Comparison of continuous versus pulsed ultraviolet light emitting diode use for the inactivation of *Bacillus globigii* spores

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ABSTRACT

Light emitting diodes (LEDs) in the ultraviolet (UV) range offer a promising alternative for the disinfection of water. LEDs have many advantages over conventional UV lamps but there are concerns related to the operating life of the LED lamps. In this project *Bacillus globigii* was inactivated using UV LED technology. The experimental strategy included using pulsed ultraviolet (PUV) output rather than continuous UV (CUV) current in order to reduce the power requirements and extend the life of the lamps. The kinetic profiles for CUV experiments reached 6-log inactivation faster than PUV at 9.1% duty cycle (approx. 840 vs. 5,000 s) but the PUV required lower fluence (365 vs. 665 J/m^2). In addition, the inactivation rate constants associated with PUV were generally higher than those of CUV (4.6–5.1 vs. 3.6–4.4 m^2/J), which supports the notion that high energy bursts are more effective at causing cellular damage. Multi-target kinetics applied to most of the kinetic observations and tailing effects were generally observed. PUV LED appears to have potential to extend the lifetime of the LEDs for inactivation of spore-forming pathogens.

Key words | *Bacillus globigii*, disinfection, light emitting diodes (LEDs), UV light, water treatment

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INTRODUCTION

Ultraviolet (UV)-based water disinfection is a well-established technology for water treatment (Bolton & Cotton 2008; Crittenden *et al.* 2012). Unlike chlorine or other chemical processes, UV systems produce very few disinfection byproducts (Metcalf & Eddy 2003; Crittenden *et al.* 2012). UV works for two reasons. The first and most well-known mechanism is photochemical in nature. Nucleic acids absorb UV energy in the wavelength range between 200 and 300 nm, a range overlaps with short range (i.e., 100–280 nm, UVC) and medium range (280–315 nm, UVB) energy (Wang 1976). DNA and RNA are damaged when the UV energy is absorbed, thus interrupting normal cell function. The second mechanism is a photothermal effect. When UV energy increases the cell temperature, proteins unfold and the cell membrane can become deformed (Arami *et al.* 1997; Ito & Ito 1983; Takeshita *et al.* 2003). At present, there is a variety of configurations that can be used to implement UV disinfection, and there are a growing number of full-scale UV treatment systems around the world. As the technology becomes more cost competitive this trend is expected to continue.

UV radiation can be delivered with light emitting diodes (LEDs), which are two-lead semiconductor light sources. These devices release energy in response to the generation of electrical current. LEDs are typically small (5–9 mm in diameter) and have several advantages over incandescent light sources, including lower energy consumption and faster switching (Schubert 2006; Bettles *et al.* 2007). LEDs can also be configured for many wavelengths in the UV spectrum, and they may provide several advantages over conventional mercury lamps for water disinfection applications (Gaska *et al.* 2007). For example, conventional systems employ lamps based on emission lines of mercury; since LEDs do not contain the toxic element mercury, they do not have to be disposed

of as hazardous waste. LEDs do not require warm-up time, permitting them to start up faster and they can be turned on and off with greater frequency. In spite of these advantages, the technology requires further development in order to have the lifetimes and power efficiencies that are necessary in order to compete as direct replacements of mercury lamps.

