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Violet 405 nm Light: A Novel Therapeutic Agent Against Common Pathogenic Bacteria

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Running Title: Visible light treatment on pathogenic bacteria

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ABSTRACT

Background:
The increasing incidence hospital-associated infections (HAI) and multidrug-resistant organisms demonstrate the need for innovative technological solutions. *Staphylococcus aureus*, *Streptococcus pneumonia*, *Escherichia coli*, and *Pseudomonas aeruginosa* in particular are common pathogens responsible for a large percentage of indwelling medical device-associated clinical infections. The bactericidal effects of visible light sterilization (VLS) using 405 nm is one potential therapeutic under investigation.

Materials and Methods:
405 nm light-emitting diodes were used to treat varying concentrations of *S. aureus*, *S. pneumonia*, *E. coli*, and *P. aeruginosa*. Irradiance levels between 2.71 ± 0.20 to 9.27 ± 0.36 mW/cm² and radiant exposure levels up to 132.98 ± 6.68 J/cm² were assessed.

Results:
Dose dependent effects were observed in all species. Statistically significant reductions were seen in both Gram-positive and Gram-negative bacteria. At the highest radiant exposure levels, bacterial log₁₀ reductions were: *E. coli* – 6.27 ± 0.54, *S. aureus* – 6.10 ± 0.60, *P. aeruginosa* – 5.20 ± 0.84, and *S. pneumoniae* – 6.01 ± 0.59. Statistically significant results (<0.001*) were found at each time point.

Conclusions:
We have successfully demonstrated high efficacy bacterial reduction using 405 nm light sterilization. The VLS showed statistical significance against both Gram-positive and Gram-negative species with the given treatment times. The β-lactam antibiotic-resistant *E. coli* was the most sensitive to VLS, suggesting light therapy could a suitable option for sterilization in drug-resistant bacterial species. This research illustrates the potential of using VLS in treating clinically relevant bacterial infections.

KEYWORDS

405 nm Light; Antibiotic Resistance; Visible Light; Sterilization; Gram-Positive; Gram-Negative
1. INTRODUCTION

Hospital Acquired infections (HAIs) are the most frequent adverse event in health-care delivery worldwide. They occur in 5-10% of acute care hospital admissions, costing the healthcare system an estimated $28-$45 billion annually (1, 2). While in recent years improved hand hygiene compliance has been shown to reduce HAIs, they remain one of the top preventable causes of morbidity and mortality in part due to the recent rise of antimicrobial-resistant (AMR) bacteria (3, 4). There is a need for an alternative solution that is safe and effective at treating these bacterial infections.

The National Healthcare Safety Network (NHSN) at the CDC reported that *S. aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* were among the leading causes of HAIs based on reports from 2,039 hospitals that reported one or more HAIs during 2009-2010 (5, 6). In this two-year period there were a total of 69,475 instances of HAIs reported with *S. aureus* found to be the most common pathogen overall (5). *E. coli* was found to be the second most common HAI pathogen and *P. aeruginosa* the fifth most common isolate overall. In addition, *Streptococcus pneumoniae* is the leading cause of pneumonia worldwide and is a common isolate of ventilator associated pneumonia (7-9).

Many infections are treated with broad spectrum antibiotics followed by more specific antibiotic regimens based on culture sensitivity. The increasing prevalence of multidrug-resistant bacteria often necessitates the use of second-line antibiotics with a greater side-effect profile and may lead to loss of efficacy for these critical therapeutics (5). Approximately 90% of *Staphylococcus aureus* strains are resistant to penicillin and similar antibiotics to which they
were originally susceptible (10). The (NHSN) reported up to 83% of blood stream infections and 20-40% of all HAIs were caused by antibiotic-resistant species (5).

Medical devices are a major conduit for HAIs with repeated cycles of antibiotic treatment leading to resistance. Manufacturers have attempted several approaches to reduce the incidence of HAI’s including increased emphasis on sterile insertion techniques, antibiotic impregnation of devices, and coating of at-risk surfaces with bacteriostatic and bactericidal agents. These coatings are intended to inhibit bacterial and fungal colonization of the device. Unfortunately, they have shown minimal effectiveness in the clinical setting at reducing device-associated infections (11, 12).

The frequency and severity of HAIs demonstrate the need for innovative technological solutions. There has recently been increased interest in the prevention and treatment of infections using visible light sterilization (VLS) due to the advantages of having a focused delivery (local application) and the lack of systemic side effects. VLS uses high intensity light-emitting diodes (LEDs) and lasers emitting non-ultraviolet light in the visible spectrum (380 nm to 700 nm). Examples of investigated applications using VLS include the treatment of \textit{H. pylori}-mediated gastritis and environmental decontamination of burn wards (13-15). VLS has shown substantial antimicrobial efficacy against pathogenic skin flora, \textit{Propionibacterium acnes}, and has received United States Food and Drug Association (FDA) clearance as a clinical treatment for acne vulgaris (16).

