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ORIGINAL ARTICLE

Investigation of critical inter-related factors affecting the efficacy of pulsed light for inactivating clinically relevant bacterial pathogens

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Abstract

Aims: To investigate critical electrical and biological factors governing the efficacy of pulsed light (PL) for the *in vitro* inactivation of bacteria isolated from the clinical environment. Development of this alternative PL decontamination approach is timely, as the incidence of health care–related infections remains unacceptably high.

Methods and Results: Predetermined cell numbers of clinically relevant Gram-positive and Gram-negative bacteria were inoculated separately on agar plates and were flashed with ≤60 pulses of broad-spectrum light under varying operating conditions, and their inactivation measured. Significant differences in inactivation largely occurred depending on the level of the applied lamp discharge energy (range 3·2–20 J per pulse), the amount of pulsing applied (range 0–60 pulses) and the distance between light source and treatment surface (range 8–20 cm) used. Greater decontamination levels were achieved using a combination of higher lamp discharge energies, increased number of pulses and shorter distances between treatment surface and the xenon light source. Levels of microbial sensitivity also varied depending on the population type, size and age of cultures treated. Production of pigment pyocynanin and alginate slime in mucoid strains of *Pseudomonas aeruginosa* afforded some protection against lethal action of PL; however, this was evident only by using a combination of reduced amount of pulsing at the lower lamp discharge energies tested. A clear pattern was observed where Gram-positive bacterial pathogens were more resistant to cidal effects of PL compared to Gram negatives. While negligible photoreactivation of PL-treated bacterial strains occurred after full pulsing regimes at the different lamp discharge energies tested, some repair was evident when using a combination of reduced pulsing at the lower lamp discharge energies. Strains harbouring genes for multiple resistances to antibiotics were not significantly more resistant to PL treatments. Slight temperature rises (≤4·2°C) were measured on agar surfaces after extended pulsing at higher lamp discharge energies. Presence of organic matter on treatment surface did not significantly affect PL decontamination efficacy, nor did growth of PL-treated bacteria on selective agar diminish survival compared to similarly treated bacteria inoculated and enumerated on nonselective agar plates.

Conclusions: Critical inter-related factors affecting the effective and repeatable *in vitro* decontamination performance of PL were identified during this study that will aid further development of this athermal process technology for applications in health care and in industry. Very rapid reductions (*c.* 7 $\log_{10}$ CFU cm^{-2}

within ≤10 pulses) occurred using discharge energy of 20 J for all tested clinically relevant bacteria under study when treated at 8 cm distance from xenon light source. While no resistant flora is expected to develop for treatment of microbial pathogens on two-dimensional surfaces, careful consideration of scale up factors such as design and operational usage of this PL technique will be required to assure operator safety.

Significance and Impact of the Study: Findings and conclusions derived from this study will enable further development and optimization of this decontamination technique in health care and in food preparation settings, and will advance the field of nonthermal processing technologies.

Introduction

There is growing international concern regarding the unacceptably high levels of health care–related infections due in part to the continued development of antimicrobial resistance. Prevention of infection is a superior approach compared to the cost and consequences of treatment of infection, with strong emphasis placed on hand hygiene compliance and proper cleaning regimens that include use of effective surface decontamination techniques (Solberg 2000). However, conventional surface decontamination approaches such as chemical biocides have recognized limitations that include unwanted microbial adaptation, subsequent cross-resistance to front line antibiotics, and lingering chemical residues (Lambert *et al.* 2001; Randall *et al.* 2001; Gebel *et al.* 2002). Numerous studies have also highlighted limitations of decontamination techniques such as conventional, continuous-wave (CW) UV mercury lamps of low pressure designed to produce energy at 254 nm (called monochromatic or germicidal light) that include microbial repair and the necessity for lengthy durations of exposure to obtain suitable levels of decontamination (Bintsis *et al.* 2000). More recently, medium-pressure mercury UV lamps have been used because of their much higher germicidal UV power per unit length and because of their ability to emit polychromatic light comprising germicidal wavelengths from 200 to 300 nm (Bolton and Linden 2003).

Pulsed light (PL) is an approach that has received considerable attention as a strategy for decontaminating food, packaging, water and air (Dunn *et al.* 1997; Gómez-López *et al.* 2007). However, PL technology is also a strong candidate for contact surface decontamination in the health care setting. This approach kills micro-organisms by using ultrashort-duration pulses of an intense broadband emission spectrum that is rich in UV-C germicidal light (200–280 nm band). PL is produced using techniques that multiplies power manifold by storing electricity in a capacitor over relatively long times (fractions of a second) and releasing it in a short time (millionths or thousandths of a second) using sophisticated pulse compression techniques (Rowan *et al.* 1999; Gómez-López *et al.* 2007). The emitted flash has a high peak power and usually consists of wavelengths from 200 to 1100 nm broad-spectrum light enriched with shorter germicidal wavelengths (MacGregor *et al.* 1998; Gómez-López *et al.* 2007). This technology has received several names in the scientific literature: pulsed UV (PUV) light (Anderson *et al.* 2000; Sharma and Demirci 2003; Wang *et al.* 2005), high-intensity broad-spectrum PL (Roberts and Hope 2003), PL (Rowan *et al.* 1999), intense PL (Gómez-López *et al.* 2007) and pulsed white light (Marquenie *et al.* 2003). Seminal developments pertaining to these next generation light-flashing technologies has been the subject of recent review (Elmnasser *et al.* 2007; Gómez-López *et al.* 2007), with emphasis strongly placed on decontamination efficacy for food and water applications that aptly reflects the focus of research in this field of study to date. A strong advantage of using pulsed xenon lamps over continuous low-to-medium pressure conventional UV lamps is that the latter has a characteristic high peak power dissipation, which allows for more rapid microbial inactivation. A continuous 10 W lamp needs to be operated for 10 s to achieve the same decontamination efficacy (supplying same energy) as a pulsed lamp of typically 1 MW operated for just 100 μs. Otaki *et al.* (2003) also reported that adaptive microbial survival (tailing phenomenon) occurs when samples are treated in high turbidity solutions using continuous UV sources, whereas tailing did not occur when similar samples were treated with pulsed xenon lamp.

While current findings from the literature suggests that development of a pulse light approach appears promising, most of the studies to date have focused on food or water applications using a limited range of electro-physical or biological parameters, such as use of a single lamp discharge energy (J) or fluence (UV dose cm^{-2}) and/or employing a single distance from light source to target treatment area. These landmark *in vitro* and *in vivo* PL

studies have been recently reviewed for efficacy in terms of inactivating food-related spoilage organisms and potential microbial pathogens (Elmnasser *et al.* 2007; Gómez-López *et al.* 2007), and include studies carried out using lamp discharge energies of 3 J (MacGregor *et al.* 1998; Rowan *et al.* 1999), 7 J cm^{-2} (Marquenie *et al.* 2003 and Gómez-López *et al.* 2005), 0·99 J cm^{-2} (Krishmanmurthy *et al.* 2004, 0·7 J cm^{-2} (Takeshita *et al.* 2003) and 1 J cm^{-2} (Wekhof *et al.* 2001). These studies demonstrated that factors such as number of light pulses applied, lamp discharge intensity, distance from lamp to treatment surfaces, shading, microbial species, age and density affected the efficacy of PL decontamination performances. To the best of our knowledge, no published study to date has investigated the *in vitro* decontamination efficacy of varying critical electro-physical parameters and biological conditions on PL-treated bacteria that were originally isolated from patients in health care environment.

Despite advances highlighted earlier, more extensive basic research is required to understand comprehensively how PL affects clinically relevant bacteria with view to maximizing the potential of this decontamination technique for application in various settings. This study therefore (i) includes a critical evaluation of the effect of varying key electrical parameters such as lamp discharge energy and pulse number on the sensitivity of a wide range of bacteria isolated from patients from clinical environment to PL treatments, (ii) the influence of bacterial population size, age and type on the level of decontamination by PL, (iii) the influence of bacterial production of light absorbing pigments and alginate slime on PL decontamination efficacy, (iv) the influence of antimicrobial drug resistance status on treated bacteria and presence of organic mater on sensitivity to PL treatments, (v) investigate how the relative position of the sample with respect to xenon lamp affects lethality of PL using different discharge energies, (vi) verifying the existence of the photoreactivation phenomenon and possible development of resistance in flashed clinically relevant bacteria, (vii) the heating of supporting agar medium by PL irradiation; emphasizing the implications of these findings for application of PL in health care settings.