There has been previous research using UV LEDs to disinfect water. For example, 4-log inactivation has been observed for *Escherichia coli* in batch or continuous reactors equipped with LEDs (Bowker *et al.* 2011; Oguma *et al.* 2013). Previous data show up to 2.2 log-inactivation for MS-2 bacteriophage inactivation and up to 4-log removal has been reported for T7 bacteriophage (Bowker *et al.* 2011) using UV LEDs. Würtele *et al.* (2011) reported up to 6-log reduction of *Bacillus subtilis* spores. Collectively, these results show that UV LEDs can inactivate a variety of microorganisms using batch or continuous flow reactors. However, these reports also contain concerns related to the lifetime of the LEDs. Thus, there is a need for a strategy that can exploit the effectiveness of the UV LED technology while also extending the lifetime. One such strategy is the use of pulsed UV (PUV). PUV is an alternate drive technique in which the light is periodically activated and then turned off, as opposed to being left on continuously. PUV will lead to lower power consumption and should extend the life of the LEDs, in part because pulsing the LEDs helps maintain a junction temperature below a critical damage threshold (Lenk & Lenk 2011). It is also likely that the drive technique can influence the inactivation effectiveness. For example, Gadelmoula *et al.* (2009) observed 3-log change inactivation of *E. coli* DH5α for both a continuous UV (CUV) output and a PUV 9.1% duty cycle (i.e., 10 ms on and 100 ms off) output. Bohrerova *et al.* (2008) found that PUV inactivated *E. coli* and phages T4 and T7 more efficiently than CUV. Similar conclusions have been reached by Sommer *et al.* (1998) for the disinfection of *E. coli* 11229 strain and by Fine & Gervais (2004) for the disinfection of microorganisms growing on glass beads. Thus, it is likely that a pulsed system could be designed to provide equal or better inactivation while significantly extending the life and reducing the power requirement of LEDs compared with CUV. This study extends the research of PUV LED disinfection to *Bacillus globigii*, a common surrogate for *Bacillus anthracis* due to their similar physiology and cellular composition. The objective of this work is to compare continuous versus PUV LED use in water disinfection on *Bacillus globigii* spores. We also

model the microbial inactivation kinetics to determine parameters that reveal important mechanistic details.

MATERIALS AND METHODS

Preparation of *Bacillus atrophaeus globigii* cultures

Bacillus atrophaeus globigii (ATCC® 9372™), formerly known as *Bacillus subtilis niger*, was used for the research. The stock culture was obtained from Central State University in Wilberforce, Ohio, USA and was prepared using the method published by Coroller *et al.* (2001) and Nicholson & Setlow (1990). The first step in preparation was to streak the stock cultures onto Tryptic Soy Agar and incubate at 30–37 °C for 24 hours. Next, generic spore media was prepared with 8 g of nutrient broth, 40 mg $MnSO_4$, 100 mg of $CaCl_2$, in 1,000 mL of sterile distilled water. Baffle flasks (500 mL size) containing 100 mL generic spore media with a culture of vegetative *Bacillus globigii* cells were incubated with continuous, gentle shaking at 35 °C until slide preparation revealed an adequate spore suspension. Spores were aseptically transferred into sterile 35 mL tubes and centrifuged at 7,540 rpm, equivalent to 5,900 rcf (relative centrifugal force), at 4 °C for 10–20 min using a fixed-angle rotor. The supernatant was removed, and spores were resuspended in ice-cold water by repeated pipetting. Spores were then pelleted by centrifugation at 7,450 rpm for 10–20 min at 4 °C. The supernatant was discarded, and the spores resuspended in a 40% ethanol solution and stored at 4 °C. The process yielded 20 mL at a concentration greater than 10^8 cfu/ml. A serial dilution of 1 mL of spore solution was added to 99 mL of sterile distilled water to obtain the concentration of 10^6 to 10^7 cfu/mL required for each disinfection test.

UV LED apparatus

A UV LED irradiation system contained an array with seven LEDs symmetrically placed at the base of the apparatus (Figure S1, available online at http://www.iwaponline.com/wst/070/395.pdf). Characterization of this irradiation system appears elsewhere (Spencer 2014; Richwine 2014). Briefly, a circular base 7.3 cm in diameter was encased in a waterproof enclosure of reflective stainless steel. During the experiment, the apparatus was securely fastened and then placed on a shaker table with an orbital motion at 115–120 rpm. The LEDs were in direct contact with the water, which permitted the energy emitted from the LED to be completely transferred into the water. This permitted

the power output to match the output, which was experimentally confirmed with an integrating sphere.

LED characterization

LEDs with a median wavelength of 269.3 nm were acquired from Sensor Electronic Technology, Inc. (Columbia, South Carolina, USA) were selected for the study (see Figure S2 for the emissions spectra, available online at http://www.iwaponline.com/wst/070/395.pdf). The 269 nm wavelength has been successfully used previously (e.g., Würtele *et al.* 2011). The LEDs were driven by a power board connected to a power box controlled by DASYLab software (Measurement Computing Corporation, Norton, Maine, USA) in both CUV and PUV mode. For either drive condition, the current through each LED was adjusted to 20 mA throughout the experiment. For the CUV configuration setup, each LED was wired in series with a 50-ohm resistor and 25-ohm variable resistors or potentiometers (POTs), which were adjusted to achieve the 20 mA current. For the PUV configuration, constant current resistors (DynaOhm, Randolph, Vermont, USA) were utilized to achieve the desired current of 20 mA instead of the use of POTs. The voltage across each LED was measured using a multimeter or oscilloscope. Current was measured using the digital multimeter and verified through calculation by measuring the voltage drop across the 50-ohm resistor.

