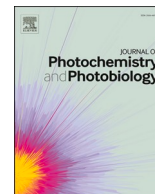




## **A low acriflavine dose strongly potentiates the antimicrobial effect of blue light on *Staphylococcus aureus***

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Author(s)	Allen, Rachel;Somorin, Yinka;Slemon, Matthew;Zekaite, Eva;Haugh, Conall;Hobbs, Chloe;Zeden, Merve S.;O'Gara, James P.;O'Byrne, Conor
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## A low acriflavine dose strongly potentiates the antimicrobial effect of blue light on *Staphylococcus aureus*

Rachel Allen<sup>a,#</sup>, Yinka Somorin<sup>b,c,#</sup>, Matthew Slemon<sup>a</sup>, Eva Zekaite<sup>a</sup>, Conall Haugh<sup>a</sup>,  
Chloe Hobbs<sup>a</sup>, Merve S. Zeden<sup>a</sup>, James P. O'Gara<sup>a</sup>, Conor O'Byrne<sup>a,\*</sup>

<sup>a</sup> Bacterial Stress Response Group, Microbiology, School of Biological and Chemical Sciences, University of Galway, Ireland

<sup>b</sup> National Centre for Laser Applications, School of Natural Sciences, University of Galway, Ireland

<sup>c</sup> Irish Photonic Integration Centre (IPIC), Tyndall National Institute, Cork, Ireland

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### ABSTRACT

*Staphylococcus aureus* infections are difficult to treat in chronic wounds due to biofilm formation and are frequently compounded by antibiotic resistance, necessitating the development of alternative therapeutic approaches. This study investigated the antimicrobial effect of 470 nm blue light, alone and in combination with the photosensitizer, acriflavine, against *S. aureus*. Planktonic cells and preformed biofilms of *S. aureus* SH1000 (methicillin-sensitive) and BH1CC (methicillin-resistant) strains were exposed to 470 nm blue light at varying intensities. A reduction of 4.9 and 5.3 log<sub>10</sub> was observed in the viability of BH1CC and SH1000 planktonic cells respectively when exposed to blue light at 28 mW cm<sup>-2</sup> for 4 h compared to unexposed cells. The effectiveness of blue light inactivation was reduced at 14 and 7 mW cm<sup>-2</sup>, and no inactivation was observed at 3.5 mW cm<sup>-2</sup>. Exposure to a combination of 5 μM acriflavine and blue light (3.5 mW cm<sup>-2</sup>) significantly reduced BH1CC viability by 6 log<sub>10</sub> ( $p = 0.0079$ ) when compared to blue light alone after 1 h. No SH1000 cells survived 1 h exposure to 3.5 and 1.75 mW cm<sup>-2</sup> combined with 5 μM acriflavine. Incubation of *S. aureus* strains with any of the tested concentrations of acriflavine in the dark produced no loss of viability, confirming the synergistic action of blue light combined with acriflavine. These results demonstrate that 470 nm blue light is lethal to *S. aureus* even at very low intensities and that this antimicrobial activity can be significantly enhanced by acriflavine at much lower concentrations than previously reported. These data also suggest that the antimicrobial mode of action for acriflavine is likely to be at least partly light mediated, a finding that has not previously been recognised.

### 1. Introduction

*Staphylococcus aureus* is a Gram-positive, opportunistic pathogen. Approximately 30 % of people are colonised by *S. aureus*, where it generally exists as a member of the nasal and skin microflora [1]. However, this bacterium can cause an array of infections in both immunocompromised and immunocompetent individuals. *S. aureus* is the leading cause of skin and soft tissue infections such as abscesses and cellulitis [2]. It is also capable of causing more severe ailments including endocarditis, osteomyelitis, pneumonia, and septicemia [3]. The bacterium possesses an array of virulence factors that facilitate the infection and proliferation in a diversity of niches within the body. These include binding proteins that facilitate adherence to different cell types and host secreted proteins [4,5] and a variety of secreted toxins and enzymes that

aid in host invasion [6]. In addition, *S. aureus* can form biofilm utilising a variety of mechanisms [7–9], which facilitates its long-term persistence by protecting it against the host immune system and increasing antibiotic tolerance [10]. Additionally, many clinically significant strains of *S. aureus* carry antibiotic resistance genes, usually encoded on mobile genetic elements which make the treatment of infections much more challenging. Resistance to all antibiotics currently licenced to treat *S. aureus* infections has been reported including all first and second line antibiotics including methicillin, vancomycin and flucloxacillin [11,12]. Thus, there is a pressing need for the development of novel strategies to combat these infections.

Light therapy for the treatment of bacterial infections has been investigated for over 100 years with Niels Ryberg Finsen winning the Nobel Prize for physiology and medicine in 1903 by demonstrating that

\* Corresponding author.

E-mail address: [conor.obyrne@universityofgalway.ie](mailto:conor.obyrne@universityofgalway.ie) (C. O'Byrne).

