1   Photoinactivation of Bacteria Attached to Glass and Acrylic Surfaces by 405 nm Light:

2   Potential Application for Biofilm Decontamination

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26 **ABSTRACT**

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28 Attachment of bacteria to surfaces and subsequent biofilm formation remains a major cause

29 of cross contamination capable of inducing both food related illness and nosocomial

30 infections. Resistance to many current disinfection technologies means facilitating their

31 removal is often difficult. The aim of this study was to investigate the efficacy of 405 nm

32 light for inactivation of bacterial attached as biofilms to glass and acrylic. *Escherichia coli*

33 biofilms (10^3-10^8 cfu mL^{-1}) were generated on glass and acrylic surfaces and exposed for

34 increasing times to 405 nm light (5-60 minutes) at ~140mW cm^{-2}. Successful inactivation of

35 biofilms has been demonstrated, with results highlighting complete/near complete

36 inactivation (up to 5 $\log_{10}$ reduction on acrylic and 7 $\log_{10}$ on glass). Results also highlight

37 inactivation of bacterial biofilms could be achieved whether the biofilm was on the upper

38 'directly exposed' surface or 'indirectly exposed' underside surface. Statistically significant

39 inactivation was also shown with a range of other microorganisms associated with biofilm

40 formation (*Staphylococcus aureus, Pseudomonas aeruginosa* and *Listeria monocytogenes*).

41 Results from this study have demonstrated significant inactivation of bacteria ranging from

42 monolayers to densely populated biofilms using 405 nm light, highlighting that with further

43 development, this technology may have potential applications for biofilm decontamination in

44 food and clinical settings.

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47 Key words: biofilms; disinfection; antimicrobial; visible light; bacteria

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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 (1,2,3,4)

Biofilm formation is a well-recognised problem within food and healthcare 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

76   biofilm development (5). A recent study highlighted the presence of *P. aeruginosa* biofilms

77   on sink areas directly resulted in 36 patients acquiring infection of which a 33% death rate

78   was observed (9). Further studies have demonstrated that biofilm formation on indwelling

79   medical devices and implanted prosthetics may account for up to 25% of patient morbidity

80   and mortality, with over one million related infections in the United States in 2010 (10,11).

81

82   Numerous decontamination technologies have been developed and integrated into industry to

83   help minimise microbial contamination. Methods for reducing microbial contamination such

84   as chemical disinfection are still heavily used, however poor penetrability of chemical agents

85   through biofilms is a major limitation, allowing bacterial survival, re-dispersal and further

86   contamination (2,12,13). The continuous use of biocides, often at sub-lethal concentrations is

87   an important factor contributing to bacterial resistance, limiting the availability of effective

88   disinfectant agents (14).   Genetic adaptations in many bacterial species has led to the

89   development of resistance against many chemical cleaning agents, thereby preventing

90   sufficient disinfection and increasing the potential risk of pathogen transmission (14,15).

91   Consequently many biocides are failing to effectively disinfect open work surfaces and novel

92   methods of decontamination are continually being sought.

93

94   Recent studies have demonstrated the bactericidal properties of violet-blue 405 nm light

95   against a range of both Gram-positive and Gram-negative bacterial species

96   (16,17,18,19,20,21,22,23,24). Although not as bactericidal as ultraviolet light, 405 nm light

97   has benefits relating to its higher safety and increased transmissibility. Photodynamic

98   inactivation (PDI) of bacteria by exposure to 405 nm light has been attributed to the

99   excitation of intracellular photosensitive porphyrin molecules. Excitation of these molecules

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100 with 405 nm light, results in the production of reactive oxygen species (ROS), most

101 predominately singlet oxygen, and consequently oxidative damage and cell death (18,22).

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103 Previous studies utilising 405 nm light for inactivation of bacterial pathogens have

104 demonstrated significant reductions of bacterial populations both in liquid suspension and

105 seeded onto solid surfaces (20,23,25). This study investigates for the first time the significant

106 bactericidal effect of 405 nm light on *E. coli* biofilms of varying maturity, generated on glass

107 and acrylic surfaces. The transmissibility of 405 nm light is demonstrated by successful

108 inactivation of bacterial biofilms after transmission through the transparent glass and acrylic

109 surfaces. Results also highlight the effect of 405 nm light for inactivation of biofilms of other

110 problematic biofilm-forming bacteria including *Pseudomonas aeruginosa*, *Staphylococcus*

111 *aureus* and *Listeria monocytogenes*.

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114 **MATERIALS AND METHODS**

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116 **Bacterial Preparation:** The bacteria used in this study were: *Escherichia coli* NCTC 9001;

117 *Staphylococcus aureus* NCTC 4135 (obtained from National Collection of Type Cultures,

118 Colindale UK); *Listeria monocytogenes* LMG 19944; and *Pseudomonas aeruginosa* LMG

119 9009 (obtained from the Laboratorium voor Microbiologie, Universiteit Gent, Belgium).

120 Bacteria were incubated in 100 mL nutrient broth (*E. coli, S. aureus* and *P. aeruginosa*) or

121 tryptone soya broth (*L. monocytogenes*), (Oxoid Ltd, UK) at 37°C for 18 hours under rotary

122 conditions (120 rpm). Broths were then centrifuged at 3939×g for 10 minutes, and the cell

123 pellets re-suspended in 100 mL phosphate buffer saline (PBS; Oxoid Ltd, UK), and diluted to

124 a population density of 10^7 CFUmL^{-1} for experimental use.