UV disinfection experiment and analysis

The UV dose or fluence (J/m^2) is dependent on the collective effects of the exposure time of the system, the UV light intensity and the UV transmittance (UVT) of the water. The fluence was a product of the irradiance, exposure time, and UVT. A value of 0.98 UVT for deionized (DI) water was experimentally determined and is in agreement with Würtele *et al.* (2011). Irradiance (W/m^2 or J/m^2-sec) was calculated by multiplying the total input power (sum of the powers of each LED from manufacturer's specification) and then dividing by the cross-sectional area of the apparatus. The apparatus was filled with 100 mL of the spore solution at the start of the test. Spore samples (100 μl, 200 μl, or 1.1 ml) were transferred with a pipette from the reactor. The UV experiments were conducted at room temperature of approximately 23 °C. Spore samples exposed to UV LEDs were subject to serial dilutions and then aliquots were placed on an agar plate using the spread plate technique (see Figure S3, available online at http://www.iwaponline.com/wst/070/395.pdf). After incubation, the colonies were counted and reported according to the American Society for Testing and Materials

standard D 5465-93 (ASTM 1998). All the experiments were conducted and plated three times for a total of nine replicates for each data point. The inactivation response or log reduction (log N/N_0) < CE: chk N_0 or N_o> was calculated, where N is the concentration (in cfu/ml) of spores at a given fluence and N_0 is the initial concentration of spores. Re-growth experiments confirmed that negligible reactivation occurred at fluence values equal to or greater than 100 J/m^2 (Marcum 2014).

A multi-target kinetic model was used to determine the apparent values of the intrinsic inactivation rate and the number of critical targets. In multi-target kinetics, the cell contains a discrete number of critical targets that must be damaged by UV photons in order to cause inactivation (Severin *et al.* 1983). After a threshold is reached, inactivation occurs with first order kinetics during the exponential decay phase. These multi-target survival functions are typically implied when a lag phase (also known as a 'shoulder') is observed at low UV fluence values (i.e., shortly after initial exposure of the UV radiation). Let n be the number of critical targets and k be the inactivation rate constant (m^2/J). Multi-target kinetics are described by the following:

$$\frac{N}{N_o} = 1 - (1 - \exp(-kF))^n \tag{1}$$

N is the concentration of spores (cfu/ml) and F is fluence (J/m^2). The parameters n and k can be extracted from the log (N/N_o) vs. F plot by determining the slope and y-intercept. Note that $n = 1$ reduces Equation (1) to a single-target model. Statistical analysis (i.e., p values) was carried out using JMP software (SAS Institute Inc., Cary, North Carolina, USA).

