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Comparison of UVB and UVC irradiation disinfection efficacies on *Pseudomonas Aeruginosa (P. aeruginosa)* biofilm

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ABSTRACT

Disinfection routines are important in all clinical applications. The uprising problem of antibiotic resistance has driven major research efforts towards alternative disinfection approaches, involving light-based solutions. *Pseudomonas aeruginosa* (*P. aeruginosa*) is a common bacterium that can cause skin, soft tissue, lungs, kidney and urinary tract infections. Moreover, it can be found on and in medical equipment causing often cross infections in hospitals. The objective of this study was to test the efficiency, of two different light-based disinfection treatments, namely UVB and UVC irradiation, on *P. aeruginosa* biofilms at different growth stages. In our experiments a new type of UV light emitting diodes (LEDs) were used to deliver UV irradiation on the biofilms, in the UVB (296nm) and UVC (266nm) region. The killing rate was studied as a function of dose for 24h grown biofilms. The dose was ramped from 72J/m2 to 10000J/m2. It was shown that UVB irradiation was more effective than UVC irradiation in inactivating *P. aeruginosa* biofilms. No colony forming units (CFU) were observed for the UVB treated biofilms when the dose was 10000 J/m2 (CFU in control sample: 7.5×10^4). UVB irradiation at a dose of 20000J/m2 on mature biofilms (72h grown) resulted in a 3.9 log killing efficacy. The fact that the wavelength of 296nm exists in daylight and has such disinfection ability on biofilms gives new perspectives for applications within disinfection at hospitals.

Keywords: Disinfection, Ultraviolet light, UVC, UVB, light emitting diodes (LEDs), *Pseudomonas aeruginosa* (*P. aeruginosa)*, sterilization.

1. INTRODUCTION

Biofilm-contaminated medical devices are believed to be a common cause for hospital acquired infections[1]. Moreover, biofilms are implicated in chronic infections such as chronic wounds and tissue filler-, implant- and catheter-associated infections[2]. Traditionally microbial infections are treated by antibiotics that inhibit the expansion of the contaminated area or kill the microbe[3]. However the problem with this approach is that antibiotic resistance is developed[4]. Furthermore, biofilms exhibit greater tolerance to antibiotics and antimicrobial stressors than planktonic organisms of the same species[5]. As a consequence researchers have turned their interest towards alternative disinfection approaches, including bacteriophage[6], bacteriocins[7] and light based treatments[8]. Ultraviolet (UV) radiation[9,10], photodynamic therapy[11,12], blue[13,14] and near infrared light[15,16] have been reported to have the advantage of non-invasiveness and the ability to inactivate microorganisms. Moreover, the expectation that the bacteria will develop light-resistant genes is low[8]. On the other hand, light-based solutions demand effective light delivery. Thus, the potential applications are confined by penetration depth of light to the region of interest. For this reason, branches of medicine like dermatology and dentistry were the first to adopt the technology, due to the easier optical access to the region of interest.

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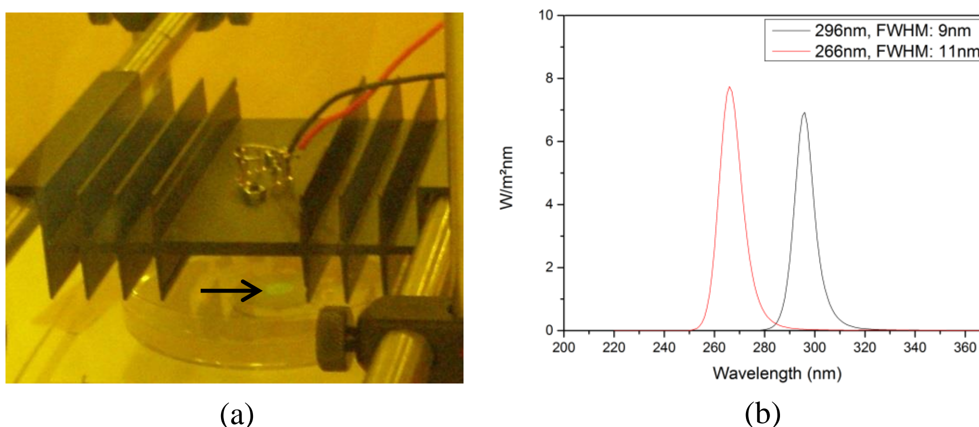
In this work the efficiencies of two different light-based disinfection treatments were tested, namely UVB and UVC irradiation, on *P. aeruginosa biofilms* at different growth stages. Their antibacterial action was studied as a function of dose. The survival curves were modelled using GInaFiT[17], a freeware tool to assess non-log-linear microbial survivor curves.

