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Inside-Out Ultraviolet-C Sterilization of *Pseudomonas aeruginosa* Biofilm *In Vitro*

Cameron C Jones^{1,*}, Steffi Valdeig¹, Raymond M Sova², and Clifford R Weiss¹

¹The Russell H. Morgan Department of Radiology and Radiological Science, Johns Hopkins University School of Medicine, Baltimore, MD

²Photonics Division, Johns Hopkins University Applied Physics Lab, Laurel, MD

Abstract

Biofilms are difficult to eradicate due to a protective architecture and create major challenges in patient care by diminishing both host immune response and therapeutic approaches. This study investigated a new strategy for treating surface-attached biofilms by delivering germicidal UV through a material surface in a process referred to as “inside-out sterilization” (IOS). Mature *Pseudomonas aeruginosa* (ATCC® 27853™) biofilms were irradiated with up to 1400 mJ cm⁻² of germicidal UV from both ambient and IOS configurations. The lethal dose for the ambient exposure group was 461 mJ cm⁻² 95% CI [292, 728] compared to the IOS treatment group of 247 mJ cm⁻² 95% CI [187, 325], corresponding to 47% less UV dosage for the IOS group ($p < 0.05$). This study demonstrated that with IOS, a lower quantal dosage of UV energy is required to eradicate biofilm than with ambient exposure by leveraging the organizational structure of the biofilm.

Graphical Abstract

*Corresponding author: cjone169@alumni.jh.edu (Cameron Jones).

INTRODUCTION

Bacterial biofilms

Biofilms are complex structures derived from surface-adherent microorganisms that protect internal cellular constituents from external insults, and are prevalent in medical, industrial, and environmental settings. Mature biofilms utilize several defensive mechanisms such as restricted penetration, altered microenvironments, and upregulation of stress-response genes, which can increase antimicrobial resistance a thousand-fold over biocidal concentrations effective against planktonic (free-floating) bacteria (1–3). This resistance is distinct from adaptive resistance (i.e., conventional antimicrobial resistance that develops due to genetic alterations), and unique to biofilm-encapsulated bacteria, as the biofilm phenotype imparts a protective advantage (4).

The initial formation of the biofilm occurs in two general stages: the first phase consists of a reversible process of bacterial adhesion accomplished predominantly through non-covalent bonding and hydrophobic interactions with a conditioned surface; and a secondary locking phase between specific adhesins and the surface, which is solidified by the production of the glycocalyx (2, 5–7). After the irreversible adhesion of surface-attached microorganisms, molecules in the immediate environment begin to aggregate onto the growing gelatinous exopolysaccharide matrix, which envelopes and anchors the surface-bound bacteria. For many biofilms, non-cellular components (water channels and extracellular polymeric substances (EPS)) exceed cellular constituents by a factor of three, with the latter being more consolidated near the attached surface (8). A sophisticated transport system for nutrient delivery and waste removal allows the biofilm to continue to grow in size without resulting in cell death to outer layers. When the biofilm reaches equilibrium between nutritional supply and size, signaling molecules cue the outer EPS matrix to begin budding planktonic organisms to colonize new surfaces (2).

Biofilms create major challenges in patient care by limiting both the host immune response and targeted drug therapy, and may also lead to diminished antimicrobial efficacy long-term through genetic biocide resistance from chelated antimicrobial agents trapped within the matrix (1, 9, 10). Due to their protective architecture, biofilms are especially difficult to completely eradicate, which often leads to chronic and systemic infections. According to the National Institutes of Health, biofilms are estimated to account for over 80% of all nosocomial infections, and are particularly common with device implants, such as: contact lenses, ventricular assist devices, vascular and urinary catheters, and endotracheal tubes (10–12). One of the most common bacterial pathogens predominant in both community-acquired and hospital-acquired infections is the Gram-negative *Pseudomonas aeruginosa* (13–15). *P. aeruginosa* forms a highly virulent biofilm that has been associated with higher mortality rates compared with other bacterial pathogens, and there are growing concerns over its increased antimicrobial and multidrug resistance (16, 17, 15).

Current pharmacologic and therapeutic efforts targeting bacterial biofilms and/or their microenvironment include disabling the phenotype of persister cells, degrading the biofilm matrix, and disrupting quorum sensing mechanisms, as well as a number of physical approaches in concomitant use with antibiotics, which are well-reviewed elsewhere (1).