Materials and methods

PL equipment

These studies used a bench-top pulsed power source (PUV-1; Samtech Ltd, Glasgow, UK), a low-pressure (60 kPa) xenon-filled flashlamp (Heraeus Noblelight XAP type NL4006 series constructed from a clear UV transparent quartz tube; Heraeus Noblelight Inc., Duluth, GA) that produced a high-intensity diverging beam of polychromatic PL as per the system arrangement described recently by Wang *et al.* (2005). The solid-state pulsed power source utilizes power-compression to transfer stored electrical energy to the xenon flashlamp in a short duration but with peak power. When the pulsed power source operates at 1 kV, as in this study, the energy of 20 J is stored in a 40 μF capacitor, and the energy is dissipated in the flashlamp within 30 μs, thereby producing a peak power of 1 MW. The PL has a broadband emission spectrum extending from the UV to the infrared region with a rich UV content and its intensity also depends on the level of the voltage applied as shown by the UV/visible lamp spectra in Fig. 1 as reported by the fabricant. The fabricant stated that the discharge tube represents a line source of limited length and as a consequence of this, the light formed an elliptical, equi-intensity profile over the sample plane eliminating shading effects. This resulted in a *c.* 30% variation in luminous intensity between the centre and the edge of the sample. The light emission spectrum was monitored using a four channel Ocean Optics SQ2000 fibre optic spectrometer (Ocean Optics, Dunedin, FL) (Fig. 1). The spectral resolution was 1·25 nm on each channel and the optical detector (Sony 1LX511; StellarNet Inc., Tampa, FL) had been enhanced to allow UV detection. The pulsed broadband emission spectra exhibit rich output in the UV-C region from 220 to 280 nm comprising three distinct peaks at 229, 247 and 260 nm. This xenon lamp is therefore capable of providing high-energy UV output using a small number of short-duration pulses. The light source has an automatic frequency-control function that allows it to operate at 1 pulse per second that was used throughout this study. Light exposure was homogenous as the xenon lamp (9·0 cm × 0·75 cm in length and width, respectively) was longer than the 8·5 cm diameter polystyrene Petri dishes used in the tests, which were placed directly below the lamp. For standard treatments (unless

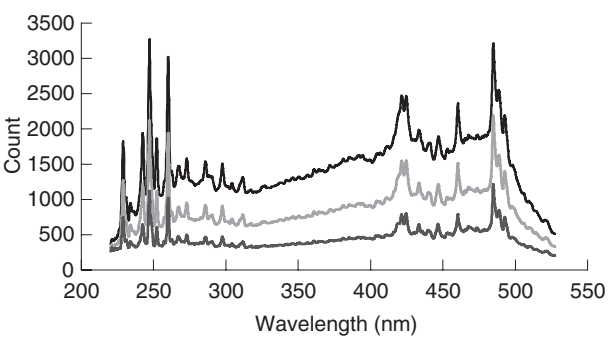

Figure 1 Spectral distribution of used xenon lamp at different applied voltages with equivalent discharge energies. (—) 1000 V or 20 J; (–) 800 V or 12·8 J and (—) 600 V or 7·2 J.
Source and permission: Samtech, Glasgow, UK.

otherwise noted), the light source was mounted at 8 cm above the treatment area that was designed specifically to accommodate a standard Petri dish.

PL inactivation procedures

In this study, standard treatments involved surface spread-inoculating 8·5 cm Petri dishes that were positioned at 8 cm distance from the light source (unless otherwise noted), and separately subjected to lamp discharge energies of 3·2, 5, 7·2, 9, 12·8 and 20 J. The number of pulses of light applied ranged from 0 (control) to 60 pulses. The influence of varying distance from the light source from the inoculated Petri dishes (range 8–20 cm) on inactivation efficacy was also investigated by rotating the treatment shelf in the PL system to the desired distance. The PL system was manually adjusted to deliver 1 pulse per second for all studies. The lethality of the PL process under varying experimental conditions was determined by treating predetermined numbers of test organisms that were inoculated on agar surfaces and by enumerating survivors post treatments (expressed in terms of $\log_{10}$ CFU cm^{-2}). Subtraction of the logarithm of the count after different combinations of pulsing, discharge energies and distances from light source, from the logarithm of the count before processing provided a measure of process lethality. As it is not appropriate to determine log-linear death rate kinetic data from PL-treated test bacteria as a function of time (as total duration of treatment comprises the pulse-pause sequence as well as the actual duration of pulsing), inactivation plots were measured following a modified first-order dependence formulae of Kühn *et al.* (2003): $(\log_{10}\ (N_P/N_0) = -k_p$, where k = rate constant). N_0 is the initial concentration of bacteria and N_p is the microbial concentration after applying a defined number of pulses at discharge energy (J). This rate constant k defines the sensitivity of a micro-organism to a defined PL treatment and is unique to each microbial species; the higher the k value the more sensitive the test strain to the treatment process. The former approach was employed to determine k values for PL-treated test bacteria, as individual fluence values ($\mu W\ cm^{-2}$) were not available for all combinations of lamp discharge energy and distance from light source . All experiments were carried out in triplicate using same culture to avoid sample variability.

Bacterial strains used and preparation

The species of test bacteria used in these experiments, their origin, clinical relevance and corresponding selective culture media are summarized in Table 1. Initially (and unless otherwise stated), emphasis was placed on using an extensive number of *Pseudomonas aeruginosa* test strains for investigating electrical and biological factors affecting PL decontamination efficacy with subsequent comparisons of tolerances to a range of other clinically significant Gram-positive and Gram-negative bacteria isolated from patients in health care environment. Test bacteria also comprised multiple antimicrobial drug–resistant (MDR) strains, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* species (VRE) and *Klebsiella pneumoniae* ATCC 700721. Confirmation of MDR strain status was performed in a clinical laboratory using recommended disc diffusion susceptibility testing as per standard procedures (Clinical and Laboratory Standards Institute 2008). *Staphylococcus aureus* D3187 was shown to be resistant to erythromycin, cefoxitin 30, fucidin, ofloxacin and oxacillin/flucloxacillin, while sensitive to chloramphenicol, linezolid and vancomycin. *Enterococcus faecium* J5616 and J5810 were resistant to amoxicillin/ampicillin and vancomycin, while sensitive to gentamicin, linezolid and quinupristin/dalfopristin. E-testing confirmed resistance to vancomycin for both *Ent. faecium* J5616 and J5810, where minimum inhibitory concentration was shown to be >256 $\mu g\ ml^{-1}$. *Klebsiella pneumoniae* ATCC 700721 was resistant to ampicillin, ticarcillin, trimethoprim–sulfamethoxazole and gentamicin, but was susceptible to amikacin, ciprofloxacin and imipenem. The vegetative form of *Bacillus cereus* were prepared as per methods described previously (Rowan *et al.* 1999). All test strains were maintained in Microbank storage vials (Cruinn Diagnostics, Dublin 12, Ireland) at −70°C. The identity of three randomly selected isolates of each bacterial pathogen was confirmed before and after experimental studies as per methods described by Barrow and Feltham (2003). Strains were stored at 4°C on agar slopes of tryptone soya agar supplemented with 0·6% yeast extract (TSYEA) (Oxoid, Basingstoke, UK) and monthly checked for purity and renewed. To prepare the test samples, bacterial test strains were streaked to purity from porous beads taken from Microbank vials, and an isolated colony then transferred to 50 ml tryptone soya broth supplemented with 0·6% yeast extract and cultivated with shaking at 125 oscillations per minute for 24 h at 37°C to reach early stationary phase. The optical density was then adjusted at 540 nm to 2·0 (*c.* 10^9 CFU ml^{-1}) by spectrophotometric (Model UV-120-02 instrument; Shimadzu Corp., Kyoto, Japan) determination using 0·1 mol l^{-1} phosphate buffered saline (PBS) (pH 7·2) (confirmed via aerobic plate count). Using the spread plate technique, 0·5 ml aliquots of the 24-h OD_{540}-adjusted cultures were then inoculated onto triplicate TSYEA agar plates. After drying for 1 h on the laboratory bench to avoid light attenuation because of PBS, plates were flashed and then incubated without further treatment at 37°C for 48 h. Care was taken to ensure that samples were centrally inoculated on each agar plate and were subsequently spread