# These authors contributed equally to the study.

blue light could be used to treat lesions caused by tuberculosis on the skin [13]. After the discovery of antibiotics research into photoinactivation of bacteria fell mostly out of favour. However, with the rise of antimicrobial resistance, the field is receiving renewed interest. Although it has been extensively demonstrated that blue light is capable of killing a variety of organisms regardless of the antibiotic resistance profiles [14–20] the mechanisms behind this killing are often not well understood. The generally accepted hypothesis is that bacteria may be killed through the light-mediated generation of reactive oxygen species (ROS) [21–23]. ROS are formed when photons of blue light interact with endogenous photosensitisers in the cell which causes them to enter an excited singlet state. The photosensitizers next transition to the excited triplet state via intersystem crossing which facilitates their reaction with cellular components or molecular oxygen yielding highly toxic radicals, collectively termed ROS, including hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), superoxide ( $\text{O}_2^-$ ) and hydroxyl radicals (OH) or singlet oxygen ( $^1\text{O}_2$ ). ROS then cause cellular membrane damage, DNA damage and protein denaturation and aggregation via the formation of disulfide bonds and lipid peroxidation [24]. The ROS produced can overwhelm the native detoxification pathways present in the cell whose normal function is to detoxify ROS produced through aerobic metabolism [25,26]. Cells treated with ROS scavengers such as glutathione or powerful antioxidants such as ascorbic acid are completely rescued from damage induced by blue light [21,27,28], lending further support to this hypothesis. Several studies have previously validated the efficacy of blue light usually at lower wavelengths (400–450 nm) as an antimicrobial strategy active against a variety of methicillin resistant *S. aureus* (MRSA) and methicillin susceptible *S. aureus* (MSSA) strains [15,29–36].

The antimicrobial activity of light can be potentiated through the addition of exogenous photosensitizers [37]. Exogenous photosensitisers are usually dyes that have excitation peaks at the same or similar wavelength to the light source utilised. These molecules are not sufficient to cause cellular death in the dark but during irradiation, become excited and increase the total amount of ROS produced. This will either increase the speed at which cells can be killed by light or, sensitize cells to a wavelength of light that was not sufficient to cause cell death on its own [38]. For example, one study showed that phenothiazium dyes, including methylene blue and azure B, could enhance the inhibitory effect of broad spectrum white light on the growth of MRSA strains of *S. aureus* [39]. The presence of photosensitizers facilitates the use of longer wavelength light, which is lower energy and therefore likely to be safer for use in clinical applications. As the damage caused to the cell by light is non-specific in nature, it is hypothesised that emergence of resistance to this treatment is less likely. Indeed, Tomb *et al.* showed that while continuous culture in low light conditions ( $<1\text{mW}/\text{cm}^2$ ) did seem to marginally increase bacterial oxidative stress resistance, it did not increase survival following exposure to lethal ( $60\text{mW}/\text{cm}^2$ ) 405 nm light [40].

The present study sought to determine whether blue light at 470 nm could be an effective treatment against *S. aureus* and whether the antimicrobial effects of blue light could be enhanced in the presence of the acridine dye acriflavine. Acriflavine has been in clinical use since 1912 as an antibacterial agent [39] and has FDA approval for topical use in the treatment of wounds. Its antimicrobial activity has largely been attributed to its ability to intercalate into nucleic acids [41,42] but its role as a photosensitiser has received only limited attention [38]. Previous work has shown that high concentrations of acriflavine induced cell wall changes that are lethal for *S. aureus* [40–42]. Here we show that *S. aureus* can be killed by blue light irradiation at 470 nm and show that acriflavine dramatically enhances the killing effect. Overall, the data suggest that acriflavine has potential to be used in combination with blue light irradiation to augment the treatment of topical infections caused by this pathogen.

## 2. Materials and methods

### 2.1. Bacterial strains and culture

Methicillin sensitive *Staphylococcus aureus* strains (8325–4 and SH1000) and the methicillin resistant strains DAR45 [43] and BH1CC [44] were used in this study. *S. aureus* 8325–4 is laboratory strain and SH1000 originates from 8325–4 strain but has the defective *rsbU* gene repaired [45]. Both 8325–4 and SH1000 are methicillin-sensitive while BH1CC and DAR45 are both methicillin-resistant clinical isolates. All strains were stored at  $-80\text{ }^\circ\text{C}$  in Tryptone Soy broth (TSB) (Sigma) containing 7 % DMSO and recovered on Tryptone Soy agar (TSA) grown overnight at  $37\text{ }^\circ\text{C}$ . To prepare an inoculum, a single, isolated colony on TSA plate was inoculated into 5 mL TSB, in 50 mL Falcon tubes and incubated for 18 h on an orbital shaker at 150 RPM. After overnight incubation (18–20 h), 1 mL of the culture was washed twice in 1 mL of PBS and was then centrifuged at 5500 g for 2 min, and the resultant pellets were re-suspended in 1 mL PBS. The suspension was normalised to an optical density of 600 nm ( $\text{OD}_{600\text{nm}}$ ) of 0.1, corresponding to approximately  $1 \times 10^8\text{ CFU ml}^{-1}$  (confirmed by CFU enumeration on TSA plates), using a UV/visible spectrophotometer (LLG-uniSPEC 2 Spectrophotometer, LLG Labware, Germany). All incubations were carried out at  $37\text{ }^\circ\text{C}$ . Experiments investigating the involvement of acriflavine included the addition of  $5\text{ }\mu\text{M}$  acriflavine (Sigma) to PBS. All experiments were conducted in independent biological triplicate with three technical replicates each.

### 2.2. Light source

A mounted light-emitting diode (LED) (model M470L 3-C1, Thorlabs) was used to investigate the effect of blue light on planktonic cells suspended in phosphate-buffered saline (PBS) (Sigma-Aldrich, USA) in a 96-well polystyrene plate. The LEDs emitted continuous blue light at a wavelength of 470 nm with a full width at half maximum (FWHM) of 25 nm. The LED beam irradiated an area of  $19.63\text{ cm}^2$  (5 cm beam diameter) which resulted in equal exposure to 12 wells of a standard 96-well plate. The bacterial suspensions and biofilm were irradiated with 14, 7, 3.5 and  $1.75\text{ mW cm}^{-2}$  resulting in different fluences ( $\text{J cm}^{-2}$ ), depending on the time exposed. The irradiance was adjusted by a LED driver (M98L01, ThorLabs) and was measured using a power meter (PM100D, Thorlabs).