VLS has an advantage over the use of ultraviolet light (UV) for the clinical use of bacterial inactivation due to the different mechanisms by which they operate. UV light is shorter wavelength radiation, with the peak potency around a wavelength of 250 nm, causing
cellular damage by breaking the strong O–H, P–O, and N–H bonds (17). These chemical bonds make up the backbone of DNA and tertiary bonds of DNA and protein. Therefore, the use of UV light in vivo is limited due to its detrimental effects on living mammalian tissue (18). A 57% loss of keratinocytes was noted by Dai, et al. while investigating central line infection treatments using UV light (19). This cytotoxicity illustrates that while UV light is an effective antimicrobial agent, it may not be best suited for use near living tissue.

In contrast, slightly longer wavelength 405 nm light does not affect cellular DNA in bactericidal doses (20). Instead, 405 nm VLS bacterial inactivation results mainly from the production of highly cytotoxic ROS (21-23). Bacteria are more susceptible to this oxidative stress than human tissue due to fewer antioxidants and less efficient repair mechanisms (24). Therefore, 405 nm VLS is less detrimental to mammalian cells than UV irradiation (25). This increased bacterial susceptibility and low mammalian cell toxicity provides a wide therapeutic index that could be beneficial in a clinical setting.

While other research groups have demonstrated the bactericidal efficacy of 405 nm light on Gram-positive and Gram-negative bacteria, few studies have been conducted to demonstrate a comparison of clinically relevant pathogenic bacteria while independently assessing for time and exposure dependence (13, 14, 17, 26-30). We present here a comprehensive in vitro evaluation of the effectiveness of specific wavelength VLS treatment on S. aureus, S. pneumoniae, E. coli and P. aeruginosa by varying treatment time, irradiation level, bacterial concentrations and photokinetic dosing levels.

2. MATERIAls AND METHODS
The bacterial preparation, general experimental setup, digital plate analysis and statistical
analysis conducted on *S. aureus*, *S. pneumoniae* and *P. aeruginosa* has been described
previously (31). Some data previously gathered in that study concerning a K-12 strain of *E. coli*
(fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17)
was re-analyzed and is presented along with these three bacterium for comparative purposes.
Here, we summarize the methods with appropriate variations.

2.1. **Bacteria Preparation**

Three clinically relevant pathologic bacterium, specifically *S. aureus* (ATCC 29213), *S.
*pneumoniae* (ATCC 49619), and *P. aeruginosa* (ATCC 27853) were used in all experiments. As
previously described, the *E. coli* was transformed with the pCIG mammalian expression vector,
conferring ampicillin resistance via expression of the β-lactamase gene. Standard sterile
polystyrene Petri dishes (100 mm x 15 mm) were plated with Luria-Bertani (LB) broth (Lennox
L3022). LB selective media containing 0.1 mg/ml ampicillin was used in experiments on *E. coli*,
while regular LB was used for growth of the other three bacteria.

Dilution series were made from cultures inoculated with 3-4 well-separated bacterial
colonies and grown to saturation for consistency in counting colony-forming units (CFUs)
between experiments. Serial dilutions were prepared by taking 0.5 mL of the saturated
bacterial solution and adding it to 4.5 mL of the LB broth stock solution. Time and power trial
plates were diluted in this fashion by a factor of $10^5$ for each control and treated plates. Log$_{10}$
reduction plates were diluted by a factor of $10^3$ for control and between $10^1$ and $10^3$ for
treated. Each plate was seeded with 200 µL aliquots and the liquid was spread until uniformly
distributed. Optimal plating densities were determined in pilot experiments in order to yield 10
to 15 CFUs/cm$^2$.

2.2. Experimental Setup

The 405 nm LED (Bivar #: UV5TZ-405-15) wavelength was verified using a visible light spectrometer (Ocean Optics USB2000). The measured wavelength was centered at 403.24 nm ± 0.69 with a spectrum of 21.24 nm ± 4.52 at 50% relative peak intensity. Nine LEDs were mounted to a parallel circuit board and arranged in a 3 x 3 grid pattern. This setup was placed onto a tray customized for an incubator, with one LED focused on a single treatment plate. An opaque polyethylene sleeve was placed to isolate the control half of the plate from the treatment side, which was left uncovered. In addition, custom partitions were created to isolate individual plates and prevent light transference between treatment plates.

A digital power meter (PM100USB, Thorlabs, Newton, New Jersey, USA) and sensor (S140C, Thorlabs, Newton, New Jersey, USA) were used to determine LED output power. Diode intensity was measured for each LED before and after each treatment to determine irradiance and monitor for variability during treatment. Irradiance is defined as power per unit area, in this context having units of mw/cm$^2$. In addition, radiant exposure is defined as the intensity of light per unit area for a specific time, with units of mW·sec/cm$^2$ or J/cm$^2$. Varying light intensity and irradiation time led to several different radiant exposure levels tested.

Light sources were placed approximately 3.75 cm above the uncovered surface of the plate. This distance was chosen both for space constraints during incubation and to achieve the desired sterilization irradiance. Circular areas with a radius of 1.02 cm were marked on both the control and treated sides of the plate to provide a general light treatment target area. With the
LED beam centered and focused within these circles, total LED radiant flux was measured and averaged across the 1.02 cm radius circle.

