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APPLICATION NOTE

DISINFECTION OF WATER BY FLOW-THROUGH PULSED ULTRAVIOLET LIGHT STERILIZATION SYSTEM

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ABSTRACT
Disinfection of water is an important task for semiconductor, pharmaceutical, food or other industries for various purposes. Pulsed ultraviolet light is a novel technology which offers a rapid and effective solution to achieve sterilization of water and provide reductions in organic load of the water. In this study, efficacy of pulsed UV light was studied for inactivation of *Bacillus subtilis* spores by using a flow through pulsed UV light chamber. Various flow rates up to 14 L/min were evaluated. The pulsed UV treatment results demonstrated the complete inactivation of *B. subtilis* for all the flow rates evaluated, which yielded 5.5 Log_{10} CFU/ml reduction or more. Furthermore, there was no growth observed after enrichment in dark or under light, which indicated that there were no injured cells and no recovery of the spores due to the photorepair mechanism. After pulsed UV-light treatment, the UV-light absorption at 254 nm reduced significantly for most of the cases suggesting that the turbidity of the water reduced. Therefore, this study clearly demonstrated that pulsed UV-light has a potential to be utilized for sterilization of water.

INTRODUCTION

Water is used abundantly in several industries. This water contains several pathogenic microorganisms including *Vibrio cholerae*, *Salmonella*, *Shigella*, *Campylobacter*, and *Escherichia coli*. Therefore, it is necessary to disinfect the water before using it for industrial applications to ensure the safety and purity of water. Ultraviolet (UV) light disinfection is gaining interest among public water systems and commercial applications to disinfect the drinking water. UV-light does not form harmful by-products while inactivating pathogenic microorganisms (1). Therefore, UV-light is viewed as an alternative to chemicals such as chlorine. UV-light has been used for disinfection of drinking water from 1906. In addition to the disinfection of drinking water, UV-light can also used for disinfection of water used in semiconductor, pharmaceutical, food or other industries and for sanitation of waste water.

UV-light can be applied in two different modes; namely continuous and pulsed modes. Recently the use of pulsed UV-light is getting attention as it can inactivate pathogenic microorganisms in a short period of time as it has better penetration depth than continuous UV-light. Pulsed UV-light is a broad spectrum radiation covering UV, visible, and infrared regions with typical wavelength range from 100 nm to 1100 nm. Energy is stored in a capacitor and released as very short intermittent pulses (typical pulse duration ranges from several hundreds of microseconds to nanoseconds), so the peak energy will be in the order of several Megawatts. However, the total energy is comparable to continuous UV-light (in the order of several watts). Therefore, by using same amount of total energy, one can achieve better inactivation using pulsed UV-light due to higher peak energy and constant disturbance caused by pulses. Krishnamurthy et al. (2004) reported that pulsed UV-light can inactivate *Staphylococcus aureus,* a

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foodborne pathogenic microorganism within several seconds (3). Within 5 second treatment up to 8.5 log_{10} CFU/ml reduction was achieved. This clearly indicates the effectiveness of pulsed UV-light.

The inactivation modes of pulsed UV-light can be hypothesized as: i) Photochemical effect: UV-light component of the pulsed UV-light damages the bacterial DNA by forming thymine dimers, leading to cell death or spore inactivation, ii) Phtothermal effect: Visible and infrared portion of the pulsed UV-light have a thermal effect on bacterial cell/spore. The rate of cooling or heating of bacterial cell/spore is different from the surrounding media which leads to localized heating of the bacterial cell resulting in inactivation the enzymes and other cellular contents (4), and iii) Pulse effect: constant disturbance caused by intermittent pulses may damage the cell wall and other cellular constituents. The objective of this research is to investigate the efficacy of the pulsed UV-light for sterilization of water during continuous water treatment. As spores are more resistant to UV-light than vegetative cells, *Bacillus subtilis* spores were used in the study to evaluate the efficacy of the system in a worst case scenario.