While there are many encouraging approaches to combatting bacterial biofilms in the healthcare setting, most require penetrating the biofilm matrix, and some may adversely contribute to adaptive resistance. This paper investigated a new strategy for destroying *P. aeruginosa*, a clinically relevant biofilm producing bacteria. It is proposed that the following strategy is less subjective to the protective mechanisms of the EPS matrix and may provide a unique advantage in certain applications either independently or in combination with existing therapeutic approaches.

Inside-out UV sterilization

The use of ultraviolet (UV) radiation (100–400 nm), particularly in the UVC range (200–280 nm), for germicidal applications has been well-known for decades, and its use in clinical settings for over 75 years (18). Throughout this manuscript, ‘UV’ and ‘UVC’ are used interchangeably in the context of germicidal activity, and unless otherwise noted, refer to wavelengths in the 200–280 nm range.

Absorption of UVC by cell nuclei causes a photochemical change in DNA nucleotides by creating pyrimidine dimers and other photoproducts that lead to failure in replication and protein synthesis (19). Microorganisms rely on various strategies of photo-protection including UV-absorptive biomolecules, repair mechanisms, and for some, the mobility to move towards lower UV exposure (20). The assembly of bacterial biofilms affords additional intrinsic protection against UV radiation by limiting UV transmission and may undergo sacrificial cell death of outer cell layers to protect internal constituents (21, 20).

While biofilms may be highly heterogeneous in both microbial content and matrix composition, multi-layered, surface-attached biofilms might be conceptualized in a dome-like architecture, with the protective EPS matrix shielding internal cellular constituents (Fig. 1) (8, 22). In natural settings, the defensive mechanisms of bacterial biofilms are generally directed at providing protection from mostly external insults, such as exposure to environmental UV. However, rarely would biocidal threats stem from a conditioned surface, and therefore the defensive mechanisms of the biofilm architecture may not be as developed to mitigate surface-derived challenges. This paper presents evidence that biofilm sequestered bacteria are more susceptible to germicidal UV when exuded from a surface than when challenged with ambient UV exposure, using a novel method of inside-out sterilization (IOS).

MATERIALS AND METHODS

Bioreactor

A custom bioreactor was assembled to allow germicidal UV to irradiate bacterial biofilms from either the conventional outside-in (OUT) or IOS orientation (Fig. 2). The UV source was a narrowband 254 nm mercury lamp (XX-15S bench lamp, UVP, Upland, CA) and was rotated from a mounted position above the biofilm samples to below for the OUT and IOS configurations, respectively. A reversible sub-assembly consisted of a 35 mL UV-transparent fused quartz culture dish (Quartz Scientific, Inc., Fairport Harbor, OH) and 1500 grit light diffuser (DGUV10–1500, Thorlabs, Inc., Newton, NJ), positioned between the UV source

and culture dish, creating a uniform illumination of the dish surface. The reversible sub-assembly was placed on rail-mounted shelves with adjustable height to ensure the culture dish was equidistant from the UV source in either configuration.

UV energy imparted to the biofilm samples was quantified by a benchtop lightmeter (ILT1700, International Light Technologies, Peabody, MA) and photodetector (SED240, International Light Technologies) calibrated at a peak responsivity wavelength of 254 nm. The diffusion profile from the two parallel mercury lamps permitted tandem placement of two reversible sub-assemblies approximately 15 cm from the UV bulbs along the longitudinal center axis of the ballast. The baseline uniform irradiance on the surface of the dish was measured to be 0.63 mW cm^{-2} , with approximately 40% attenuation through 3 mL of 90% v/v heparinized saline and broth media (Fig. 3). Attenuation through the quartz dish was 6.2%.

Biofilm preparation

P. aeruginosa (ATCC® 27853™), a clinical isolate, was used in this study. An overnight suspension ($1.0 \times 10^6 \text{ CFU mL}^{-1}$) was prepared by inoculating a single colony in 5 mL BBL™ Trypticase™ Soy Broth (TSB; Becton, Dickinson and Company (BD), Sparks, MD) at 37 °C. Two hundred microliters of the overnight suspension was seeded in the center of the quartz dishes and incubated for 18 h at 37 °C to allow initial bacterial adhesion to the dish surface. Following this attachment phase, 100 µL of the overnight suspension was added to the initial seed and incubated for another 24 h at 37 °C. Three milliliters of sterile heparinized saline (10 USP units mL^{-1} , BD, Franklin Lakes, NJ) was added to the biofilm culture and incubated for a final 24 h at 37 °C. Total biofilm preparation took 4 days.

