of these genes in soil was much lower for the treated WW. Untreated WW had up to 5 orders of magnitude higher number of *E. coli* than the TWW and thus it seems that this has a major impact on AR dissemination into soil communities. Any future regulatory frameworks cannot exclude the concentration of viable bacteria in the wastewater since it is clear that molecular methods alone cannot predict the changes in the downstream environment.

As for the direct comparison between the two treatments, HPC did not produce benefits that justify its elevated cost. While chlorination is known to have issues

with toxic by-products such as trichloromethane and other chlorinated by-products such as trichloroethane, these were not so phytotoxic as to affect plant growth. At residual chlorine levels close to the Italian regulatory level of <0.2 mg/L, stunt plant growth has been observed.[143] While deleterious effects on plant growth can take place even at this low level, modelling studies with trichloromethane and trichloroethane as model compounds show a low risk of absorption into plant biomass and transfer to humans via the food chain.[148] These compounds have high Henry's law constants and thus partition to the gas phase. Weber*, et al.*[148] modelled the partitioning of these two compounds and estimate that 95% and 79% of trichloromethane and trichloroethane respectively would partition to the gas phase with the remainder being almost exclusively found in the soil. Thus these compounds are more of an issue to plant growth rather than to human health. Different plants are expected to have different tolerance levels which would make chlorination not so much of an issue for irrigation of these crops.

At least for the genes selected in this study, chlorination is the more feasible option, while HPC most probably has benefits associated with an overall higher level of water treatment, with regards to ARGs chlorination did the job at a lower cost and is a simpler process. Mandating too strict requirements in WW reuse will inevitably result in a lower adoption of this practice, limiting its utility for combating water scarcity. Having said that other factors may well justify the higher treatment in reactivity. An angle that was out of the scope of this study, but highly relevant, was the accumulation of chemical micropollutants in plant biomass from wastewater irrigation. Paltiel*, et al.*[149] demonstrated that humans eating produce which have been grown irrigated with wastewater show an increased level of carbamazepine in their blood stream. While this is at levels much lower than therapeutic doses, the presence of cocktail effects and chronic exposure pose a potential hazard. Thus, this study should be used as part of a larger body of evidence for a comprehensive risk assessment of wastewater reuse in edible crop irrigation.

5. Project-Wide Concluding Remarks

The application of photocatalysis for antibiotic resistance abetment has been a topic of high interest and relevance throughout the PhD project such as 76 research papers have been published since 2016[‡] and over 2000 for photocatalytic wastewater treatment in general.[§]

The research work conducted in *WP A - Selection of a suitable photocatalyst* produced an improved efficiency photocatalyst with low cost starting materials, facile synthesis and of widespread usability for photocatalytic processes.

In *WP B - Catalyst immobilisation* it was demonstrated that the photocatalyst can be easily immobilised on common materials again with simple methods of preparation. The photocatalytic coating was active both against two selected model antibiotics as well as bacterial disinfection, including in real wastewater.

Finally the use of this optimised catalyst was directly contrasted with a conventional method in *WP C –*. Here it is noted that the ecological boundaries were of a significant obstacle for AR to thrive after being introduced through wastewater for irrigation but did not prevent ARG increases even after treatment. Direct empirical data on copy numbers of selected antibiotic resistance genes and associated genes after irrigating with different water types were obtained. Here it was shown that no evidence supports the use of HPC for agricultural wastewater reuse over chlorination at least over one growth cycle for lettuce crops in sandy soil.

[‡] Scopus search for: TITLE-ABS-KEY (photocatalysis AND antibiotic AND resistance) AND (LIMIT-TO (PUBYEAR , 2020) OR LIMIT-TO (PUBYEAR , 2019) OR LIMIT-TO (PUBYEAR , 2018) OR LIMIT-TO (PUBYEAR , 2017) OR LIMIT-TO (PUBYEAR , 2016))

[§]Scopus search for: TITLE-ABS-KEY (photocatalysis AND + AND wastewater) AND (LIMIT-TO (PUBYEAR , 2020) OR LIMIT-TO (PUBYEAR , 2019) OR LIMIT-TO (PUBYEAR , 2018) OR LIMIT-TO (PUBYEAR , 2017) OR LIMIT-TO (PUBYEAR , 2016))

5.1. Recommendations for Future Work

Much research remains to be done in order to be able to holistically asses the risk associated with antibiotic resistance during wastewater irrigation. This includes:

- Assessing if different ARGs show differences in soil accumulation between treatments.
- Assessing if different soil types (clay, loam etc.) affect significantly the accumulation of ARGs from wastewater irrigation.
- Assessing any cumulative effects between treatments when irrigation is carried out over multiple growth cycles over a period of a few years.
- Assessing the benefit of co-treatments i.e. if sequential or combined treatments produce better results than the sum of the separate treatments. An example of such treatments is the combined effect of ozonation and photocatalysis or the sequential application of chlorination at low levels followed by photocatalysis.
- Assessing the dissemination of AR to soil using treated wastewater stored at ambient temperature since that might result in regrowth.
- Disinfected wastewater is often stored for numerous days before reuse, this allows for bacterial regrowth and an altered bacterial community. Using solar based disinfection treatments, a pre-irrigation additional disinfection stage could be implemented as a delocalised system. That is a disinfection step taking place directly on the fields just prior to drip irrigation in order to remove the risk of regrowth.

6. References

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