Susceptibility of microcosm subgingival dental plaques to lethal photosensitisation

I.U. Allan[1]*, J. F. O'Neill[2] and C.K. Hope[3]

[1]School of Pharmacy and Biomolecular Sciences, University of Brighton, UK

[2]Eastman Dental Institute, University College London, UK

[3]School of Dental Sciences, University of Liverpool, UK

* Corresponding author. Present address: School of Pharmacy and Biomolecular Sciences, University of Brighton, Cockcroft Building, Lewes Rd, Brighton, UK, BN2 4GJ.

E-mail address: i.u.allan@brighton.ac.uk

Abstract

Photodynamic therapy (PDT) offers potential as a non-invasive treatment of periodontal disease. In this study, microcosm biofilms were grown *in vitro* under conditions designed to mimic subgingival plaques typically found in patients with periodontitis. To investigate potential PDT modalities, biofilms were exposed to light from a helium/neon laser in conjunction with a photosensitiser, toluidine blue (TBO), at varying output and concentration, respectively. To determine cytotoxic effects, viability profiling was undertaken on whole biofilms using standard plating methods, and on horizontal cross-sections of biofilms using confocal laser-scanning microscopy (CLSM) in conjunction 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

application to treat bacterial infections is gaining momentum – the treatment of periodontal disease being one of the most promising (Meisel & Kocher, 2005). A major benefit of PDT is that the bactericidal effect is localised to areas which are treated with both photosensitiser and light, preventing disruption of the indigenous microflora at sites distal to the treated area (Wilson, 2004). The non-specific mode of action of PDT, expressly the generation of singlet oxygen, means that the acquisition of bacterial resistance to these agents is unlikely.

Previous studies have examined the effects of PDT on single-species biofilms using streptococci to model supragingival plaque in conditions associated with onset of dental caries (Zanin et al 2005, Zanin et al 2006) and *Streptococcus pyogenes* to model a skin biofilm (Hope & Wilson, 2006). However, PDT also offers a promising, non-invasive treatment for periodontal disease. Previously, it was reported that toluidine blue and helium/neon laser light could kill significant numbers of bacteria associated with periodontal disease, using a simple system where bacteria were grown on nitrocellulose membranes (O'Neill et al 2002). In this investigation we describe the modelling of subgingival plaque using a sophisticated *in vitro* model more representative of *in vivo* conditions.

The aim of this study therefore was to investigate the susceptibility of microcosm subgingival periodontal plaques grown in a constant-depth film fermenter (CDFF) to PDT using a range of biofilm viability profiling techniques, and to compare the effectiveness of PDT to the commonly used oral antimicrobial, CHL, representative of a conventional, non-invasive modality.

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

was added to each biofilm for sensitisation. Laser light intensities of 63 J (163.8 J cm^{-2}) and 95.4 J (248 J cm^{-2}) were employed (L+S+). Controls were TBO in the absence of laser light (L-, S+), and addition of 10 μl sterile saline, with or without laser light (L+S- and L-S-, respectively). In addition to controlling for laser light and TBO exposure, L-S- also served to determine whether exposure to an aerobic atmosphere for the duration of the experiment had any effect on bacterial viability. A pre-exposure time of 5 minutes (in the absence of light) was applied to allow the solutions to penetrate into the biofilm. The L+S+ and L+S- samples were then exposed to laser light for 15 min. Discs were then immersed in 1 ml pre-reduced BHI broth and biofilms re-suspended by vortexing for 60 sec. The total number of viable, culturable bacteria (CFU ml^{-1}) from re-suspended biofilms before and after treatment was determined by viable counting on Fastidious Anaerobe Agar (FAA) (Bioconnections, Leeds, UK) containing 5 % (v/v) defibrinated horse blood (E & O Laboratories, Bonnybridge, UK). Duplicate 25 μl aliquots were spread over the surface of FAA plates and these incubated for 7 days at 37ºC in an anaerobic atmosphere, and resultant colonies counted.

CLSM of biofilms subjected to PDT

Biofilms were subjected to either PDT using 63 J HeNe laser light and 81.7 μM TBO, or no treatment as described above. Post-PDT biofilms were then placed into a miniature Petri dish, biofilm uppermost and stained using the *Bac*Light™ bacterial viability kit (Molecular Probes, Oregon, USA) with 5 min stain penetration time in darkness. Scans were taken of the biofilms using a Leica DMLFS fixed stage microscope with a Leica TCS SP confocal scan-head. The objective lens was a 63x HCX water

immersion dipping lens. Confocal image stacks were captured of both the viable (488nm) and non-viable (568nm) fluorescent emissions. Data are from two biofilms, one an untreated control (L-S-), and the other subjected to PDT (L+S+).

