Photocatalytic inactivation of *E. faecalis* in secondary wastewater plant effluents

Karin Backhaus, Javier Marugán, Rafael van Grieken and Carlos Sordo

ABSTRACT

Photocatalytic inactivation of *Enterococcus faecalis* using TiO_2 suspensions was investigated and compared to the inactivation of the most commonly used faecal indicator strain *Escherichia coli*. In contrast to the inactivation in pure deionized water, disinfection of effluents from the biological process of an urban wastewater plant showed a longer initial lag phase and higher survival fractions after several hours of irradiation. Moreover, the fluctuation of the composition of the effluents strongly affects the overall inactivation rate, not directly related to changes in the values of organic matter content. Additionally, it was found that *E. faecalis* seems to be more resistant than *E.coli* towards the photocatalytic treatment. These results could be related to the differences in the cell wall structure of both microorganisms. The main conclusion of this work is that attention must be paid when transferring results obtained for model organism to real bacteria consortia and from laboratory experiments with deionized water to effluents from sewage plants.

Key words | disinfection, *Enterococcus faecalis*, *Escherichia coli*, photocatalysis, wastewater

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INTRODUCTION

Provision of safe drinking water is nowadays a serious problem to be solved properly. In Southern Europe, the Middle East, Central Asia and Africa, water availability is limited and, due to the worldwide climate change, decreases, while in large parts of the northern hemisphere higher amounts of water will be available. Due to poverty and population growth in addition to decreasing water availability, developing countries are especially affected by safe drinking water shortage. However, problems are not only caused by water shortage but also by contamination. It is assumed that about 80–90% of water related diseases, such as diarrhoea, are caused by contamination with pathogenic microorganisms and represent a significant problem and cause of death.

Chlorine has been widely used as a water disinfection agent for more than one century now. During the last years, the advantages of efficient pathogenic microorganism inactivation and growth inhibition are put into perspective with human health because of the generation of, for example, trihalomethanes (THMs) and haloacetic acids (HAAs) by reaction with natural organic matter (NOM) in surface water and effluent organic matter (EfOM) in wastewater which have a high carcinogenic and mutagenic potential (Gopal *et al.* 2007; Yang *et al.* 2007). Ozone (O_3) treatment of water has proven to be an alternative to chlorination, only generating halogenated by-products if there are halogens present in the wastewater (Wert *et al.* 2007). Furthermore, ozone is relatively unstable and therefore has to be produced at its usage site, requiring installation of expensive production equipment. Moreover, because of the fact that ozone autodecomposes, there is a lack of residual disinfection effect. UV-Irradiation alters the structure of DNA so that the microorganism can no longer replicate. This method is still under consideration for drinking water provision (Madigan *et al.* 2003), but bacteria should be considered only as temporally inactivated (not death) due

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to the existence of DNA repairing mechanisms in the cells. To this account, there is a demand for the development of cheap disinfection technologies with low energy consumption (Herrera Melián *et al.* 2000; Madigan *et al.* 2003). Among the alternatives, heterogeneous photocatalysis has shown important advantages, such as the use of sunlight as cheap light source. Moreover, there is no need for additional hazardous chemical reactants, although the addition of some salts and acidic pH for example has shown to improve disinfection efficiency (Rincón & Pulgarín 2004). Photocatalysis has already been well investigated for the degradation of organic compounds, being its application for the disinfection of water by pathogenic microorganism inactivation of significant interest (McCullagh *et al.* 2007).

Photocatalysis is the topic of a wide range of heterogeneously catalyzed reactions that use radiation to activate the solid catalytic function. Irradiation is most commonly performed by UV light or natural sunlight that consists of about 5 to 7% of UV radiation, being the main disadvantage of the process the low quantum yield in the use of radiation (usually about 4%) There is a wide range of possible semiconductor catalysts, but among them, titanium dioxide (TiO_2) is the most usual, due to its high photo activity, lack of toxicity and low cost (Herrmann 2005; Benabbou *et al.* 2007). Irradiation leads to the formation of electron-hole pairs, which need to reach the semiconductor surface to contact the adsorbed species. The hole in the valence band generates radicals by oxidation of water or hydroxyl ions. The electron in the conduction band reduces oxygen to generate superoxide radical ions that can be partially protonated. The pollutant is then attacked by $OH\cdot$, $HO_2\cdot$ and $O_2^{-\cdot}$ radicals, commonly known as reactive oxygen species (ROS), although $HO_2\cdot$ and $O_2^{-\cdot}$ are very weak reactants, and consequently almost all of the reactivity of the ROS is related to the OH radicals. Overall, the reaction rate is determined by the rates of electron-hole pair formation, formation of ROS from adsorbed species and electron-hole recombination, in addition to the possible control of the adsorption and/or mass transfer from the bulk solution (Rincón & Pulgarín 2003; Sun *et al.* 2003; Adesina 2004; Herrmann 2005).

