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Photoinactivation of Bacteria Attached to Glass and Acrylic Surfaces by 405 nm Light: Potential Application for Biofilm Decontamination

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ABSTRACT

Attachment of bacteria to surfaces and subsequent biofilm formation remains a major cause of cross-contamination capable of inducing both food-related illness and nosocomial infections. Resistance to many current disinfection technologies means facilitating their removal is often difficult. The aim of this study was to investigate the efficacy of 405 nm light for inactivation of bacterial attached as biofilms to glass and acrylic. *Escherichia coli* biofilms (10^3–10^8 CFU mL^{-1}) were generated on glass and acrylic surfaces and exposed for increasing times to 405 nm light (5–60 min) at *ca* 140 mW cm^{-2}. Successful inactivation of biofilms has been demonstrated, with results highlighting complete/near-complete inactivation (up to 5 $\log_{10}$ reduction on acrylic and 7 $\log_{10}$ on glass). Results also highlight that inactivation of bacterial biofilms could be achieved whether the biofilm was on the upper "directly exposed" surface or "indirectly exposed" underside surface. Statistically significant inactivation was also shown with a range of other microorganisms associated with biofilm formation (*Staphylococcus aureus, Pseudomonas aeruginosa* and *Listeria monocytogenes*). Results from this study have demonstrated significant inactivation of bacteria ranging from monolayers to densely populated biofilms using 405 nm light, highlighting that with further development this technology may have potential applications for biofilm decontamination in food and clinical settings.

INTRODUCTION

Bacterial attachment to surfaces under the correct environmental conditions often leads to biofilm formation. The structure of a biofilm can range from simple monolayers to vast complex multicellular structures, either of single or mixed species and can act as a protective barrier against hostile environmental conditions providing resistance to both physical and chemical stresses (1–4).

Biofilm formation is a well-recognized problem within food and health-care industries, with their presence having detrimental effects on both food quality and safety, and infection control. Multiple bacterial species including *Escherichia coli, Salmonella, Staphylococcus, Listeria* and *Pseudomonas* are all capable of attaching and inducing biofilm formation on various surfaces including metals, glass and plastics (5,6), allowing for a continuous bacterial reservoir often leading to further contamination.

Food production premises provide an ideal environment for biofilm formation. These, often moist, environments have a continuous supply of nutrients from various food products, as well as vast surface areas for attachment and continuous supply of inoculum to initiate biofilm formation (1,7,8). Their existence in food environments is not only problematic in terms of consumer health but also has massive financial implications in terms of product loss, justifying the need for investment in novel disinfection technologies.

Within clinical environments formation of microbial biofilms is a notable problem, contributing to the transmission of hospital-acquired infections. It has been suggested that the presence of patient fluids such as blood, urine and saliva influence bacterial adhesion and biofilm development (5). A recent study highlighted that the presence of *Pseudomonas aeruginosa* biofilms on sink areas directly resulted in 36 patients acquiring infection of which a 33% death rate was observed (9). Further studies have demonstrated that biofilm formation on indwelling medical devices and implanted prosthetics may account for up to 25% of patient morbidity and mortality, with over one million related infections in the United States in 2010 (10,11).

Numerous decontamination technologies have been developed and integrated into industry to help minimize microbial contamination. Methods for reducing microbial contamination such as chemical disinfection are still heavily used, however, poor penetrability of chemical agents through biofilms is a major limitation, allowing bacterial survival, redispersal and further contamination (2,12,13). The continuous use of biocides, often at sublethal concentrations, is an important factor contributing to bacterial resistance, limiting the availability of effective disinfectant agents (14). Genetic adaptations in many bacterial species has led to the development of resistance against many chemical cleaning agents, thereby preventing sufficient disinfection and increasing the potential risk of pathogen transmission (14,15). Consequently, many biocides are failing to effectively disinfect open work surfaces, and novel methods of decontamination are continually being sought.

Recent studies have demonstrated the bactericidal properties of violet–blue 405 nm light against a range of both Gram-positive and Gram-negative bacterial species (16–24). Although not as bactericidal as UV light, 405 nm light has benefits relating to its higher safety and increased transmissibility. Photodynamic inactivation (PDI) of bacteria by exposure to 405 nm light has

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*Corresponding author email: karen.mckenzie@strath.ac.uk (Karen McKenzie) been attributed to the excitation of intracellular photosensitive porphyrin molecules. Excitation of these molecules with 405 nm light, results in the production of reactive oxygen species (ROS), most predominately singlet oxygen, and consequently oxidative damage and cell death (18,22).

Previous studies utilizing 405 nm light for inactivation of bacterial pathogens have demonstrated significant reductions in bacterial populations both in liquid suspension and seeded onto solid surfaces (20,23,25). This study investigates for the first time the significant bactericidal effect of 405 nm light on *E. coli* biofilms of varying maturity, generated on glass and acrylic surfaces. The transmissibility of 405 nm light is demonstrated by successful inactivation of bacterial biofilms after transmission through the transparent glass and acrylic surfaces. Results also highlight the effect of 405 nm light for inactivation of biofilms of other problematic biofilm-forming bacteria including *P. aeruginosa*, *S. aureus* and *L. monocytogenes*.

