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1 **Susceptibility of microcosm subgingival dental plaques to lethal photosensitisation**

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13

14 **Abstract**

15 Photodynamic therapy (PDT) offers potential as a non-invasive treatment of periodontal

16 disease. In this study, microcosm biofilms were grown *in vitro* under conditions designed

17 to mimic subgingival plaques typically found in patients with periodontitis. To

18 investigate potential PDT modalities, biofilms were exposed to light from a helium/neon

19 laser in conjunction with a photosensitiser, toluidine blue (TBO), at varying output and

20 concentration, respectively. To determine cytotoxic effects, viability profiling was

21 undertaken on whole biofilms using standard plating methods, and on horizontal cross-

22 sections of biofilms using confocal laser-scanning microscopy (CLSM) in conjunction

23 with a differential viability stain. A light energy dose of 94.5 J in combination with 81.7

μM TBO was found to be optimal, achieving significant kills of over 97%. CLSM enabled visualisation of the effects of PDT in three dimensions. Viability profiling of the CLSM images revealed that lethal photosensitisation was most effective in the upper layers of biofilm. PDT was found to reduce the viability of subgingivally-modelled plaques *in vitro* by a similar magnitude to chlorhexidine digluconate (CHL), which is commonly used to treat periodontal disease. The findings of this study indicate that PDT may be an effective alternative to conventional modalities in the treatment of periodontal disease.

Keywords: Lethal photosensitisation, Subgingival plaque, Oral Biofilm**,** Constant-depth film fermenter, Photodynamic therapy

Introduction

Periodontal disease is caused by the accumulation of subgingival plaque biofilm near the gingival margin and is responsible for up to 60% of tooth loss in the UK. Chronic marginal gingivitis probably affects the whole dentate population at some stage and is characterised by inflammation of the gum margin. If the build-up of gum marginal plaque is left unchecked, gingivitis can develop into the more serious condition of chronic adult periodontitis (Pihlstrom et al 2005). This condition is characterised by the migration of the junctional epithelium tissue at the base of the gingival crevice down the tooth to form a periodontal pocket. The cause of this pocketing has been attributed to tissue destruction as a consequence of bacterial stimulation of host degradative enzyme production (e.g. matrix metalloproteinases) and invasion of host cells (Bodet et al 2007,

47 Amano, 2003). The (probing) depth of the periodontal pocket is used as an indicator of
48 the spread of the disease and The Adult Dental Health Survey (1998) revealed that 54%
49 of the UK adult dentate population had probing depths greater than 3.5 mm (moderate
50 periodontal disease) whilst 8% of the population had a loss of attachment over 5.5 mm
51 (severe destructive periodontitis) (Morris et al 2001). The annual cost of NHS
52 periodontal therapy in 2001/2002 was £174 million on 15.5 million treatment episodes
53 (Chapple, 2004). Conventional periodontal therapy routinely involves root scaling /
54 planing which may be complemented with adjunct antimicrobial chemotherapy, such as
55 doxycycline (Tuter et al 2007).

56 Bacteria harboured within biofilms are notoriously recalcitrant to antimicrobial
57 agents (Costerton et al 2003, Anwar et al 1990, Millward & Wilson, 1989) and their
58 eradication represents a formidable challenge where they are associated with disease.
59 The mechanisms by which biofilms resist conventional antimicrobial agents are generally
60 considered to be multifactorial, although current understanding is that the production of
61 high numbers of essentially invulnerable persister cells, in response to shock proteins or
62 'alarmones', is the most significant of these factors (Gilbert et al 2002b).

63 Treatment of bacterial infections by PDT offers an alternative to the use of
64 antimicrobial agents (Malik et al 1990) and to invasive dental procedures which are
65 associated with patient trauma. The bactericidal effect associated with PDT (known as
66 lethal photosensitisation) is caused by the generation of cytotoxic free radicals, such as
67 singlet oxygen, when a photo-reactive agent (photosensitiser) is excited by light of an
68 appropriate wavelength (Malik et al 1990). PDT has been used clinically to treat a
69 number of systemic conditions including tumor (Choi et al 2006). Interest in its

70 application to treat bacterial infections is gaining momentum – the treatment of

71 periodontal disease being one of the most promising (Meisel & Kocher, 2005). A major

72 benefit of PDT is that the bactericidal effect is localised to areas which are treated with

73 both photosensitiser and light, preventing disruption of the indigenous microflora at sites

74 distal to the treated area (Wilson, 2004). The non-specific mode of action of PDT,

75 expressly the generation of singlet oxygen, means that the acquisition of bacterial

76 resistance to these agents is unlikely.