### 2.3. Blue light survival assay

Overnight stationary phase cultures of *S. aureus* SH1000 and BH1CC were washed in PBS twice and adjusted to  $\text{OD}_{600\text{nm}}$  of 0.1 culture in PBS. Then,  $250\text{ }\mu\text{l}$  of the normalised bacterial suspension was added in triplicate to three designated wells on two polystyrene 96-well plates. One of the 96-well plates (designated “Light”) was exposed to blue light (at the specified irradiance) while the second plate (designated “Dark”) was wrapped in aluminium foil to prevent exposure to ambient light. Both plates were incubated at  $37\text{ }^\circ\text{C}$ . Aliquots ( $40\text{ }\mu\text{l}$ ) were taken from each well at designated time points, serially diluted in PBS and plated on TSA plates in triplicates. All plates were incubated at  $37\text{ }^\circ\text{C}$  for 24 h. Colonies were then counted manually and the number of viable cells ( $\text{CFU ml}^{-1}$ ) was determined.

### 2.4. Antimicrobial effect of blue light treatment on preformed biofilms

Overnight cultures of *S. aureus* SH1000 and BH1CC were grown in 5 mL of TSB at  $37\text{ }^\circ\text{C}$  for 18 h. Stationary phase *S. aureus* SH1000 and BH1CC normalized to  $\text{OD}_{600\text{nm}}$  of 0.02 (approx  $10^7\text{ CFU ml}^{-1}$ ) in fresh TSB supplemented with 1 % glucose. Thereafter, the medium and planktonic cells in each well was removed and washed with PBS twice. Then,  $200\text{ }\mu\text{l}$  of PBS only and PBS with  $5\text{ }\mu\text{M}$  acriflavine was added to quadruplicate wells in two 96-well plate, with one plate exposed to light

(3.5 mW cm<sup>-2</sup> and 14 mW cm<sup>-2</sup>) for 2 h and the other plate unexposed to light (Dark Control).

### 2.5. Crystal violet staining

After incubation, biofilm biomass in each well was assessed using crystal violet staining according to [46] with slight modifications. Briefly, the medium and planktonic cells in each well was removed and biofilms were washed with sterile de-ionized water three times and dried at 60 °C for 1 h. Then, 200 µl of 0.1 % w/v crystal violet was added to each well and incubated at room temperature for 30 min on a rocker at 70 oscillations/min. Each well was rinsed three times with de-ionized water to remove unbound crystal violet. Thereafter, 200 µl of 4 % v/v acetic acid was added to the wells and incubated for 30 min. Then, absorbance of the destained mixture was measured at a wavelength of 600 nm.

### 2.6. Colony enumeration of irradiated biofilms

For enumeration of colony forming units 100 µl PBS was added to each well and biofilm disrupted with pipette tip and pipetting up and down. The plates were then pulse-sonicated (Elmasonic P 30H, Germany) for 5 min at a frequency of 37 kHz and a 100 % power output. The suspension from each well was transferred to a separate sterile 1.5 ml tube, which were then vortexed for 30 s. Aliquots (50 µl) were taken from each tube, serially diluted in PBS and plated on TSA plates in triplicates. All plates were incubated at 37 °C for 48 h. Colonies were then counted manually and the number of viable cells (CFU ml<sup>-1</sup>) was determined.

### 2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.0.2 for Windows software (GraphPad Software, California, USA). Unpaired *t*-tests were used to compare differences survival for the light

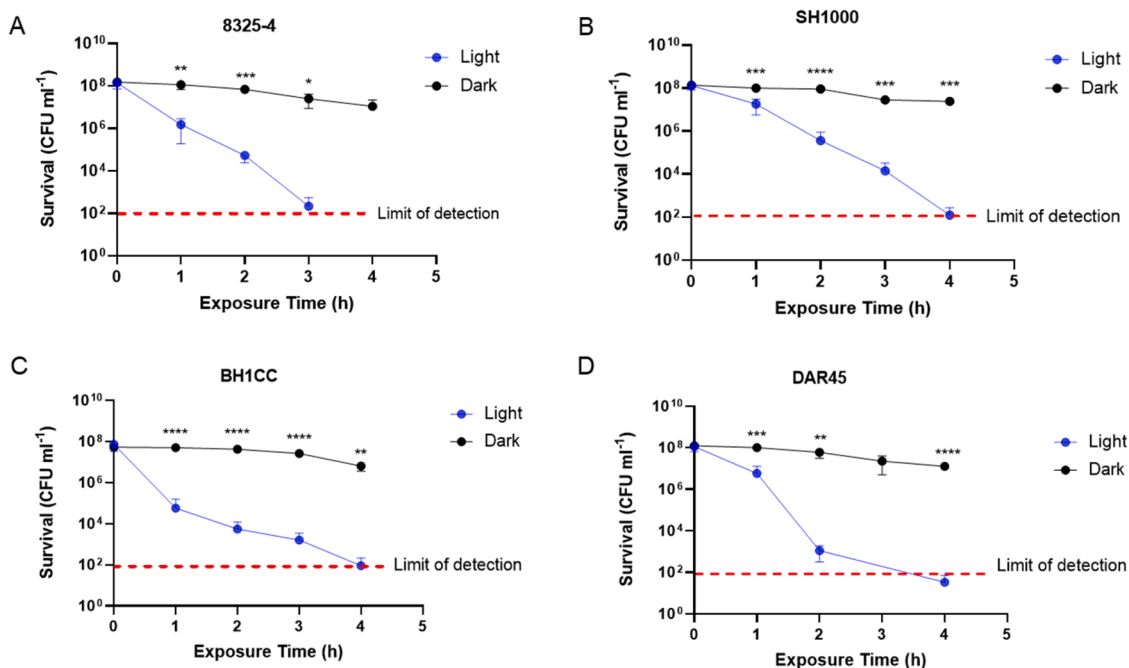
vs dark treated cells in Figs. 1 and 2 and, for the acriflavine + light and acriflavine + dark cells in Fig. 3. Differences with a *P* value < 0.05 were considered statistically significant. For Fig. 4, statistical analysis was carried out using a two way analysis of variance (ANOVA) followed by a *post hoc* Tukey test for multiple comparisons. Differences with a *P* value < 0.05 were considered statistically significant.