Planktonic studies were also used for benchmark validation of the bioreactor. Samples were prepared from 100 µL of the overnight suspension and added to 5 mL of sterile saline.

Study design (a priori data)

A small pilot study ($n = 64$) was initially conducted to validate the biofilm model and identify the range of UVC dosage necessary to achieve sterilization of the two treatment groups. Regression curves were fit to the data and showed an exponential trend with most of the germicidal action occurring within the first 100 mJ cm^{-2} , with complete sterilization of the IOS and OUT samples approximated at 600 mJ cm^{-2} and 1000 mJ cm^{-2} , respectively (data not shown). Emphasis in the present study was therefore placed on collecting a higher proportion of data points during the earlier phases of UV radiation ($0\text{--}170 \text{ mJ cm}^{-2}$) and extending the study beyond the anticipated maximum sterilization dosage required (1400 mJ cm^{-2}).

The sample size for the present study was also skewed-right, mirroring the distribution of discrete UV radiation timepoints and accounting for sample variance over the initial slope of the survival curve. During the preliminary study, a number of the irradiated samples (9%) were identified as outliers, and therefore required sample sizes for each of the irradiation timepoints were increased by at least 10% in order to account for potential outliers. The number of samples in each timepoint ranged from 3 to 15, with the greater number of samples collected at lower UVC doses.

UV treatment and viable counts

P. aeruginosa biofilms were irradiated with UVC using the custom bioreactor at room temperature. Each biofilm sample was pre-assigned to a treatment group and discrete germicidal duration ranging from 2.5 min up to 60 min (corresponding to approximately 60 mJ cm⁻² up to 1400 mJ cm⁻², accounting for media attenuation). The biofilm treatment groups were divided by the orientation of the UV source relative to the surface-attached biofilm, with the two configurations being OUT (n = 70) and IOS (n = 72); there were 8 controls that received no UVC exposure.

The UVC exposure for the planktonic group ranged 3–20 s (0.9–6.0 mJ cm⁻²), with 10 test samples and 3 controls.

Samples were transferred to Falcon® tubes by thoroughly pipetting all contents of the culture dish. The harvested samples were vortexed for 10–15 s to disperse any biofilm and evenly mix the suspension. Colony counts were obtained following standard serial dilution techniques.

Regression model

The data was checked for outliers by calculating the DFFITS value for each sample prior to performing any regression analysis. The DFFITS parameter is a measure of the degree of influence a specific data point has on the predicted value when the point in question is removed from the sample, and is calculated as (23):

$$\text{DFFITS} = \frac{\hat{y}_i - \hat{y}_{i(i)}}{s_{(i)} - \sqrt{h_{ii}}}, \quad (1)$$

where $\hat{y}_i$ and $\hat{y}_{i(i)}$ are the predictions for point i with and without point i included in the regression fit, $s_{(i)}$ is the standard error estimated without point i , and h_{ii} is the leverage for the particular point. In the case of a Gaussian distribution, the leverage for each point is p/n , which p is the number of parameters of the regression model divided by the number of points n , and where outliers are considered for values greater than

$$\pm 2 \sqrt{\frac{p}{n}}. \quad (2)$$

The DFFITS value was calculated for each time point and normalized by Eq. 2, where values greater than ± 1 were removed from the regression model.

A quantal dose-response curve was obtained by a least-squares fit of the data to a four parameter logistic equation of the form

$$y = \min + \frac{\max - \min}{1 + \left(\frac{x}{LD_{50}}\right)^{-B}}, \quad (3)$$

where B is the Hill Slope, LD_{50} is the lethal dose corresponding to the midway point between max and min dose-response, and for $y = [0, 1]$.

