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TiO_2 Application for the Photocatalytical Inactivation of *S. enterica*, *E. coli* and *M. luteus* Bacteria Mixtures

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***Abstract* – Water contamination by various bacteria, viruses and other pathogens is a great threat to human health. Amongst other Advanced Oxidation Processes TiO_2 photocatalysis is considered as one of the most efficient treatment for the polluted wastewater disinfection. Usually, the wastewater produced by higher risk objects, such as hospitals, implicates diverse contaminants, but efficiency of most of the Advanced Oxidation Processes is tested by using only single pathogens and information on inactivation of bacteria mixtures is still limited. In this study, photocatalytical inactivation of three commonly found bacterial pathogens (gram-positive (*Micrococcus luteus*) and gram-negative (*Salmonella enterica, Escherichia coli*)) was investigated. Efficiency of traditional photocatalytic disinfection process using single bacterial pathogens was compared to the one observed for their mixtures. The impact of photocatalytical process parameters and treatment time on bacteria disinfection efficiency was studied. Photocatalytic disinfection efficiency testing with bacteria mixtures revealed, that in the presence of TiO_2 photocatalyst and UV irradiation tested gram-positive cells were inactivated slower than gram-negative cells. Another important finding was that an overall photocatalytic disinfection efficiency of bacteria mixtures is not a straight forward sum of inactivation rates of individually tested pathogens but has a strong relationship to the properties of their competitive growth.**

***Keywords* – Bacteria mixture; disinfection; *E. coli*; *M. luteus*; pathogens; photocatalysis; *S. enterica*; TiO_2**

Nomenclature		
AOP	Advanced oxidation processes	–
CFU	Colony forming unit	–
DP	Drop plate	–
LB	Luria Bertani	–
SP	Spread plate	–

1. INTRODUCTION

An adequate wastewater cleaning is equally important from the environmental and sanitarian points of view because wastewater cleaning also acts as the protecting measure

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from the spreading of various bacterial and viral diseases. The efficient treatment and disinfection of wastewater is especially important at hospitals, health centres, farms, slaughterhouses and other potentially higher risk objects where relatively large numbers of infected individuals can produce highly bio-contaminated wastewater with several types of viruses and/or bacteria [1], [2]. Usually, such objects are obliged to treat wastewater with strong chemicals (mostly chlorine based), UV sterilisers and/or antibiotics. Unfortunately, all of these traditional measures have large drawbacks. After treatment by antibiotics, water could be highly polluted by hardly biodegradable, toxic and bio-persistent xenobiotics [3]. Therefore, after treating wastewater with strong chemicals their residuals have to be somehow isolated and extracted. This increases operating cost and only transfers environmental pressure elsewhere. UV sterilisation in most cases is not 100 % efficient and the surviving bacteria and viruses reproduce their population [4]. Meanwhile, treatment with antibiotics is becoming highly undesirable due to the rising immunity of the treated species especially at such antibiotic rich users as hospitals, farms and others [3], [4]. Seeking for the efficient disinfection alternatives exponentially increasing number of scientists are focusing at the implementation of the Advanced Oxidation processes (AOP) which have a huge potential to efficiently remove both biological (virus, bacteria and other microorganisms) and industrial (dyes, pesticides, pharmaceutical etc.) contaminants [6]–[8]. Due to its relative simplicity and potentially low operation costs photocatalysis is one of the most attractive AOP. The advantage of the photocatalysis process is that it can completely mineralise recalcitrant pollutants into simpler compounds that are benign or can be processed by natural mechanisms to harmless constituents. Moreover, this method does not transfer the pollutant from one phase to another, as in a case of certain conventional treatment techniques such as adsorption but rather eliminates the target compound [6], [7].

