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The antibacterial synergetic effect of titanium dioxide and blue light

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The antibacterial synergetic effect of titanium dioxide and blue light

It has been demonstrated, that certain wavelengths of visible blue light have strong antimicrobial tendencies. Unlike with ultraviolet radiation the underlying mechanism is not based on dispersal of nucleic material, but instead on photochemical reactions via bacterial photosensitizers. These reactions create reactive oxygen species (ROS) which bring about the partial mineralization of endogenous substances concluding in the inactivation of the cell in question; once the required level of oxidative stress is met. Visible antimicrobial blue light (aBL) is multi-functional and safe method to eradicate all forms of vegetative microbes, a promising option of disinfection in the current times when the medical community is working hard to reduce the excess use of antibiotics.

Microcrystalline titanium dioxide nanoparticles are also known to generate oxygen radicals, which react with surrounding organic compounds including components of the microbial cytoplasmic membrane concluding in the eruption of the cell. This effect is called photocatalytic disinfection and it can be activated by high energy photons, *i.e.* light of specific wavelength and intensity. Recent modifications in colloidal solutions of titanium dioxide have enabled activation to take place also with light from the visible region of the electromagnetic spectrum, including antimicrobial blue light.

This thesis has been compiled as a part of cooperative CDIO *Capstone Innovation* -project between LED TAILOR INNOVA7ION and Turku University of Applied Sciences. The project in question was the third of its kind between these entities, previous projects have included observation and analysis of the bactericidal effect of antimicrobial blue light on model bacteria *Escherichia coli*. This time, objective of the project was to observe the combined bactericidal activity of photon and photocatalytic disinfection. This thesis consists of the theory behind these two methods of disinfection, detailed compilation of an operational test protocol, depiction of the experimentation and obtained inactivation data with attendant discussion.

The compiled test protocol was confirmed to generate applicable inactivation data. In addition, it greatly increased the yield with an experimental surface simulation method compared to conventional swap samples. Distinct bactericidal effect of visible blue light and the accelerating impact of titanium dioxide were both observed, thus supporting the hypothesis.

KEYWORDS: Antibacterial activity, Photon disinfecting, Photocatalytic disinfection, Oxidative stress, Endogenous photosensitization, Titanium dioxide, *Escherichia coli*

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Titaanidioksidi-pinnoitteen ja sinisen valon antibakteerinen yhteisvaikutus

Tietyillä sinisen valon aallonpituuksilla on todettu olevan voimakkaita antimikrobisia vaikutuksia. Toisin kuin ultraviolettisäteilyn, näkyvän sinisen valon desinfioiva vaikutus ei perustu nukleiinihappojen tuhoutumiseen vaan valokemiallisiin reaktioihin mikrobeissa esiintyvien valoherkisteiden kanssa. Näissä reaktioissa syntyy happiradikaaleja, joiden aikaan saama oksidatiivinen stressi aiheuttaa endogeenisten yhdisteiden osittaisen mineralisaation johtaen kyseisen solun tuhoutumiseen sisältä päin. Sininen valo on monikäyttöinen ja turvallinen tapa tuhota aktiiveja mikrobeja ja lupaava vaihtoehtoinen desinfiointimetodi aikana, jolloin liiallisesta antibioottien käytöstä halutaan lääketieteellisen yhteisön piirissä kiivaasti päästä eroon.

Tunnetusti myös titaanidioksidi molekyylistä mikrokiteytyneet nanopartikkelit tuottavat mikrobeille vahingollisia radikaaleja happiyhdisteitä. Nämä yhdisteet reagoivat kohtaamiensa orgaanisen materiaalin – mukaan lukien mikrobin sytoplasmisen kalvon komponenttien – kanssa, lopulta puhkaisten sen. Tämän ilmiön nimi on fotokatalyyttinen desinfiointi ja sen vaikutus aktivoidaan käyttämällä korkea energisiä fotoneita; toisin sanoen sopivia elektromagneettisen säteilyn aallonpituuksia, sekä riittävää intensiteettiä. Viimeaikaiset modifikaatiot titaanidioksidi kolloidiliuoksissa ovat mahdollistaneet mekanismin aktivoitumisen myös näkyvän valon aallonpituuksilla, mukaan lukien fotonidesinfioinnissa käytetyn sinisen valon hyödyntämillä aallonpituuksilla.

Tämä opinnäytetyö on laadittu osana LED TAILOR INNOVA7ION :n ja Turun Ammattikorkeakoulun yhteistä *Capstone Innovation* - CDIO projektia. Tämänkertainen projekti oli kolmas tahojen välinen yhteistyöprojekti, aikaisempina vuosina opiskelijat ovat mm. todenneet fotonidesinfioinnin tuhoavan vaikutuksen *Escherichia* kolibakteeriin. Tämänkertaisen projektin tarkoituksena on todentaa fotonidesinfioinnin ja fotokatalyyttisen desinfioinnin antibakteerisesta yhteisvaikutuksesta saatava hyöty. Tämä opinnäytetyö sisältää teorian edellä mainittujen desinfiointimetodin takana, testiprotokollan laadinnan, kuvauksen testauksen suorittamisesta, sekä aikaansaadut testitulokset niihin liittyvän syventävän osion kanssa.

Laadittu testiprotokolla havaittiin toimivaksi ja sen kokeellista pinta-simulaatio metodia seuraten näytteiden saantoa saatiin merkittävästi nostettua tavanomaisiin sivelynäytteisiin verrattuna. Testausten aikana sekä sinisen valon antibakteerinen vaikutus, että titaanidioksidin desinfiointia nopeuttava vaikutus demonstroitiin.

ASIASANAT: Antibakteerinen vaikutus, Fotonidesinfiointi, Fotokatalyyttinen desinfiointi, Oksidatiivinen stressi, Endogeeninen valoherkistys, Titaanidioksidi, *Escherichia coli*

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Abbreviations

Conceptual terms

aBL	Antimicrobial blue light
aPDT	Antimicrobial photodynamic therapy
B-LETB	Blue light-emitting toothbrush
CFU	Colony forming unit
ESBL	Extended-spectrum beta-lactamase
LED	Light-emitting diode
MSC II	Microbiological safety cabinet, class two
NP	Nano particle
OD_{600}	Optical density, at a wavelength of 600 nm
PCD	Photocatalytic disinfection
R.I.	Refractive index
ROS	Reactive oxygen species
UV	Ultra violet
VOC	Volatile organic compounds

Chemical formulas

Ag	Silver
H_2O	Oxidane
H_2O_2	Hydrogen peroxide
N	Nitrogen
NO_x	Oxide of nitrogen
O_2	Allotrope of oxygen
O_2^-	Superoxide anion
TiO_2	Titanium dioxide

Cell lines

E. coli	Escherichia coli
hGEP	Human gingival epithelial
P. aeruginosa	Pseudomonas aeruginosa
S. aureus	Staphylococcus aureus

Reagents

PBS	Phosphate buffered saline
TSA	Trypticase soy agar
TSB	Trypticase soy broth

Measure / unit symbols

E	Energy	Hz	Hertz
λ	Wavelength	J	Joule
		eV	Electron-Volt
cb	Conduction band	W	Watt
vb	Valance band		
e^-	Electron	rpm	Rounds per minute
h^+	Vacancy hole		

1. ANTIMICROBIAL BLUE LIGHT

For a long time, a growing worry in the medical and biomedical community has been the rising threat of antibiotic resistant bacteria. *Center of Disease Control* has named it to be "one of the biggest public health challenges of our time." The number of cases in US alone break the limit of 2 million each year (CDC 2018). In Finland there were about 7 000 cases of antibiotic resistant infections in 2016 according to THL. Most of the cases were caused by ESBL-stains, usually consisting of *Escherischia coli* and *Klebsiella pneumoniae* bacterial strains which have developed a capacity to produce enzymes to break down antibiotics (THL 2018). In turn, chemical disinfectants are often volatile, and their byproducts can cause significant harm to humans. In addition to high toxicity some of the byproducts are also proven to be carcinogenic (CDC 2016). Especially for hospital environment it has become vital to come up with new innovative methods to neutralize bacteria without the use of antibiotics or chemical disinfectants.

