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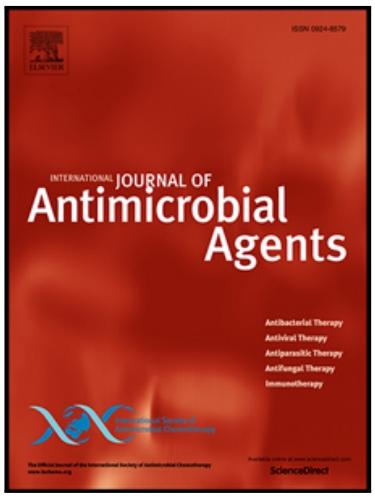

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Highlights

- Bacterial spores contribute to cross-transmission of infection within healthcare
- Current sporicidal agents possess several drawbacks such as user toxicity
- Photosensitisers supplemented with spore germinants provide rapid spore eradication
- Photosensitiser-QAC-germinant mixtures maintain activity in low-light conditions

Development of a high-level light-activated disinfectant for hard surfaces and medical devices

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Abstract

Background: Bacterial spores are an important consideration in healthcare decontamination, with cross-contamination highlighted as a major route of transmission due to their persistent nature. Their containment is extremely difficult due to the toxicity and cost of first-line sporicides.

Methods: Susceptibility of *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Escherichia coli* to phenothiazinium photosensitisers and cationic surfactants under white- or red-light irradiation was assessed by determination of MICs, MBCs and time-kill assays. *B. subtilis* spore eradication was assessed via time-kill assays, with and without nutrient and non-nutrient germinant supplementation of photosensitiser, surfactant and photosensitiser-surfactant solutions in the presence and absence of light.

Results: Under red light irradiation, >5-$\log_{10}$ cfu/mL reduction of vegetative bacteria was achieved within 10 minutes with Toluidine Blue O (TBO) and methylene blue (MB). Cationic surfactant addition did not significantly enhance spore eradication by photosensitisers ($p>0.05$). However, addition of a nutrient germinant mixture to TBO achieved 6-$\log_{10}$ reductions after 20 minutes irradiation, while providing 1-2 $\log_{10}$ improvements in spore eradication for MB and Pyronin Y.

Conclusions: Light-activated photosensitiser solutions in the presence of surfactants and germination-promoting agents provide a highly effective method to eradicate dormant and vegetative bacteria. These solutions could provide a useful alternative to traditional chemical agents used for high-level decontamination and infection control within healthcare.

Keywords: *Bacillus*, photosensitisers, germinants, bacterial spores, photodynamic therapy, cationic surfactants

1. Introduction

Cross-contamination is a major route of infection transmission within hospitals due to the ability of bacteria to survive on hard surfaces for several weeks and the persistent nature of spore-forming bacteria which makes their eradication extremely difficult [1, 2]. This highlights a significant problem for hospital environmental hygiene, with many current cleaning agents showing decreased efficacy against common nosocomial pathogens [3]. Presently, peroxy and aldehyde-based disinfectants are viewed as the gold-standard for sterilisation in healthcare settings. However, these agents possess several disadvantages, such as short shelf-life, material incompatibilities and can cause irritation of mucous membranes, eyes and skin upon acute or chronic exposure [4, 5]. Additionally, aldehyde-based disinfectants require up to 10 hours for spore eradication and also require suitable ventilation and recovery systems to ensure their safe use [6]. Medical instruments, such as endoscopes, possess intricate designs and often comprise a mixture of metal and polymeric materials, thus necessitating complicated reprocessing procedures to allow their safe re-use, with many imcompatible with oxidising disinfectants. Thus, it is evident that current high-level chemical disinfectants lack convenience and can pose a health risk to personnel and patients.

With the aim of developing a user-friendly, stable high-level disinfectant, this study describes a novel formulation using a combination of quaternary ammonium compounds (QACs), germinant mixtures and photosensitisers to exploit the reduced chemical resistance of spore cells upon spore germination to enable rapid spore eradication. QACs are non-sporicidal compounds used for general disinfection of surfaces but have exhibited activity against *C. difficile* spores when supplemented with nutrient germinants [7, 8]. Similarly, improved inactivation of *Bacillus anthracis* and *C. difficile* spores by chemical agents in the presence of nutrient germinants has also been reported [9, 10].

Phenothiazinium photosensitisers, such as methylene blue (MB) and toluidine blue O (TBO), possess potent broad-spectrum antibacterial activity when irradiated with white or red light, which has been attributed to their light-activated production of cytotoxic singlet oxygen ($^1O_2$) [11, 12]. The phenothiazinium-like xanthene dye pyronin Y (PY) has also shown potent antibacterial properties despite an extremely low $^1O_2$ yield efficiency relative to MB [13]. Phenothiazinium photosensitisers also possess potent antifungal and antiviral properties when irradiated with light, and have been shown to inactivate *Bacillus* spores [14].

This study describes how the combination of QACs, germinants and photosensitisers can exploit the reduced chemical resistance of spores upon germination to enable rapid spore eradication comparable to first-line high-level disinfectants while minimising the detrimental effects on medical devices and occupational health hazards commonly seen with high-level disinfectants.

2. Materials and Methods

2.1.Materials

Toluidine Blue O (80%) (TBO), Methylene Blue (MB), Pyronin Y (PY), Benzalkonium chloride (≥95%) (BZK), Benzethonium chloride (≥95%) (BZT), Hexadecyltrimethylammonium bromide (≥99%) (CTAB), Cetylpyridinium chloride (≥99%) (CPC), L-Asparagine ≥98%, D-Fructose ≥99%, D-Glucose ≥99%, Potassium chloride (≥99%) (KCl), L-Valine ≥98%, L-Alanine ≥98%, Calcium chloride ≥97% ($CaCl_2$) and dipicolinic acid ≥99% (DPA) were purchased from Sigma-Aldrich (Poole,

UK). Phosphate-buffered saline (PBS), Nutrient broth (NB), Mueller-Hinton broth (MHB), Nutrient agar (NA) and Mueller-Hinton Agar (MHA) were supplied by Oxoid Ltd. (Basingstoke, UK) and Letheen broth modified (LBM) was purchased from VWR Ltd (Dublin, Ireland).