The researchers showed that many materials can be successfully used as photocatalyst for wastewater disinfection, such as ZnO [9], Bi_2O_3 [10], TiO_2 [11], Fe_2O_3 [12], etc. Nevertheless, TiO_2 is still considering as one of the most promising photocatalysts due to its unique characteristics [13]. Moreover, its disinfection capacity was confirmed with various bacteria, viruses and other contaminants: *Escherichia coli* [14], *Salmonella enterica* [15], *Hepatitis B* [16], *Tordon 2.4-D herbicide* [17] and others. The potentially higher risk objects can produce wastewater with diverse contaminants, but majority of the research studies were performed by using one-type contaminant. This suggests that insufficient investigations were made in order to evaluate disinfection of diverse contaminants. Only few scientist groups have been researching wastewater with various type contaminants. Moreira *et al*. performed photocatalysis experiments with urban wastewater, which involves organic micropollutants, human pathogen indicators, antibiotic resistant bacteria and related genes [18]. They showed that TiO_2-P25 assisted with H_2O_2 is the most efficient process on the degradation of the chemical organic micropollutants. Tototzintle *et al*. treated *Pseudomonas aeruginosa* and *Bacillus subtilis* containing wastewater by UVA/TiO_2-P25/H_2O_2 photocatalysis [19]. It was observed that both P. *aeruginosa* and *B. subtilis* develop different resistance mechanism to photocatalytic disinfection. Presumably, this can limit the decomposition ability of specific contaminants when several types are implicated into wastewater. Zheng investigated possibility to decompose virus/bacteria system by Cu-TiO_2 nanofibers under visible light. The results showed that virus (*Bacteriophage f2*) exhibited stronger resistance to photocatalytic oxidation than *Escherichia coli* [20]. However, these and majority of other diverse contaminants containing wastewater disinfection experiments can be applied in specific conditions only. Also, there is still a lack of knowledge about disinfection of Gram-positive and Gram-negative containing suspensions.

Therefore, this study is focused on the analysis of gram-positive (*Micrococcus luteus*) and gram-negative cells (*Salmonella enterica, Escherichia coli*) containing water photocatalytic disinfection. Several influencing factors such as disinfection time and treatment conditions (UV only, TiO_2 only, and their combination) in single-type cells and their mixture were studied.

2. METHODOLOGY

In current study for the photocatalytical disinfection of biologically contaminated solutions we used combination P25 TiO_2 powders (99.5 % purity, obtained from Sigma-Aldrich) and 365 nm LED UV light source (M365LP1-C1, Thor labs). P25 TiO_2 powders were chosen for their well-documented physical and photocatalytical properties and credible comparison to the related studies. Efficiency of photocatalytic disinfection process was evaluated using spread plate (SP) technique and drop plate (DP) methods. Samples from the cuvettes were diluted 1000 or 4000 times in PBS buffer and about 750 cells were spread on LB agar Petri dish for spread plate method and formed colonies were counted manually. Drops of 10 µL of diluted samples were plated for DP method.

2.1. Bacteria Cultivation

Gram-negative *Salmonella enterica* ser. Typhimurium SL1344, obtained from prof. Séamus Fanning (Institute of Food and Health, University College Dublin, Ireland), *S. enterica* DS88 and *E. coli* DH5α cells were grown on Luria Bertani agar (LB-Agar, Roth, Karlsruhe, Germany) at 37 °C. For experiments the cells were grown in 10 mL sterile Luria Bertani (LB) medium (LB-medium Roth, Karlsruhe, Germany) in 30 mL tube, incubated with shaking at 220 rpm for 18–22 h. The overnight culture was diluted with fresh LB medium to optical density (OD_{600}) of 0.15 and grown until the OD_{600} of 0.8–1.0. The obtained bacterial suspension was centrifuged at 3000 g for 10 min at 4 °C (Heraeus™ Megafuge™ 16R, Thermo Scientific, Germany). The pellet was resuspended in 300 µL of PBS buffer, pH 7.4 (Roth, Karlsruhe, Germany). During the experiments bacterial suspension was kept on ice, not longer than 4 hours.

Gram-positive *Micrococcus luteus* cells were transferred from LB agar to 10 mL of sterile LB medium and incubated with shaking for 18–22 h at 30 °C. The overnight culture was diluted with fresh LB medium to give an initial OD_{600} of 0.15 and grown to OD_{600} of 0.8–1. The obtained suspension was centrifuged (Heraeus™ Megafuge™ 16R, Thermo Scientific, Germany) at 3000 g for 10 min at 16 °C. The pellet was resuspended in 300 µL PBS buffer. This bacterial suspension during the experiments was stored at room temperature not longer than 4 hours.

2.2. Optimization of Experimental Conditions

Prior to the photocatalytic disinfection distance between vessel and light source, dose of UVB light, and concentration of P25 TiO_2 powder were optimised. The goal of the optimisation was to determine specific set of parameters when UV light irradiation as well as TiO_2 powders alone (in the dark) would not severally affect the bacterial viability, i.e. acting individually neither UV nor TiO_2 should not reduce bacteria viability by more than 50–60 %. It was determined that suitable conditions are 10 cm distance from the light source, 2 mW/cm^2 irradiation, and 1 mg/mL of P25 TiO_2 concentration.