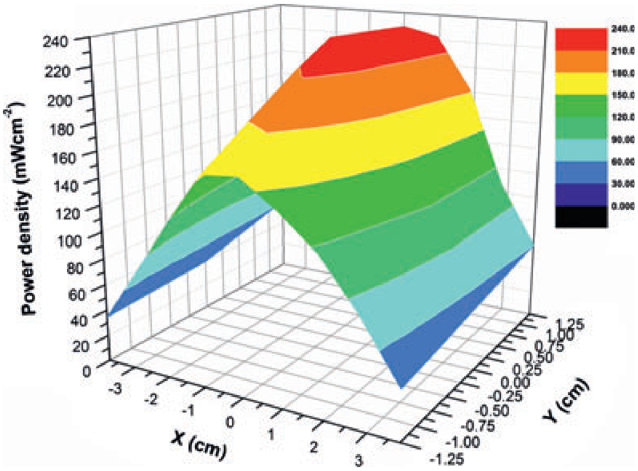

Figure 1. Three-dimensional model simulating the irradiance distribution of the 405 nm light across the glass and acrylic test surfaces (60 × 25 mm), plotted using OriginPro 8.1 software.

MATERIALS AND METHODS

Bacterial preparation. The bacteria used in this study were as follows: *E. coli* NCTC 9001; *S. aureus* NCTC 4135 (obtained from National Collection of Type Cultures, Colindale, UK); *L. monocytogenes* LMG 19944 and *P. aeruginosa* LMG 9009 (obtained from the Laboratorium voor Microbiologie, Universiteit Gent, Belgium). Bacteria were incubated in 100 mL nutrient broth (*E. coli, S. aureus* and *P. aeruginosa*) or tryptone soya broth (*L. monocytogenes*), (Oxoid Ltd, UK) at 37°C for 18 h under rotary conditions (120 rpm). Broths were then centrifuged at 3939 × ***g*** for 10 min, and the cell pellets resuspended in 100 mL (Oxoid Ltd) and diluted to a population density of 10^7 CFU mL^{-1} for experimental use.

Biofilm formation. Biofilms were prepared on glass and acrylic slides (60 × 25 mm). These materials were selected for two reasons: (1) they represent hydrophilic and hydrophobic surfaces; respectively, and (2) their transparency allowed transmission of the 405 nm light. To prepare biofilm samples, glass and acrylic slides were first cleaned with ethanol to sterilize and remove grease. For development of single-species monolayer biofilms, for both direct and indirect exposure, slides were fully immersed in 125 mL 10^7 CFU mL^{-1} bacterial suspension for 1 h to facilitate initial attachment. The bacterial suspension was then discarded and replaced with growth media (1.0 g bacteriological peptone and 0.7 g yeast extract L^{-1} in sterile distilled water) in which the slides were left for a further 4 h to allow development of a monolayer biofilm (method adapted from [1]). For development of more mature biofilms, slides were left in the growth media for increasing time periods (24, 48, 72 h). After biofilm development, slides were aseptically removed from the growth media and left to dry for 10 min in sterile conditions at room temperature prior to light exposure. For development of mixed-species biofilms, slides were immersed in a 125 mL 10^7 CFU mL^{-1} bacterial suspension containing 62.5 mL *E. coli* suspension and 62.5 mL *S. aureus* suspension for 1 h. Slides were then placed in growth media for a further 24 h to allow sufficient biofilm formation.

405 nm light source. An ENFIS Quattro Mini Air Cooled Light Engine (ENFIS Ltd, UK), containing an array of 144 light emitting diodes (LED) with a light emission of 405 nm (±5 nm), was used for exposure of bacterial biofilms. The light engine incorporated a heat sink and cooling fan to permit continuous ventilation and prevent overheating of the LED array, and was powered by a 48 V power supply. For exposure, biofilm sample slides were positioned directly below the LED array at a distance of 5 cm. Irradiance from the LED array at this distance was measured using a radiant power meter and photodiode detector (LOT Oriel). The variation in irradiance was measured across the dimensions of the slide, and measurements indicated greatest irradiance at the midpoint of the slide, with a gradual decrease toward the outer edges (Fig. 1). The average irradiance across the entire slide surface was calculated, using OriginPro 8.1 software package, to be 141.48 mW cm^{-2}. Test samples were light exposed for 5–60 min, giving a range of average doses from 42 to 504 J cm^{-2}. Control slides were set up and left on the laboratory bench with no 405 nm illumination.

In addition to directly exposing biofilms, the potential for 405 nm light to transmit through glass and acrylic slides and inactivate biofilms on the underside of slides was investigated. To do this, it was important to determine the transmissibility of the light through these slides. This was measured by placing the power meter detector head on the underside of the exposed surfaces: transmission of the 405 nm light through these materials was found to result in an approximate 4% loss in irradiance.

Swabbing and enumeration. Following light exposure, surviving bacteria were recovered from the slide using a sterile cotton-tipped swab moistened in PBS. The swabbing procedure involved rolling the swab forward and backward multiple times across the entire surface of the illuminated side of the slide to ensure maximum recovery of bacterial biofilm sample (1). This protocol was kept consistent for all sample slides. The swab was then immersed in a 10 mL volume containing 9 mL PBS and 1 mL 3% Tween-80 suspension and vortexed for 1 min to allow resuspension of bacteria from swab into suspension. The suspension was serially diluted, by transfer of 1 mL volumes into 9 mL of PBS. Samples were plated using the pour plate method, with 1 mL sample volumes overlayed with nutrient agar (*E. coli, S. aureus* and *P. aeruginosa*) or tryptone soya agar (*L. monocytogenes*): this method provided a detection limit of 1 CFU mL^{-1}. For enumeration of bacteria in a mixed biofilm population, in addition to pour-plating samples in nutrient agar to obtain the total viable counts (TVC), bacterial samples (100–500 μL) were plated onto mannitol salt agar (MSA) and violet red bile agar (VRBA), which allowed the selective growth of *S. aureus* and *E. coli* respectively. Plates were then incubated at 37°C for 18–24 h. Plates were enumerated manually by counting the bacterial colony-forming units (CFU) present on the plate. Results in figures are reported as bacterial CFU count per milliliter ($\log_{10}$ CFU mL^{-1}) as a function of time (minutes).