Until now, there is no exact understanding of the photocatalytic microorganism killing mechanism. Direct oxidation of intracellular components, disruption of the microorganism cell wall and disruption of the cytoplasm membrane are considered to be the most probable ones, all of them as consequence of different types of reactions since the ROS have a high and non-selective reactivity. According to Huang *et al.* (2000), a damage of the cell wall should lead to increase the accessibility of the cell membrane, followed by membrane damage and intrusion of titania particles and release of cell content. These authors found evidences for a couple of different mechanisms finally leading to cell death. Their permeability analysis of whole *E. coli* cells and spheroplasts and a their analysis of the lysed cells showed that first damages to outer membrane and peptidoglycan layer lead to a slow viability decrease and then result in faster viability decrease by disruption of the cell membrane. They also demonstrated the direct oxidation of enzymes.

Most of the photocatalytic disinfection research until now lay on the inactivation of *E. coli* as an indicator strain for faecal contaminations. Due to the fact that the cell wall is usually considered to be the initial point of attack, attention must be paid to the differences in the nature of pathogenic microorganisms' cell walls and, as a result, to possible differences in inactivation efficiency (Maness *et al.* 1999; Madigan *et al.* 2003). Prokaryotic microorganisms are divided into two groups by the setup of their cell wall: Gram(+) microorganisms have a thick cell wall of multiple layers of repetitive structures of *N*-acetyl-glucosamine and *N*-acetylmuraminic acid, and amino acids, called peptidoglycan or murein; Gram(−) microorganisms' cell walls are thinner but more complex. They consist of a thin peptidoglycan layer and an additional outer membrane consisting of polysaccharides and proteins in addition to phosphate lipids (Madigan *et al.* 2003).

The aim of this work was to elucidate possible differences in the photocatalytic inactivation behaviour between the most commonly investigated faecal indicator strain, the Gram(+) *E. coli*, and the less intensively studied Gram(−) *E. faecalis* in real wastewater treatment plant effluents.

METHODS

Lyophilized microorganisms were acquired from the CECT (*Colección Española de Cultivos Tipo*). The strains used in

the experiments were *Enterococcus faecalis* CECT 5142, corresponding to ATCC 11700 and *Escherichia coli* K12 CECT 4624, corresponding to ATCC 23631. *E. faecalis* was grown in Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) as liquid and solid culture media whereas Luria Bertani (LB) Broth and LB Agar were used for *E. coli*. For experiments, 100 μL of a mother culture in the stationary growing phase were added to 20 ml of fresh culture broth and incubated at 37°C for 24 hours on a rotary shaker.

Pure water (18 MΩ cm) was obtained from a *Millipore Milli-Q*® System with a filter pore size of 0.22 μm and distilled water feed. This water was directly used for reactions as possible contaminations were considered to be negligible in comparison with the inoculated microorganism cell number.

On the other hand, a different set of inactivation experiments were performed using as aqueous matrix the effluent of the wastewater treatment plant of the Rey Juan Carlos University in Móstoles (Spain). Prior to the experiments, the outgoing water from the biological treatment was treated by coagulation with aluminium sulfate to reduce turbidity below 10 NTU. To investigate the influence of variation in composition, the water used as matrix was taken from the plant at different days, with total organic carbon (TOC) values in the range 8–25 mg_C/L and conductivities in the range 450–750 μS/cm.

The photoreactor consist of a 1 litre glass vessel irradiated from one side at a distance of 8 cm by a *Radium* SupraBlack HBT 125 W UV-A bulb lamp which has its highest emission at 365 nm (Figure 1). The UV-A incident photon flow, determined by ferrioxalate actinometry, was 5.6×10^{-7} Einstein/L s. According to previous studies (Marugán *et al.* 2008), the maximum activity corresponding to an optimal light utilization can be achieved for a TiO_2 concentration of 0.1 g/L. The catalysts (Degussa P25 TiO_2) was used in slurry and maintained in suspension by magnetic stirring. All experiments were conducted at the natural pH value of the water without further adjustment.

