Development of a Protocol for the Determination of the Ultraviolet Sensitivity of Microorganisms Suspended in Air

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ABSTRACT

Referring to the standardized method applied to bench scale sensitivity study of waterborne microorganism, a batch reactor system was built, using a collimated beam system and a small-scale cylindrical vessel to measure the UV susceptibility constants of aerosolized Bacillus subtilis spores. The average UV fluence rate inside the cell could be determined using the same method as in water sensitivity studies. Since there is essentially no absorption of 254 nm UV light in air, no media factor is required. The aerosolized spores were emitted from a nebulizer and introduced into and trapped inside the vessel, and collected into impingers after exposure to UV irradiation for the desired time.

The spore UV inactivation efficiency data versus fluence (UV dose) was fitted using the multi-target kinetic model. A statistical comparison of the kinetic models showed that there was no significant difference between the k value of the aerosolized spores and the k value of waterborne spores. The results also indicated that, within the relative humidity range of 55-75%, the relative humidity did not affect the spores UV sensitivity.

Key words: Ultraviolet; UV; air treatment; fluence; UV dose; collimated beam; disinfection.

INTRODUCTION

Due to its ability to inactivate aerosolized microorganisms, Ultraviolet Germicidal Irradiation (UVGI) at 254 nm is widely accepted as an effective disinfection technique in indoor air quality control. The parameters in survival curves that describe how various species of microorganism respond to the UVGI fluence or dose are called susceptibility constants. In UVGI air disinfection system design, the target species' susceptibility constants are critical parameters in sizing the system. Without a standard testing protocol, aerosolized microorganism UVGI inactivation kinetics studies, in which susceptibility constants are measured, have been carried out with various methods and different kinds of apparatus. Recent work (Fletcher et al. 2003; Miller and Janet 2000; Peccia et al. 2001; Xu et al. 2003) showed the diversity of the methods and apparatus in the aerosolized microorganism UV inactivation studies. **Table 1** summarizes the recent studies in terms of the apparatus and the irradiance measurements. These kinetic studies covered not only the microorganism losses due to UVGI inactivation, but also ventilation losses, and deposition losses. To obtain the UV susceptibility constants, experiments were carried out in such a way that other losses could be measured and deducted from the overall decay effects. By measuring a number of points in the UV irradiation field, the average fluence rates of the UV field were calculated. Assumptions were made in these studies that the flow regimes in the study UV vicinity were completely mixed ones in order to calculate the UV dose.

Source	Flow apparatus and flow regime	UV irradiance quantification
(Peccia et al. 2001)	Pilot scale completely mixed flow cubic chamber (0.8 m^3)	25 KI/KIO_3 actinometry spherical quartz cells to measure the average irradiance
(Fletcher et al. 2003)	Pilot scale flow through chamber(4L)	Radiometer reading through an access port to estimate the irradiance
(Miller and Janet 2000)	Complete mixed room (36 m3) with ventilation	12 point radiometer readings to estimate the average irradiance
(Xu et al. 2003)	Complete mixed room (87 m^3) with ventilation	20 actinometry spherical cells (1 cm diameter) and radiometer readings

Table 1: Recent air suspended microorganism UV susceptibility studies

In the 'Standard for Laboratory testing of UVGI Air and Surface Rate constants' proposed by the UV Air Treatment Topical Group of the International Ultraviolet Association (IUVA 2005a,b), laboratory apparatus is recommended to simulate or scale down the full scale air handling units to simulate the environment of the working condition of the full scale unit. By doing this, the susceptibility study can account for the effects of the UV inactivation and other

physical and self decay losses. This proposed standard is different from the standard protocol used in waterborne microorganism UV inactivation kinetic studies. In waterborne microorganism UV inactivation kinetic studies a standardized protocol (Bolton and Linden 2003) is used, in which a Petri dish is used as a completely mixed batch reactor to hold the microorganism suspension in the collimated UV radiation field. The UV inactivation kinetic study is independent of the geometry and fluid regime of any UV reactor. This reactor independency is of great value in UV reactor design, especially when the Computational Fluid Dynamics (CFD) simulation technique is used to model the reactor.

