

Relationship between physiochemical properties, aggregation and u.v. inactivation of isolated indigenous spores in water

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ABSTRACT

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Aims: The objective of the study was to compare ultraviolet (u.v.) inactivation kinetics of indigenous aerobic spores in surface water with their laboratory-cultured spore isolates and to investigate the relationship between physicochemical characteristics and u.v. inactivation kinetics of spore isolates.

Methods and Results: Lake water samples were analysed for the presence of indigenous aerobic spores. Different bacterial isolates from the heterogeneous indigenous population were genetically characterized, resporulated and examined for hydrophobicity, surface charge, particle size distribution and survival at different u.v. 254 nm fluence levels. Cultured isolated spores exhibited a three-stage inactivation curve consisting of shoulder, first order and tailing regions whereas indigenous spores exhibited only one stage of linear kinetics. Hydrophobicity of the *Bacillus* spore isolates was inversely related to the extent of u.v. inactivation before tailing occurred.

Conclusions: Tailing in the u.v. inactivation curves results from aggregation of a portion of the spore population because of hydrophobic interactions, supporting the link between aggregation of spores, hydrophobicity and u.v. inactivation.

Significance and Impact of the Study: Evidence of the link between spore physicochemical parameters and u.v. disinfection performance furthers the understanding of factors that affect inactivation of microbes in natural waters supplied to drinking water treatment plants.

Keywords: *Bacillus* spore, hydrophobicity, mathematical model, particle size, shoulder, tailing.

INTRODUCTION

Spores are ubiquitous in natural waters, originating from the soil. They are generally nonpathogenic, heterogeneous in species and very much resistant to disinfection (Rice *et al.* 1996; Barbeau *et al.* 1999; Nieminski and Bellamy 2000). Although indigenous spores naturally occur in water, in research studies, spores are typically prepared for study using laboratory culturing techniques. Within the same spore strain, culture conditions of many organisms can affect their sensitivity to ultraviolet (u.v.) (Severin *et al.* 1983; Sommer and Cabaj 1993; Nicholson and Law 1999) or to

chlorine dioxide disinfection (Chauret *et al.* 2001). Under the same culture conditions, researchers found a fivefold difference in CT values necessary for 2 log inactivation of various strains of isolated aerobic spores from water sources exposed to chlorine (Barbeau *et al.* 1999). The level of spore u.v. resistance was attributed to the environment where spores were sporulated. Spores extracted from the natural soil environment were more resistant to u.v. when compared with their laboratory-cultured isolates, which were also comparable with typical laboratory strains (Nicholson and Law 1999). Clearly, the inactivation of spores depends not only on the disinfection strategy but also on the nature of spores (indigenous vs cultured), the spore strain and the culturing method.

The kinetics of u.v. inactivation of microbes in water is often more complex than a simple log-linear inactivation.

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Many organisms exhibit a u.v. fluence–response curve characterized by shoulder behaviour at low u.v. fluence, log-linear inactivation at mid-range fluence and tailing at high u.v. fluence, as illustrated in Fig. 1. Laboratory cultured *Bacillus subtilis* spores were described as a micro-organism with a shouldered u.v. irradiation survival curve that could be mathematically modelled (Cabaj *et al.* 2001); however, it is complex to predict the tailing phenomenon mathematically (Chiu *et al.* 1999). Spore repair systems operate during spore germination (Setlow 1992) and up to certain u.v. fluence may repair damage and result in a shoulder characterized by the multi-hit theory or by the multi-target theory (Harm 1980). Tailing can be regarded as an artefact because of heterogeneity of the population of micro-organisms, heterogeneity of treatment, lack of precision in enumeration of low concentration of survivors, presence of aggregates in spore suspension (Cerf 1977) or microbes associated with particles without a direct exposure pathway to u.v. light (Loge *et al.* 2001).

Hydrophobicity and surface charge play a role in microbial surface adhesion; however, the magnitude of surface charge in increasing or decreasing attachment of microbes to surfaces is not well understood (Flint *et al.* 2000). It was previously shown that different spore species possess different hydrophobic characteristics (Rosenberg *et al.* 1980; Doyle *et al.* 1984; Koshikawa *et al.* 1989) and hydrophobicity plays a major role in attachment or adhesion of *bacillus* spores to surfaces with the most hydrophobic spores having greater affinity towards hydrophobic surfaces (Ronner *et al.* 1990; Faille *et al.* 2002). However, the relationship between hydrophobicity and surface charge of spores to the pattern of the u.v. inactivation curve is currently unknown. It was hypothesized that a spore provides a surface for attachment of another spore and, therefore, hydrophobic spores can aggregate with each other. Consequently, the presence of tailing in the fluence–response curve of spores, if, because of spore–spore or

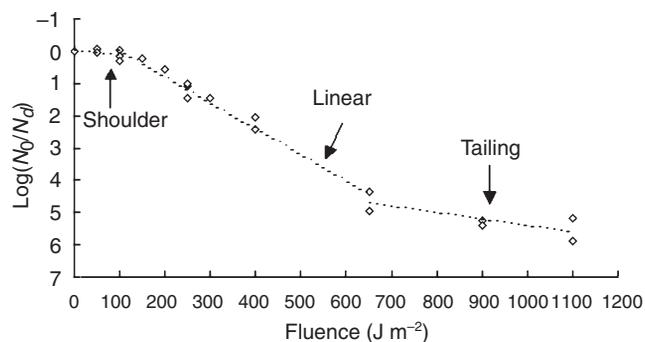


Fig. 1 Ultraviolet fluence–response curve characterized by shoulder behaviour at low u.v. fluence, log-linear inactivation at mid-range fluence, and tailing at high u.v. fluence for isolated spore ENV 1

spore–particle aggregation, may be a characteristic of the surface properties of the spores.