5

Biofilm Formation: Biofilms were prepared on glass and acrylic slides (60×25 mm). These materials were selected for two reasons: (i) they represent hydrophilic and hydrophobic surfaces, respectively, and (ii) their transparency allowed transmission of the 405 nm light. To prepare biofilm samples, glass and acrylic slides were first cleaned with ethanol to sterilise 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 CFUmL^{-1} bacterial suspension for 1 hour 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 hours 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 hours). After biofilm development, slides were aseptically removed from the growth media and left to dry for 10 minutes 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 hour. Slides were then placed in growth media for a further 24 hours 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 48V 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

150   Oriel, USA). The variation of irradiance was measured across the dimensions of the slide,

151   and measurements indicated greatest irradiance at the midpoint of the slide, with a gradual

152   decrease towards the outer edges (Figure 1). The average irradiance across the entire slide

153   surface was calculated, using OriginPro 8.1 software package, to be 141.48 mW cm^{-2}. Test

154   samples were light exposed for 5-60 minutes, giving a range of average doses from 42 J cm^{-2}

155   to 504 J cm^{-2}. Control slides were set up and left on the laboratory bench with no 405 nm

156   illumination.

157

158   <FIGURE 1>

159

160   In addition to directly exposing biofilms, the potential for 405 nm light to transmit through

161   glass and acrylic slides and inactivate biofilms on the underside of slides was investigated. To

162   do this, it was important to determine the transmissibility of the light through these slides.

163   This was measured by placing the power meter detector head on the underside of the exposed

164   surfaces: transmission of the 405 nm light through these materials was found to result in an

165   approximate 4% loss in irradiance.

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168   **Swabbing and Enumeration:** Following light exposure, surviving bacteria were recovered

169   from the slide using a sterile cotton-tipped swab moistened in PBS. The swabbing procedure

170   involved rolling the swab forward and backward multiple times across the entire surface of

171   the illuminated side of the slide to ensure maximum recovery of bacterial biofilm sample (1).

172   This protocol was kept consistent for all sample slides. The swab was then immersed in a

173   10 mL volume containing 9 mL PBS and 1 mL 3% Tween-80 suspension and vortexed for 1

174   minute to allow re-suspension of bacteria from swab into suspension. The suspension was

175 serially diluted, by transfer of 1 mL volumes into 9 mL of PBS. Samples were plated using

176 the pour plate method, with 1 mL sample volumes overlayed with nutrient agar (*E. coli,*

177 *S. aureus* and *P. aeruginosa*) or tryptone soya agar (*L. monocytogenes*): this method provided

178 a detection limit of 1 CFU mL^{-1}. For enumeration of bacteria in a mixed biofilm population,

179 in addition to pour-plating samples in nutrient agar to obtain the total viable counts (TVC),

180 bacterial samples (100μl-500μl) were plated onto mannitol salt agar (MSA) and violet red

181 bile agar (VRBA), which allowed the selective growth of *S. aureus* and *E. coli,* respectively.

182 Plates were then incubated at 37°C for 18-24 hours. Plates were enumerated manually by

183 counting the bacterial colony-forming units (CFU) present on the plate. Results in Figures are

184 reported as bacterial CFU count per millilitre (log$_{10}$ CFU mL^{-1}) as a function of time

185 (minutes).

186

187 **Statistical Analysis:** Experimental data is an average of a minimum of triplicate independent

188 experimental results, with triplicate samples taken from each experiment. All data were

189 analysed using one way ANOVA test with Minitab 15 statistical software, where significant

190 difference was accepted at $P < 0.05$. Weibull statistics (26) were used to analyse the

191 inactivation behaviour of monolayer bacterial biofilms cultured on glass and acrylic surfaces,

192 methodology for this analysis is described later.

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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 show 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 minutes exposure, and complete kill (3.55 $\log_{10}$ CFU mL^{-1} reduction) following 20 minutes exposure, as shown by the 4-hour trendline on Figure 2. After 24 hours in the growth medium, bacterial biofilm populations on glass were shown to be approximately 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 minutes respectively. Biofilms on glass developed over 48 and 72 hour periods had increased cell densities, with starting populations of between 7-8 $\log_{10}$ 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 minutes, and a further ~2 $\log_{10}$ CFU mL^{-1} reduction after a further 20 minutes. Near complete inactivation of the 48-hour biofilms and complete inactivation (<1 CFU mL^{-1} surviving) of the 72-hour biofilms was achieved following 60 minutes exposure to 405 nm light.

<FIGURE 2>

Figure 3 demonstrates the inactivation kinetics of *E. coli* biofilms on acrylic. Monolayer biofilms on acrylic surfaces were reduced by approximately 0.5 $\log_{10}$ CFU mL^{-1} after 10 minutes exposure, significantly less (P=0.002) than the 2.52 $\log_{10}$ CFU mL^{-1}

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225 reduction observed on glass. After 15 minutes exposure, there was however a 3.33 $\log_{10}$

226 CFU mL^{-1} reduction in the biofilm population on acrylic, statistically similar to that achieved

227 on glass at the same time point. After 24 hours of biofilm development, bacterial populations

228 were approximately 4.7 $\log_{10}$ CFU mL^{-1} on acrylic slides. Biofilm inactivation occurred at a

229 steady and consistent rate when applied with increasing exposure times of 405 nm light (20,

230 30, 40 and 60 minutes), resulting in reductions of 2.30, 3.07, 3.67 and 4.69 $\log_{10}$ CFU mL^{-1},

231 respectively. Development of biofilms over a 48 hour period generated a bacterial population

232 of ~5.1 $\log_{10}$ CFU mL^{-1}, where near complete inactivation was achieved following exposure

233 for 60 minutes (<1 CFU mL^{-1}). Bacterial biofilm formation on acrylic surfaces after 72 hours

234 growth period demonstrated no significant increase in bacterial count from that recorded after

235 48 hour growth period (P= 0.06), therefore biofilms grown for 72 hours on acrylic were not

236 investigated.