RESULTS AND DISCUSSION

UV LED experiments

Figure 1 gives the log inactivation for the spores as a function of time. We observed exponential decay of live spores followed by a tailing phase characterized by slower inactivation kinetics. During the exponential phase, the effective pseudo-first order rate constant (k_d) was approximately 0.80 min^{−1}; this value compares favorably with recently published rate constants associated with the inactivation of *Bacillus subtilis* spores using conventional UV and peroxide (Zhang *et al.* 2014). During the tailing phase, we observed a slower pseudo-first order rate constant (i.e., 0.12 min^{−1}). These data did not
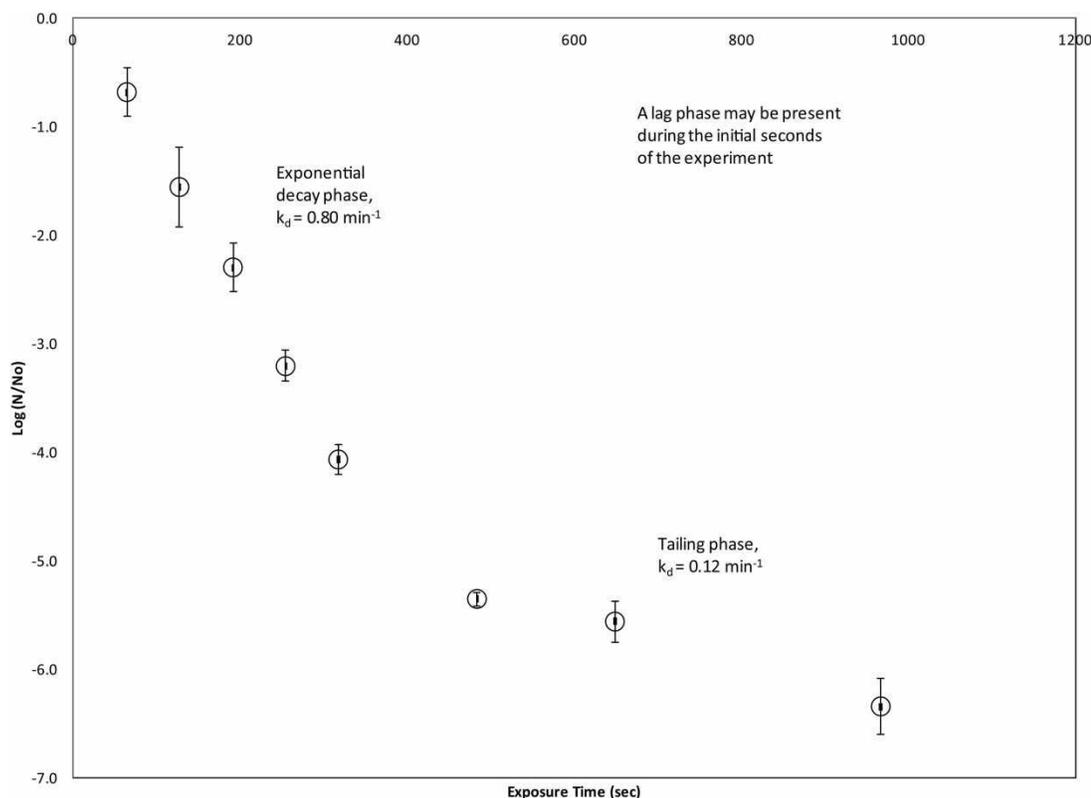
Figure 1 | Inactivation of *Bacillus globigii* spores as a function of time.

demonstrate the noticeable lag period that is expected when multi-target kinetics apply (Xue & Nicholson 1996). The 'tailing phase' that is presented in Figure 1 occurs after approximately 7 min. Tailing can be caused by a number of factors. It may be due to a highly resistant sub-population with enhanced capability to avoid UV-induced damage. It also may be due to clumping of spores, which would effectively shield spores. It is also possible that crevices in the test apparatus may have provided a measure of protection from the UV radiation (Warriner *et al.* 2001; Pennell *et al.* 2008). The inactivation phases observed in Figure 1 are common for UV disinfection of pathogens. It is also important to note that 6-log inactivation was achieved in these experiments, which compares favorably to the previous UV disinfection research with *Bacillus* spores (Würtele *et al.* 2011). Fluence-inactivation response curves are shown in Figure 2 for three CUV LED experiments. For CUV 1 and 3, the lag phase is not noticeable but for CUV test 2 a 'shoulder' is present, which indicates the presence of a lag period. The exponential decay phase is present between fluence values of 50–380 J/m² and a long tailing effect at the higher fluence values (> 380 J/m²) is observed. As described earlier, the slope and *y*-intercept of these curves can be used to determine two underlying

model parameters, namely the *n* and *k* as shown in the figure inset. Two of three experiments were associated with *n* ¼ 1, which is consistent with the absence of a lag phase; in this case, the multi-target model is reduced to a single-target model. For CUV test 2, the number of targets in this case was 5, consistent with the presence of the 'shoulder'. Although it is not clear why the 'shoulder' was only present during CUV test 2, it is clear that the inactivation rate constants and the number of targets revealed here are in keeping with what has been previously observed for *Bacillus subtilis* spores (Gardner & Shama 1998). These data show that 6-log inactivation can be achieved with kinetics that are consistent with either single- or multi-target models. It should be noted that, in general, the inactivation rate constants are impacted by the experimental conditions, the reactor design, and the biological species of interest.