MATERIALS AND METHODS

Microorganism

Bacillus subtilis (ATCC 6633) was obtained from American Type Culture Collection (Manassas, Va.) and kept as frozen culture at -80°C. The culture was transferred to 150 ml of Tryptic soy broth (TSB, Difco, Sparks, Md.) and grown for 24 h at 37°C and then transferred to tryptic soy agar (TSA) slants. After incubating at 37°C for 24 h, the slants were stored in the refrigerator until further use. Sub-culturing was performed on TSA slants every other week in order to ensure the culture viability.

Spore preparation

Four different methods of spore preparation were tested by changing the growth media, wash buffer, and/or number of days of incubation in order to maximize the spore count. Using the culture stored on TSA slants at 4°C, streak plating was done on TSA followed by incubation at 37°C for 24 h. A single colony from the plate was transferred to 10 ml of TSB and incubated. All the incubation was done at 37°C for 24 h unless noted.

Method 1: The prepared culture was spread plated on TSA plates and incubated for 3 days. Then, the plates were rinsed with 5 ml of KCl/0.5 M NaCl solution and disturbed gently with a sterile spreader in order to remove the spores from the plates. Rinsing was repeated with another 5 ml of KCl/0.5 M NaCl solution and the rinse solution was transferred to sterile centrifuge bottles. The solution was vortexed in order to maintain the homogeneity followed by centrifugation at 3,800 x g, at 4°C for 10 min (Sorvall Super T 21, ST-H750, Kendro Lab Products, Newton, Conn.), After centrifugation, the cells were washed with 250 ml of 950 mM Tris-HCl/EDTA buffer and re-centrifuged. Washing with 250 ml of 950 mM Tris-HCl/EDTA buffer was repeated two more times. The washed cells were resuspened in phosphate buffer (supplemented with Tween 20, pH 7.4, Sigma-Aldrich, St. Louis, Mo.) and the cells were heat shocked to produce spores at 80°C for 10 min. The spore suspension was stored at 4°C until further use. It was believed that the high amount of Tris-HCl and EDTA in the buffer resulted in injury to the cell leading to very low final spore concentration. Therefore, a lower concentration of Tris-HCl and EDTA were used in method 3.

Method 2: The prepared culture was grown in TSB for 7 days at 37°C. The sample was centrifuged at 3,800 x g at 4°C. After centrifugation, the cells were washed with 250 ml of phosphate saline buffer and re-centrifuged. Washing with 250 ml of phosphate saline buffer was

repeated two more times. The washed cells were resuspened in phosphate saline buffer and the cells were heat shocked at 80°C for 10 min to produce spores. The spore suspension was stored at 4°C until further use.

Method 3: The prepared culture was spread-plated on TSA plates and incubated for 7 days at 37°C. The plates were rinsed with 5 ml of KCl/0.5 M NaCl solution and disturbed gently with a sterile spreader. In order to remove the spores from the plates, rinsing was repeated with another 5 ml of KCl/0.5 M NaCl solution and the rinse solution was transferred to sterile centrifuge bottles. The solution was vortexed in order to maintain the homogeneity followed by centrifugation at 3,800 x g, at 4°C for 10 min. After centrifugation, the cells were washed with 250 ml of 10 mM Tris-HCl/EDTA buffer and re-centrifuged. Washing with 250 ml of 10 mM Tris-HCl/EDTA buffer was repeated two more times. The washed cells were resuspened in phosphate saline buffer and the cells were heat shocked to produce spores at 80°C for 10 min. The spore suspension was stored at 4°C until further use.

Method 4: The prepared culture was spread-plated on TSA plates (50 plates/batch) and incubated for 7 days at 37°C. The plates were rinsed with 5 ml of KCl/0.5 M NaCl solution and disturbed gently with a sterile spreader in order to remove the spores from the plates. Rinsing was repeated with another 5 ml of KCl/0.5 M NaCl solution and the rinse solution was transferred to sterile centrifuge bottles. The solution was vortexed in order to maintain the homogeneity followed by centrifugation at 3,800 x g, at 4°C for 10 min. After centrifugation, the cells were washed with 250 ml of phosphate saline buffer and re-centrifuged. Washing with 250 ml of phosphate saline buffer was repeated two more times. The washed cells were resuspened in phosphate saline buffer and the cells were heat shocked to produce spores at 80°C for 10 min. The spore suspension was stored at 4°C until further use.