In a previous study, indigenous aerobic spores occurring naturally in lake water were found to exhibit first order linear u.v. inactivation kinetics without shoulder or tailing observed (Mamane-Gravetz and Linden 2004). However, the heterogeneity of the indigenous spore population studied, raised the question of the relative u.v. resistance of their pure cultured isolates. In this research, the goal was to characterize the u.v. fluence response behaviour of indigenous natural spores in unfiltered water sources compared with their pure, laboratory-cultured isolates. The specific objectives were to (i) isolate the heterogeneous population of indigenous aerobic spores producing various environmental spore strains, (ii) compare and mathematically model the u.v. inactivation kinetics of isolated spores, (iii) compare the inactivation kinetics between isolated spores with the indigenous aerobic spores and (iv) investigate the role of surface charge, hydrophobicity, and particle size distribution (PSD) on u.v. inactivation kinetics for each isolated spore type.

MATERIALS AND METHODS

Indigenous and isolated spore preparation and enumeration

Indigenous spore preparation, described by Nieminski and Bellamy (2000), consisted of preincubating 100 ml of raw water at 35–37°C for 30 min, followed by pasteurization for 15 min at 65°C with a shaking water bath and then placing the flasks in ice water. Samples were membrane-filtered (0.45 µm, 47 mm) and placed on a pad, to which 1.45 ml tryptic soya broth (TSB) was added, to determine colony counts within 22–24 h of incubation at 35°C as adapted from Barbeau *et al.* (1997). The total count of heterogeneous population of indigenous spores that germinated to vegetative cells on the membrane filter were determined by enumerating all the colonies that grew on the membrane (Mamane-Gravetz and Linden 2004). Indigenous spores were sampled from the raw water of Lake Michie, NC, a source for the two primary water treatment plants in Durham (NC, USA). Various colony types appeared on the membrane filter and were isolated into different bacterial strains according to the different colony morphologies. A wrinkled big colony with an irregular noncircular shape was isolated and named ENV 1, a slimy 2–3 mm colony was isolated and called ENV 2, while a yellow small 1 mm colony that appeared on the filter was isolated and named ENV 3. A small amount of inoculum was collected from a single colony on the membrane and streaked on solid agar. The pure culture was inoculated into TSB and after overnight incubation, the germinated spores were inoculated

on Schaeffer solid media and left to sporulate for 6 days at 35°C. Spores were collected and harvested by washing with deionized water three times at 5000 g. The suspension was placed in a water bath at 75–78°C for 15 min and refrigerated at 4°C (Sommer and Cabaj 1993). A total of three colonies with different morphologies were collected separately and isolated to produce spore stocks of ENV 1, ENV 2 and ENV 3. Spores of *B. subtilis* ATCC 6633 produced by fermentation technique were obtained freeze dried and named fermented spores (F6633). *Bacillus subtilis* ATCC 6633 cultured on Columbia agar plates were named surface spores (S6633).

Spores were stained by the malachite-green dye and visualized under light microscopy to confirm spore morphology and purity. Isolated spores were spiked separately in treated drinking water to provide a suspension at an initial concentration of 10^6 – 10^7 colony-forming units per ml (CFU ml⁻¹) (Table 1). Water from the Wilson water treatment plant in Durham (NC, USA) was collected prior to chlorination, filtered with 0.22 µm nylon membrane filter (Millipore, Bedford, MA, USA) prior to use and kept at 4°C. Ten millilitres of the spore suspension was distributed to sterile 60 mm Petri dishes and irradiated under u.v. 253.7 nm radiation with constant stirring. Tenfold serial dilutions of the spore samples were prepared and low cell counts were determined by spread plating 1 ml aliquots of the undiluted suspension on plate count agar (PCA) and incubated overnight at 37°C. The spot droplet technique (Collins *et al.* 1989; Lindsay *et al.* 2002) was used for higher cell counts by spotting 50 µl droplets of the 10-fold serial dilutions on agar.

Low-pressure u.v. irradiation system and radiometry

u.v. inactivation of spore isolates spiked in water was conducted using a low-pressure (LP) lamp emitting monochromatic (253.7 nm) u.v. light. The quasi-parallel beam bench scale u.v. apparatus consisted of four 15-W LP mercury vapour germicidal lamps (ozone-free; General Electric G15T8, General Electric Co., Fairfield, CT, USA) emitting u.v. radiation directed through a circular opening. u.v. irradiance (mW cm⁻²) was measured with a radiometer and a calibrated u.v. detector, according to

Bolton and Linden (2003). The average irradiance in the mixed suspension was determined by the u.v. absorbance of the test suspension, the sample depth and the incident average irradiance (Morowitz 1950). Required exposure times were calculated by dividing the desired u.v. fluence by the average u.v. irradiance.