237

238 <FIGURE 3>

239

240   The population densities of all non-exposed control biofilm samples, on both glass

241 and acrylic, remained consistent throughout, with no significant differences recorded over the

242 duration of the experiment, indicating that inactivation was a direct result of 405 nm light

243 exposure. It is likely that the reason for no loss of viability in the control populations was due

244 to the relatively short periods involved (up to 1 hour periods) as well as the protective effect

245 of the biofilm structure.

246

247   It is also worth noting that no significant temperature build up was observed on test

248 samples during light exposure. Temperatures of both glass and acrylic surfaces were

249 measured across the slide to give accurate representation of heat distribution across the entire

250 area. For each material, temperatures were measured after maximum exposure periods using

251 a thermocouple, which was pressed onto the test surface. The maximum temperature recorded

252 was 33°C, indicating bacterial kill was not a result of direct thermal kill or desiccation

253 through prolonged heat treatment.

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

257 Experiments were carried out to establish whether the 405 nm light could transmit through

258 the glass and acrylic and inactivate biofilms on the underside of the slides. For 'indirect'

259 biofilm exposure, after removal from the growth medium, the upper side of the slide was

260 wiped clean to ensure biofilm formation was only on the underside of the slide. The

261 inactivation data for these 'indirectly' exposed biofilms is presented in Figures 4a and 4b as a

262 comparison to the directly exposed biofilms.

263

264   Glass microscope slides have high transmittance in the visible light region around 400

265 nm. As a result, there was negligible differences between the inactivation for both direct and

266 indirectly exposed biofilms on glass slides as can be seen from Figure 4a,. There was no

267 significant difference (P= 0.738) between the direct and indirect $\log_{10}$ CFU mL^{-1} reductions

268 following 10 minutes exposure, and complete inactivation (<1 CFU mL^{-1} survivors) was

269 achieved for both samples after 20 minutes exposure. Similarly, Figure 4b demonstrated

270 similar inactivation curves for both direct and indirect exposure of biofilms on acrylic, with

271 no significant difference in the population reductions achieved with direct and indirect

272 exposure after 10 minutes (P=0.421) and 20 minutes (P=0.507) which also indicates that the

273 acrylic slides used in the present work transmit well visible 405 nm light. As noted,

274 irradiance measurements determined that transmission of the 405 nm light through the glass

275 and acrylic slides with monolayer growth, resulted in an approximate 4% reduction in

276 irradiance, however, this reduction was insufficient to cause significant differences in the

277 inactivation rates of the directly and indirectly exposed biofilms on either the glass or the

278 acrylic surfaces.

279 <FIGURE 4>

280

281 **Weibull Analysis**

282

283 The inactivation behaviour of monolayer bacterial biofilms grown on glass and acrylic

284 surfaces was analysed using the Weibull statistical approach, which can help in the

285 identification of potential differences in inactivation mechanisms. The Weibull distribution

286 has been employed for the analysis of microbial inactivation kinetics, including PDI studies

287 (27,28), however, a paper by Schenk et al. (29) which investigates UV microbial inactivation,

288 states that "there is no information about the application of a Weibullian-type model to

289 survival curves corresponding to microorganisms inoculated onto a solid surface". Therefore,

290 it was interesting to examine the potential applicability of this statistical model to 405 nm

291 light inactivation of biofilms cultured on solid surfaces.

292

293 In the Weibull's approach, the 405 nm light dose, D_c, which is required to kill a single

294 microorganism from an entire population is considered as a measure of resistance of this

295 organism to the light. It is also assumed that D_c is Weibull distributed. The survival rate of

296 microorganisms which form biofilms, $S(D)$, is defined as the number of microorganisms

297 surviving at a specific 405 nm light dose, $N(D)$, divided by the initial number of

298 microorganisms, N_0:

299 $$S(D) = N(D)/N_0 \tag{1}$$

12

300   In these conditions the survival rate, *S(D)*, can be described by the Weibull's cumulative

301   distribution function, (26) and satisfies the following equation:

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

302   (2)

303   where α and β are parameters of the Weibull distribution.

304

305   Experimental inactivation data for monolayer biofilms on acrylic and glass surfaces

306   exposed to direct and indirect 405 nm light have been represented as *log₁₀ (S(D))*. These

307   experimental date points and corresponding analytical fit lines obtained by Equation (2) are

308   shown in Figures 4a and 4b (inset graphs). Analytical lines show downward concavity for

309   the acrylic surface and upward concavity for the glass surface.

310

311   Coefficient β which determines the shape of the Weibull's distribution has been

312   obtained for both surfaces and both types of the light treatment as shown in Figure 4. For

313   acrylic surfaces (Figure 4a) the shape parameter, β, is higher than 1: $\beta = 3$ in the case of the

314   direct exposure and $\beta = 3.6$ in the case of the indirect exposure. $\beta > 1$ indicates that with an

315   increase in 405 nm light dose, microorganisms in the biofilm become increasingly damaged

316   and can be killed at a higher rate. In the case of the glass surface (Figure 4b) the shape

317   parameter is smaller than 1: $\beta = 0.378$ and $\beta = 0.396$ for direct and indirect exposures

318   respectively. $\beta < 1$ means that the rate of inactivation is higher at lower 405 nm light doses,

319   and this rate decreases with an increase in the light dose. Potentially such inactivation

320   behaviour may indicate that remaining (surviving) microorganisms in the biofilm become

321   more resistive to the external stress (405 nm light).