Staphylococcus aureus (ATCC 6538) and *Bacillus subtilis* (NCTC 10073) were used as model Gram-positive bacteria and *Pseudomonas aeruginosa* (PA01) and *Escherichia coli* (ATCC 11303) as model Gram-negative bacteria. *B. subtilis* spores (NCTC 10073) were prepared by inoculating 100 mL NB with an overnight culture of *B. subtilis* and incubating for 6 hours at 30°C and then grown on sporulation media [Beeby & Whitehouse agar containing NB (6 g/L), $MnSO_4$ (0.03 g/L), KH_2PO_4 (0.25 g/L) and Agar No. 1 (12 g/L)] for 5 days at 30°C. Spores were harvested by addition of 10 mL sterile distilled water to the media surface and scraping with sterile glass beads, and purified by washing the spore suspension three times with sterile distilled water and centrifuging (3000 rpm, 15 minutes). Purified spores were resuspended in sterile distilled water and stored at 4°C until use. *B. subtilis* endospores were not heat-shocked prior to testing. While this is common practice in literature to enhance spore germination, it does not replicate the conditions of spores encountered in a hospital setting, whereby a disinfectant is likely administered onto surfaces of ambient temperature or heat-sensitive materials [15]. Furthermore, while it also prevented elimination of vegetative bacteria, this provided a scenario more representative of surfaces contaminated with a mix of vegetative and spore cells.

2.2. Methods

2.2.1. Measurement of light source intensity

Irradiation was conducted with white (FloLight™, Microbeam 1024 daylight spot, Markertek, UK) or red (Prakasa, LED Seven Spectrum grow light, Growlights, UK) LED arrays. The light sources were maintained at fixed heights (White LED at 50 cm, red LED at 32 cm) above samples to maintain a constant light intensity. This was measured between 450-700 nm (corresponding to the absorbance range of the three photosensitisers) using an Ocean Optics Jaz spectrometer and analysed using SpectraSuite software. A power intensity of 6.57 mW/cm^2 (450-700 nm) and 9.28 mW/cm^2 (450-700

nm) was recorded for the white and red arrays and used for up to 60 minutes providing a maximum light dose of 23.65 and 33.41 J/cm^2, respectively.

2.2.2. Concentration dependent photoinactivation of vegetative bacteria

B. subtilis, S. aureus, E. coli and *P. aeruginosa* were grown in NB or MHB in an orbital incubator at 37°C for 18 hours, centrifuged (3000 rpm, 12 mins) and resuspended in PBS to an OD_{550} of 0.3 (~10^8 cfu/mL). Control samples were tested in the absence of photosensitiser. Samples were irradiated with red light (or white light – PY) or kept in dark conditions for 5 minutes. TBO, MB and PY concentrations of 0.5, 1, 2.5, 5, 10, 15, 20 and 25 μg/mL were assessed. Following exposure, 20 μL aliquots were removed to a sterile 96-well microtitre plate, serially diluted using sterile PBS, plated on NA or MHB plates to allow determination of bacterial viability by the Miles & Misra technique [16], and incubated at 37°C for 24 hours.

2.2.3. Antibacterial activity of cationic surfactants

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined for BZK, CPC, CTAB and BZT using the broth microdilution test as described previously [17]. A stock solution of 0.2% w/v of each QAC was freshly prepared with Tryptic soy broth (TSB) as the diluent, filter sterilised (0.22 μm membrane), and serial two-fold dilutions prepared to provide a concentration range of $4.88x10^{-5}$ – 0.1% w/v. *S. aureus* and *P. aeruginosa* were grown in MHB and *B. subtilis* and *E. coli* grown in NB (18 hours, 37°C) in an orbital incubator, and adjusted to an OD_{550} of 0.3 ($1x10^8$ cfu/mL). Further dilution in TSB provided a final inoculum of approximately $1x10^6$ cfu/mL, verified by viable count.

To each well of a microtitre plate, 100 μL inoculum was added and the plates incubated at 37°C in an orbital incubator for 24 h. Positive (100 μL TSB / 100 μL inoculum) and negative controls (200 μL TSB) were included in each assay. MIC was taken as the lowest concentration that produced no visible growth at 24 h. MBC was then determined by spreading 20 μL of suspension from wells exhibiting no growth onto MHA or NA plates, which were incubated for 24 h in a static incubator at 37°C and examined for 99.9% killing.

2.2.4. Concentration dependent photoinactivation of *B. subtilis* spores with varying photosensitiser concentration

0.2 mL *B. subtilis* spore suspension (~10^8 viable spores/mL) was added to 19.8 mL of a solution consisting of varying photosensitiser concentrations in PBS, giving an inoculum of 10^6 viable spores/mL. Control samples were tested in the absence of photosensitiser. Samples were incubated in the dark at 37°C for 10 minutes. 200 µL of each sample was then dispensed into a sterile 96-well microtitre plate and subjected to either light (white light – PY; red light – TBO, MB) or dark conditions. The contents of each well were mixed thoroughly before sampling at 0, 5, 10, 15, 30, 60, 120 and 180 minutes, followed by serial dilution in sterile PBS. Viable counts were performed using the Miles & Misra technique [16] on NA plates, incubated at 37°C for 36 hours.

2.2.5. Sporicidal activity of cationic surfactants

The sporicidal activity of 20 µg/mL solutions of BZK, CPC, CTAB and BZT in PBS was assessed as described in section 2.2.4. except LBM was used as a diluent during serial dilutions to inactivate QACs, and the efficacy of neutralisation was confirmed against each QAC, and non-toxicity of the neutraliser towards spores was confirmed.

2.2.6. Effect of the addition of cationic surfactants to photosensitiser solutions on spore viability

BZK, CPC, CTAB and BZT were added to the most effective photosensitiser solutions (TBO 25 µg/mL; MB 12.5 µg/mL; PY 25 µg/mL) to investigate the effect on spore eradication. Assessment of sporicidal activity was conducted using the same parameters described in section 2.2.5. Serial dilutions were made using LBM to neutralise surfactant activity.