2. MATERIALS AND METHODS

2.1 UV Irradiation system

UV light emitting diodes (LEDs) were used to deliver UV irradiation on the biofilms, in the UVB (296nm) and UVC (266nm) region. The setup for exposure of the biofilms is shown in Fig. 1a. The distance between the biofilm and the light sources was kept constant in all exposures. The spectral power distribution of the irradiation sources is shown in Fig. 1b. The LEDs were purchased from Sensor Electronic Technology, Inc (SETi, Columbia, SC, USA; TO3 package, hemispherical lens window, half angle of 20-25 degrees). Details about the irradiance measurements protocol and the setup can be found in Barnkob et al. [18].



(a) (b)

Figure 1. (a): The setup for exposure of biofilms to UVB and UVC irradiation, using LEDs. The biofilm is fluorescing under UV irradiation (greenish spot on the agar plate, arrow) (b): Spectral irradiance of the UVC (red curve) and UVB LEDs (black curve) used for the irradiation of the biofilms.

2.2 Biofilm preparation

The biofilms were grown on AB-trace glucose (0.5%) and incubated for 24 hours (h) and 72 h at 37°C. The *P. aeruginosa* (strain PAO1) used for the experiments was purchased from the *Pseudomonas* Genetic Stock Center (www.pseudomonas.med.ecu.edu). The biofilm was plated on cellulose nitrate membrane filters (Whatman™). The biofilms were kept in a UV free environment before the exposures. The biofilm was visible with naked eye and was fluorescing under UV exposure (see Fig. 1a, arrow). For a mature biofilm to develop (72 h grown), the membrane filter (containing the developing biofilm) was cautiously released and transferred to a fresh, agar plate every 24 h. In this way the biofilm had access to fresh media and enabled a growth comparable to biofilm growth attained by more complicated methods[19].

2.3 Colony forming units (CFU)

After the UV exposure, the (biofilm) plated cellulose nitrate membrane filters were immersed in sterile saline and degassed for 5 min before 5 min ultra-sonication. Serial dilutions (tenfold dilution for each step, 8 steps) were made and plated onto lysogeny broth medium. Control samples, *i.e.* biofilms which were not exposed to UV irradiation, were plated every hour and included in the study (tenfold dilutions, 8 steps); as a reference for growth (Fig. 2). All samples after treatment were kept in a UV free environment. CFUs for determining growth were counted after 24h of incubation

in the dark at 37°C. Zero counts of CFUs on all replicas after treatment were indicating total disinfection. Note that CFUs are per ml.

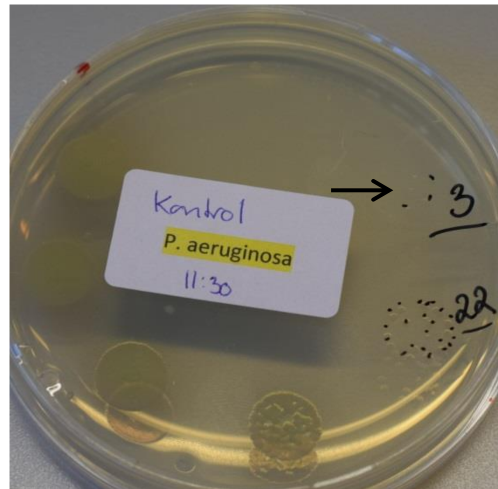


Figure 2. Picture of control sample serial dilutions' generated at 11:30. The control samples (not UV exposed) are used as a reference for growth. At higher dilutions, countable and well separated colony forming units were formed (arrow).