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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 minutes of 405 nm light to determine the comparative levels of bacterial inactivation. Using the stipulated 4-hour 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 minute, resulted in between 0.6-1.5 $\log_{10}$ 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 minutes 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 minutes exposure were similar between the two Gram-negative bacteria (~3.6 $\log_{10}$ CFU mL^{-1}), with which complete inactivation was achieved, and between the two Gram-positive bacteria (~2.6 $\log_{10}$CFU mL^{-1}).

<TABLE 1>

14

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 (Figure 5) of a biofilm slide which had been Gram-stained in order to visualise 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 (24h growth period) of *E. coli* and *S. aureus*, to 405 nm light. Results show that after a 30-minute 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 post-exposure biofilm populations using VRBA and MSA selective media demonstrated significant inactivation of both bacterial species present in the biofilm, with approximately 1.2 and 1.7 $\log_{10}$ CFU mL^{-1} reductions in *E. coli* and *S. aureus*, respectively, being observed.

<FIGURE 5>

<TABLE 2>

DISCUSSION

Despite the development of new antimicrobial agents and novel sterilisation 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

375 of 405-nm light on bacterial biofilms, with results demonstrating successful inactivation of

376 biofilms on both glass and acrylic surfaces, and that the bactericidal effect was observed with

377 both monolayer and mature biofilm populations. Overall, results showed that successful

378 inactivation was achieved with all complexities of *E. coli* biofilms generated on both glass

379 and acrylic, with the general trend demonstrating that the more densely populated the biofilm,

380 the greater the time (and consequently, the greater the dose) required for inactivation.

381

382 As previously discussed, bacterial biofilms can readily form on both glass and plastic

383 surfaces, with production of an extracellular matrix in as little as 4 hours (1,5,30,31,32).

384 Studies have reported stronger initial adhesion between bacteria and hydrophobic surfaces,

385 such as plastics, compared to that of hydrophilic materials, including glass, which may

386 account for initial variations in *E. coli* monolayer biofilm populations. Experimental data

387 from this study highlighted that after 1 hour, *E. coli* attachment to acrylic was greater when

388 compared to that on glass surfaces, however statistical analysis showed this to be

389 insignificant (P = 0.098). Slight variation in *E. coli* monolayer biofilm starting populations,

390 after 4 hours development, ($\sim$0.5 $\log_{10}$ CFU mL^{-1}) was observed between the glass and

391 acrylic surfaces, which may be an influence of bacterial interactions with surface material

392 properties. Despite this slight difference, investigation into the 405-nm light inactivation of

393 *E. coli* monolayer biofilms on glass and acrylic demonstrated successful results, with near

394 complete bacterial inactivation observed following exposure 20 minutes (approximate dose of

395 168 J cm^{-1}), highlighting the susceptibility of monolayer biofilms to 405 nm light.

396

397 In addition to monolayer biofilms, results demonstrated the successful inactivation of more

398 mature *E. coli* biofilms on both on glass and acrylic surfaces. Population densities of these

399 biofilms ranged from approximately 10^3–10^8 CFU mL^{-1}, with the more densely populated

400   biofilms requiring increasing exposure periods for complete inactivation. Biofilms generated

401   on acrylic surfaces over a 24 hour time period required increased exposure time for complete

402   inactivation when compared to those on glass surfaces, despite having significantly lower

403   starting bacterial populations (~1.5 $\log_{10}$ CFU mL^{-1} lower). This may be an artefact of the

404   physical adhesive properties between bacteria and specific materials. Chmieleweski and

405   Frank (33) reported that although initial bacterial adherence to hydrophobic surfaces is likely

406   to be stronger, greater maximum bacterial adhesion is achieved on hydrophilic surfaces, as a

407   result of high free surface energy, allowing for generation of denser biofilm populations

408   (33,34,35). This information correlates with data shown in this study, with results

409   demonstrating greater adhesion and increased biofilm formation on hydrophilic glass

410   surfaces, following development of mature biofilm structures. Results highlighted that mature

411   biofilms developed over 24 and 48 hour periods had bacterial densities of approximately 4.5-

412    $\log_{10}$ versus 6-$\log_{10}$ CFU mL^{-1} and 5-$\log_{10}$ versus 8-$\log_{10}$ CFU mL^{-1} for hydrophobic and

413   hydrophilic surfaces, respectively.

414

415   Bacterial biofilms generated on both glass and acrylic surfaces over 48 and 72 hours appeared

416   to have similar population densities, suggesting that after the 48-hour growth period, bacterial

417   attachment was maximised and had consequently plateaued. This may be attributed to a lack

418   of nutrients present in the growth media after extended time periods, suggesting media must

419   be replenished to generate increased biofilm populations.

420

421   Successful inactivation of bacterial biofilms on the underside of the glass and acrylic surfaces

422   was also shown, demonstrating the ability of the 405 nm light to transmit through these

423   transparent materials whilst maintaining its antimicrobial activity. With regards to the results

424   of the Weibull analysis, the difference in the inactivation behaviour of "young", 4-hour

425   monolayer biofilms could potentially be attributed to the different degree of adhesion of

426   microorganism to these acrylic and glass surfaces, with the hydrophobic and hydrophilic

427   interactions between the monolayer biofilms and the surfaces to some extent influencing the

428   inactivation behaviour. However, mature, 24 h, 48 h, and 72 h biofilms don't show similar

429   tendencies. Moreover, their inactivation curves, shown in Figures 2 and 3, demonstrate

430   almost linear behaviour and cannot be fitted with the Weibull's curves (Equation 2).