The experiments were performed using 15 ml volume of magnetically stirred bacterial suspension in PBS buffer (pH 7.4) incubated at 22 °C in thermostated vessel with, the suspension layer thickness of 1.5 cm. Before the experiment TiO_2 powders were stored in dark. During the experiment, PBS buffer, containing 1 mg/mL of TiO_2, was irradiated with UV light for 10 min before addition of bacteria. For each experiment the initial concentration of added *S. enterica* or *E. coli* cells was 2.7×10^6 cfu/mL; and for *M. luteus* cells concentration was 3×10^6 cfu/mL. Every 40 min of incubation samples were taken from the vessel and plated on LB agar. Plates were incubated for 18–22 hours at 37 °C and after incubation formed colonies were counted. In all cases, experiments were performed with UV irradiation and TiO_2 exposure separately and in a combination of these factors.

2.3. Statistical Analysis

The data was analysed for one-way ANOVA with Dunnett's method for comparison between control and treated groups statistical analysis at $a = 0.05$ significant level by using *Sigmaplot 12.5* software. The statistical analysis was carried out to determine the significant differences between the control groups without TiO_2 and UV effect and after photocatalytic disinfection. P values ≤ 0.05 were considered significant. The amount of colony forming units was indicated as means ± standard deviation.

3. RESULTS AND DISCUSSION

3.1. The Effect of Treatment on Gram-Negative Bacteria

3.1.1. TiO_2 Effect on E. coli Cells

The results obtained using spread plate technique with the gram-negative *E. coli* cells showed that under selected conditions both TiO_2 (Fig. 1c) and UV light (Fig. 1b) separately had a limited effect on bacterial viability.

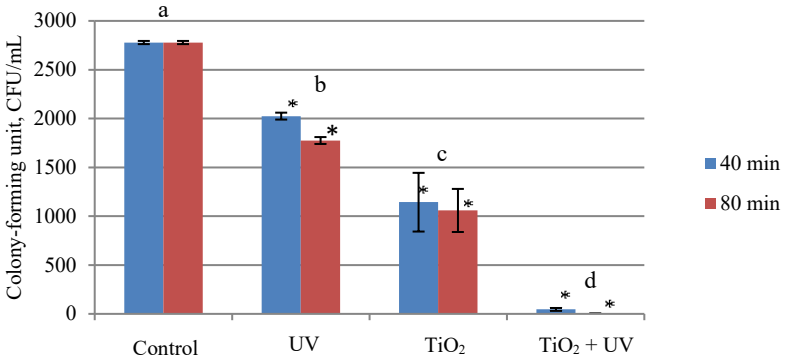


Fig. 1 Viability of *E. coli* cells assayed by spread plate technique ($n = 3$): a) negative control with not irradiated cells; b) effect of UV; c) effect of TiO_2; d) effect of TiO_2 + UV. The experiments were performed at 22 °C, the initial number of bacteria was 2.7×10^6 cfu/mL, dilution factor 1000. 1 mg/mL of TiO_2 was used and the intensity of UV irradiation was 2 mW/cm^2. *statistically significant difference compared to control group, using one-way ANOVA, $p < 0.05$.

On the other hand, the combination of these factors already after 40 min significantly reduced the number of formed colonies (Fig. 1d). The buffer with bacteria became sterile

after 80 min irradiation. It is also important to note that UV-exposed bacteria formed smaller colonies of variable size (Fig. 1b) compared to the control group (Fig. 1a).

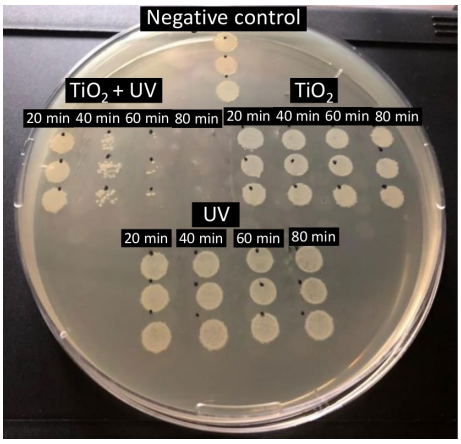


Fig. 2 The ability of *E. coli* to form colonies assayed by DP method. Obtained results after overnight incubation at 37 °C.