Analysis of CLSM data

Fluorescence intensity profiles (Hope et al 2002) through the biofilms were generated by measuring the brightness of each optical section in the confocal image stack, for both the viable (488 nm) and non-viable (568 nm) channels, using ImageJ computer software (ImageJ 1.36d, The National Institutes of Health, USA, http://rsb.info.nih.gov/ij/). To enable an effective comparison between the viable and non-viable channels, these data were normalised against the maximum image intensity for each individual channel (maximum value = 1) and plotted against depth into the biofilm. Zero-depth was taken as the top of the confocal image stack.

Projection images were constructed by combining all confocal optical sections into one image.

Susceptibility of biofilms to chlorhexidine digluconate

Two pans, each containing five biofilms, were removed aseptically from the CDFF and placed in a sterile glass container. To this was added 5 ml 0.2% CHL (Sigma) or, in the case of the control pan, 5 ml PBS (Oxoid, Basingstoke, UK) and incubated for 1 or 15 min. Biofilms were then prepared for viable counting as described above. In parallel, PDT was applied to biofilms of the same age using a regime of 2.1 J HeNe laser light with 81.7 μM TBO for 1 min and then prepared for viable counting. In addition, the

15 min CHL exposure results were compared to the data generated using the PDT modalities described above (15 min treatment).

Statistical analyses

The statistical significance of the data was ascertained using the two-tail t-test assuming unequal variance. For viable counting following PDT, four individual biofilms were used to determine CFU ml^{-1} by plating in duplicate, n=8. For CHL and TBO studies, three biofilms were plated in duplicate, n=6. For ease of interpretation, viability is recorded as percentage kill compared to untreated control.

Results

Effect of PDT on biofilm viability

Table 1 shows the effect of increasing light dose and photosensitiser concentration on the efficacy of lethal photosensitisation in terms of the percentage of bacteria killed. The most effective combination of light and photosensitiser was 94.5 J and 81.7 μM TBO, which produced a significant kill of 97.4%. A reduced TBO concentration of 65.4 μM with the same light dosage still produced a significant kill of 95.0%. Increasing photosensitiser concentration to 163.4 μM but still using 94.5 J laser light reduced the efficacy of the procedure, producing a diminished, but significant, kill of 74.4%. A photosensitiser concentration of 32.7 μM in combination with a light dose of 94.5 J was sufficient to produce a significant bactericidal effect, with a kill of 82.7%.

Reducing the light dosage from 94.5 to 63 J reduced the bactericidal efficacy of PDT. 63 J laser light with a photosensitiser concentration of 81.7 μM produced a

significant kill of 69.0%, however this kill was less than that observed at the higher light dosage using the same photosensitiser concentration, albeit the difference was not significant. 63 J of laser light in conjunction with 32.7 μM TBO was insufficient to produce a significant lethal effect.

CLSM studies on biofilms subjected to PDT

Microcolony formations interspersed with water channels can be seen in the control biofilm shown in figure 1a. Viable cells (green) appeared to be far more numerous than the non-viable cells (blue). (It should be noted that the red, non-viable stain used in the procedure was digitally altered to appear blue using ImageJ for ease of interpretation). Figure 1b shows a multi-species biofilm that was subjected to lethal photosensitisation with 63 J of HeNe laser light in the presence of 10 μl of 81.7 μM TBO. Visually, there was a discernible difference between this biofilm and the control (figure 1a). The structure of the biofilm seems to have been disrupted by exposure to the light in the presence of the TBO. In addition, there appeared to be a larger proportion of non-viable to viable cells compared to the control biofilm.

The normalised fluorescence image intensity profile through the control biofilm (figure 2) conformed to those observed previously (Hope et al 2002, Hope & Wilson, 2006) in similar supragingival and subgingival plaque biofilm models. Biofilm which was exposed to 63 J of HeNe laser light in the presence of TBO (figure 3) showed a broader distribution of non-viable fluorescence, particularly in the upper layers closest to the illumination source.

Effect of chlorhexidine digluconate on biofilm viability

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

Discussion

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

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

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

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

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

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

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

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

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

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

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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

455
456

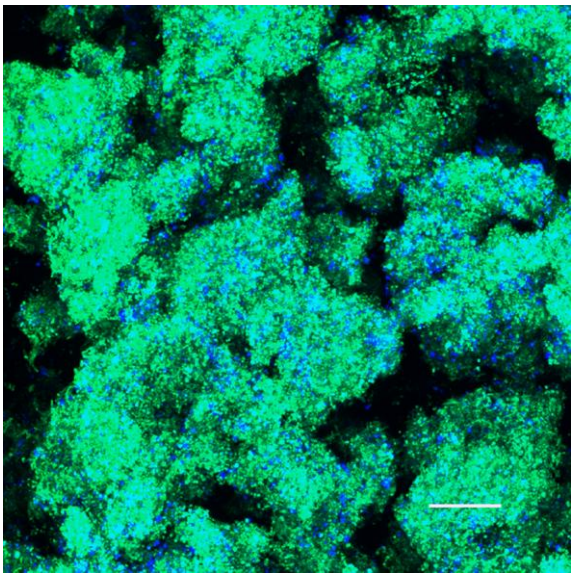

Figure 1a: Projection of confocal image stack of a control CDFF-cultivated multi-species biofilm (L-S-). Bar represents 20 µm. Viable cells = green, non-viable cells = blue.