Hydrophobicity, zeta potential and particle count

Surface hydrophobicity of spores was measured by the assay of microbial adhesion to hydrocarbon (Rosenberg *et al.* 1980). Spores were suspended in deionized water and 0.6 ml hexadecane was added to 3 ml of spore suspensions in test tubes (18 mm × 150 mm). The phases were mixed on a vortex mixer for 30 s and allowed to separate for 15 min. The aqueous phase was removed with a Pasteur pipette for absorbance measurements with a UV-VIS spectrophotometer (Model Cary 100bio; Varian, Vic., Australia). The decrease in absorbance (450 nm) of the aqueous phase is a measure of spore surface hydrophobicity (Doyle *et al.* 1984). Measurements of zeta potential of isolated spores were performed with a zeta meter (model 3.0+ unit; Zeta-Meter, Inc., Staunton, VA, USA). A Multisizer 3 (Beckman Coulter, Miami, FL, USA) was used to size spores. Particles suspended in an electrolyte solution (Isotone II; Beckman Coulter) are drawn although 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 or volume per ml (µm³ ml⁻¹) were obtained.

Microscopy

Spore cells were observed by phase contrast microscopy (400×, Nikon E600; Nikon, Melville, NY, USA) and imaged without staining the sample. Samples for scanning electron microscopy (SEM) were fixed with 2% (w/v) glutaraldehyde for 1 h. Spore samples were collected by filtration with 0.22 and 3 µm polycarbonate filters (Millipore) or by absorbance to a poly-L-lysine coated cover slip in a humid chamber for 30 min (Becton-Dickinson, Biocoat Cellware, Bedford, MA, USA). Subsequently, the samples were dehydrated through a graduated series of 30, 50, 75 and 100% (twice) ethanol solution. Following dehydration,

Table 1 Number of viable spores at the start of the tailing zone

Spore type	Initial spore count (CFU ml ⁻¹)	Fluence before entering tailing zone (J m ⁻²)	Spore concentration at entrance to tailing zone (CFU ml ⁻¹)	Percent of residual spores at tailing zone (%)
ENV 1	6×10^6 to 3×10^7	650	245–342	0.0029 ± 0.0026
ENV 2	3×10^5 to 2×10^6	250	2860–20 600	1.03 ± 0.43
ENV 3	8×10^6 to 1×10^7	250	2360–7760	0.05 ± 0.02

samples were transferred to the critical point dryer, mounted on aluminium stub using silver paste, coated with gold–palladium alloy and viewed by SEM (Cambridge S200; LEO Electron Microscopy Inc., Thornwood, NY, USA).

16S rRNA gene sequencing

Genetic identity was determined by amplifying and sequencing *ca* 600-bp fragment from the 16S rRNA gene from spore isolates. The isolates were incubated for 48 h at 37°C on PCA. A small amount of cells were transferred with a sterile toothpick from a bacterial colony directly into the PCR reaction mix (modified from Gussow and Clackson 1989). Each 20 µl reaction mix contained 2 µl 10× buffer, 1.6 µl dNTP (1.25 mmol l⁻¹), 2 µl MgCl (25 mmol l⁻¹), 0.2 µl F primer (10 µmol l⁻¹), 0.2 µl R primer (10 µmol l⁻¹), 1 µl Taq polymerase, 13 µl sterile ddi water. The primers were BSF 343/15 (5'-TACGGRAGGCAG-CAG-3') and BSR 926/20 (5'-CCGTC AAT TTYTT-TRAGTTT-3') (Wilmotte *et al.* 1993). The cycling protocol was: 10 min at 94°C, followed by 35 cycles of 94°C for 60 s, 50°C for 30 s and 72°C for 60 s. For purification and concentration, the PCR reaction mix was diluted to 450 µl with sterile water, loaded onto a Microcon filter device (Millipore 42410) and centrifuged at 1200 rev min⁻¹ for 6 min to a volume of 25–30 µl. Water was added (450 µl) and the process was repeated twice. The cleaned PCR products were quantified by agarose gel electrophoresis through comparison with known DNA standards. Both strands of each PCR product (20–40 ng) were sequenced with the BSF and BSR primers using Applied Biosystems (ABI, Foster City, CA, USA) protocols and fluorescently labelled dideoxynucleotides. The reactions were processed with an ABI 3700 DNA analyser.

Data presentation

Mean concentration (CFU ml⁻¹) of indigenous spores from source water or isolated spores spiked in suspension without u.v. exposure was taken as the initial concentration, N_0 . Duplicates of 10 ml samples of spore–water suspension or source water were irradiated under predetermined u.v. fluence. Each duplicate was diluted twice and plated three times to give a total of 12 repetitions per u.v. fluence. All the data fields from each repetition were organized in columns, one for fluence and one for spore concentration. The arithmetic mean concentration per fluence (N_d) and standard deviation were summarized. The log₁₀ transformation for N_0/N_d was plotted as a function of the u.v. fluence (H). Regression analysis and 95% CI was performed on all the data fields used to fit the linear sections of the log inactivation curve. The linear curve was described by the following equation:

$$\log_{10} \frac{N_0}{N_d} = k \cdot H \quad (1)$$

The fluence-based inactivation rate coefficient (k) was determined for each experimental run.

RESULTS

Ultraviolet fluence–response curves of indigenous and isolated spores

The concentration of indigenous spores examined in unfiltered surface waters varied from 10² to 10⁴ spores 100 ml⁻¹, with heterogeneity in species, as observed by the different colony types growing on the membrane filter after incubation (Mamane-Gravetz and Linden 2004). Surface water (Lake Michie, Durham, NC, USA), exposed to different levels of u.v. irradiation, was pasteurized and cultured to observe if a mixed indigenous spore population surviving high u.v. fluence are more resistant after isolation and culturing methods. After exposure to a u.v. fluence of 900 J m⁻², a yellow small 1 mm colony that appeared on the filter was chosen from the various colonies, isolated and named ENV 3. With u.v. irradiation of 250 J m⁻², a slimy 2–3 mm colony was isolated and called ENV 2, while a wrinkled big colony with an irregular noncircular shape was isolated and named ENV 1. Isolated, cultured spores were spiked into the filtered surface water at a concentration ranging from 10⁶ to 10⁷ CFU ml⁻¹.