Pulsed UV-light treatment system

Pulsed UV-light treatment was carried out with SteriPulse®-RS 4000 pulsed light sterilization system (Xenon Corporation, Wilmington, Mass.) (Figure 1). The system generated 1.27 J/cm^2/pulse of radiant energy at 0.7″ below the lamp surface and produced a polychromatic radiation in the wavelength range of 100 to 1100 nm, with 54% of the energy being in the UV-light region. The system produced 3 pulses of 360 μsec duration per second. The system chamber has an annular cylinder arrangement with UV lamp being placed at the center (Figure 2). The water disinfection system was made up of stainless steel and had 4″ outer diameter and 16″ length. A site glass is provided to facilitate the observation of water flow and to measure the UV-light intensity. The annular space between the UV-lamp and the outer wall of the vessel were separated by a quartz sleeve to enhance the transmission of UV-light to water. The maximum volume of water in the disinfection chamber at any given time is 0.7725 gallons (i.d. of the outer vessel was 3.86″, o.d. of the inner quartz tube was 0.97″, length of the chamber was 16″, and added volume due to site glass was 0.0132 gal). The water was pumped through using a centrifugal pump (TE5-5C-MD, Emerson motor company, St. Louis, Md.) and the flow rate was adjusted by a control valve. The water from a 10 or 20 L carboy container (Cole-Parmer, Vernon Hills, IL) was

Figure 1. Pulsed UV-light sterilization system

pumped through the water vessel. The flow of the water was measured and adjusted using a flow meter (F-41017L, Blue white industries, Huntington Beach, Calif.).

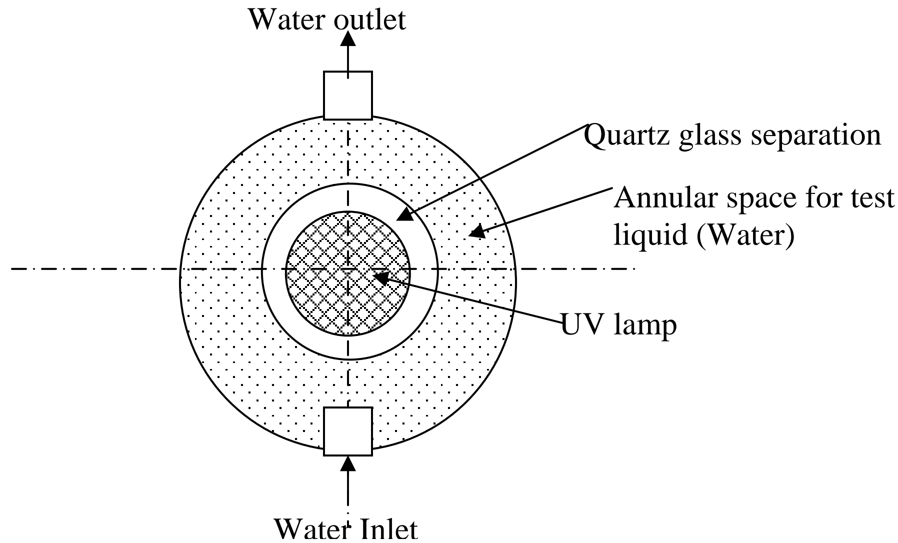

Figure 2. Schematic diagram of cross section of the SteriPulse® RS-4000 chamber.

Cleaning of the flow through pulsed UV system

In order to avoid any cross contamination in the pulsed UV system, pulsed UV-light was coupled with chlorine solution followed by sterile D.I. water rinse to remove any chlorine residues after several different combinations were investigated to get a sterile system. The final cleaning procedure was determined as follows: i) circulate 10 L sterile deionized (D.I.) water with pulsed UV-light on for 10 L/min for 10 min for 10 L of water and 30 min for 20 L of water, ii) Circulate 10 L of 200 ppm chlorine solution for 10 min, iii) Circulate 10 L of sterile D.I. water for 10 min, iv) pump sterile D.I. water to adjust the target flow rate.

