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Influence of the Metabolic Activity of Microorganisms on Disinfection Efficiency of the Visible Light and P25 TiO_2 Photocatalyst

Sandra Sakalauskaite [1,2,*], Deimante Vasiliauske [1,2], Emilija Demikyte [1,2], Rimantas Daugelavicius [1,2] and Martynas Lelis [2]

[1] Faculty of Natural Sciences, Vytautas Magnus University, 8 Vileikos St., 44404 Kaunas, Lithuania; deimante.galalyte@alumni.vdu.lt (D.V.); emilija.demikyte@alumni.vdu.lt (E.D.); rimantas.daugelavicius@vdu.lt (R.D.)

[2] Centre for Hydrogen Energy Technologies, Lithuanian Energy Institute, 3 Breslaujos St., 44403 Kaunas, Lithuania; martynas.lelis@lei.lt

* Correspondence: sandra.sakalauskaite@vdu.lt; Tel.: +370-69646691

Abstract: The beneficial photocatalytic properties of UV light activated TiO_2 powder are well-known and have been demonstrated with various pollutants and pathogens. However, traditionally observed photocatalytic activity of visible light activated pristine TiO_2 is insignificant but there are a few studies which have reported that under some specific conditions commercially available TiO_2 powder could at least partially disinfect microorganisms even under visible light. To better understand this phenomenon, in the current study we focused on bacteria response to the treatment by visible light and P25 TiO_2 powder. More specifically, we analyzed the relationship between the bacteria viability, outer membrane permeability, metabolism, and its capacity to generate intracellular reactive oxygen species. During the study we assayed the viability of treated bacteria by the spread plate technique and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method. Changes in bacterial outer membrane permeability were determined by measuring the fluorescence of N-phenyl-1-naphthylamine (NPN). To detect intracellular reactive oxygen species formation, the fluorescence of dichlorodihydrofluorescein diacetate (DCFH-DA) was assayed. Results of our study indicated that TiO_2 and wide spectrum visible light irradiation damaged the integrity of the outer membrane and caused oxidative stress in the metabolizing bacteria. When favorable conditions were created, these effects added up and unexpectedly high bacterial inactivation was achieved.

Keywords: *S.* Typhimurium; bacteriophages; metabolic activity; disinfection; reactive oxygen species; oxidative stress; photocatalysis; TiO_2; visible light

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1. Introduction

Accumulating scientific reports and increasing weather extremes have forced society to recognize man made climate change [1]. Currently this issue is attracting most of the public attention, but it is not the only environmental problem causing big concern. According to UNICEF and WHO [2], almost one third of the global population has a deficiency of safe drinking water. In some areas the problem is the absence of fresh water sources, but in other regions it is related to water contamination by various pollutants including bacteria, viruses, and other pathogens. The necessity to clean the contaminated water and to prevent polluted wastewater release to the environment requires a new generation of efficient and environmentally friendly water cleaning and disinfection technologies.

Advanced oxidation processes (AOP) rely on in situ generation of strong but short-lived oxidants and are seen as a potential solution in dealing with both biological and chemical contamination [3]. Photocatalytic water treatment is arguably the most widely investigated AOP method. With this method a photocatalyst (mainly comprised of a

semiconductor material with a specific electron level structure) is irradiated by intensive ultraviolet (UV) or visible (vis) light. When valence band electrons absorb light, they are excited up to the conduction band and electron-hole pairs are produced. In contact with water molecules and dissolved oxygen, excited charges generate reactive oxygen species (ROS), that are universal oxidants and can neutralize various contaminants to form neutral compounds [4]. In comparison to the traditional wastewater treatment methods like UV irradiation and chlorination, photocatalytic water treatment has superior oxidation power [5], does not form carcinogenic disinfection by-products [6], and can be used for the treatment of pathogens which are resistant to chlorine [7].

Looking at the specific materials which have been used for photocatalytic water treatment, TiO_2—and its modifications—stands out as the most important representative. Its photocatalytic properties were discovered nearly a hundred years ago, but significant interest in their practical application was inspired only after the report by Fujishima and Honda [8]. Since this pioneering work, TiO_2 has been used for both, photocatalytic oxidation of inorganic pollutants, and for inactivation of microorganisms (bacteria, fungi, and viruses) [9,10]. However, TiO_2 application in practice has encountered several challenges. First, TiO_2 has a band gap of approximately 3.2 eV, therefore it is active under UV light, but its response to visible light is limited. Although significant progress has been achieved, many research groups are still trying to improve the photocatalytic properties of visible light activated TiO_2. Currently, this challenge is being solved by applying dopants, dye sensitization, and other methods [11].

The second challenge is more complicated and is related to the discrepancies between how photocatalytic treatment neutralizes inorganic pollutants, and how it deals with microbiological contamination. In general, it is considered, that the main factor of any photocatalytic water treatment process is the formation of ROS, namely, hydrogen peroxide (H_2O_2), superoxide ($O2\bullet-$), and hydroxide ($OH\bullet$) radicals. In solution ROS are expected to act as strong oxidizing agents and fully oxidize harmful inorganic compounds, mineralize organic molecules, or cause fatal damage to the microbial membranes, proteins, and nucleic acids [12]. However, there is a fundamental difference in how strong oxidants react with various inorganic compounds and how they affect live microorganisms which can actively respond to the external oxidation.

For example, deeper analysis of the photocatalytic process showed that under some conditions activated TiO_2 can produce short-lived singlet oxygen which is a strong oxidant and can significantly contribute to the efficiency of photocatalysis. However, diffusion of short-lived radicals into the cells is weak, therefore the singlet oxygen effect for the viability of bacteria is small [13]. On the other hand, there are reports which show that at a TiO_2 surface, short-lived ROS can be transformed into long-lived radicals [14]. Subsequently. long-lived superoxide, hydroxide, and hydrogen peroxide radicals can cause damage to the bacterial membranes [15]. Other reports [16–18] showed that rupture of the plasma membrane can occur after cell treatment with nano-TiO_2. If such damage occurs, electron transport along the respiratory chain is impaired and generation of intracellular ROS is triggered [18]. At a first glance, all these factors should increase the efficiency of photocatalytic disinfection, but bacteria are constantly adapting to their environment and have many mechanisms to enable them to survive under oxidative stress conditions. For instance, some bacteria have well developed antioxidant systems which have been comprehensively reviewed in refs. [19,20]. Different bacteria transcription factors, such as OxyR, PerR, OhrR, and SoxRS can be activated by direct oxidation of their sensor proteins, and then can adjust the bacterial response appropriately [21]. Moreover, bacteria with and without specific antioxidant systems, can reduce the suffered oxidative damage by remodeling their metabolism [21]. Altogether this means that photocatalytic disinfection efficiency of microorganisms depends on at least the following three factors: photocatalyst properties, irradiation characteristics, and specific microorganism response to the oxidative stress. Frequently, the integral affected will not be the linear sum of separate factors [22]. Therefore, the extrapolation of photocatalysis efficiency estimations from relatively simple

tests, like methylene blue or rhodamine B solution bleaching, to much more complex objects like bacteria can be misleading.