Table 1 Origin, clinical significance and culture media of used test bacteria

Bacterium	Code	Source*		Clinical relevance†	Selective media (solid)‡
		Origin	Clinical site		
Gram-positive bacteria					
Enterococcus faecium	J952	NUIG	Yes	Bloodstream	ESA
	J5616	NUIG	Yes	VRE	ESA
	J5810	NUIG	Yes	VRE	ESA
Enterococcus faecalis	D46209	NUIG	Yes	Bloodstream	ESA
Enterobacter cloacae	04B3311	DIT	Yes	Bloodstream	VRBGA
Enterobacter sakazakii	8155	NCTC	No	Food poisoning	HESA
Staphylococcus aureus	25923	ATCC	No	None reported	BPA
	5624	NUIG	Yes	Bloodstream	BPA
	J2860	NUIG	Yes	Bloodstream	BPA
	D3187	NUIG	Yes	MRSA	BPA
Staphylococcus epidermidis	10221	NUIG	Yes	Bloodstream	CA
	03B2511	DIT	Yes	Device implant	CA
Bacillus cereus	11143	NCTC	No	Vomit	BCSA
	22728	LMG	No	Food poisoning	BCSA
Listeria monocytogenes	11994	NCTC	No	Meningitis	LSA
	9863	NCTC	No	Meningitis	LSA
Gram-negative bacteria					
Acinetobacter baumanii	D3953	NUIG	Yes	Bloodstream	MLAA
Escherichia coli	25922	ATCC	No	None reported	MacCA
	411	NUIG	Yes	–	MacCA
	02B1173	DIT	Yes	UTI	MacCA
Pseudomonas aeruginosa	5449	NUIG	Yes	–	KAA
	R5137	NUIG	Yes	Sputum	KAA
	R1460	NUIG	Yes	Sputum	KAA
	2605	NUIG	Yes	–	KAA
	2633	NUIG	Yes	Sputum	KAA
	02B7570	DIT	Yes	UTI	KAA
	2502	NUIG	Yes	–	KAA
	02B710	DIT	Yes	Otitis media	KAA
	03B8474	DIT	Yes	–	KAA
	03B6908	DIT	Yes	–	KAA
	03B3922	DIT	Yes	Bloodstream	KAA
	04B2325	DIT	Yes	Sputum	KAA
	03B3694	DIT	Yes	–	KAA
	03B3845	DIT	Yes	Bloodstream	KAA
	27853	ATCC	No	None reported	KAA
Klebsiella pneumoniae	04B4415	DIT	Yes	Liver abscess	SCAI
	700721	ATCC	Yes	Sputum (MDR)	SCAI
Proteus mirabilis	02B1121	DIT	Yes	UTI	TMSA

–, not known.

*Code for cultures used: ATCC, American Type Culture Collection, Rockville, MD, USA; NCTC, National Collection of Type Cultures, Colindale, London, UK; LGM, Laboratorium Microbiologie, Universiteit Gent, Belgium; NUIG, National University of Ireland Galway, Department of Bacteriology, Ireland; DIT, Dublin Institute of Technology, Kevin Street, Dublin, Ireland.

†Clinical codes: VRE, vacomycin-resistant enterococcus; MRSA, methicillin-resistant *Staph. aureus*; UTI, urinary tract infection; MDR, multiple drug resistant.

‡Selective media codes and sources: ESA, enterococcus selective agar (SIFIN, Berlin, Germany); VRBGA, violet red bile glucose agar (Oxoid, Hampshire, UK); BPA, Baird Parker agar (Oxoid); MLAA, modified Leeds *Acinetobacter* agar (Oxoid); MacCA, MacConkey agar (Oxoid); BCSA, *B. cereus* selective agar (Oxoid); LSA, *Listeria selective* agar (Oxoid); SCAI, Simmons citrate agar with 1% (w/v) inositol (Difco); TMSA, Thayer–Martin selective agar (Difco); HESA, hi-crome *Enterobacter sakazaki* agar (Biochemika); CA, Chapman agar (Merck).

up to a distance of *c.* 0·5 cm from the perimeter of the polystyrene Petri dish in or to minimize or eliminate any shading effects. The latter procedure was adopted as it also is recognized that polystyrene absorbs considerably in the UV range. 15 ± 0·1 ml of molten agar was aseptically transferred by pipette to Petri dishes thus maintaining

standardized depth of agar for all studies. After incubation, separate colonies were enumerated and survivors were expressed in terms of $\log_{10}$ CFU cm^{-2}. Samples were also similarly spread inoculated on selective agar media for each respective test bacteria to investigate the influence of antimicrobial agents and/or dye present in selective agars on survival of PL-treated samples (Table 1). The limit of detection was one colony. Typical colonies of each test strain were randomly selected from respective agar plates after 24 and 48 h at 37°C with the highest dilution, and were confirmed by use of appropriate physiological and biochemical tests as described earlier.

Influence of population size and age on decontamination efficacy

To determine the influence of bacterial population size on the decontamination efficacy of PL, several agar media in Petri dishes were inoculated with 0·5 ml aliquots of the tenfold dilution of 24 h OD_{540} adjusted culture on TSYEA plates to give initial cell populations of *c.* 3, 5, 7 and 9 $\log_{10}$ CFU cm^{-2}. Plates were removed from the PL treatment chamber after predetermined number of flashes and survivors were enumerated by means of the above plating method. To determine the influence of population age on sensitivity of test strains to PL treatments, cultures were grown for 16 h (late exponential) and the OD was spectrophotometrically adjusted to *c.* 2·0 at 540 nm giving an initial cell population of *c.* 9 $\log_{10}$ CFU ml^{-1} (confirmed by plate count). Plates were then inoculated, PL treated and survivors enumerated as per method described earlier.

Media heating

Heating of the surface of agar plates inoculated with test bacteria was investigated using thermal imaging (IRI 4010; InfraRed Integrated Systems Ltd, Northampton, UK) as per modified method of Nugent and Higginbotham (2007). Plates were positioned 8 cm from light source and were flashed with 60, 45 and 20 pulses of 3·2, 12·8 and 20 J, respectively. An image was taken of the treated plates immediately post treatment and examined for thermal data using relevant software (IRISYS 4000 Series Imager, ver. 1.0.0.14; Infra Red Integrated Systems Ltd, Northampton, UK). Each data point represents a mean value of 12 randomly selected points over three replicate treatments.

Influence of photoreactivation on decontamination efficacy

This experiment was designed to investigate the effect of sunlight on the reactivation of damaged bacteria under typical real-life conditions as per method described by Gómez-López *et al.* (2005) with modifications. To test the possibility that photoreactivation occurs, agar plates were inoculated with test bacteria and were exposed to PL treatments according to standard method and then subjected to the following. The first three plates were immediately wrapped in aluminium foil post treatment; the other three similarly treated plates were exposed to direct sunlight by placement on laboratory bench illuminated by sunlight for 4 h. The plates were incubated for 48 h at 37°C as described earlier and survivors enumerated (expressed as $\log_{10}$ CFU ml^{-2}). The experiment was conducted in triplicate.