In this study, we have adapted the standardized protocol from water susceptibility studies to determine UV susceptibility constants for *Bacillus subtilis* spores suspended in air with a similar method and apparatus.

METHODOLOGY AND MATERIALS

Quartz-capped air vessel

A bath reactor was built from a 20 cm long PCV duct, with an internal diameter of 100 mm. The bottom of the duct was sealed with a PVC plate as the reactor base. A small fan (12 VDC, Brushless, Taiwan) was installed on the bottom plate to mix the aerosol inside the duct. The top of the duct was sealed with a quartz plate (11 cm × 11 cm). Various ports were drilled into the duct, including the aerosol inlet port, the dry air inlet port, the sampling/waste outlet port, the flushing air inlet port, and Relative Humidity (RH) probe port. Swage-lok® fitting and PVC valves (Edmonton Valves and Fittings Inc., Edmonton AB, Canada) were installed onto these ports and connected with Teflon tubing to the air supply sources and impingers. There were two impingers used in the system. A Midget impinger (Standard Midget, Supelco Inc.) with 5 mL D.I. water was used as sampling impinger to collect the spores from the air. A bubbling impinger (500mL PYREX®, Fisher Sci.) was used as waste vessel to collect the spores in the effluent air. The nebulizer (Collison 3-jet; BGI Inc., Waltham, Mass.) was driven by a compressed air cylinder (Extra-dry, Praxair, Edmonton, AB, Canada) with 2-stage regulators (Fisher Sci. Canada) to generate the spore aerosol. A barometer (Winters, Cole Palmer Inc.) and a flow meter (Cole Palmer. Inc.) were installed in the Teflon tubing to monitor the pressure condition of the nebulizer and the flow rate. Dry air was supplied by another compressed air cylinder (Extra-dry, Praxair, Edmonton, AB, Canada) to dilute the water-saturated air from the nebulizer to the designated relative humidity level. A flow meter (Watchman II, Cole Palmer) was also used to monitor the dry air flow rate.

The quartz-capped air vessel was placed in the UV-beam position in a collimated beam system (RAYOX®, Calgon Carbon Corporation, USA), in which a low-pressure mercury arc lamp (G12T6L, Atlantic Ultraviolet, USA) was used as UV source. The construction schematic of the quartz vessel is shown here in **Figure 1**.