### 2.3. Digital Plate Analysis

Standardized images were taken of each plate using a digital 18-megapixel camera (Canon T2i DLSR) mounted on a stand with the lens 61 cm from the plate surface after 24 hours of growth. The images were processed and analyzed using ImageJ (National Institute of Health). Untreated and treated (exposure to 405 nm light) areas within the same plate were measured for CFUs in order to control for potential plate-to-plate differences in bacterial plating densities. Bacterial growth analysis was limited to a 0.5 cm radius circle in order to minimize possible non-uniform exposures near the edge of the treatment area. This prevented possible concomitant confounding of the results. Previously acquired images of *E. coli* growth were re-analyzed based on these constraints for direct comparison.

For digital plate analysis, individual colonies were selected based on a size and circularity protocol. The colony size was determined by pixel count, with a range of 200-4000 pixel$^2$ based on the average isolated colony size. A circularity value range of 0.2 to 1.0 was selected, with 1.0 indicating a perfect circle and 0.2 indicating an elongated polygon. The resulting counted CFUs were then plotted as a survival fraction (based on a log$_{10}$ normalization) versus radiant exposure.

### 2.4. Statistical Analysis

Similar to previous descriptions, individual survival fractions are defined as the quantity of colony forming bacterial units normalized by taking logarithm of said value in treated and untreated control groups (31). Differences in individual survival fractions were assessed using a
Wilcoxon signed-rank test using JMP software (SAS, Cary, NC). Data was binned based on variable component in each experimental setup. For example, in time variable testing, data groups were binned by time, in logarithm reduction testing, groups were binned based on concentration and time. Differences in binned data were assessed using a paired t-test using Microsoft Excel 2013. A $P$-value of less than 0.001 was set as the \textit{a priori} level of significance and marked by a single asterisk (*).

3. \textbf{RESULTS}

3.1. \textbf{Variable Irradiance with Constant Exposure Time Analysis}

In order to determine the effect of altering LED output irradiance, analysis of the bactericidal effect of 405 nm light on both Gram-negative and Gram-positive bacteria was conducted with constant time (120 minutes) analyses. Irradiance was modulated by adjusting the current to a quartile percentage (25\%, 50\%, 75\% and 100\%) of the manufacturer’s suggested forward current (3.4 V, 30 mA). This produced approximately 9.43 mA ± 0.72, 16.05 mA ± 0.79, 26.15 mA ± 2.30 and 30.30 mA ± 1.25 across each diode.

Figure 1 summarizes the bactericidal effect of 405 nm light exposure with constant time and variable irradiance on \textit{S. aureus}, \textit{S. pneumoniae}, \textit{E. coli}, and \textit{P. aeruginosa}. As previously stated, physical experiments with \textit{E. coli} were conducted previously with the data here reanalyzed and published for comparison (31). A 120-minute treatment averaging 9.27 mW/cm$^2$ (64-70 J/cm$^2$) resulted in a mean reduction of 86.93\%, 93.89\%, 100.00\%, and 95.41\% in bacterial CFUs for \textit{S. aureus}, \textit{S. pneumoniae}, \textit{E. coli} and \textit{P. aeruginosa}, respectively. These results illustrate VLS dose dependent reduction of each bacterium. Table 1 lists the average
bacterial counts and percent reductions observed at each quartile treatment intensity (25%, 50%, 75% and 100%). A one-way ANOVA and Wilcoxon signed-rank test found statistically significant differences ($P<0.001$) between bacterial CFU counts, indicating that variable irradiance results in bactericidal effects for all strains.

3.2. **Variable Exposure Time with Constant Irradiance Analysis**

In order to compare the effect of varying exposure time while holding irradiance constant to the results of the previous section, analysis of the bactericidal effect of 405 nm light on the four bacteria of interest was then determined. Manufacturers recommended voltage and current (3.4 V, 30 mA) were used in this analysis. Treatment times were approximately 10 minute, 50 minute, 120 minute and 250 minute for all bacteria. The average irradiation during these protocols was $9.01 \pm 0.51 \text{ mW/cm}^2$.

Figure 2 illustrates the results of these time dependent trials. In all cases, a 250-minute treatment at an average of $9.01 \text{ mW/cm}^2$ (127.81-136.91 J/cm$^2$) resulted in a 100.00% reduction of bacteria. No growth was observed within the treatment area of these plates. Table 2 lists the radiant exposures, average bacterial counts and percent reductions observed at treatment duration (10 minute, 50 minute, 120 minute and 250 minute). Statistically significant results ($P<0.001$) were found at both the 120 and the 250-minute exposure times for all strains, indicating that bactericidal effects are time dependent.