Determination of alginate levels from *Pseudomonas aeruginosa* test strains

The purpose of this experiment is to investigate whether production of mucoid (or slime) by *Ps. aeruginosa*, which is directly attributed to synthesis of exopolysaccharide alginate, confers resistance to PL exposure. Fifteen strains of *Ps. aeruginosa* comprising seven mucoid and eight nonmucoid strains as noted in Table 1 were grown on MacConkey agar supplemented with 5% (v/v) glycerol and *Ps. aeruginosa* chromogenic agar (bioMérieux, La Balme-les-Grottes, France) at 37°C and visually checked thereafter for mucoid colony development after 24 and 48 h incubation as recommended by Laine *et al.* (2009) (Table 2). Isolated colonies of mucoid strains (and nonmucoid control strain ATCC 27853) were then separately grown with shaking at 150 rev min^{-1} for 20 h at 37°C in 50 ml of chemically defined alginate promoting medium of Terry *et al.* (1991) that was adjusted to pH 7 before media sterilization. To confirm alginate production in culture suspensions for PL studies, cells were pelleted after incubation by centrifugation at 10 000 rev min^{-1} for 10 min at 4°C. Thereafter, the culture supernatant was precipitated using an equal volume of 2% (w/v) cetylpyridinium chloride (Sigma) followed by centrifugation at 10 000 rev min^{-1} for 10 min at 4°C (Knutson and Jeans 1968). The alginate pellet was resuspended in 5 ml of 1 mol l^{-1} NaCl and precipitated with 5 ml of 2-propanol and centrifuged at 10 000 rev min^{-1} for 10 min at 4°C. The final alginate pellet was resuspended in 500–4000 μl PBS depending on the quantity of alginate recovered. The amount of alginate was determined using a borate/carbazole method for which sodium alginate (Sigma) was used to create a standard curve. A 30 μl solution of purified alginate was mixed with 1·0 ml borate/sulfuric acid reagent (10 mmol l^{-1} H_2BO_2 in concentrated H_2SO_4) and 30 μl carbazole reagent (Sigma) [0·1% (v/v) in ethanol] was added. The mixture was heated to 55°C for 30 min and

Table 2 Comparison of the relationship between pyocyanin and alginate slime and *in vitro* inactivation of *Pseudomonas aeruginosa* strains treated at 8 cm distance from light source with discharge energy of 7·2 J

Test strain	Pyocynanin (μg 5 ml^{-1})	Alginate slime	Population reduction* Pulse number 5	10	15	20	Inactivation rate† at 7·2 J *k* value	R^2
5449	–	–	3·4 ± 0·1	6·8 ± 0·3	7·2 ± 0·4	7·2 ± 0·1	0·64 ± 0·04D	0·99
R1460	–	–	2·4 ± 0·1	5·5 ± 0·2	5·6 ± 0·4	6·4 ± 0·4	0·54 ± 0·02B	0·97
R5137	–	+	1·4 ± 0·1	4·1 ± 0·2	5·8 ± 0·3	6·7 ± 0·5	0·41 ± 0·03A	0·99
2605	8·3 ± 0·3	+	2·0 ± 0·1	4·2 ± 0·2	5·9 ± 0·3	6·3 ± 0·2	0·37 ± 0·01A	0·99
2633	–	+	1·9 ± 0·1	4·8 ± 0·1	5·4 ± 0·4	6·1 ± 0·3	0·42 ± 0·03A	0·96
02B7570	2·9 ± 1·1	+	1·5 ± 0·1	4·0 ± 0·2	5·9 ± 0·2	6·7 ± 0·5	0·41 ± 0·03A	0·99
02B710	12·2 ± 0·2	+	2·0 ± 0·1	4·4 ± 0·2	5·8 ± 0·3	6·8 ± 0·4	0·39 ± 0·02A	0·99
03B8474	3·7 ± 1·1	–	2·5 ± 0·2	5·5 ± 0·3	5·7 ± 0·4	6·5 ± 0·5	0·54 ± 0·01B	0·98
03B6908	4·6 ± 1·0	–	3·6 ± 0·1	7·0 ± 0·2	7·2 ± 0·2	7·2 ± 0·3	0·67 ± 0·02D	0·99
03B3694	2·4 ± 0·9	–	3·5 ± 0·2	6·9 ± 0·1	7·1 ± 0·3	7·2 ± 0·1	0·64 ± 0·02D	0·96
03B3922	4·3 ± 0·3	–	3·0 ± 0·1	5·6 ± 0·2	6·2 ± 0·2	6·4 ± 0·3	0·58 ± 0·02C	0·98
04B2325	–	+	3·8 ± 0·2	6·0 ± 0·2	6·9 ± 0·4	7·0 ± 0·3	0·56 ± 0·03C	0·98
2508	–	+	3·3 ± 0·2	6·3 ± 0·3	6·5 ± 0·1	7·0 ± 0·4	0·61 ± 0·03C	0·98
27853	6·5 ± 0·5	–	3·4 ± 0·1	6·3 ± 0·2	6·6 ± 0·4	6·9 ± 0·3	0·61 ± 0·02C	0·98
03B3845	2·2 ± 0·3	–	3·3 ± 0·2	6·3 ± 0·1	6·6 ± 0·2	7·0 ± 0·2	0·61 ± 0·02C	0·98

(–), Not detected; (+) detected.

*Population reduction, expressed in $\log_{10}$ CFU cm^{-2}, achieved after pulsing at 7·2 J where initial population was inoculated on agar surfaces at *c.* 9 $\log_{10}$ CFU cm^{-2}.

†Reduction rate constant (*k*) is the mean rate (±SD) of log cell reductions per pulse, which is measure of inactivation of test strain per pulse. The higher the *k* value the more sensitive the strain to pulsed light treatments. R^2 is correlation co-efficient from linear regression data. Values with different capitalized letter in same column are significantly different at $P \leq 0{\cdot}05$ level.

the alginate concentration was determined spectrophotometrically at 500 nm.

Determination of pyocyanin levels from *Pseudomonas aeruginosa* test strains

The relationship between production of the light absorbing pigment pyocyanin by some *Ps. aeruginosa* strains and sensitivity to PL irradiation was examined. The method of Essar *et al.* (1990) was followed with slight modifications. The pyocyanin assay is based on the absorbance of pyocyanin at 520 nm in acidic solution. The strains were grown with shaking (125 rev min^{-1}) at 37°C for 24 h in 50 ml Cetrimide broth medium (Watman, Kent, UK) that is similar in formulation to King's A medium and known to promote pyocyanin production in *Ps. aeruginosa* (Brown and Lowbury 1965). A 5 ml aliquot was transferred to a sterile universal and centrifuged at 4000 rev min^{-1} for 10 min at 4°C. The supernatant was filtered through a 0·2-μm filter (Millipore), and pyocyanin was subsequently extracted from the filtered supernatant with 3 ml of chloroform. The solution was vortexed to mix before centrifugation at 4000 rev min^{-1} for 10 min at 4°C. The upper layer was carefully removed using a micropipette and discarded. The pyocyanin, if present in the bottom layer, was subsequently transferred to a fresh universal and 1 ml of 0·2 mol l^{-1} HCl was added. The solution was vortexed and centrifuged as described earlier. The resulting pink-coloured upper layer was carefully extracted and transferred to a cuvette and the absorbance was determined at A_{520}. Micrograms quantities were calculated by multiplying the absorbance at 520 nm by 17·072 as per method of Kurachi (1958).

Statistical analyses

Analysis of variance, balanced model (Minitab software release 13; Minitab Inc., State College, PA, USA) was used to compare the effects of relationship of independent variables on light treatments.

Results

Sensitivity of test bacteria to PL treatments

The sensitivity of all clinical isolates of *Ps. aeruginosa* to PL treatments was initially investigated at lamp discharge energy of 7·2 J and is summarized in Table 2. Based on PL inactivation rates shown in Table 2, all test *Ps. aeruginosa* strains were reduced by *c.* 7 $\log_{10}$ CFU cm^{-2} within 20 pulses at 7·2 J. There were marked variations in sensitivity to treatments amongst strains of *Ps. aeruginosa*.