2.4 Experimental design

The objective was to determine how UV range (UVB versus UVC) and dose influence the viability of *P. aeruginosa* biofilms. The dose was ramped from 72J/m2 to 10000J/m2. The total irradiance among the different doses was varied between 18,6 W/m2 and 108,4 W/m2 with the UVC diode. All UVC exposures lasted less than 1 minute and 32 seconds. For the UVB diode the total irradiance was varied between 1 W/m2 and 14,8 W/m2. In all UVB treatments the exposure time was less than 12 minutes (clinical treatment should not exceed 15 minutes for convenience). All tests, on 24h and 72h grown biofilms, were repeated respectively 3 and 2 times (triplet or doublet determination).

2.5 Modelling of biofilm survival curves

GInaFiT[17], (Geeraerd and Van Impe Inactivation Model Fitting Tool), a freeware Add-inn for Microsoft® Excel, was used to model the biofilm survival curves. The tool supports testing of nine types of microbial survival models, and five statistical measures (i.e., sum of squared errors, mean sum of squared errors and its root, R2, and adjusted R2) are provided to monitor the best fit (*f* function). Here, a choice of five suitable models [20-24] was applied to the mean values of log survival (obtained from experimental data):

$$\text{Log survival} = \text{Log}\,(N_{treated}/N_{control}) = f\,(\text{dose}) \quad (1)$$

where $N_{treated}$ is the number of CFUs after a UV fluence (J/m^2) is delivered to the biofilm, $N_{control}$ is the number of CFUs on the controls (not UV exposed). Since log(0) is not defined; when zero counts are observed in $N_{treated}$, log survival is equal to –x, where x is the order of magnitude of the $N_{control}$ (example if $N_{control}$ is of the order 10^6, and $N_{treated}$ had zero counts, log survival=-6).

3. RESULTS

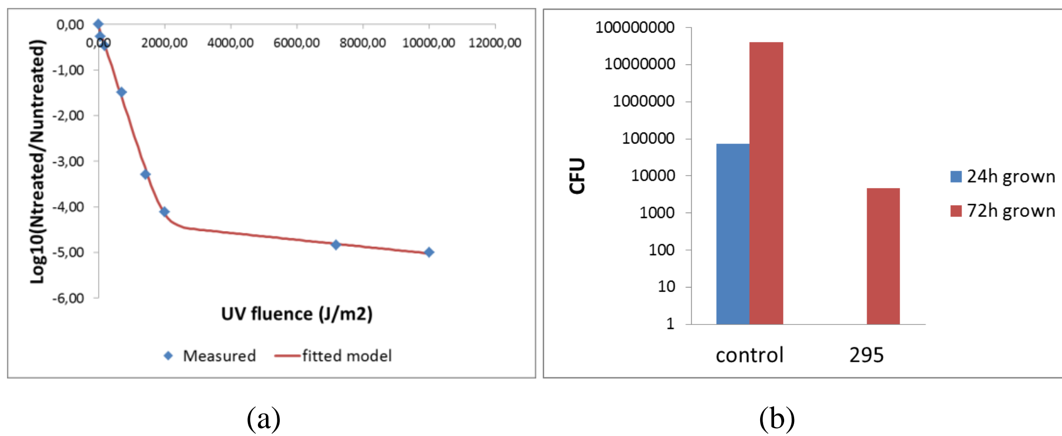
3.1 UVB antibacterial action

The log survival values of *P. aeruginosa* biofilms, after being exposed to UVB irradiation (see spectral power distribution in Fig. 1b, black curve) with a central wavelength at 296nm, as a function of UV fluence (dose) are presented in Fig. 3a. Most models demonstrated a good fit to the experimental data ($R^2 \geq 0{,}9$) except the linear model [20]. The "biphasic" model[24] fitted best to the data (Fig. 3a). Though, this model requests minimal 10 points for assuring validity. The statistical measures of the models applied are presented in Table 1. If we use the concept of reliability engineering the reliable dose d_R[25] (dose needed to reduce number of microorganisms by a factor of 10) is calculated to be