3.3. **Log$_{10}$ Reduction Analysis**

Log$_{10}$ reduction analysis was performed in order to assess the bacterial reduction potential of 405 nm light *in vitro*. Using the ASTM E2315-03 protocol (Standard Guide for Assessment of
Antimicrobial Activity), S. aureus, S. pneumoniae, E. coli and P. aeruginosa were plated in high confluent concentrations and irradiated with increasing exposure durations (60, 120 and 250 minutes). The results of these tests are illustrated in Figure 3. A 250-minute treatment resulted in ≥ 5.5 log$_{10}$ reduction for all four bacteria ($P<0.001$). The radiant exposures, CFU counts and mean log$_{10}$ reductions are summarized in Table 3. Statistically significant results ($P<0.001$) were found at all exposure times, indicating that variable radiant exposure results in proportional bactericidal effects for all strains.

3.4. Equivalent Radiant Exposure Analysis

The potential difference between high irradiance with short duration (HI-SD) times and low irradiance long duration (LI-LD) times treatments of equivalent radiant exposures (J/cm$^2$) was also investigated. Irradiance was modulated by adjusting the radiant flux percentage (25% or 100%) of the manufacturer’s suggested forward current (3.4 V, 30 mA) and duration was modified (250 minutes or 1000 minutes) to produce an equivalent radiant exposure for HI-SD and LI-LD groups. HI-SD groups were run at 100% power and 250 minutes. Radiant exposure of HI-SD groups averaged 135.32 J/cm$^2$ ± 6.84, and LI-LD groups averaged 130.54 J/cm$^2$ ± 7.39. Figure 4 shows a typical set of HI-SD and LI-LD plates after treatment.

4. Discussion

This study has demonstrated that 405 nm VLS exhibits a significant bactericidal effect on four clinically relevant bacteria (two Gram-positive and two Gram-negative), namely S. aureus, S. pneumoniae, E. coli and P. aeruginosa. This study set out with four aims: (1) to compare the relative susceptibility of a clinically significant Gram-positive and Gram-negative microbes to
405 nm VLS, (2) to examine the effect of bacterial antibiotic resistance on VLS efficacy, (3) to assess the potential log$_{10}$ reduction capability of 405 nm VLS, (4) to determine the potential difference in efficacy between a brief high-intensity and a long low-intensity treatment of equivalent irradiant dose and (5) to propose VLS as an alternate method to combat clinically relevant bacteria.

4.1. **Gram-positive vs Gram-negative Bactericidal Effect of 405 nm VLS**

In order to assess the potential of 405 nm VLS as a treatment for medically significant microbes, it is necessary to examine and compare the relative susceptibility of Gram-positive and Gram-negative organisms. The efficacy of the broad-spectrum action of violet light against both types of bacterial species has been well-documented (17, 26, 29, 30, 32, 33). In one study, multiple Gram-positive bacteria were shown to exhibit 2-3 times the 405 nm VLS sensitivity compared to multiple Gram-negative bacteria (27). However, both groups of bacteria were susceptible to the VLS. A 2.6-5.0 log$_{10}$ reduction in Gram-positive species and 3.1-4.7 log$_{10}$ reduction in Gram-negative species was observed (27). In other studies, Gram-negative bacteria have been shown to exhibit similar, if not slight more, sensitivity to 405 nm VLS compared to Gram-positive species (17, 32).

In this study, we observed similar sensitivity to 405 nm VLS in Gram-negative bacteria compared with Gram-positive bacteria. The most VLS sensitive bacteria in this study was ampicillin-resistant *E. coli* and, followed by, *S. pneumoniae, S. aureus* and finally *P. aeruginosa* with log$_{10}$ reductions of $6.27 \pm 0.54$, $6.10 \pm 0.60$, $6.01 \pm 0.59$, and $5.20 \pm 0.84$ respectively. The discrepancies between sensitivities found in this and other studies may be due to staining family or species variability. It has been previously described that the difference in the
photoinactivation rate was due to the distribution and amounts of the various porphyrins, including coproporphyrin, in Gram-positive versus Gram-negative bacterial strains (34). Alternatively, the variation in sensitivity could be strain specific. Even within the same Gram staining family, studies have illustrated significant species dependent variable sensitivity to VLS (27, 30). Regardless, percent reduction differences between the species test analyzed were not statistically different.

4.2. Susceptibility of Antibiotic Resistant Bacteria to VLS

VLS offers an alternate bactericidal approach that avoids known mechanisms of antibiotic resistance. Common methods of antibiotic resistance include chemical or enzymatic drug modification, intracellular drug concentration reduction via transmembrane pumps, and drug target modification (35). In contrast, VLS bacterial toxicity is due to a non-specific and generalized killing mechanism. In particular, the accepted hypothesis involves photon absorption, photoexcitation of endogenous intracellular porphyrins and ultimately the production of highly cytotoxic reactive oxygen species (ROS) (21-23). Cytotoxic singlet oxygen is the most notable ROS produced (22). This intracellular accumulation of ROS causes generalized widespread damage to vital cellular structures (36). Compared to more targeted methods of action employed by most antibiotics, the overwhelming generation of ROS and associated pervasive damage reduces the ability of bacteria to develop resistance (35-37). In addition, bacteria are more susceptible to oxidative stress than human tissue due to fewer antioxidants and less efficient repair mechanisms (24).