Similarly to SP, when using DP method (Fig. 2), we determined that individually TiO_2 and UV had almost no effect on *E. coli* viability. Whereas, after 40 min of treatment by TiO_2 + UV *E. coli* bacteria formed less colonies than the control cells and after 80 min colony formation was not observed at all.

3.1.1 TiO_2 Effect on S. enterica Cells

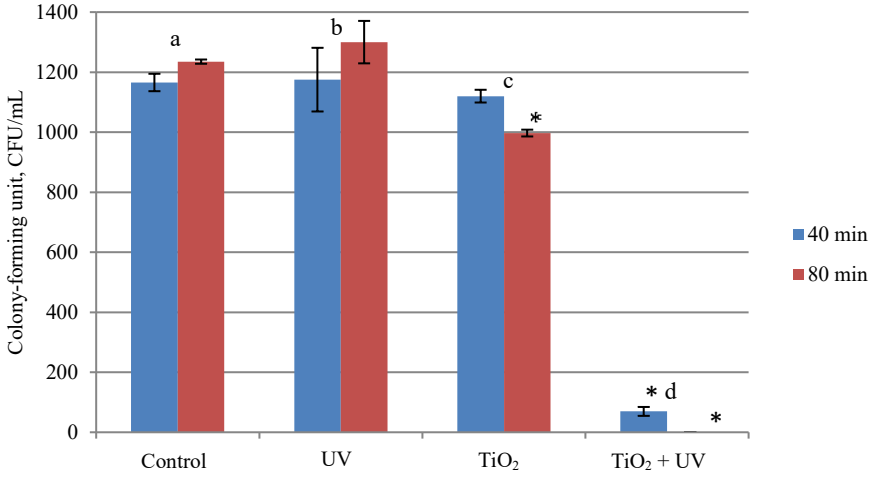


Fig. 3 Viability of *S. enterica* cells evaluated by SP method ($n = 3$): a) Negative control with none-irradiated cells; b) effect of TiO_2; c) effect of UV; d) effect of TiO_2 + UV. The experiments were performed at 22 °C, the initial amount of bacteria was 2.7×10^6 cfu/mL, dilution factor 1000. 1 mg/mL of TiO_2 was used and the intensity of UV irradiation was 2 mW/cm^2. *statistically significant difference compared to control group, using one way ANOVA, $p < 0.05$.

SP technique with the gram-negative *S. enterica* (Fig. 3) cells showed that under the selected conditions both, TiO_2 and UV light, used separately do not lower bacterial viability

(Fig. 3b) and Fig. 3c), but combination of these measures already after 40 min effectively reduced the number of formed colonies by more than 93 %. After 80 min of irradiation (Fig. 3d) the PBS buffer with bacteria did not became sterile but had only 0.25 % of the colonies formed at the negative control sample.

Using DP method, we also obtained qualitatively the same results – after 40 min and 80 min of treatment by only TiO_2 or UV *S. enterica* viability was not affected. Whereas after 40 min of treatment by TiO_2 + UV combination *S. enterica* bacteria was reduced significantly and after 80 min of combined effect no bacteria colonies were found. In general, these results indicate that gram-negative *E. coli* and *S. enterica* cells are similarly sensitive to the photocatalytic disinfection by TiO_2 + UV.

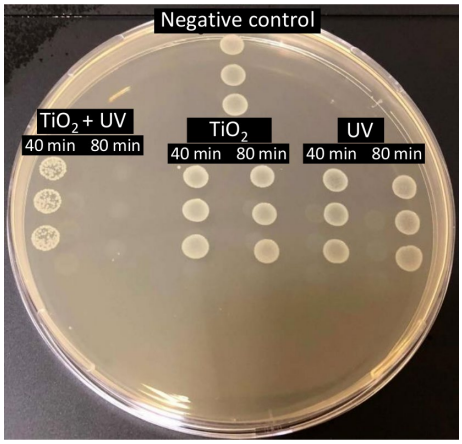


Fig. 4 The ability of *S. enterica* cells to form colonies determined by DP method. Obtained results after overnight incubation at 37 °C.