Figure 2 illustrates the log inactivation of isolated spore ENV 3 as a function of u.v. fluence. Initially, a shoulder is observed at a low u.v. fluence of 50 J m⁻² for ENV 3. Subsequently, a first order linear relationship is observed between the isolated spore logarithmic survival rate and u.v. fluence between 50 and 250 J m⁻². With increasing u.v. fluence above 250 J m⁻², a reduced inactivation rate was observed. Ultraviolet inactivation of the spore isolates compared with the indigenous spores is presented in Fig. 3. The u.v. fluence required for 3 log inactivation was 500, 740 and 220 J m⁻² for ENV 1, 2 and 3 respectively. In comparison, the original indigenous spores reached only 1 log inactivation at u.v. fluence of 600 J m⁻².

Genetic characterization of isolated spores

A 600-bp long section of the 16S rRNA gene from three different environmental isolates were sequenced and compared with sequences in the public database. ENV 2 and ENV 3 had identical sequences, differing at four bases from the ENV 1 sequence. Using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>), each of the two sequence variants was found to have perfect matches to 16S rDNA sequences within the complete genomic sequence of *B. subtilis* (Kunst

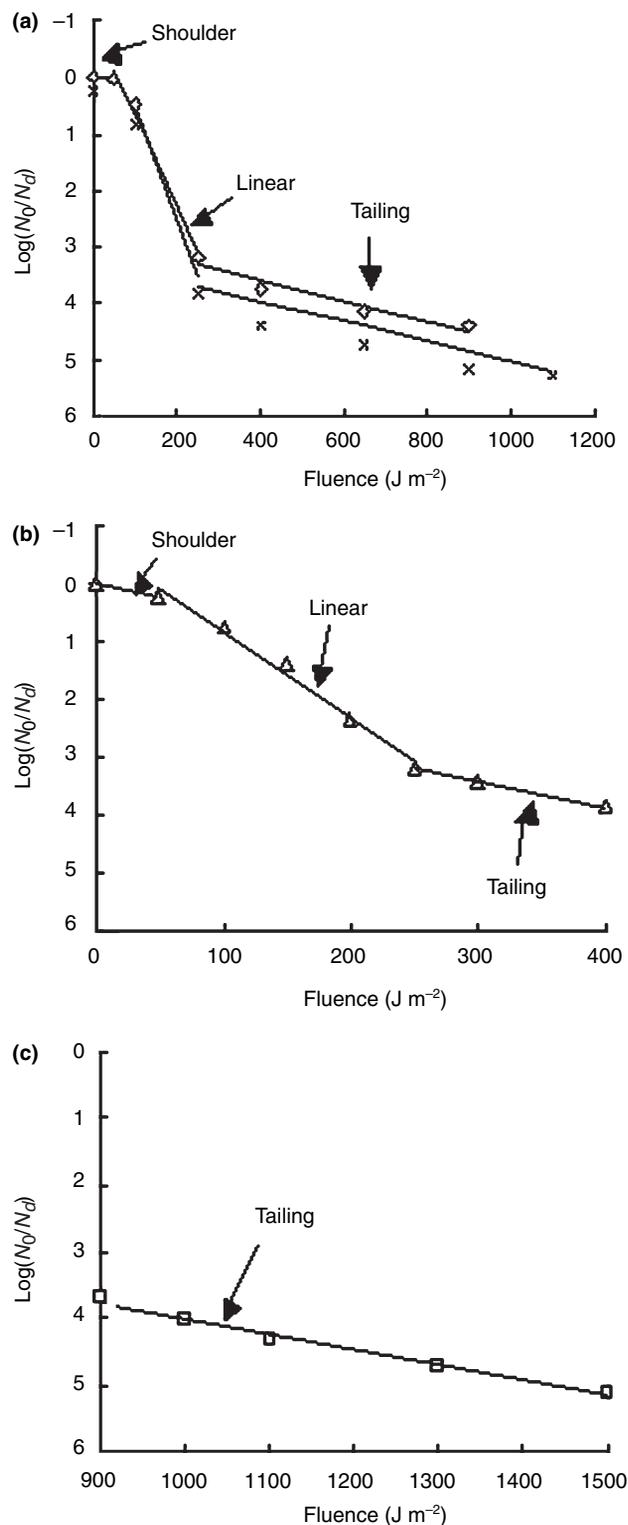


Fig. 2 Ultraviolet fluence–response curves for isolated spore ENV 3: (a) shoulder, linear and tailing zone at fluence of 0–1100 J m^{-2} , (b) shoulder and linear zone with more data points at fluence of 0–400 J m^{-2} and (c) tailing region with more data points at fluence of 900–1500 J m^{-2} . Experiment type: (\diamond) 1, (\times) 2, (\triangle) 3, (\square) 4

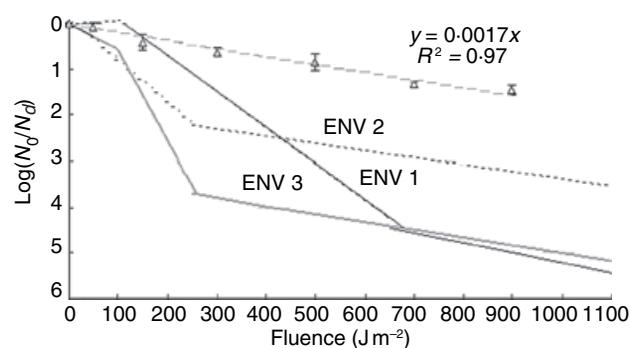


Fig. 3 Log inactivation of indigenous aerobic spores (\triangle) and pure isolates ENV 1 (—), ENV 2 (---) and ENV 3 (—) as a function of UV Fluence

et al. 1997). This identifies the three as *B. subtilis*. The sequences were deposited in GenBank with the following accession numbers: ENV 1, AY616159; ENV 2, AY616160; ENV 3, AY616161.