638 J/m^2 (from the "Weibull model" the hazard rate was found to be α=54,86 and the shape parameter β=0,34). The GInaFiT tool predicts that the needed dose for achieving a 4log reduction in the CFUs is 1900J/m^2, in accordance with the experimentally observed value (at 2000J/m2, 4.1 log reduction). No CFU were observed when a dose of 10000J/m^2 was delivered to the biofilm, indicating total disinfection. Mature biofilms (grown for 72h) were much more resistant to the UVB treatment (Fig. 3b). A dose of 20000J/m2 on mature biofilms (72h grown) resulted in a 3.9 log reduction. Higher resistance of mature *P. aeruginosa* biofilms (grown for 48h) has also been reported with various antibiotic treatments (tobramycin, colistin, ciproflox)[19].

3.2 UVC antibacterial action

The antibacterial action of UVC irradiation, as a function of UV fluence (dose), with a power spectral distribution as shown in Fig. 1b (red curve) is shown in Fig. 4. The "biphasic" model fitted best to the data (not shown). From the "Weibull model[22]" (Fig. 4) with α=9870 and β=0,44 the reliable dose d_R[25] was calculated to be 65722J/m^2. The statistical measures of the models applied are shown in Table 1. The GInaFiT tool in this case was unable to predict the needed dose for achieving a 4log reduction (maximum reduction achieved experimentally was log1).



(a) (b)

Figure 3.(a): UVB fluence dependent antibacterial action on *P. aeruginosa* biofilms. "Biphasic model" fitted exceptionally to the experimental data (statistical measures of fitted models presented in Table 1). However, this model requires minimal 10 points for assuring validity. (b): Comparison of effectivity of UVB treatment on mature-72h grown (blue columns) and non-mature-24h grown (red columns) *P. aeruginosa* biofilms. Total disinfection from a non-mature biofilm was achieved with a dose of 10000J/m^2. A dose of 20000J/m^2 resulted in a 3.9 log reduction on mature biofilms.

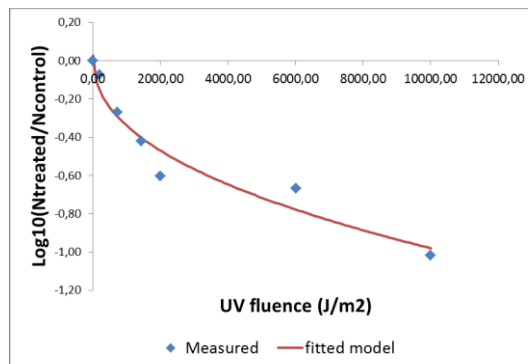


Figure 4. UVC fluence dependent antibacterial action on *P. aeruginosa* biofilms (experimental data and Weibull fit).

Table 1. Statistical measures of the models applied to the experimental data for log reduction of *P. aeruginosa* by UVB (and UVC).

Fitted model	**MSE**	**RMSE**	**R^2**	**R^2-adjusted**	**SSE**	**D 4log**
Log-linear	1,6 (0,03)	1,28 (0,16)	0,69 (0,84)	0,63 (0,80)	9,84 (0,13)	8700 (NA)
Weibull	0,60 (0,01)	0,78 (0,1)	0,90 (0,95)	0,87 (0,92)	2,99 (0,04)	3300 (NA)
Biphasic	0,01 (0,01)	0,09 (0,08)	0,9989 (0,98)	0,9980 (0,95)	0,04 (0,02)	1900 (NA)
Log+tail	0,014 (0,02)	0,12 (0,12)	0,9978 (0,92)	0,9970 (0,88)	0,07 (0,06)	1900 (NA)
Albert (Weibull+tail)	0,017 (NA)	0,13 (NA)	0,9978 (NA)	0,9960 (NA)	0,07 (NA)	1900 (NA)

