Evaluation of *Bacillus* Spore Survival and Surface Morphology Following Chlorine and Ultraviolet Disinfection in Water

Hadas Mamane[1]; Zuzana Bohrerova[2]; and Karl G. Linden, M.ASCE[3]

Abstract: The objective of this paper is to evaluate the change in *Bacillus subtilis* spore survival and dimensions following ultraviolet and chlorine disinfection in water. Disinfection was monitored by using tools such as atomic force microscopy (AFM), particle sizing by the electrozone sensing technique and fluorescence of spores after staining with an optical brightener. Results indicated that there was a change in the adsorbed fluorescence following chlorine; however, the magnitude of this change was only approximately twofold at 90% of spore kill. In addition, changes in spore particle-size distribution following chlorine occur at above 99.9% of spore kill. Even the roughness (RMS), width, and length of spores as measured by AFM change only after about 99% of spore killing with chlorine. Use of optical brighteners, AFM, and sizing are not sensitive enough for detecting the disinfection of chlorine-resistant spores and as expected no changes occurred with ultraviolet treated spores. Even though, these techniques may have the potential for determining oxidative disinfection and for the development of monitors and sensors of chemical disinfection for chlorine-sensitive microorganisms.

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CE Database subject headings: Disinfection; Particle size; Water treatment; Chlorine.

Introduction

Spores are ubiquitous in natural waters, are heterogeneous in species, and are very resistant to chemical and physical disinfections (Rice et al. 1996; Barbeau et al. 1999; Nieminski and Bellamy 2000). *B. subtilis* spores are generally resistant to chemical disinfection as the coat, a multilayered structure encasing the spore, serves as a barrier for chemical and enzymatic attack by sieving out such molecules (Driks 1999). Yet, ultrastructural and chemical changes in the outer spore coat were observed during chemical disinfection (Kulikovsky et al. 1975). However spore killing or injury by oxidative chemical species such as chlorine dioxide and hypochlorite are likely through oxidative damage to the proteins in the spore inner membrane, which may undergo conformational changes and become more permeable (Young and Setlow 2003; Cortezzo et al. 2004). The spore resistance properties against disinfectants do not depend solely on the sieving protection mechanism of the spore coat. The spore core contains the DNA which is protected against ultraviolet damage by small acid soluble DNA binding proteins (Setlow 1994; Atrih et al. 1996). However upon exposure to ultraviolet irradiation, a DNA photoproduct can accumulate in spores which is the unique thymine dimer 5-thyminyl-5,6-dihydrothymine, often referred to as the "spore photoproduct" (Spizizen 1958) and this photoproduct results in spore inactivation.

Because oxidants such as chlorine and ultraviolet irradiation have fundamentally different activities for water disinfection, it is likely that their physical effects on the spore surface would also differ. There are a number of methods to investigate the surface morphology of spores and gain insights into mechanisms of spore disinfection. Flow cytometry combined with fluorescent stains could potentially provide information on a variety of microbial cell properties. Particularly, fluorescent optical brighteners have tremendous potential as they are very bright fluorescent dyes when excited with ultraviolet light (Davey and Kell 1997). These stains have been used effectively for staining of fungal cell walls (Hejtmanek et al. 1990) and for the determination of cellular (Berglund et al. 1987) and bacterial (Mason et al. 1995) viability by flow cytometry. Atomic force microscopy (AFM) is a type of scanning probe microscopy that can resolve structural changes at the nanometer scale, with minimal sample preparation or alteration. Changes in cell surface morphology as a result of chemical treatment have been previously studied by AFM. Kasas et al. (1994) studied the morphology of *B. subtilis* cells in their exponential phase exposed to penicillin and observed in air by the contact mode, which first resulted in height change and then in release of the cell contents into the environment. Camesano et al. (2000) found that chemical treatments altered the bacterial surface roughness and correlated those values to the qualitative shape change in the cells while Chada et al. (2003) used AFM to characterize spore surface features. Previous studies validated the use of electrical sensing measurements of bacteria to obtain the mean cell volume (Kubitschek and Friske 1986). Schneider et al. (1997) suggested three methods to measure cell volume of endothelial cells which included electrical property methods, interference light microscopy, and AFM. They described the advantage

[1]Faculty Fellow and Lecturer, School of Mechanical Engineering, Faculty of Engineering, Tel-Aviv Univ., Tel-Aviv, Israel 69978 (corresponding author). E-mail: hadasmg@post.tau.ac.il

[2]Research Associate, Dept. of Civil and Environmental Engineering, Duke Univ., Durham, NC 27708-0287. E-mail: bohrerz@duke.edu

[3]Professor, Civil, Environmental, and Architectural Engineering, Univ. of Colorado at Boulder, Boulder, CO 80309. E-mail: karl.linden@colorado.edu

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of cell volume determination by AFM as it allows obtaining three dimensions with nanometric resolution and simultaneous surface and volume information especially for cells attached to a solid support.