431   Therefore, it is possible to conclude that with an increase in biofilm 'age' (biofilm thickness

432   and/or number of microorganisms), the influence of substrate material on the inactivation

433   process becomes significantly reduced or disappears completely.

434

435   It is necessary to note that although Equation (2) can be used to fit the experimental

436   inactivation data shown in Figure 4, it is not possible to conclude that the proposed analytical

437   lines are incorrect due to the limited number of experimental data points (only two 405 nm

438   light doses have been used in the present inactivation tests). The present work is aimed only

439   at identification of potential differences in the inactivation behaviour and does not involve a

440   full-scale statistical analysis which requires larger number of experimental data points. It is

441   planned to conduct further direct and indirect tests using a greater number of 405 nm light

442   doses in order to validate the proposed statistical model.

443

444   A variety of methods have been used for biofilm sampling in previous studies including

445   sonication and swabbing (1,36,37,38). Despite all being successful and well utilised methods

446   for recovering microorganisms, each presents its own limitations. Swabbing is a well-

447   recognised method for sampling bacterial contamination within health care and food

448   industrial settings as well as for recovery for bacterial biofilms in laboratory experiments

449   (1,37,38). This method was used throughout this study as a viable and effective technique for

450   bacterial biofilm removal from both glass and acrylic surfaces. Regardless of all inaccuracies/

451   limitations associated with swabbing for bacterial removal, test and control samples in this

452   study were recovered identically using a standard swabbing technique, allowing for directly

453   comparable results.

454

455   The methodology for biofilm formation used in this study was adapted from previous work

456   by Gibson and colleagues (1). The possibility of including a rinsing stage prior to light

457   exposure to ensure all non-attached microorganisms were removed was investigated. Results

458   demonstrated that there was no significant difference between biofilm populations on rinsed

459   and non-rinsed slides, for both monolayer and more mature biofilms (P>0.08). Rinsing of test

460   surfaces provided conclusive evidence of biofilm formation as weakly attached cells would

461   have been removed during the rinsing stage. Non-significant reduction in bacterial count

462   following rinsing suggested bacteria were protected, most likely by the presence of an

463   exopolysaccharide matrix which has been shown to protect cells concealed within biofilm

464   layers from harsh environmental conditions such as flowing water (39).

465

466   Previous studies investigating 405-nm light exposure of bacterial suspensions and bacteria

467   seeded onto nutritious surfaces have demonstrated its bactericidal effects (20,40). Studies

468   have indicated that inactivation of Gram-positive bacteria require less exposure time than that

469   of Gram-negative bacteria. Possible explanations for this trend have been accredited to

470   cellular structure, where penetration of light through more structurally complex cells is

471   reduced (41), and variation in the levels of different intracellular porphyrin molecules

472   (40,42). Previous studies have identified numerous porphyrin molecules involved in the

473   photodynamic inactivation of bacteria. A recent study by Dai et al (43) highlighted the

474   presence of both corproporphyrin and uroporphyrin in *P. aeruginosa*. Similarly,

475   photoinactivation studies have demonstrated the presence of corpoporphyrin in *S. aureus* and

476   protoporphyrin in *E. coli*, whilst a range of various porphyrin molecules have been associated

477   with *L. monocytogenes* (43,44,45).

478

479   Looking at the results of this study for the inactivation of the four different bacterial species,

480   it can be seen that successful inactivation was achieved with all organisms, with approximate

481      $\log_{10}$ CFU mL^{-1} reductions of 3.6 and 2.6 for Gram-negative and Gram-positive biofilm

482   populations, respectively. It is, however, difficult in the current study to directly compare the

483   efficacy of 405 nm light for the inactivation of the different bacterial species due to the

484   differences in starting populations observed within the monolayer biofilms. The

485   methodology for preparing the biofilms was kept consistent for the different bacterial species,

486   and this method resulted in the generation of varying populations, possibly reflecting

487   differences in propensity for attachment and/or the rate of multiplication of the attached

488   populations. Variance in bacterial inactivation between the Gram-positive and Gram-negative

489   species (Table 1 and Table 2) may have been a direct effect of the increased adherence of the

490   Gram-positive cells causing increased starting populations, and consequently requiring a

491   greater exposure for complete inactivation, compared to the lower populated Gram-negative

492   biofilms. However, recent data published by Murdoch et al (20) showed that even at similar

493   starting populations, Gram-negative *Salmonella enterica* was inactivated 30% more

494   effectively than Gram-positive *L. monocytogenes* when exposed to 405 nm light whilst

495   seeded onto plastic surfaces (20).

496

497   In addition to investigating single species biofilms, initial tests were carried out to assess the

498   antimicrobial activity of 405 nm light against mixed species biofilms. After a 24h growth

499   period, the population of mixed species biofilm was lower than the populations achieved

when compared to 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 ~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 coloniser (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 colonise 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 (Figure 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 colonised *E. coli* populations, suggesting that *E. coli* may act as a primary coloniser 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 oxidative damage may directly affect cellular membranes, resulting in reduced membrane stability (47). Interference with the cell

525 membrane and its components may consequently reduce biofilm stability, leading to biofilm

526 degradation through bacterial inability to remain attached to surfaces. It may also be plausible

527 that following 405 nm light exposure, alterations in structural membrane components may

528 possibly prevent cellular attachment and thus prevent biofilm formation. Although this study

529 simply investigated the effects of 405 nm light on the viability of bacterial biofilms once

530 attached to glass and acrylic surfaces, it would be of great interest to investigate the

531 degradative properties of 405 nm light on bacterial biofilms as well as investigating the

532 specific effects of 405 nm light on cellular adhesion. A previous study by Mussi et al. (48)

533 investigated the use of longer blue light wavelengths for inhibition of biofilm formation.