The first demonstration of the antimicrobial properties of UV activated TiO_2 was presented in 1985 [7]. Over the years many more studies of photocatalytic disinfection by UV light activated TiO_2 have been reported. More recently, researchers started to focus on visible light activated photocatalysis with modified TiO_2, which has a narrower band gap and is capable to efficiently generating ROS under visible light [23,24]. At the same time, some researchers reported that visible light [25] and pristine TiO_2 [26], as separate factors, affected the viability of bacteria. Still, the number of studies on bacteria inactivation by the combination of pristine TiO_2 and visible light remains scarce [27,28].

Our recent study [29] showed that under certain conditions the combination of gram-negative *Salmonella enterica* bacteria, visible light, and pristine P25 TiO_2 photocatalyst can provide an unexpectedly high disinfection efficiency, although under the same conditions photocatalytic bleaching of methylene blue solution was found to be negligible. Photocatalytic disinfection of bacteria mixtures by UV light activated P25 TiO_2 also indicated interesting behavior of the competing bacteria cells and showed a non-linear response [30]. To better understand the mechanisms behind the observed results, we performed in depth analysis of the role of bacteria metabolism in its response to the oxidative stress caused by pristine P25 TiO_2 photocatalysts and visible light. By conducting various experiments, we demonstrated that in metabolizing bacteria. TiO_2 and visible light affected the integrity of the bacterial outer membrane and caused internal oxidative stress. When acting together, the effect of these factors is strengthened and an effective inactivation of bacteria can be achieved.

2. Results

2.1. Effect of TiO_2 and Irradiation on the Viability and the Outer Membrane Permeability of S. Typhimurium Cells

In order to evaluate the effects of combined light and activated TiO_2 treatment on bacteria, initially we determined the individual effects of these factors on *S.* Typhimurium cell viability and its outer membrane (OM) permeability. Measurements were carried out with two sets of conditions: the cells were (i) irradiated with light, but without TiO_2, or (ii) in the dark treated with two different concentrations of TiO.

Results of bacteria plating on agar showed, that the capability of the cells to form colonies after 1 h of UV irradiation decreased by 20%, after interaction with 0.3 mg/L of TiO_2 by 30%, and 1 mg/L of TiO_2 decreased the cell viability by 40%. Figure 1a demonstrates that visible light had no significant effect on the viability of *S.* Typhimurium cells.

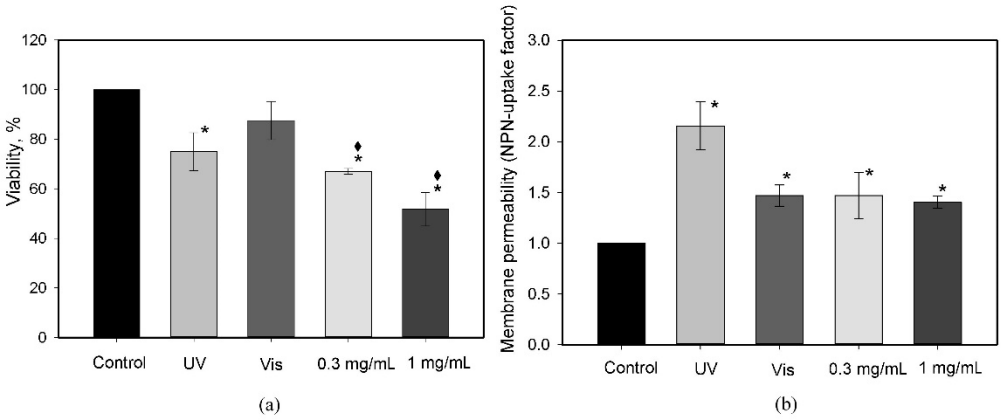

Figure 1. Effects of light and TiO_2 on *S.* Typhimurium cell: (**a**) viability; (**b**) outer membrane permeability. The experiments were performed at 37 °C in PBS buffer (pH 7.4), containing 0.1% of glucose. The initial *S.* Typhimurium cell concentration was 3×10^7 cfu/mL. Concentrations of TiO_2 are indicated in the figure. The cells were incubated for 60 min, the intensities of UV and Vis light were 30 and 40 mW/cm^2 respectively. * and ♦ mark experimental values which are significantly different from the control sample and other samples respectively (more details on statistical analysis are provided in Section 4.5).

N-phenyl-1-naphthylamine (NPN) is a hydrophobic fluorescent probe. The fluorescence of 1-NPN is significantly enhanced in a hydrophobic environment (e.g., membrane lipid bilayer), rendering it an appropriate dye to probe OM integrity of gram-negative bacteria. It is known [31] that TiO_2 can affect the integrity of bacteria OM, so the cell wall permeabilization of bacteria was assayed by the cellular uptake of this compound. From Figure 1b it is clear, that UV light increased by more than twice the NPN binding to the cells, meanwhile visible light or incubation with TiO_2 was only increased by half of that. Determining the capacity of this compound to damage the bacteria, TiO_2 concentrations of 1 mg/mL and 0.3 mg/mL were compared, but no significant difference was observed. NPN fluorescence in suspension of UV-treated cells was the highest among all tested samples. These results show that UV radiation permeabilizes the outer membrane stronger than TiO_2, but TiO_2 affects the cell viability more than the permeability of the bacteria envelope.

2.2. MTT Reduction

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay was used for the estimation of metabolic activity of the viable cells. The test is based on the enzymatic reduction of the lightly colored tetrazolium salt to intense purple-blue color formazan, which can be quantified spectrophotometrically. Under properly optimized conditions, the obtained absorbance value is directly proportional to the number of viable cells [32]. Usually, the MTT assay is used in eukaryotic cell lines, however it has recently become increasingly employed to determine the viability of bacteria and fungi. In bacteria, the conversion of MTT into formazan crystals is catalyzed by several bacterial dehydrogenases, mostly located in the respiratory chain. In other words, the ability to convert MTT into formazan shows the efficiency of the cellular electron transport in bacteria [33].