1.1 Photon disinfection

One of the most promising new innovations is the utilization of photon disinfection with visible light. This disinfecting method exploits irradiation with visible blue light (λ 400-500 nm) near the ultraviolet region (λ 100-400 nm) of the electromagnetic spectrum. Strong enough electromagnetic radiation at apposite wavelengths trigger a strong antimicrobial effect inside the exposed microbial cell. Disinfection mechanism in question, works through a completely different biochemical mechanism than the well-established antimicrobial effect of ultraviolet light. Especially the irradiation with UV type C light induces damage to exposed genomic material by breaking down RNA or DNA. For this reason, high energy UV is not only harmful to microbes but also to animal cells, including human. On the contrary, antimicrobial blue light does not directly attack nucleic compounds but increases the overall oxidative stress inside the organism (Figure 1). The cytotoxic effect of aBL does not significantly affect animal cells, this is due to the specifics of the inactivation mechanism. Photon disinfection using aBL is still a quite unknown disinfection method to the public, but it has ignited interest in the medical community for its high biocompatibility.

1. Cell is exposed to blue light irradiation
2. Light is absorbed by light-sensing compounds
3. Absorption of blue light initiates generation of ROS
4. ROS reacts with vital endogenous compounds
5. Destruction and partial mineralization of the cell

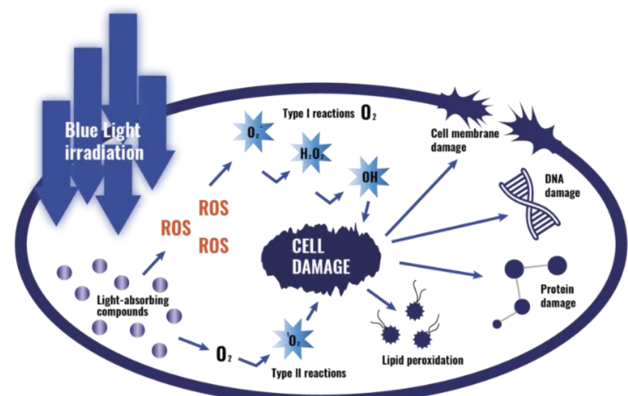


Figure 1. Cytotoxic reaction chain
Illustration of the different endogenous cell damage inducing effects of aBL from the moment of absorption.

1.2 Antimicrobial effect

In the last ten years, the inactivation capacity of aBL has been tested with a wide range of microbial spices. Studied strains include gram-positive and gram-negative bacteria, molds and yeasts (Wang Y. *et al.* 2017). Combined results concerning the inactivation of microbes display that the microbicidal activity is extensive and virtually affect all types of microbes to a varying degree. Some sections of the available test data are inconsistent and difficult to interpret, this is due to the high number of contributing factors that affect the underlying inactivation mechanism. This mechanism is tightly linked to the metabolic activity of the targeted microbes, therefore microbes that lack this activity can survive the photon disinfection process.

Inactivation studies that have been conducted with viruses demonstrate, that the virucidal effect is closely linked to the concentration of photosensitizers in the used media. This correlation was demonstrated in a 2014 study by exposing bacteriophages to λ 405 nm light in minimal and high-nutrient media (Tomb R. *et al.* 2014). Further experimentation was conducted with a norovirus surrogate resulting in surprisingly high levels of virucidal efficacy, even for the samples in minimal media (Tomb R. *et al.* 2017). In all reviewed test cases, minimal media has resulted in comparably lower virucidal action, due to the absence of photosensitizing compounds. In its totality, data indicates aBL inactivation to be affective for all metabolically active microbes. Inactivation of spores and other non-vegetative microbes and viruses may be better accomplished with a tandem disinfection method that would utilize some modification or another disinfection technique in addition to pure aBL.

1.3 Underlying biochemical mechanism

The current and widely shared notion about the mechanism behind the antimicrobial activity of aBL is that the effect is caused by endogenous photosensitizers, present in the cytoplasm of all strains of bacteria and in most vegetative microbial cells. It has been hypothesized that these photosensitizers primarily comprise of two groups of metabolic compounds; porphyrins and flavins. Absorption of energy from light at specific wavelengths by these compounds results in the formation of reactive oxygen species (Hessling M. *et al.* 2017). This generation of ROS happens when absorbed energy surpasses the threshold and breaks free an electron thus increasing oxidative stress within the cell *i.e.* by reacting with hydroxyl ions to form hydroxyl radicals; compounds with high reactivity (De Freitas L. & Hamblin M. 2016). When in contact with organic material, ROS are highly reactive and induce partial mineralization of vital endogenous components of the microbial cell, these include nucleic material, proteins and lipids.

1.4 Biosafety

In animal cells the concentration of photosensitizing porphyrins is extremely low compared to the concentration in bacterial cells. Therefore, the potential concentration of ROS generated by the photosensitizing mechanism is also significantly lower. In addition, animal cells have an inherent background concentration of ROS present in their cytoplasm for redox signaling (Schieber M. & Chandel N. 2014). In general, animal cells are more resistant to the oxidative stress compared to smaller microbial cells. No significant DNA damage is induced by exposing mammalian cells to high intensity aBL. This was demonstrated in 2013 by examining exposed mouse epidermal cells for possible DNA-fragmentation via TUNEL assay. Even after exposure of 195 J/cm^2 at λ 415 nm, irradiation led to almost no apoptotic cells (Zhang Y. *et al.* 2014). Same level of exposure of UV-light would have severely damaged the genomic material.

Effects of irradiation at different wavelengths on human skin cells were discussed in a study published by the Institute of Molecular Medicine in Düsseldorf, Germany (Liebmann J. *et al.* 2010). It was discovered that blue light at λ 453 nm does not cause any cytotoxic effects up to the extreme dose of 500 J/cm^2. On the contrary, high doses of blue light at λ 412-426 nm exerted dose-dependent as well as cell-type-dependent cytotoxic effects on the targeted cell cultures. These effects were most prominent after exposure in three doses of 33 J/cm^2 at λ 412, on consecutive days; which resulted in a cell loss of about 80 % with the exposed endothelial cells. Reduction rate furtherly rose to the total loss of viability when the energy of the given doses was increased to 100 J/cm^2. However, in the conclusion of the study in question it was stated that: “there is currently no evidence that the (aPDT) lamps may cause harm to the users” (Liebmann J. *et al.* 2010). Regarding to some of the more concerning result, there is some suspicion amongst the research community about the chance of “false positives”, due to the presence of some light-sensing compounds in the used test medium.

Biocompatibility of aBL was again demonstrated in a paper published in 2016 about dental resin formulations. In the underlying experiment, *E. coli* and *hGEP* cells were exposed to 46.3 J/cm^2 at λ 485 nm. Results confirmed the antibacterial effect but also showed increased ROS production in *hGEP* cells although without any cytotoxicity was observed with the tested concentrations. This increase in ROS was not considered prohibitive towards the use of aBL in dental applications (Zane A. *et al.* 2016). The datum that aBL is not notably harmful to human cells gives it a substantial advantage over UV-light. Especially regarding medical applications where high biocompatibility is necessary.

1.5 The effects of different conditions

There has been exhaustive and detailed experimentation of aBL inactivation, nevertheless a large portion of the test data lacks consistency as detailed in a 2016 review of the existing data (Hessling M. *et al.* 2017). Reason for this ambiguity, is the significant influence of multiple conditional parameters to the underlying photon disinfection mechanism. The primary conditional factor is naturally the aBL-source itself, involving such parameters as the intensity of the irradiation, illuminance uniformity of the test area, the wavelength maxima and the form of the spectrum spike or spikes. The utilization of different types of light sources including incandescent, fluorescent and LED; has a substantial effect on the before mentioned parameters. To achieve the narrowest possible spectrum spike, so that the optimal absorption wavelength would be targeted with the maximal percentage of the expended energy; the utilization of LED technology is necessary (Figure 2). The emitted light from specially manufactured diodes is the best way to target extremely narrow sections of the electromagnetic spectrum without the use of optical filters.

