INACTIVATION OF *KLEBSIELLA PNEUMONIAE* IN SEWAGE BY SOLAR PHOTOCATALYSIS AND INVESTIGATION OF CHANGES IN ANTIBIOTIC RESISTANCE PROFILE

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ABSTRACT

The constant presence of various and virulent microorganisms in sewage imposes the application of proper and efficient disinfection techniques in order to control possible transmission of waterborne diseases. *Klebsiella pneumoniae* is considered as an emerging human pathogen and can be found in wastewater, but it has been merely studied as far as its resistance against disinfection is concerned. It is an enteric, gram-negative, lactose-fermenting bacillus with a prominent capsule, recognized as an opportunistic pathogen, well known for its ability to develop resistance profiles against various antibiotics. The objectives of the present work were (i) to investigate the efficiency of cobalt- and manganese-doped titania materials to inactivate *K. pneumoniae* in real wastewater under solar radiation, (ii) to study the catalysts efficiency under natural sunlight, (iii) to compare inactivation rates between solar photocatalysis and chlorination and (iv) to study possible changes in *K. pneumoniae* antibiotic resistance profile through treatment.

Dopants significantly enhanced the photocatalytic activity of TiO_2 under solar irradiation, in terms of *K. pneumoniae* inactivation. The process was retarded under natural solar light and longer periods were required for total bacterial removal from the reaction solution. Catalysts with the binary dopant exhibited the best photocatalytic activity in all cases, highlighting the fact that composite dopants induce a synergistic effect. On the other hand, chlorine concentrations ranging from 0.05 to 0.3 mg/L proved to be satisfactory for an overall 7-Log reduction of *K. pneumoniae* population after 90 min of treatment. Nevertheless, the possible production of toxic by-products during chlorination raises certain concerns regarding this method and its use for wastewater disinfection. The effect of disinfection on bacterial antibiotic resistance profile and on MICs varied, depending on the tested antibiotic and on the applied treatment method. Further research is required for the application of effective treatment/disinfection methods for the complete inactivation/elimination of ARB in WWTPs.

Keywords: sewage disinfection; TiO_2; photocatalysis; solar irradiation; *K. pneumoniae*; antibiotic resistance; MIC

1. Introduction

In recent years considerable attention has been drawn towards many opportunistic pathogens contained in raw and treated wastewater, among which *Klebsiella pneumoniae* is prominently included (Makris et al., 2014). This bacillus is gram-negative, lactose-fermenting and it belongs to the broad family of Enterobacteriaceae (Burke et al., 2009). *K. pneumoniae* accounts for a significant proportion of hospital-acquired infections, like pneumonia, septicaemia, and soft tissue infections and is responsible for numerous nosocomial outbreaks worldwide (Samra et al., 2007). The ability of this bacterial strain to spread rapidly in the hospital, as well as in the aquatic environment and its resistant nature against bactericidal factors enlist *K. pneumoniae* among the highly virulent and pathogenic microorganisms, especially for susceptible population groups and immunocompromised patients (Yousef et al., 2012).

The frequency of reports concerns mainly the multi-drug resistance of this specific strain, while equally significant is the resistance of *K. pneumoniae* isolates to environmental stressed conditions and deleterious agents, such as disinfectants (Baroud et al., 2013; Vijay et al., 2013). The prominent polysaccharide capsule that they possess increases their virulence through protection from phagocytosis and prevents cellular destruction by bactericidal factors (Venieri et al., 2014).