Statistical analysis. Experimental data are an average of a minimum of triplicate independent experimental results, with triplicate samples taken from each experiment. All data were analyzed using one-way ANOVA test with Minitab 15 statistical software, where significant difference was accepted at $P < 0.05$. Weibull statistics (26) was used to analyze the inactivation behavior of monolayer bacterial biofilms cultured on glass and acrylic surfaces, methodology for this analysis is described later.

RESULTS

Inactivation of *E. coli* biofilms on glass and acrylic surfaces

Results have demonstrated that *E. coli* biofilms on glass and acrylic surface materials can be successfully inactivated by 405 nm light exposure. Figure 2 shows results for the inactivation of *E. coli* biofilms on glass. The most rapid inactivation was observed with *E. coli* monolayer biofilms, with a 2.52 $\log_{10}$ CFU mL^{-1} reduction following 10 min exposure, and complete kill (3.55 $\log_{10}$ CFU mL^{-1} reduction) following 20 min exposure,

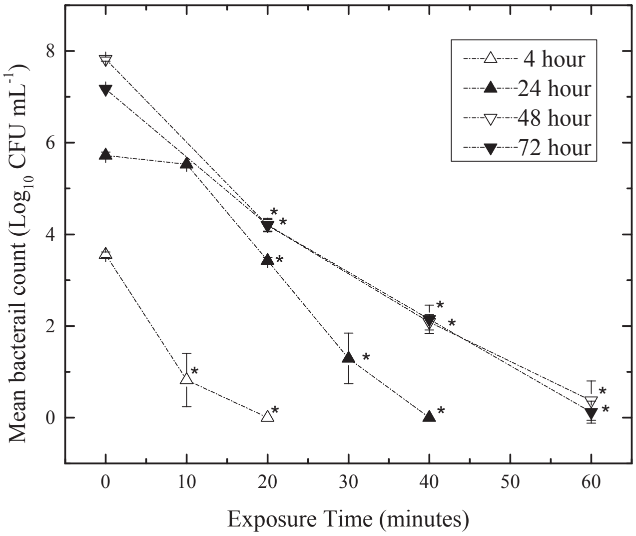

Figure 2. Inactivation of *Escherichia coli* biofilms on glass surfaces following exposure to 405 nm light with an irradiance of *ca* 140 mW cm^2, given as a function of time. Biofilms were allowed to develop for 4, 24, 48 and 72 h before light exposure. *Indicates statistically significant differences when compared with control samples ($P \leq 0.05$).

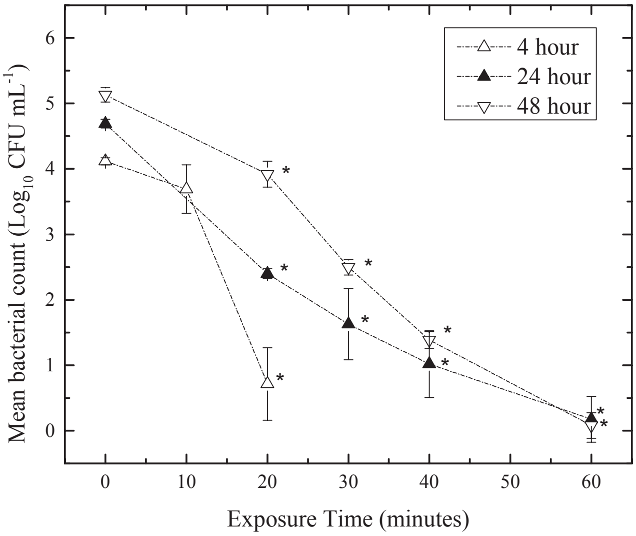

Figure 3. Inactivation of *Escherichia coli* biofilms on acrylic surfaces following exposure to 405 nm light with an irradiance of *ca* 140 mW cm^2, given as a function of time. Biofilms were allowed to develop for 4, 24 and 48 h before light exposure. *Indicates statistically significant differences when compared with control samples ($P \leq 0.05$).

as shown by the 4 h trend line on Fig. 2. After 24 h in the growth medium, bacterial biofilm populations on glass were shown to be *ca* 5.7 $\log_{10}$ CFU mL^{-1}. Inactivation of these biofilms occurred at a relatively linear rate, with reductions of 2.27, 4.41 and 5.7 $\log_{10}$ CFU mL^{-1} following exposure to 20, 30 and 40 min respectively. Biofilms on glass developed over 48 and 72 h periods had increased cell densities, with starting populations of between 7 and 8 $\log_{10}$ CFU mL^{-1} prior to light exposure. The rate of inactivation for these biofilms was very similar, with 3–3.5 $\log_{10}$ CFU mL^{-1} reductions achieved when exposed for 20 min, and a further *ca* 2 $\log_{10}$ CFU mL^{-1} reduction after a further 20 min. Near-complete inactivation of the 48 h biofilms and complete inactivation (<1 CFU mL^{-1} surviving) of the 72 h biofilms were achieved following 60 min exposure to 405 nm light.