2.2.7. Effect of the addition of nutrient and non-nutrient germinants to photosensitiser solutions on spore viability

Solutions containing photosensitisers (TBO 25 µg/mL; MB 12.5 µg/mL; PY 25 µg/mL) and QACs (20 µg/mL) were used in investigation of the effect of germinant addition, described below.

2.2.7.1. Nutrient germinants (NG)

A NG solution consisting of *L*-asparagine, *D*-fructose, *D*-glucose and K^+ (termed collectively as AFGK) was prepared in PBS, each at a concentration of 10 mM. *L*-alanine and *L*-valine were also added at a concentration of 0.4 mM [18]. Samples were incubated and investigations conducted as per section 2.2.5. Serial dilutions were made using LBM to neutralise surfactant activity.

2.2.7.2. Non-nutrient germinants (NNG)

A NNG solution consisting of Ca^{2+} (from $CaCl_2$) (60 mM) and DPA (60 mM) was prepared in sterile PBS based on previously published optimal germinant results [19]. Samples were incubated and investigations conducted as per section 2.2.5. Serial dilutions were made using LBM to neutralise surfactant activity.

2.2.8. Effect of light intensity on *Bacillus* spore eradication in the presence of light activated disinfectant solutions

The most effective solutions from section 2.2.7 were chosen for further study. These were:

Solution 1: TBO (25 µg/mL), BZK (20 µg/mL) and NG

Solution 2: TBO (25 µg/mL), CTAB (20 µg/mL) and NG

Solutions were tested as described in section 2.2.5, however, the light source was fitted with a series of 0.15, 0.3, 0.6 neutral density filters to reduce light intensity to 75%, 50% and 25% respectively to assess the effect of reduced light intensity on the eradication of spores. Serial dilutions were made using LBM to neutralise surfactant activity.

2.2.9. Statistical analysis

Statistical analysis was carried out using two-way ANOVA with Tukey's multiple comparisons test, with GraphPad Prism 8 (GraphPad Software, California, USA). The number of replicates (n) was 3, and statistical significance was denoted by $p < 0.05$.

3. Results

3.1. Phenothiaziniums possess broad-spectrum antibacterial activity

The viability of vegetative bacteria in the presence of increasing concentrations of TBO after 5-10 minutes of irradiation is shown in Figure 1. TBO (25 μg/mL) achieved a 6-$\log_{10}$ reduction after 5 minutes exposure to all bacteria except *P. aeruginosa,* which required 10 minutes irradiation. In contrast, MB required 2-10-fold higher concentrations to achieve similar efficacy (Figure S1). PY was least effective, achieving 6-$\log_{10}$ reduction only against *E. coli* and *B. subtilis* (Figure S2). *P. aeruginosa* and *S. aureus* were the least and most susceptible species to PS-mediated toxicity, respectively.

3.2. QACs do not possess sporicidal activity

The antibacterial activity of BZK, CTAB, CPC and BZT against vegetative bacteria was examined by determination of MIC and MBC (Table 1). Each QAC required considerably higher concentrations to kill Gram-negative bacteria compared to Gram-positive bacteria, requiring a 256-fold higher concentration of CTAB and CPC for *P. aeruginosa* inhibition compared to MIC for *S. aureus*. CPC was the most effective against Gram-positive bacteria, while BZK provided the most potent effect against both Gram-negative species.

Based on the MIC data (Table 1), the effect of a QAC concentration 2-10 x MIC on *B. subtilis* spore viability was assessed (Figure 2). QACs activity against *B. subtilis* spores after 60 minutes was not statistically significant.

3.3. Red wavelength light enhances sporicidal activity of photosensitiser solutions

B. subtilis spores were exposed to varying concentrations of TBO, MB and PY. The effect of TBO on *B. subtilis* spore viability upon irradiation with white light is shown in Figure 3. All PSs possessed concentration-dependent sporicidal action. Upon irradiation, only 12.5, 25 and 50 µg/mL TBO obtained a >6-$\log_{10}$ reduction in spore viability by 180 minutes when exposed to white light. Above 25 µg/mL, sporicidal activity was reduced and almost negligible at >500 µg/mL compared to control. An inverse concentration-activity relationship was also observed above 25 µg/mL MB (Figure S3). PY was least effective, with <2-$\log_{10}$ reduction at 180 minutes (Figure S4).

The effect of irradiation source on PS-induced sporicidal action against *B. subtilis* spores is shown in Figure 4. A significant reduction ($p<0.0001$) in eradication time was observed for TBO when switching from white to red-light, reducing time for a 6-$\log_{10}$ spore reduction from 180 minutes to 30 minutes respectively. Conversely, no significant differences ($p>0.05$) were observed in MB with contrasting irradiation sources while PY sporicidal action significantly ($p<0.0001$ decreased under red light irradiation. There was no significant reduction ($p>0.05$) in spore viability with any photosensitiser in dark conditions.

3.4. Nutrient germinants enhance the photoinactivation efficacy of photosensitiser solutions against *B. subtilis* spores

The effect of germinant mixtures on *B. subtilis* spore viability was evaluated. A previously reported NG mixture[18] was added to PS and PS-QAC solutions, and spore viability monitored over 60 minutes irradiation with red light. Similarly, the effect of the NNG, Ca^{2+}-DPA, on spore viability was also assessed. Results for TBO, MB and PY are shown in **Error! Reference source not found.**, S5 and S6, respectively.

Addition of NGs significantly ($p<0.001$) reduced viable spore count for all PS and PS+QAC solutions after 10 and 20 minutes irradiation compared to solutions without NG. With NNG, the effect on spore

viability was attenuated in solutions containing TBO and MB, with reduced spore eradication observed at all time points compared to solutions with no germinants. TBO, TBO+BZK and TBO+CTAB solutions supplemented with NG achieved >5-$\log_{10}$ spore eradication within 20 minutes, while all TBO and MB-containing solutions supplemented with NG achieved similar potency within 30 minutes. In contrast, the addition of either germinant to PY and PY-QAC solutions provided similar reductions in spore viability and significantly improved spore eradicating activity.