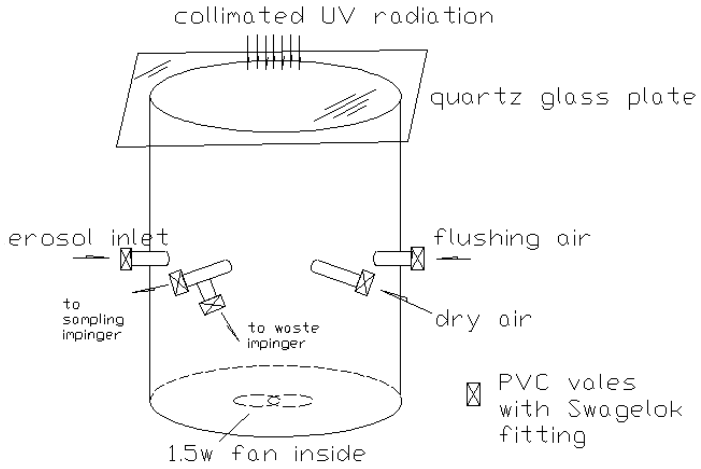

Figure1: Quartz-capped air vessel construction schematic

Determination of the average fluence rate in the quartz-capped vessel

Following the protocol of Bolton and Linden (2003), there are three critical factors in the calculation of the average fluence rate inside the quartz-capped vessel: the Petri dish factor, the divergence factor and the reflection factor. The Petri dish factor is a concept used in collimated beam testing in waterborne microorganism inactivation studies. In this case, the Petri dish factor is defined as the ratio of the average fluence rate passing through the quartz top of the quartz vessel to fluence rate at the central point of the top quartz plate. The Petri dish factor was determined by the grid reading method (Bolton and Linden 2003) with a radiometer (Model P-9710 with detector UV3718, Gigahertz-Optik, Germany). The divergence factor was actually determined by applying the inverse square law along the collimated beam path (Bolton and Linden 2003). The reflection factor was measured by conducting KI/KIO3 actinometry tests. Dishes with diameter of 91 mm and 8 mm depth were used to hold the actinometry solution mixed by 3 mm × 12 mm magnetic stirring bars. The solution was exposed to UV radiation with and without the quartz vessel cap covering above the 91 mm dishes for the same exposure time. The ratio of the irradiance values from the actinometry tests under the two covering conditions is the reflection factor of the quartz cap. A medium factor is not applicable in this case because the absorbance in air within the testing relative humidity for UV irradiance at 254 nm is negligible.

The average fluence rate in the quartz-capped vessel is given by:

$$[1]\ E_{avg} = E_0 \times F_{petri} \times F_r \times F_d$$

Where $E0$ is the incident fluence rate, as read by the radiometer with the detector at the center of the quartz plate (mW/mJ); F_{petri} is the Petri factor; F_r is the reflection factor and F_d is the divergence factor.

The average fluence (mJ/cm^2) delivered inside the vessel is then just the product of E_{avg} and the exposure time (s).

Bacillus subtilis spore culture and enumeration

Bacillus subtilis spore culture

Bacillus subtilis spores (ATCC 6633) were cultured with the Modified Schaeffer method. Frozen dry *B. subtilis* was pre-cultured in a 100 mL nutrient solution. The components of the nutrient broth solution are: 8.00 g/L, $MgSO_4$ï$7H_2O$; 0.25 g/L, KCl; 1.00 g/L Peptone(Fisher Sci. Canada) The flask was put into a shaker incubator to re-hydrate the frozen dry *B. subtilis* for 24 h at 37.5 ̣C at 180 rpm. In the biohazard fume hood, 1 mL of pre-cultured *B. subtilis* suspension was then inoculated into Modified Schaeffer (MS) nutrient media, consisting of $FeSO_4$ (1 μM), $MnCl_2$ (10 μM), $CaCl_2$ (1 μM). The baffled flasks were put into the shaker incubator (Innova, New Brunswick Sci) to culture the spores for 24 h at 37.5 ̣C and 180 rpm; the culture was allowed to grow for 15 days. Spore stain was used to verify the domination of the spores in the nutrient media. 5% Malachite green and 1% Safranin red stain solutions were used to stain the samples on a slide for 1 min and 30 s, respectively. The slide was then observed with a microscope to determine the domination of the spores. Spores are green and vegetative cells are red. Once the spores dominate the culture MS media, a centrifuge (Sorvall Refrigerated Superspeed Centrifuge, Mandel Sci) was used to harvest the spores (centrifugation time 20 min at 7500 rev/min). An 80˚C water bath was used to kill the vegetative cells. *B. subtilis* spores were stored in 50% ethanol in a 1L autoclaved bottle.

Bacillus subtilis spore enumeration

A pour plate method was used to enumerate the concentration of spores in the collecting liquids in the impingers. 9 mL of 0.1% Peptone (Fisher Sci. Canada) solution was transferred into each glass tube. A serial of 1/10 dilutions of the sample were made by transferring 1 mL of the sample into the 9 mL of the peptone solution. 15 mL of Nutrient agar (8 g nutrient broth powder, 16 g agar, 1 L MilliQ water) was transferred to each glass tube. The tubes filled with autoclaved agar were kept in a water bath at 50˚C.