These data were compared to those of Würtele *et al.* (2011), who recently carried out experiments on *Bacillus subtilis* spores using CUV LEDs at wavelengths of 269 nm and 282 nm (Figure S4, available online at http://www. iwaponline.com/wst/070/395.pdf). They found that UV LEDs at 269 nm reached a higher level of inactivation for the same applied fluence than the 282 nm UV LEDs.
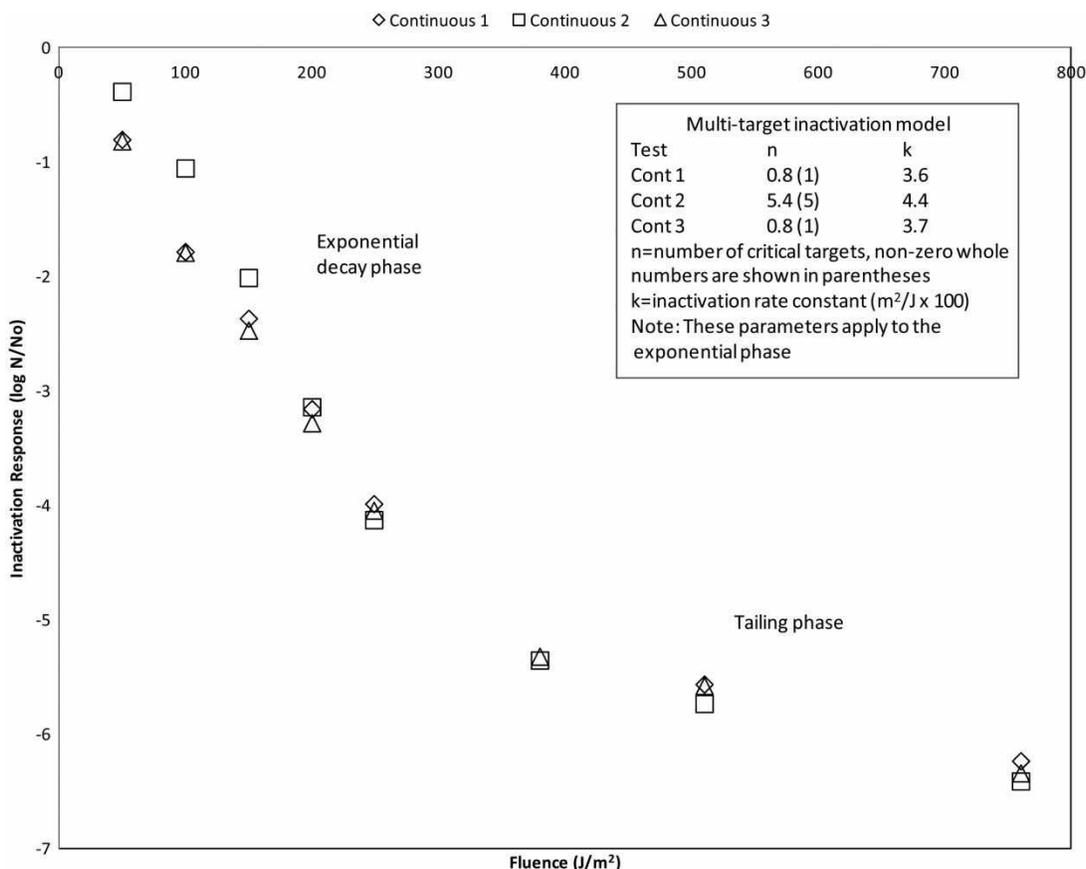
Figure 2 | The effect of fluence on the inactivation of *Bacillus globigii* spores: continuous LED tests.

Multi-target kinetics applied to the 282 nm LED data (i.e., $n \frac{1}{4}$ 5), and its inactivation profile contains a noticeable lag because the exponential decay profile is not in alignment with the origin. The k values for all three data sets are in good agreement. The single-target kinetic model applies to the data from the current study (at 269 nm) as well as the 269 nm data of Würtele *et al.* (2011). This finding is expected because 269 nm is very close to 260 nm, the optimum wavelength for absorption of UV radiation by DNA (Wang 1976).