3.5. Light-activated photosensitiser solutions can maintain spore eradication in low light conditions but require prolonged irradiation

The two most effective solutions in spore eradication from section 3.4; 1) TBO/BZK/NG and 2) TBO/CTAB/NG, were further investigated to determine if diminishing light intensity adversely affected spore eradication. Light intensities of 75%, 50% and 25%, compared to the unfiltered light, were applied and the effect on *B. subtilis* spore viability is shown in Figure 6. Both solutions showed reduced spore eradication with decreasing light intensity. The time required for a >6-$\log_{10}$ reduction doubled from 20 minutes under unfiltered light (100%) to 40 minutes in 50% and 25% light conditions. In the absence of light (0%), sporicidal activity was significantly reduced to ~2-$\log_{10}$ reduction after 60 minutes compared to control. However, this result indicated that the addition of NG improved the activity of BZK and CTAB significantly compared to surfactants alone, which displayed no sporicidal activity as shown in Figure 2.

4. Discussion

Cross-transmission of pathogens from inanimate objects within the healthcare environment is a major contributor to the development of life-threatening nosocomial infections [20, 21]. High-level disinfectants play a crucial role in eradicating bacteria, fungi and viruses, and importantly can inactivate bacterial spores. However, most FDA-approved high-level disinfectants are based on chlorine, aldehyde or peroxy agents which are associated with several drawbacks, such as the need for specialised equipment, user health risks, limited shelf-life and material incompatibilities [22]. A primary aim of this

study was to formulate a non-toxic high-level disinfectant which eliminated such occupational health hazards.

The sporicidal activity of TBO and MB with white light irradiation was significantly enhanced by switching to a red light source, reducing the time for a TBO-induced 6-$\log_{10}$ reduction in *B. subtilis* viability from 180 to 30 minutes, supporting previous findings on the importance of wavelength for photodynamic activity [14, 23]. The paradoxical relationship observed between TBO or MB concentration and sporicidal efficacy can be attributed to a quenching phenomenon whereby increased PS concentration increases pigmentation of the solution, with similar trends previously reported [14]. Reduced light penetration limits TBO excitation to the outer regions of the solution, limiting $^1O_2$ generation and subsequent sporicidal activity. The poor activity of PY under both irradiation conditions was likely due to its Type I-mediated reaction to produce superoxide and hydroxyl radicals. These require access to DNA and protein substrates for cidal activity, which are not readily accessible in bacterial spores. Photosensitisers were capable of achieving >4-$\log_{10}$ reductions in the viability of bacteria commonly implicated in healthcare conditions (*S. aureus, B. subtilis* and *E. coli*) within 10 minutes which matches the antibacterial performance reported in recent studies on MB [24, 25], although only TBO significantly reduced *P. aeruginosa* viability within this time. TBO and PY possess $^1O_2$ quantum yields of 0.86 and 0.05 respectively, relative to MB [26], however TBO displayed improved performance compared to MB despite its lower $^1O_2$ generation efficiency. This may be due to the improved dimer formation of TBO and its ability to attach to LPS in Gram-negative bacterial cells [11, 27, 28]. PY has, in contrast to this study, previously demonstrated efficacy against both *S. aureus* and *E. coli*, possessing the lowest minimum lethal concentration of several phenothiazinium dyes, including TBO [26]. The required 30 minute irradiation implies, however, that the hypothesised Type I-mediated PY mechanism may occur at a slower rate with Type I target sites within the bacterial cell.

The combination of photosensitisers with commonly used QACs was hypothesised as a simple method to enhance the previously reported sporicidal efficacy of phenothiazinium photosensitisers. QACs, while commonly stated as safe in acute medical scenarios, may pose occupational hazards with repeated use, e.g. irritant or allergic dermatitis [29, 30]. Thus, a QAC concentration of 20 μg/mL,

substantially below concentrations associated with irritation or *in vitro* toxicity, was chosen to reduce the potential risk of adverse effects upon repeated exposure. QACs are commonly used alone or in combination with other chemicals for low-level disinfection. They act as membrane-active agents, causing disruption of bacterial cell membranes and leakage of intracellular material and possess broad-spectrum efficacy against bacteria and viruses [31, 32]. All QACs displayed decreased antibacterial efficacy against *P. aeruginosa* compared to Gram-positive bacteria. The increased potency of CTAB and CPC towards Gram-negative bacteria compared to BZK and BZT may be explained by their long 16-carbon chain preventing penetration and disruption of the Gram-negative cell wall due to obstruction by membrane proteins and lipopolysaccharides, with similar findings highlighted by Adair et al. [33]. In this study, all four QACs were sporistatic after 60 minutes which agrees with previous literature [34]. However, the addition of QACs did improve the sporicidal efficacy of photosensitisers; it has been postulated that some QACs may damage the spore inner membrane, with CTAB previously shown to trigger dipicolinic acid release from *Bacillus* spores [35]. Inner membrane damage could additively enhance the effects of photosensitiser-generated $^1O_2$ and facilitate movement of photosensitiser into the vulnerable spore core, however in most instances the improved activity was not significant.

An emerging strategy in spore eradication involves pre-treatment or *in situ* addition of germinants to trigger germination and outgrowth of resistant spores into vegetative cells, increasing their vulnerability to the toxic effects of biocides [8, 10]. NG significantly enhanced spore eradication for all photosensitiser solutions with or without QAC after 10- and 20-minutes irradiation. Spore germination occurs upon binding of specific germinant compounds to spore-specific protein receptors located within the inner membrane, triggering significant changes in its permeability to macromolecules. Once initiated, it is non-reversible, even upon germinant removal [36]. This process likely increased vulnerability to the effects of PS and/or QAC, facilitating photosensitiser movement beyond the inner membrane to exert direct damage to the spore core [37]. In contrast to NGs, Ca^{2+}-DPA decreased TBO and MB-induced killing. This is likely due to its visible precipitation in PBS pH7.4, in

turn preventing activation of germination when undissolved and increasing solution turbidity, which may have encouraged light scattering and decreased light transmission, reducing $^1O_2$ generation.