The ability to convert MTT into formazan directly depends on the activity of respiration and correlates with the viability of cells [32]. Results, presented in Figure 2, show that MTT reduction increased by approximately 20% during the experiment in a control sample in the cuvette without external factors. UV irradiation had no significant effect on the reduction of accumulated MTT, while after exposure to visible light it was 20% lower compared with the control. TiO_2 alone decreased the ability of cells to convert MTT to formazan by almost 30%. Combination of TiO_2 with visible light or UV irradiation reduced MTT reduction below 20%.

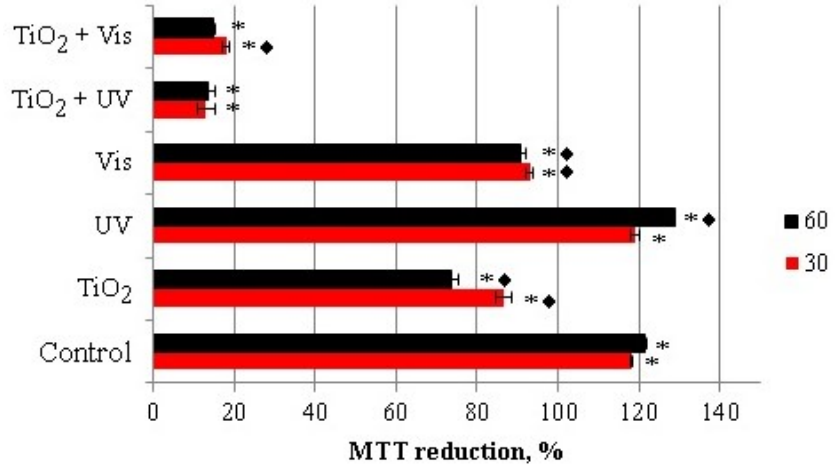

Figure 2. MTT reduction in *S.* Typhimurium cells. The experiments were performed at 37 °C in PBS buffer, pH 7.4, containing 0.1% glucose. The cell concentration was 3×10^7 cfu/mL. TiO_2 was added to 0.3 mg/L. Duration of the treatment was 30 min and 60 min. * and ♦ mark experimental values which are significantly different from the control sample and other samples respectively (more details on statistical analysis are provided in Section 4.5).

2.3. *Effects of Cell Metabolic Activity on Bactericidal Properties of TiO_2*

Results of our previous studies [29,34] showed, that after *S.* Typhimurium treatment by UV activated TiO_2, high intracellular ROS levels are formed. Potentially, this also could be one of the reasons why bacteria viability decreases after treatment by visible light and TiO_2. Therefore, in the following experiments, changes in OM permeability and intracellular ROS level were studied with varying parameters, which affect the intracellular ROS formation rate.

2.3.1. Viability of Bacteria

The bactericidal properties of TiO_2 photocatalyst in the presence (or absence) of UV/visible light and glucose were measured at 22 °C and 37 °C. The obtained results (Figure 3) revealed that in comparison to the control group, *S.* Typhimurium bacteria incubation at 22 °C with TiO_2 (without glucose) increased the viability of cells by 18%. With glucose the increase in cell viability was lower and reached 10%. Whereas, after incubation without and with glucose at 37 °C, the viability of the cells was decreased by approximately 10% and 30%, respectively (Figure 3b).

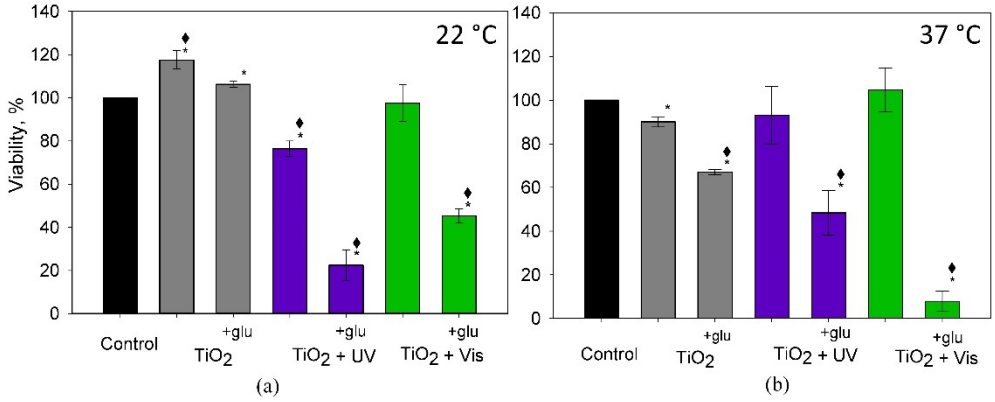

Figure 3. The impact of incubation medium temperature, (**a**) 22 °C; (**b**) 37 °C, on bactericidal properties of TiO_2. Experiments were performed in PBS buffer, pH 7.4, without and with (+glu) the addition of 0.1% glucose. The initial concentrations of *S.* Typhimurium cells and TiO_2 photocatalyst were 3×10^7 cfu/mL and 0.3 mg/mL, respectively. * and ♦ mark experimental values which are significantly different from the control sample and other samples respectively (more details on statistical analysis are provided in Section 4.5).

At 22 °C TiO_2 irradiation by UV light significantly reduced the ability of bacteria to form colonies. In the presence of glucose, the drop in cell viability was 80%, without the glucose it was 22%. Meanwhile, at 37 °C the reduction of cell viability by the combined TiO_2 and UV treatment was considerably smaller. As expected from a photocatalyst which is generally not active under visible light, without the glucose at both tested temperatures, treatment by visible light irradiated TiO_2 did not change the viability of the bacteria cells. However, addition of glucose changed the situation completely and strong reduction in cell viability was observed. At 22 °C the viability reduction was 50%, whereas at 37 °C the viability was reduced by 93% which was even larger than under UV light.