Fluence-based inactivation rate coefficient

The difference in fluence-based inactivation rate coefficient (k) between isolated spores was determined by comparing the first order and tailing inactivation rate coefficients (Tables 2 and 3). The transition between first order and tailing is not precisely discernable. Three to five data points, following the shoulder or lag in inactivation, were taken to develop the linear first order line and an additional three to five data points, using the highest u.v. fluence, while providing reasonable counts, were used to create the tailing linear line. The intersection between those lines provided the approximate point where first order ends and tailing starts. The first order linear region exhibits a very high linear correlation coefficient ($r^2 > 0.97$) for all spore isolates indicating a strong correlation between the log inactivation and the u.v. fluence. Average first order k was $0.0079 \text{ m}^2 \text{ J}^{-1}$ for ENV 1; $0.0088 \text{ m}^2 \text{ J}^{-1}$ for ENV 2 and $0.0171 \text{ m}^2 \text{ J}^{-1}$ for ENV 3. The k of the first order zone is different for each spore strain. The tailing region also exhibited a good linear fit ($r^2 = 0.63\text{--}0.94$) and average k of $0.00195 \text{ m}^2 \text{ J}^{-1}$ for ENV 1 and ENV 2 and $0.00196 \text{ m}^2 \text{ J}^{-1}$ for ENV 3. At the tailing zone, lower counts effect reproducibility as seen by the lower r^2 was compared with linear first order inactivation zone.

Mathematical equation to describe shoulder and tailing of spores

The survival function of micro-organisms with shoulder and tailing can be modelled using eqn (2) with four parameters (k_1 , k_2 , d , a) to describe the survival curve (Cabaj and Sommer 2000). Equation (2) was developed for a case of a mixture of

Fluence-based inactivation rate coefficient of first order linear zone	ENV 1 (m ² J ⁻¹)	ENV 2 (m ² J ⁻¹)	ENV 3 (m ² J ⁻¹)
Experiment 1	0.0084 (0.99)	0.0088 (0.98)	0.0200 (0.99)
Experiment 2	0.0079 (0.99)	0.0095 (0.98)	0.0163 (0.99)
Experiment 3	0.0074 (0.97)	0.0090 (0.97)	0.0151 (0.98)
Experiment 4		0.0078 (0.97)	
Average linear	0.0079 ± 0.0005	0.0088 ± 0.0007	0.0171 ± 0.0026

Shoulder was apparent only at fluence of 0–50 J m⁻² for ENV 2 and ENV 3 and at fluence of 0–100 J m⁻² for ENV 1.

*r*² values are given in parentheses.

Fluence-based inactivation rate coefficient of tailing zone	ENV 1 (m ² J ⁻¹)	ENV 2 (m ² J ⁻¹)	ENV 3 (m ² J ⁻¹)	Indigenous spore
Experiment 1	0.00203 (0.87)	0.00219 (0.92)	0.00173 (0.88)	0.00165 (0.97)
Experiment 2	0.00213 (0.63)	0.00155 (0.92)	0.00181 (0.89)	
Experiment 3	0.00170 (0.65)	0.00266 (0.72)	0.00235 (0.94)	
Experiment 4		0.00141 (0.93)		
Average linear	0.00195 ± 0.0002	0.00195 ± 0.0006	0.00196 ± 0.0003	

*r*² values are given in parentheses.

two micro-organisms with different sensitivities to u.v. irradiation; one more sensitive with a shoulder (*d*) and the other less sensitive. The survival function was adapted to describe each of the isolated spores with different sensitivities to u.v. within the same suspension. Each spore isolate exhibited two different fluence-based inactivation rate constants (*k*) where the first order inactivation zone corresponds to the sensitive portion and the tailing zone corresponds to the less sensitive portion of the spore population.

$$\frac{N_d}{N_0} = \frac{1 - (1 - 10^{-k_1 \cdot H_0})^{10^d} + a \cdot 10^{-k_2 \cdot H_0}}{1 + a} \quad (2)$$

$$a = \frac{N_{0,2}}{N_{0,1}} \quad (3)$$

where *k*₁ is the absolute value of *k* of the first order linear zone in the fluence–response curve plotted as log(*N*_{*d*}/*N*₀) vs *H*₀ (m² J⁻¹); *k*₂, absolute value of *k* of the tailing zone in the fluence–response curve plotted as log(*N*_{*d*}/*N*₀) vs *H*₀ (m² J⁻¹); *d*, intercept with *y*-axis [log(*N*_{*d*}/*N*₀)] of the first order linear zone in the fluence–response curve; *N*₀, initial concentration of sensitive (*N*_{0,1}) and less sensitive (*N*_{0,2}) micro-organism; log *a*, intercept with *y*-axis [log(*N*_{*d*}/*N*₀)] of the tailing zone in the fluence–response curve

Table 2 Comparison of the first order linear fluence-based inactivation rate coefficient (*k*) for isolates ENV 1, ENV 2 and ENV 3 with respect to the average kinetics and least square best fit

Table 3 Comparison of the tailing fluence-based inactivation rate coefficient (*k*) for indigenous spores and isolates ENV 1, ENV 2 and ENV 3 with respect to the average kinetics and least square best fit

(*N*_{0,2} ≪ *N*_{0,1}); *H*₀, the fluence at 253.7 nm (m² J⁻¹); *N*_{*d*}/*N*₀, survival rate.