Given the persistence of this bacterium in the environment it is imperative to explore effective and reliable disinfection techniques, which would eventually inactivate it, providing appropriate control measures of such pathogens. Although the beneficial effects of many treatment processes of water and wastewater have been well addressed, *K. pneumoniae* has been merely mentioned in research studies as far as its resistance against disinfection is concerned. Conventional wastewater disinfection involves chlorination, which is widely used to remove effectively an extended variety of microorganisms (Hu et al., 2012). Yet, the diverse structural and physiological features of the latter lead to different response in the course of treatment, raising some concerns regarding the suitability of chlorination. Another approach towards efficient inactivation of persistent pathogens in wastewater seems to be the application of titanium dioxide photocatalysis, which has already demonstrated high reduction rates of viruses and bacteria in water/wastewater samples under ultraviolet (UV) irradiation (Cho et al., 2011). Further applications are based on the exploitation of total solar light spectrum and the use of metal dopants, which have been explored for visible light-induced photocatalysis (Karunakaran et al., 2010). The exploitation of total solar light spectrum expands practical applications of TiO_2 photocatalysis, which has been successfully adopted as a disinfection technique (Ishiguro et al., 2013).

Complete bacterial inactivation, which is mostly desired, is highly dependent upon operating parameters of each sewage disinfection process that uses natural or simulated solar irradiation. However, even in the case of residual cells post treatment, the extent of DNA damage and the induced oxidative stress may have various consequences, among which is the alteration of the microbial antibiotic resistance profile. The emergence and rapid spread of antibiotic resistant bacteria (ARB) has led to an increasing concern about the potential environmental and public health risks. ARB have been detected extensively in wastewater samples. Available data show significantly higher proportion of antibiotic resistant bacteria contained in raw and treated wastewater relative to surface water. The conditions in wastewater treatment plants (WWTPs) are favourable for the proliferation of ARB. A significant proportion of these pathogens, carried in water/wastewater, develop resistance mechanisms and remain present either in water supplies, or in treated effluents (Maier et al., 2009). As a result, there is a widespread danger to public health from these hardy environmental microorganisms. The level of removal is driven by many factors, such as the bacterial cell structure and molecular properties of the bacteria (Walsh, 2000) and the design of the WWTP (Batt et al., 2006). In general, chemical or biological processes alone are inefficacious in removing and/or biodegrading ARB during wastewater treatment (Ingerslev et al., 2000; Adams et al., 2002).

In this perspective, the objectives of the current study comprise the following: (a) testing of manganese- (Mn-), cobalt- (Co-), and binary (Mn/Co-)-doped titania materials, which were prepared in previous work (Venieri et al., 2014), in terms of *K. pneumoniae* inactivation in real wastewater samples under simulated solar irradiation. Disinfection effectiveness was evaluated in relation to influential operating parameters, like catalyst type and dopant concentration; (b) study the catalysts efficiency under natural sunlight; (c) comparison of inactivation rates between solar photocatalysis and chlorination and (d) study of possible changes in *K. pneumoniae* antibiotic resistance profile after disinfection treatment.

2. Materials and methods

Metal-doped TiO_2 nanoparticles were prepared by a co-precipitation method with molar ratio in different concentrations in the range of 0.02 to 1 wt%. Details regarding synthesis and characterization of all catalysts used in the present study can be found in previous studies (Binas et al., 2012; Venieri et al., 2014). The crystal structure, particle size, and morphology were examined with powder X-ray diffraction (XRD), SEM, and TEM, respectfully.

Disinfection experiments were carried out with real wastewater samples collected from the municipal wastewater treatment plant (117,500 equivalent inhabitants) located in Chania, W. Crete, Greece. Sampling took place from the effluent of the biological treatment process (activated sludge) sludge unit prior to disinfection (chlorination). Average values of wastewater chemical parameters were as follows: the chemical oxygen demand and dissolved organic carbon were 26 and 7.8 mg/L, respectively, the concentration of chlorides, sulphates, nitrates, nitrites, bicarbonates and total solids were 222.1, 60.3, 25.9, 57.1, 182.1 and 7 mg/L, respectively, while the pH was 7.8. All samples were filtered through membranes with pore size 0.45μm prior to disinfection treatment and were inoculated with the desired titre of bacterial stock cultures.