77 Previous studies have examined the effects of PDT on single-species biofilms

78 using streptococci to model supragingival plaque in conditions associated with onset of

79 dental caries (Zanin et al 2005, Zanin et al 2006) and *Streptococcus pyogenes* to model a

80 skin biofilm (Hope & Wilson, 2006). However, PDT also offers a promising, non-

81 invasive treatment for periodontal disease. Previously, it was reported that toluidine blue

82 and helium/neon laser light could kill significant numbers of bacteria associated with

83 periodontal disease, using a simple system where bacteria were grown on nitrocellulose

84 membranes (O'Neill et al 2002). In this investigation we describe the modelling of

85 subgingival plaque using a sophisticated *in vitro* model more representative of *in vivo*

86 conditions.

87 The aim of this study therefore was to investigate the susceptibility of microcosm

88 subgingival periodontal plaques grown in a constant-depth film fermenter (CDFF) to

89 PDT using a range of biofilm viability profiling techniques, and to compare the

90 effectiveness of PDT to the commonly used oral antimicrobial, CHL, representative of a

91 conventional, non-invasive modality.

92

Materials and Methods

Subgingival plaque inoculum

Subgingival plaque samples were obtained from ten patients attending the Eastman Dental Hospital, London, for treatment of moderate-to-advanced adult periodontitis. Each sample consisted of the contents of at least two periodontal pockets from each patient. The samples were pooled and mixed in pre-reduced BHI broth (Oxoid, Basingstoke, UK) containing 10% glycerol and then aliquoted into 1 ml volumes and stored at -70ºC, to generate a bank of homogenous innocula for use throughout this series of experiments. Two vials of homogenised plaques were used as the inoculum to produce the biofilms *in vitro*. Compositional studies (using culture and molecular methods) on biofilms produced from this pooled inoculum using the method described below, demonstrated the presence of many of the genera found in subgingival plaque including; *Veillonella, Streptococcus, Proprionibacterium, Eubacterium, Micrococcus, Actinomyces*, *Fusobacterium* and *Porphyromonas* (O'Neill, unpub. Ph.D thesis, Univ. London, 2006), the latter three genera being especially associated with periodontal disease.

Production of microcosm subgingival dental plaques

Operation of a constant-depth film fermenter (CDFF) (University of Wales, Cardiff, UK) to produce multi-species oral biofilms modelling those associated with periodontal disease has previously been described (Allan et al 2002, Hope & Wilson 2006). In this study, to model the microaerophillic conditions found in periodontal pockets (Mettraux et al 1984), the CDFF was operated by continuous flushing (60 cm^3

116 min^{-1}) with a gas mixture consisting of 95% N_2, 3% CO_2, 2% O_2. The growth medium
117 used to enable propagation of biofilms from the microcosm plaques consisted of RPMI
118 (60%) (Sigma, Poole, UK) / horse serum (40%) (Oxoid) supplemented with 0.5 μg ml^{-1}
119 menadione and 5.0 μg ml^{-1} hemin. The CDFF was housed within an incubator at 37ºC.
120 The substratum for subgingival plaque is enamel/cementum, the mineral component of
121 which is similar to hydroxyapatite (HA). HA discs were therefore used as the substratum
122 for biofilm formation. The CDFF housed fifteen sample pans on a rotating turntable, each
123 pan containing five HA discs, of 5 mm diameter. Discs were recessed to a depth of 100
124 μm.
125 The inoculum was added to 500 ml growth medium and pumped onto the rotating
126 HA substrata for 8 h, at a rate of 1 ml min^{-1}. The CDFF was then connected to fresh
127 medium which was supplied at the same flow rate and biofilms were then grown for 7
128 days before being removed for experimentation.
129
130 Photosenitisation and laser application
131 TBO (Sigma) was used at concentrations of 32.7, 65.4, 81.7 or 163.4 μM in 0.85%
132 saline. The laser used was a Helium/Neon (He/Ne) gas laser (Spectra-Physics, Darmstadt-
133 Kranichstein, Germany) with a measured power output of 35 mW. Light was emitted in a
134 collimated beam of 3.5 mm diameter, with wavelength of 632.8 nm. To irradiate biofilms,
135 laser light was passed through a lens (x2 magnification), allowing light coverage of the
136 entire biofilm surface.
137 To test biofilm susceptibility to TBO/HeNe laser light, pans were removed and
138 the biofilm-containing discs removed and placed in microtitre plate wells. 10 μl of TBO