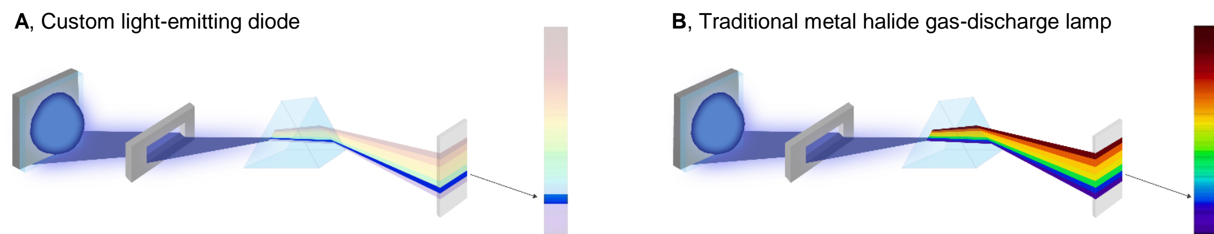


Figure 2. Spectral differences between light sources
Illustration of the narrow spectral spike of the custom LED (A) compared to the spectrum of a light source traditionally used in inactivation experiments (B). The utilization of LED technology saves energy by removing the need for a filter.

In addition to these light specific parameters, other factors like the prevailing temperature, concentrations of nutrients, the level of dissolved oxygen and the growth phase of the exposed bacteria all have a notable impact on the rate of inactivation. To produce comparable test results, all these parameters need to be taken in consideration. This is done by exact control over suspensions properties and the prevailing environmental conditions, during the photon disinfection process. Subsequent overviews of different conditional parameters and genomic characteristics are all factually based on the previously done research about these topics.

Photosensitizer concentrations correlate the level of metabolic activity in microbes. With most strains of bacteria inactivation takes less time with increased metabolism, still there are some exceptions. This trend has been verified by comparison between test results from setups with nutrition available during the photon disinfection process to those without it. Metabolic activity is also directly impacted by the prevailing temperature. However, test data from aBL inactivation experiments – which compared inactivation efficiency in different solution temperatures – are vastly contradictory or show no noticeable difference (Wang Y. *et al.* 2017).

Additional contributing factor is the level of available oxygen. High microbial concentration in the suspension might lead to an untenable consumption rate of the diffused oxygen, leading to a lower level of bacterial photoinactivation. The high dependence of the prevailing oxygen concentration on the rate of inactivation has been demonstrated *e.g.* with *S. aureus* in 2008. In this experimentation, the oxygen enhanced sample required a 3.5 times smaller dose of aBL to achieve the same level of inactivation than did the non-enhanced control (Maclean M. *et al.* 2008). It has also been proposed by the research community that a major part of the inconsistencies in aBL test data could be explained by the varying oxygen levels and/or neglected oxygen resupply (Hessling M. *et al.* 2017). Major part of the published photoinactivation research papers do not mention anything about prevailing oxygen concentrations during photoinactivation process, however most of them used nutrient-limited PBS or NaCl solutions resulting most likely in low consumption rates of oxygen.

In an experiment conducted in 2017 with *E. coli*, depleted oxygen levels at the timepoint of 8 hours allowed remaining cells to start a recovery process, and in 24 hours the volume of the population was identical to the dark control (Vollmerhausena T. *et al.* 2017). Aforementioned results were attained with initial bacterial concentration of 10^5 CFU/ml. The recovery was possible due to the facultative anaerobic nature of *E. coli* and tightly links the photon disinfection process to available oxygen. The overall results from this experiment suggest that growth supporting nutrient-rich conditions lead in time to the depletion of dissolved oxygen and able a fraction of the *E. coli* cells to survive. Discussion about dissolved oxygen, is only relevant when targeted microbes are suspended at the moment of aBL irradiation. On solid surfaces – where most of the inactivation takes place in the real world – supply of oxygen is near limitless and hypoxia is not an issue. But when aBL is used to disinfect solutions *e.g.* waste water or fluid food products, level of soluble oxygen and its sufficient resupply is essential to be taken into consideration.

Russian ministry of the health conducted a comparison between bacteria's acceptability to aBL inactivation in different phases of growth. In this experimentation three bacterial strains were exploited *E. coli*, *S. aureus* and *P. aeruginosa*. It was observed, that for all these strains the rate of inactivation was significantly lower for bacteria in logarithmic phase than for bacteria in latent phase (Keshishyan E. *et al.* 2015). In a similar study conducted in 2017, inactivation of five *E. coli* strains, all from different phylogenetic groups were examined in different phases of growth (Abana C. *et al.* 2017). Results from this experimentation greatly varied between tested substrains, some of which were – congruently with the Russian experiment – more suitable for aBL in stationary phase, but for others the effect was adverse. This finding suggests that the photosensitizer activity and the rate of inactivation are greatly depend on the specific characteristics of the exploited strains genome. The causal factors for these differences in growth phase specific inactivation rates are not yet understood. Varying metabolic activity and subsequently varying concentrations of endogenous photosensitizers could conceivably explain these differences. Further research on

these topics could clarify the connection between certain attributes in microbial genome and the acceptability to aBL inactivation, which would also help to better understand the underlying mechanism of photon disinfection.

1.6 Possible applications

In addition to basic interior surface disinfection, aBL can also be used in more targeted applications. Possible use cases might vary all the way from waste water treatment and mold prevention to produce preservation. However, the most advantage feature of aBL is clearly its biocompatibility, which makes it suitable for all kinds of medical applications few of which are briefly explained in subsequent paragraphs.

One of the most exciting possible applications for photon disinfecting is called antimicrobial photodynamic therapy, aPDT; which means the treatment of pathogenic tissue with aBL. In 2013 this experimental treatment was tested against multidrug-resistant *Acinetobacter baumannii* via mouse burn model. Infected tissue was exposed to 55.8 J/cm^2 of light at λ 415 nm. Results were highly encouraging; bacterial burden was significantly reduced, and no signs of developing resistance was detected even after ten sub-lethal cycles of photon disinfection (Zhang Y. *et al.* 2014). This research indicates that aPDT has a great potential to be a significant medical weapon against tissue infections, including antibiotic resistant flesh-eating bacteria.

In another study about lights effects on human skin cells, new possible use application for aPDT was discussed. Photocatalytic generation of NO_x compounds from nitrosated proteins was observed, when the cells were exposed to aBL at wavelengths less than λ 453 nm. The presence of nitric oxide is known to initiate skin cell differentiation. According to this study, aBL might provide a molecular mechanism to reduce proliferation, thus making it possible to treat hyperproliferative skin conditions (Liebmann J. *et al.* 2010). More research is still needed to determinate the extend and specifics of the tissue treatment capacity of aPDT *e.g.* the penetration depth of the bactericidal effect and the exact levels of cytotoxicity to each cell type when exposed to specific wavelengths.

Urinary tract infections caused by the usage of catheters, lead the charts in hospital-acquired infections. These types of infections are generally caused by *E. coli* so the risk for ESBL mutation is present. In 2017 aBL inactivation tests were performed to silicone matrix attached coli bacteria in urine mucin media. CFU concentrations were successfully lowered under the detection level after 6 hours with aBL at λ 420 nm (Vollmerhausena T. *et al.* 2017). This suggests aBL to be a promising antibacterial technology for biofilm prevention in catheters.

In the field of dental research, the effect of aBL to tooth plaque has been tested and observed. Dental applications like blue light-emitting toothbrushes could decrease the formation of biofilm and improve oral hygiene without causing any excess harm to teeth or oral tissue. In 2015 a controlled clinical trial was conducted to demonstrate the potency of B-LETB use in improving oral health (Genina E. *et al.* 2015). The intensity and wavelength of the used light was 2 mW/cm^2 and λ 405-420 nm respectively. Up to 50 % improvement was observed in comparison to the control group by the set baseline, complied from dental indices. Major improvement was observed in all used index markers, which were the prevalence of plaque, gingival bleeding, and inflammation. B-LETBs might be the most practical and easy means for the public to utilize photon disinfection technology in their everyday life.

The disinfecting potential of aBL has clearly peaked interest amongst the developers of medical instruments. Due to the substantial impact of prevailing conditions to the level of inactivation, precise testing is necessary in order to detect the gained benefit in each use applications. Nevertheless, currently available test data gives us no reason to question the biosafety of aBL.

2. ANTIMICROBIAL EFFECT OF TITANIUM DIOXIDE

Titanium dioxide is ultra-white, inorganic, non-toxic and vastly researched compound. TiO_2 nanoparticles are well known for their optical and photocatalytic properties, resulting in wide range of use applications. Optical properties of TiO_2 have widely been exploited in construction, where TiO_2 is better known by the name *Pigment White 6*. Currently it is the most important pigment used in coatings and paints, due to its efficiency in scattering visible light. In addition, the use of this pigment increases materials resistance against heat, light and nearly all kinds of weather conditions. The feature that makes TiO_2 optically so unique, is its combination of high transparency in the visible region of the electromagnetic spectrum, and its high refractive index of over 2.5. Although this number varies slightly between different crystal structures of TiO_2.