The goal of this research was to investigate mechanistic differences between chemical (oxidants) and physical (ultraviolet) modes of inactivation on spore survival, through the measurement of spore surface morphology. Techniques used to monitor the surface of spores following disinfection included changes in the florescence of stained spores, changes in cell dimension obtained by AFM, and electrical property methods for volume measurement using the Coulter counter. These techniques were evaluated for use in monitoring disinfection.

Materials and Methods

Spore Preparation and Enumeration

Liquid cultivated *B. subtilis* spores (ATCC 6633) were prepared and obtained freeze-dried by Sommer, Institute of Hygiene, Medical University of Vienna, according to the method described in Sommer and Cabaj (1993). The spore batch was certified for ultraviolet validation testing of reactors by the Austrian standard (ONORM 2001). A working solution was prepared by resuspending the freeze-dried spores in buffered phosphate buffer saline (PBS) water (Fisher Scientific, United States) to yield a spore concentration of 10^6 CFU/mL. Spore concentration was determined after serial 10-fold dilutions, followed by distributing 1 mL aliquots of the suspension on empty agar plates, pouring the agar medium (plate count agar) at 45°C into the plates (about 15–20 mL), and incubating for 48 ± 4 h at 37°C. The purity of sporulation was between 90 and 95% when the plate counts were compared to count by phase contrast microscopy.

Low-Pressure Ultraviolet Irradiation System and Radiometry

A quasi-parallel beam bench scale ultraviolet apparatus (collimated beam) consisted of four 15 W low-pressure (LP) mercury vapor germicidal lamps emitting nearly monochromatic ultraviolet radiation at 253.7 nm. Ultraviolet exposures for producing standard ultraviolet fluence-response curves were performed in a bench scale "collimated beam" LP ultraviolet system (monochromatic output of 253.7 nm). A volume of 10 mL sample was placed in Petri dishes and samples were completely mixed. ultraviolet absorbance (200–400 nm) of the samples spiked with spores was measured in a spectrophotometer (Cary 100-bio, Varian Corp., Houston, Tex.). Ultraviolet fluence (mJ/cm^2) was calculated as the average irradiance multiplied by the exposure time. Ultraviolet incident irradiance (mW/cm^2) was measured at the surface of the liquid suspension using a radiometer (International Light IL1700 SED240/W, Peabody, Mass.) calibrated at 254 nm. The sensor is traceable to the National Institute of Standards and Technology. The average ultraviolet irradiance in the mixed sample was determined using the protocol described by Bolton and Linden (2003).

Chlorination

A freshly prepared chlorine stock solution from household bleach (Clorox; diluted in chlorine demand free water) was added to the PBS samples spiked with *B. subtilis* spores at a beginning target concentration of 4.5 mg/L free chlorine. Generally, 250 mL Erlenmeyer flasks were used with a working volume of 200 mL. The procedure involved sufficient mixing by shaking the samples at room temperature (19–22°C) at 200 rpm. The flasks were wrapped in aluminum foil. Samples were collected at a minimum of eight targeted contact times (1, 10, 30, 60, 120, 200, 250, and 300 min), and free and total chlorine residuals were measured using the N,N-diethyl-p-phenylenediamine chlorine assay kit (HACH, Loveland, Colo.) following the manufacturer's instructions. Sterile sodium thiosulfate (0.5 mL of 10% Na_2SO_3 solution for 5 mL of sample) was added to stop the action of chlorine. The samples were plated after serial dilutions. Ammonium was measured using Hach DR/2000 spectrophotometer methods (the low range test of 0–2.5 mg/L N-NH_3) and was not detected in any sample. The free chlorine dose Ct (mg min/L) was calculated as the time averaged chlorine concentration (mg/L) multiplied by the contact time T (min). The averaged chlorine concentration was obtained by integrating free chlorine residuals over time using the trapezoidal rule, as detailed in Bohrerova and Linden (2006).

Data Presentation

The mean concentration [colony forming units (CFU)/mL] of spores spiked in suspension without ultraviolet or chlorine exposure was taken as the initial concentration, N_0 (approximately 10^6 CFU/mL), while the mean concentration per ultraviolet fluence or chlorine dose was taken as N_d. The level of inactivation was expressed as the $\log_{10}$ transformation for N_0/N_d plotted as a function of the ultraviolet fluence or chlorine dose. Regression analysis was used to fit the linear sections of the log inactivation curve for disinfection experiments because *B. subtilis* spores follow the delayed Chick-Watson model both with chemical and physical disinfection (Sommer and Cabaj 1993; Barbeau et al. 1999).