1 mL of the desired dilution sample was transferred into a 100 mm culture plate. A methane flame was used to sterilize the top of the agar tubes. The agar was poured into the plate and the plate tilted until the agar-sample mixture was evenly distributed in the plate. It took 3 to 5 min for the agar to solidify. Culture dishes were put into the incubator (Forma Series II, Thermo, USA) upside down to incubate for 36 h. The spores were retained in their positions by the solidified agar. The colony forming units (CFU) in the culture dishes were counted. The valid range of the CFU counting was 30 to 300. For high log reduction rates, a CFU count below 30 was also treated as a valid count.

Inactivation tests

Inactivation tests of the aerosolized spores were performed under two relative humidity conditions: 50 to 60% and 70 to 83%. The RH conditions controlled by adjusting the ratios of the dry air to the saturated air from the nebulizer. The Relative Humidity meter probe (RH411 Thermohygrometer, Omega Inc.) was plugged into the quartz cell to measure the RH in the quartz cell.

The quartz-capped air vessel was run in a batch reactor mode to measure the UV inactivation rate of the *B. subtilis* spores. Spore-laden air was trapped in the quartz-capped vessel for designated time with or without UV radiation. Spore-free dry air from the flushing port was then injected to direct the spores toward the sampling impinger for collection. The inactivation levels were determined by enumerating the number of spores collected in the sampling impingers. The inactivation experiment was run under two RH levels: 50-60%, which is the typical relative humidity in living rooms (IUVA 2005b). The other RH was set to be 70-83% to see how the RH level affects the UV inactivation to the spores. For each RH level, the entrapped spores were exposed to different UV doses by controlling the exposure time. Cross contamination is a big concern in these inactivation tests. In order to avoid residual spores trapped in the quartz cell and in the valves and tubing, UV light was used to disinfect the residual spores in the cell, and high velocity spore-free dry air was used to flush the quartz cell and the valves and the tubings.

Model fitting

In most of the UV inactivation studies for air suspended microorganisms, the single stage exponential model is often used to fit the UV response curve. In UVGI system design, this model is regarded as sufficient to determine the UV output for the system. The single stage exponential model is given as:

$$[2] \quad S = e^{-kE_{avg}t}$$

Where S is the surviving fraction of the microorganism population, k is the UV susceptibility constant (cm^2/mJ), E_{avg} is the average UV fluence rate (mW/cm^2) in the reactor assuming complete mixing and t is the exposure time in seconds.

As an alternative model, a multi-target model has also been used by researchers to model the microorganisms' response to UV irradiance. It can account for the shoulder characteristics in the response curve. The form of the multi-target model was given by Severin et al. (1983) as:

$$[3] \quad S = 1-(1- e^{-k_m E_{avg} t})^{Nc}$$

Where k_m is the UV susceptibility constant (cm^2/mJ) and Nc is the number of the critical sites in the microorganism particles. According to the multi-target model hypothesis, the last site of the critical sites has to be hit by UV radiation to achieve inactivation in a microorganism or a microorganism clump. The multi-target model is commonly used to model clumping effects of the microorganisms in UV inactivation studies (Severin et al. 1983). Uvbiama (2005), used the multi-target model to describe the UV inactivation kinetics of the aggregated *Bacillus subtilus* spores in water. Since the spores used in this study and the ones used in the Uvabiama's study were cultured from the

same mother stock with the same method, the multi-target model was chosen to model the UV inactivation kinetics for easy comparison. The parameters k_m and N_C in the multi-target were estimated by applying least square criteria regression to the logarithm form of equation [3].

RESULTS

Fluence calculation

Petri dish factor, divergence factor and reflection factor were measured or calculated as per the Bolton and Linden (2003) protocol. By multiplying the average fluence rate by the exposure time, the fluence that the suspended spores received inside the cell was calculated.