To prepare the reaction suspension of approximately 10^6 CFU ml^{-1}, 5 ml of the microorganism liquid cultures were centrifuged for 15 minutes at 3,000 rpm, removing the liquid culture medium. After resuspension in autoclaved pure water, 1 ml of the microorganisms' suspension was added to water and made up to 1 L. The required amount of the photocatalyst (Degussa P25 TiO_2) was placed in the reactor before water with bacteria was added, followed by 15 minutes of stirring in the dark to homogenize the system before being exposed to UV light. Experiments with pure water were performed for 2 hours whereas experiments with secondary wastewater treatment effluents lasted 4 hours. Samples of 2 ml were taken at time intervals, quantifying the concentration of viable bacteria through a standard serial dilution procedure. Known volumes of each diluted solution were dropped on agar plates and incubated for 24 h at 37°C before counting.

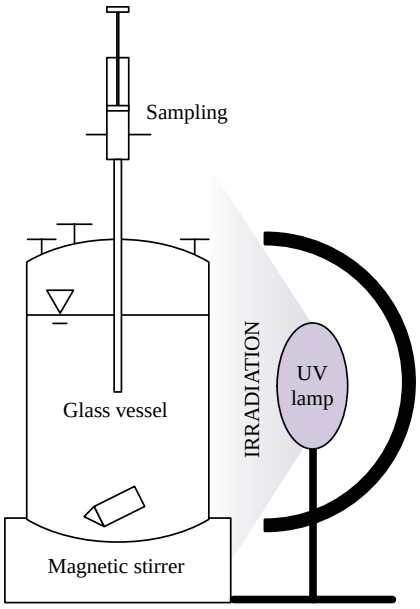

Figure 1 | Representation of the experimental setup.

RESULTS AND DISCUSSION

E. faecalis inactivation

Figure 2 shows the results of the bacteria inactivation profiles obtained in the experiments with effluents taken in three different days in comparison with the results in pure deionized water. Preliminary experiments of dark adsorption and UV disinfection without TiO_2 showed that both effects can be neglected in comparison with the TiO_2-based photocatalytic inactivation, in agreement with a previous work for *E. coli* inactivation (Marugán *et al.* 2008).

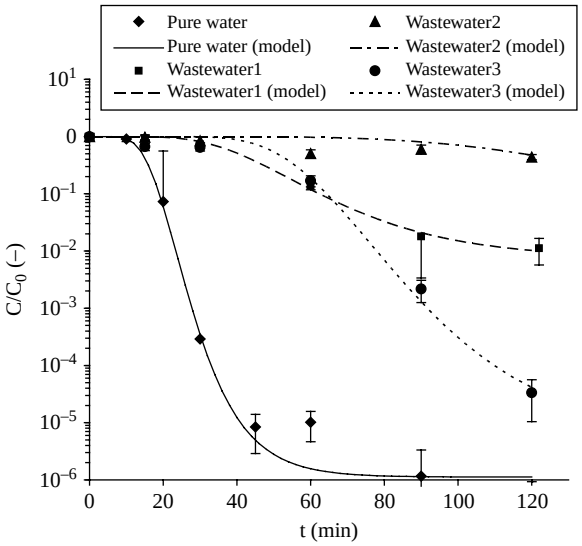

Figure 2 | Experimental data and modified Hom model fitting for the inactivation of *Enterococcus faecalis* in four different types of water (Catalyst concentration: 0.05 g TiO_2 L^{-1}). The 95% confidence level error bars have been estimated from the standard deviations of the cell numbers.

As it can be seen, bacteria inactivation strongly depends on the type of water. The composition of the effluent from the wastewater plant is different each few hours. It contains effluent organic matter (EfOM) as well as inorganic compounds with varying proportions that affects the overall inactivation behaviour. However, lower inactivation rates do not correlate with higher values of macroscopic parameters such as TOC or conductivity, indicating the strong importance of the specific compounds present in the water, in agreement with previous studies (Marugán *et al.* 2008).

In agreement with previous studies (Huang *et al.* 2000; Dunlop *et al.* 2002; Sun *et al.* 2003) inactivation curves show three different regions that cannot be described by the simple, but commonly used, first-order inactivation model derived from the Chick's Law. Instead, the modified Hom model has shown to be in good agreement with description of the initial shoulder and the ending tail of the experimental microorganism inactivation profiles (Cho *et al.* 2003). This model is given by Equation (1).