The bacterial strain used in the present study was *K. pneumoniae* NCTC 5056. Solar irradiation experiments were carried out in a solar radiation simulator system (Newport, model 96000) equipped with a 150 W xenon ozone-free lamp and an Air Mass 1.5 Global Filter (Newport, model 81094), simulating solar radiation reaching the surface of the earth at a zenith angle of 48.2^o. Temperature was maintained at 25±2 ^{o}C with a temperature control unit. The external reaction vessel was covered with aluminum foil to reflect irradiation exerting the outer wall of the reaction vessel. In a typical experimental run, the bacteria suspension was spiked in 200 mL of wastewater, which were then loaded in the reaction vessel with the appropriate amount of catalyst.

Photocatalytic experiments were also carried out under natural sunlight within the period of June - July 2014 at the Technical University of Crete campus located at 38^{o}31΄ N and 24^{o}04΄ E, in clear sunny days. During those runs the recorded ranges of solar irradiance and temperature were 12.7-13.4 W/m^2 and 29-32.7^{o}C, respectively. Each sample was introduced into borosilicate glass bottle, which was magnetically stirred during all experiment. The total volume was 200 mL. The photocatalyst was added to the sample and after 30 min of stirring in the dark for homogenisation it was exposed to natural solar irradiation.

Chlorination was performed with the use of NaOCl testing three different concentrations, namely 0.05, 0.1 and 0.3 mg/L.

Initial bacteria concentration was 10^7 CFU/mL in every case. At specific time intervals samples of about 1.5 mL of the reaction solution were withdrawn and were immediately analysed with respect to viable bacterial cells applying conventional culture method. The detection and enumeration of and *K. pneumoniae* were performed using Nutrient Agar (HiMedia Laboratories) and the serial dilution streak plate procedure. Incubation was performed at 37 ^{o}C for 20-24 h before viable counts were determined.

Antibiotic resistance was studied with Minimum Inhibitory Concentration (MIC) technique, using Ampicillin, Cefaclor, Sulfamethoxazole and Tetracycline. Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The broth microdilution method was performed using microplate wells of broth containing dilutions of an antimicrobial agent. Resistance profile was studied in intact cells of *K. pneumoniae* and in residual cells after each treatment.

3. Results and discussion

3.1. Inactivation of *K. pneumoniae* during photocatalysis

Bacterial inactivation in wastewater samples was recorded under solar irradiation in the presence of the prepared doped catalysts. Metal-doped catalysts showed satisfactory inactivation, as may be observed in Figure 1. Dopants improved the photocatalytic activity of pure titania (P25), which achieved a 1-Log reduction after 90 min of treatment. It is clearly shown that the catalyst contained the binary (0.04%wt Mn/Co:TiO_2) and the Co-dopant demonstrated high inactivation rates, as there was an overall 7-Log reduction in microbial population within 60 min of irradiation. The finding regarding the Mn/Co binary-doped TiO_2 verifies the general aspect that composite dopants induce a synergistic effect surpassing the disadvantages of the individual components (Pelaez et al., 2012). The superiority of binary-doped catalysts has already been addressed in other cases with *E. coli* being the testing microorganism and an average 4-Log reduction of its density after 90 min of exposure to visible light (Wu et al., 2010). The ratio of doping level (0.04 %wt of Mn/Co) proved to be optimum for rapid killing of *K. pneumoniae* in wastewater samples, although it should not be ignored that dopants have the potential to cause photo-induced corrosion and promote charge recombination at some metal sites (Papadimitriou et al., 2011).

Regarding the two Mn- catalysts, they exhibited different behaviour, which could be attributed to their structural features. Higher performance was recorded with composite sponge based titania (0.04%wt Mn:TiO_2 – sponge) synthesized by alkali solution treatment.