139 was added to each biofilm for sensitisation. Laser light intensities of 63 J (163.8 J cm^{-2})
140 and 95.4 J (248 J cm^{-2}) were employed (L+S+). Controls were TBO in the absence of
141 laser light (L-, S+), and addition of 10 µl sterile saline, with or without laser light (L+S-
142 and L-S-, respectively). In addition to controlling for laser light and TBO exposure, L-S-
143 also served to determine whether exposure to an aerobic atmosphere for the duration of
144 the experiment had any effect on bacterial viability. A pre-exposure time of 5 minutes (in
145 the absence of light) was applied to allow the solutions to penetrate into the biofilm. The
146 L+S+ and L+S- samples were then exposed to laser light for 15 min. Discs were then
147 immersed in 1 ml pre-reduced BHI broth and biofilms re-suspended by vortexing for 60
148 sec. The total number of viable, culturable bacteria (CFU ml^{-1}) from re-suspended
149 biofilms before and after treatment was determined by viable counting on Fastidious
150 Anaerobe Agar (FAA) (Bioconnections, Leeds, UK) containing 5 % (v/v) defibrinated
151 horse blood (E & O Laboratories, Bonnybridge, UK). Duplicate 25 µl aliquots were
152 spread over the surface of FAA plates and these incubated for 7 days at 37ºC in an
153 anaerobic atmosphere, and resultant colonies counted.
154
155 CLSM of biofilms subjected to PDT
156 Biofilms were subjected to either PDT using 63 J HeNe laser light and 81.7 µM
157 TBO, or no treatment as described above. Post-PDT biofilms were then placed into a
158 miniature Petri dish, biofilm uppermost and stained using the *Bac*Light™ bacterial
159 viability kit (Molecular Probes, Oregon, USA) with 5 min stain penetration time in
160 darkness. Scans were taken of the biofilms using a Leica DMLFS fixed stage microscope
161 with a Leica TCS SP confocal scan-head. The objective lens was a 63x HCX water

162 immersion dipping lens. Confocal image stacks were captured of both the viable (488nm)
163 and non-viable (568nm) fluorescent emissions. Data are from two biofilms, one an
164 untreated control (L-S-), and the other subjected to PDT (L+S+).
165
166 Analysis of CLSM data
167 Fluorescence intensity profiles (Hope et al 2002) through the biofilms were
168 generated by measuring the brightness of each optical section in the confocal image
169 stack, for both the viable (488 nm) and non-viable (568 nm) channels, using ImageJ
170 computer software (ImageJ 1.36d, The National Institutes of Health, USA,
171 http://rsb.info.nih.gov/ij/). To enable an effective comparison between the viable and
172 non-viable channels, these data were normalised against the maximum image intensity
173 for each individual channel (maximum value = 1) and plotted against depth into the
174 biofilm. Zero-depth was taken as the top of the confocal image stack.
175 Projection images were constructed by combining all confocal optical sections
176 into one image.
177
178 Susceptibility of biofilms to chlorhexidine digluconate
179 Two pans, each containing five biofilms, were removed aseptically from the
180 CDFF and placed in a sterile glass container. To this was added 5 ml 0.2% CHL (Sigma)
181 or, in the case of the control pan, 5 ml PBS (Oxoid, Basingstoke, UK) and incubated for 1
182 or 15 min. Biofilms were then prepared for viable counting as described above. In
183 parallel, PDT was applied to biofilms of the same age using a regime of 2.1 J HeNe laser
184 light with 81.7 μM TBO for 1 min and then prepared for viable counting. In addition, the

185 15 min CHL exposure results were compared to the data generated using the PDT
186 modalities described above (15 min treatment).
187
188 Statistical analyses
189 The statistical significance of the data was ascertained using the two-tail t-test
190 assuming unequal variance. For viable counting following PDT, four individual biofilms
191 were used to determine CFU ml^{-1} by plating in duplicate, n=8. For CHL and TBO
192 studies, three biofilms were plated in duplicate, n=6. For ease of interpretation, viability
193 is recorded as percentage kill compared to untreated control.
194
195 **Results**
196 Effect of PDT on biofilm viability
197 Table 1 shows the effect of increasing light dose and photosensitiser concentration on the
198 efficacy of lethal photosensitisation in terms of the percentage of bacteria killed. The
199 most effective combination of light and photosensitiser was 94.5 J and 81.7 µM TBO,
200 which produced a significant kill of 97.4%. A reduced TBO concentration of 65.4 µM
201 with the same light dosage still produced a significant kill of 95.0%. Increasing
202 photosensitiser concentration to 163.4 µM but still using 94.5 J laser light reduced the
203 efficacy of the procedure, producing a diminished, but significant, kill of 74.4%. A
204 photosensitiser concentration of 32.7 µM in combination with a light dose of 94.5 J was
205 sufficient to produce a significant bactericidal effect, with a kill of 82.7%.
206 Reducing the light dosage from 94.5 to 63 J reduced the bactericidal efficacy of
207 PDT. 63 J laser light with a photosensitiser concentration of 81.7 µM produced a