Statistical methods

This study analyzed the two treatment methods by comparing the calculated LD_{50} values obtained from the quantal dose response curve (Eq. 3). Statistical significance was determined if the confidence interval for the difference between the two sample means does not contain zero (24). At the $\alpha = 0.05$ level, this can be arranged as:

$$|\bar{x}_1 - \bar{x}_2| > 1.96 \sqrt{SE_1^2 + SE_2^2}, \quad (4)$$

where $\bar{x}$ is the LD_{50} value expressed in logarithmic form, with subscripts identifying the two different samples, and SE is the standard error of the regression (or standard error of the estimate), calculated as:

$$SE = \sqrt{\frac{\sum (y_i - \hat{y}_i)^2}{n-2}}, \quad (5)$$

where y_i is the experimental value, and $\hat{y}_i$ as before, is the predicted value, and where $n-2$ degrees of freedom are used for the standard error of the regression equation. Values and statistical results corresponding to the dose-response curves (e.g., confidence intervals) presented in this manuscript have been back-transformed from the logarithmic scale for clarity.

RESULTS

Biofilm formation

The *P. aeruginosa* biofilms developed over a course of 4 days and following gentle agitation appeared to be attached to the quartz surface by the end of the Day 3. Uniformity of the biofilms was assessed by gross observation, with some variability in shape attributed to natural formation of the biofilm in the absence of a shear-induced model. Samples were randomly assigned to either the OUT or IOS treatment groups and both groups had approximately equal representation of the various biofilm phenotypes. Throughout the course of this study, 8 samples were randomly selected as controls to receive no UVC exposure, and plate counted with an average of $8.83 \pm 0.16 \log_{10} \text{ CFU mL}^{-1}$.

For the respective treatment groups, surviving CFU values were separated into bins according to duration of UV exposure (ranging 3–15 samples per bin). Within each bin,

normalized DFFITS values were calculated and samples whose value was greater than ± 1 were considered outliers and removed from the regression model (Fig. 4). The number of outliers removed was 6 OUT and 5 IOS.

Planktonic studies

In order to verify the bioreactor setup did not impart an unaccounted bias between the two UV source configurations, planktonic *P. aeruginosa* (seed concentration of 1.0×10^5 CFU mL⁻¹) were irradiated from either the OUT or IOS orientation. Survival curves for the OUT and IOS approaches are shown in Fig. 5. As expected, there was no difference between the two orientations since the bacteria in suspension are equally vulnerable from all directions. Given the small duration of time between sample prep and total UV exposure (maximum exposure time was 20 s), it was assumed the bacterial suspension did not settle due to gravitational effects at an appreciable degree. Thus, for samples positioned equidistant from the UV lamp, the only additional attenuation in UV exposure accounted for between the two orientations was that passing through the bottom of the quartz dish for IOS. The total germicidal UV exposure required to reach complete sterilization of all samples was 4.3 mJ cm⁻².

UVC exposure for complete biofilm sterilization

The quantal dose-response curve in Fig. 6 shows the relative efficacy for the IOS and OUT methods in sterilizing mature *P. aeruginosa* biofilms. The graph illustrates that the biofilms were much more susceptible to germicidal UV in the IOS configuration than in the OUT orientation, as 15% of the IOS samples were sterilized within the first 5 minutes (108 mJ cm⁻²) and the first recorded OUT sterilization was not until 15 min (347 mJ cm⁻²) of UV exposure. After approximately 350 mJ cm⁻² of UV radiation, 60% of the IOS samples were sterilized, whereas only 29% of the OUT samples had negative plate cultures.

The experimental results were fit to the four parameter logistic model in Eq. 3, and there was a higher correlation of fit for the IOS method than the OUT method. As noted earlier, there was some observed inhomogeneity in the biofilms that were produced, having various degrees of opacity in the visible EPS matrix. It is probable this accounted for the larger variance in the OUT samples due to the higher density of the biofilm matrix in outer layers, which would be less influential in the IOS configuration.

The LD₅₀ for the OUT treatment group was 461 mJ cm⁻² 95% CI [292, 728] and the LD₅₀ for the IOS treatment group was 247 mJ cm⁻² 95% CI [187, 325] (Fig. 7), demonstrating a reduction of 47% in sterilizing UV dosage for the *P. aeruginosa* biofilms using the IOS approach ($p < 0.05$). In this study, the durations of UVC exposure corresponding to the LD₅₀ values above were 20 min and 11.4 min, respectively. These exposure times reflect a sterilizing dose that was required to achieve a 9-log reduction in viable bacteria versus controls.