## 3. Results

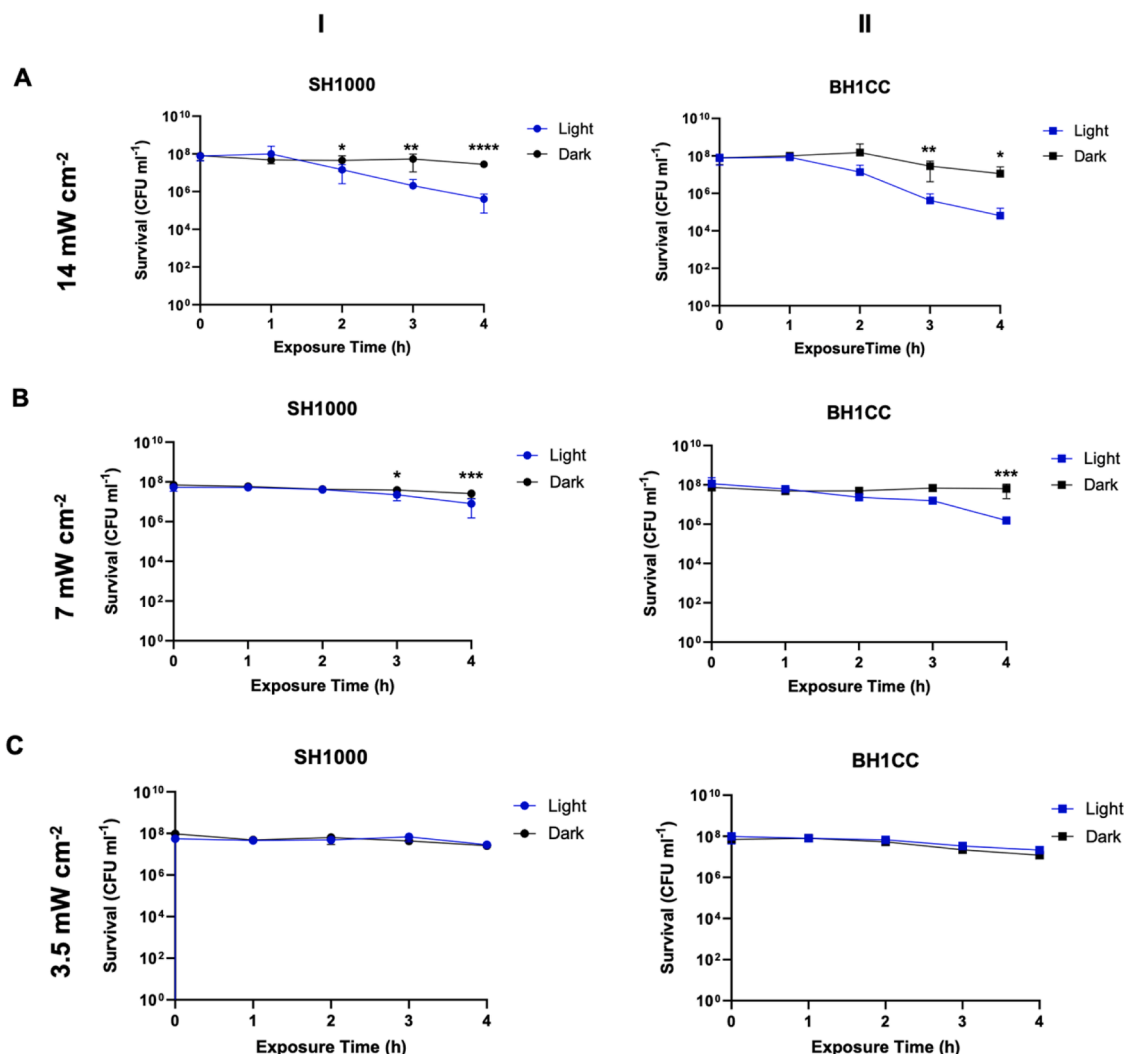
### 3.1. Blue light (470 nm) inhibits *Staphylococcus aureus* strains at varying intensities

We investigated the effect of the 470 nm blue light on the viability of planktonic cell suspensions of four strains of *S. aureus* at 28 mW cm<sup>-2</sup>. The survival rate of continuously irradiated planktonic suspensions of *S. aureus* strains in phosphate buffered saline (PBS) for 4 h was measured (Fig. 1). Viable cell counts of all the planktonic *S. aureus* strains were significantly reduced (*p* < 0.05) when exposed to 470 nm blue light for 1 h (dose 100.8 J cm<sup>-2</sup>) compared to unexposed cells (dark control). After 4 h of irradiance at 470 nm (dose 403.2 J cm<sup>-2</sup>), there were no detectable survivors of *S. aureus* 8325-4 (Fig. 1A). Similarly, when compared to dark control, the viability of *S. aureus* SH1000 was reduced by 5.3 log<sub>10</sub> (Fig. 1B), while BH1CC and DAR45 were reduced below the detection limit (Fig. 1C, D).