3.2. The Effect of Treatment on Gram-Positive Bacteria

3.2.1 TiO_2 Effect on Micrococcus luteus Cells

The results obtained with Gram-positive *M. luteus* bacteria showed that in comparison to the negative control group bacteria treatment by UV light alone (Fig. 5c) had a mediocre effect on their viability and after 80 min of radiation bacteria population was reduced by approximately 40 %. On the other hand, without UV radiation TiO_2 (Fig. 5b) had almost no effect on the viability of the cells. Interestingly, the combined treatment by TiO_2 and UV light (Fig. 5d) showed that after 40 min of treatment *M. luteus* were less affected than the investigated gram-negative bacteria but after 80 min *M. luteus* formation was completely absent.

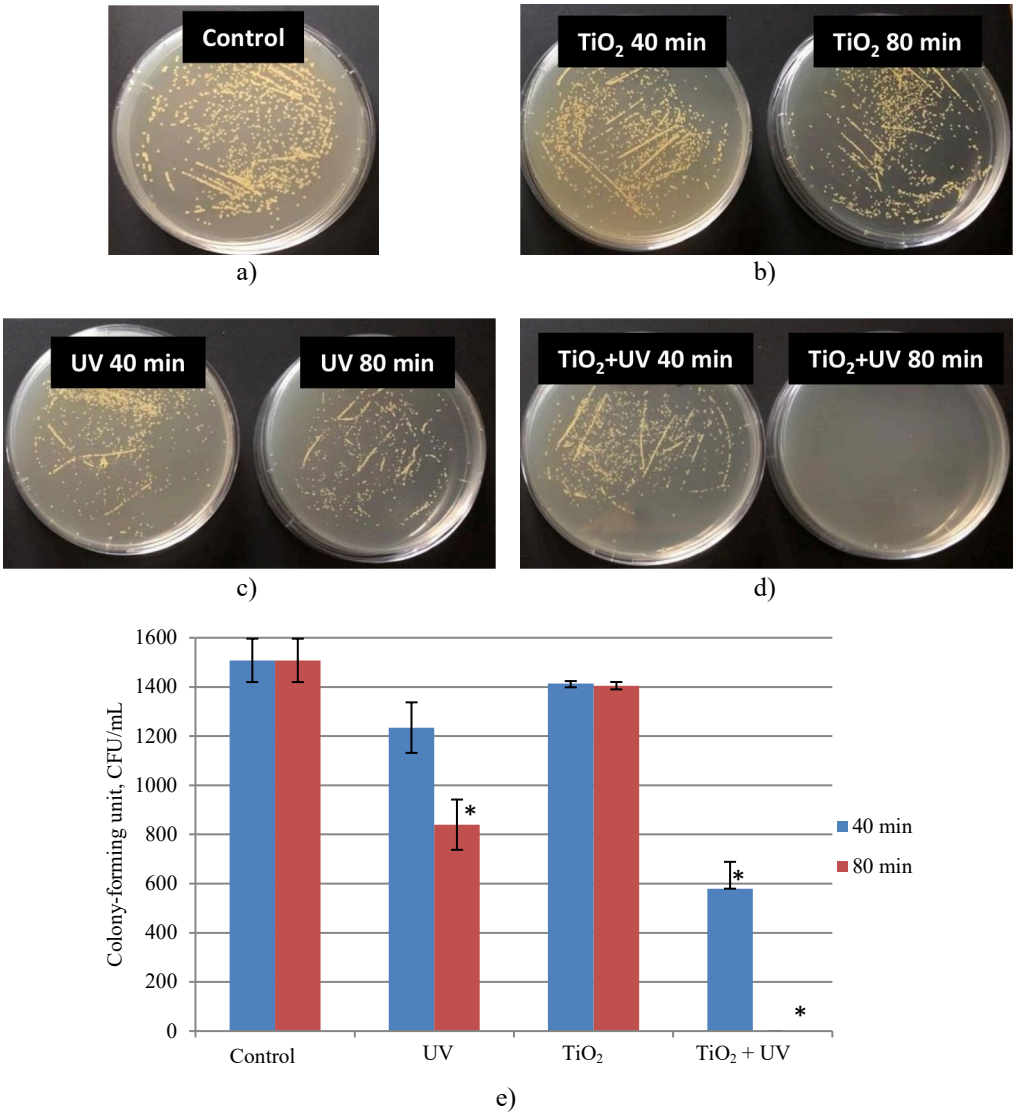


Fig. 5 Viability of *M. luteus* cells determined by SP method ($n = 3$): a) Negative control with none-irradiated cells; b) effect of TiO_2; c) effect of UV; d) effect of TiO_2 + UV. The experiments were performed at 22 °C, the initial amount of bacteria was 2.7×10^6 cfu/mL, dilution factor 1000. 1 mg/mL of TiO_2 was used and the intensity of UV irradiation was 2 mW/cm^2. *statistically significant difference compared to control group, using one-way ANOVA, $p < 0.05$.