208 significant kill of 69.0%, however this kill was less than that observed at the higher light
209 dosage using the same photosensitiser concentration, albeit the difference was not
210 significant. 63 J of laser light in conjunction with 32.7 μM TBO was insufficient to
211 produce a significant lethal effect.
212
213 CLSM studies on biofilms subjected to PDT
214 Microcolony formations interspersed with water channels can be seen in the
215 control biofilm shown in figure 1a. Viable cells (green) appeared to be far more
216 numerous than the non-viable cells (blue). (It should be noted that the red, non-viable
217 stain used in the procedure was digitally altered to appear blue using ImageJ for ease of
218 interpretation). Figure 1b shows a multi-species biofilm that was subjected to lethal
219 photosensitisation with 63 J of HeNe laser light in the presence of 10 μl of 81.7 μM TBO.
220 Visually, there was a discernible difference between this biofilm and the control (figure
221 1a). The structure of the biofilm seems to have been disrupted by exposure to the light in
222 the presence of the TBO. In addition, there appeared to be a larger proportion of non-
223 viable to viable cells compared to the control biofilm.
224 The normalised fluorescence image intensity profile through the control biofilm
225 (figure 2) conformed to those observed previously (Hope et al 2002, Hope & Wilson,
226 2006) in similar supragingival and subgingival plaque biofilm models. Biofilm which
227 was exposed to 63 J of HeNe laser light in the presence of TBO (figure 3) showed a
228 broader distribution of non-viable fluorescence, particularly in the upper layers closest to
229 the illumination source.
230

231 Effect of chlorhexidine digluconate on biofilm viability

232 Exposure of biofilms to 0.2% CHL for 1 min did not result in a statistically
233 significant reduction in the number of recovered viable organisms post-exposure when
234 compared to the control, whereas exposure for 15 min with this treatment resulted in a
235 significant reduction of 99.7%. 1 min exposure to PDT using a combination of 2.1 J of
236 laser light and 81.7 μM TBO did not produce a significant kill, whereas (as described
237 above) combinations of 63 J (163.8 J cm^{-2}) with 81.7 μM TBO and 94.5 J (248 J cm^{-2})
238 with 32.7, 65.4, 81.7 and 163.4 μM TBO produced significant reductions in biofilm
239 viability.
240

241 **Discussion**

242 PDT was performed using two light doses, 63 J (163.8 J cm^{-2}) and 95.4 J (248 J
243 cm^{-2}), with a range of photosensitiser concentration, the latter light dosage proving the
244 most effective.

245 Inconsistencies in effect of laser and photosensitiser activity alone on biofilm
246 viability were observed. In all but one instance, these were not significant. However, 94.5
247 J laser light, and 81.7 μM TBO alone, produced significant kills (54.4% and 57.1%
248 respectively), although these kills were much lower than those achieve in combination
249 (97.4%). It is possible that the variability encountered in these experiments was due to the
250 heterogeneous nature of these multispecies biofilms, where perhaps unculturable bacteria
251 accounted for varying proportions of biofilm biomass. Alternatively, the treatments alone
252 may have contributed a small antimicrobial effect. Further experiments, with larger
253 sample sizes are required to build on this preliminary study.

254 A combination of 63 J laser light and 81.7 μM TBO produced the greatest
255 bactericidal effect, as determined by viability studies using plate culture. An increase in
256 the photosensitiser concentration (to 163 μM) did not effect an increase in bactericidal
257 activity of PDT. This observation seems counter-intuitive; one would have envisioned
258 that increasing the concentration of photosensitiser applied to the biofilm would result in
259 an increase in kill. However, if the TBO solution was too concentrated, penetration of the
260 solution by the laser light would be impeded. Thus, a shielding effect (Herzog et al 1994)
261 could occur, preventing the excitation of TBO proximal to the biofilm bacteria, with a
262 consequent reduction in efficacy of killing. Alternatively, TBO may also aggregate at
263 higher concentrations localising its presence and preventing adequate pervasion of the

264 target material. This may also account for the lack of increased efficacy at higher
265 concentrations (Sternberg & Dolphin, 1996). If the concentration of TBO used is sub-
266 optimal for the light dose administered, photo-bleaching will occur (Rigaut & Vassy,
267 1991) resulting in degradation of photosensitiser, and diminished bactericidal activity.
268 Thus, there appears likely to be a concentration of TBO optimal for photosensitisation –
269 sufficient to prevent bleaching but below a threshold where its presence becomes
270 detrimental to the procedure.

271 CLSM studies utilised the *Bac*Light™ bacterial viability kit (LIVE/DEAD) to
272 determine the viability profile of bacteria within the biofilms and revealed biofilms of
273 typical architecture, similar to those observed in other CLSM studies e.g. Costerton et al
274 (1994), Hope et al (2002). Microcolonies interspersed with water channels were visible
275 (figure 1a).