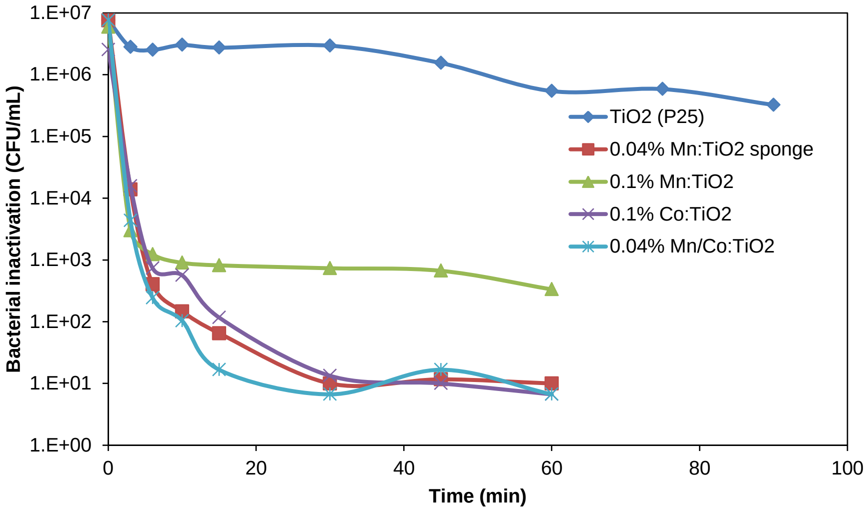

Figure 1: *K. pneumoniae* inactivation in the presence of different Mn, Co and binary Mn/Co-doped TiO_2 catalysts in wastewater under solar irradiation. Catalyst concentration is 250 mg/L.

Moreover, it can be noticed that bacterial inactivation reached a plateau and became slow, leading in residual cells at the end of the process. Similar findings were obtained in other study referred to *K. pneumoniae* inactivation with the use of reactive plasma processed nanocrystalline TiO_2 powder (Vijay et al., 2013). Vijay et al. noticed deceleration of the decay of this bacterial strain in the course of treatment, attributing this outcome to the released metabolites from the killed bacteria, which form a screen and protect the remaining active cells. The same conclusion was drawn in the work of Yousef et al., who investigated the effect of CuO/TiO_2 nanofibers on *K. pneumoniae* elimination under visible light (Yousef et al., 2012). Despite that this specific emerging pathogen is considered persistent during various treatments and disinfection techniques, photocatalysis with metal-doped catalysts seems quite promising, since it may demonstrate complete inactivation in short periods with initial cell densities as high as 10^7 CFU/mL in “complex” matrices, like wastewater.

3.2. Natural sunlight and NoOCl-mediated *K. pneumoniae* inactivation

Additional disinfection experiments were performed in natural sunlight in order to compare inactivation results with those obtained with simulated solar irradiation. Under perfectly sunny conditions wastewater samples, inoculated with *K. pneumoniae*, were exposed outdoors in sunlight for 1 h and results are shown in Figure 2. During sunlight experiments ranges of solar irradiance and temperature were 12.7-13.4 W/m^2 and 29-32.7 ^{o}C, respectively, with average values of 13.1 W/m^2 and 30.1 °C. Also, no inactivation was recorded a) in dark conditions with or without the catalysts in question and b) under natural sunlight without the use of any catalyst.