The observed results are clinically relevant in reaffirming the susceptibility of antibiotic resistant bacterial strains to 405 nm VLS. Other studies have also shown 405 nm VLS efficacy to
antibiotic-resistant species, including methicillin-resistant *S. aureus* (MRSA) and multi-
antibiotic-resistant *H. pylori* (13, 27, 29). Here we extend these observations to an *E. coli* strain expressing β-lactamase, which confers resistance to penicillin-class antibiotics (38).

The mechanism of action of 405 nm VLS is relevant when assessing the potential for microbial resistance. Although resistance to 405 nm light has not been observed or specifically assessed, investigations into microbial resistance to photodynamic therapy have been conducted. These studies indicate that bacterial species failed to develop resistance to the photodynamic process after 10 and 20 partially therapeutic cycles (36, 39). It is possible that a lack of resistance may be observed due to the ROS mediated damage on various vital cellular structures, compared to the specific targets of antibiotics. Further studies are necessary to determine the extent to which bacteria are able to develop resistance. However, the significant toxicity demonstrated against antibiotic-resistant bacterium may illustrate the potential of VLS as an alternative therapy for certain antibiotic-resistant infections.

4.3. Log$^{10}$ Reduction

This study has established the potential log$^{10}$ reduction of 405 nm light on the clinically relevant *S. aureus*, *S. pneumoniae*, *E. coli* and *P. aeruginosa*. The FDA requires that a minimum of a four-log$^{10}$ reduction be achieved in order to be considered an antibacterial solution (40). The FDA recommends following ASTM E2315-03 Standard Guide for Assessment of Antimicrobial Activity Using a Time-Kill Procedure or an equivalent method during testing for approval.

Using the ASTM protocol, we have demonstrated a 6.10, 6.01, 6.27 and 5.20 log$^{10}$ reduction in *S. aureus*, *S. pneumoniae*, *E. coli* and *P. aeruginosa*, respectively. All of the
treatments were found to exhibit ≥ 15 times the log_{10} reduction required for a bactericidal claim from the FDA. *P. aeruginosa*, at a 5.20 log_{10} reduction, is still over 10 times the reduction necessary. The results from this study also illustrate the time and dose dependence of 405 nm VLS, suggesting that this technology could be a suitable clinical option for bacterial sterilization. This bacterial reduction information, along with those previously published, may help direct future applications of VLS in a clinical setting (31).

4.4. Equivalent Radiant Exposure Dose

This study also examined the potential difference in efficacy between high irradiance with short duration (HI-SD) times and low irradiance long duration (LI-LD) times of equivalent radiant exposure dose, which is of clinical significant for treatment. Significant differences have been observed in the bactericidal effect on *Listeria monocytogenes* at equivalent radiant exposure levels while adjusting irradiance and exposure time (30). We performed similar preliminary testing on *S. aureus*, *S. pneumoniae*, *E. coli* and *P. aeruginosa*.

Additionally, we compared equivalent radiant exposures occurring between the time dependent and irradiance dependent trials to assess for potential variations. In all four bacteria, similar log_{10} reductions inside the treated area were observed for each equivalent radiant exposure available. Figure 4 shows petri dishes with a treated area inside of the circle. This circle section showed with no growth in either the HI-SD or LI-LD plate. However, they differ in the small perimeter area outside of the treated circle. Due to light scattering outside the treatment area we see differences in bacterial growth between the two groups. Qualitatively we observed that HI-SD treatments have isolated colonies outside the treatment area while LI-LD treatments formed a clear confluent boarder. Isolated colonies on the perimeter of the HI-
SD treatments suggests migration of bacteria toward nutrient rich media after light treatments were finished. These results suggest that longer duration treatments even with lower irradiance may provide better overall bactericidal and bacteriostatic effect due to a continuous VLS.

5. CONCLUSION
The antibacterial potential of 405 nm light on clinically significant bacteria has been established. This study specifically examined the efficacy and dosing regimens of using 405 nm light to inactivate both Gram-positive and Gram-negative bacterium including one antibiotic-resistant strain. The increasing incidence of these HAIs illustrate that current antimicrobial technologies are inadequate. By showing high efficacy against the top bacterial causes of the four most common HAIs, this study illustrates the potential for clinical use of 405 nm VLS. The results provide the ideal dosing parameters for bacterial inactivation. Future studies would need to explore these dosing regimens on various mammalian cells to better understand the safety of 405 nm VLS and the clinical translatability of this technology.

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7. DISCLOSURE STATEMENT
This research was paid in part from a grant through the National Science Foundation Innovation Corps proposal number 1347345. This team grant was provided partially based on technology commercialization potential. One (or more) of the authors recognizes their equity interest and patent applications/registrations related to the commercialization of this research.