The PUV operating strategy involves implementing a duty cycle, which is defined as the percentage of time in which the signal is 'on'. For example, a duty cycle of 9.1% is associated with 10 ms 'on' and 100 ms 'off'. Preliminary studies were conducted with LEDs at different duty cycles. We found that the optical power associated with the 18.2 and 27.3% duty cycles was respectively two and three times the optical power required for the 9.1% duty cycle, which means that there is a linear relationship between the duty cycle and the optical power (Bates 2014). This work also demonstrated that the sum of the time of 'on' during PUV had the same optical power as the equal time in the CUV configuration (Bates 2014).

Figure S5 (available online at http://www.iwaponline.com/wst/070/395.pdf) gives the log inactivation results from the PUV configuration for the 9.1% duty cycle as a function of time. The curves show an exponential decay phase and a tailing phase, and there is a clear indication of a lag period as the exponential phase trend line is not aligned with the origin of Figure S5. The apparent first order rate constants for the exponential decay and tailing phases were 0.1 and 0.02 min^{-1}, respectively, which are smaller than those associated with the CUV experiments described in Figure 1. Disinfecting spores in the 9.1% duty cycle takes more time than it does in CUV mode. It is also interesting to note that the lag period is more obvious in the 9.1% duty cycle than it is when CUV mode is used. This makes sense, because it takes a longer period of time to apply enough UV radiation to overcome DNA repair mechanisms and cause damage (e.g., Xue & Nicholson 1996). Figure 3 shows the log inactivation-fluence curves for both PUV and CUV operation. For a given fluence, the PUV configuration generally demonstrated lower log (N/N_o) than the CUV configuration. In order to achieve 6-log inactivation, a fluence of approximately 665 J/m^2 was required for CUV exposure,

while approximately 365 J/m^2 was required with PUV. There-fore, PUV was approximately 1.8 times more efficient for inactivation of *Bacillus globigii* when trying to achieve a 6-log inactivation. PUV LED disinfection is slower but more energe-tically effective than CUV LED disinfection. This increase in energetic efficiency suggests that LED lifetimes may be extended by operating in PUV mode. It is noteworthy that the multi-target kinetic model parameters shown in Figure 3 are consistent with previous research (e.g., Sugawara *et al.* 1981, Gardner & Shama 1998). Because the PUV experiments have $n ¼ 2$ or 4, these data prove the applicability of multi-target kin-etics while also suggesting that the PUV kinetic profiles contain relatively brief lag periods. The inactivation rate constants of the PUV model were higher than those observed for the CUV mode, a finding that is consistent with the idea that these higher energy bursts are more efficient at causing cellular damage.