534 Studies have shown many bacteria possess blue light receptors, capable of causing photo-

535 regulated behaviour upon exposure to blue light, and it has been suggested that direct

536 interactions between blue light and subsequent receptors may have inhibitive properties

537 relating to biofilm formation (24,48). However, Mussi and colleagues stated that the effect of

538 light on biofilm formation was inconclusive; highlighting that further work is required to

539 identify any relationship between blue light, blue light receptors and biofilm formation.

540

541 The light irradiance produced by LED array used in this study has a Gaussian distribution, as

542 with many LED-based light systems (49). Highest irradiance is found directly below the

543 centre of the LED array distributing gradually towards the outer edges. Irradiance over the

544 entire test slide was measured (as shown in Figure 1) and the average irradiance was

545 calculated. Although there was a large difference in the measured irradiance at the centre and

546 the outer edges of the slide, the fact that (i) complete inactivation of the biofilm populations

547 could be achieved and (ii) swabs were used to recover bacteria from the entire slide not just

548 the centre point, demonstrated that the inactivation effect was achieved across the entire slide,

549 regardless of the non-uniformity of the light exposure. Although average doses could be

550 calculated, due to the non-uniformity of the irradiance, results (Figures 2, 3 and 4) have been

551 expressed as a function of exposure time. Previous work (25) has shown that 405 nm light

552 microbial inactivation is dose-dependent therefore due to dose being the product of irradiance

553 × exposure time, the inactivation rates of these biofilms will be increased if an increased

554 irradiance of light is used for exposure.

555

556 The use of light for biofilm decontamination has been extensively investigated, with

557 particular focus on UV-light due to its highly bactericidal properties (8,50). Evidence has

558 suggested that UV-C radiation is largely absorbed by organic materials, such as biofilms,

559 resulting in poor penetration of light and insufficient decontamination (50,51). Short

560 wavelengths in the UV spectrum (200-280nm) present poor penetrability, when compared to

561 that of the visible spectrum. Data shown in Figure 4 highlights the penetrability of 405-nm

562 light, where both direct and indirect exposure of biofilm on glass and acrylic surfaces to 405-

563 nm light produced almost identical inactivation curves. Detrimental properties associated

564 with the use of UV-light are also well recognised, greatly limiting its use for open surface

565 decontamination. With regards to human safety, human exposure to UV radiation is limited

566 due to the associated risk with development of skin and eye cancer (51). Polymer degradation

567 is a further limitation of UV light, imposing financial restraints for continuous repair and

568 replacement of degraded machinery and equipment (52). The safety and operational benefits

569 of 405 nm violet-blue visible light make it suitable for development for both food-related and

570 biomedical decontamination applications for both biofilm and general disinfection purposes.

571 Previous studies have demonstrated the efficacy of 405 nm light for general environmental

572 disinfection (53), but potential uses of violet-blue light also include disinfection of medical

573 devices, and wound decontamination, with a recent study by Dai et al (43) demonstrating the

574 application of blue light for disinfection of *P. aeruginosa* infected burns in mice with no

575 significant damage noted in skin cells.

576

577 In summary, this study has demonstrated for the first time the use of 405-nm light for the

578 inactivation of bacterial biofilms. Biofilms of varying maturity and also of varying bacterial

579 species have been shown to be susceptible to inactivation, demonstrating the ability of the

580 405 nm light to inactivate even densely populated biofilm communities. The ability to

581 inactivate bacterial biofilms present on inert surfaces using 405-nm light is of great

582 significance, and the penetrability of 405-nm light through transparent materials highlights

583 further advantages of this bactericidal light, and demonstrates the potential for development

584 of technologies using 405-nm light for practical decontamination applications within both the

585 food industry and healthcare settings.

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794   **Table 1:** Inactivation of bacterial monolayer biofilms on glass surfaces following exposure to

795   405-nm light.

796

Bacterial species	Time (min)	Dose (J cm^{-2})	Non exposed sample (mean log$_{10}$ CFU mL^{-1})	Exposed sample (mean log$_{10}$ CFU mL^{-1})	Log reduction (* P≤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

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802 **Table 2:** Inactivation of *E. coli, S. aureus,* and mixed *E. coli* and *S. aureus* bacterial biofilms

803 formed on glass surfaces over a 24 hour period. Counts are provided for total viable counts

804 (TVC), and in the case of the mixed biofilms, selective counts of *S. aureus* and *E. coli* have

805 been provide using selective media: (MSA and VRBA).

806

Biofilm Species	Bacterial count	Non-exposed biofilm (mean $\log_{10}$ CFUmL^{-1})	Exposed biofilm (mean $\log_{10}$ CFUmL^{-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*

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FIGURE CAPTIONS

820

821   **Figure 1:** Three dimensional model simulating the irradiance distribution of the 405 nm light

822   across the glass and acrylic test surfaces (60×25 mm), plotted using OriginPro 8.1 software.

823

824   **Figure 2:** Inactivation of *E. coli* biofilms on glass surfaces following exposure to 405 nm

825   light with an irradiance of ~140 mW cm^2, given as a function of time. Biofilms were allowed

826   to develop for 4, 24, 48 and 72 hours before light exposure.   * Indicates statistically

827   significant differences when compared to control samples ($P \leq 0.05$).