8. REFERENCES


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9. FIGURE CAPTIONS

9.1. Figure 1. Variable Irradiance with Constant Treatment Time

Survival fraction of each bacterium in treated areas with averaged control is shown. Trendlines
are added to show the general relationship between control and treated plates as a function of radiant exposure. Treatment times were held at 120 minutes and irradiance was varied between 2.38 mW/cm$^2$ ± 0.08 and 9.71 mW/cm$^2$ ± 0.08. Variable irradiance groups (25%, 50%, 75%, and 100%) were binned and tested for statistical significance in treated vs. species specific control. Statistical significance of $P<0.001$ is noted with an asterisk (*).

9.2. **Figure 2. Variable Exposure Time with Constant Irradiance Analysis**

Survival fraction of each bacterium with averaged control is shown. Light was delivered at a constant irradiance (9.2 mW/cm$^2$ ± 0.51) with varying durations of time: 10 minutes, 50 minutes, 120 minutes and 250 minutes and radiant exposure levels from 5.48 J/cm$^2$ ± 0.26 to 132.98 J/cm$^2$ ± 4.03. Trendlines are added to show the general relationship between control and treated plates as a function of radiant exposure. Statistical significance of $P<0.001$ is noted with an asterisk (*).

9.3. **Figure 3. Log$_{10}$ Reduction at Variable Radiant Exposure**

Inactivation of confluent concentrations of each bacterial species upon exposure to 405 nm light as a function time duration. Light was delivered at a constant irradiance (8.38 mW/cm$^2$ ± 0.55) with varying durations of time: 60 minutes, 120 minutes and 250 minutes. Statistical significance of $P<0.001$ is noted with an asterisk (*).

9.4. **Figure 4. Equivalent Radiant Exposure Dose**

Representative Petri dishes from equivalent radiant exposure testing showing a high irradiance-short duration (HI-SD, Left) and low irradiance-long duration (LI-LD, Right). Radiant exposure of
HI-SD groups averaged $135.32 \pm 6.84$ J/cm$^2$, and LI-LD groups averaged $130.54 \pm 7.39$. 

10. **Tables**

10.1. **Table 1: Variable Irradiance with Constant Treatment Time**

Table 1 Caption: Variable irradiance with constant treatment time analysis of *S. aureus*, *S. pneumoniae*, *E. coli*, and *P. aeruginosa* CFU reduction following exposure to 405 nm light. Light treated colony forming unit (CFU) counts and control CFU are also shown with a mean reduction. Statistical significance of $P<0.001$ is noted with an asterisk (*).

10.2. **Table 2: Variable Treatment Time with Constant Irradiance**

Table 2 Caption: Variable treatment time with constant irradiance analysis of *S. aureus*, *S. pneumoniae*, *E. coli*, and *P. aeruginosa* CFU reduction following exposure to 405 nm light. Light treated colony forming unit (CFU) counts and control CFU are also shown with a mean reduction. Statistical significance of $P<0.001$ is noted with an asterisk (*).

10.3. **Table 3: Log$_{10}$ Reduction**

Table 3 Caption: Mean log$_{10}$ reduction analysis of *S. aureus*, *S. pneumoniae*, *E. coli*, and *P. aeruginosa* CFU reduction following exposure to 405 nm light. Light treated colony forming unit (CFU) counts and control CFU are also shown with a mean reduction. Statistical significance of $P<0.001$ is noted with an asterisk (*).
Table 1 Variable Irradiance with Constant Treatment Time