Figure 3 demonstrates the inactivation kinetics of *E. coli* biofilms on acrylic. Monolayer biofilms on acrylic surfaces were reduced by *ca* 0.5 $\log_{10}$ CFU mL^{-1} after 10 min exposure, significantly less ($P = 0.002$) than the 2.52 $\log_{10}$ CFU mL^{-1} reduction observed on glass. After 15 min exposure, there was, however, a 3.33 $\log_{10}$ CFU mL^{-1} reduction in the biofilm population on acrylic, statistically similar to that achieved on glass at the same time point. After 24 h of biofilm development, bacterial populations were *ca* 4.7 $\log_{10}$ CFU mL^{-1} on acrylic slides. Biofilm inactivation occurred at a steady and consistent rate when applied with increasing exposure times of 405 nm light (20, 30, 40 and 60 min), resulting in reductions of 2.30, 3.07, 3.67 and 4.69 $\log_{10}$ CFU mL^{-1} respectively. Development of biofilms over a 48 h period generated a bacterial population of *ca* 5.1 $\log_{10}$ CFU mL^{-1}, where near-complete inactivation was achieved following exposure for 60 min (<1 CFU mL^{-1}). Bacterial biofilm formation on acrylic surfaces after 72 h growth period demonstrated no significant increase in bacterial count from that recorded after 48 h growth period ($P = 0.06$), therefore biofilms grown for 72 h on acrylic were not investigated.

The population densities of all nonexposed control biofilm samples, on both glass and acrylic, remained consistent throughout, with no significant differences recorded over the duration of the experiment, indicating that inactivation was a direct result of 405 nm light exposure. It is likely that the reason for no loss of viability in the control populations was due to the relatively short periods involved (up to 1 h periods) as well as the protective effect of the biofilm structure.

It is also worth noting that no significant temperature build up was observed on test samples during light exposure. Temperatures of both glass and acrylic surfaces were measured across the slide to give accurate representation of heat distribution across the entire area. For each material, temperatures were measured after maximum exposure periods using a thermocouple, which was pressed onto the test surface. The maximum temperature recorded was 33°C, indicating bacterial kill was not a result of direct thermal kill or desiccation through prolonged heat treatment.

Inactivation of *E. coli* monolayer biofilms through transmissible materials

Experiments were carried out to establish whether the 405 nm light could transmit through the glass and acrylic and inactivate biofilms on the underside of the slides. For "indirect" biofilm exposure, after removal from the growth medium, the upper side of the slide was wiped clean to ensure biofilm formation was only on the underside of the slide. The inactivation data for these "indirectly" exposed biofilms are presented in Fig. 4a,b as a comparison to the directly exposed biofilms.

Glass microscope slides have high transmittance in the visible light region around 400 nm. As a result, there were negligible differences between the inactivation for both direct and indirectly exposed biofilms on glass slides as can be seen from Fig. 4a. There was no significant difference ($P = 0.738$) between the direct and indirect $\log_{10}$ CFU mL^{-1} reductions following 10 min exposure, and complete inactivation (<1 CFU mL^{-1} survivors) was achieved for both samples after 20 min exposure. Similarly, Fig. 4b demonstrated similar inactivation curves for both direct

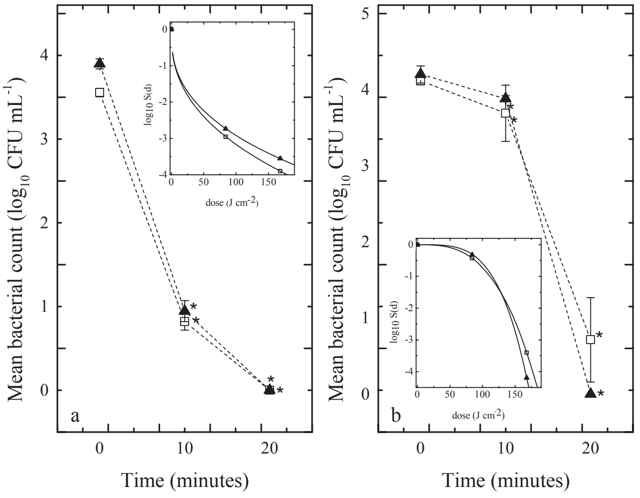

Figure 4. Inactivation of *Escherichia coli* monolayer biofilms on (a) glass and (b) acrylic, by direct (▲) and indirect (□) exposure to 405 nm light. Indirect exposure investigated transmission of the 405 nm light through the slides to inactivate biofilms on the underside of the slides. The average irradiance of 405 nm light across the slides was *ca* 140 mW cm^{-2}, reducing by *ca* 4% when transmitted through the slide. Results are given as a function of time. *Indicates a statistically significant difference when compared with control samples ($P \leq 0.05$). No significant difference was observed between direct and indirect inactivation on both surfaces. Weibull analytical fit lines are shown as inset graphs and were obtained using Eq. (2).

and indirect exposure of biofilms on acrylic, with no significant difference in the population reductions achieved with direct and indirect exposure after 10 min ($P = 0.421$) and 20 min ($P = 0.507$) which also indicates that the acrylic slides used in the present work transmit well visible 405 nm light. As noted, irradiance measurements determined that transmission of the 405 nm light through the glass and acrylic slides with monolayer growth resulted in an approximate 4% reduction in irradiance, however, this reduction was insufficient to cause significant differences in the inactivation rates of the directly and indirectly exposed biofilms on either the glass or the acrylic surfaces.

Weibull analysis

The inactivation behavior of monolayer bacterial biofilms grown on glass and acrylic surfaces was analyzed using the Weibull statistical approach, which can help in the identification of potential differences in inactivation mechanisms. The Weibull distribution has been employed for the analysis of microbial inactivation kinetics, including PDI studies (27,28), however, a study by Schenk *et al.* (29) which investigates UV microbial inactivation, states that "there is no information about the application of a Weibullian-type model to survival curves corresponding to microorganisms inoculated onto a solid surface." Therefore, it was interesting to examine the potential applicability of this statistical model to 405 nm light inactivation of biofilms cultured on solid surfaces.