828

829   **Figure 3:** Inactivation of *E. coli* biofilms on acrylic surfaces following exposure to 405 nm

830   light with an irradiance of ~140 mW cm^2, given as a function of time. Biofilms were allowed

831   to develop for 4, 24 and 48 hours before light exposure.   * Indicates statistically significant

832   differences when compared to control samples ($P \leq 0.05$).

833

834   **Figure 4:** Inactivation of *E. coli* monolayer biofilms on (a) glass and (b) acrylic, by direct

835   (▲) and indirect (□) exposure to 405 nm light. Indirect exposure investigated transmission

836   of the 405 nm light through the slides to inactivate biofilms on the underside of the slides.

837   The average irradiance of 405 nm light across the slides was approximately 140 mW cm^{-2},

838   reducing by ~4% when transmitted through the slide. Results are given as a function of time.

839   * Indicates a statistically significant difference when compared to control samples ($P \leq 0.05$).

840   No significant difference was observed between direct and indirect inactivation on both

841   surfaces. Weibull analytical fit lines are shown as inset graphs and were obtained using

842   Equation (2).

843

844   **Figure 5:** Microscopic visualisation of a Gram stain of a mixed species biofilm consisting of

845   *S. aureus* and *E. coli* after 24 hour development. Cells were viewed under oil immersion at

846   ×1000 magnification.
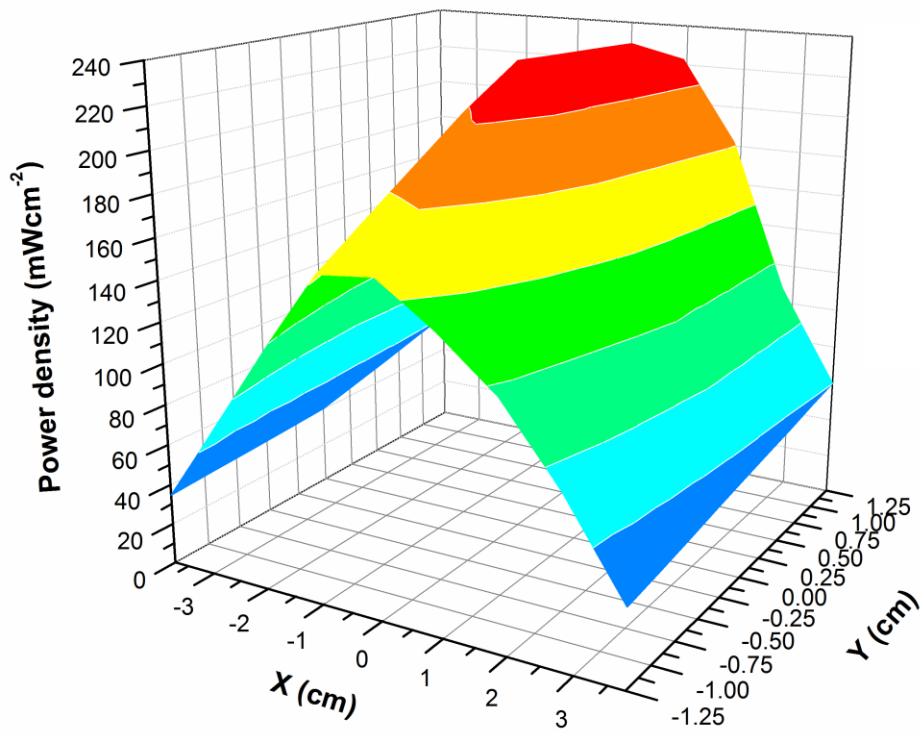
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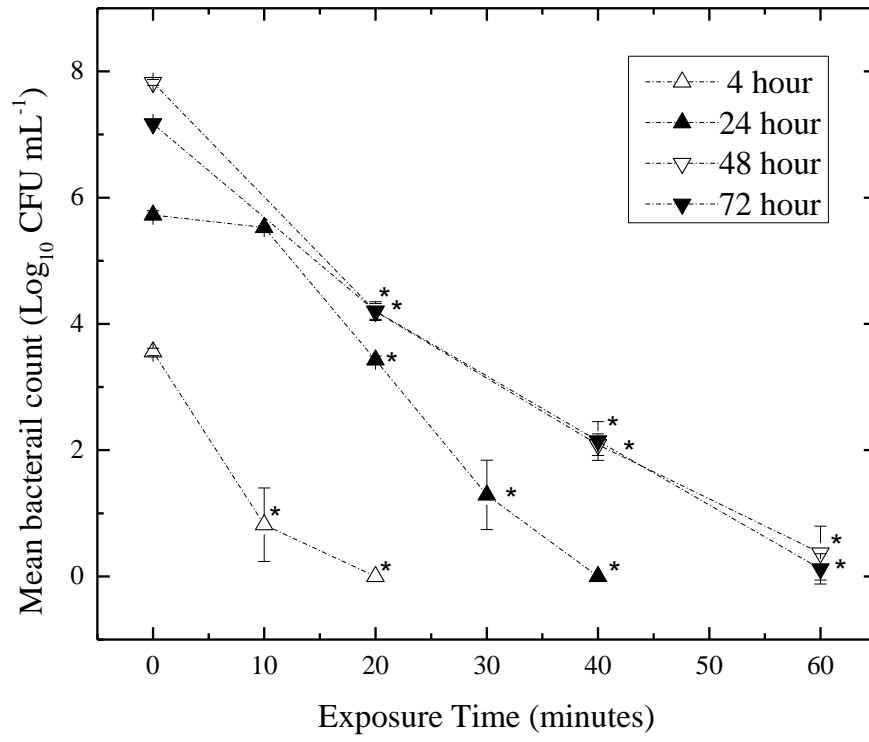857