Staining of Spores with Optical Brightener

The method described herein was developed to monitor disinfection efficiencies of spores stained with a fluorescent dye. The method of staining spores with optical brightener was adapted from Davey and Kell (1997) with a slight modification with dye concentration and filtering of stained spores prior to analysis. *B. subtilis* spores at a concentration of 10^6 CFU/mL were suspended overnight in ethanol to a final ethanol concentration of 70%. Fixed spores were centrifuged at 5,000 g for 10 min and washed in PBS (Fisher Scientific, BP399), which is the sheath fluid for flow cytometry. A stock solution of Tinopal CBS-X was prepared at a concentration of 1000 μg/mL and spores were suspended in a Tinopal concentration of 25 μg/mL. Due to fluorescence background and dye carryover during flow cytometry analysis, the fluorescent solution was removed after staining. Thus, after 2-min contact time, spores were filtered on a 0.2-μm Nylon filter, and the filter was resuspended in PBS to release the dyed spores into suspension. Filtering the stained spores with the fluorescent suspension, and resuspending the fluorescent spores in cold PBS yielded the brightest fluorescence. Control studies were performed to optimize (a) volumetric ratio of Tinopal to spore at a spore concentration of 10^6 CFU/mL (1:20–10:20); (b) concentration of Tinopal (20–200 μg/mL); and (c) spore concentration at fixed dye concentration.

Flow Cytometry

The fluorescence of the *B. subtilis* spores dyed with Tinopal was measured by flow cytometry. The flow cytometer used was a BD FACSVantage that included Turbosort, three lasers, and six fluorescence detectors (Becton Dickinson Immunocytometry Systems, San Jose, Calif.), housed at the Duke University Comprehensive Cancer Center. Spores were excited by a 15-mW argon air-cooled laser at 488-nm wavelength. The fluorescence intensity emitted by each stained spore was detected by a FL1 detector after passing through a 405-nm band-pass filter (395–415 half peak width). Gating was performed in a spore dot plot using the characteristic forward (FSC) versus orthogonal light scatter (SSC) to eliminate background noise or other particles in the sample. FSC is related to the particle size, while SSC is related to the particles' internal granularity and complexity. Data for at least 10,000 spores were acquired per sample. These flow cytometer data were gated and extracted using software WinMDI Version 2.8.

Volume and Size Measurements

AFM

B. subtilis spores at a volume of 50 μl and concentration of 10^8 CFU/mL were deposited on freshly cleaved mica (Muscovite Mica-V1, EMS, Washington, Pa.) and allowed to air dry before imaging. In the second sample preparation, a filter was placed on a stainless steel filter holder for 13-mm disk filters. Spores were trapped by passing a spore suspension through a 0.4-μm, 13-mm-diameter polycarbonate Isopore membrane filter (Millipore, Bedford, Mass.). The filter was attached to a cover glass slide using double-faced tape that is fixed on a metallic disk. AFM imaging was done immediately.

Tapping mode images were collected with a multimode Nanoscope IIIIa (Digital instrument, Veeco, Santa Barbara, Calif.) with nanoscope 6.12r1 software. In the tapping mode the cantilever scans the surface vertically at constant root mean square (RMS) amplitude. Roughness analysis is applied to an image, according to the relative heights of each pixel in the image based upon least-squares calculations. Specifically, RMS roughness is determined with a best fit of all height points. All experiments were conducted with etched silicon probes, model TESP7 cantilevers, length of 110–140 μm, resonant frequency of 230–410 μm, a spring constant of 20–80 N m^{-1}, and a rectangular shape tip. A real-time planefit image was applied. The volume of the cell by AFM (V_{AFM}) was calculated assuming an elliptical cross section (Nishino et al. 2004) by the following equation:

$$V_{AFM} = \frac{\pi h w}{4} l \quad (1)$$

where w=width, h=height, and l=length of spore cell in micrometers.

Particle-Size Counter

A Multiziser 3 Coulter Counter (Beckman Coulter, Miami, Fla.) was used to obtain the size and volume of spores and compare it to results obtained by AFM. Particles (spores) suspended in an electrolyte solution (Isotone II, Beckman Coulter, Miami, Fla.) are drawn through an aperture with electrodes on the sides that result in increased resistance, when current is applied, proportional to the actual volume of the particle. Distributions of counts, count per mL (μm/mL) or volume per mL (μm^3/mL), were obtained as a function of particle diameter.

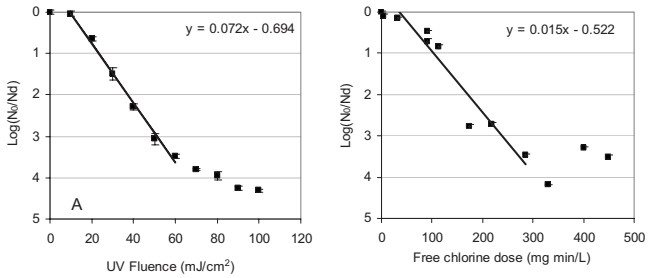

Fig. 1. Dose-response curves for *B. subtilis* spores exposed to (a) ultraviolet disinfection; (b) chlorine disinfection. Error bars represent standard deviation.