In the Weibull approach, the 405 nm light dose, D_c, which is required to kill a single microorganism from an entire population, is considered as a measure of resistance of this organism to the light. It is also assumed that D_c is Weibull distributed. The survival rate of microorganisms which form biofilms, *S(D)*, is defined as the number of microorganisms surviving at a specific 405 nm light dose, *N*(*D*), divided by the initial number of microorganisms, N_0:

$$S(D) = N(D)/N_0 \tag{1}$$

In these conditions the survival rate, *S*(*D*), can be described by the Weibull cumulative distribution function, (26) and satisfies the following equation:

$$\log_{10}(S(D)) = -0.4343\alpha D^{\beta} \tag{2}$$

where α and β are parameters of the Weibull distribution.

Experimental inactivation data for monolayer biofilms on acrylic and glass surfaces exposed to direct and indirect 405 nm light have been represented as $\log_{10}$ (*S*(*D*)). These experimental date points and corresponding analytical fit lines obtained by Eq. (2) are shown in Fig. 4a,b (inset graphs). Analytical lines show downward concavity for the acrylic surface and upward concavity for the glass surface.

Coefficient β which determines the shape of the Weibull distribution has been obtained for both surfaces and both types of the light treatment as shown in Fig. 4. For acrylic surfaces (Fig. 4a), the shape parameter, β, is higher than 1: $\beta = 3$ in the case of the direct exposure and $\beta = 3.6$ in the case of the indirect exposure. $\beta > 1$ indicates that with an increase in 405 nm light dose, microorganisms in the biofilm become increasingly damaged and can be killed at a higher rate. In the case of the glass surface (Fig. 4b), the shape parameter is smaller than 1: $\beta = 0.378$ and 0.396 for direct and indirect exposures respectively. $\beta < 1$ means that the rate of inactivation is higher at lower 405 nm light doses, and this rate decreases with an increase in the light dose. Potentially, such inactivation behavior may indicate that remaining (surviving) microorganisms in the biofilm become more resistive to the external stress (405 nm light).

Comparison of the inactivation of different bacterial monolayer biofilms on glass surfaces

As a comparison to *E. coli*, the bactericidal efficacy of 405 nm light was tested against a range of other bacterial biofilms. *S. aureus, L. monocytogenes* and *P. aeruginosa* monolayer biofilms attached to glass surfaces were exposed to 5, 10 and 20 min of 405 nm light to determine the comparative levels of bacterial inactivation. Using the stipulated 4 h development period, it was found that the initial starting populations varied considerably between the different bacterial species, with higher populations found for the Gram-positive species. Results in Table 1 show that successful bactericidal effects were recorded with all the tested biofilms. Initial exposure for 5 min, resulted in between 0.6 and 1.5 $\log_{10}$ CFU mL^{-1} reductions for *S. aureus*, *L. monocytogenes* and *P. aeruginosa*, whereas at the same time point little change in population was observed for the *E. coli* biofilms. After 10 min exposure, 405 nm light achieved a 2.4–2.5 $\log_{10}$ CFU mL^{-1} reduction in bacterial population in both *E. coli* and *P. aeruginosa*, compared to 1.1 and 1.9 $\log_{10}$ CFU mL^{-1} reductions in *L. monocytogenes* and *S. aureus* biofilms respectively. Overall, the population reductions achieved following 20 min exposure were similar between the two Gram-negative bacteria (*ca* 3.6 $\log_{10}$ CFU mL^{-1}), with which complete inactivation was achieved, and between the two Gram-positive bacteria (*ca* 2.6 $\log_{10}$ CFU mL^{-1}).

Table 1. Inactivation of bacterial monolayer biofilms on glass surfaces following exposure to 405 nm light.

Bacterial species	Time (min)	Dose (J cm^{-2})	Nonexposed sample (mean log_{10} CFU mL^{-1})	Exposed sample (mean log_{10} CFU mL^{-1})	Log reduction (*$P \leq 0.05$)	*P*-values
E. coli	0	0	3.55 (±0.02)	3.55 (±0.02)	0	
	5	42	3.52(±0.05)	3.33(±0.05)	0.19	0.07
	10	84	3.39(±0.07)	0.89(±0.58)	2.50*	0.002
	20	168	3.41(±0.11)	0(±0)	3.41*	0.00
P. aeruginosa	0	0	3.58(±0.03)	3.58(±0.03)	0	
	5	42	3.47(±0.01)	1.97(±0.05)	1.5*	0.00
	10	84	3.59(±0.05)	1.16(±0.06)	2.43*	0.00
	20	168	3.72(±0)	0(±0.29)	3.72*	0.00
L. monocytogenes	0	0	4.14(±0.27)	4.14(±0.27)	0	
	5	42	4.10(±0.08)	3.49(±0.57)	0.61*	0.047
	10	84	4.24(±0.16)	3.15(±0.08)	1.09*	0.002
	20	168	4.14(±0.27)	1.66(±0.41)	2.48*	0.001
S. aureus	0	0	5.36(±0.07)	5.30(±0.13)	0	
	5	42	5.32(±0.33)	4.75(±0.06)	0.61*	0.004
	10	84	5.89 (±0.9)	3.45(±0.33)	1.87*	0.00
	20	168	5.36(±0.07)	3.14(±0.09)	2.75*	0.002

Inactivation of mixed-species biofilms

Mixed-species biofilms containing both *E. coli* and *S. aureus* were prepared on glass slides. Confirmation of the mixed population was obtained by microscopic view (Fig. 5) of a biofilm slide which had been Gram-stained to visualize the presence of both *E. coli* (pink rods) and *S. aureus* (purple cocci). Table 2 displays results from the exposure of these mixed biofilm populations, and also single-species complex biofilms (24 h growth period) of *E. coli* and *S. aureus*, to 405 nm light. Results show that after a 30 min exposure period, significant inactivation was achieved in all cases. Exposure of single-species biofilms induced a 4.37 log_{10} CFU mL^{-1} reduction in *E. coli* and a 2.97 log_{10} CFU mL^{-1} reduction in *S. aureus* biofilms. Successful inactivation was also observed in the case of the mixed biofilm population, with a 2.19 log_{10} CFU mL^{-1} reduction in TVC. Analysis of the pre- and postexposure biofilm populations using VRBA and MSA selective media demonstrated significant inactivation of both bacterial species present in the biofilm, with *ca* 1.2 and 1.7 log_{10} CFU mL^{-1} reductions in *E. coli* and *S. aureus*, respectively, being observed.