Inactivation model parameter estimation

The inactivation results were used to fit the multi-target model and the curves are plotted as **Figure 2:**

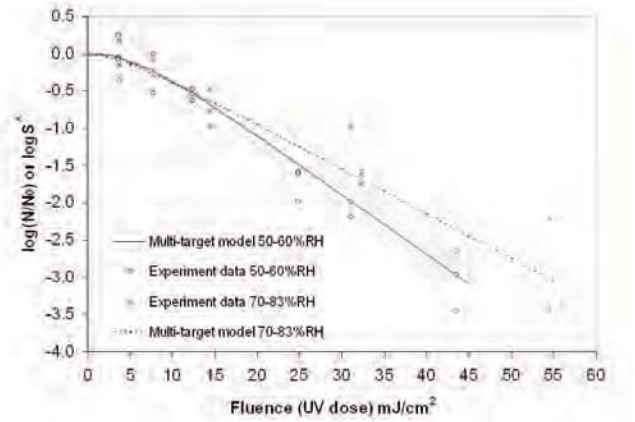

Figure 2: Aerosolized *Bacillus subtilis* spores UV response curves

k_m and N_C were estimated by the lease squares regression to be 0.17 cm²/mJ and 3 at RH = 50 to 60%, and 0.14 cm²/mJ and 2 at RH = 70 to 83%. k_m values in both RH ranges are close to some researchers' results with an average value of 0.153 cm2/mJ (Kowalski et al. 2005). The inactivation curves at RH = 70 to 83% and RH = 50 to 60% indicate that the susceptibility of *B. subtilis* spores in aerosol form is not sensitive to the relative humidity. Unlike vegetative microorganisms suspended in air, viable cells of the suspended spores are protected by the proteinaceous spore coats. During UV irradiation, these spore coats prevent the moisture in ambient environment from entering the cells; it is perceived that spores remain their metabolic activities and repairing ability from UV damage at the same level regardless of the outside RH conditions.

The k_m value is also closed to the K_m = 0.18 cm²/mJ reported by Uvbiama using the same batch of *B.subtilis* spores, which were inactivated by UV in water (Uvbiama 2005). This agreement of results shows that the *B.subtilis* spores' susceptibility to UV is not affected by the media: air or water. Note that at inactivation level of 3 log reduction, the CFU number was very low, with 1 CFU per dish in 6 dishes out of 9 Petri dishes. These data are not very reliable because CFU values per Petri dish below 10 are not considered as reliable colony counts.

The Joint Confidence Regions (JCR) of the estimation of the parameters at the 95% confidence level shows the Nc value ranges from 1 to 15 in this study. Typical N_C values of the *B.subtilis* spores suspended in water were reported as 8 to 9 (Uvbiama 2005), hence there is no statistical difference between the N_C values in the two media.

DISCUSSION

The well-mixed batch reactor apparatus used in this study has the following advantages for determining UV inactivation kinetics of airborne microorganisms compared to the approaches used by other researchers.

1. Because of the simple geometry of the well-mixed batch reactor, the average fluence rate can be determined easily and accurately, using the standardized protocol established for collimated beam systems (Bolton and Linden, 2003). The factors that affect the distribution of the fluence rate inside the quartz cell can be determined precisely by applying the protocol to the reactor. In contrast, the geometries used by other researchers (Fletcher et al. 2003; Miller and Janet 2000; Peccia et al. 2001) are more complex and the fluence rates were either measured by carrying actinometry at certain spatial points (Peccia et al. 2001), or by a series of radiometer readings within the UV reactor (Miller and Janet 2000). With these approaches many measurements must be made, and it is hard to determine the appropriate number of and location of the points where measurements should be made in order to calculate the average fluence rate of the reactor accurately.

2. The well-mixed reactor was operated in a batch mode. The fan installed on the bottom of the reactor ensured complete mixing of the spores suspended in the air. Vigorous re-circulation of the microorganisms ensured each of suspended microorganisms received an identical UV dose. The inactivation kinetics determined in the well-mixed batch reactor are independent of reactor geometry and mixing. They are, therefore, suitable for application in for CFD modeling, in which the stereotype of a UV reactor is virtually divided into numerous finite volume cells so that the local flow regime in each finite volume cell is regarded as completely mixed. In contrast other researchers used continuous-flow apparatuses in which a UV dose-distribution exists. In these systems, the measured inactivation is function of both the inactivation kinetics of the particular microorganism and the flow regime in the reactor.