Results

Disinfection by Ultraviolet and Chlorine

Fig. 1(a) illustrates the ultraviolet dose-response curve for *B. subtilis* spores. A shoulder is observed up to a ultraviolet fluence of 10 mJ/cm^2. Subsequently, a first order linear relationship is observed between the spore logarithmic survival rate and ultraviolet fluence up to 60 mJ/cm^2. With a further increase in ultraviolet fluence, a reduced inactivation rate (tailing) was observed. The fluence-response data in the first order region were fitted using linear regression resulting in a ultraviolet sensitivity coefficient of 0.072 cm^2/mJ. Ultraviolet spore inactivation kinetics is in agreement with previously published data [U.S. Environmental Protection Agency (USEPA) 2006].

Total chlorine in the amount of 4.5 mg/L was added to the spore suspension. After 10 min of contact time the free chlorine dropped to 2.5 mg/L. The *p*H of the spore suspension remained constant throughout the experimental exposure time at a level of 7.5 ± 0.06. The inactivation of the spores by chlorine is illustrated in Fig. 1(b). At low chlorine doses, a shoulder or lag in inactivation occurred for the spores indicating that a minimum dose is needed to initiate inactivation. After the shoulder, inactivation of spores followed first order kinetics resulting in a chlorine sensitivity coefficient of 0.015 L/mg min. Tailing seemed to occur after a free chlorine dose of approximately 280 mg min/L. In previous studies, the inactivation of *B. subtilis* spores disinfected by chlorine showed a similar pattern of lag, linear first order, and tailing (Barbeau et al. 1999). For three-log inactivation (99.9%) of the spores used in this study, an ultraviolet fluence of 50 mJ/cm^2 or a chlorine dose (Ct) of 240 mg min/L is required. For comparison, the Ct values for three-log inactivation of viruses and Giardia cyst using chlorine are 4 and 104 mg min/L, respectively, based on a free chlorine residual and under specified conditions (USPEA 1991). Chlorine is an effective virucide however it was limited for inactivating protozoa and spores. The typical application dose for ultraviolet is between 20 and 100 mJ/cm^2 and chlorine is 1–6 mg/L (Crittenden et al. 2005); however, in this study due to the long contact times required with chlorine, ultraviolet is more effective for inactivation of liquid cultivated *B. subtilis* spore.

Impact of Disinfection on Fluorescence of Stained Spores

The effect of disinfection on spore morphology was determined by direct staining of the spores with a fluorescent optical brightener (Tinopal) after exposure to ultraviolet or to chlorine to allow the measurement of fluorescence. The spore suspension after sev-

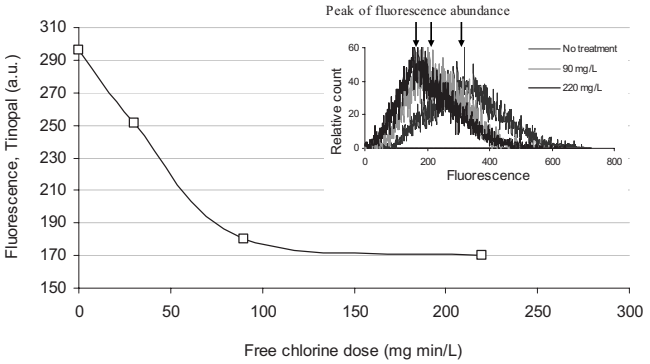

Fig. 2. Change in peak of fluorescence abundance as a function of free chlorine dose (Ct) for *B. subtilis* spore. The insert represents fluorescent histogram overlays of spores treated with different chlorine doses.

eral procedures (described in methods section) fluoresces blue under a flow cytometer equipped with an argon laser (488 nm). Plotting spore count versus Tinopal fluorescence yielded a correlation between fluorescence and chlorine dose. The change in fluorescence with the highest count number termed "peak of fluorescence abundance" as a function of chlorine dose (Ct) for *B. subtilis* spores is illustrated in Fig. 2. Up to about one-log spore inactivation (about 100 mg/L chlorine), the fluorescence decreases linearly with a slope value of -1.27 and intercept of 293 units, and above that the florescence of cells remains constant (tails) at about 170 units. The peak fluorescence of the control (spore without ultraviolet or chlorine exposure) and the spore sample exposed to ultraviolet disinfection fluence of 60 mJ/cm^2 were nearly identical.