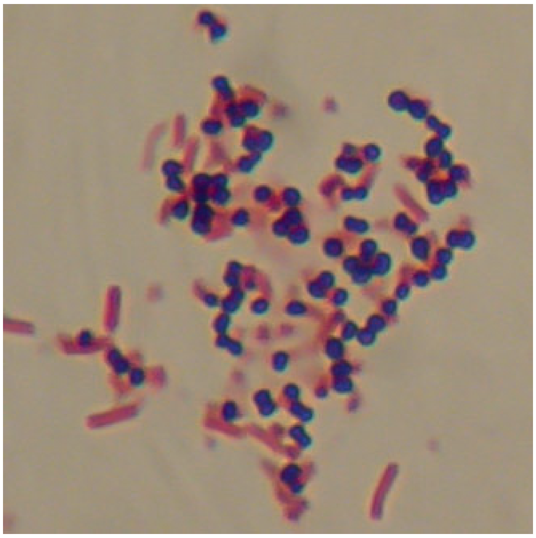

Figure 5. Microscopic visualization of a Gram stain of a mixed-species biofilm consisting of *S. aureus* and *E. coli* after 24 h development. Cells were viewed under oil immersion at ×1000 magnification.

DISCUSSION

Despite the development of new antimicrobial agents and novel sterilization and disinfection technologies, bacterial biofilms remain a significant problem in both the food industry and clinical settings. The current study has investigated, for the first time, the bactericidal effects of 405 nm light on bacterial biofilms, with results demonstrating successful inactivation of biofilms on both glass and acrylic surfaces, and that the bactericidal effect was observed with both monolayer and mature biofilm populations. Overall, results showed that successful inactivation was achieved with all complexities of *E. coli* biofilms generated on both glass and acrylic, with the general trend demonstrating that the more densely populated the biofilm, the greater the time (and consequently, the greater the dose) required for inactivation.

As previously discussed, bacterial biofilms can readily form on both glass and plastic surfaces, with production of an extracellular matrix in as little as 4 h (1,5,30–32). Studies have

Table 2. Inactivation of *E. coli, S. aureus* and mixed *E. coli* and *S. aureus* bacterial biofilms formed on glass surfaces over a 24 h period. Counts are provided for total viable counts, and in the case of the mixed biofilms, selective counts of *S. aureus* and *E. coli* have been provided using selective media: (MSA and VRBA).

Biofilm species	Bacterial count	Nonexposed biofilm (mean log_{10} CFU mL^{-1})	Exposed biofilm (mean log_{10} CFU mL^{-1})	Log_{10} reduction (*$P \leq 0.05$)
E. coli	TVC	5.66	1.29	4.37*
S. aureus	TVC	6.31	3.34	2.97*
Mixed	TVC	5.29	3.09	2.19*
	MSA	4.30	2.63	1.67*
	VRBA	2.29	1.10	1.19*

MSA = mannitol salt agar; TVC = total viable counts; VRBA = violet red bile agar.

reported stronger initial adhesion between bacteria and hydrophobic surfaces, such as plastics, compared with that of hydrophilic materials, including glass, which may account for initial variations in *E. coli* monolayer biofilm populations. Experimental data from this study highlighted that after 1 h, *E. coli* attachment to acrylic was greater when compared with that on glass surfaces, however, statistical analysis showed this to be insignificant ($P = 0.098$). Slight variation in *E. coli* monolayer biofilm starting populations, after 4 h development, (*ca* 0.5 $\log_{10}$ CFU mL^{-1}) was observed between the glass and acrylic surfaces, which may be an influence of bacterial interactions with surface material properties. Despite this slight difference, investigation into the 405 nm light inactivation of *E. coli* monolayer biofilms on glass and acrylic demonstrated successful results, with near-complete bacterial inactivation observed following exposure 20 min (approximate dose of 168 J cm^{-1}), highlighting the susceptibility of monolayer biofilms to 405 nm light.

In addition to monolayer biofilms, results demonstrated the successful inactivation of more mature *E. coli* biofilms on both on glass and acrylic surfaces. Population densities of these biofilms ranged from approximately 10^3–10^8 CFU mL^{-1}, with the more densely populated biofilms requiring increasing exposure periods for complete inactivation. Biofilms generated on acrylic surfaces over a 24 h time period required increased exposure time for complete inactivation when compared with those on glass surfaces, despite having significantly lower starting bacterial populations (*ca* 1.5 $\log_{10}$ CFU mL^{-1} lower). This may be an artifact of the physical adhesive properties between bacteria and specific materials. Chmieleweski and Frank (33) reported that although initial bacterial adherence to hydrophobic surfaces is likely to be stronger, greater maximum bacterial adhesion is achieved on hydrophilic surfaces, as a result of high free surface energy, allowing for generation of denser biofilm populations (33–35). This information correlates with data shown in this study, with results demonstrating greater adhesion and increased biofilm formation on hydrophilic glass surfaces, following development of mature biofilm structures. Results highlighted that mature biofilms developed over 24 and 48 h periods had bacterial densities of *ca* 4.5 $\log_{10}$ *versus* 6 $\log_{10}$ CFU mL^{-1} and 5 $\log_{10}$ *versus* 8 $\log_{10}$ CFU mL^{-1} for hydrophobic and hydrophilic surfaces respectively.