The survival function of micro-organisms exhibiting a shoulder and first order inactivation without tailing (eqn 4) can be derived from eqn (2) using two parameters (*k*₁, *d*), as *k*₂ and *a* are both equal to zero when tailing is not present.

$$\frac{N_d}{N_0} = 1 - (1 - 10^{-k_1 \cdot H_0})^{10^d} \quad (4)$$

The survival function of micro-organisms exhibiting first order inactivation without shoulder or tailing (eqn 5), the Chick–Watson inactivation model can be derived from eqn (4); with one parameter (*k*₁), because parameter *d* is zero when no shoulder is present.

$$\frac{N_d}{N_0} = 10^{-k_1 \cdot H_0} \quad (5)$$

Therefore, eqn (2) (with tailing and shoulder) is the general case of the first order inactivation equation (eqn 5). The model described by eqn (2), was used with the data obtained from the isolated spores u.v. inactivation experiments. Figure 4 illustrates the experimental and theoretical model-based u.v. fluence–response curve for ENV 2 (experiment 2). The inputs for eqn (2) were the *k* and *d* values that were measured experimentally. The equation was

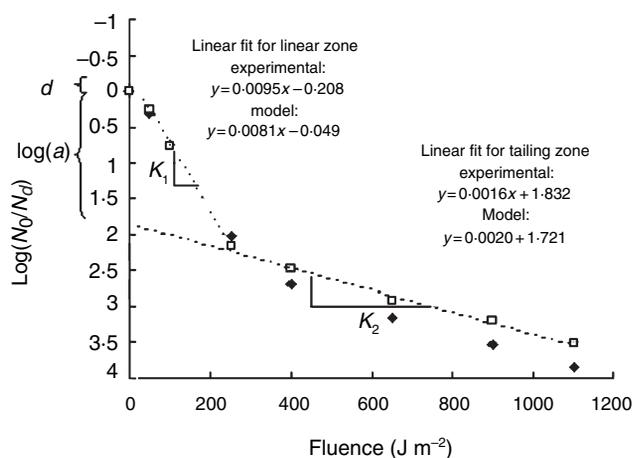


Fig. 4 Experimental results (\square) and theoretical model (\blacklozenge) based u.v. fluence–response curve for ENV 2 (experiment 2)

also applied to ENV 1 and ENV 3 strains and the same similarity existed between the experimental and theoretical outcome.

Statistical analysis

ANOVA analysis indicated that the differences between the fluence-based inactivation rate coefficients of the tailing portion of isolated spores ENV 1, 2 and 3, as groups, are not statistically significant ($P > 0.10$). Moreover, the mean fluence inactivation rate coefficients of indigenous spores were compared with the mean fluence rate inactivation coefficients of the tailing zone of isolated spores (ENV 1, 2 and 3) and ANOVA indicated that the differences are also not significant ($P > 0.10$). However, differences between the mean fluence inactivation rate coefficient of indigenous spores and the fluence-based inactivation rate coefficients of the tailing portion of isolated spores within most individual experiments (i.e. ENV1 experiment 1, ENV1 experiment 2...ENV2 experiment 3...) are statistically significant. Figure 5 shows the 95% confidence interval (CI) of the tailing portion for the fluence-based inactivation rate coefficient (k) of indigenous and of the individual experiments of cultured isolated spores. The individual 95% CI of experiments ENV 2 experiments 1 and 3 and ENV 3 experiment 3 do not overlap with 95% CI of indigenous spores, indicating significant differences between these inactivation rates.

Physicochemical measurements of isolated spores

Comparison between the different spores (isolated and ATCC spores) with regard to zeta potential, mean particle size, specific-surface area, hydrophobicity, PSD and the

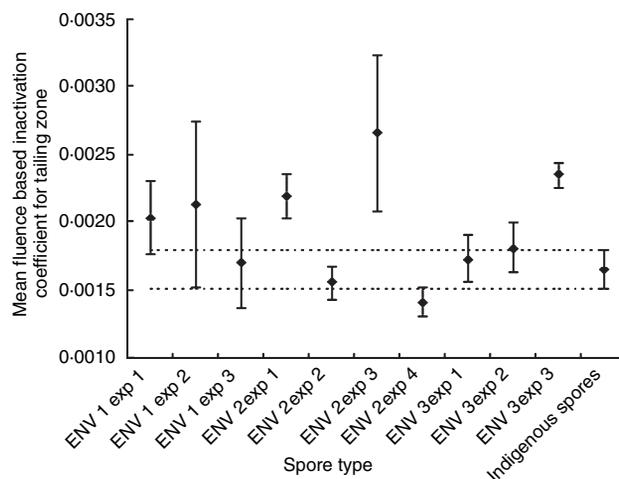


Fig. 5 Mean (\blacklozenge) and 95% confidence interval (—) for the slope of indigenous spores in Lake Michie sample compared with tailing slope of its isolates. The error bars represent upper and lower 95% confidence interval (t -test)