Photocatalysis is the acceleration in photoreactive rate by added catalytic substance. To this date, photocatalytic properties of titanium dioxide have been generally exploited in solar cell technology. Thin layers of TiO_2 nanoparticles have long been used in dye-sensitised solar cells and in recent years there has also been promising research about futuristic TiO_2 applications *e.g.* nanowires and quantum-dots. Current TiO_2 use applications are vast and include dental implants, cosmetics, textiles, food packaging, food colouring, plastics and the protection of marble (TDMA 2017). For many use cases, the protection from the UV radiation is the reason that explains the high popularity for the compound in question. Effectual absorption of UV-light enables the photocatalytic activity, that is also the source of the antimicrobial and anti-VOC properties of TiO_2.

2.1 Crystal structure

For titanium dioxide, there are two commercially available crystal structures; *anatase* and *rutile*. These two slightly differ in their spectral reflectance curves and refractive index values. Unlike anatase, rutile absorbs some percentage of the visible light – *i.e.* about 50 % of light at λ 400 nm gets absorbed (The Chemours Company 2016). This is important for the photocatalytic activity, which is only activated when enough energy gets absorbed. In addition to these two more commonly used TiO_2 crystal structures, there is a third one called *brookite*. In a study published in 2018, the sample with the highest level of photocatalytic activity also contained the highest *brookite* concentration. In contrast, the lowest effect was observed for the sample containing pure *rutile* (Allena N. *et al.* 2018). In outdoor use, UV-radiation from the sun contains the required energy to activate any of these three existing crystal structures.

$$R.I. = \frac{Speed\ of\ light\ in\ vacuum}{Speed\ of\ light\ in\ substance}$$

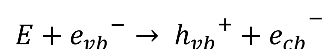
2.2 Antimicrobial and anti-VOC effect

In 2011 a review with an overview of all previous data about antimicrobial effects of TiO_2 was published (Foster H. *et al.* 2011). It collected together test data from inactivation tests performed with over 60 species of bacteria, and in addition with some types of fungi, algae, protozoa and viruses. Joined results show that the photocatalytic activity of TiO_2 is overall very capable of inactivating all vegetative forms, but faces more resistance with endospores, fungal spores and protozoan cysts. The occurring inactivation is due to cell wall degradation caused by increased ROS presence. Increased wall thickness of non-vegetative microbes protects them from the oxidative stress and longer or stronger influence is needed to achieve inactivation. Data also showed that gram-positive bacteria were commonly more resistant to the antibacterial effect, possible due to their thicker peptidoglycan layer.

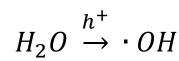
In addition to titanium dioxides microbicidal properties, its photocatalytic activity has been demonstrated to have strong anti-VOC and anti-NO_x effects. Measured rate of air purification for TiO_2-coating is 200 m^3/day for NO_x compounds and 60 m^3/day for typical VOCs. The used unit: *m^3/day* represents the volume of air that 1 m^2 of catalytic TiO_2-film can clean per day (Lawrence Berkeley National Laboratory 2008). Generated ROS reacts with all organic and some oxide compounds that come in contact with it. Therefore, environmental factors *e.g.* the quality of air or excess other organic material might degrease the microbicidal capacity TiO_2, by exhausting some percentage of the available ROS.

2.3 Photocatalytic disinfection mechanism

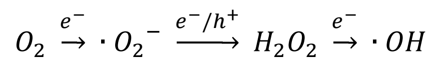
Mechanism behind the PCD activates when TiO_2 NP encounters a photon carrying the sufficient amount of energy to trigger electron promotion. To achieve promotion, energy carried by the photon must match the energy difference of the band cap. The required energy and consequently the maximum wavelength differ between two most common crystal structures; E = 3.2 eV, λ ≤ 385 nm for *anatase* and E = 3.0 eV, λ ≤ 415 for *rutile*. Absorbed energy promotes an electron from the valance band into previously vacant conduction band. In this process promoted electron leaves behind a positively charged vacancy hole. These pairs of electrons and vacancy holes further react with oxygen and atmospheric water – more specifically with hydroxyl ions – to procedure ROS. TiO_2 NPs themselves are not depleted by this process, they only catalyze it. Created vacancy holes are in time refilled and the same reaction chain can restart. These chain reaction cycles in time produce a prominent ROS presence.



$$E + {e_{vb}}^{-} \rightarrow {h_{vb}}^{+} + {e_{cb}}^{-}$$



$$H_2O \overset{h^+}{\rightarrow} \cdot OH$$



$$O_2 \overset{e^-}{\rightarrow} \cdot {O_2}^- \xrightarrow{e^-/h^+} H_2O_2 \overset{e^-}{\rightarrow} \cdot OH$$

Created hydroxyl radicals H_2O_2 and O_2^-, in addition to positively charged vacancy holes are extremely reactive when in contact with organic compounds. In the vicinity of cells, they induce severe damage to the cell wall and to the cytoplasmic membrane. Damaged sites start to leak cellular material (Figure 3), cell lysis and finally the left behind debris are also oxidized by the photocatalytic reaction products completing the mineralization of the organism (Ghosha S. & Dasa A. 2015). This reaction chain can continually inactivate microbes and eliminate VOC and NO_x compounds given that it has the necessary radiance. Since photocatalytic mechanism only activates with UV-light or light close to the UV-band its use as a disinfection agent has traditionally been limited to outdoor use; to be activated by the suns UV-rays.

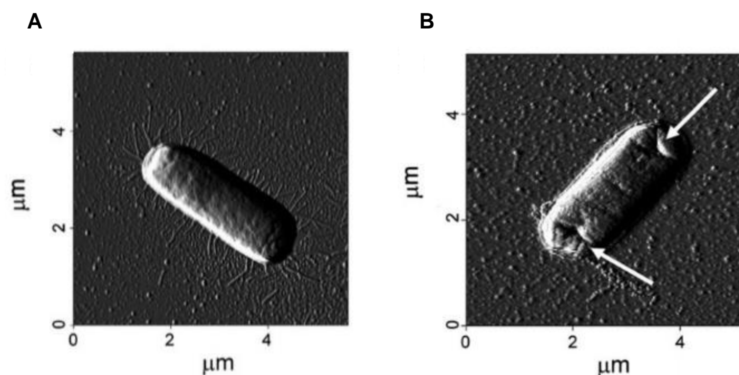


Figure 3. Induced damage
Atomic force microscopic images of E. coli cells before (A) and after (B) 1 min PCD treatment. Arrows indicate the sites of damaged cell wall. (Liou J. & Chang H. 2012)

2.4 Impurity doping

To extend the disinfection applications of TiO_2, various modified substrates have been created. These modifications widen the range of apposite wavelengths and allow photocatalytic activity to be triggered with light from the visible region as well as from the UV-region (Figure 4). Substrate modifications have been manufactured by impurity doping via metal coating and controlled calcination. Common doping agents in colloidal solutions of visible light-responsive TiO_2 photocatalysts are Ag and N. Added impurities create intraband gaps inside the energy band gap, which makes stepwise excitation possible for the lower energy photons (Liou J. & Chang H. 2012). In addition to their visible light-responsiveness, some Ag_2O doped species have been observed to trap conduction band electrons during the photocatalytic reaction, thus inhibiting recombination and further increasing the bactericidal activity (Wu P. *et al.* 2010).

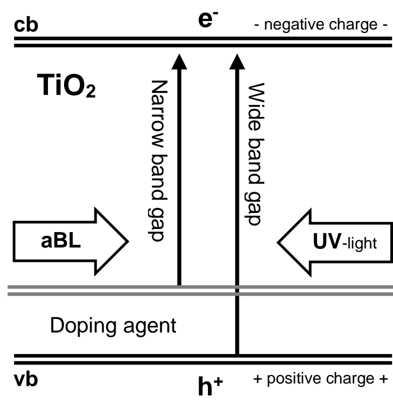


Figure 4. Photocatalytic reaction
Illustration of the difference in the amount of energy needed to promote a electron, between non-doped and doped and titanium dioxide sol-gel.