Impact of Disinfection on Size Parameters of Spores

AFM can be used to determine quantitatively the effect of chemicals on depth, length, width, and surface roughness by measuring the height deviation (Camesano et al. 2000). A representative AFM image of *B. subtilis* spores prior to any treatment is illustrated in Fig. 3 and alterations in spore dimension, topography, and RMS due to exposure to chlorine are shown in Fig. 4. The data were collected from thirty individual intact cells and averaged. Cells that were totally disintegrated were not considered in the size averaging.

An increase in chlorine dose resulted in a decrease in surface roughness and size parameters as analyzed by AFM. A Ct of 90 mg min/L which corresponds to approximately 0.75-log inactivation of spores does not result in any change in the average dimensions of spores. A Ct of 220 mg min/L is required for three-log inactivation of spores, which does not change the average width or depth of spores, but results in a small change in spore average length from 1.5 to 1.44 μm, and in an increase in STD. Even if the standard deviation (STD) overlaps, the larger STD in length means that exposure to chlorine results in a larger variation which is observed first in cell length but also in cell width and depth. Kasas et al. (1994) investigated the impact of antibiotic on growing bacteria as analyzed in air by AFM and observed first changes in bacteria length and after that release of cell content. The average RMS for *B. subtilis* spores prior to any treatment was 230 nm, and a decrease in RMS value occurred with an increase in chlorine dose. Moreover, a substantial decrease in length, width and depth of spores occurred only at a Ct above 400 mg min/L,

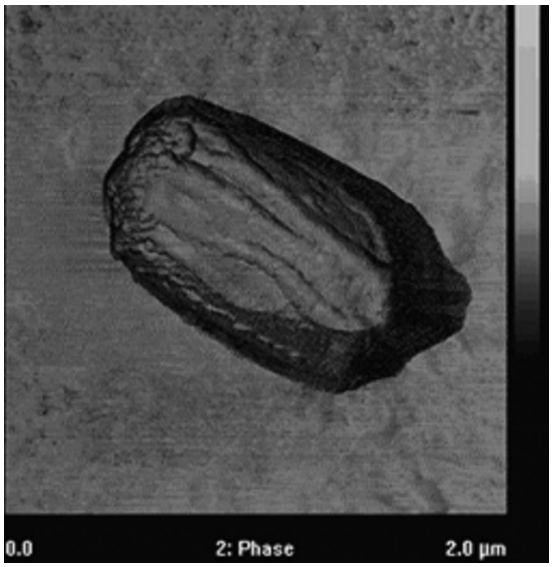

Fig. 3. Tapping mode AFM image of *B. subtilis* spore cell at phase mode

while RMS changes occurred at doses lower than that. With exposure to an ultraviolet fluence of 60 mJ/cm^2 no difference occurred in any of the measured parameters.

Fig. 5 illustrates tapping mode AFM images of *B. subtilis* spores on 0.4 μm Isopore filters, prior and after exposure to chlorine at concentrations of 220 and 2,400 mg min/L. As previously mentioned, a Ct of 240 mg min/L is required for a three-

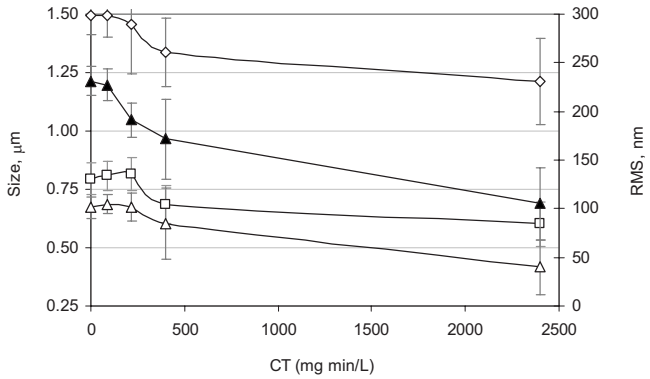

Fig. 4. *B. subtilis* spore dimension and RMS changes due to exposure to chlorine, as analyzed by AFM. Error bars represent standard deviation ($n \geq 30$).

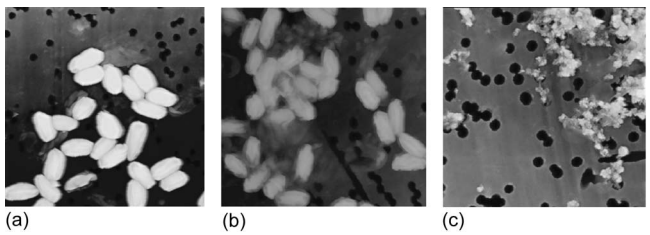

Fig. 5. Changes in *B. subtilis* spore surface morphology studied by AFM: (a) no treatment; (b) cells exposed to 220 mg min/L chlorine; and (c) cells exposed to extreme chlorine concentration of 2400 mg min/L