Next we investigated reduced doses of blue light to determine the lower boundary for cell inactivation. For 14 mW cm<sup>-2</sup> irradiance, there was no significant reduction in viable count of SH1000 and BH1CC after 1 h (dose 50.4 J cm<sup>-2</sup>) (Fig. 2A). After 2 h continuous exposure (dose 100.8 J cm<sup>-2</sup>), there was a significant 0.5 log<sub>10</sub> reduction (*p* = 0.0201) in SH1000 compared to the dark control, whereas the 1 log<sub>10</sub> reduction measured for BH1CC was not significantly different (*p* > 0.05). When 7 mW cm<sup>-2</sup> irradiance was used, there was only significant inactivation after 3 h (dose 75.6 J cm<sup>-2</sup>) and 4 h (dose 100.8 J cm<sup>-2</sup>) for SH1000 and only after 4 h (dose 100.8 J cm<sup>-2</sup>) for BH1CC (Fig. 2B). There was no detectable inactivation of either strain after 4 h continuous irradiance at



**Fig. 1.** Blue light (470 nm) inhibits *Staphylococcus aureus* strains at 28 mW cm<sup>-2</sup>. Overnight cultures were normalized to an OD<sub>600</sub> of 0.1 (approximately 1 × 10<sup>8</sup> CFU ml<sup>-1</sup>). *S. aureus* strains were exposed to continuous illumination at 28 mW cm<sup>-2</sup> for 4 h (Light) and those not exposed to light was the control (Dark), with aliquots Cells were incubated at 37 °C for 24 h. The values represent the means of the results from three independent replicates. The error bars represent the standard deviations between replicates. Student's *t* test was carried out to determine the statistical difference (*P* ≤ 0.05, indicated with an asterisk) between cultures grown in light and dark.



**Fig. 2.** Blue light (470 nm) inhibits *Staphylococcus aureus* strains at lower light intensities. White bars represent growth following continuous illumination, and the black bars represent a dark control. The top graph shows the final OD<sub>600</sub> after 24 h, and the bottom graph shows the difference in lag times between the two conditions. Overnight cultures were standardized to an OD<sub>600</sub> of 1.0 and diluted to 10–5 (approximately 104 cells ml<sup>-1</sup>). Cells were incubated at 37 °C for 24 h. The values represent the means of the results from three independent replicates. The error bars represent the standard deviations between replicates. Student's t-test was carried out to determine the statistical difference ( $P \leq 0.05$ , indicated with an asterisk) between cultures grown in light and dark.

3.5 mW cm<sup>-2</sup> (dose 50.4 J cm<sup>-2</sup>) (Fig. 2C). Thus a minimum dose of approximately 100 J cm<sup>-2</sup> of blue light is required to inactivate a planktonic suspension of stationary phase *S. aureus* cells.

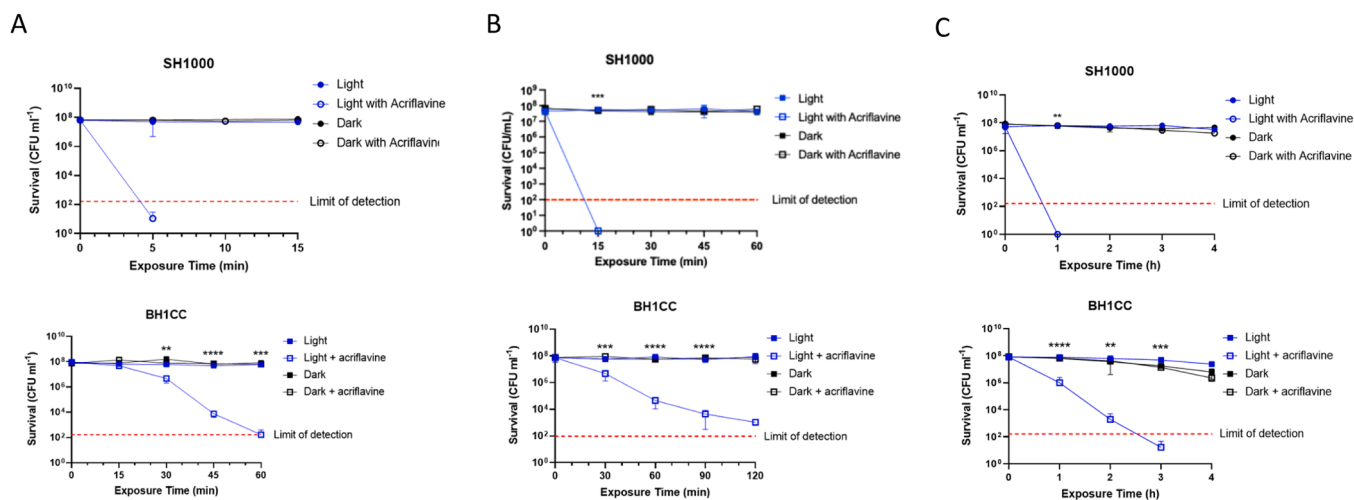
### 3.2. Acriflavine potentiates the antimicrobial activity of 470 nm blue light on planktonic *Staphylococcus aureus*