In comparison with planktonic cultures, the *P. aeruginosa* biofilm matrix substantially increased the resistance of viable cells to UV. When considering traditional methods of UV sterilization (i.e., ambient exposure), the *P. aeruginosa* biofilms required over two orders of magnitude more UV energy than in the planktonic state. And although the IOS method was

significantly more efficient than the OUT approach, complete sterilization still required over 50 times more UV radiant exposure for biofilm sequestered bacteria.

DISCUSSION

Bacterial biofilms are particularly challenging to eradicate due to intrinsic defensive mechanisms against a variety of environmental insults. Although they are largely inhomogeneous in both composition and architecture, there does appear to be a gross orientation with respect to surface-attached structures. In its simplest form, this 3-D arrangement may be described as an internal cellular region shielded by an outer EPS matrix. Prior to this study, it was unknown if this organizational structure could be leveraged to increase effectiveness of germicidal UV in destroying viable cell counts.

The work presented in this paper demonstrated that a clinically relevant bacterial biofilm was more susceptible to UV radiation when that energy is emitted from a surface versus equal levels of radiation arising from ambient sources. *P. aeruginosa* biofilms were grown *in vitro* and exposed to various doses of UVC radiation from different directions. It was shown that 47% less UV exposure was required to completely inactivate the mature biofilms when delivered directly to the attached base layer than when administered externally to the outer biofilm matrix. Although it was not within the scope of this study to visualize the EPS scaffold, these results suggest a few possible insights into the topographical structure of the biofilm given the lower UV resistance observed in the IOS approach.

As shown by Lawrence *et al.*, a higher portion of cellular material in *P. aeruginosa* biofilms is consolidated in lower layers relative to the surface, with a higher EPS content in outer layers (8). Purely with regards to linear attenuation, vital cellular constituents will receive an overall higher delivery dosage in the IOS setup since a greater proportion of cellular material would receive the peak UV energy. A similar pyramidal layering has been characterized in *Escherichia coli* and others which suggest this method may be effective against other clinically-relevant organisms (8, 25–27, 22).

During the early stages of *P. aeruginosa* biofilm development, the glycocalyx encases surface-attached bacteria, helping to anchor the growing biofilm to the material surface (22). As the matrix grows, channels develop for the transport of nutrient and waste products. Although the matrix extends throughout the biofilm structure, density of the non-cellular construction decreases deeper towards the material surface (28, 27, 8, 26). Therefore, an accurate illustration of the base layer of a bacterial biofilm assembly would depict cell adhesins in an EPS suspension, rather than a fully exposed layer of cellular material. This portrayal of the biofilm would support the results of this study which found that although the IOS method required roughly half the dosage as the OUT method, it still displayed substantial UV resistance, requiring over 50 times more UV energy than planktonic cultures for complete sterilization.

Mature *P. aeruginosa* biofilms have also been shown to use programmed cell death (apoptosis) and autolysis to form an internal cavity to facilitate dispersal of planktonic cells (22). In theory, a pocket within the biofilm would result in less scattering/absorption of the

incident UV irradiation and allow deeper penetration within the biofilm (whether outside-in or inside-out). Although this study did not investigate the topology of the matrix or assess its maturity through the presence of biofilm-derived colonies, it is reasonable that the maturity of the biofilm would be a factor in the relative efficacy between the OUT and IOS methods, perhaps suggesting an optimal window for therapeutic intervention.

One limitation of this work was the use of a pure *P. aeruginosa* biofilm growth model. While this model allowed for increased reproducibility and the ability to isolate the variable of UV source orientation, it is not suitable for predicting the behavior of natural biofilms which are vastly more complex due to the assimilation of biomolecules and other microbial species from the local environment.

A second limitation of the study was that the *P. aeruginosa* biofilms were grown in the absence of shear forces in order to assist with attachment to the quartz surface. Earlier runs growing biofilm under low shear resulted in unattached biofilm development, resulting in completely encapsulated free-floating structures with no partiality to UV source orientation (data not shown). While the thickness and density of *P. aeruginosa* biofilms does not appear to be influenced by flow, biofilms grown under shear are characterized by stronger adhesion strength and are phenotypically different (29, 30). Future research addressing various adhesion strengths and bacterial phenotypes is needed to determine how these results may translate to clinical settings in order to better approximate potential therapeutic benefit.