3. COMPILATION OF THE TEST PROTOCOL

The objective of the collaborative between the client company, *Capstone Innovation* - project team and the author of this thesis was to determinate the antibacterial advantage gained form the titanium dioxide coating during the photon disinfection process. Prior to experimentation, adequate test protocol had to be prepared and tested. The main objective of this thesis was to develop an operational test protocol for this experimentation. The given hypothesis was as follows; during photon disinfection bacteria on top of the TiO_2-coated surface would inactivate faster and with a higher rate, compared to control bacteria on non-coated surface. Common objective amongst all participant was to confirm the hypothesis and to determinate the level of gained advantage.

Composed test protocol consists of introduction, preparation and seven operations. Preparations included ordering, collecting and sterilizing of necessary laboratory material, and the preparation of TSB medium and PBS solution. Each test cycle took place during two successive workdays, first of which contained the disinfection operation and sample processing; the second day was reserved for counting of the CFUs and data analysis. Printed protocol acted additionally as a documentation of possible changes and/or defects – and was to be signed by the executor of each operation.

Protocol was compiled under supervision from the company behind the assignment; LED TAILOR INNOVA7ION. The protocol can be found in its entirety in the *attachments* section of this thesis. Test protocol was refined during the test cycles according to the acquired test data. Stand-alone protocol was composed for the pre-cultivation and freeze-drying operations in the beginning of this project. However, this protocol includes only standard microbiological laboratory methods and for that reason was not worth including.

3.1 Basis for the experimentation

The central idea was to compare the inactivation effectiveness of aBL on bacterial suspension divided between TiO_2-coated and non-coated stainless-steel test tiles. Photon disinfection process itself was laid out in three two-hour periods. In every timepoint, samples from the test area under aBL and controls from the dark stowage were processed. Firstly, the dried bacteria mass was suspended from each test tile into spate solution tube via vortexing operation. Following the preparation of dilution series all samples were spread on TSA filled Petri-dishes and incubated overnight. By counting resulting colonies and by comparing samples to controls – and coated samples to non-coated – it was possible to calculate the gained advantage.

Three replicates were prepared for each sample and control used, to determine repeatability and to increase reliability. According to the protocol; one test cycle included total of 48 samples – 12 pcs per timepoint, half of which were controls.

3.2 Exploited bacterial strain

Model bacteria strain *Escherischia coli* was selected for its prevalence in cases of antibiotic resistance. Cultivation of *E. coli* is extremely rapid, at its optimal cultivation temperature of 37 ^{0}C. Selected genotype was *JM109*, a modified substrain part of the phylogenetic subgroup A1 and originally designed for *M13 cloning system*. In an experiment conducted by comparing aBL inactivation rates of *E. coli* substrains from different phylogenetic groups, substrain *DH5α* from the subgroup A1 displayed the highest level of overall reduction (Abana C. *et al.* 2017). Substrain *JM109,* also from A1 was selected to ensure observable reduction within the 6-hour disinfection period. Metabolic profile of substrain *JM109* differs only slightly from the *E. coli* type strain by its deficiency in β-galactosidase activity due to deletion of *lacZ* gene (Promega 2019).

Biosafety level of the selected strain is one on a four-step scale, denoting the lowest possible risk. However, there is a high probability that ESBL-producing *E. coli* would exhibit similar acceptability to aBL inactivation or PCD. In an educational laboratory environment, handling of high-risk microbes is not possible let alone practical for this kind of laboratory experimentation. For the project in question, low risk strain was an obvious choice.

3.3 Selected TiO_2-coating and test tiles

According to the test protocol; during the photon disinfection, dried bacteria film is spread onto identical test tiles. Tiles were made out of inox steel and were 2 x 2 cm^2 in size. This material was selected because of its uniformly plane surface texture. Porosity would increase the surface area thus increasing the amount of TiO_2 particles able to attach to it. Inox steel simulates the most challenging case for PCD. For this experiment, selected coating was *anatase* based Ag/N co-doped titanium dioxide sol-gel, intended for indoor use (TitanPE Technologies 2018). Half of the available tiles *i.e.* 25 pcs were coated on both sides and carefully kept separate from the non-coated ones. Before the test cycles began, TiO_2-coated and non-coated test tiles were pretested vis-à-vis the operations specified in the test protocol. Photocatalytic coating should not deteriorate during normal use, but accidentally inflicted scrapes or dents undoubtedly would have an effect on the photocatalytic potential. Therefore, the tiles were handled with great care.

3.4 Utilized light source

Photon disinfection was conducted in a closed space inside a class II biological safety cabinet. 3D model of the MSC II was prepared according to the premeasured physical dimensions, and specifics of lighting were designed using *DIALux* software (Figure 5). To maximize inactivation, selected aBL-source utilized LED technology and its spectrum incorporated narrow spikes at carefully selected wavelength(s) to optimally target endogenous photosensitizers. The exact wavelength(s) that were targeted during this experimentation are kept classified.

Before the experimentation began, glass walls of the MSC II were covered with black plastic film to keep all excess light from entering. Same treatment was repeated to second safety cabinet, where control samples were stored during the photon disinfection. Test area of 30 cm x 30 cm was defined and carefully marked below the light source, so that uniformity ratio of illuminance was >0.9. During test cycles two different irradiance intensities were used, 0.7 and 1.17 mW/cm^2. At the last timepoint of 6 hours, total amount of photonic energy that had made contact with the test area were 15.12 and 25.40 J/cm^2 respectively. Effective light pollution coverage and desired aBL irradiance levels were verified with a high-grade illuminance meter.

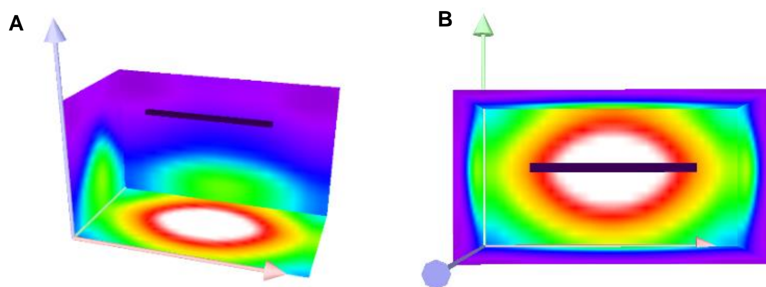


Figure 5. Lighting design, DIALux software
MSC II from the user's perspective (A) and from above (B).
Illustration of the illuminance distribution of aBL.

3.5 Pre-cultivation and freeze-drying

First step in the laboratory sequence was the pre-cultivation and freeze-drying of the selected bacterial strain. Prepared pre-culture was equally divided into glass ampules before freeze-drying, from which it was resuspended at the beginning of each test cycle. This process was later repeated to increase the cell concentration. Successful freeze-drying process eliminated the need to regrow the culture before each cycle and increased repeatability. However, separate pre-cultures were prepared for last three test cycles. During pre-cultivation and freeze-drying self-made TSA plates, TSB medium and D-lactose based freeze-drying medium were utilized.

When initiating the pre-cultivation, *E. coli* substrain *JM109* was revived from bacterial glycerol stock and plated. After one-night incubation formed colonies were selected and transferred into 10 ml cultivation tubes; incubation was continued for another 20 hours at 250 rpm. After incubation culture was gathered, vortexed and again divided into falcon tubes for centrifugation. Finally, the pellets were resuspended into freeze-drying medium and pipetted into ampules. Due to the uncertainty about the rate of cell mortality during the freeze-drying operation; first time ampules were prepared with two different cell counts. Approximation of the pre-cultures cell concentration was calculated before centrifugation from the average of measured OD_{600} values. In order that the required concentration would be attained during resuspension of the pellet.

$$1.0_{OD_{600}} = 8E8\ \frac{cells_{E.coli}}{ml}$$

$$Cell\ count = \frac{\sum_{i=1}^{n} \mathrm{x}_{OD\,600_i}}{n} \cdot 8E8\ \frac{cells_{E.coli}}{ml} \cdot \mathrm{y}_{volume}\ ml$$

3.6 Viability testing

After both rounds of the freeze-drying process, the viability of the dried culture was qualified. Viable cell count was obtained by counting the colonies and multiplying the result according to the used level of dilution. Results showed that about 65 % of the cells survived the freeze-drying process, although this might significantly depend on the cell concentration and does not include the inactivation taking place during retention. According to the gained viability results, the initial cell count and sample dilution ratio of the test protocol were determined.