The antimicrobial effect of 470 nm blue light alone or in combination with the photosensitizer, acriflavine, was compared against *S. aureus*. Acriflavine (5 μM) was added to planktonic *S. aureus* suspensions and exposed to 7 mW cm<sup>-2</sup>. Strikingly, no viable *S. aureus* SH1000 cells were detected after 1 h (dose 25.2 J cm<sup>-2</sup>) in the presence of 5 μM acriflavine compared to light alone (Fig. 3A). Furthermore, it was observed that reducing the dosage to 2.1 J cm<sup>-2</sup> (7 mW cm<sup>-2</sup> for 5 min) was also effective in reducing the viability of SH1000 below the limit of detection (Fig. 3A). Similar to SH1000, 5 μM acriflavine in combination with 7 mW cm<sup>-2</sup> blue light (25.2 J cm<sup>-2</sup>) led to a significant 5.4 log<sub>10</sub> reduction ( $p = 0.0087$ ) in BH1CC cells compared to exposure to light only (Fig. 3A). However, no potentiating effect of acriflavine was observed when BH1CC cells were exposed to acriflavine and 7 mW cm<sup>-2</sup> blue light for up to 15 min (6.3 J cm<sup>-2</sup>) (Fig. 3A).

Exposure to acriflavine reduced the intensity of blue light required to inactivate *S. aureus*, with SH1000 cells being more sensitive than BH1CC cells. Exposure to a combination of 5 μM acriflavine and blue light (3.5 mW cm<sup>-2</sup> at 1 h; dose 12.6 J cm<sup>-2</sup>) completely inactivated SH1000 (Fig. 3B) and was accompanied by a 3.1 log<sub>10</sub> reduction in BH1CC (Fig. 3B), when compared to only light treatment. Further analysis showed that as low as 3.15 J cm<sup>-2</sup> (3.5 mW cm<sup>-2</sup> for 15 min) in combination with 5 μM acriflavine was sufficient to completely eradicate SH1000 (Fig. 3B), whereas longer irradiance (at least 3.5 mW cm<sup>-2</sup> for 30 min; dose 6.3 J cm<sup>-2</sup>) was needed to significantly reduce BH1CC (Fig. 3B). Furthermore, similar to irradiation at 3.5 mW cm<sup>-2</sup>, there were no detectable SH1000 cells after 1 h exposure to 1.75 mW cm<sup>-2</sup> (dose 6.3 J cm<sup>-2</sup>) when combined with 5 μM acriflavine (Fig. 3C).

### 3.3. Acriflavine potentiates the activity of blue light against preformed *S. aureus* biofilms in a strain-dependent manner

Since the results demonstrated that blue light can efficiently inactivate *S. aureus* strains in planktonic suspensions in the presence of acriflavine, we sought to investigate if the same treatment could also be effective for inactivating preformed *S. aureus* biofilms. Biofilms were



**Fig. 3.** Acriflavine potentiates inhibition of *Staphylococcus aureus* by 470 nm blue light at 7 mW cm<sup>-2</sup> (A), 3.5 mW cm<sup>-2</sup> (B) and 1.75 mW cm<sup>-2</sup> (C). Inactivation of planktonic cells of *S. aureus* SH1000 and BH1CC either resuspended in PBS or in PBS with 5 μM acriflavine measured during irradiance with 470 nm blue light at 7 mW cm<sup>-2</sup> (A), 3.5 mW cm<sup>-2</sup> (B) and 1.75 mW cm<sup>-2</sup> (C). Cells resuspended in PBS but not exposed to light (Dark) and cells resuspended in PBS with 5 μM acriflavine but not exposed to light but (Dark + acriflavine) served as the controls. The values represent the means of the results from three independent replicates. The error bars represent the standard deviations between replicates. Student's *t* test was carried out between dark + acriflavine and light + acriflavine to determine the statistical difference ( $P \leq 0.05$ , indicated with an asterisk).

grown in 96-well plates for 24 h, before the medium was removed and replaced with PBS containing 5 μM acriflavine, which was then subjected to blue light exposure while the control was kept in the dark. When SH1000 was exposed to (14 mW cm<sup>-2</sup> for 2 h; dose 100.8 J cm<sup>-2</sup>), light had no significant impact on preformed biofilms, but addition of acriflavine significantly ( $p = 0.0004$ ) increased the biomass by 2-fold (Fig 4A). However, for BH1CC biofilm, while exposure to light alone significantly increased the biomass when compared to dark control, combination with acriflavine did not significantly impact biomass compared to light alone (Fig 4B).

When cell viability within the biofilms was measured, the combination of light (14 mW cm<sup>-2</sup> for 2 h; dose 100.8 J cm<sup>-2</sup>) and acriflavine significantly inactivated *S. aureus* SH1000 and BH1CC by 1.1 log<sub>10</sub> (92.8 % reduction) and 1.4 log<sub>10</sub> (96 % reduction) respectively (Fig. 4C,D). Blue light alone had no inactivating effect on the viability of *S. aureus* SH1000 and BH1CC biofilms indicating that biofilm-associated cells are intrinsically more resistant to blue light.