276 Analyses of the control biofilms (L-S-) (figure 2) revealed that in general there
277 was little difference in the spatial distribution of live cells relative to dead cells, with the
278 exception that at the margins of the upper and, in particular, lower biofilm interfaces
279 there seemed to be a slight increase in dead cells. In the case of the upper surface it is
280 possible that obligately anaerobic bacteria were killed during the sampling process (Hope
281 & Wilson, 2006). At the biofilm/substratum interface there may have been nutrient
282 limitation or a build up of toxic products (especially in areas relatively distant from water
283 channels) that may have resulted in the death of some cells.

284 Biofilms exposed to laser light and photosensitiser showed evidence of lethal
285 photosensitisation, as indicated by an increasing proportion of dead cells, particularly in
286 the upper portion of the biofilm (0 to 10 µm), suggesting that PDT was most effective in

287 this area (figure 3). In addition to the possible limitations associated with photosensitiser
288 efficacy discussed above, it is also possible that with increasing depth, biofilms became
289 increasingly more resistant to PDT. This may be due to reduced growth rate of cells with
290 increasing depth in the biofilm, a known contributor to antimicrobial resistance in biofilm
291 populations (Evans et al 1990, Roberts & Stewart, 2004).

292 CHL is used in the treatment of a wide range of oral infections, including
293 periodontitis (Pietruska et al 2006). Its efficacy against subgingivally-modelled *in vitro*
294 biofilms was compared to that of PDT to provide an indication of the suitability of PDT
295 as a putative therapeutic regime in treating periodontal disease. CHL (at 0.2%) did not
296 produce a significant kill when applied to the biofilms for 1 min, suggesting that this may
297 be an ineffective treatment when directed against periodontal plaques of similar
298 dimensions *in situ*. CHL did produce a statistically significant kill after 15 min, although
299 this would be an impractical exposure period clinically. However, it is possible that a
300 similar kill could have been achieved with an exposure period somewhere between 1 and
301 15 min or at increased concentration (not tested). When PDT was employed with a low
302 light dosage (2.1 J) for 1 min, no significant reduction in viability of the biofilms was
303 observed. However, as discussed above, comprehensive, significant kills were achieved
304 with 15 min exposure at increased light intensities, and optimally with a light dose and
305 photosensitiser concentration of 94.5 J and 81.7μM, respectively. Hence, from these
306 preliminary *in vitro* data one can conclude that PDT (using toluidine blue and HeNe laser
307 light) can be as effective as CHL when directed against biofilms similar to those found in
308 periodontal disease.

309 The site-specific clinical application of PDT would enable avoidance of the use of
310 antibiotics (which are often used following periodontal surgery) and the associated
311 problem of perturbation of the indigenous systemic microflora e.g. gastrointestinal upset.
312 The increasing prevalence of bacterial antibiotic resistance necessitates the development
313 of alternative antibacterial therapies. Sublethal concentrations of biocides may also select
314 for mutants expressing multidrug efflux pumps which can also render them antibiotic
315 resistant (Gilbert et al 2002a). Thus, routine use of antimicrobial oral healthcare products
316 could conceivably select for a population of bacteria with an intrinsic ability to resist
317 antibiotic therapy, and consequently limit the effectiveness of antibiotic treatment
318 following periodontal surgery. Development of resistance to chlorhexidine has been
319 demonstrated in some Gram-negative bacteria including *Psuedomonas aeruginosa*
320 (Thomas et al 2000) and *Klebsiella pneumoniae* (Fang et al 2002). With PDT, lethal
321 photosensitisation mediates bacterial killing via singlet oxygen and free radicals. Thus,
322 resistance development would be unlikely due to the multiplicity of target sites which
323 include the outer and plasma membranes of Gram-negative bacteria, DNA, and
324 photolabile surface-associated proteins (Bhatti et al 1998, Bhatti et al 2001) against which
325 the generated singlet oxygen and free radicals are active.
326

327 **References**

328 Allan, I., Newman, H. & Wilson, M. (2002). Particulate Bioglass reduces the viability of
329 bacterial biofilms formed on its surface in an *in vitro* model. *Clinical Oral Implants*
330 *Research* **13**, 53-8
331