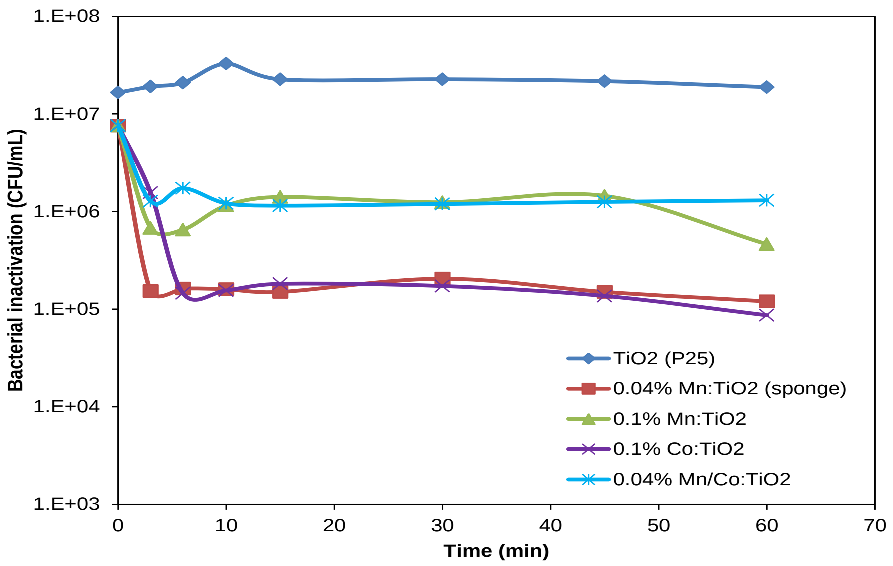

Figure 2: *K. pneumoniae* inactivation in the presence of different Mn, Co and binary Mn/Co-doped TiO_2 catalysts in wastewater under natural sunlight. Catalyst concentration is 250 mg/L.

Comparing rates between simulated (Figure 1) and natural solar radiation (Figure 2), it may be observed that there is an overall retardation of the process. Under the operational conditions of the current study bacterial decay was delayed when disinfection was performed outdoors, resulting in 1 or 2-Log reduction after 60 min of exposure. Similar retardation periods were shown by Suri et al., who studied inactivation rates of *E. coli* in water with Pt- and Ag-doped titania. Although sunlight was effective for activating the photocatalysts and eliminating the bacterial indicator, the decay rates were slower compared to those obtained with artificial light (Suri et al., 2012).

Apart from the light intensity dependence, there are other factors that may differentiate the course of the process, of which the composition is enlisted among the most determinants. Photocatalytic activity may be outweighed by other effects, reducing or varying the observed microbial inactivation rates. In our case experimental matrix was wastewater, whose chemical parameters concentration did not vary significantly among the samples tested in each case. Nevertheless, it is considered as a “complex” sample that affects photocatalytic process to a considerable extent (Ditta et al., 2008). However, based on our results and in accordance to other studies, photocatalysis proved to be effective method for wastewater disinfection in artificial and natural conditions. Additional asset of this technique is the fact that it may contribute in reducing the precursors responsible for the formation of disinfection by-products (DBPs), which are often produced, especially if wastewater disinfection practice involves chlorination (Suri et al., 2012). In the present study chlorination with the use of NaOCl at concentrations of 0.05, 0.1 and 0.3 mg/L exhibited high inactivation rates of *K. pneumoniae* in sewage samples. An almost 7-Log reduction was observed in all cases within 90 min of treatment. However, the complex composition of wastewater samples raises certain concerns regarding chlorination and its overall effect in terms of public health protection.

3.3. Antibiotic resistance profile of *K. pneumoniae* and MICs after treatment

According to many studies, disinfection processes seem to influence the proportion of ARB in wastewater. The effect varies in each case, depending on the kind and dosage of disinfectant. In the current study antibiotic resistance and MIC of the selected antibiotics were tested in intact cells of *K. pneumoniae* and in residual cells after each treatment and the results are shown in Figure 3.