#### 4. Discussion

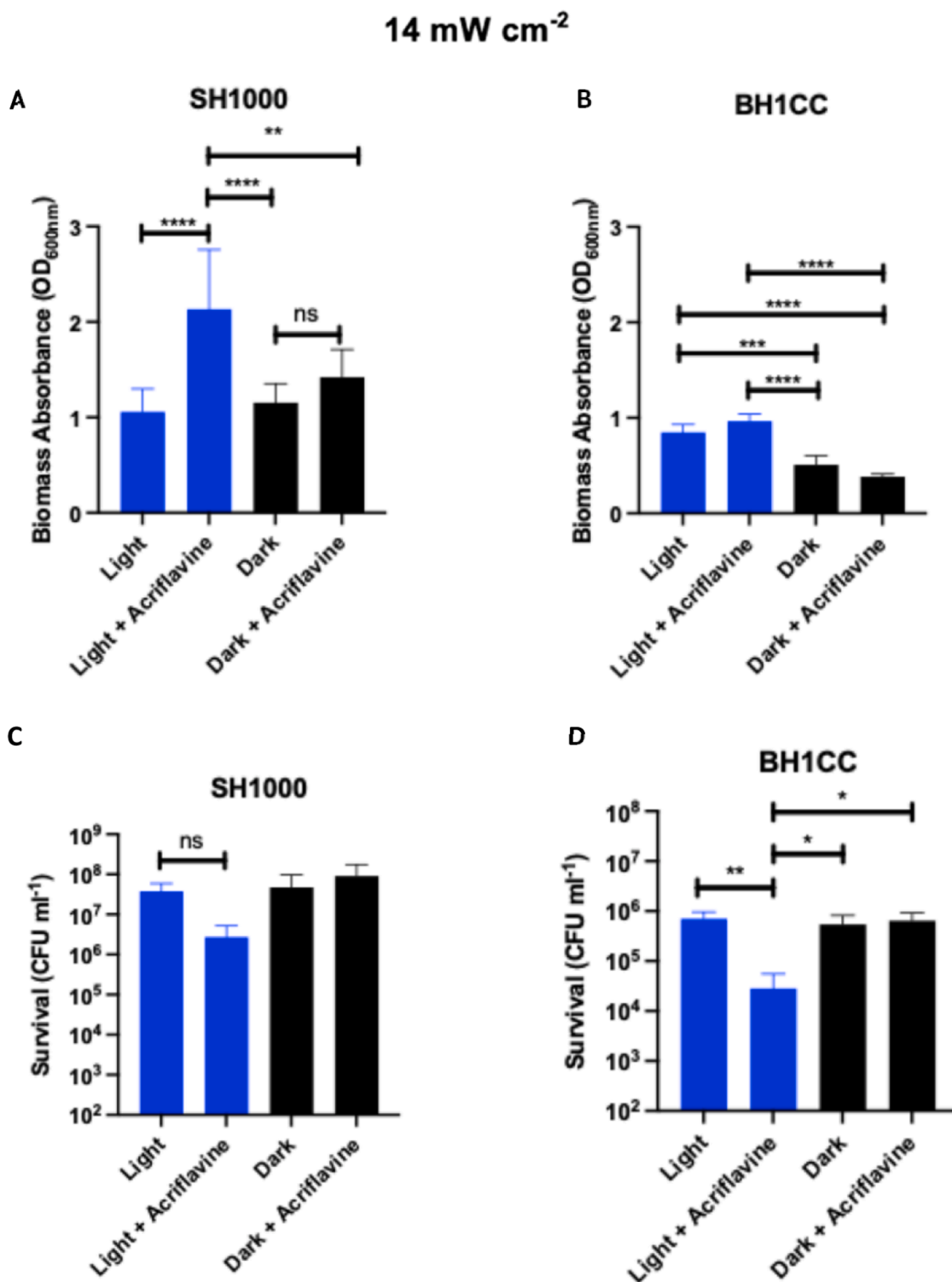
MRSA represents a significant public health threat and novel, effective alternatives to existing antibiotics are urgently needed [47,48]. In this study we explored the efficacy of 470 nm blue light both on its own and in conjunction with the photosensitizer acriflavine hydrochloride at inducing cell death in planktonic *S. aureus* cultures as well as those embedded in a preformed biofilm. Our results demonstrated that light on its own could induce significant killing of planktonic *S. aureus* where, a 6-log reduction in cell viability could be achieved in all four genetic backgrounds by 4 h of continuous treatment. When the treatment was combined with acriflavine, we demonstrated a substantially increased magnitude and rate of cell killing using just 25 % of the power density that was effective in the absence of photosensitiser. We further demonstrated that there were significantly fewer viable MRSA (BH1CC) cells remaining in the biofilm after light treatment combined with acriflavine compared to those exposed to light alone.

The efficacy of blue light as an alternative strategy to antibiotic therapy for *Staphylococcus* infections has been previously investigated with a variety of strains, wavelengths of light and light intensities utilised. In addition, a range of photosensitizers have also been screened with significantly increased killing efficiencies achieved for; flavin

mononucleotide [49], Hypocrellin B [50], Curcumin [51], Resveratrol [52], Riboflavin [53] and Coproporphyrin III [54]. The mechanism of cell death via irradiation has been studied and a model based on the generation of ROS has been widely proposed. However, where exogenous photosensitizers are used, the mechanisms of killing tend to be less clear than simply stimulating an increase in total ROS levels. A 2009 study [55] demonstrated that acriflavine caused minor cell wall thickening but was not sufficient to induce cell death, at similar concentrations to those used in our study. Increased cell wall thickness was correlated to potent bactericidal activity at the highest tested concentrations which, was six times greater than our tested concentrations [55]. However, it is important to note that, in that study the authors did not mention whether they controlled for light exposure. We have observed antimicrobial effects of acriflavine even with ambient light present in the lab (data not shown). While we did not specifically look at cell wall changes, we only observed cell death when acriflavine was combined with light. This raises the possibility that acriflavine, when used at lower concentrations, may exert its antimicrobial effects on bacteria as a result of its photoactivation. The thickening of the cell wall induced by acriflavine may also help to explain why we saw increases in biomass in SH1000 biofilm treated with acriflavine and light (Fig. 4A).