332 Amano, A. (2003). Molecular interaction of *Porphyromonas gingivalis* with host cells:
333 implication for the microbial pathogenesis of periodontal disease. *Journal of*
334 *Periodontology* **74**, 90-6
335
336 Anwar, H., Dasgupta, M.K. & Costerton, J.W. (1990). Testing the susceptibility of
337 bacteria in biofilms to antibacterial agents. *Antimicrobial Agents and Chemotherapy* **34**,
338 2043-6
339
340 Bhatti, M., MacRobert, A., Meghji, S., Henderson, B. & Wilson, M. (1998). A study of
341 the uptake of toluidine blue O by *Porphyromonas gingivalis* and the mechanism of lethal
342 photosensitization. *Photochemistry and Photobiology* **68**, 370-6
343
344 Bhatti, M., Nair, S.P., Macrobert, A.J., Henderson, B., Shepherd, P., Cridland, J. &
345 Wilson, M. (2001). Identification of photolabile outer membrane proteins of
346 *Porphyromonas gingivalis*. *Current Microbiology* **43**, 96-9
347
348 Bodet, C., Andrian, E., Tanabe, S. & Grenier, D. (2007). *Actinobacillus*
349 *actinomycetemcomitans* lipopolysaccharide regulates matrix metalloproteinase, tissue
350 inhibitors of matrix metalloproteinase, and plasminogen activator production by human
351 gingival fibroblasts: a potential role in connective tissue destruction. *Journal of Cell*
352 *Physiology* **212**, 189-94
353

354 Chapple, I.L. (2004). Management of chronic periodontal diseases within the NHS.
355 *British Dental Journal* **196**, 505
356
357 Choi, Y., Wiessleder, R., & Tung, C.H. (2006). Selective antitumor effect of novel
358 protease-mediated photodynamic agent. *Cancer Research* **15**, 7225-9
359
360 Costerton, J.W., Lewandowski, Z., DeBeer, D., Caldwell, D., Korber, D. & James, G.
361 (1994). Biofilms, the customized microniche. *Journal of Bacteriology* **176**, 2137-42
362
363 Costerton, W., Veeh, R., Shirtliff, M., Pasmore, M., Post, C. & Ehrlich, G. (2003). The
364 application of biofilm science to the study and control of chronic bacterial infections.
365 *Journal of Clinical Investigation* **112**, 1466-77
366
367 Evans, D.J., Brown, M.R., Allison, D.G. & Gilbert, P. (1990). Susceptibility of bacterial
368 biofilms to tobramycin: role of specific growth rate and phase in the division cycle.
369 *Journal of Antimicrobial Chemotherapy* **25**, 585-91
370
371 Fang, C.T., Chen, H.C., Chuang, Y.P., Chang, S.C. & Wang, J.T. (2002). Cloning of a
372 cation efflux pump gene associated with chlorhexidine resistance in *Klebsiella*
373 *pneumoniae*. *Antimicrobial Agents and Chemotherapy* **46**, 2024-2028
374

375 Gilbert, P., Allison, D.G. & McBain, A.J. (2002). Biofilms *in vitro* and *in vivo*: do
376 singular mechanisms imply cross-resistance? *Journal of Applied Microbiology* **92** Suppl,
377 98S-110S
378
379 Gilbert, P., Maria-Litran, T., McBain, A.J., Rickard, A.H., & Whyte, F.W. (2002). The
380 physiology and collective recalcitrance of microbial biofilm communities. *Advances in*
381 *Microbial Physiology* **46**, 202-56
382
383 Herzog, M., Moser, J., Wagner, B. & Broecker. J. (1994). Shielding effects and hypoxia
384 in photodynamic therapy. *International Journal of Oral Maxillofacial Surgery* **23**, 406–8
385
386 Hope, C.K., Clements, D. & Wilson, M. (2002). Determining the spatial distribution of
387 viable and nonviable bacteria in hydrated microcosm dental plaques by viability
388 profiling. *Journal of Applied Microbiology* **93**, 448-55
389
390 Hope, C. K. & Wilson, M. (2006). Biofilm structure and cell vitality in a laboratory
391 model of subgingival plaque. *Journal of Microbiological Methods* **66**, 390-8
392
393 Malik, Z., Hanania, J. & Nitzan, Y. (1990). Bactericidal effects of photoactivated
394 porphyrins--an alternative approach to antimicrobial drugs. *Journal of Photochemistry*
395 *and Photobiology* B. **5**, 281-93
396

397 Meisel, P. & Kocher, T. (2005). Photodynamic therapy for periodontal diseases: state of
398 the art. *Journal of Photochemistry and Photobiology* B **79**, 159-70
399

400 Mettraux, G.R., Gusberti, F.A. & Graf, H. (1984). Oxygen tension (pO2) in untreated
401 human periodontal pockets. *Journal of Periodontology* **55**, 516-21
402

403 Millward, T.A. & Wilson, M. (1989). The effect of chlorhexidine on *Streptococcus*
404 *sanguis* biofilms. *Microbios* **58**, 155-64
405

406 Morris, A.J., Steele, J. & White, D.A. (2001). The oral cleanliness and periodontal health
407 of UK adults in 1998. *British Dental Journal* **191**, 186-192
408

409 O'Neill, J.F., Hope, C.K. & Wilson, M. (2002). Oral bacteria in multi-species biofilms
410 can be killed by red light in the presence of toluidine blue. *Lasers in Surgery and*
411 *Medicine* **31**, 86-90
412