2.3.2. Permeability of the Outer Membrane

Figure 4 indicates that, at 22 °C in the presence of glucose, a slight increase in the outer membrane permeability was detected only in the samples with UV irradiated TiO_2. The effect of glucose was stronger at 37 °C. At this temperature, after bacteria treatment by TiO_2 and UV light, the NPN uptake factor increased by up to 1.5. However, it did not react to the presence of glucose. The dependency of glucose was observed when TiO_2 was irradiated by visible light. In the absence of glucose, the NPN accumulation was the same as in the control sample, but it increased to 3 after the addition of glucose. These results agree with

previous results on *S.* Typhimurium cell viability (Figure 3). The increase in permeability of the outer membrane for the combination of visible light and glucose suggests that the energy metabolism of the cells could be the factor strengthening the antibacterial efficiency of TiO_2. On the other hand, the outer membrane permeability was not sensitive to glucose when the cells were irradiated by UV light. These results indicate different mechanisms of bacterial inactivation by UV and visible light.

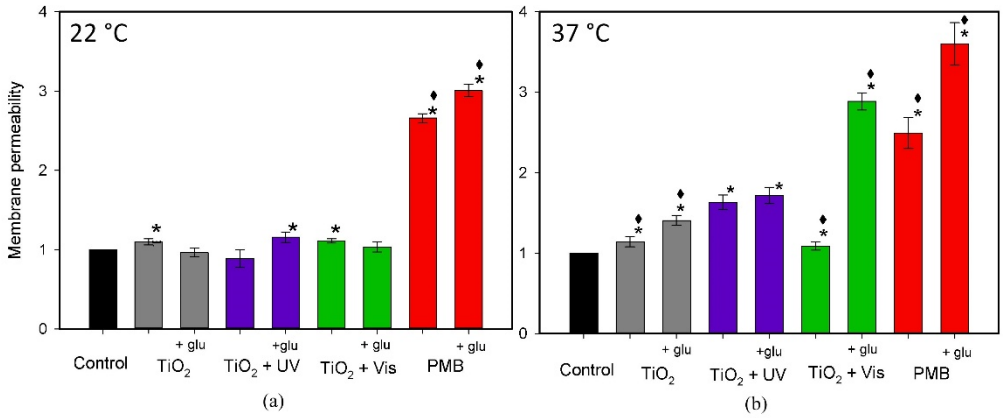

Figure 4. Influence of the incubation temperature and the irradiation source on the outer membrane permeabilizing activity of TiO_2: (**a**) 22 °C and (**b**) 37 °C. The experiments were performed in PBS buffer, pH 7.4, with/without 0.1% glucose. Concentration of *S.* Typhimurium cells was 3×10^7 cfu/mL, and concentration of TiO_2 was 0.3 mg/L. * and ♦ mark experimental values which are significantly different from the control sample and other samples respectively (more details on statistical analysis are provided in Section 4.5).

2.4. Intracellular ROS Generation in S. Typhimurium

It is generally accepted, that the bactericidal activity of TiO_2 is based on the production of ROS in the incubation medium, causing oxidative stress to the bacteria [7]. After registration of the unexpectedly high cell inactivation efficiency of TiO_2, irradiated by visible light, we decided to assay the intracellular ROS concentration, measuring the intensity of dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence. ROS, including hydrogen peroxide, are produced during aerobic growth and are toxic to bacterial cells [35]. To determine, if intracellular ROS are the cause of the high efficiency of visible light activated TiO_2, we chose bacterial strains, expressing the antioxidant system at different intensities. Bacteriophages, as nonmetabolizing biological agents were used for the control.

2.4.1. Concentration of ROS in Oxidative Stress Resistant *S.* Typhimurium Cells

At conditions optimal for *S.* Typhimurium growth (i.e., glucose containing medium at 37 °C), the bacterial antioxidant system is active and can reduce ROS concentration. Meanwhile, at lower temperatures or in the absence of glucose, processes which protect bacteria from oxidative stress are very slow. Under these conditions the intensity of respiration is lower and generation of ROS should be decreased. The results of experiments, in Figure 5a,c, show that ROS levels, were higher in samples without glucose, where the intensity of respiration should be lower. In addition, the intensity of DCFH-DA fluorescence was the highest in samples with TiO_2, and UV activated TiO_2. Samples with Vis light activated TiO_2 showed increased fluorescence compared to the control samples. It was found that temperature had no effect on the intensity of the fluorescence in samples without glucose. However, the addition of glucose fundamentally changed the picture (Figure 5b,d). At 22 °C the fluorescence considerably increased when glucose was added to the samples with visible light activated TiO_2 and decreased in the samples activated by UV. At conditions optimal for *S.* Typhimurium growth (37 °C, with glucose), ROS levels were lower than in other samples. The increased intensity of fluorescence in the sample

with visible light activated TiO_2 was obtained only with an addition of glucose. On the other hand, fluorescence in the sample with UV activated TiO_2 decreased.