3.7 Pretesting of the test protocol

In addition to the basic microbiological methods and operations, test protocol included an experimental operation of re-dissolution of the bacterial mass from the test tile into PBS solution. In practice, test tiles were transferred from the photon disinfection chamber into individual falcon tubes containing the buffer, for the vortexing operation. This experimental method was developed to increase the yield, which is only about 10-30 % for swab samples. Before implementation tile transfer method was pretested and assessed to increase the yield and possibly enhance the uniformity of the sampling process.

The experimental operation was performed and the obtained CFU counts were compared to directly plated control samples. Results were promising: bacterial yield was close to 70 % and parallel samples gave no significant deviation. One disadvantage for this operation is the

demanding and meticulous pipetting of the suspension onto small test tiles. Especially when dealing with coated, highly hydrophobic surfaces. Suspension beads, which differ in their form also differ in their required drying time; which needs to be taken in consideration. To ensure reliable results, all pipetted bacteria needs to be adequately dry before the transfer to falcon tubes can take place. This is in part because successful transfer would be near impossible with liquid still on top of the test tiles and in part because during the evaporation of the liquid substantial cell loss – of some 20 % – occurs which would distort the results if not taken into consideration.

In addition to these pretests, aBL inactivation experiment was performed to *E. coli JM109*, while the cell suspension was divided onto the surfaces of TSA plates. Thus, the antibacterial activity was confirmed and the later data comparison to surface samples was made possible. With the light intensity of 1.17 mW/cm^2, viable cell count was diminished from about 50 million to 80 000 during a 6-hour exposure period. This is equivalent to a survival rate of less than 0.152 %.

3.8 Execution of the test cycles

During the test cycles, workload in the microbiology laboratory was shared between participants of the *Capstone Innovation* -project group. Author of this thesis acted as a supervisor for the precise execution of the composed test protocol and stayed in active communication with the company in charge of the assignment. During the test cycles, protocol was actively updated according to the gained results and accrued experience.

Total of 8 test cycles where completed. The entire experiment was carried out in the microbiological laboratory of Turku University of Applied Sciences at *Lemminkäisenkatu* campus. Starting from the 5th week of 2019, one test cycle was performed weekly excluding 8th and 9th week for winter holiday and preparations. Photon disinfection and sample processing took place on Thursdays, and Fridays were reserved for CFU counting and result analysis. Incubation time for all samples was maintained in 20 hours minimum, so the exact timing for Friday's counting was scheduled accordingly.

To maximize effectiveness, sample processing operations occurred simultaneously one by one in three separate workstations. These three operations were performed at every timepoint: dissolving of the bacteria into buffer solution, preparation of the dilution series and plating. Due to the 6-hour photon disinfection and limited laboratory time-window it was important to make efficient use of the time available.

3.9 Feedback and modification

Inactivation of bacteria by photon or photocatalytic disinfection is dependent on multiple factors as mentioned in a previous chapter. Some of those factors are environmental and are sufficiently controlled in a laboratory setup. Other factors like; cell density, the effect of death cells and other organic material, should be taken into consideration while planning the test protocol. Due to the current, incomplete state of the research in regard to disinfection methods explored in this thesis, these factors had to be mediated with when faced. Therefore, every test cycle prompted suggestions for possible protocol improvement.

After the first test cycle, intensity of aBL was increased from 0.7 to 1.17 mW/cm^2. This modification was due to the inactivation rate much lower than expected. In pilot studies, intensity of 0.7 mW/cm^2 was enough to decrease the bacterial concentration by multiple logarithms. This increase in intensity significantly improved the inactivation and at the same time demonstrated the massive difference caused, in part by the decelerated metabolism rate and in part by the shielding effect of the excess biomass. A description for the mentioned shielding process can be found in the discussion chapter of this thesis.

During first four test cycles, varying concentrations of freeze-dried bacteria were utilized. In this process, significant percentage of the bacterial cells are inactivated due to the extreme physical stress. As the rounds progressed, it was assessed that the high percentage of death bacteria might act as a “bio shield”, protecting the live bacteria from aBL and from the external ROS. In addition, suspending solution was changed from TSB medium to PBS solution. At start TSB medium was selected to provide the suspended cells with nutrient-rich conditions to increase their level of metabolism. However, this contributed into the level of excess biological compounds hindering the function of both disinfection mechanisms.

This effect has an influence only if – like in this experiment – bacteria is tightly spread on top of a small surface area. After these cycles were completed, adjustments were made to the test protocol and instead of freeze-dried bacteria, freshly grown bacteria was used. Pre-cultivation was initiated on the previous day and grown slowly at 25 ^{o}C, to minimize the number of dead cells. In addition, centrifuged bacterial pellet was resuspended in PBS solution to avoid any extra organic material on top of the test tiles.

However, the best inactivation results were achieved with the higher level of aBL irradiation before the other modifications. There was no sufficient time to fine-tune the test protocol for aforementioned changes. More research is needed to observe and determinate “bio shielding” - effects significate in relation to inactivation with high cell concentrations.

4. RESULTS

Successful test cycles produced a coherent data set with four subsets of samples; two of which were exposed to aBL, the rest being dark controls. From these subsets, four graphs were comprised depicting the percentual time-decline in the concentration of viable bacterial cells.

Figures 6-8 display the inactivation data from a successful test cycle that utilized freeze-dried bacteria. Percentual inactivation of bacteria, was manifestly higher in both; aBL exposed and TiO_2-coated samples compared to the non-coated dark control. Notable feature of these graphs are the similar forms of TiO_2-coated and non-coated diagrams. The highest level of inactivation was achieved with the sample set depicting the tandem method, thus supporting the thesis.

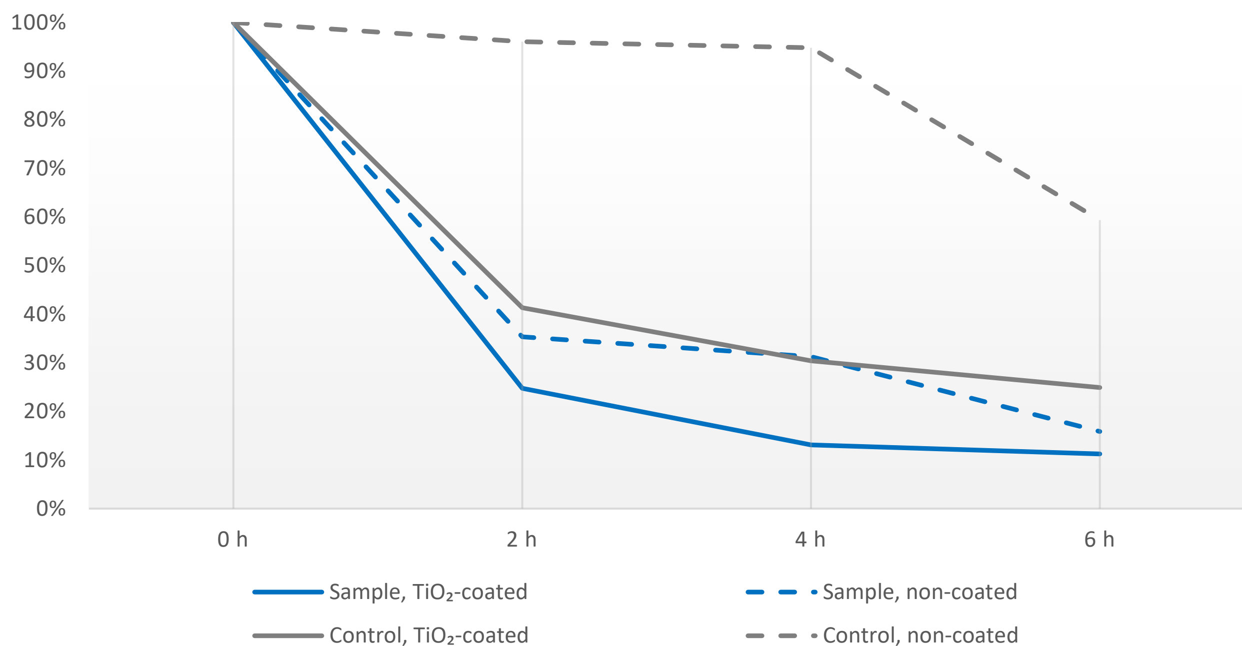


Figure 6. Percentual survival rate
Inactivation test results, 08.02.2019. Samples were exposed to 1.17 mW/cm^2 of aBL, controls were kept in the dark.