Table 3 Comparison of inactivation rate constant *k* values obtained for test bacteria using increasing lamp discharge energies ranging from 3·2 to 20 J at 8 cm distance from light source to sample

Test bacterium	Code	Reduction rate (*k*) at different discharge energies* (J)			
		3·4	7·2	12·8	20
Gram-positive bacteria					
Enterococcus faecium	J952	0·07 ± 0·03A	0·24 ± 0·02C	0·55 ± 0·02H	0·93 ± 0·05J
	J5616†	0·06 ± 0·02A	0·24 ± 0·01C	0·50 ± 0·04H	0·95 ± 0·04J
	J5810†	0·10 ± 0·01A	0·29 ± 0·04D	0·61 ± 0·03G	0·98 ± 0·03J
Enterococcus faecalis	D46209	0·09 ± 0·01A	0·29 ± 0·02D	0·65 ± 0·02G	1·07 ± 0·03K
Enterobacter cloacae	04B3311	0·16 ± 0·03B	0·39 ± 0·03E	0·89 ± 0·04J	1·21 ± 0·02L
Enterobacter sakazakii	8155	0·17 ± 0·04B	0·47 ± 0·02F	1·11 ± 0·03K	1·33 ± 0·03M
Staphylococcus aureus	25923	0·13 ± 0·01B	0·38 ± 0·03E	0·90 ± 0·02J	1·29 ± 0·03M
	5624	0·16 ± 0·02B	0·49 ± 0·01F	1·07 ± 0·04K	1·42 ± 0·04N
	J2860	0·15 ± 0·03B	0·37 ± 0·04E	0·81 ± 0·02I	1·42 ± 0·05N
	D3187†	0·14 ± 0·02B	0·40 ± 0·03E	1·12 ± 0·05K	1·45 ± 0·05N
Staphylococcus epidermidis	10221	0·13 ± 0·01B	0·37 ± 0·03E	0·90 ± 0·03J	1·33 ± 0·02M
	03B2511	0·15 ± 0·02B	0·40 ± 0·02E	0·88 ± 0·05J	1·29 ± 0·03M
Bacillus cereus	11143	0·09 ± 0·01A	0·24 ± 0·02C	0·56 ± 0·02H	1·06 ± 0·03K
	22728	0·14 ± 0·02B	0·25 ± 0·02C	0·57 ± 0·04H	1·08 ± 0·03K
Listeria monocytogenes	11994	0·16 ± 0·03B	0·39 ± 0·02E	0·92 ± 0·03J	1·41 ± 0·02N
	9863	0·18 ± 0·03B	0·40 ± 0·03E	0·90 ± 0·04J	1·33 ± 0·03M
Gram-negative bacteria					
Acinetobacter baumanii	D3953	0·15 ± 0·02B	0·35 ± 0·02E	0·77 ± 0·02I	1·19 ± 0·04L
Escherichia coli	25922	0·19 ± 0·02B	0·52 ± 0·04F	1·41 ± 0·03N	1·61 ± 0·05O
	411	0·17 ± 0·03B	0·44 ± 0·03E	0·93 ± 0·04J	1·35 ± 0·04M
	02B1173	0·24 ± 0·02C	0·51 ± 0·03F	1·31 ± 0·02M	1·65 ± 0·03O
Pseudomonas aeruginosa	02B710	0·14 ± 0·02B	0·39 ± 0·02E	0·86 ± 0·04J	1·36 ± 0·04K
	2633	0·16 ± 0·03B	0·42 ± 0·03E	0·91 ± 0·02J	1·33 ± 0·03K
	2605	0·16 ± 0·01B	0·37 ± 0·01E	1·10 ± 0·03K	1·35 ± 0·03K
	R5137	0·23 ± 0·02C	0·67 ± 0·02G	1·49 ± 0·04N	1·59 ± 0·02O
Klebsiella pneumoniae	04B4415	0·24 ± 0·01C	0·53 ± 0·03F	0·88 ± 0·02J	1·36 ± 0·03M
	700721†	0·23 ± 0·01C	0·50 ± 0·02F	1·29 ± 0·2M	1·42 ± 0·04N
Proteus mirabilis	02B1121	0·25 ± 0·03C	0·67 ± 0·02G	1·31 ± 0·3M	1·61 ± 0·03O

*Inactivation kinetic *k* data. Values with different capitalized letter are significantly different at $P \leq 0{\cdot}05$.
†Designates strains that harbour genes for multiple resistance to antibiotics.

A clear pattern was observed regarding strains expressing mucoid or slime phenotype as these in general were shown to be less sensitive to treatments at 7·2 J. The sensitivity of a select number of mucoid and nonmucoid *Ps. aeruginosa* strains was then compared to other clinically relevant bacteria over an extended range of energy densities (Table 3). A clear significant pattern was observed where more rapid reductions in predetermined populations of all test bacteria occurred with increased levels of pulsing and with use of higher discharge energies. For instance, rapid reductions in all test organisms (*c.* 7 log orders) occurred in ≤10 pulses at 20 J compared to a requirement to use up to 45 pulses to attain same level of inactivation for these test pathogens at the lower 3·2 J. This overall trend of greater susceptibility to PL with use of increasing discharge energies for test bacteria is clearly shown for *Ps. aeruginosa* 2605 in Fig. 2, which illustrates determination of the death rate constant *k* values for

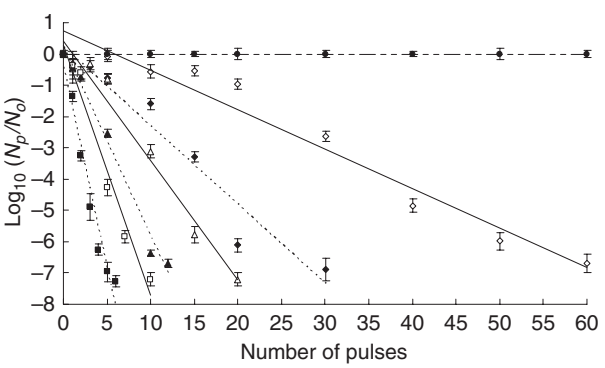

Figure 2 Determination of inactivation rate constant *k* value for *Staphylococcus aureus* D3187 (methicillin-resistant *Staph. aureus*) after pulsing at 8 cm using decreasing lamp discharge energies: (●) untreated control; (■) 20 J; (□) 12·8 J; (▲) 9 J; (△) 7·2 J; (◆) 5 J and (◇) 3·2 J.

linearized inactivation data where the slope (or k value) provides a measure of the log reduction in cell population ($\log_{10}$ CFU cm^{-2}) per pulse. While k value determinations where established for all test strains in a similar manner, it is neither practical nor beneficial to illustrate all linearized data plots for each strain in this study. However, the lower the k value the greater the resistance to the applied PL treatment, and this means of evaluating *in vitro* inactivation performance was used to compare sensitivities of all clinical bacteria under similar studies using increasing discharge energies at 8 cm from light source (Table 3).

Staphylococci and enterococci were significantly less sensitive to lethal action of PUV at all discharge energies tested compared to other Gram-positive and Gram-negative bacteria under study. The level of resistance, expressed in terms of k value determinations, of test bacteria to PL treatments differed significantly ($P < 0{\cdot}05$) and are listed in order of decreasing resistance: *Enterococcus* spp., *Staphylococcus* spp., *B. cereus*, *Enterobacter* spp., *Listeria monocytogenes*, *Acinetobacter baumanii*, *Ps. aeruginosa*, *Escherichia coli*, *Kl. pneumoniae* and *Proteus mirabilis* (Table 3). In particular, there was no significant difference in inactivation levels amongst all *Ps. aeruginosa* test strains at the uppermost test discharge energy of 20 J, irrespective of production of slime or pigments ($P < 0{\cdot}05$). The harbouring of genes encoding multiple resistances to antibiotics in certain test bacteria, such MRSA, VRE and MDR-*Kl. pneumonia*e, did not confer greater resistance to pulse light treatment when compared to similar strains of the same species or different species of test bacteria (Table 3). However, studies were not carried out to determine whether these microbial antibiotic resistance genes were plasmid or chromosomally carried. The influence of the relative position between test sample and light source on decontamination efficacy was also investigated. A clear pattern was observed where Gram-positive and Gram-negative bacteria treated at a shorter distance from treatment surface to the PL light source were more sensitive to this decontamination approach (Fig. 3). An even distribution of microbial killing was produced on the PL-treated plates as observed by the random locations of survivor colonies on agar surfaces, indicating that the central positioning of the agar plates directly under the light source coupled with the spread plate technique carried out in this study helped to minimize any possible shading effects.

Influence of microbial population size and age on decontamination efficacy

Test bacteria may react differently to PL exposure depending on population size and stage in growth cycle.