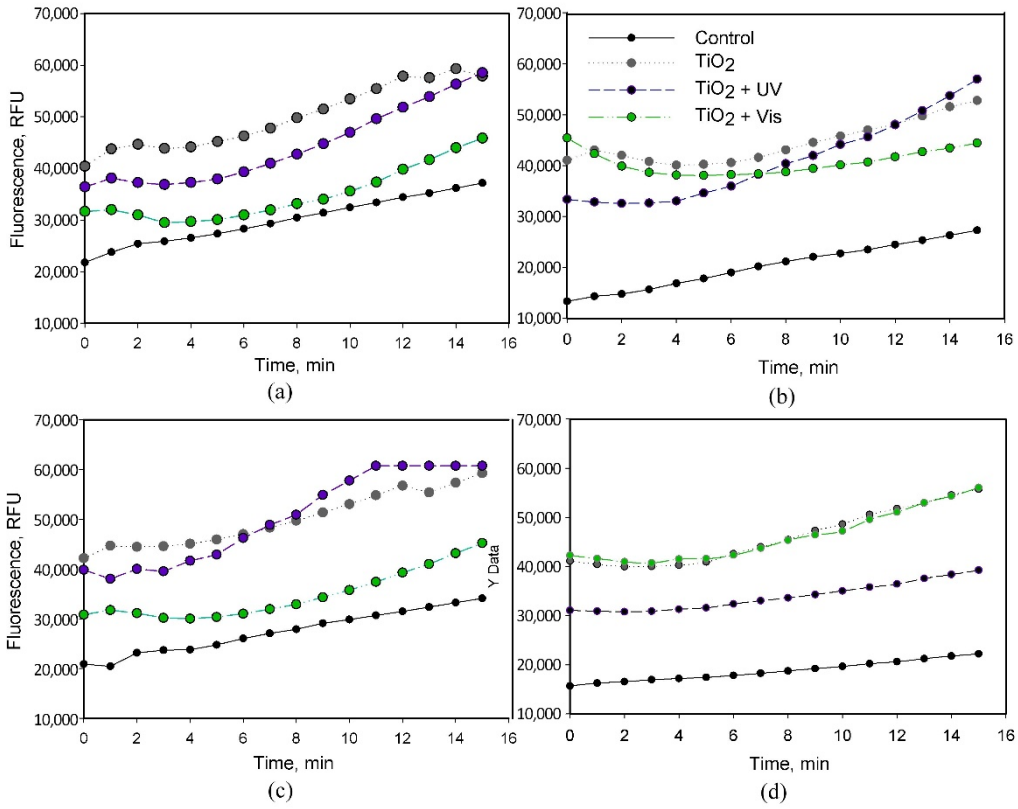

Figure 5. Intracellular ROS generation in *S.* Typhimurium cells. The experiments were performed at: (**a**,**b**) 22 °C or (**c**,**d**) 37 °C; (**a**,**c**) in absence of glucose or (**b**,**d**) in the medium containing 0.1% glucose. One hundred μL of concentrated cell suspension (OD_{600} of 0.1) contained 10 μM DCFH-DA. The excitation wave was λ_{ex} = 492 nm, and emission λ_{em} = 535 nm.

It should be mentioned, that TiO_2 induces very high levels of intracellular ROS, but the cell viability and the outer membrane permeability remain only moderately impaired when compared to the other factors we studied. This could be due to activation of the antioxidant system inside the cells which neutralizes ROS. To examine this possibility more carefully, we performed experiments with *S.* Typhimurium ΔTolC cells that have a less active antioxidant system [36] and Escherichia coli cells, which are less resistant to oxidative stress compared to Salmonella.

2.4.2. Effects of Visible Light Activated TiO_2 on Oxidative Stress Sensitive Bacteria

TolC is an important protein channel located in the bacterial outer membrane which participates in drug resistance of *S.* Typhimurium and *E. coli* cells. The deficiency of TolC leads to increased sensitivity of a wide range of molecules, including bile salts, detergents, and antibiotics. It is known [36], that TolC is involved in protection against oxidative stress in *S.* Typhimurium. Accordingly, ΔTolC mutant cells (i.e., TolC-deficient bacteria strains) should be more sensitive to oxidative stress compared to wild types. To determine, whether the intracellular ROS can be considered as the key factor behind the unexpectedly high efficiency of treatment by visible light irradiated TiO_2, we repeated the tests (viability, outer membrane permeability and the intracellular ROS level assessment after irradiation of TiO_2) with *S.* Typhimurium ΔTolC mutant cells and compared it to the wild type of *S.* Typhimurium. For more comprehensive comparison we also did the same measurements with *E. coli* cells which are less resistant to oxidative stress, than *S.* Typhimurium [37].

Figure 6a shows that TiO_2 powder increased NPN fluorescence by nearly 0.4 units, indicating the increase in permeability of the outer membrane. Treatment by TiO_2 irradiated with UV and visible light slightly reduced membrane permeability of the cells and this decreased NPN uptake to 1.2–1.3 units. Intracellular ROS production was the highest in the sample with TiO_2 (Figure 5b). In UV and Vis light irradiated samples the intensity of DCFH-DA fluorescence did not change during the first 8 min, but later it started to increase (Figure 5b). The cell viability results, presented in Figure 5c, show a high sensitivity of the ΔTolC cells to TiO_2, but UV irradiation did not improve it. On the other hand, visible light irradiation enhanced the bactericidal effect and was lethal for nearly all the ΔTolC cells.

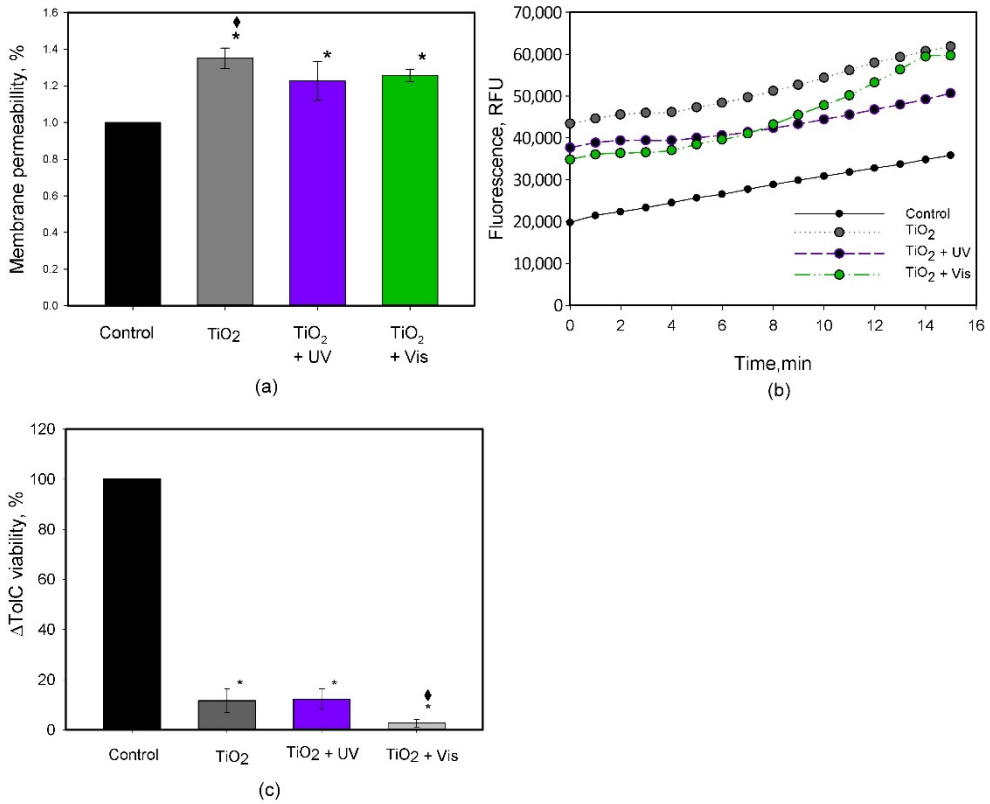

Figure 6. Effects of light and TiO_2 on *S.* Typhimurium ΔTolC cells: (**a**) outer membrane permeability; (**b**) intracellular ROS concentration and (**c**) viability of *S.* Typhimurium ΔTolC mutant cells. The experiments were performed in PBS buffer, pH 7.4, containing 0.1% glucose. Concentrations of the cells and TiO_2 were 3×10^7 cfu/mL and 0.3 mg/m, respectively. * and ♦ mark experimental values which are significantly different from the control sample and other samples respectively (more details on statistical analysis are provided in Section 4.5).