3. The volume of the quartz-capped vessel apparatus is relatively smaller (1.5 L) compared to apparatuses used by other researchers (Fletcher et al 2003; Miller and Janet 2000; Peccia et al. 2001). A small vessel is preferred in bioassay testing, especially when pathogenic microorganisms are the study objects, because it is easier to control possible leakage of the microorganisms into the environment.

However, the apparatus also has its own limitations compared to the apparatuses used by other researchers. Since the volume of the quartz cell is small and the spore concentration was limited by the capacity of the nebulizer, the spore concentration in the trapped air was low. Thus the colony counts at 3 log reduction level were so low that the results were not regarded as very reliable results. Increasing the spores concentration in the nebulizer reservoir and using a 6-jet nebulizer may compensate for this limitation. As suggested by IUVA (2005a), a scaled-down or a full-scale physical model should be built if the purpose of the susceptibility study of a UVGI system is to account for the effect of flow conditions

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REFERENCES

Bolton, J. R., and Linden, K. G. 2003. "Standardization of methods for fluence (UV dose) determination in bench-scale UV experiments." J. Environ. Eng. - ASCE, 129(3): 209-215.

Fletcher, L. A. N., C J, Beggs. C B, Sleigh, P A, Donnelly, J and Kerr, K. 2003. " The Ultraviolet Susceptibilty of Aerosolised Microorganisms and the Role of Photoreactivation." CDROM Proceedings of the 2nd International Congress on Ultraviolet Technologies, Vienna, Austria, International Ultraviolet Association, Ayr, ON, Canada N0B 1E0.

IUVA. 2005a. "General Guideline for UVGI Air and Surface Disinfection System" (Vol. IUVA-G01A-2005), International Ultraviolet Association, Ayr, ON, Canada N0B 1E0.

IUVA. 2005b. "Guideline for Design and Installation of UVGI In-Duct Air Disinfection System" (Vol. IUVA Draft Guideline IUVA-G03A-2005), International Ultraviolet Association, Ayr, ON, Canada N0B 1E0.

Miller, S. L. and Janet, M. 2000. "Evaluation of a Methodology for Quantifying the Effect of Room Air Ultraviolet Germicidal Irradiation on Airborne Bacteria", Aerosol. Sci. Technol., 33(3): 274-294.

Kowalski, W.J., Bahnfleth, W.P., and Mistrick, R.G. 2005. "A Specular Model for UVGI Air Disinfection Systems", IUVA News, 7(1): 19-26.

Peccia, J. and Hernandez, M. 2001. "Photoreactivation in airborne Mycobacterium parafortuitum." Appl. Environ. Microbiol., 67(9): 4225-4232.

Peccia, J., Werth, H. M., Miller, S. and Hernandez, M. 2001. "Effects of relative humidity on the ultraviolet induced inactivation of airborne bacteria.", Aerosol Sci. Techol., 35(3): 728-740.

Severin, B. F., Suidan, M. T. and Engelbrecht, R. S. 1983. "Kinetic modeling of U.V. disinfection of water." Wat. Res., 17(11): 1669-1678.

Uvbiama, R.D. 2005. "The Effect of Upstream Treatment Processes On UV Inactivation of Microorganisms in Filtered Drinking Water", MSc. Thesis. Deptartment of Civil and Environmental Engineering, University of Alberta, Edmonton, AB Canada.

Xu, P., Peccia, J., Fabian, P., Martyny, J. W., Fennelly, K. P., Hernandez, M. and Miller S.L. 2003. "Efficacy of ultraviolet germicidal irradiation of upper-room air in inactivating airborne bacterial spores and mycobacteria in full-scale studies." Atm. Environ., 37(3): 405-419.