Table 1. Effect of Chlorine and Ultraviolet Treatment on *B. Subtilis* Spore Volume and Average Diameter as Measured by AFM or Particle Coulter

	V_{AFM} (μm^3)	V_{PAR} (μm^3)	Dp_{AFM} (μm)	Dp_{PAR} (μm)
No treatment	0.63	0.34	1.06	0.82
90 mg min/L	0.65	0.43	1.07	0.83
220 mg min/L	0.63	0.42	1.06	0.84
400 mg min/L	0.44	0.31	0.94	0.81
2400 mg min/L	0.25	0.34	0.78	0.81
60 mJ/cm^2	0.64	0.35	1.07	0.81

Note: V_{AFM}=volume of the cell obtained by AFM; V_{PAR}=volume of the cell obtained by particle counter; Dp_{AFM}=diameter of the cell obtained by AFM; and Dp_{PAR}=diameter of the cell obtained by particle counter.

log inactivation of spores, which does not change the average dimensions of the spores significantly; however, the surface of the spores under AFM appears cloudy as can be observed qualitatively in the image of Fig. 5(b). Increasing CT to very high and non realistic values with regard to chlorine disinfection (2,400 mg min/L) will cause cells to decrease in dimension, as chlorine impairs the cell membrane and also results in leakage of cell components that seem to increase stickiness and aggregation [Fig. 5(c)].

Table 1 summarizes the effect of 60 mJ/cm^2 ultraviolet and 90–2,400 mg min/L chlorine on the average diameter and volume of *B. subtilis* spores. High resolution AFM images of the spores revealed an average size of approximately 1.5 μm (length) $\times$ 0.8 μm (width) $\times$ 0.7 μm (depth) with an oval shape (Table 1, no treatment). As a comparison, Chada et al. (2003) sized spores by AFM and found that *B. subtilis* spores were in the size range of 1.2×0.8 μm, while *B. anthracis* was larger in the size range of 1.6×0.9 μm. Zolock et al. (2006) also sized bacterial spores with AFM and found that they were generally ellipsoidal, 1.5–2 μm long and 750–1 mm wide. An average spherical diameter of approximately 1 μm is obtained for *B. subtilis* spores by AFM while a smaller average of 0.82 ± 0.22 μm was obtained with the Coulter counter analyzer.

Fig. 6 illustrates the particle-size distribution obtained with the Coulter counter for *B. subtilis* spores as a function of chlorine dose and ultraviolet fluence. This figure shows that the shape of the distribution curve and the average particle size is consistent across an increase in chlorine dose. However based on particle sizing, the total particle count of the spores (number/mL) at a Ct value of 400 mg min/L and above decreased. As expected with ultraviolet, no change was observed in cell size using the Coulter counter.

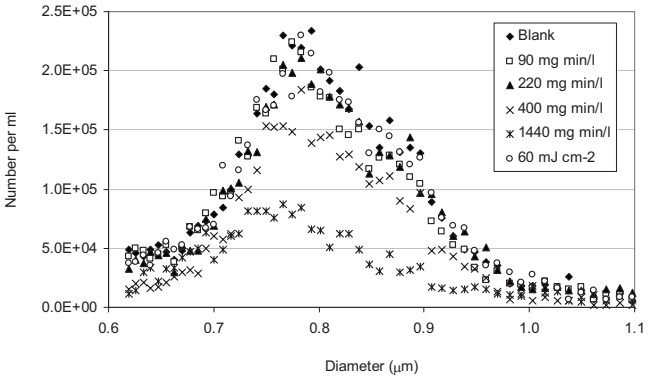

Fig. 6. Particle-size distribution of *B. subtilis* spores as a function of chlorine dose and ultraviolet fluence

Discussion

Impact of Disinfection Monitored through Cell Volume Change

Both AFM and the Coulter counter were used to compare the impact of disinfection through changes in cell volume dimensions. AFM provides height, width, and depth of spores from which volume can be calculated according to Eq. (1). The Coulter counter provides the volume of the spore that actually displaces a volume of the conducting liquid in which the spores was suspended.