The results of Escherichia coli treatment (Figure 7) revealed that in comparison to the control sample, TiO_2 increases NPN uptake factor by slightly over 0.1 (Figure 7a). UV light activated TiO_2 did not change the outer membrane permeability and NPN factor remained similar to the control, whereas visible light irradiated TiO_2 increased the NPN uptake to 1.3. Measurements of DCFH fluorescence (Figure 7b) showed generally similar results to *S.* Typhimurium ΔTolC cells. The intracellular ROS concentration was the highest in the sample treated by TiO_2 and in the sample treated by TiO_2 and visible light combination. On the contrary, the viability test, presented in Figure 7c, showed that TiO_2 alone does not have a significant effect on *E. coli* growth, but when TiO_2 was irradiated by UV light the cells were not able to form colonies. Visible light irradiated TiO_2 decreased the viability of *E. coli* by 80%.

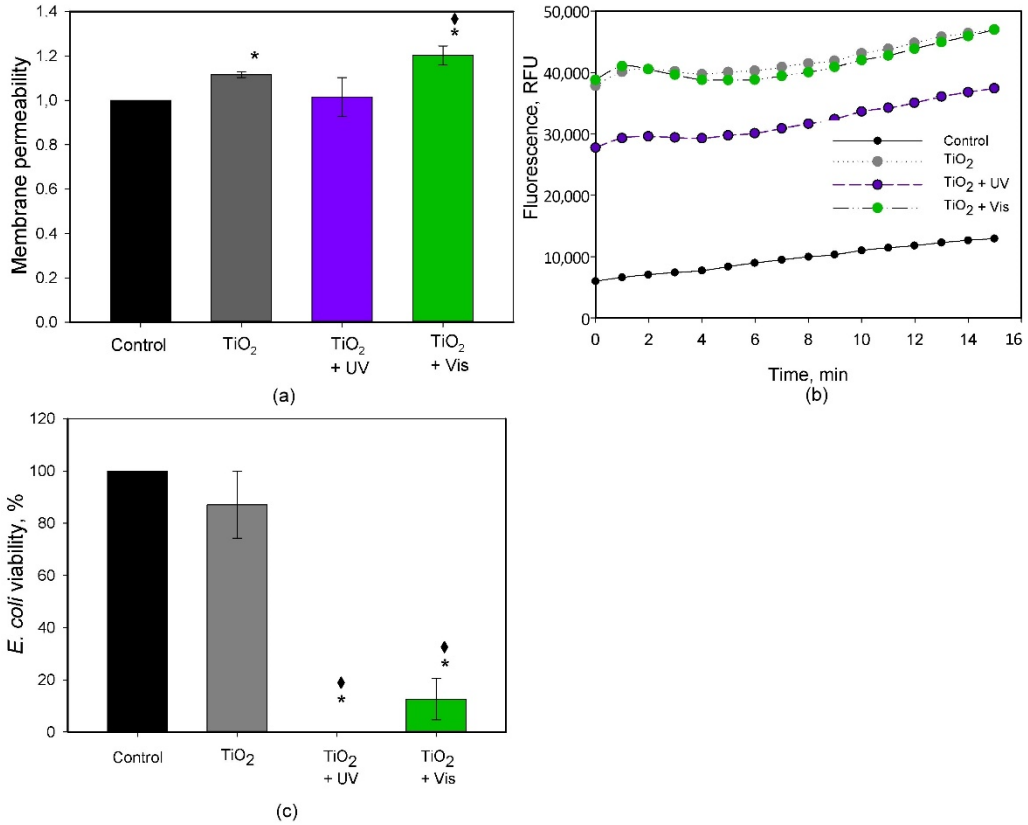

Figure 7. Effects of light and TiO_2 on *E. coli* cells: (**a**) outer membrane permeability; (**b**) intracellular ROS concentration and (**c**) viability of *E. coli*. The experiments were performed in PBS buffer containing 0.1% glucose, pH 7.4. Concentrations of the cells and TiO_2 were 3×10^7 cfu/mL and 0.3 mg/m, respectively. * and ♦ mark experimental values which are significantly different from the control sample and other samples respectively (more details on statistical analysis are provided in Section 4.5).

2.4.3. The Effect of Visible Light Activated TiO_2 on Bacteriophages

In the current study we used the Myoviridea family representing bacteriophage T4 and the membrane containing member of Tectiviridea family bacteriophage PRD1 as relatively inert biological control objects which are not able to produce ROS. The experiments were performed in the glucose containing medium and in the absence of it. The effect of titanium dioxide and irradiation was determined. In general, results, presented in Figure 8, did not show any sensitivity of these two phages to TiO_2 and/or irradiation. More specifically, we would like to emphasize, that in contrast to the examined bacteria, neither visible light nor TiO_2, acting together or separately, affected the infectivity of these two dsDNA bacterial viruses.

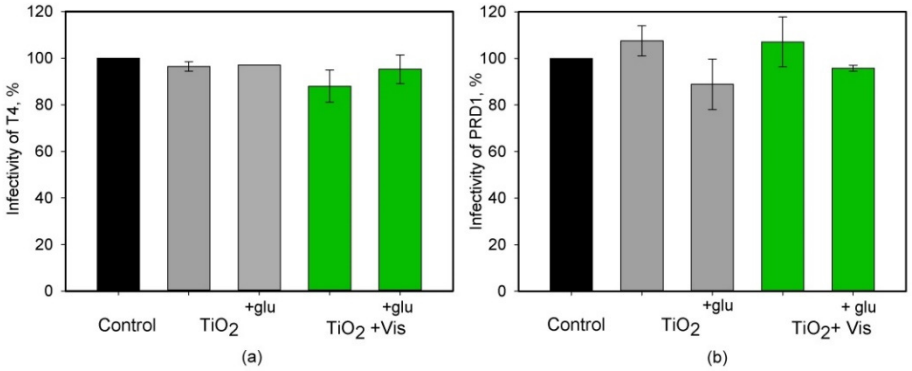

Figure 8. The effect of glucose on antimicrobial properties of TiO_2 against bacteriophages: (**a**) T4; (**b**) PRD1. Experiments were performed in PBS buffer, pH 7.4. with (+glu)/without 0.1% of glucose. The concentration of bacteriophages was 1×10^3 pfu/mL (plaque-forming unit/mL) and concentration of TiO_2–0.3 mg/L.