Efficacy in varying light conditions is a crucial consideration with photoactivated approaches. Ideally, a photoactive disinfectant would be used in a well-lit environment allowing activation of phototoxic pathways, however, the device size and shape, or lack of proper lighting equipment, must be considered. Digital endoscopes require regular high-level disinfection to prevent transmission of spores and other microbes between patients. They possess complex designs with several narrow opening and exit ports connected by multijointed channels. Adequate light provision can be achieved with fibre optics [38], however, small joints within the device may create localised areas in which microbes may gather and where light transmission may be limited. The ability of PS-NG-QAC formulations to maintain effective spore eradication in conditions mimicking poor light transmission is therefore significant, only requiring an increase from 20 to 40 minutes irradiation. In the absence of light, and therefore PS-generated $^1O_2$, spore eradication was reduced to ~2-$\log_{10}$ reductions compared to control after 60 minutes. This indicates the disinfectant solution can still maintain modest sporicidal and antibacterial activity in dark conditions due to the synergistic combination of NG and QAC which persists in the absence of photogenerated singlet oxygen. This can be attributed to the initiation of spore outgrowth by the presence of germinants making *Bacillus* spores more susceptible to QACs. A similar sensitisation has also been reported for *Clostridium difficile* spores upon exposure to QACs in the presence of germinants [8]. The preservation of a modest sporicidal and antibacterial activity in dark conditions provided by the QAC ensures the disinfectant solution remains capable of disinfecting surfaces when an appropriate light source may not be available or when used to disinfect complex products in which it may be difficult to fully irradiate all surfaces.

5. Conclusions

In conclusion, the addition of NGs can significantly enhance the eradication of *Bacillus* spores by photosensitisers and QACs. This study presents an alternative approach to high-level disinfection

utilising compounds previously shown to be safe in the treatment of human disease, achieving >6-log_{10} reductions in spore count within 20 minutes of red-light irradiation. Clinically relevant efficacy was maintained in low light and dark conditions. This strategy can avoid common health hazards associated with high-level disinfectants and is unlikely to corrode or damage metal or plastic materials. Further studies to determine efficacy against a broader range of microbial species and to assess important concerns, such as the effect of potential staining of medical devices on repeated disinfection or the effect of organic matter will be conducted in future studies. Furthermore, the feasibility of constructing an illuminated disinfection chamber/bath, will require further investigation to fully determine its applicability to routine healthcare high-level medical device disinfection.

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Declarations

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Declaration of interest: None.

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Figure Captions

Figure 1. The effect of varying concentrations of TBO, after 5-10 minutes irradiation with red LED light at 37°C, on the viability of 10^6 cfu/mL of (a) *S. aureus*, (b) *P. aeruginosa*, (c) *E. coli* and (d) *B. subtilis*. Dashed line indicates a limit of detection of 50 cfu/mL, * indicates no detectable growth was observed.

Figure 2. Effect of quaternary ammonium compounds on *B. subtilis* spore viability over a period of 60 minutes. All samples were incubated for 60 minutes at 37°C with red light irradiation. *B. subtilis* spores were challenged with 20 µg/mL solutions of each quaternary ammonium compound. Error bars represent ± SD.

Figure 3. Effect of changing concentration of TBO on *B. subtilis* spore viability upon irradiation with white LED light. All samples were incubated at 37°C. Spores were challenged with 12.5µg/mL, 25µg/mL, 50µg/mL, 100µg/mL, 500µg/mL and 1000µg/mL of each PS. Error bars represent ± SD. Dashed line indicates a limit of detection of 50 spores/mL.

Figure 4. Effect of changing from a white LED (dotted line) to red LED (solid line) light source when *B. subtilis* spores were challenged with 25µg/mL TBO (circle), 12.5µg/mL MB (square) and 25µg/mL PY (triangle). All samples were incubated at 37°C. Error bars represent ± SD. LED light intensity - white – 6.57 mW/cm^2, red – 9.28 mW/cm^2. Dashed line indicates a limit of detection of 50 spores/mL.

Figure 5. The effect of addition of 20 µg/mL QACs and/or germinant mixtures to TBO (25µg/mL) solutions on the reduction in spore viability upon irradiation with red light at 37°C for (a) 10, (b) 20, (c) 30, and (d) 60 minutes.

Figure 6. Effect of changing light intensity on the viability of *B. subtilis* spores exposed to a solution containing (a) 25 ug/mL TBO, 20 ug/mL BZK and nutrient germinants, and (b) 25 ug/mL TBO, 20 ug/mL CTAB and nutrient germinants. All samples were incubated at 37°C and irradiated with red light of appropriate intensity relative to an unfiltered red-light intensity of 9.28 mW/cm^2. Light percentage values relate to the portion of light transmitted through a filter onto a sample relative to the unfiltered red light irradiation (100%) and absence of irradiation/darkness (0%). Error bars represent ± SD. Dashed line indicates a limit of detection of 50 spores/mL.

Table 1. Minimum inhibitory concentration and minimum bactericidal concentration for four quaternary ammonium compounds (QAC) against *S. aureus, B. subtilis, P. aeruginosa* and *E. coli*.

Strain			QACs			
			BZK	CTAB	CPC	BZT
Gram +ve	*S. aureus*	MIC (μg/mL)	2	2	<1	2
		MBC (μg/mL)	8	8	4	8
	B. subtilis	MIC (μg/mL)	4	4	2	8
		MBC (μg/mL)	16	4	4	16
Gram -ve	*P. aeruginosa*	MIC (μg/mL)	64	512	256	64
		MBC (μg/mL)	64	2048	512	64
	E. coli	MIC (μg/mL)	16	256	128	64
		MBC (μg/mL)	16	256	128	64

Figure 1.