The average volume of the spores obtained by AFM (V_{AFM}) was 0.63 ± 0.06 μm^3 and by the Coulter counter (V_{PAR}) was 0.34 ± 0.02 μm^3 (Table 1, no treatment). Nishino et al. (2004) measured for vegetative *B. subtilis* cells an average V_{AFM} of 0.18 μm^3 and an average V_{PAR} of 0.51 μm^3 with *Bacillus* in the late-exponentially growth phase. However average volume data are not comparable to spores as they were obtained for vegetative cells and also for different *B. subtillis* strain. Carrera et al. (2006) sized *B. subtillis* 1,031 by transmission electron microcopy (TEM) and found that length varied between 0.89 and 1.53 μm and width between 0.41 and 0.67 μm and resulted in a volume of 0.160 μm^3. To calculate volume they used also the ellipsoid equation but since TEM does not provide depth measurement they used the equation: $V=\pi LW^2/6$, in which L represents the length and W the width. Using this equation and the data on spore sizing by Chada et al. (2003) (i.e., 1.2×0.8 μm) will yield a spore volume of 0.4 μm^3 and a volume of 0.5 μm^3 for the spores used in this study. Thus spore volume measurements will depend on method for measurement, equations used for obtaining volume, spore strain, and artifacts in measurements for each technique used. Volume artifacts could occur in both techniques. The Coulter analyzer is widely used for cell sizing however it requires suspending cells in an electrolytic solution that can alter the size of particles. With AFM, dehydration could occur after filtration and imaging in air. On the other hand, AFM images can appear larger than their actual size because the cantilever cannot trace the surface of the material at the exact edge. Binnig et al. (1986) stated that if bacterial cells are placed on Mica then the depth measurements are more reliable. In this research depth measurements were conducted with spore samples placed on mica; however, it is not clear how the overall artifacts impacted the measured cell volume by AFM. It is suggested to use the filter holes to check the shape of the AFM cantilever tip (Kasas et al. 1994) and quantify artifacts in the cell length and width. A measured average size of 0.43 μm (n=14) was observed, while the calibrated size of the filter pores as stated by datasheet obtained from the manufacturer was 0.4 μm, which is a difference of 6%. Taking into consideration 6% of the length and width measurements of AFM (Table 1) will result in a corrected spore volume of 0.55 μm^3 instead of 0.63 μm^3, which is somewhat closer to the volume obtained by the particle analyzer.

Fluorescence for Monitoring of Spores Disinfection Efficiency

Tinopal and other optical brighteners are used to discriminate between microorganisms and could be used to possibly detect

pathogens (Davey and Kell 1997) and for cell viability studies (Mason et al. 1995). Moreover, Widmer et al. (2002) found that the scatter signal during the flow cytometry of ozone exposed Giardia cysts confirmed particle disappearance (decrease in cyst concentration) with an increase in ozone dose. Thus, the use of flow cytometry measurements of microorganisms stained with optical brighteners could potentially be an easy and useful technique for monitoring chemical disinfection, microorganism concentration, and for development of sensor technologies.

The total change in fluorescence as measured in our study was a function of the chlorine concentration, and the florescence of cells decreased linearly with an increase in chlorine dose up to one-log spore inactivation (Fig. 2). Fluorescence measurements were very sensitive to up to one-log inactivation of spores by chlorine at a dose of 100 mg min/L, which is much higher than typical chlorine doses. Thus the fluorescence of stained spores can apparently detect up to one-log spore inactivation, but this method may have more potential with chlorine sensitive microorganisms such as vegetative bacteria because a larger range of inactivation can be determined.

No observed changes in spore fluorescence have occurred with an increase in the ultraviolet fluence up to 60 mJ/cm^2. Thus, the fluorescence method cannot be used to detect disinfection efficiency of spores exposed to ultraviolet at a monochromatic 254 nm, and spores exposed to ultraviolet can serve as a control for chlorine based disinfectants with this fluorescence dye method.

Spore membrane oxidation by chlorine can possibly result in intermediate states of injury that may not be detected in spore viability tests. For example, Berney et al. (2007) used live/dead fluorescent commercial kits with flow cytometry to detect intermediate states of cell injury. Accordingly, optical brighteners combined with flow cytometry may be used to potentially study injury of membrane due to oxidation. More research however should be conducted to correlate between the levels of inactivation by chlorine as determined by plate counting to cell death or intermediate injury steps.

AFM for Monitoring of Spores Disinfection Efficiency

Camesano et al. (2000) found that exposing spores to most chemical treatments resulted in an increase in RMS, indicating that surfaces became rougher. Stoimenov et al. (2002) found holes in the spore cell wall, created by chlorinated metal oxide nanoparticles that significantly changed spore profile, height, and increased RMS, as compared to total destruction of *E. coli*. It lost its distinctive cell membrane shape and the cell contents leaked out. Both studies showed that chemical treatment increased the RMS while in the present study the average RMS did not change at typical chlorine doses for spores. RMS however decreased only at high chlorine exposure (Fig. 4) indicating that a smoother surface was probably created because of the high surface oxidation leaving cells smoother only for the few remaining intact cells prior to lysis. Only the intact spores that retained a rigid cell structure were evaluated for RMS; thus the cells that were damaged and lysed were not evaluated. The extreme exposure to chlorine that resulted in almost complete cell rupture is shown in Fig. 5. Kasas et al. (1994) described this release of cell content to the environment as a change to ghost appearance and after that amorphous cell material remains. This ghost appearance [Fig. 5(b)] followed by amorphous cell remains Fig. 5(c)] was also apparent in this study. In addition, possibly the dehydration effect on bacterial surface characteristics during the ex situ imaging process may have impacted spores cells; however, all cells were imaged similarly.