Qualitative DP test confirmed that TiO_2 alone does not affect the viability of *M. luteus* cells. The colonies formed after 40 min of UV exposure were smaller compared to the negative control group and after 80 min their number was reduced significantly. Similarly, after 40 min of TiO_2 and UV *M. luteus* formed less and smaller colonies, whereas after 80 min of treatment *M. luteus* was unable to form colonies (Fig. 6). Altogether, the observed differences from gram-negative bacteria indicate that although *M. luteaus* is more sensitive to the UV light it also requires noticeably more time before bacteria became incapable to form colonies.

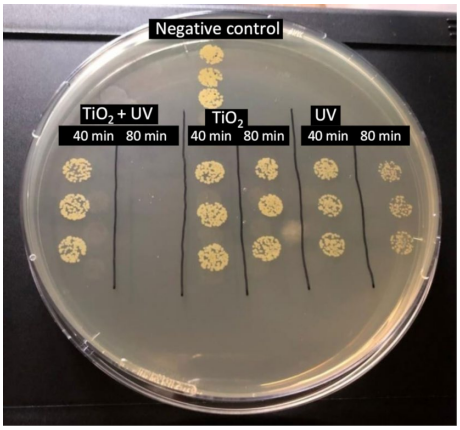
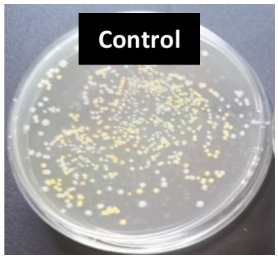


Fig. 6 *M. luteus* capability to form colonies determined by DP method. Obtained results after overnight incubation at 37 °C.

3.3. TiO_2 Effect on the Bacterial Mixtures

In order to evaluate the efficiency of bacterial mixtures disinfection by UV photocatalyzed TiO_2, experiments were performed with two types of samples containing cell mixtures: 1) *E. coli* with *M. luteus* (Fig. 7 and Fig. 8); and 2) *S. enterica* with *M. luteus* (Fig. 9 and Fig. 10). First of all, it should be noted that although initial concentrations of selected bacteria were the same as in the individual testing described above (concentrations of *S. enterica* and *E. coli* were 2.7×10^6 cfu/mL, of *M. luteus* – 3×10^6 cfu/mL), after being cultivated in mixtures their control samples had formed up to 2–7 times less colonies of each type of the bacteria. The observed competitive growth of the cells has resulted that both tested mixtures had significantly lower overall concentrations of the used pathogens.



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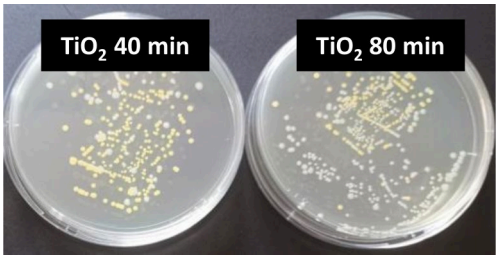

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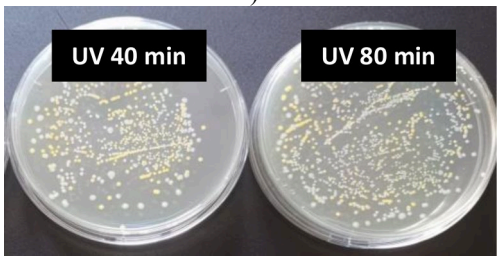