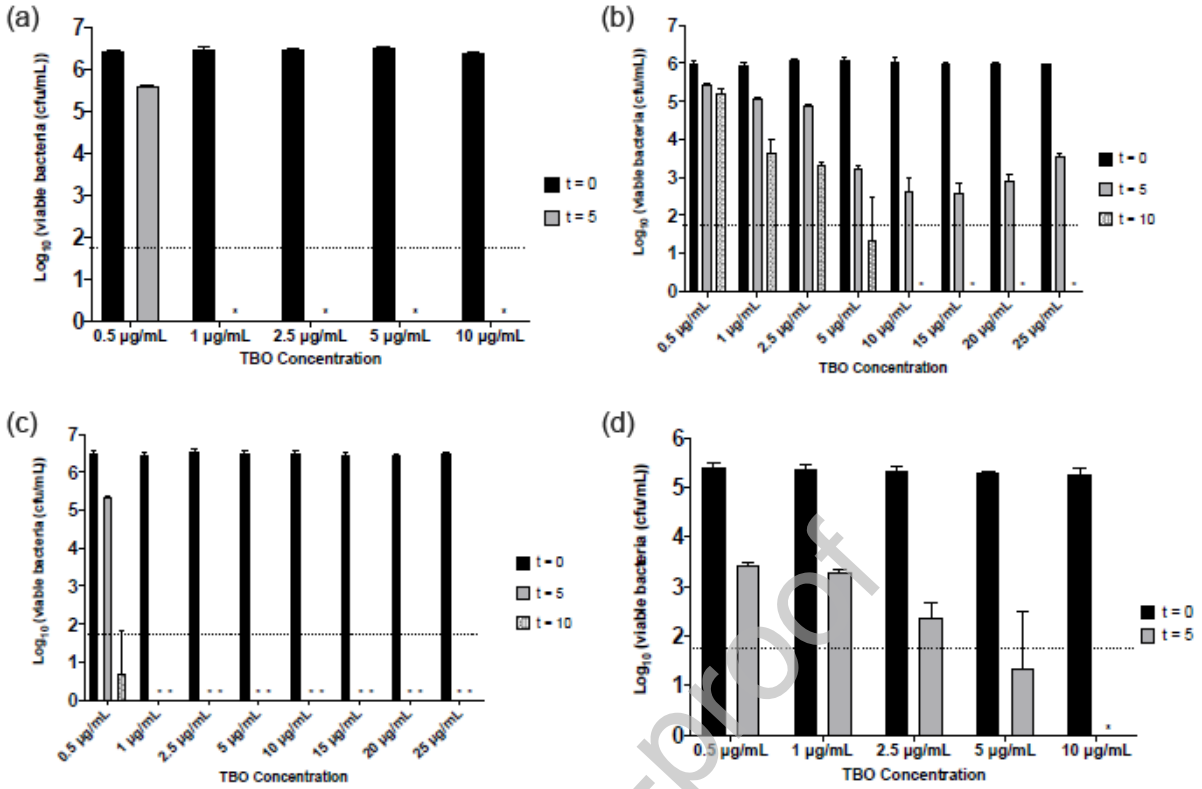
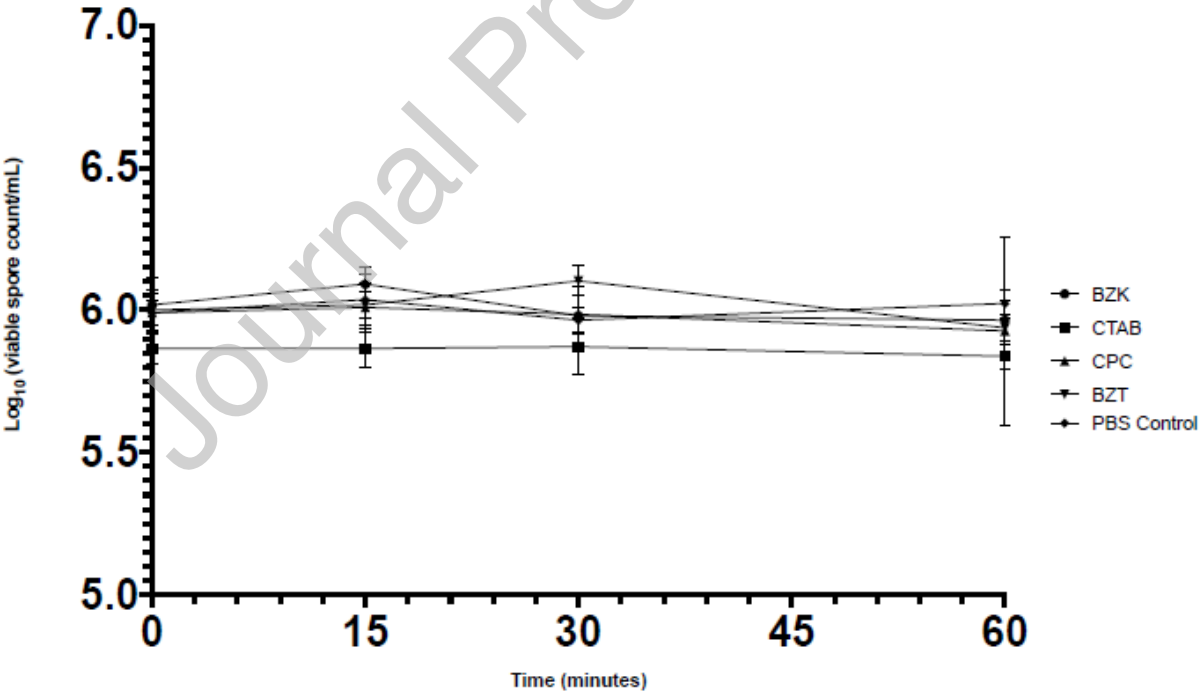


Figure 2.