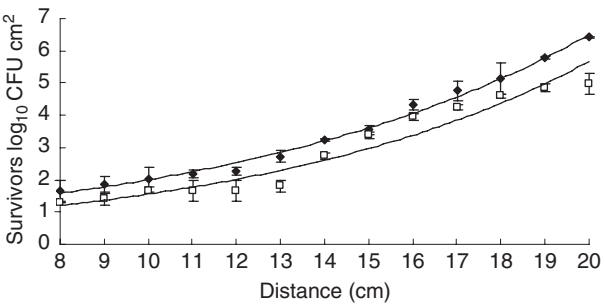

Figure 3 Effect of the distance between the lamp source and the surface of spread-inoculated Petri dishes on the inactivation of *Staphylococcus aureus* D3187 (◆) and *Escherichia coli* 411 (□) at lamp discharge energy of 12·8 J. Bars indicate ±SD. N and N_o: number of test bacteria, respectively, before and after flashing. Solid line is the trend line.

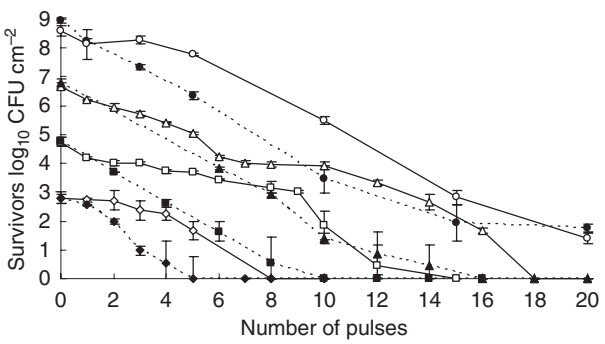

Figure 4 Effect of initial population size (expressed in $\log_{10}$ CFU cm^{-2}) and pulse number on the inactivation of *Staphylococcus aureus* D3127 and *Pseudomonas aeruginosa* 2605 using lamp discharge energy of 7·2 J at 8 cm distance from xenon light source. Bars indicate ±SD. Initial count for *Ps. aeruginosa* 2605, (◇) 3 $\log_{10}$; (□) 5 $\log_{10}$; (△) 7 $\log_{10}$ and (○) 9 $\log_{10}$. Initial count for *Staph. aureus* D3127, (◆) 3 $\log_{10}$; (■) 5 $\log_{10}$; (▲) 7 $\log_{10}$ and (●) 9 $\log_{10}$.

Figure 4 shows changes in level of inactivation as a function of the number of *Staph. aureus* D3127 (MRSA) and *Ps. aeruginosa* cells present on the surface of agar before treatment. A clear pattern emerged where the lower the initial starting cell population treated, the faster the reduction in numbers observed. PL-treated *Staph. aureus* cells produced an initial shoulder effect in sigmoidal-shaped kinetic inactivation plots. With the exception of samples treated at the lamp lowest discharge of 3·2 J, this shoulder effect was absent from similarly treated *Ps. aeruginosa* cells that exhibited linear inactivation data during early to mid ranges of treatment using discharge energies ≥5 J. The presence of this shoulder effect was a pattern that was observed for all tested Gram-positive bacteria, whereas all Gram negatives were devoid of this shoulder phenomenon. It is important to note that death

rate kinetic data was determined from Gram-positive bacteria using log-linear portion of curve avoiding use of the shoulder section. Figure 4 also illustrated a distinct tailing or tolerance effect in the inactivation data for both *Staph. aureus* and *Ps. aeruginosa*, and this is a clear pattern that emerged for all test bacteria where initial cell population inoculated on plates was *c.* 9 $\log_{10}$ CFU cm^{-2}. The effect of microbial population size had a significant effect on the sensitivity of slime and pigment producers of *Ps. aeruginosa* to PL treatments. At starting cell populations of $\leq 10^5$ $\log_{10}$ CFU cm^{-2}, all strains were equally sensitive to PL, but at high starting cell populations, slime and pigment producers were more tolerant to PL (Fig. 4). Figure 5 shows that *Ps. aeruginosa* was more sensitive to lethal action of PL treatments during late exponential (16 h) phase of growth compared to similarly treated samples that had been grown and treated in their stationary (24 h) phase when exposed to combinations of low number of pulses using lower lamp discharge energies. No significant difference in inactivation data was observed between 16 and 24 h cultures exposed to full treatment regimes of flashing at different range of discharge energies used in this study (data not shown).

Influence of selective antimicrobial composition of agar, soiling of agar and photoreactivation on PUV decontamination efficacy

Antimicrobial or selective nature of agar

To test the possibility that the presence of antibiotics and dyes present in selective agar may impact negatively on the survival of subpopulations of PL-treated test bacteria, samples were separately inoculated on both nonselective TSYEA and selective agar (listed in Table 1) and similarly pulsed at 7·2 J at 8 cm from light source and thereafter enumerated after 48 h incubation at 37°C. A clear pattern emerged where presence of selective agents in agar did not significantly affect levels of survivors compared to similarly treated samples grown and enumerated on TSYEA plates. For example, Fig. 6 shows similar inactivation kinetic data for *E. coli* 411 initially inoculated separately on selective MacConkey agar and nonselective TSYEA, flashed at 7·2 J at 8 cm distance from light source, and survivors enumerated after 48 h. Data are not presented for the other clinical bacteria treated and subsequently enumerated on respective selective agar (as per Table 1) and on nonselective agar (TSYEA), as there was no significant difference observed for survivor counts using different plating media ($P > 0{\cdot}05$).

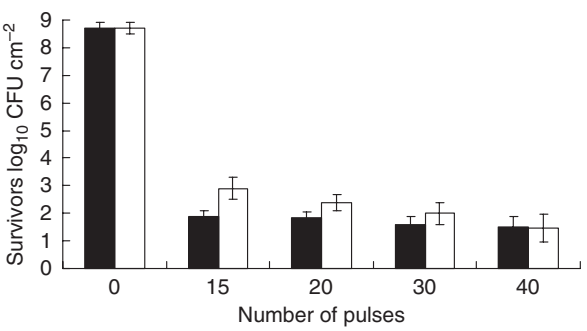

Figure 5 Effect of population age on sensitivity of *Escherichia coli* 411 to lethal action of pulsed light at 7·2 J at 8 cm from light sources. (■) 16 h and (□) 24 h cultures. Bars indicate ±SD.

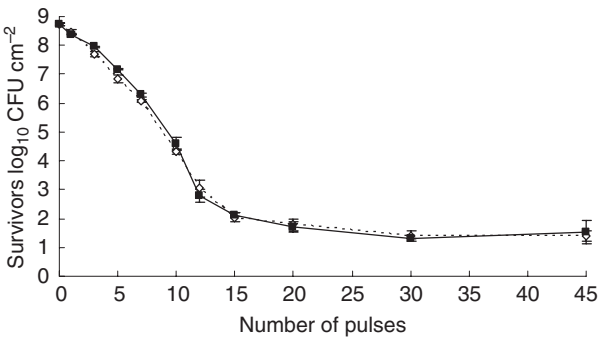

Figure 6 Survivor data (expressed in terms of $\log_{10}$ CFU cm^{-2}) for *Escherichia coli* 411 inoculated separately on selective MacConkey agar and nonselective tryptone soya agar supplemented with 0·6% yeast extract (TSYEA) plates and flashed at 7·2 J at 8 cm from light source. (■) MacConkey count and (□) TSYEA count. Bars indicate ±SD.

Artificial soiling of agar surface

To test the possibility that presence of organic matter artificially seeded on agar surfaces may impact negatively of PL inactivation efficacy, test populations of bacteria were suspended in PBS solutions containing increasing concentrations (0% control, 5%, 10% and 20% v/v) of foetal calf serum (FCS) and were subjected to 50 pulses using the mid range discharge energy of 7·2 J. No significant difference in levels of inactivation was evident based on these agar surface seeding studies ($P = 0{\cdot}445$). However, a significant difference in PL efficacy was observed when similar suspensions of test organisms were treated in corresponding PBS–FCS solutions (separate 10 ml volumes treated in Petri dish) (Fig. 7). PL treatments reduced test populations by 7·2 ± 0·3 $\log_{10}$ CFU cm^{-2} (PBS control; $P = 0{\cdot}545$), 7·0 ± 0·3 $\log_{10}$ CFU cm^{-2} (5% FCS; $P = 0{\cdot}475$), 6·5 ± 0·2 $\log_{10}$ CFU cm^{-2} (10% FCS; $P = 0{\cdot}035$) and 3·6 ± 0·4 $\log_{10}$ CFU cm^{-2} (20% FCS; $P = 0{\cdot}001$). Figure 8 shows that there is a clear linear correlation in increased absorbance with increasing concentrations of FCS at wavelengths of known biocidal efficacy (254, 260, 265 and 270 nm).