Bacterial biofilms generated on both glass and acrylic surfaces over 48 and 72 h appeared to have similar population densities, suggesting that after the 48 h growth period, bacterial attachment was maximized and had consequently plateaued. This may be attributed to a lack of nutrients present in the growth media after extended time periods, suggesting media must be replenished to generate increased biofilm populations.

Successful inactivation of bacterial biofilms on the underside of the glass and acrylic surfaces was also shown, demonstrating the ability of the 405 nm light to transmit through these transparent materials while maintaining its antimicrobial activity. With regards to the results of the Weibull analysis, the difference in the inactivation behavior of "young," 4 h monolayer biofilms could potentially be attributed to the different degree of adhesion of microorganism to these acrylic and glass surfaces, with the hydrophobic and hydrophilic interactions between the monolayer biofilms and the surfaces to some extent influencing the inactivation behavior. However, mature, 24, 48 and 72 h biofilms do not show similar tendencies. Moreover, their inactivation curves, shown in Figs. 2 and 3, demonstrate almost linear behavior and cannot be fitted with the Weibull curves Eq. (2). Therefore, it is possible to conclude that with an increase in biofilm "age" (biofilm thickness and/or number of microorganisms), the influence of substrate material on the inactivation process becomes significantly reduced or disappears completely.

It is necessary to note that although Eq. (2) can be used to fit the experimental inactivation data shown in Fig. 4, it is not possible to conclude that the proposed analytical lines are incorrect due to the limited number of experimental data points (only two 405 nm light doses have been used in the present inactivation tests). The present work is aimed only at identification of potential differences in the inactivation behavior and does not involve a full-scale statistical analysis which requires larger number of experimental data points. It is planned to conduct further direct and indirect tests using a greater number of 405 nm light doses to validate the proposed statistical model.

A variety of methods have been used for biofilm sampling in previous studies including sonication and swabbing (1,36–38). Despite all being successful and well-utilized methods for recovering microorganisms, each presents its own limitations. Swabbing is a well-recognized method for sampling bacterial contamination within health-care and food industrial settings as well as for recovery for bacterial biofilms in laboratory experiments (1,37,38). This method was used throughout this study as a viable and effective technique for bacterial biofilm removal from both glass and acrylic surfaces. Regardless of all inaccuracies/limitations associated with swabbing for bacterial removal, test and control samples in this study were recovered identically using a standard swabbing technique, allowing for directly comparable results.

The methodology for biofilm formation used in this study was adapted from previous work by Gibson and colleagues (1). The possibility of including a rinsing stage prior to light exposure to ensure all nonattached microorganisms were removed was investigated. Results demonstrated that there was no significant difference between biofilm populations on rinsed and nonrinsed slides, for both monolayer and more mature biofilms ($P > 0.08$). Rinsing of test surfaces provided conclusive evidence of biofilm formation as weakly attached cells would have been removed during the rinsing stage. Nonsignificant reduction in bacterial count following rinsing suggested bacteria were protected, most likely by the presence of an exopolysaccharide matrix which has been shown to protect cells concealed within biofilm layers from harsh environmental conditions such as flowing water (39).

Previous studies investigating 405 nm light exposure of bacterial suspensions and bacteria seeded onto nutritious surfaces have demonstrated its bactericidal effects (20,40). Studies have indicated that inactivation of Gram-positive bacteria require less exposure time than that of Gram-negative bacteria. Possible explanations of this trend have been accredited to cellular structure, where penetration of light through more structurally complex cells is reduced (41), and variation in the levels of different intracellular porphyrin molecules (40,42). Previous studies have identified numerous porphyrin molecules involved in the photodynamic inactivation of bacteria. A recent study by Dai *et al.* (43) highlighted the presence of both coproporphyrin and uroporphyrin in *P. aeruginosa*. Similarly, photoinactivation studies have demonstrated the presence of coproporphyrin in *S. aureus* and protoporphyrin in *E. coli*, whereas a range of various porphyrin molecules have been associated with *L. monocytogenes* (43–45).

Looking at the results of this study for the inactivation of the four different bacterial species, it can be seen that successful inactivation was achieved with all organisms, with approximate $\log_{10}$ CFU mL^{-1} reductions of 3.6 and 2.6 for Gram-negative and Gram-positive biofilm populations respectively. It is, however, difficult in the current study to directly compare the efficacy of 405 nm light for the inactivation of the different bacterial species due to the differences in starting populations observed within the monolayer biofilms. The methodology for preparing the biofilms was kept consistent for the different bacterial species, and this method resulted in the generation of varying populations, possibly reflecting differences in propensity for attachment and/or the rate of multiplication of the attached populations. Variance in bacterial inactivation between the Gram-positive and Gram-negative species (Tables 1 and 2) may have been a direct effect of the increased adherence of the Gram-positive cells causing increased starting populations, and consequently requiring a greater exposure for complete inactivation, compared with the lower populated Gram-negative biofilms. However, recent data published by Murdoch *et al.* (20) showed that even at similar starting populations, Gram-negative *Salmonella enterica* was inactivated 30% more effectively than Gram-positive *L. monocytogenes* when exposed to 405 nm light while seeded onto plastic surfaces (20).