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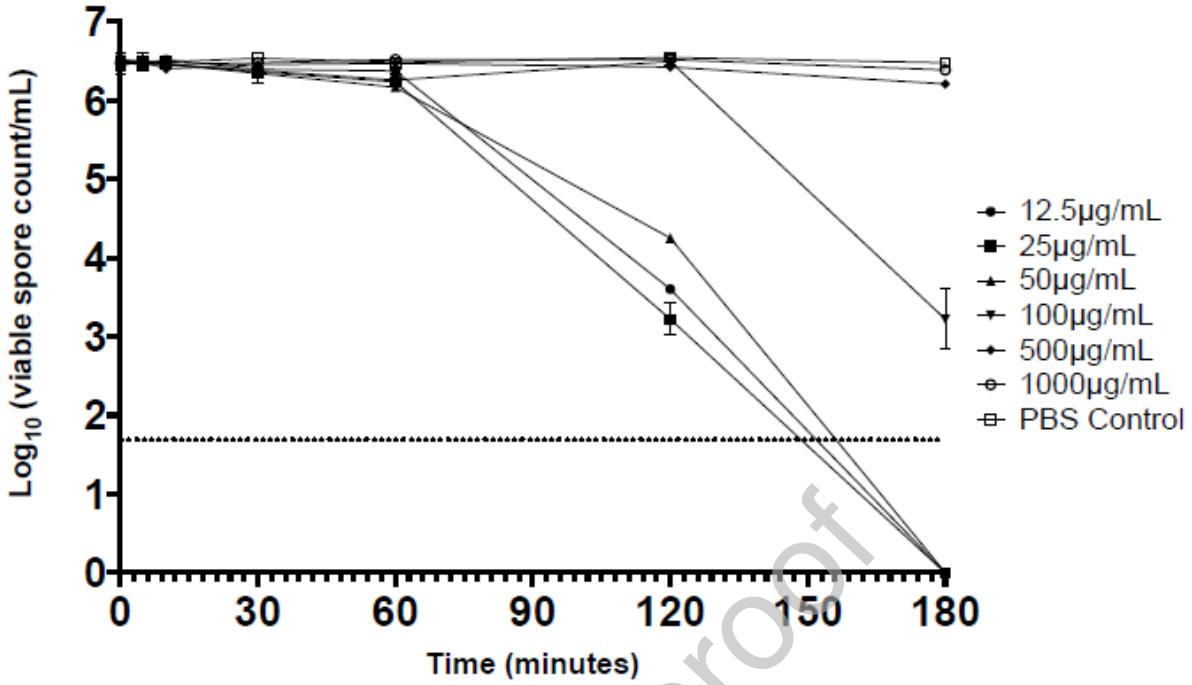

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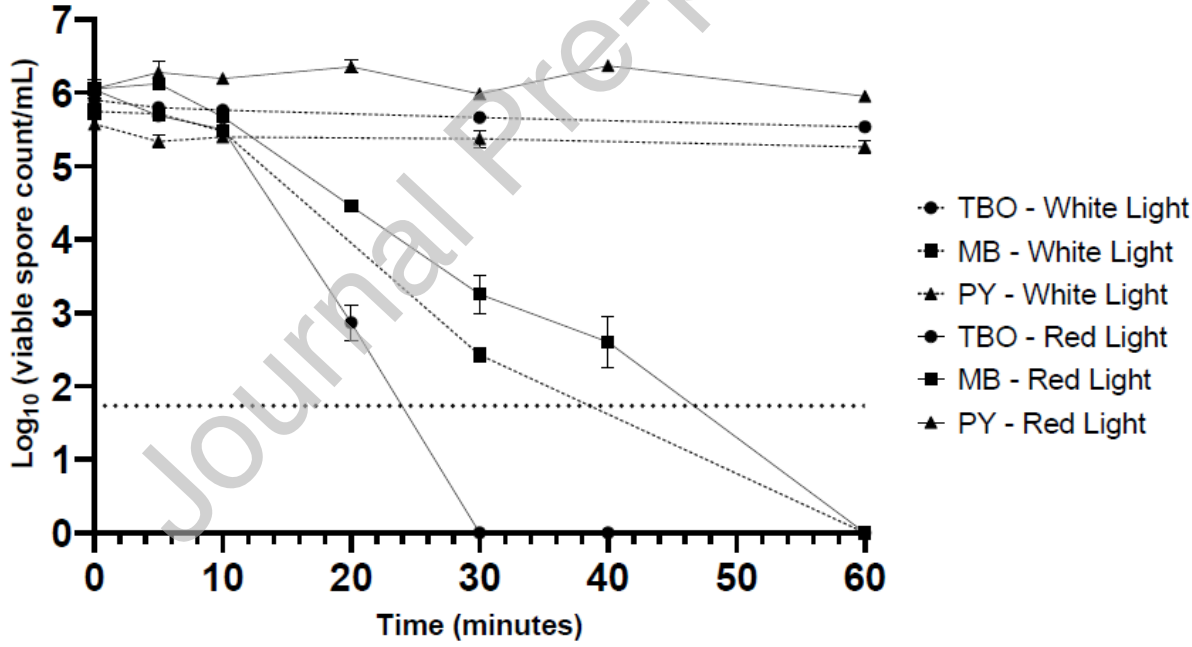