3. Discussion

Our earlier study with visible light activated carbon-doped TiO_2 photocatalyst showed effective inactivation of PRD1 and T4 bacteriophages [38]. C-doped TiO_2 traditionally has a narrower band gap and additional electron energy levels [23,24]. Therefore, it is active under visible light and phage inactivation can be attributed to the traditional photocatalytic disinfection model which relies on the oxidative power of photocatalytically generated ROS. More specifically, it can be supposed that during the photocatalysis, generated radicals cause common damage to virus capsid proteins which in turn inactivates the virus [39,40]. However, our current study with commercially available P25 TiO_2 powder under visible light did not show inactivation of bacteriophages. Such results indicate that visible light irradiated pristine TiO_2 does not generate ROS, and this observation is in good agreement with results of other studies [39–41].

The example with PRD1 and T4 bacteriophages showed that photocatalytic treatment of these relatively simple biological objects can be compared to the mineralization of inorganic compounds. However, as it was discussed above, photocatalytic treatment of live bacteria is much more complicated. It is well known that UV activated pristine TiO_2 has strong bactericidal properties, but only a few studies have discussed its photocatalytic activity under visible light. Moreover, even those few studies have usually involved additional factors which might have influenced the final results. For example, Suyama et al. [42] observed that TiO_2 can be activated by visible light of 380–520 nm wavelength, but this was achieved only in the presence of H_2O_2 in the incubation medium.

Current experiments with bacteria revealed that under conditions suitable for bacterial growth (i.e., with elevated medium temperature and addition of glucose), visible light irradiated TiO_2 in bacteria cause oxidative stress which leads to the cell death. Naturally, it can be argued that these results are caused by secondary damages, because the damage of bacterial membranes by TiO_2 powder have already been observed and reported in the literature [31]. Nevertheless, our results indicate that it is necessary to consider how particular bacteria respond (adopt) to the external stress factors, like intensive UV/Vis irradiation and TiO_2. For instance, bacteria can generate intracellular ROS which could leak to the media through the outer membrane porins. In this way the bacteria itself can be the source of H_2O_2 and other forms of ROS. Such a process might end up with H_2O_2 in the media and produce a bactericidal effect [42], However, this reaction pathway describes just one of the possibilities.

The phenomenon of visible light activated TiO_2 could be compared to some effects of antibiotics: if primary damage of the specific targets is severe enough, it can result in death directly. Otherwise, primary damage stimulates a pathway that leads to the accumulation of ROS, causing secondary damage [21] leading to the possible death of bacteria. However, bacteria are constantly adapting to their environment and they have a well-developed

antioxidant system which allows them to survive. For example, species dependent transcription factors such as OxyR (i.e., in *S. enterica*, *Francisella tularensis*, and *Porphyromonas gingivalis*), PerR (i.e., in *S. aureus* and *B. subtilis*), OhrR (i.e., in *B. subtilis* and *Mycobacterium smegmatis*), and SoxRS (i.e., in *E. coli* and *S. aureus*) can be activated by direct oxidation of their sensor proteins, and then appropriately adjust the bacterial response [21]. A new pathway used by *S.* Typhimurium to detoxify extracellular H_2O_2 using an ABC family multidrug efflux pump MacAB secreting a linear siderophore, metabolite of enterobactin was also proposed by ref. [43]. Similar to ABC family pumps, TolC, component of RND family efflux pumps, can also play a role in reduction of oxidative stress [36]. Accordingly, in the absence of TolC, *S.* Typhimurium mutant cells are very sensitive to intracellular ROS. Our experiments support this published data: high level of intracellular ROS causes the death of the ΔTolC cells, while wild *S.* Typhimurium cells are able to form colonies at the same levels of ROS. We also determined that *S.* Typhimurium treatment by titanium dioxide and visible light reduces the activity of its efflux pumps [44].

Although *Escherichia coli* and *Salmonella* enterica ser. Typhimurium are closely related, the latter cells are more resistance to oxidative stress. A possible explanation was provided by M.Rhen who stated that Salmonella bacteria are equipped with an array of enzymes and reducing compounds aimed at detoxifying ROS and repairing ROS-induced damage [37]. The differences between the photocatalytic process effect of the reduction of the viability of *S.* Typhimurium, *S.* Typhimurium ΔTolC, and *E. coli* bacteria as well as inactivation of PRD1 and T4 phages, suggest that intracellular ROS is the main cause of bactericidal properties of the visible light irradiated TiO_2. Such an assumption is supported by the earlier reports that the mechanisms of TiO_2 based photocatalyst action are related to the decreased expression of a wide array of genes or proteins specific for signaling, regulatory, and growth functions in parallel with subsequent selective effects on ion homeostasis, coenzyme-independent respiration, and cell wall structure [45].

Apart from antioxidant defense systems, in order to survive under unfavorable conditions, metabolism remodeling in bacteria also plays a pivotal role in dealing with oxidative damage. Metabolic adaptions can reduce the oxidative burden by retarding respiration [21]. Our results of MTT reduction after exposure with titanium agree with this model. Reduced MTT reduction, due to slowed respiration followed by cell death, was determined. Combined TiO_2 and irradiation treatment at 22 °C has a smaller effect on bacteria viability, than the corresponding process performed at 37 °C. The reduced bactericidal efficiency at lower temperature (22 °C) in the presence of glucose, further supports the statements of slower respiration and smaller generation of ROS.

4. Materials and Methods

4.1. Bacteria Cultivation

Gram-negative *Salmonella enterica* ser. Typhimurium SL1344 (wild-type) and ΔTolC (mutant strain of RND-type efflux pumps) cells were obtained from prof. Séamus Fanning (Institute of Food and Health, University College Dublin, Dublin, Ireland). *Escherichia coli* KMY strain cells were obtained from prof. Edita Suziedeliene (Institute of Biosciences, Vilnius University, Vilnius, Lithuania). Bacteria preparation and cultivation were performed following the procedure described in [30].

4.2. Experimental Conditions

Three types of experiments were performed: (i) treatment by light without TiO_2; (ii) treatment by two different concentrations of TiO_2 without light; and (iii) combining TiO_2 and light factors. For the treatment of bacteria and bacteriophages, we used well documented P25 commercial titanium dioxide powder (Sigma-Aldrich), narrow band 365 nm UV light LED (M365LP1-C1, Thor labs, Mölndal, Sweden) and wide spectrum cold white LED sources (SOLIS-3C, Thor labs, Mölndal, Sweden). The approximate intensity of UV and visible light irradiation at the solution surface was 30 and 40 mW/cm^2, respectively.