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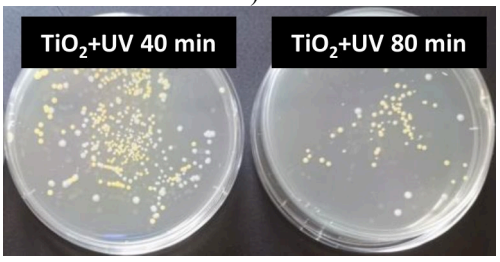
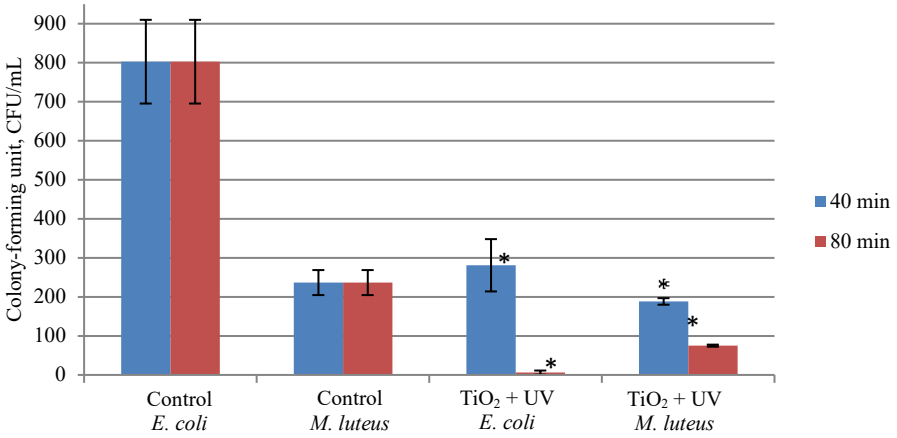


d)



e)

Fig. 7 Viability of *E. coli* and *M. luteus* (yellow) cells in mixture evaluated by SP method (n=3): a) negative control with none-treated cells; b) effect of TiO_2; c) effect of UV; d) effect of TiO_2 + UV. The experiments were performed at 22 °C, the initial amount of bacteria was 2.7×10^6 cfu/mL, dilution factor 1000. 1 mg/mL of TiO_2 was used and the intensity of UV irradiation was 2 mW/cm^2. *statistically significant difference compared to control group, using one-way ANOVA, $p < 0.05$.

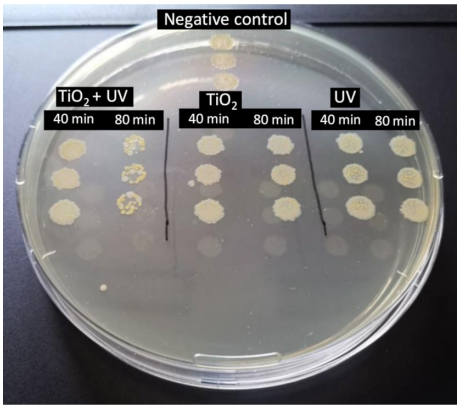


Fig. 8 The ability of *E. coli* and *M. luteus* cell mixture to form colonies assayed by DP method. Obtained results after overnight incubation at 37 °C.

The results obtained for *E. coli* with *M. luteus* bacteria mixture disinfection showed that acting separately, neither TiO_2 nor UV light did not have significantly different effect on bacteria in mixtures and cells formed colonies in generally the same proportional manner as in the control experiments with individual pathogens. However, then the mixtures were treated by combination of TiO_2 and UV light the disproportions became more evident. Individual viability of *E. coli* and *M. luteus* bacteria after 40 min of combined treatment decreased by 99.9 % and 62 %, respectively. After the same duration of their mixture treatment corresponding numbers were 64.9 % and 30.4 %. In comparison to the control group this represents approximately two times lower disinfection efficiency than by using

individual pathogens. Prolonged (80 min) combined treatment of *E. coli* with *M. luteus* bacteria mixture has reduced *E. coli* viability by 99.3 % but the final reduction *M. luteus* viability was just 68.3 %. The later value is particularly low considering that the corresponding viability reduction for both bacteria treated separately was 100 %. It could be that because of competition between these two types of bacteria, colonies of *M. luteus* form after *E. coli* stopped to form. So the photocatalytical effect on *M. luteus* is observed only after prolonged time of treatment.