average log inactivation at the entrance to the tailing zone is presented in Table 4. A zeta potential close to -38 mV was observed for spore isolates suspended in deionized water at pH of *ca* 6.5 with 0 conductivity. The conductivity of the filtered surface water used for u.v. inactivation experiments was $100\text{--}110 \mu\text{S cm}^{-1}$ at pH of *ca* 7; therefore, with the higher ionic strength of lake water compared with deionized water, the zeta potential of isolated spores was expected to decrease and resulted in a zeta potential between -24 and -27 mV. Sizing of the spores was performed with a particle size analyser and mean diameter and specific surface area of each spore type was determined. A suspension of ENV 2 spores had a lower surface area and a larger particle size than suspensions of ENV 1 and ENV 3. ENV 1 and ENV 3 were least hydrophobic, with *ca* 7 and 10% adherence, respectively, and ENV 2 was the most hydrophobic isolated spore, tested with *ca* 70% adherence. Hydrophobicity values were also measured before and after exposure to u.v. fluence of 1000 J m^{-2} to test if aggregates may be produced during u.v. irradiation; however, no measured difference in hydrophobicity was observed. PSD of the cultured isolated spores as a fraction of total spore count and count of particles larger than $3 \mu\text{m}$ divided in size bands are illustrated in Fig. 6. ENV 2 has the largest fraction of particles at $\text{Log}(dp)$ larger than 0.3 (or dp larger than $2 \mu\text{m}$), while for ENV 1 and ENV 3 spores, particles are not apparent at $\text{log}(dp)$ larger than 0.3. As shown in the insert of Fig. 6, the distribution of particles larger than $3 \mu\text{m}$ was an order of magnitude smaller for ENV 1 and ENV 3 when compared with ENV 2 and no particles are present at size bands larger than $9 \mu\text{m}$. When analysing the particle volume, *ca* 70% was distributed in the diameter range

Table 4 Comparison between the different spores with regard to mean particle size, specific surface area, zeta potential, hydrophobicity and the average log inactivation at the entrance to the tailing zone

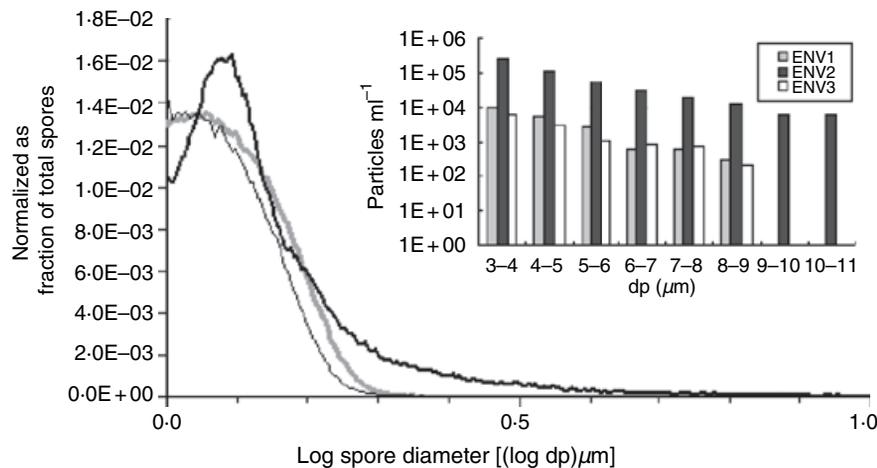
Characteristics	ENV 1	ENV 2	ENV 3	S6633*	F6633
Mean particle size (μm)	1.06 \pm 0.03	1.23 \pm 0.74	1.01 \pm 0.27	0.93 \pm 0.18	0.94 \pm 0.15
Specific surface area ($\mu\text{m}^2 \text{ml}^{-1}$)	46851	18007	51163	59466	60127
Zeta potential, (mV; suspended in DI water [†]).	-37.4 \pm 2.3	-37.8 \pm 4.5	-39.6 \pm 4.8	-46.6 \pm 9.4	-25.4 \pm 5.0
Zeta potential, (mV; suspended in surface water [‡]).	-23.5 \pm 0.8	-27.4 \pm 1.4	-27.4 \pm 2.3	NA	NA
Hydrophobicity (%)	7.2 \pm 0.7	69.7 \pm 3.8	10.3 \pm 1.3	3.7 \pm 0.16 \S	15.1 \pm 0.1 \S
Average log inactivation at entrance to the tailing zone	4.65 \pm 0.43	2.03 \pm 0.17	3.30 \pm 0.21	No tailing	3.51 \pm 0.02
Fluence level that isolated spores enter tailing zone (J m^{-2})	650	250	250	No tailing	600

*S6633 showed a shoulder up to 50 J m^{-2} and linear inactivation kinetics with a fluence of 300 J m^{-2} resulting in about 5.5 log inactivation.

[†]Deionized water.

[‡]Surface water filtered with 0.22 μm .

\S deviation from value obtained by linear regression to actual data point.

**Fig. 6** Normalized fraction of spore count as a function of particle diameter (dp) of cultured isolated spores ENV 1 (—) ENV 2 (---) and ENV 3 (····). The insert represents distribution of particle counts larger than 3 μm within different diameter bands

larger than 3 μm for ENV 2, while <6% of total particle volume was distributed in the same diameter range for ENV 1 and ENV 3 spores. The hydrophobicity of *B. subtilis* spores (ATCC 6633) cultured by surface (S6633) and fermentation (F6633) technique and the average log inactivation at the entrance to the tailing zone was evaluated. Surface spores with low hydrophobicity values (3.7%) showed linear kinetics (no tailing) throughout the fluence tested (data not shown) while fermented spores with higher hydrophobicity showed tailing.