$$\log\left(\frac{C}{C_0}\right) = -k_1(1 - \exp(-k_2 t))^{k_3} \tag{1}$$

The three adjustable parameters of this equation have no physical meaning and cannot be related to specific inactivation curve characteristics. To this account, it is not useful for predictions and scale up, but it can be used for comparison among experiments performed under different conditions. For that reason, the maximum inactivation rate (V_{max}) and the time at which it is achieved (t_{max}) were calculated to make easier the quantitative comparison of inactivation experiments. V_{max} is computed from the expression of the first derivative of the model equation (Equation 1) at t_{max}, whereas t_{max} is calculated by making zero the second derivative. The analytic expression of both parameters is given by Equations (2) and (3) being summarized in Table 1 the values of the kinetic parameters of two replicates of the experiment of *E. faecalis* inactivation in pure water to show the reproducibility of the measurements.

$$V = \frac{\mathrm{d}\log(C/C_0)}{\mathrm{d}t} = \frac{-k_1 \cdot (1 - \mathrm{e}^{-k_2 t})^{k_3} \cdot k_3 \cdot k_2 \cdot \mathrm{e}^{-k_2 \cdot t}}{1 - \mathrm{e}^{-k_2 \cdot t}} \tag{2}$$

$$\therefore\ V_{max} = V(t_{max})$$

$$\frac{\mathrm{d}^2\log(C/C_0)}{\mathrm{d}t^2} = 0 \Rightarrow t_{max} = \frac{\ln(k_3)}{k_2} \tag{3}$$

From Figure 2, the length of the first lag region, the inactivation rate in the second region and the concentration of viable bacteria for high reaction times strongly depends on the type of water. For the wastewater plant effluent experiments, longer initial delays, lower inactivation rates and higher concentration of survival bacteria are generally observed, although the differences between each

Table 1 | Kinetic parameters of *E. faecalis* inactivation in pure water

Replicate	C_{cat} (g TiO_2 L^{-1})	k_1 (–)	k_2 (min^{-1})	k_3 (–)	V_{max} (min^{-1})	t_{max} (min)
1	0.05	5.95	0.102	11.4	0.234	23.9
2	0.05	5.71	0.135	8.02	0.303	15.4
Mean	0.05	5.83 ± 0.12	0.119 ± 0.016	9.77 ± 1.67	0.269 ± 0.49	19.7 ± 6.0

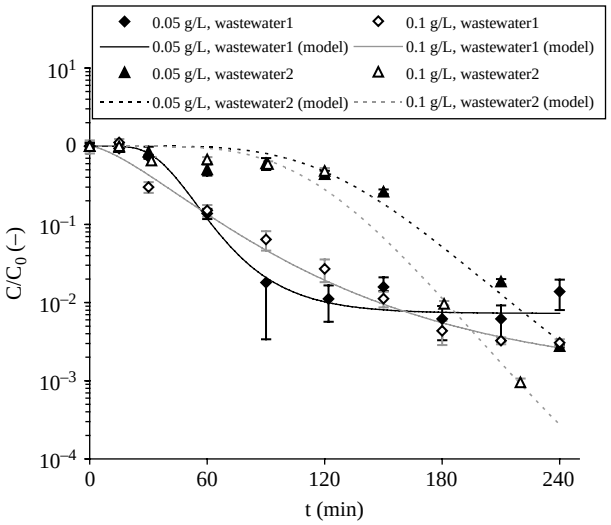

Figure 3 | Inactivation of *Enterococcus faecalis* for two different concentrations. Lines correspond to the modified Hom model fitting and error bars to the 95% confidence level.

experiment are quite significant. The longer initial region can be conveniently explained considering the competition between bacteria and EfOM for reaction with the photogenerated ROS (Rincón & Pulgarín 2004). As a result, the number of damages necessary for bacteria death is reached after a longer period of time. Slower decrease and therefore longer duration of the logarithmic inactivation phase and higher living cell density in the third phase can also be explained by the forenamed competition. Additionally, inorganic compounds can modify the catalyst and microorganism surfaces or reduce the availability of catalytic active sites for generation of ROS (Marugán *et al.* 2008). Moreover, the organic compounds released to the medium after the cell lysis can compete with the catalysts for the absorption of photons, reducing the efficiency of the process.

Figure 3 shows the comparison of *E. faecalis* inactivation for two different catalyst concentrations and two of the tested effluents, the values of the kinetic parameters being summarized in Table 2.

It can be noticed that similar results are obtained when increasing the concentration of TiO_2 from $0.05\,g\,L^{-1}$ to $0.1\,g\,L^{-1}$ in both effluents, with changes in the values of V_{max} and t_{max} not significantly different from the experimental error shown in Table 1. This effect is in agreement with previous results for the inactivation of bacteria in pure water using the same experimental device (Marugán *et al.* 2008), suggesting that the catalyst concentration in these range is not a limiting factor and that the disinfection activity cannot be improved without increasing the irradiation power.