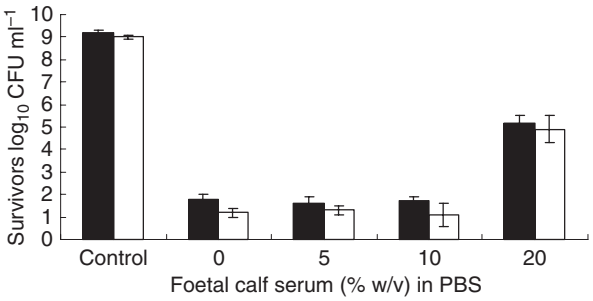

Figure 7 Effect of presence of organic matter on sensitivity of *Pseudomonas aeruginosa* 2605 (■) and *Staphylococcus aureus* D3127 (□) to pulsed light after 40 pulses using 7·2 J at 8 cm distance from light source.

Photoreactivation

To test the possibility that photoreactivation occurs, inoculated plates of *Ps. aeruginosa* were pulsed at 20, 7·2 and 3·2 J and then were exposed for 4 h of sunlight post treatment. Untreated and treated control plates (wrapped in aluminium foil for equivalent 4 h photoreactivation period) were incubated and enumerated post treatment. Some photoreactivation was evident after 15 and 45 pulses at the lower discharge energies of 3·2 and 7·2 J, respectively (Fig. 9). However, a clear pattern was demonstrated where no photoreactivation effect was evident after full flash treatment regimes at all discharge energies tested. No photoreactivation was evident after 5 and 10 pulses at 20 J for *Ps. aeruginosa* (Fig. 6).

Heating of agar surface during PUV treatments

Because of the presence of visible and infrared light (together with UV light) in the pulsed spectrum emitted by the xenon lamp, test samples and surrounding surface media can warm up during flashing. Significant change in surface media temperature can contribute in part to inactivation performance achieved by PUV system. Findings from this study showed that limiting pulse number to 60, 50, 40, 30 and 15 pulses at lamp discharge energies of 3·2, 5, 7·8, 12·2 and 20 J respectively did not significantly alter surface temperature beyond 4·2 ± 0·2°C. A distance of 8 cm between the light source and the treatment agar surface was used for these studies.

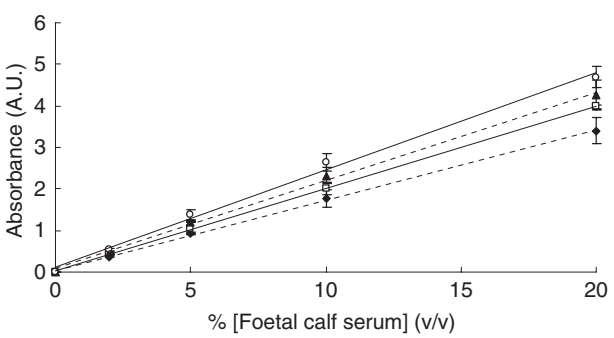

Figure 8 Best fit plots of absorbance *vs* increasing percentage of foetal calf serum (v/v). (◆) 254 nm; (□) 260 nm; (▲) 265 nm and (○) 270 nm. Bars indicate ±SD.

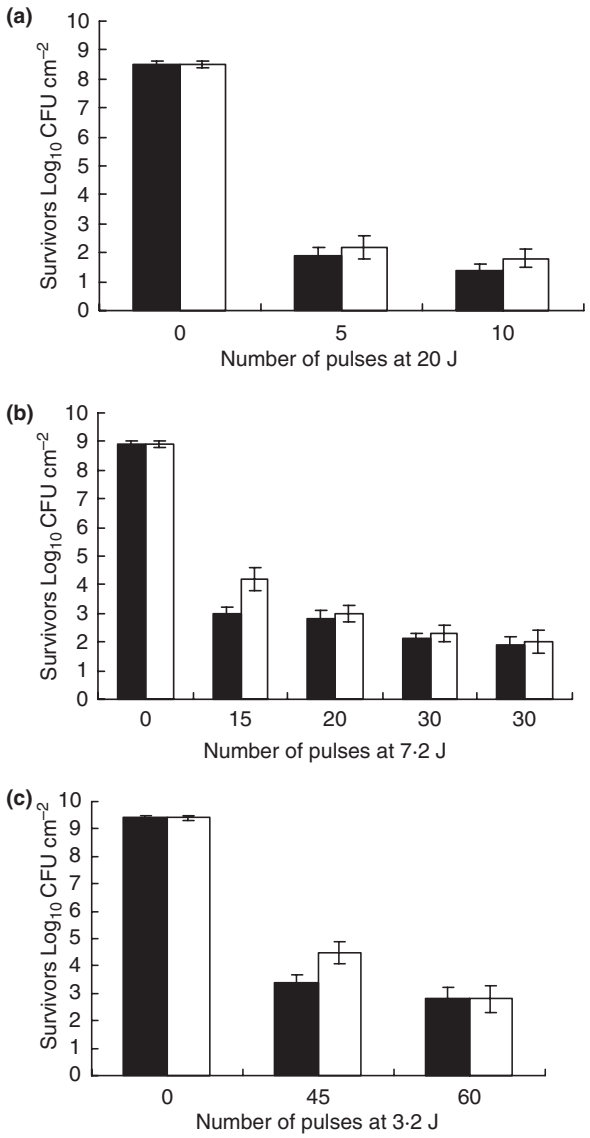

Figure 9 Effect of photoreactivation and repair for *Pseudomonas aeruginosa* 2605 treated at lamp discharge energies of (a) 20 J, (b) 7·2 J and (c) 3·2 J. Bars indicate ±SD. Reductions in populations expressed in $\log_{10}$ CFU cm^{-2} with (□) and without provision for conditions inductive to (■) photoreactivation.

Discussion

Results presented in this study showed that all clinically relevant bacteria tested were reduced by *c.* 7 $\log_{10}$ CFU cm^{-2} on artificially seeded agar surfaces by the lethal action of pulsed UV light. The inactivation rates achieved for all test bacteria was shown to be directly related to intensity of the lamp discharge energy, the number of pulses applied, the distance from the light source, microbial type and microbial population size and age studied. Greater variations in sensitivity of test bacteria to PL treatments was observed using combinations of low discharge energies and reduced pulse number. For instance, ≤40 and 30 pulses were required to reduce predetermined populations by *c.* 7 $\log_{10}$ CFU cm^{-2} using 3·2–5 J, respectively, at 8 cm from light source, compared to use of ≤10 pulses to achieve similar levels of inactivation for samples treated at 20 J. Gram-positive bacteria were shown to be more resistant than Gram negatives, a similar trend was reported by Anderson *et al.* (2000). All Gram positives tested exhibited a characteristic shoulder effect in their sigmoid shaped inactivation kinetic data. However, the latter shoulder feature was absent from similarly PL-treated Gram negatives. Other researchers reported that the shape of inactivation curves for microbial inactivation by CW UV light and by PL was sigmoid in shape, but did not report specific differences in the shape of death rate kinetic data as a function of Gram reaction. Previous researchers have reported that the initial plateau in death rate plots is because of injury phase and once the maximum amount of injury has been surpassed, minimal additional UV exposure would be lethal for micro-organisms with commensurate rapid reductions in survivor numbers (Gómez-López *et al.* 2007). The latter section of the death rate plot has been characterized by a tailing phase for which there are several possible explanations: lack of homogenous population, multi-hit killing phenomena, varying abilities of cells to repair DNA damage, shading effect that may have been produced by the edge of the Petri dishes used in some experiments (Yaun *et al.* 2003). Use of large initial cell populations inoculated on agar plates will provide a shading protection effect because of heterogeneous sample seeding, where cells in the underneath layers are not illuminated because of overcrowding effect. However, in this present study, tailing was not evident for lower cell population sizes, particularly when studying inactivation plots of treated Gram-negative bacteria. Complete inactivation of micro-organisms and absence of tailing have also been reported (Otaki *et al.* 2003; Wang *et al.* 2005); however, Gómez-López *et al.* (2007) noted in their review of these findings that the effect on the detection limit of the enumeration method should have been better assessed. The latter authors also cautioned about the potential for overestimation of the lethality because of the possibility that two or more survivors situated very close to each other on agar surfaces can form just one colony, and therefore be enumerated as one survivor. Bacterial strains harbouring multiple resistance to antimicrobial agents were not more tolerant to PL irradiation compared to similar strains of the same bacteria species or different types of test bacteria. This is a promising finding as these particular MDR pathogens have been reported to exhibit cross-resistance to chemical biocides and to other antibiotics (Lambert *et al.* 2001; Randall *et al.* 2001; Gebel *et al.* 2002).