Most of the light-based studies in *S. aureus* have focused on the killing efficiency of light in planktonic cell cultures or, in the early stages of biofilm development where inhibition of biofilm formation is the goal as opposed to biofilm disruption. The cell density effect on the efficacy of blue light treatment also has a role to play on how successful light could be as a biofilm disrupting agent. Additionally, the efficacy of light treatment likely relies on the accumulation and diffusion of ROS through the medium. Thus, it is possible that the biofilm matrix helps shield cells from this oxidative stress exposure. In order to make the cells more vulnerable to blue light treatment, these challenges would need to be overcome. A recent study demonstrated the antibiofilm activity of curcumin, which is also a photosensitizer. The authors showed that curcumin could degrade the extracellular matrix as well as decreasing the viability of *S. aureus* cells isolated from a patient wound sample [56]. This study did not utilise light in addition with curcumin so, it is possible that if the two therapies were combined, similar levels of killing and disruption of biofilm could be achieved with lower concentrations of curcumin.

The use of blue light to treat infections caused by *S. aureus*, has been



**Fig. 4.** Disruption of preformed staphylococcal biofilm exposed to blue light at 14 mW cm<sup>-2</sup> for 2 h. Biomass levels by crystal violet staining of *S. aureus* biofilms treated with and without 5  $\mu$ M acriflavine and exposed to 14 mW cm<sup>-2</sup> light for 2 h compared to dark control (A, B). Biofilm was established in a 96-well plate prior to exposure to light in either fresh PBS or PBS with 5  $\mu$ M acriflavine. Cell viability of biofilms that received the same treatment in (A, B) are shown in (C, D). The values represent the means of the results from three independent replicates. The error bars represent the standard deviations between replicates. A two-way ANOVA was carried out with a *post hoc* Tukey test to determine the statistical difference ( $P \leq 0.05$ , indicated with an asterisk) between cultures grown in light and dark.

explored previously in other studies. One such study assessed three different wavelengths of light, 420, 455 and 470 nm for their efficacy at reducing the bacterial burden of *S. aureus*, *S. epidermidis* and *P. aeruginosa* as well as the light protocols effect on *in vitro* human

dermal fibroblasts. In this study they found that none of the wavelengths at any intensity were effective at killing *S. aureus*. More interestingly, blue light treatment at 455 nm showed only slight cell toxicity to immortalised keratinocytes after receiving a dose of 180 J/cm<sup>2</sup>, whereas

light treatment at 480 nm was non-toxic at any dose [57]. This is especially promising considering, this study utilised 470 nm light in combination with acriflavine at doses ranging from 6–50 J/cm<sup>2</sup> to achieve a 6-log<sub>10</sub> reduction in cell density. Another study utilised pyocyanin against MRSA and vancomycin-resistant *S. aureus* with 405 nm light, the authors needed to utilise pyocyanin in combination with light treatment at 108 J/cm<sup>2</sup> to achieve a 4-log<sub>10</sub> reduction in MRSA burden [58] whereas, in the present study acriflavine in combination with 6 J/cm<sup>2</sup> achieved a 6-log<sub>10</sub> reduction in 15 min of MRSA strain BH1CC. While there have been no clinical trials specifically accessing the toxicity and safety of acriflavine, 100 mg of acriflavine was administered daily to HIV patients over a three-month period and no adverse effects on trial participants were reported [59,60]. Taking the data together, the longer wavelength and the doses of light utilised in this study are well below what has been reported in the literature as toxic to human cells.

Differences in strain sensitivity to blue light treatment were observed in the present study. The most sensitive strain was 8325–4, a methicillin sensitive strain that is closely related to SH1000. Interestingly, there was a significant difference in the dose required to reduce the cell density of both by 6-log<sub>10</sub>. SH1000 has 3 single nucleotide polymorphisms compared to 8325–4, a 63-bp deletion upstream of the *spa* gene, as well as a repaired RsbU mutation that provides SH1000 with a functional SigB-mediated general stress response system that is absent in 8325–4 [61]. The functional SigB in SH1000 likely explains why a slightly more prolonged exposure of light is required to kill this strain when compared to 8325–4 but killing is still achieved below the limit of detection with both strains within a four-hour timeframe. This could represent a useful avenue to investigate the molecular methods that may aid *S. aureus* in surviving this treatment. The MRSA strains were more resistant to the treatment than their methicillin sensitive counterparts, which has been observed previously in environmental stress studies, however the exact mechanisms remain to be elucidated [62].

## 5. Conclusion

This study is a proof of concept demonstrating that acriflavine in combination with light can be used as a photosensitizer for inducing *S. aureus* cell death at a much lower concentration of acriflavine and lower dose of blue (470 nm) light than has previously been reported. The study also demonstrated that concentrations of acriflavine that have been reported as antimicrobial previously show no killing effect unless combined with light, suggesting that a light activation mechanism provides the principal antimicrobial mode of action. This study offers a promising cornerstone for future work in this area where, the exact molecular mechanisms underlying the antimicrobial effect should be studied, as well as assessing why strain specific differences arise and the genetic and physiological basis for those. Combining blue light treatment with a photosensitizer such as acriflavine could prove to be a valuable therapeutic option for topical infections, especially at a time where antibiotic resistant infections are posing an increasing challenge for clinicians.

## CRedit authorship contribution statement

**Rachel Allen:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Yinka Somorin:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Matthew Slemon:** Validation, Methodology, Investigation. **Eva Zekaite:** Visualization, Validation, Methodology, Investigation, Formal analysis. **Conall Haugh:** Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Chloe Hobbs:** Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Merve S. Zeden:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation. **James P. O’Gara:** Writing – review & editing, Writing – original draft,

Supervision, Resources, Methodology, Formal analysis. **Conor O’Byrne:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Data availability

Data will be made available on request.

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