Another area of potential interest is investigating whether a biofilm's defensive mechanisms are predisposed to an outward polarity, since a conditioned surface is typically benign to a growing biofilm community. Although novel surface characteristics may provide initial microbial resistance, they are limited in effective duration when active agents are depleted or the surface chemistry is altered due to the adsorption of environmental molecules. Focus in this area may give clues to other therapeutic approaches that may leverage the biofilm's organizational structure.

In the present study, it is unknown whether the UV sterilization had any effect on the physical biofilm architecture. Although both methods of UV exposure were demonstrated to be successful in killing the *P. aeruginosa* biofilms, the clinical application of UV as a standalone therapeutic may be limited if it does not have a significant effect on destroying the biofilm matrix, since some studies have suggested that the biofilm scaffold may be easily recolonized by subsequent contaminations (31, 32). Future work will be required to investigate the application of UV for *in situ* device/implant sterilization in the context of individual versus concomitant use.

Finally, additional work is needed to overcome both the practical and technical challenges of delivering UV from a material surface, including size, attenuation, and material degradation. However, with the development of narrowband UV LEDs, there may be opportunities for delivery platforms with more control over light output, and safer instruments for clinical application (33–35). Further, by investigating methods such as IOS and others that increase the efficiency of germicidal UV, some of the challenges for implementation may be more attainable.

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A new method for treating surface-attached *Pseudomonas aeruginosa* biofilms using ultraviolet-C light required 47% less UVC dosage to achieve a 9-log reduction in viable bacteria versus ambient exposure. By leveraging the organizational structure of the biofilm, this work may provide direction for the development of new technologies for use in clinical applications.

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Figure 1.

The figure shows a cartoon schematic of a surface attached biofilm and orientation of the germicidal UV source (D). The primary defensive means of the biofilm (B) is an outer EPS matrix (A) which protects internal cellular constituents (C). With ambient ("outside-in", or OUT) exposure, UV rays must pass through the outer EPS matrix to reach internal cells and cell signaling molecules. Although the EPS matrix envelops much of the biofilm, bacterial adhesins provide initial surface attachment. Germicidal UV exuding through the surface ("inside-out" sterilization, or IOS) may have less resistance through the surface-attached cells than the EPS matrix.

Figure 2.

The custom bioreactor enabled repeatable biofilm sterilization using germicidal UVC radiation from OUT and IOS orientations. The front panel of the bioreactor has been removed in the photos to visualize the internal assembly of the unit.

Figure 3.

Plot shows UVC attenuation through the test media (90% v/v heparinized saline with 10% v/v TSB). UV irradiance at the surface of the culture dish was measured to be 0.63 mW cm⁻². Attenuation through 3 mL of media resulted in 42.1% attenuation of the source energy and 52.3% attenuation through 5 mL. UV energy imparted to the IOS samples was attenuated an additional 6.2% by the quartz culture dish. Error bars show standard deviation of measurements.

Figure 4. Normalized DFFITS values are shown above. Values greater than ± 1 were considered outliers and removed from the regression analysis. There were 6 outliers removed from the OUT treatment group and 5 removed from the IOS group. The removed outliers included samples that were on both extremes of the surviving CFU vs. dose curve.

Figure 5.

Figure shows the survival curve for planktonic *P. aeruginosa* when exposed to germicidal UV from the OUT (solid circles) and IOS (open circles) orientation.

Figure 6.

Quantal dose-response curve for OUT (solid circles) and IOS (open circles) configurations on *P. aeruginosa* biofilms. Within the first 5 min (108 mJ cm⁻²), 15% of IOS samples were completely sterilized, whereas the first observed sterilization of OUT samples did not occur until 15 min (347 mJ cm⁻²).

Figure 7.

Lethal dose (LD₅₀) as calculated from the dose-response curve in Fig. 6 shows a 47% reduction for the IOS method (light gray) over the OUT method (dark gray). For the irradiance received by the biofilm samples, the LD₅₀ values correspond to 20 minutes for OUT and 11.4 minutes of UVC radiation for IOS. These exposure times reflect a sterilizing dose that was required to achieve a 9-log reduction in viable bacteria versus controls. Bars show 95% confidence intervals. Single asterisk represents significance at the $p < 0.05$ level.