Pseudomonas aeruginosa is notorious for its resistance to antibiotics primarily due to the permeability barrier afforded by its Gram-negative outer membrane. *Pseudomonas aeruginosa* strains produce two types of soluble pigments, the fluorescent pyoverdin and the blue green pigment pyocyanin (often referred to as 'blue pus') that is a characteristic of suppurative infections caused by this pathogen. Previous researchers have demonstrated that pyocyanin, a water soluble pigment, helps to protect *Ps. aeruginosa* strains from UV-C exposure because of their capacity to absorb wavelengths of light in this germicidal region (Kerr *et al.* 1999). This present study showed that production of pyocyanin by certain *Ps. aeruginosa* test strains provided some protection against germicidal action of PL exposure where low levels of flash discharge energies (3·2–5 J) were used to treat initial cell populations of ≤10^7 CFU cm^{-2}. The ability of pigments and/or slime to afford protection against PL exposure was observed to be a direct function of cell population, lamp discharge energy and number of pulses applied. It is possible that only beyond a significant cell population threshold does *Ps. aeruginosa* produce protective pigmentation, which is tightly regulated via complex quorum sensing and inter-cell signalling. A recent *in vivo* study carried out by Feuilloley *et al.* (2006) reported that a single light pulse of a dose of 1 J cm^{-2} is sufficient to entirely reduce a 6 log CFU ml^{-1} population of *Ps. aeruginosa* suspended in ampoules of solution for injection.

Mucoid strains of *Ps. aeruginosa* produce an exopolysaccharide (alginate) slime that forms the matrix for *Pseudomonas* biofilm, which anchors this bacterium to various contact environments. In medical situations, the exopolysaccharide protects the pathogen from the human host immune defences. A clear pattern emerged where mucoid-producing test strains of *Ps. aeruginosa* at high population densities (*c.* 10^9 CFU cm^{-2}) may exhibit increased tolerance perhaps related to additional shading via formation of complex slime. However, there was no significant evidence of a protective effect from pigments and/or slime in *Ps. aeruginosa* test strains when discharge energies of ≥12·8 J were used at a distance of 8 cm from

inoculated agar surfaces. The contributing role of functional housekeeping enzymes involved in either mopping up toxic free radicals produced during PUV irradiation and/or chaperoning (protecting) critical internal cell constituents cannot be discounted. Anderson *et al.* (2000) demonstrated that the darker pigmented fungal spores of *Aspergillus niger* were more resistant than spores of *Fusarium culmorum* due perhaps to the pigment of the former absorbing more in the UV-C region, thus protecting *Aspergillus* in part from UV damage. However, the authors did not corroborate these assumptions by obtaining absorbance data for pigments extracted from spores. In contrast, Gómez-López *et al.* (2005) did not observe any sensitivity pattern among different groups of bacteria, yeast and moulds to intense pulse light treatments. However, *Ps. aeruginosa* was not included in their study.

The lethal effects of PL can be attributed to its rich broad-spectrum UV-C content, its short duration, high peak power, and its ability to regulate both the pulse duration and frequency output of the flash lamps, which plays a major role in microbial destruction. Despite advances in applied research for this nonthermal processing approach, findings from same of the effects of PUV are frequently explained using current knowledge from studies on the effect of continuous conventional UV irradiation. However, despite PUV containing a UV-C enriched component in its broad spectrum, the mechanistic underpinnings governing microbial inactivation are not equivalent for both approaches (Gómez-López *et al.* 2005). Considerable research has been performed to elucidate the different mechanisms underpinning microbial inactivation by PL, all of them emphasizing pivotal role of UV component of the pulsed spectrum along with intimating other minor photochemical and photothermal effects (Anderson *et al.* 2000; Wekhof *et al.* 2001; Takeshita *et al.* 2003; Wuytack *et al.* 2003; Wang *et al.* 2005). Gómez-López *et al.* (2007) astutely emphasized the duality or co-existence of both mechanisms, and that the relative importance of each one would depend on the fluence and target micro-organism. The germicidal effect of UV light on treated bacteria is mainly because of formation of pyrimidine dimers inhibiting formation of new DNA that ultimately derails the vital process of cell replication (called clonogenic death). Without sufficient repair mechanisms, such damage results in lethal irreversible mutations. Thus, the UV region is crucial to the decontamination efficiency of PL treatments. It has been confirmed that no microbial inactivation occurs if a filter is included to remove the UV wavelength region lower than 320 (Takeshita *et al.* 2003). Wang *et al.* (2005) also corroborated this finding, where they demonstrated that the filtered removal of broadband emission spectrum of light at wavelengths above 300 nm had no significant bearing on microbial inactivation performance of a pulsed xenon flashlamp. Maximum lethality to PL-treated *E. coli* occurred at around 270 nm. The latter corroborated earlier work of Wekhof (2000) who demonstrated that UV-C fluxes alone are sufficient for full inactivation using a flash lamp.

However, it is also important to note that the photothermal effect can occur and that intercellular heating may contribute significantly to overall germicidal efficacy of PL. Although the increase in surface temperature of the inoculated agar did not exceed 4·2 ± 0·2°C after full treatments in this present study, localized overheating of the internal cellular constituents cannot be dismissed. For example, Wekhof (2000) proposed that with a fluence exceeding 0·5 J cm^{-2}, the disinfection is achieved through rupture of the bacteria during their momentous overheating caused by absorption of all UV light from a flash lamp. Wekhof *et al.* (2001) later illustrated electron micrographs of deformed and ruptured PL-treated *A. niger* spores that provided evidence of the escape of overheated contents of these fungal spores. However, Takeshita *et al.* (2003) reported that the process of irreversible electropermeabilization of cell membrane induced by action of PL exposure cannot be ruled out, particularly as the yeast cell structure of CW UV light–treated cells was almost the same as that of nonexposed cells. It is likely that the mechanism of cellular killing is multi-targeted as this is also in line with relative inactivation of clinical bacteria plated on selective and nonselective agars in this study. This also corroborates findings of Wuytack *et al.* (2003) who concluded that pulsed white light inactivation should be regarding as a multi-hit process based on enumeration of treated *Salmonella* cells plated on selective and nonselective agar. This is in marked contrast to recovery and enumeration of microorganisms exposed to deleterious stress commonly associated with conventional decontamination approaches, such heating or use of chemical preservatives in food processing, where sublethal damage of treated cells occurs (Rowan *et al.* 1999; Yaqub *et al.* 2004).

It must also be stated that use of PL enriched in UV has operational limitations because of its potential detrimental effects on skin tissue and components of the eye. While such operational issues can be addressed, other research groups are alternatively exploring the potential of photodynamically inactivating bacterial pathogens using visible light at 400–420 nm (Ashkenazi *et al.* 2003; Ganz *et al.* 2005; Lukšiene 2005). The mechanism of cellular destruction attributed to photosensitization involves using visible light where blue wavelength component photoactivates endogenous porphyrins (photosensitizers) that accumulate within the bacterial cells in oxygen dependent process that ultimately kill the illuminated

cells. This porphyrin excitation leads to energy transfer and ultimately, the production of highly cytotoxic, oxygen-derived free-radical species (Wainwright 1998). While photosensitization is another milder technique that might be useful to decontaminate surfaces and air using light, a comparison of inactivation data reported previously by other researchers as a function of wavelength suggests that visible light inactivation of bacteria (Guffey and Wilborn 2006) is a significantly less efficient process than is ultraviolet light inactivation (Wang *et al.* 2005).

Acknowledgements

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