In addition to investigating single-species biofilms, initial tests were carried out to assess the antimicrobial activity of 405 nm light against mixed-species biofilms. After a 24 h growth period, the population of mixed-species biofilm was lower than the populations achieved when compared with the single-species biofilms of *E. coli* and *S. aureus.* Analysis of mixed biofilm populations using VRBA and MSA selective media highlighted that although the total population was *ca* 5 $\log_{10}$ CFU mL^{-1}, it was found that the ratio between *S. aureus* and *E. coli* was uneven, with *S. aureus* being the dominant colonizer (data shown in Table 2). This is likely a direct result of interactions between the bacterial species and competition for attachment. When these mixed biofilms were exposed to 405 nm light, successful inactivation was achieved, with a 2.2 $\log_{10}$ reduction in total population demonstrated. Use of selective media also allowed assessment of the specific populations of *E. coli* and *S. aureus* within the mixed biofilm. VRBA and MSA were chosen for this purpose as they facilitated the selective isolation of *E. coli* and *S. aureus*, respectively, and importantly, these bacteria also act as negative controls when used on the alternative media (46). Results demonstrated that *S. aureus* was the predominant organism to colonize the biofilm, however, successful inactivation of both bacterial species was achieved, with significant reductions achieved in the case of both species. Microscopic examination of the mixed biofilm (Fig. 5) highlighted that biofilm distribution was not linear across the entire surface area, but instead displayed many large cellular communities with individual cells dispersed randomly between. Interestingly, it was also noted that attachment of *S. aureus* was largely present on top of previously colonized *E. coli* populations, suggesting that *E. coli* may act as a primary colonizer during biofilm formation, highlighting possible roles of cellular interactions during biofilm formation.

As discussed, the antimicrobial activity of 405 nm light can be attributed to excitation of porphyrin molecules within the cell, leading to production of ROS and oxidative cellular damage (18,22). Recent studies have suggested that oxidative damage may directly affect cellular membranes, resulting in reduced membrane stability (47). Interference with the cell membrane and its components may consequently reduce biofilm stability, leading to biofilm degradation through bacterial inability to remain attached to surfaces. It may also be plausible that following 405 nm light exposure, alterations in structural membrane components may possibly prevent cellular attachment and thus prevent biofilm formation. Although this study simply investigated the effects of 405 nm light on the viability of bacterial biofilms once attached to glass and acrylic surfaces, it would be of great interest to investigate the degradative properties of 405 nm light on bacterial biofilms as well as investigating the specific effects of 405 nm light on cellular adhesion. A previous study by Mussi *et al.* (48) investigated the use of longer blue-light wavelengths for inhibition of biofilm formation. Studies have shown many bacteria possess blue-light receptors, capable of causing photoregulated behavior upon exposure to blue light, and it has been suggested that direct interactions between blue light and subsequent receptors may have inhibitive properties relating to biofilm formation (24,48). However, Mussi and colleagues stated that the effect of light on biofilm formation was inconclusive; highlighting that further work is required to identify any relationship between blue light, blue-light receptors and biofilm formation.

The light irradiance produced by LED array used in this study has a Gaussian distribution, as with many LED-based light systems (49). Highest irradiance is found directly below the center of the LED array distributing gradually toward the outer edges. Irradiance over the entire test slide was measured (as shown in Fig. 1) and the average irradiance was calculated. Although there was a large difference in the measured irradiance at the center and the outer edges of the slide, the fact that (1) complete inactivation of the biofilm populations could be achieved and (2) swabs were used to recover bacteria from the entire slide not just the center point demonstrated that the inactivation effect was achieved across the entire slide, regardless of the nonuniformity of the light exposure. Although average doses could be calculated, due to the nonuniformity of the irradiance, results (Figs. 2–4) have been expressed as a function of exposure time. Previous work (25) has shown that 405 nm light microbial inactivation is dose dependent, therefore, due to dose being the product of irradiance × exposure time, the inactivation rates of these biofilms will be increased if an increased irradiance of light is used for exposure.

The use of light for biofilm decontamination has been extensively investigated, with particular focus on UV light due to its highly bactericidal properties (8,50). Evidence has suggested that UV-C radiation is largely absorbed by organic materials, such as biofilms, resulting in poor penetration of light and insufficient decontamination (50,51). Short wavelengths in the UV spectrum (200–280 nm) present poor penetrability, when compared with that of the visible spectrum. Data shown in Fig. 4 highlight the penetrability of 405 nm light, where both direct and indirect exposure of biofilm on glass and acrylic surfaces to 405 nm light produced almost identical inactivation curves. Detrimental properties associated with the use of UV light are also well recognized, greatly limiting its use for open surface decontamination. With regards to human safety, human exposure to UV radiation is limited due to the associated risk with development of skin and eye cancer (51). Polymer degradation is a further limitation of UV light, imposing financial restraints for continuous repair and replacement of degraded machinery and equipment (52). The safety and operational benefits of 405 nm violet–blue visible light make it suitable for development for both food-related and biomedical decontamination applications for both biofilm and general disinfection purposes. Previous studies have demonstrated the efficacy of 405 nm light for general environmental disinfection (53), but potential uses of violet–blue light also include disinfection of medical devices, and wound decontamination, with a recent study by Dai *et al.* (43) demonstrating the application of blue light for disinfection of *P. aeruginosa* infected burns in mice with no significant damage noted in skin cells.

In summary, this study has demonstrated for the first time the use of 405 nm light for the inactivation of bacterial biofilms. Biofilms of varying maturity and also of varying bacterial species have been shown to be susceptible to inactivation, demonstrating the ability of the 405 nm light to inactivate even densely populated biofilm communities. The ability to inactivate bacterial biofilms present on inert surfaces using 405 nm light is of great significance, and the penetrability of 405 nm light through transparent materials highlights further advantages of this bactericidal light, and demonstrates the potential for development of technologies using 405 nm light for practical decontamination applications within both the food industry and health-care settings.

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