The effect of chlorine and ultraviolet treatment on *B. subtilis* spore volume and average diameter as measured by AFM or Coulter counter is presented in Table 1. It is problematic to compare the volume of spores by AFM and by the particle analyzer due to the following reasons:

1. The threshold particle size with the Coulter counter was about 0.75 μm (with aperture used in this study); thus spores smaller than 0.75 μm that underwent lysis were not sized.
2. All the spores at various sizes that were sized (above 0.75 μm) could have lysed similarly. This may explain why the average size of the spores remained similar with chlorination while total count (number of spores per mL) decreased.
3. On the other hand, shrinkage of treated spores during the drying step prior to AFM analysis may occur. Plomp et al. (2005) found that the dimensions of individual *Bacillus atrophaeus* spores decrease reversibly by 12% during a transition from fully hydrated to air-dried state.
4. AFM was limited in the number of particles it was possible to analyze but spores smaller then the threshold for particle analysis (<0.75 μm) that were still with intact shape were sized.

Schneider et al. (1997) described that AFM is also adapted to measure dynamic changes in biological samples; thus it is suggested to investigate in future studies the time based dynamic changes of chlorination on cell surface morphology and volume. Average spore dimension measured by AFM in terms of length, depth, and width cannot be used as a surrogate for spore viability as noticeable changes in these average size parameters occurs at a dose of 220 mg min/L and above, which is responsible for three-log inactivation by chlorine.

Application with Natural Water Samples

Optical brighteners can be used as a rapid indicator easily applied with flow cytometry with several constraints as follows: (a) can penetrate also living cells with perturbed membranes and (b) stains also algal and plant cell walls and can stain cellular debris (Berglund et al. 1987). However they suggested overcoming these constraints by gating out all the positive events and subtracting all nonviable signals including cell debris that may interfere with analysis. In another study Blatchley et al. (2008) used dyed microspheres combined with flow cytometry to determine ultraviolet reactor performance in surface water samples. Their goal was to capture the microspheres and discriminate them from background natural water constituents and ambient particles. Samples were prepared by filtration and resuspension in deionized water for analysis by flow cytometry. Because the size and fluorescence of the particles were known they were able to improve discrimination against background by correct gating.

Thus using stained spores as used in this study with optical brighteners have a potential to be applied also in natural water samples, provided that this method is used on relatively low turbidity waters, sufficiently large cell population, and optimized size parametrization and gating. In addition, these methods are targeted for monitoring in drinking water samples that is relatively "clean" with high ultraviolet transmittance.

To use AFM for detecting cells in natural water samples, cells have to be concentrated on filters and also differentiated from other nonliving particles that fall in the same size range as bac-

teria. Nishino et al. (2004) successfully differentiated and counted bacterial cells by AFM from nonliving particles by analysis of the cross section of particles, where rough peripheral lines indicated non living cells. However this approach is not a practical approach with natural water samples.

Conclusions

Since ultraviolet is well known to inactivate spores by DNA damage and DNA is in the spore core, it seems that analysis of the surface properties of ultraviolet inactivated spores, as expected, did not provide new information. Therefore the ultraviolet treated spores may be an excellent control for the changes seen with the chlorine-treated spores.

Results indicate that there is a change in the adsorbed Tinopal fluorescence upon spore killing by chlorine. However, the magnitude of this change is only approximately twofold, and even this is achieved with only 90% (one log) of spore killing. It seems extremely unlikely that this type of change will have any real utility in assessing spore killing for the chlorine-resistant spores used in this study.

In addition, there are no changes in spore particle count and particle-size distribution until 99.9% (approximately three logs) of the spores are killed by chlorine. In other words, the changes in spore particle-size distribution (at a Ct of 400 mg min/L and above) take place due to spore death followed by damage to spores that become leaky and lose distinctive shape. Even the roughness parameter (RMS) hardly changed (if at all) after 90% (one-log) spore killing following chlorine exposure and only at about 99% (two-log) inactivation were changes in RMS observed. These results indicate that use of optical brighteners, AFM and particle sizing are not sensitive enough for detecting disinfection of chlorine-resistant spores however changes will probably be noticeable with sensitive microorganisms. These types of studies also have the potential for determining oxidative disinfection by chlorine based disinfectants or other oxidants such as OH radicals and ozone. Since changes in cell morphology due to chlorine disinfection may be detected by surface characteristics, the development of monitors and sensors of chemical disinfection and oxidation versus cell death or injury based on these surface properties appears promising.

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