Table 2 | Kinetic parameters of *E. faecalis* inactivation in secondary wastewater treatment effluent

Wastewater	C_{cat} (g TiO_2 L^{-1})	k_1 (–)	k_2 (min^{-1})	k_3 (–)	V_{max} (min^{-1})	t_{max} (min)
1	0.05	2.14	0.0403	8.96	0.034	54
1	0.10	2.99	0.0100	1.55	0.017	44
2	0.05	5.19	0.0100	7.72	0.020	204
2	0.10	6.97	0.0100	7.06	0.028	195

It is also worth noting that despite the differences in the duration of the initial lag region and the value of t_{max}, the concentration of surviving bacteria after sufficient time is quite similar for both effluents (and for both catalyst concentrations).

Comparison with *E. coli* inactivation

E. coli inactivation experiments in secondary treatment plant effluents were performed with comparison purposes. Results for experiments with $0.05\,g\,TiO_2\,L^{-1}$ are shown in Figure 4.

When compared to *E. faecalis* inactivation, it can be seen that *E. coli* seems to be more sensitive to the

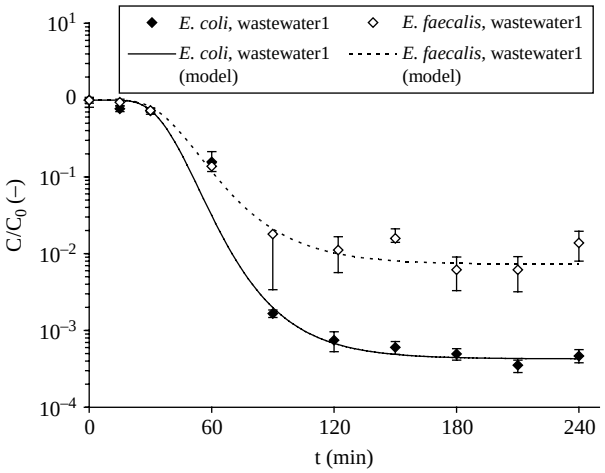

Figure 4 | Comparison between the photocatalytic inactivation of *Enterococcus faecalis* and *Escherichia coli* in secondary wastewater treatment effluents (TiO_2 concentration: $0.05\,g\,L^{-1}$). Lines correspond to the modified Hom model fitting and error bars to the 95% confidence level.

Table 3 | Kinetic parameters of *E. coli* inactivation

Water	C_{cat} (g TiO_2 L^{-1})	k_1 (–)	k_2 (min^{-1})	k_3 (–)	V_{max} (min^{-1})	t_{max} (min)
Pure water	0.05	4.81	0.104	4.36	0.208	14.2
Wastewater	0.05	3.37	0.0423	9.80	0.055	54

photocatalytic attack in the same secondary wastewater treatment effluent. Whereas the time required to reach maximum inactivation is the same, V_{max} is higher for *E. coli* (see Table 3). For long irradiation time, the fraction of surviving bacteria is 10 times higher for *E. faecalis* than for *E. coli*. However, it must be noticed that these differences should not be considered really significant considering the relative low reproducibility of the disinfection experiments and the similar values of V_{max} and t_{max} for *E. coli* (Table 3) and *E. faecalis* (Table 1) in pure water.

The different response to the photocatalytic treatment of both types of microorganisms is in agreement with other results reported in the literature (Liu & Yang 2003; Pal *et al.* 2007). The thick peptidoglycan layers of Gram(+) *E. faecalis* might have a higher affinity to the EfOM than the Gram(−) *E. coli* outer membrane. This would result in EfOM attachment to the bacteria outer surface protecting them from ROS attack after being degraded. Another possible explanation is that *Streptococci* can form capsules to protect themselves from harmful environmental conditions, what could be responsible of the higher resistance of *E. faecalis*.

CONCLUSIONS

Enterococcus faecalis has been successfully inactivated in aqueous suspensions of secondary wastewater treatment effluents by heterogeneous photocatalysis. However, bacterial inactivation profiles were found to be strongly influenced by water composition. The presence of EfOM in real effluents decrease the efficiency of the process when compared to pure water, but the extension of the inhibition is very dependent of the specific effluent. No correlation has been found between the inactivation efficiency and the macroscopic values of total organic carbon and conductivity of the effluents. Finally, it was found that *E. faecalis* seems to be less sensitive to the photocatalytic treatment than *E. coli*, what is probably related to their different cell wall structure. Consequently, attention must be paid using indicator strains for the prediction of disinfection results during the development of commercial water disinfection plants.

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