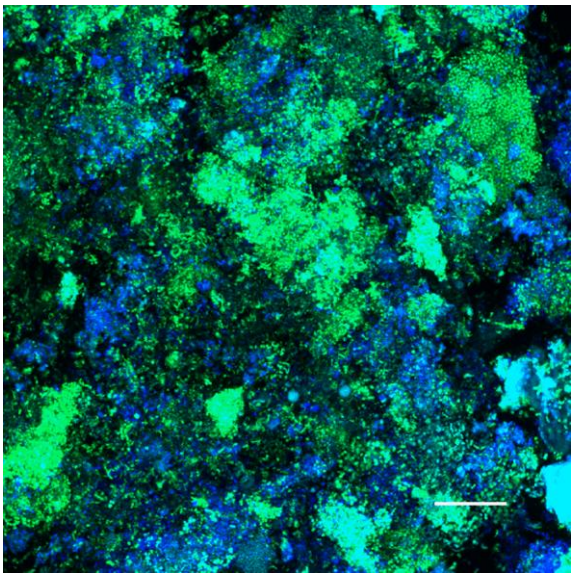

Figure 1b: Projection of confocal image stack of a CDFF-cultivated multi-species biofilm exposed to 63 J of HeNe laser light and 81.7 µM TBO (L+S+). Bar represents 20 µm. Viable cells = green, non-viable cells = blue

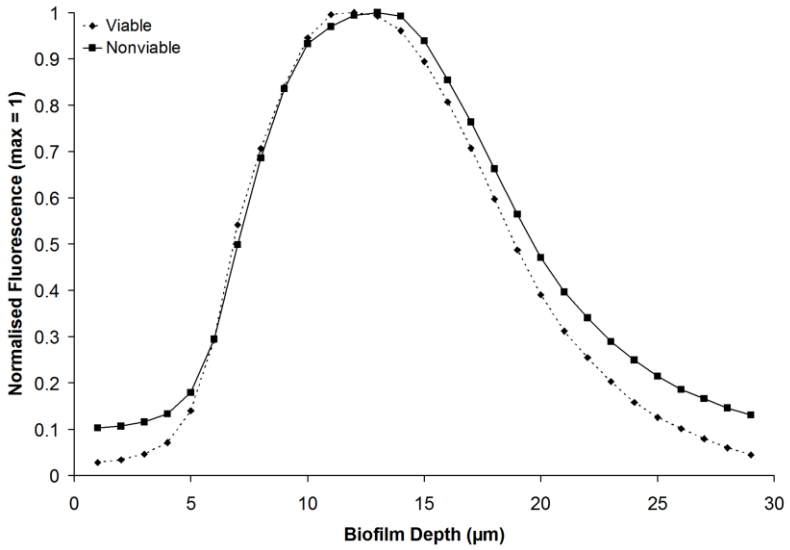

Figure 2: Normalised fluorescence intensity profile of viable and non-viable stain as a function of depth in a control biofilm (L-S-), as determined by CLSM.

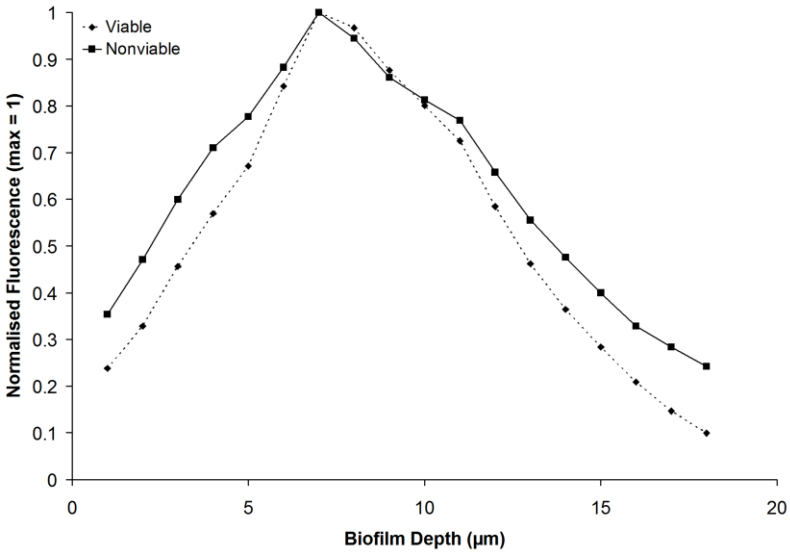

Figure 3: Normalised fluorescence intensity profile of viable and non-viable stain in biofilms exposed to 63 J of HeNe laser light and 81.7µM pTBO (L+S+), as determined by CLSM.