4. DISCUSSION

In the present study we characterized the disinfection efficacies of *P. aeruginosa* biofilms (strain PAO1) under narrowband UVB (central wavelength 296nm, FWHM 9nm) and UVC (central wavelength 266nm, FWHM 11nm) irradiation. It was demonstrated that narrowband UVB at 296nm is more efficient in killing the biofilms, than narrowband UVC at 266nm. To the contrary, several studies have shown in the past that UVC irradiation is much more effective than UVB in eliminating bacteria in solution [26,27]. When the bacteria are in planktonic state it is expected that the penetration of light will be much different, than in a biofilm where bacteria are aggregated and embedded in the extracellular polymeric matrix (EPS)[28]. Nevertheless, the defense mechanisms in a biofilm are more sophisticated due to processes like quorum sensing[29,30]. So investigations for determining the killing efficiency of various treatments against biofilms have special interest. Disinfection of catheter biofilms has been successfully reported by UVC LEDs[31]. To our knowledge, the ability to eliminate a biofilm grown for 24h and 72h, with UVB LEDs (narrowband spectral power distribution), has not previously been reported. Studies on the response of biofilm bacterial communities to various ranges of UV radiation (namely UVC, UVB and UVA) have been demonstrated before[32] with broadband light sources and have shown that UVC is more effective than UVB, while UVA has a significant effect only when photosensitizers are present. The contradiction between the results presented here and the mentioned[32] might be either due to 1) a different formation of the biofilms or 2) due to the differences between narrowband and broadband irradiation (or both 1 and 2).

Modelling of the dose dependent killing of the *P. aeruginosa* biofilms with UVC and UVB light showed that a log-linear model (based on first-order kinetics) failed to fit the experimental data and provide a satisfactory estimate for the needed dose for achieving a 4log CFU reduction. On the contrary, more complex models that take into account a “shift of behavior” (Weibull, biphasic, log+tail, Albert) for a subgroup of bacteria succeeded to fit to the experimental data ($R^2 \geq 0,9$). Moreover, the ”Weibull model was adequate for giving a rough estimation/prediction (correct order of magnitude) of the reliable dose d_R, for both UVB and UVC treatments. Weibull models have been reported to successfully predict inactivation of *Escherichia coli* and *Salmonella enterica* after exposure to ozone or pulsed light (100–1100 nm)[33]. Finally, β values were <1 both for UVB and UVC treatments meaning that there are some persistent bacteria in the biofilm that have less probability of being killed, perhaps because they adapt to the irradiation or because they are not as affected. e.g. due to limited penetration depth of light to the biofilm’s deepest layers and/or due to the shielding effect of the EPS.

5. CONCLUSSION

A 1 log killing efficacy was achieved on *P. aeruginosa* biofilms for a dose of 10000J/m2 with the UVC diode, while the UVB diode achieved a 3 log reduction at a dose of 1440J/m2 for the 24h grown biofilms. No CFU were observed for the UVB treated biofilms when the dose was 10000 J/m2 (CFU in control sample: 0,75 x 10^5). UVB irradiation, at a dose of 20000J/m2, on mature biofilms (72h grown) showed that CFU were reduced in average from 4.0 x 10^7 (untreated) to 4.65 x 10^3 (UVB treated), resulting in a 3.9 log reduction. These results show that UVB irradiation was more effective than UVC irradiation in killing *P. aeruginosa* biofilms. The efficiency of killing by irradiation is reduced when the target biofilm is mature (left to grow for 72h). That supports the hypothesis about the importance of penetration depth, since mature biofilms create a thicker matrix expected to be less penetrable by light.

The log-linear model (based on first-order kinetics) failed to fit the experimental data. The shape parameter (β) of the "Weibull model" was <1 both for UVB and UVC treatments meaning that there were some bacteria in the biofilm that had less probability of being killed.

The fact that the UVB irradiation (wavelength of 296nm) exists in daylight and has such disinfection ability on biofilms gives new perspectives for applications within disinfection at hospitals.

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