To avoid uncontrolled activation, prior to use, TiO_2 powder was always stored in a cabinet without light.

Experiments were started by adding the required amount of TiO_2 powder (0.3 mg/L, 1.0 mg/L, or 0 mg/L for treatment by light alone) into 15 mL of phosphate-buffered saline solution (PBS buffer, pH 7.4), which was continuously stirred and thermostated at 37 °C. If needed, 0.1% glucose was added together with TiO_2 powder. Before the addition of bacteria/phages, the prepared mixture was irradiated by the corresponding light source for 10 min. Only then were the bacteria/phages added to the mixture and the treatment time started to be counted.

The initial concentration of added bacterial cells was 3×10^7 cfu/mL. After 60 min of treatment, 100 µL of samples were collected from the test mixture, diluted (1250 times in PBS buffer) and plated on LB agar (spread plate (SP) method with glass beads technique, named Copacabana Method [46]). The formed colonies were enumerated after incubation of the plates at 37 °C for 18–22 h.

To remove the remaining part of the photocatalyst, bacterial suspensions from vessels were centrifuged 3000× *g* at 4 °C for 5 min (Heraeus ™ Megafuge ™ 16R, Thermo Fisher Scientific, Dreieich, Germany). The pellet was resuspended in PBS buffer, pH 7.4, to obtain 3×10^8 cfu/mL (OD_{600} of 1). Such suspensions were further used for N-phenyl-1-naphthylamine (NPN) uptake assays (bacterial outer membrane permeability assessment [47]) and for measurements of dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence (determination of intracellular ROS formation (oxidative stress) [48]).

For the experiments with bacteriophages, the initial concentration of phage T4 and PRD1 particles in the test vessel was 1×10^4 pfu/mL. *E. coli* DH5α and *S. enterica* DS88 cells were infected by bacteriophage T4 and PRD1 respectively. Samples of bacteriophage suspensions were taken after 60 min of treatment and serially diluted then, 100 µL of phage suspension (−100 phages) was poured into 4.5 mL of LB medium with 0.5% agar and 300 µL of bacterial night culture and plated on the agar. After 18–22 h of incubation at 37 °C phage-formed negative colonies were counted (plaque-forming unit (PFU) method [49]).

4.3. MTT Reduction Assay

For MTT reduction assay, after 30 and 60 min of the treatment, 100 µL samples were taken from the test vessel and syringed to 1.5 mL size microcentrifuge tubes. Then MTT solution was added to the tubes to reach 0.125 mg/mL concentration. Such test samples were incubated in the dark for 1 h at 37 °C. After the incubation samples were centrifuged for 4 min at 7000× *g*. Supernatant was removed, and the precipitated formazan was dissolved in 400 µL of sterile DMSO (Dimethyl sulfoxide) solution. To precipitate bacteria and TiO_2 powder, the samples were once again centrifuged for 4 min at 7000× *g*. Then, 200 µL of supernatant was transferred to transparent 96-well plate. The absorbance was measured immediately at 512/635 nm in TECAN GENios ProTM (Männedorf, Switzeland) plate reader, using 635 nm as the reference wavelength.

4.4. NPN-Uptake Assay and DCFH-DA Fluorescence Measurements

Samples for the NPN-uptake assessment were collected as described in Section 4.2, meanwhile the detailed description of the used analysis method and the applied parameters can be found in ref. [29].

Detection of intracellular hydroxyl (HO·), and peroxyl (ROO·) radicals and hydrogen peroxide (H_2O_2) was performed using dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich, Taufkirchen, Germany) as described in ref. [48]. DCFH-DA solution was prepared as described elsewhere [50]. After preparation of this probe, 100 µL of bacterial suspension (as described in Section 4.2) was mixed and transferred into a 96-well flat-bottom black plate, 100 µL per well, and 10 µM of DCFH was added. The relative intensity of the fluorescence was monitored for 15 min using TECAN GENios Pro (Männedorf, Switzeland) plate reader (excitation 492 nm, emission 535 nm). The plate was shaken for 5 s before each registration point.

4.5. Statistical Analysis

The data was analyzed 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. Tukey Test was used to compare significant differences between each group. Statistical analysis was carried out to determine the significant differences between the control groups without external factors and after photocatalytic disinfection. p values ≤ 0.05 were considered significant. The numbers of CFU and NPN uptake factor are presented as means $\pm$ standard deviation.

5. Conclusions

Traditionally, photocatalytic cleaning of aqueous solutions is explained by the generation of reactive oxygen species and realization of their oxidative power. For inorganic compounds and even organic molecules this model works well. Accordingly, researchers are mostly focusing on how to improve the photocatalyst and to achieve higher efficiency of ROS generation. However, living organisms, like bacteria, are constantly adapting to their environment and their reaction to intensive irradiation, contact with the photocatalysts material, and ROS can lead to non-trivial results. The results of experiments show, that under certain conditions (and for some types of bacteria) TiO_2 and visible light can affect the integrity of their outer membrane. In addition, these factors can cause internal oxidative stress in metabolizing bacteria. If direct damage to the outer membrane of the bacteria and the secondary effects caused by internal oxidative stress add up, an effective inactivation of the bacteria can be achieved. Most likely, the favorable conditions are formed when the bacteria metabolism is stimulated by higher temperature, glucose or other factors. On the other hand, in the absence of glucose or at low temperature the bactericidal effect of visible light irradiated TiO_2 is reduced. This suggest that in order to inactivate bacteria with poor metabolic activity, it is necessary to generate high amounts of ROS in the media. However, ROS generation by titanium dioxide irradiated by visible light is weak. Moreover, in most other studies, the photocatalytic efficiency of TiO_2 was tested at relatively low temperature and the metabolism of the bacteria was not stimulated. Naturally, the disinfection potential of visible light and pristine TiO_2 combination was considered as insignificant. However, creating such testing conditions potentially could well have put too much weight on photocatalyst properties and hindered the possibility to exploit the specific properties of the treated microorganisms. Accordingly, in future studies, we will try to repeat the research with various other known photocatalysts (and other compounds) whose activity in the visible light spectrum is limited. Similarly, it will be interesting to see if the currently described principles can be used for the enhancement of the disinfection efficiency of various other pathogens.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to legal issues.

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