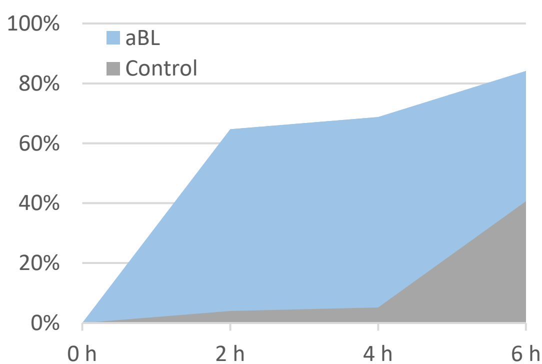


Figure 7. Percentual inactivation rate, non-coated
Inactivation test results, 08.02.2019

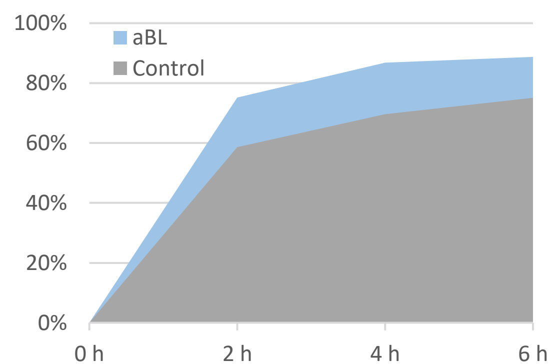


Figure 8. Percentual inactivation rate, TiO_2-coated
Inactivation test results, 08.02.2019

The test cycle in question, was performed 08.02.2019 and it produced the clearest inactivation test results of this project. The set values of two parameters make it distinct from the other cycles; optimal cell concentration of 20 000 000 cells per test tile and a method appropriate aBL intensity of 1.17 mW/cm^2. Any tuning of these parameters will result in differing, but comparable inactivation data. The highest rate of inactivation can be obtained with the tandem method, the level of the gained benefit is dependent on the specifics.

Comparable results were obtained in test cycles performed in 15.02.2019 and 08.03.2019 (Figures 9-10). During these two cycles; maximum inactivation rate varied greatly from 88 % to only 35 %, possibly due to the high surface concentration of bacteria in the later one. Regardless of the variance, the maximum inactivation was obtained in both of these cases with the tandem disinfection method. Related to these results and to the occurring variance, it is important to consider some experiment specific factors. This deliberation can be found in the discussion chapter of this thesis.

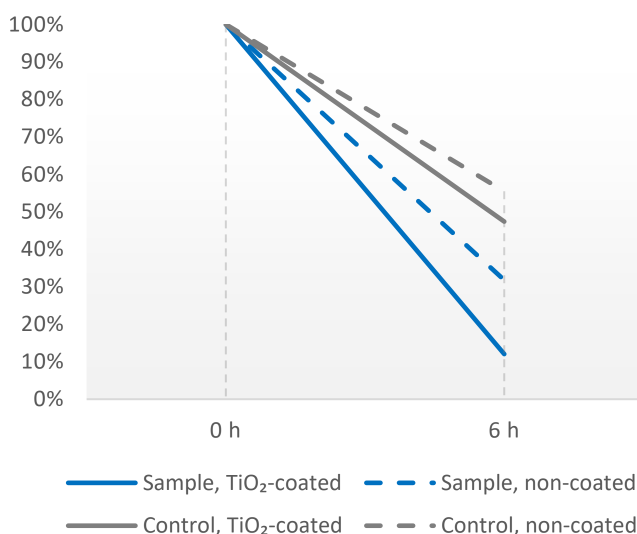


Figure 9. Percentual survival rate, 6 hours
Inactivation test results, 15.02.2019

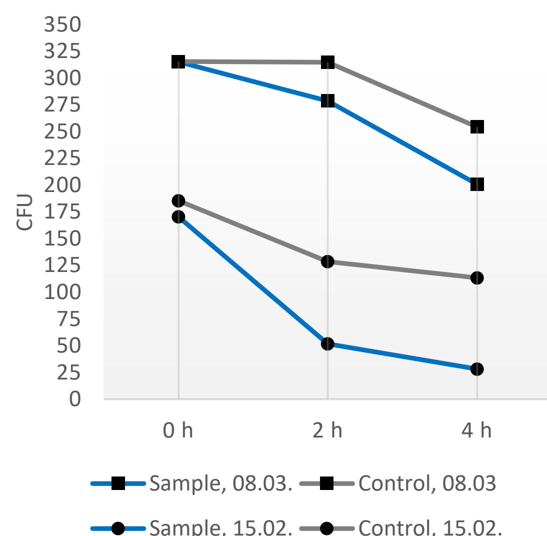


Figure 10. Percentual survival rate, TiO_2-coated
Result comparison, 15.02.2019 and 08.03.2019

4.1 Discovered benefit

The proposed benefit of the tandem disinfection method – compared to the use of pure aBL or PCD – was observed. The greatest percentual decline in the number of viable cells was attained by exposing the model bacteria to aBL in the presence of visible light sensitive photocatalyst. But stating of any percentual value for this synergetic benefit – that could apply regardless of the specific parameter values – is impossible. In this experimentation, TiO_2-coating accelerated aBL inactivation in the timepoints of 2 and 4 hours by about ten and twenty percent, respectively.

5. DISCUSSION

To properly comprehend the results obtained in this experimentation, detailed discussion about them is necessary. Specific conditional factors were assessed and observed to have a significant impact on the resulting inactivation rate. In order to better utilize photon and photocatalytic disinfection methods these influences need be thoroughly investigated, especially relating to surface disinfection. When these disinfection techniques are exploited in real-world operations, it is not adequate to blindly rely on inactivation data from any previous experimentation but instead to conduct an operation specific inactivation experiment. Subsequent subchapters expand into four factors of significant influence and discuss the future of aBL-source design and the experimental sampling method – utilized in this thesis. In their entirety, performed test cycles underlined the previous observations about the complexity of aBL inactivation tests, yet at the same time produced data supporting the given hypothesis.

5.1 Cell concentration

During the inactivation experiments conducted in association with this thesis, extremely high surface concentrations of *E. coli* were utilized. The objective was not to depict surface inactivation in the real-world, but to determinate the absolute benefit of aBL-TiO_2 co-use. However, for an experiment protocol that utilizes small test tiles, high initial bacterial inoculum might result in some unforeseen issues. According to the *Beer-Lambert Law*, higher optical density induces attenuation of light in the bacterial suspension – the level of radiance being proportional to the cell concentration – resulting in reduced inactivation (Verhoeven J. 1996). Although cells were not suspended at the time of the exposure during this experimentation, a similar effect can occur during surface disinfection if exposed cells are on top of each other.

Throughout this experimentation, surface concentrations remained between 5 and 25 million cells per cm^2, although the cell distribution presumably greatly varied in microscale due to the surface tension and the shape of the suspension droplets. The surface area of a single *E. coli* cell is about 3.8-4.42 μm^2 depending on the growth phase, therefore – when side by side – the total surface area of 25 million cells would be about one square centimeter (Prats R. & De Pedro M. 1989). Exceptionally high cell concentrations like the one in question, will distort the inactivation data with high probability. On the contrary, decreased sample sizes increase variability and can undermine credibility. Therefore, it is critical to resolve the optimal sample-specific cell count in order to generate applicable test data. Parameters that need to be taken into consideration while conducting an inactivation experiment, depend to an extend on the applied test setup. Unlike with fluid disinfection, depletion of oxygen is not an issue with surface disinfection even with high cell counts; this eliminates one of the most intricate parameters.

5.2 Adjunct antibacterial effect

The photocatalytic effect does not activate without the energy of photons, yet the TiO_2-coated test tiles exhibited strong bactericidal properties compared to non-coated ones even when they were kept in the dark. This might be caused by NPs of silver, one of the doping agents of the photocatalytic coating. Antimicrobial property of various metals known as the oligodynamic effect has been demonstrated to inactivate wide spectrum of microbes including *E. coli* bacteria. This antimicrobial effect is at least partly due to generation of intracellular oxidative stress. Although, the specific mechanism of ROS generation is not yet known (Prasher P. *et al.* 2018).