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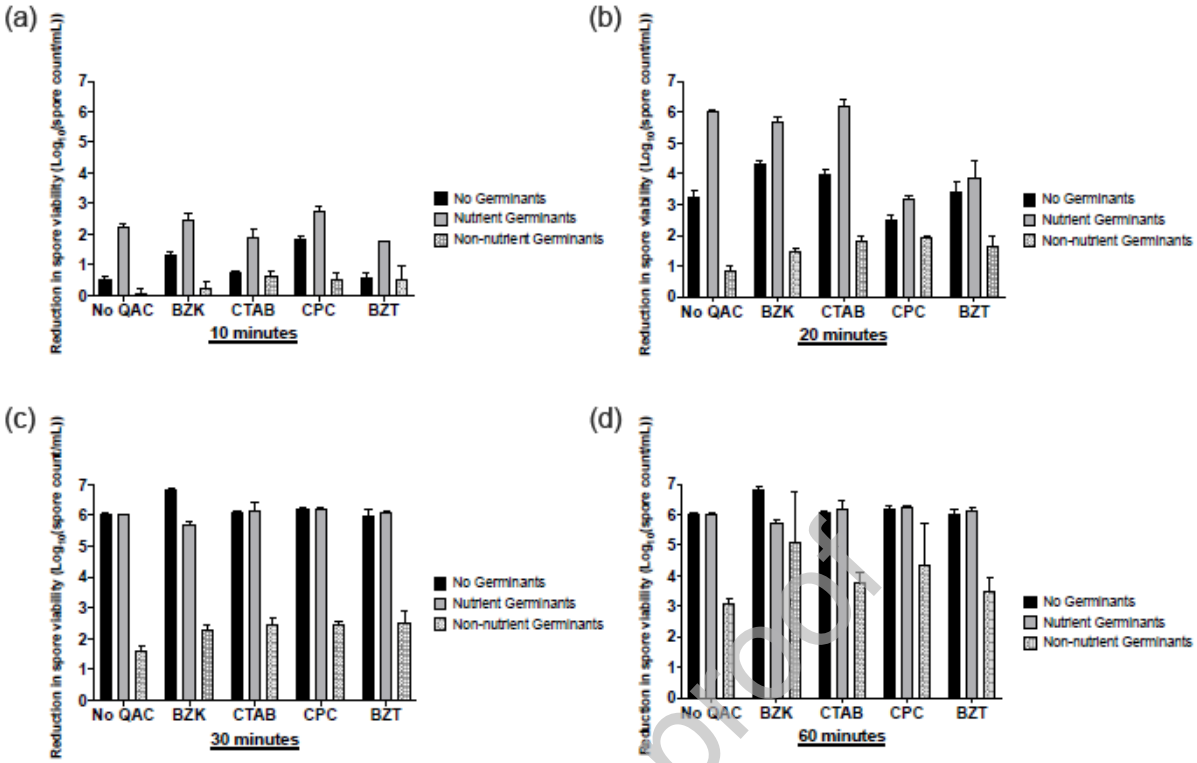

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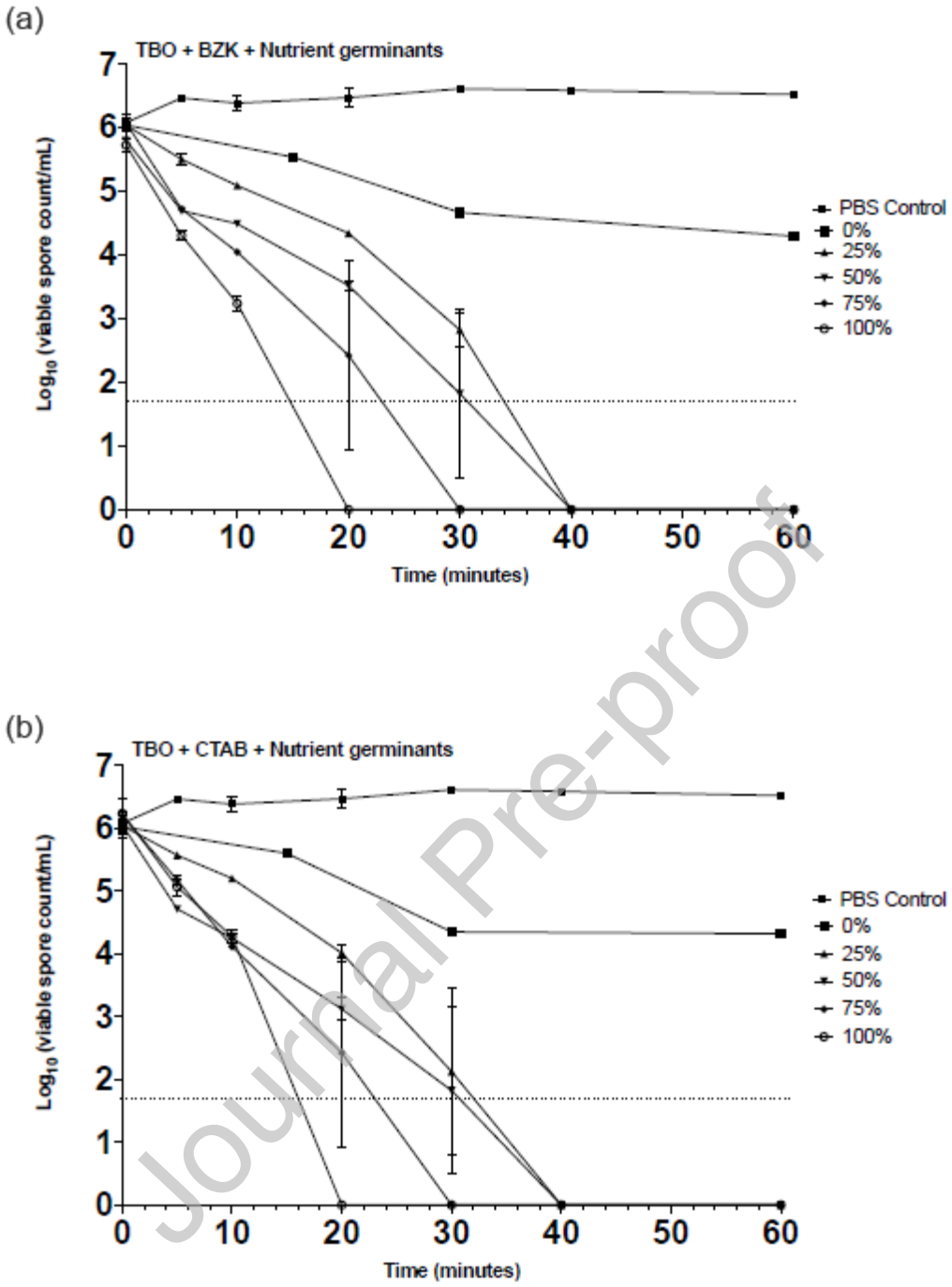

Graphical abstract