Pulsed UV light treatment

D.I. water was autoclaved at 121°C for 60 min and cooled overnight at room temperature. Ten ml of the prepared spore suspension for 10 L of D.I. water or 20 ml of the prepared spore suspension for 20 L of D.I. water was added and mixed well to ensure homogeneity. The initial spore population in water was determined by plating on TSA as 5.5-6.5 $\log_{10}$ CFU/ml. The UV-light system was turned on and the inoculated water was pumped through the system at the set flow rates of 2, 4, 6, 8, 10, and 14 L/min, which yielded 88, 44, 29, 22, 18, and 13-sec residence times in the chamber, respectively. After 50% of the water was passed through, about one liter of sample was collected. The pulsed UV-treated water was analyzed for microbial reduction by plating on TSA followed by incubation at 37°C. Two replications were performed for each treatment. Enrichment was performed for all treatments by transferring 1 ml of pulsed-UV treated water into 9 ml of TSB and incubating at 37°C for 24 h. Enrichment was performed to ensure that there were no injured cells. In order to find out that the cells will be recovered by repairing the UV damage under light exposure due to photorepair mechanism, enrichment was also performed under light.

Turbidity measurement
The absorption of the untreated and the treated samples were measured at 254 nm using a UV-Vis spectrophotometer (DU series 500, Beckman, Fullerton, Calif.) in order to monitor the turbidity of the water.
Radiant energy measurement
UV-light energy absorbed by the water was measured by using an radiometer (Ophir PE50, Ophir Optronics Inc., Wilmington, Mass.) by measuring the broadband UV-light intensity for each pulse by placing the pyroelectric detection head on the quartz window provided for light measurements on the water treatment chamber. The radiometer was calibrated at 254 nm. The UV-light intensity at 254 nm was determined by comparing the UV-light intensity obtained using another radiometer (SED240/ACT5/W detector head, International lights, Newburyport, Mass) as per the data given by Xenon Corporation (Wilmington, Mass.)

Temperature measurement
The bulk temperature of the water before and after treatment was measured by placing a thermometer at the center of the container after mixing it well. Several measurements were taken and average was reported.

RESULTS AND DISCUSSION
Method used for spore harvesting played a significant role in getting a higher spore concentration (Table A). Generally, spores grown on agar plates yielded more spores than broth as thin agar plates provide less nutrition over 7 days of incubation. Also more number of days of incubation resulted in higher spore concentration as more vegetative cells produce spores due to lack of nutrition and moisture over the period of incubation. The wash solution also played a significant role in getting higher spore concentration as some wash solution might have injured the spores resulting in lower spore counts. Since spore suspension prepared by method 4 yielded the highest spore concentration (8.34 $\log_{10}$ CFU/ml), method 4 was followed to prepare the inoculum.

The pulsed UV treatment results demonstrated the complete inactivation of *B. subtilis* for all the flow rates evaluated (2, 4, 6, 8, 10, and 14 L/min), which yielded 5.5 Log_{10} reduction or more (Table B). Initial inoculum concentration for each flow rate was slightly different (ranged from 5.5 to 6.5 $\log_{10}$ CFU/ml). Furthermore, there was no growth observed after enrichment in dark or under light, which indicated that there were no injured cells and no recovery of the spores due to the photorepair mechanism. Therefore, all the spores subjected to pulsed UV-light treatment were completely inactivated under the evaluated conditions. After pulsed UV-light treatment, the UV-light absorption at 254 nm reduced significantly for most of the cases suggesting that the turbidity of the water reduced (Table C), which suggested that pulsed UV-light treatment not only disinfects the water, but also disintegrates the organic material by oxidation which results in purer sterile water.