However, it is unlikely that this effect explains the entire difference between the results from TiO_2-coated and non-coated control samples. In a study conducted in 2009, inner surface of a pure silver test pot did not significantly reduce the bacterial load of *E. coli* before the 8-hour timepoint; total reduction was achieved in 24 hours. The bactericidal effect of silver was significantly less effective compared to copper or brass pots, which both achieved total inactivation in less than 4 hours (Shrestha R. *et al.* 2009). Oligodynamic effect might increase endogenous ROS, but being non-catalytic it therefore simultaneously exhausts AgNPs, which are essential to the photocatalytic mechanism. There are multiple available visible light sensitive TiO_2-coatings that do not contain NPs of silver. In a following experiment the influence of oligodynamic effect should be assessed by comparing the bactericidal activity of Ag-containing and non-containing photocatalytic coatings. However, the rate of silvers accumulation and the attendant bactericidal activity greatly varies between different stains (Clement J. & Jarrett P. 1994). A strong oligodynamic effect would suggest that Ag doped photocatalytic coating would decrease in its sensitivity to visible light, once AgNPs are exported into cells. Another prospect would be to utilize a strain with a high natural silver-resistance in the following inactivation experiments, *e.g.* Pseudomonas stutzeri AG259 (Mattuschka B. *et al.* 1993).

To prevent premature accumulation of ROS on the surfaces of TiO_2-coated test tiles – *i.e.* prior to the timepoint zero – all light was tightly controlled. In addition, ventilation of the MSC II was in operation during the arrangement of the test tiles, the pipetting of the bacterial suspension and the following drying period. ROS generated prior to the timepoint zero would have distorted the results by increasing the level of initial oxidative stress. Despite these actions, some additional ROS might have been present. This issue is possible to address to an extent during data analysis, by calculating the sample-specific percentual decline in the viable cell concentration compared to timepoint zero, instead of only comparing decreases in CFU counts. This would even out the start and make the visual presentations easier to read, however this would not take into consideration the possible disruptive influence of dead cell material.

5.3 Excess organic material

During the first four test cycles freeze-dried bacterial pellets were utilized to maintain the cell concentration constant. In this process – of freezing and freeze-drying – about half of the cells are neutralized leaving behind only about 50 % of viable cells to be utilized in the following experimentation. Therefore, for each viable cell in the suspension there is correspondingly one cell worth of dead, organic debris. In addition to dead bacterial biomass, nutrients like amino acids and peptides are present if TSB medium is being used to create the cell suspension. During the photon disinfection this organic material might significantly affect the rate of inactivation. Exposed viable cells are shielded from a notable portion of the incoming photons by the excess organic material. This would mean that to achieve the same level of inactivation comparative to real-world circumstances, intensity of aBL must be raised. Dead cell material and dried nutrient broth could also weaken the bactericidal effect of titanium dioxide by reacting with the generated ROS. In real-world circumstances, surface concentrations of bacteria do not reach the levels used in this experiment and shielding of this kind does not occur. In the future experiments, cells should be suspended into minimal medium or buffer solution before the exposure to the aBL. This might degrease the observed level of inactivation due to the decreased rate of metabolism, but simultaneously solve the issue of excess organic material and depict the real-world circumstances more accurately.

5.4 Comparison between high-nutrient and minimal environment

As described in the subchapter about the pretesting process, *E. coli* cells were inactivated on the surfaces of TSA plates with an immense percentage of mortality. However, to observe the synergetic effect of aBL inactivation and PCD – as the main experimentation of this thesis was – stainless steel test tiles had to be utilized to preserve the bacteria during photon disinfection. In the 6-hour exposure period, the level of surface inactivation was only about 84 % with samples from the non-coated test tiles. This translates into a survival rate of 16 %. Which is more than one hundred times the survival rate of the same bacteria exposed to the same amount energy, in a high-nutrient environment. However, it is important to keep in mind that this difference in inactivation efficiency might not be as extreme with other microbes as it was with this substrain of *E. coli* in this specific test setup. The observed difference in efficiency supports the findings of the previous inactivation studies, and the theory behind it is disclosed in the subchapter of this thesis about the effects of different conditions to aBL inactivation. Currently it is unclear, how much of this difference can be attributed to the influence of the nutrient content over the metabolic rate, or to the level of photocatalytic activity of photosensitizers in the media. More research is needed to clarify the contribution of these factors, but their influence on the inactivation rate is definite.

The significant difference in inactivation efficiency between high-nutrient and minimal environment might question the validity of the aBL as an efficient surface disinfection method. This concern applies only to environments with low or non-existent levels of nutrients and not *e.g.* to fluid disinfection or aPDT. Most of the current test data has been generated in experiments were culture medium or Petri-dishes were utilized during the photon disinfection process, both usually containing high-levels of available nutrients. The divergence in efficiency due to the aforementioned environmental factors needs to be taken in consideration when photocatalytic disinfection method is being implemented.

Even when these factors have been taken into consideration, like in a review published in the March of 2019 about the use of photon disinfection in hospitals, some questions can remain (Cabral J. & Rodrigues A. 2019). The review contains a discussion about the possible use of aBL irradiation in clinical areas, whilst citing an inactivation experiment that was conducted by suspending a bacterial pellet in PBS solution before the exposure to aBL (Maclean M. *et al.* 2009). Even when the reaction medium itself is not high in nutrients, the metabolic rate and the ensuing concentrations of endogenous photosensitizer – in all probability – need some time period to decline. Rapid change in environmental conditions might not be enough to address the issue.

5.5 The importance of aBL-source design

As previously stated in the subchapter about the effects of different conditions; when studying or experimenting with photon disinfection it is important to consider the exact properties of the utilized aBL-source. In addition to basic parameters like the wavelength maxima or the intensity of irradiance, inconsistent uniformity in illumination might lead to suboptimal test data. This parameter is highly dependent on the test setup and can be easily compromised by the utilization of too point-like light source *e.g.* a single LED or an array with insufficient surface area. A great example of this can be found in an inactivation experiment conducted utilizing 20 mm × 16 mm LED array (Murdoc L. *et al.* 2012). This resulted in highly varying power density distribution across the used test area and the need for custom-designed aBL-source – to enable more uniform intensity distribution – was acknowledged in the discussion chapter of the aforementioned study.

The inactivation experiment conducted in association with this theses, utilized a LED array specially designed and manufactured for photon disinfection. Illuminance uniformity of 0.9 was attained for the whole test area just by adjusting the distance of the light source to the surface. In addition to uniformity in power density distribution, specially designed LED based aBL-sources enable narrow spikes in optimal segments of the electromagnetic spectrum, low-energy consumption and vast possibilities for additional optimization. A study conducted in 2017 compared the bacterial inactivation rates of single and double exposure periods. Both test groups

received the total dose of 121 J/cm^2; first-group continuously, latter-group with a 30-minute interval in between two equivalent doses (Biener G. *et al.* 2017). Results showed a logarithmical decline to be significantly greater with double exposure compared to the single exposure; 1.71 to 1.05 respectively. Another light-source related study – worth mentioning in this discussion – was also published in the same year and demonstrated the possible benefit of pulsed light. This experiment utilized λ 405 nm LED aBL with a pulsing frequency of 100 Hz to 10 kHz to target *S. aureus*. Approximately the same level of inactivation was achieved with pulsed light compared to the control, while simultaneously a significant 83 % increase in optical efficiency was observed, *i.e.* higher inactivation per unit of optical energy (Gillespie J. *et al.* 2017).

5.6 The experimental method

Although the test tile surface sampling method was utilized successfully during this experimentation, lots of time and cycles were spent on parameter configuration. Further research is still needed about the correct implementation of this method and about the effects of parameter adjustment to the gathered test data. During this collaborative, available time in laboratory was very limited and thusly insufficient for extensive investigation of aforementioned effects. However, the experimental sampling method might prove itself useful in successive laboratory studies related to microbial surface concentrations due to its high comparability and yield, compared to the more traditional surface swab sampling method. When utilized, proper determination of the apposite initial cell count is critical for the success of the experimentation.

In addition to increased yield, a method that utilizes surfaces instead of Petri-dishes, mediums or buffer solutions during photon disinfection, might have some other benefits as well. Illumination setups, which contain transparent sample vessels or even transparent agar surfaces might increase irradiation levels by accidentally reflecting excess light to the exposed sample area (Hessling M. *et al.* 2017). This would be a non-issue with a uniformly flat surface, although the distortive influence of excess reflections should be assessed to estimate this possible benefit.

6. WORKS CITED

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7. ATTACHMENTS

#1 The antibacterial synergetic effect of titanium dioxide and blue light - experiment protocol