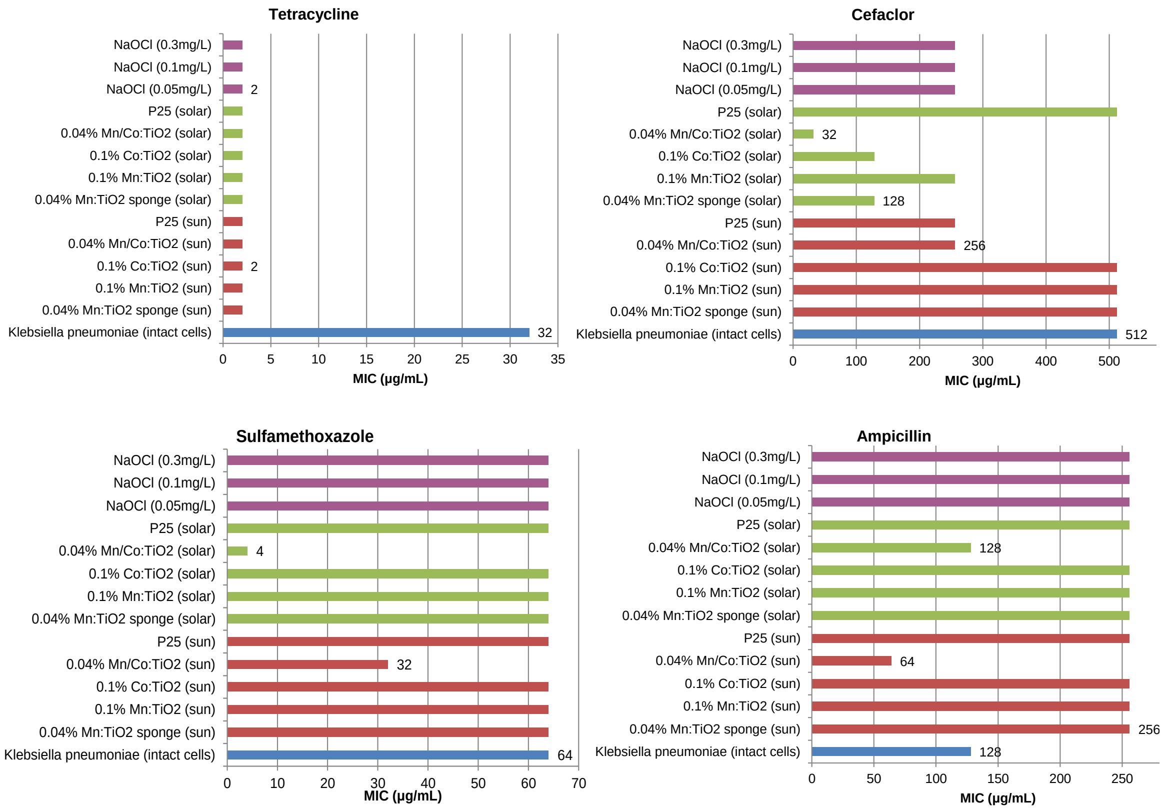

Figure 3: Minimum Inhibitory Concentration (MIC) of the selected antibiotics before (*K. pneumoniae* – intact cells) and after each treatment.

All tested bacteria remained resistant in β-lactams (cefaclor & ampicillin), according to EUCAST (European Committee on Antimicrobial Susceptibility Standards) standards, but the corresponding inhibitory concentration of those antibiotics changed through treatment. For example MIC of cefaclor decreased from 512 μg/mL in intact cells to 256 μg/mL after chlorination. On the contrary, when ampicillin was tested, in most cases MIC was higher after disinfection, resulting in higher tolerance compared to that exhibited in untreated cells. In the case of sulfamethoxazole the pattern of resistance remained almost the same, while MIC of tetracycline was highly less after all processes (from 32 to 2 μg/mL). Generally, disinfection was not selective towards ARB, but instead it induced the development of either antibiotic resistance or sensitivity.

Up to date, there is insufficient information available to reach a definite conclusion on the presence and fate of ARB in the WWTPs. It is clear that bacteria will continue to develop resistance during wastewater treatment by either new mutations or the exchange of genetic information, which is, putting old resistance genes into new hosts. There is still lack of fundamental data on their fate in WWTPs during disinfection and on their effects in the environment and consequently in public health. What is important is that any treatment process has the potential to alter antibiotic resistance profile of the bacteria causing adverse effects towards the environment and human.

4. Conclusions

- Dopants significantly enhanced the photocatalytic activity of TiO_2 under solar irradiation, in terms of *K. pneumoniae* inactivation.

- The process was retarded under natural solar light and longer periods were required for total bacterial removal from the reaction solution.
- Catalysts with the binary dopant exhibited the best photocatalytic activity in all cases, highlighting the fact that composite dopants induce a synergistic effect.
- The effect of disinfection on bacterial antibiotic resistance profile and on MICs varied, depending on the tested antibiotic and on the applied treatment method.
- Further research is required for the application of effective treatment/disinfection methods for the complete inactivation/elimination of ARB in WWTPs.

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