Table A. Evaluation and comparison of spore harvesting methods

Method #	Spore preparation method	Final spore conc. ($\log_{10}$ CFU/ml)
1	950 mM Tris-Hcl/EDTA buffer + 3 days incubation on Tryptic soy agar	1.45
2	Phosphate buffer saline procedure + 7 days incubation in Tryptic soy broth	4.33
3	10 mM Tris-HcI/EDTA buffer + 7 days incubation on tryptic soy agar	5.43
4	Phosphate buffer saline procedure + 7 days incubation on tryptic soy agar	8.34

Table B. Inactivation of Bacillus subtilis spores by pulsed UV-light treatment

Flow rate (L/min)	Population (Log_{10} CFU/ml)	Growth after enrichment
0 (Before treatment)	5.5 – 6.5	Yes
2	0	No
4	0	No
6	0	No
8	0	No
10	0	No
14	0	No

*Both under lighted and dark conditions.

Broadband energy per pulse (J/pulse), power (Watts), power per area (Watts/cm^2), estimated power at 254 nm (watts/cm^2) were reported for each tested flow rate on Table D. The energy delivered by pulsed UV-light was measured using the radiometer suggesting that approximately 0.21 Watts/cm^2 (difference between the broadband power with no water (0.56 watts/cm^2) and average broadband power with water (0.35 watts/cm^2)) of the energy is absorbed by inoculated water (Table D). Some portion of the UV-light is absorbed directly by the microorganism and some by water resulting in increase in the bulk temperature, up to 4°C under the evaluated conditions.

Table C. UV-light absorption at 254 nm

Flow rate (L/min)	UV-light absorption at 254 nm	
	Untreated	Pulsed UV treated
2	0.093±0.023	-0.285±0.054
4	-0.079±0.093	-0.146±0.113
6	0.131±0.076	-0.055±0.064
8	0.136±0.042	-0.061±0.073
10	0.052±0.039	-0.095±0.042
14	0.028±0.069	0.064±0.073

Table D. UV-light energy measurements during pulsed UV-light treatment[1]

Flow rate	Broadband energy per pulse (J/pulse)	Broadband power[2] (Watts)	Broadband power per area[3] (Watts/cm^2)	Estimated power at 254 nm[4] (watts/cm^2)
0 L/min (No water)	3.35	10.05	0.56	0.0112
2 L/min	2.13	6.39	0.35	0.0070
4 L/min	2.12	6.36	0.35	0.0070
6 L/min	2.12	6.36	0.35	0.0070
8 L/min	2.11	6.33	0.35	0.0070
10 L/min	2.10	6.30	0.35	0.0070
14 L/min	2.04	6.12	0.34	0.0068

[1]The Radiometer was calibrated at 254 nm. Broadband energy is reported throughout.
[2]Three pulses were produced per second.
[3]Radiometer had a 48 mm diameter area of exposure.
[4]Based on the comparison of measurements with SED240/ACT5/W radiometer detector head, as suggested by Xenon Corporation, 2% of the broadband energy was at 254 nm based on the comparison studies.

In conclusion, pulsed UV-light treatment has shown to be very effective in inactivating *B. subtilis* spores in this study. The results clearly show the potential of pulsed UV-light to be utilized

for water disinfection cost-effectively. Testing at higher flow rates is needed for the optimization of the system. In general, vegetative cells need less energy than spores to be inactivated and hence pulsed UV-light can be used effectively to inactivate pathogens in a short period of time with less energy. Especially pulsed UV-light can inactivate *Cryptosporidium parvum,* a protozoa of major concern in water, effectively as it is less resistant than *Bacillus subtilis* spores. Boeger et al. reported that one pulse of pulsed UV-light inactivated 1.00 and 4.60 $\log_{10}$ CFU/ml of *Bacillus subtilis* and *Cryptosporidium parvum,* respectively (5). Therefore, pulsed UV-light has a potential to be utilizied for disinfection of vegetative cells, bacterial spores, and protozoa such as *Cryptosporidium parvum*. Also pulsed UV-light provides a mercury free UV-light treatment which does not produce any hazardous by-products and environmentally friendly.

ACKNOWLEDGEMENTS
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