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Irradiance (mW/cm²)</th>
<th>Time (minutes)</th>
<th>Radiant Exposure (J/cm²)</th>
<th>Treated (CFU)</th>
<th>Control (CFU)</th>
<th>Mean % Reduction</th>
<th>P-values</th>
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<tbody>
<tr>
<td><strong>S. aureus</strong></td>
<td>2.81 ± 0.35</td>
<td>120</td>
<td>20.23 ± 2.49</td>
<td>22.67 ± 5.81</td>
<td>89.52 ± 27.01</td>
<td>73.28%</td>
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<td>16.22 ± 18.12</td>
<td>52.19 ± 16.93</td>
<td>73.10%</td>
<td>&lt;0.001*</td>
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<td></td>
<td>6.94 ± 0.29</td>
<td>120</td>
<td>49.96 ± 2.10</td>
<td>6.56 ± 3.47</td>
<td>75.61 ± 34.02</td>
<td>91.02%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>9.13 ± 0.31</td>
<td>120</td>
<td>65.74 ± 2.27</td>
<td>7.44 ± 8.62</td>
<td>54.81 ± 18.93</td>
<td>86.93%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td><strong>S. pneumoniae</strong></td>
<td>2.38 ± 0.08</td>
<td>120</td>
<td>17.13 ± 0.55</td>
<td>32.22 ± 19.80</td>
<td>61.30 ± 21.65</td>
<td>48.60%</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>5.21 ± 0.28</td>
<td>120</td>
<td>37.50 ± 2.04</td>
<td>9.44 ± 9.67</td>
<td>62.11 ± 25.23</td>
<td>87.25%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>6.86 ± 0.43</td>
<td>120</td>
<td>49.38 ± 3.07</td>
<td>6.50 ± 5.66</td>
<td>57.46 ± 20.55</td>
<td>87.18%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>9.71 ± 0.44</td>
<td>120</td>
<td>69.93 ± 3.18</td>
<td>3.44 ± 3.05</td>
<td>63.56 ± 23.40</td>
<td>93.89%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>2.89 ± 0.19</td>
<td>120</td>
<td>20.79 ± 1.39</td>
<td>3.56 ± 3.00</td>
<td>19.67 ± 3.78</td>
<td>82.32%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>4.91 ± 0.24</td>
<td>120</td>
<td>35.37 ± 1.72</td>
<td>2.11 ± 1.83</td>
<td>19.96 ± 3.80</td>
<td>89.74%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>8.07 ± 0.66</td>
<td>120</td>
<td>58.09 ± 4.78</td>
<td>0.44 ± 0.73</td>
<td>22.85 ± 3.69</td>
<td>97.27%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>9.37 ± 0.31</td>
<td>120</td>
<td>67.49 ± 2.21</td>
<td>0.00 ± 0.00</td>
<td>25.71 ± 8.19</td>
<td>100.00%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td>2.89 ± 0.19</td>
<td>120</td>
<td>20.79 ± 1.39</td>
<td>19.38 ± 10.06</td>
<td>84.75 ± 24.31</td>
<td>77.41%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>4.35 ± 0.17</td>
<td>120</td>
<td>31.33 ± 1.26</td>
<td>8.33 ± 5.57</td>
<td>39.93 ± 20.69</td>
<td>76.97%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>8.07 ± 0.66</td>
<td>120</td>
<td>58.09 ± 4.78</td>
<td>5.14 ± 1.95</td>
<td>70.01 ± 21.37</td>
<td>92.10%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>8.85 ± 0.39</td>
<td>120</td>
<td>63.69 ± 2.83</td>
<td>1.44 ± 1.01</td>
<td>37.26 ± 20.86</td>
<td>95.41%</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>
Table 2 Variable Treatment Time with Constant Irradiance

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Irradiance (mW/cm²)</th>
<th>Time (minutes)</th>
<th>Radiant Exposure (J/cm²)</th>
<th>Treated (CFU)</th>
<th>Control (CFU)</th>
<th>Mean % Reduction</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>9.55 ± 0.45</td>
<td>10</td>
<td>5.73 ± 0.27</td>
<td>54.89 ± 21.99</td>
<td>72.09 ± 21.34</td>
<td>23.50%</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>9.61 ± 0.43</td>
<td>50</td>
<td>28.83 ± 1.28</td>
<td>35.44 ± 11.74</td>
<td>63.76 ± 11.74</td>
<td>40.57%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>7.62 ± 2.35</td>
<td>120</td>
<td>54.85 ± 16.90</td>
<td>8.67 ± 8.66</td>
<td>52.40 ± 21.07</td>
<td>83.37%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>9.02 ± 0.38</td>
<td>250</td>
<td>135.30 ± 5.70</td>
<td>0.00 ± 0.00</td>
<td>48.87 ± 6.81</td>
<td>100.00%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td>8.55 ± 0.60</td>
<td>10</td>
<td>5.13 ± 0.36</td>
<td>48.33 ± 29.33</td>
<td>58.46 ± 29.47</td>
<td>18.77%</td>
<td>0.307</td>
</tr>
<tr>
<td></td>
<td>8.60 ± 0.66</td>
<td>50</td>
<td>25.81 ± 1.97</td>
<td>34.41 ± 17.11</td>
<td>80.25 ± 24.19</td>
<td>55.72%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>9.71 ± 0.44</td>
<td>120</td>
<td>69.93 ± 3.18</td>
<td>3.44 ± 3.05</td>
<td>63.56 ± 23.40</td>
<td>93.89%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>8.79 ± 0.67</td>
<td>250</td>
<td>131.91 ± 10.00</td>
<td>0.00 ± 0.00</td>
<td>85.98 ± 26.18</td>
<td>100.00%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>E. coli</td>
<td>9.32 ± 0.51</td>
<td>10</td>
<td>5.59 ± 0.31</td>
<td>18.94 ± 7.71</td>
<td>28.90 ± 15.27</td>
<td>28.59%</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>9.22 ± 0.55</td>
<td>50</td>
<td>27.67 ± 1.66</td>
<td>4.11 ± 4.02</td>
<td>31.00 ± 14.27</td>
<td>83.55%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>9.37 ± 0.03</td>
<td>120</td>
<td>67.49 ± 2.21</td>
<td>0.00 ± 0.00</td>
<td>25.71 ± 8.19</td>
<td>100.00%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>9.13 ± 0.27</td>
<td>250</td>
<td>136.91 ± 4.06</td>
<td>0.00 ± 0.00</td>
<td>40.63 ± 24.50</td>
<td>100.00%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>9.15 ± 0.40</td>
<td>10</td>
<td>5.49 ± 0.24</td>
<td>80.33 ± 24.26</td>
<td>74.65 ± 23.11</td>
<td>-10.23%</td>
<td>0.477</td>
</tr>
<tr>
<td></td>
<td>9.11 ± 0.45</td>
<td>50</td>
<td>27.33 ± 1.36</td>
<td>53.19 ± 28.02</td>
<td>68.08 ± 28.02</td>
<td>23.43%</td>
<td>0.143</td>
</tr>
<tr>
<td></td>
<td>8.85 ± 0.42</td>
<td>120</td>
<td>63.75 ± 3.02</td>
<td>1.38 ± 1.06</td>
<td>39.58 ± 21.02</td>
<td>96.17%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>8.52 ± 0.47</td>
<td>250</td>
<td>127.81 ± 6.99</td>
<td>0.00 ± 0.00</td>
<td>27.23 ± 6.84</td>
<td>100.00%</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>
Table 3 Log$_{10}$ Reduction