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859   Figure 1

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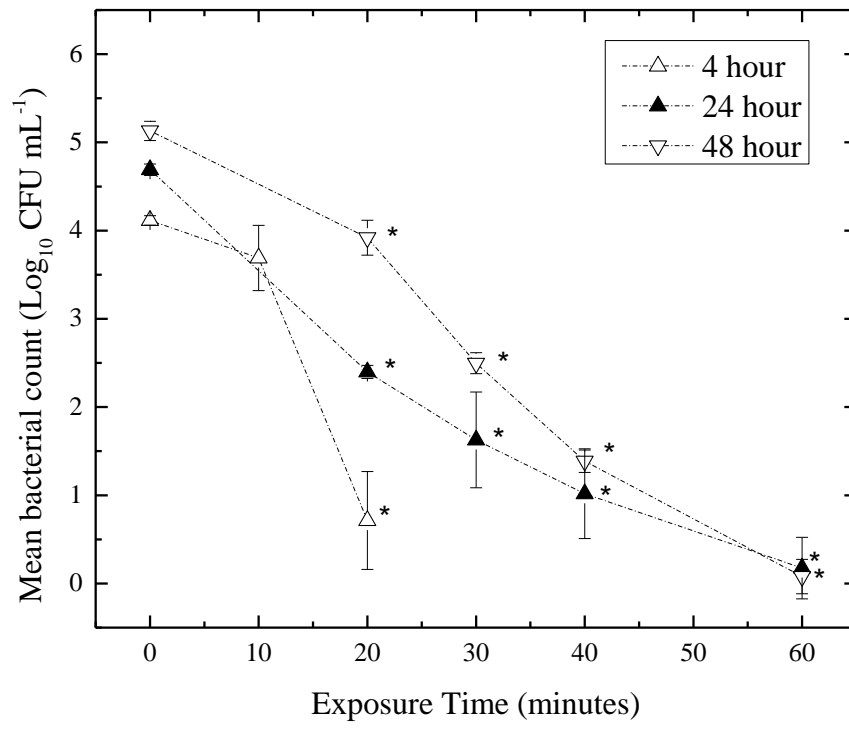
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Mean bacterail count (Log_{10} CFU mL^{-1}) vs Exposure Time (minutes)

Legend:
- 4 hour
- 24 hour
- 48 hour
- 72 hour

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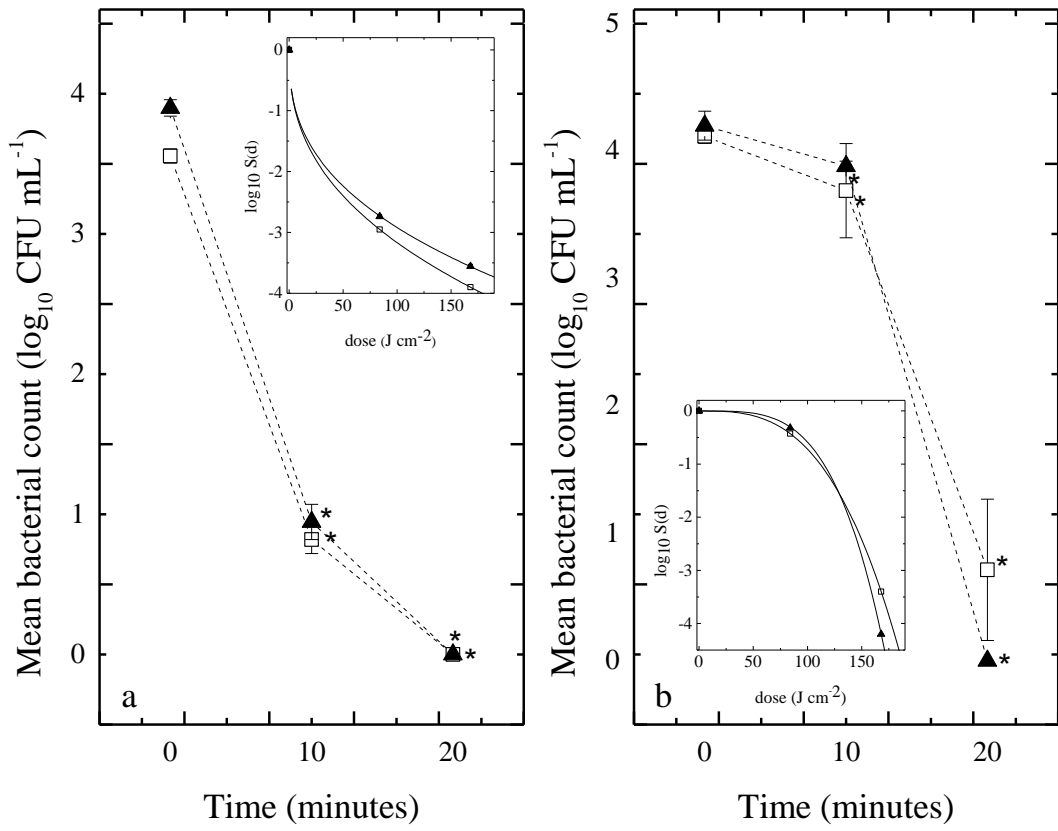870   Figure 2
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