PUV mechanisms related to disinfection and operation

The previous work in this area showed that PUV was more effective than CUV for the inactivation of bacteria and phages, and the current work showed that this finding also applied to the inactivation of *Bacillus globigii* spores. The short-duration burst of UV energy causes more damage than CUV at equivalent fluence values. The mechanistic details of this phenomenon are subject to some debate, but there are two plausible explanations. First, the rate of LED photon generation is increased in proportion to the sharp increase in current that is applied during pulsing. Thus, the PUV peak photon discharge is higher than that of CUV. The second is related to the broad wavelength spec-trum, which includes both visible and infrared regions with biocidal potency (Anderson *et al.* 2000; Wekhof *et al.* (2001)). The situational importance of these two mechanisms probably depends on the pathogen of interest and the oper-ating conditions of the PUV device, but either of these mechanisms can be associated with the photochemical reac-tions that damage nucleic acid or the photothermal effects that induce membrane disruption and protein unfolding. Indeed, Wekhof *et al.* (2001) found that PUV LED treatment caused the top of *Aspergillus niger* spores to become punctu-red, evidently because internal water was vaporized. Future
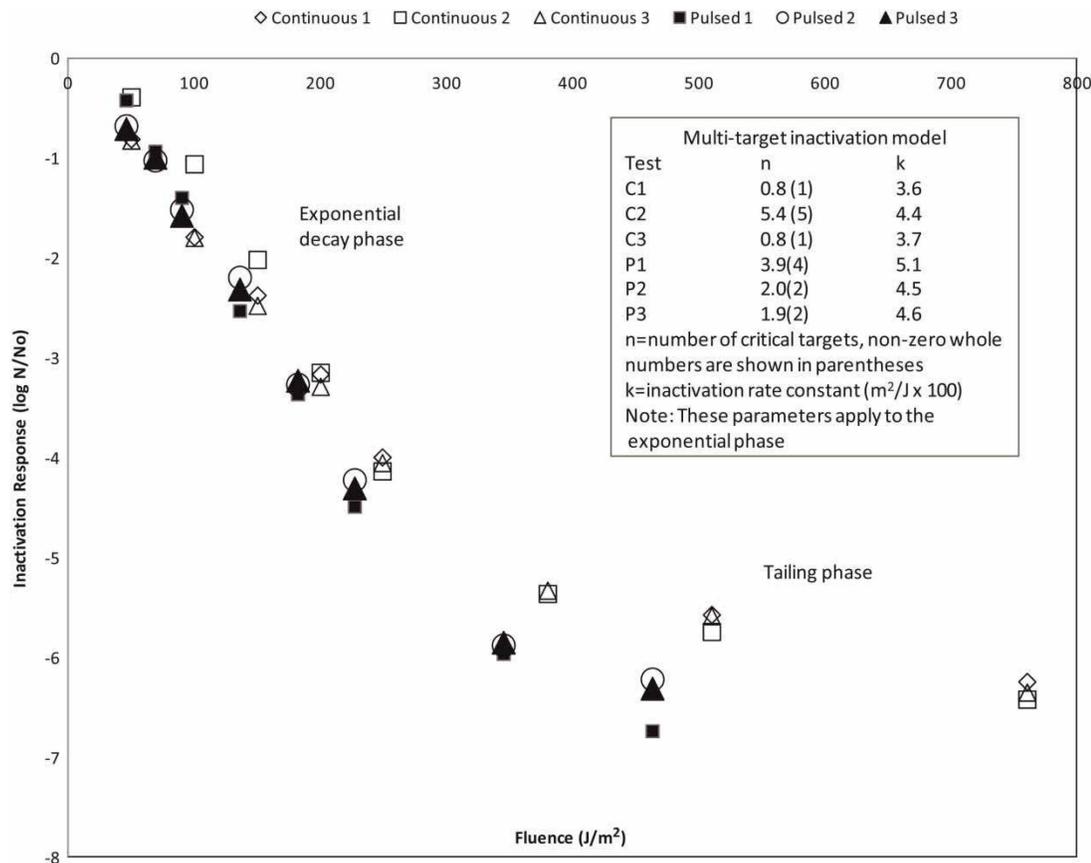

Figure 3 │ The effect of fluence on the inactivation of *Bacillus globigii* spores: pulsed and continuous tests.

PUV researchers should look for this deformation, which would be visible with a light microscope.

Mechanisms related the stability of LEDs are also of interest. Heat degrades the LED because a large amount of energy is being channeled through a very small area. LED efficiency is also partly to blame, because as much as 90% of the energy that is being generated is converted to heat (Schubert 2006). The heat can break down the crystal-line structure of the device, causing defects and leading to eroding performance. The idea that PUV extends the life of the LED is supported in part by the fact that the heat is not constantly flowing through the device. However, the contrasting argument to this is as follows: If the LED is being heated and then cooled, and if the thermal expansion constants and the electrical contacts are not well matched, these temperature changes can induce stress, which eventually causes the connections to the LED to fail. This is why previous reports have suggested that the lifetime of some LEDs are improved with pulsing, while others perhaps are degraded (Buso *et al.* 2008). In some cases, the LED may not be degraded but the interfaces to the parts around it may be compromised by the repeated heating and cooling. Future efforts should improve the durability and performance of these devices and lead toward more consistent and successful applications.

CONCLUSION

The results of this research demonstrated effective inactivation of *Bacillus globigii* spores by both PUV LEDs and CUV LEDs. Both configurations were able to achieve a 6-log inactivation; however, the CUV experiments reached 6-log inactivation faster than PUV (approx. 840 vs. 5,000 s) while the PUV required lower fluence (365 vs. 665 J/m^2). The inactivation rate constants associated with PUV were generally higher than those of CUV operation (4.6–5.1 vs. 3.6–4.4 m^2/J), which suggests that high energy bursts are more effective at causing cellular damage.

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