413 Pietruska, M., Paniczko, A., Waszkiel, D., Pietruski, J. & Bernaczyk, A. (2006).
414 Efficacy of local treatment with chlorhexidine gluconate drugs on the clinical status of
415 periodontium in chronic periodontitis patients. *Advances in Medical Sciences* **51**, 162-5
416

417 Pihlstrom, B.L., Michalowicz, B.S. & Johnson, N.W. (2005). Periodontal diseases.
418 *Lancet* **366**, 1809-20
419

420 Rigaut, J.P. & Vassy, J. (1991). High-resolution three-dimensional images from confocal
421 scanning laser microscopy. Quantitative study and mathematical correction of the effects
422 from bleaching and fluorescence attenuation in depth. *Analytical and quantitative*
423 *cytology and histology* **13**, 223-32
424
425 Roberts, M.E. & Stewart, P.S. (2004). Modelling antibiotic tolerance in biofilms by
426 accounting for nutrient limitation. *Antimicrobial Agents and Chemotherapy* **48**, 48-52
427
428 Sternberg, E. & Dolphin, D. (1996). Pyrrolic photosensitizers. *Current Medicinal*
429 *Chemistry* **3**, 239 – 72
430
431 Thomas, L., Maillard, J.Y., Lambert, R.J. & Russell, A.D. (2000). Development of
432 resistance to chlorhexidine diacetate in *Pseudomonas aeruginosa* and the effect of a
433 "residual" concentration. *Journal of Hospital Infections* **46**, 297-303
434
435 Tuter, G., Kurtis, B., Serdar, M., Aykan, T., Okyay, K., Yucel, A., Toyman, U., Pinar, S.,
436 Cemri, M., Cengel, A., Walker, S.G. & Golub, L.M. (2007). Effects of scaling and root
437 planing and sub-antimicrobial dose doxycycline on oral and systemic biomarkers of
438 disease in patients with both chronic periodontitis and coronary artery disease. *Journal of*
439 *Clinical Periodontology* **34**, 673-81
440

441 Wilson, M. (2004). Lethal photosensitisation of oral bacteria and its potential application
442 in the photodynamic therapy of oral infections. *Photochemistry and Photobiology*
443 *Sciences* **3**, 412-418
444
445 Zanin, I.C., Goncalves, R.B., Junior, A.B., Hope, C.K. & Pratten, J. (2005).
446 Susceptibility of *Streptococcus mutans* biofilms to photodynamic therapy: an *in vitro*
447 study. *Journal of Antimicrobial Chemotherapy* **56**, 324-30
448
449 Zanin, I.C., Lobo, M.M., Rodrigues, L.K., Pimenta, L.A., Hofling, J.F. & Goncalves,
450 R.B. (2006). Photosensitization of *in vitro* biofilms by toluidine blue O combined with a
451 light-emitting diode. *European Journal of Oral Sciences* **114**, 64-9
452

453 **Table 1.** Effect of laser light intensity and sensitiser concentration on kill efficacy.

Light intensity (J)	TBO concentration (μM)	% kill
63	32.7	41.9*
63	0	0*
0	32.7	72.6*
63	81.7	69.0
63	0	0*
0	81.7	36.3*
94.5	32.7	82.7
94.5	0	0*
0	32.7	0*
94.5	65.4	95.0
94.5	0	0*
0	65.4	0*
94.5	81.7	97.4
94.5	0	54.4
0	81.7	57.1
94.5	163.4	74.4
94.5	0	0*
0	163.4	0*

454 * not significant

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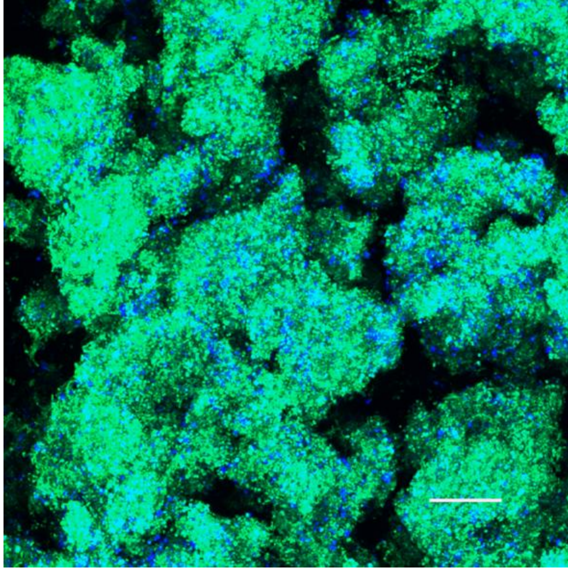
457 

458 **Figure 1a**: Projection of confocal image stack of a control CDFF-cultivated multi-species

459 biofilm (L-S-). Bar represents 20 µm. Viable cells = green, non-viable cells = blue.