In contrast, when the mixture of *S. enterica* and *M. luteus* cells was treated under the same conditions (80 min, combined TiO_2 and UV light) the viability of both pathogen cells has fallen to near 0 %. This result can be explained by the differing bacteria growth under competition and different reaction to TiO_2 and UV treatment. More specifically, one can notice that under the used conditions in control group *S. enterica* slightly stronger suppressed the growth of *M. luteus* and when *S. enterica* viability was not impaired, their whitish *Salmonella* colonies predominated. But *S. enterica* cells were more sensitive to the photocatalytic disinfection, therefore after 40 min of combined treatment they were growing much weaker and, as a result, yellowish colonies of more resistant *M. luteus* cells were formed and their number even surpassed the control sample (number of *M. luteus* colonies was 188 and 227 for control and 40 min treated samples, respectively). Nevertheless, the number of active *M. luteus* colonies was not as high the one for the individually tested cells and cumulative effect of TiO_2 and UV light was enough to stop their growing nearly completely (viability reduced by 97.9 %).

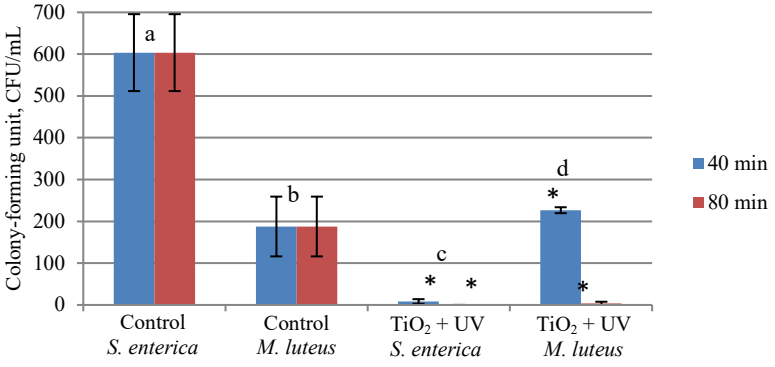


Fig. 9 Viability of *S. enterica* and *M. luteus* cells in mixture evaluated by SP method (n=3): a) negative control with none-treated cells; b) effect of TiO_2; c) effect of UV; d) effect of TiO_2 + UV. The experiments were performed at 22 °C, the initial amount of bacteria was 2.7×10^6 cfu/mL, dilution factor 1000. 1 mg/mL of TiO_2 was used and the intensity of UV irradiation was 2 mW/cm^2. *statistically significant difference compared to control group, using one-way ANOVA, $p < 0.05$.

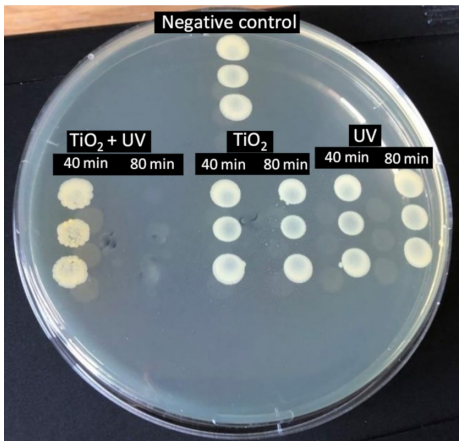


Fig. 10 The ability of mixture of *S. enterica* and *M. luteus* cells to form colonies assayed by DP method. Obtained results after overnight incubation at 37 °C.

4. CONCLUSIONS

In the current study we investigated photocatalytical inactivation of gram-positive bacterium *M. luteus*, gram-negative bacteria *S. enterica* and *E. coli* as well as their mixtures. Under the applied conditions, it was determined that *M. luteus* cells were more resistant to treatment than the other tested bacteria. Still, when treated individually, 80 min of photocatalytic processing (by P25 TiO_2 powders and UV radiation) was enough to inactivate all three types of them. However, the efficiency of the same photocatalytic treatment applied for *E. coli* with *M. luteus*, and *S. enterica* with *M. luteus* bacteria mixtures was not as efficient. This was rather surprising because due to the competitive colony formation the total number of individual bacteria colonies formed by control samples from mixtures was 2–7.5 times smaller than the ones achieved by individually grown bacteria. Furthermore, it was determined that each of the used bacteria had different sensitivity to the treatment by P25 TiO_2 powders and UV radiation. Varying sensitivity to treatment in combination with competitive growth resulted that overall efficiency of photocatalytical inactivation of bacteria mixtures was not a linear aggregate of the corresponding inactivation rates of individually treated bacteria. This means that in practical systems with mixed biological contamination it is advised that the final quality of the photocatalytically cleaned (disinfected) water should not be assessed by the straight forward extrapolation but it should be tested for each type of the pathogen individually.

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