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Irradiance (mW/cm$^2$)</th>
<th>Time (minutes)</th>
<th>Radiant Exposure (J/cm$^2$)</th>
<th>Treated (CFU)</th>
<th>Control (CFU)</th>
<th>Mean Log Reduction</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>8.74 ± 0.45</td>
<td>60</td>
<td>31.45 ± 1.62</td>
<td>158.33 ± 91.00</td>
<td>115.40 ± 29.60</td>
<td>1.80</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>8.60 ± 0.63</td>
<td>120</td>
<td>61.95 ± 4.55</td>
<td>58.83 ± 49.60</td>
<td>96.19 ± 25.10</td>
<td>3.34</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>8.32 ± 0.73</td>
<td>250</td>
<td>124.81 ± 10.88</td>
<td>0.67 ± 1.15</td>
<td>36.55 ± 2.64</td>
<td>6.10</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td>8.38 ± 0.52</td>
<td>60</td>
<td>30.16 ± 1.88</td>
<td>27.50 ± 40.21</td>
<td>51.76 ± 5.83</td>
<td>1.80</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>8.18 ± 0.54</td>
<td>120</td>
<td>58.92 ± 3.87</td>
<td>5.44 ± 7.29</td>
<td>44.50 ± 4.52</td>
<td>4.13</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>8.05 ± 0.49</td>
<td>250</td>
<td>123.41 ± 7.31</td>
<td>4.21 ± 1.04</td>
<td>53.60 ± 15.84</td>
<td>6.13</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>E. coli</td>
<td>9.45 ± 0.63</td>
<td>60</td>
<td>34.03 ± 2.28</td>
<td>105.67 ± 71.11</td>
<td>70.60 ± 7.75</td>
<td>1.98</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>9.02 ± 0.34</td>
<td>120</td>
<td>64.95 ± 2.45</td>
<td>24.57 ± 21.51</td>
<td>70.82 ± 8.34</td>
<td>3.60</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>8.71 ± 0.96</td>
<td>250</td>
<td>130.60 ± 14.37</td>
<td>0.41 ± 0.61</td>
<td>51.14 ± 14.40</td>
<td>6.27</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>7.66 ± 0.28</td>
<td>60</td>
<td>27.58 ± 1.01</td>
<td>159.39 ± 119.44</td>
<td>37.40 ± 1.25</td>
<td>1.50</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>7.60 ± 0.45</td>
<td>120</td>
<td>54.72 ± 3.21</td>
<td>29.06 ± 16.08</td>
<td>24.69 ± 5.64</td>
<td>3.00</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>7.84 ± 0.33</td>
<td>250</td>
<td>117.55 ± 4.96</td>
<td>8.06 ± 13.33</td>
<td>36.19 ± 7.84</td>
<td>5.20</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>
Figure 1

![Graph showing survival fraction Log10(CFU) vs. radiant exposure of 405 nm light (J/cm²). The graph compares control (dashed line) and treated samples (dotted lines) for different bacterial species: P. Aeruginosa, S. Pneumoniae, S. Aureus, and E. coli. The lines are marked with asterisks to indicate statistical significance.]
Figure 2

![Graph showing survival fraction vs. radiant exposure of 405 nm light (J/cm²)]

- **Control (Averaged)**
- **P. Aeruginosa Treated**
- **S. Pneumoniae Treated**
- **S. Aureus Treated**
- **P. Cali Treated**
Figure 3

![Graph showing survival fraction (log$_{10}$CFU) versus radiant exposure of 405 nm light (J/cm$^2$) for different conditions.]

- Control (Averaged)
- P. Aeruginosa Treated
- S. Pneumoniae Treated
- S. Aureus Treated
- L. C. Treated

The graph plots the survival fraction (log$_{10}$CFU) on the y-axis against the radiant exposure of 405 nm light (J/cm$^2$) on the x-axis. Different conditions are represented by various lines and markers.
**Figure 4. Equivalent Radiant Exposure Dose:** Sample petri dishes from equivalent radiant exposure testing showing a high irradiance-short duration (HI-SD, Left) and low irradiance-long duration (LI-LD, Right). Radiant exposure of HI-SD groups averaged $135.32 \pm 6.84$ J/cm$^2$, and LI-LD groups averaged $130.54 \pm 7.39$ J/cm$^2$. 