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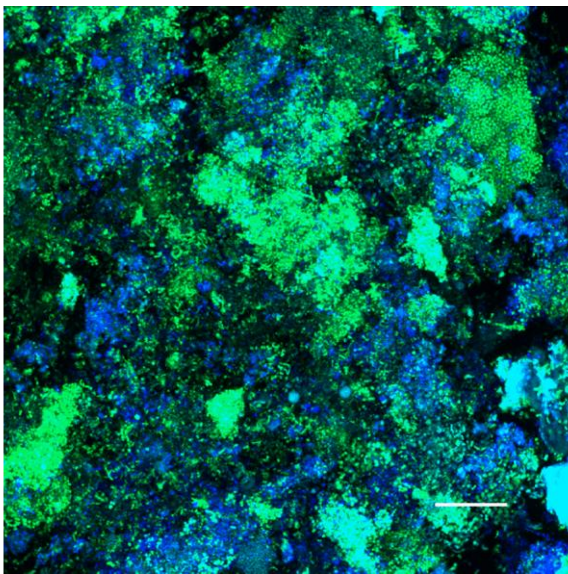
461 

462 **Figure 1b**: Projection of confocal image stack of a CDFF-cultivated multi-species

463 biofilm exposed to 63 J of HeNe laser light and 81.7 µM TBO (L+S+). Bar represents 20

464 µm. Viable cells = green, non-viable cells = blue

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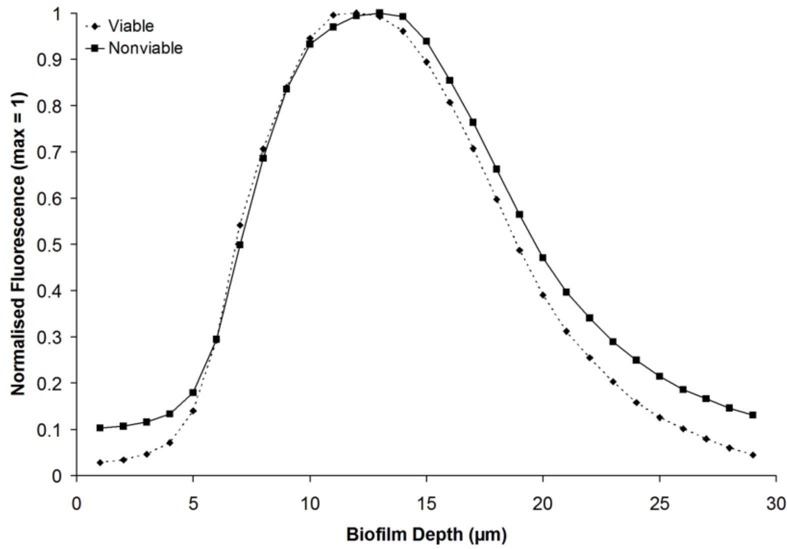

467 **Figure 2:** Normalised fluorescence intensity profile of viable and non-viable stain as a

468 function of depth in a control biofilm (L-S-), as determined by CLSM.

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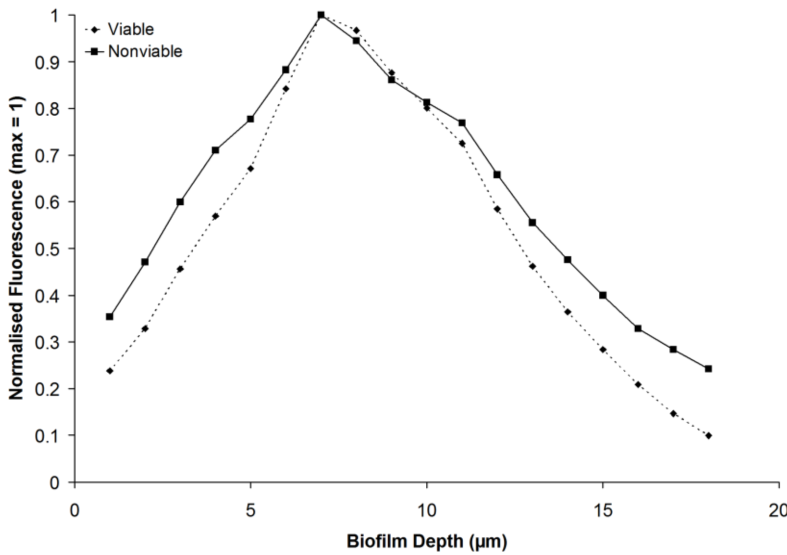

471 **Figure 3:** Normalised fluorescence intensity profile of viable and non-viable stain in

472 biofilms exposed to 63 J of HeNe laser light and 81.7µM pTBO (L+S+), as determined

473 by CLSM.