Microscopy imaging of isolated spores

Figure 7 illustrates cultured isolated ENV 2 spores as observed by phase contrast microscopy and SEM. Aggregates and mostly dispersed ENV 2 spores are observed by phase contrast microscopy (Fig. 7a). SEM micrographs show that ENV 2 spores captured on the surface of the 3 μm filters are aggregated (Fig. 7b,d), while nonfiltered ENV 2 spore samples showed dispersed spores as well (Fig. 7c).

DISCUSSION

Genetic characterization

DNA sequencing was used to define the species of the spore isolates. The three environmental isolates showed two slightly different sequence types that were all identified as environmental isolates of *B. subtilis*. Although all the isolates were identified as isolates of *B. subtilis* by sequencing 16S rDNA, the different colony morphologies of individual isolated spores were likely to be caused by other differences within their genomes. *Bacillus* spores are a common subspecies of aerobic spores and variation in spore type depends on the spatial and temporal diversity of water samples (Niemiński and Bellamy 2000).

Fluence-based inactivation kinetics

The u.v. inactivation data of isolated spores consisted of three distinct phases including shoulder, linear first order and tailing (Figs 1 and 2). *Bacillus subtilis* spores disinfected by chlorine showed the same pattern of lag, first order linear

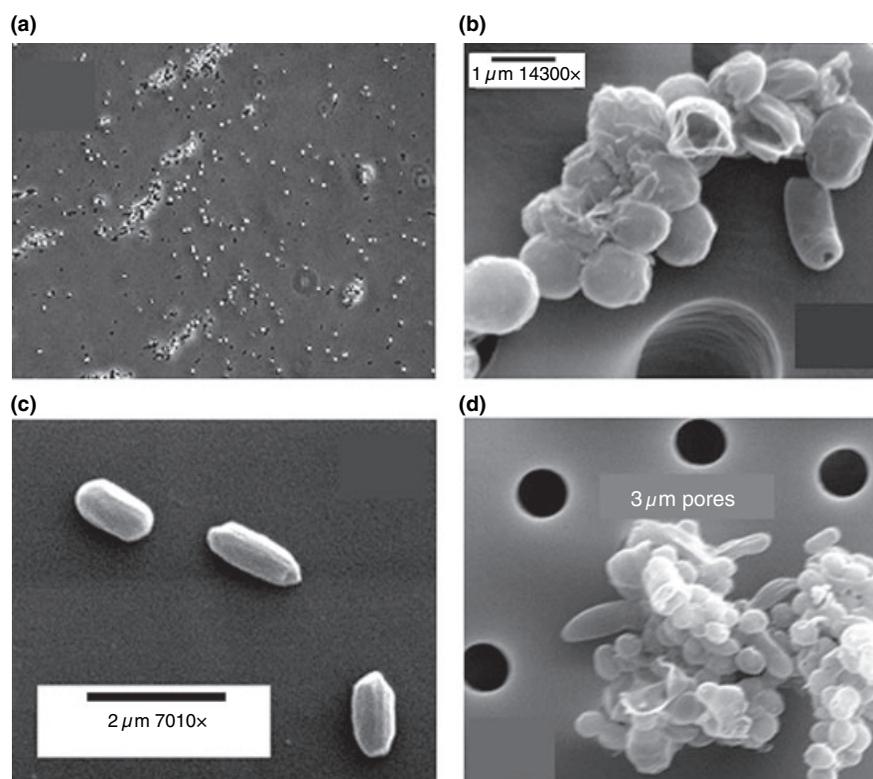


Fig. 7 Phase contrast and scanning electron microscopy (SEM) analysis of spores: (a) cultured isolated spores as observed by phase contrast microscopy (400 \times), (b) aggregates of ENV 2 spores observed by SEM, (c) dispersed ENV 2 spores observed by SEM and (d) aggregates of ENV 2 spores observed by SEM with emphasis on the aggregate size in relation to the 3 μm polycarbonate filter pores

phase and tailing (Barbeau *et al.* 1999). The shoulder phase of the spore inactivation can be explained by: (i) the series-event model, where a threshold value represents the number of sites of an organism that must be destroyed before inactivation occurs (Severin *et al.* 1983); (ii) protection of the protoplasm by a thick cortex multilayered spore coat (Broadwater *et al.* 1973); (iii) factors such as sporulation and counting medium, pH, incubation temperature and heat required for initiating germination (Yokoya and York 1965); (iv) aggregates within spore suspension (Cerf 1977); (v) DNA repair. Large shoulders of repair proficient *Escherichia coli* strains exposed to near u.v. were because of the fluence-dependent repair process, where repair decreases as fluence increases (Webb and Brown 1976), whereas repair deficient spores exhibit inactivation kinetics without shoulder (Tyrrell 1978; Munakata 1981; Sommer *et al.* 1999).

The linear first order region of the curve describes spores that follow the Chick–Watson inactivation model. Interpretation of the tailing phenomenon can be caused by individuals in a population cultured from a pure colony that was not identical with respect to disinfection; or because of similarity of resistance between individuals in a population with other factors affecting inactivation (Cerf 1977). Tailing could occur because of the following (Cerf 1977): (i) modification of spore resistance during treatment; (ii) inactivation of the same species with resistant and sensitive genetic variants; (iii) local variations in disinfection concen-

trations, pH and medium; (iv) aggregation of spore suspension that occurs during treatment; (v) artefact because of errors in method used for enumeration of survivors; and (vi) microbe-particle association (Loge *et al.* 2001).

Considering these factors, controllable experimental variables that may produce false tailing were adopted. All isolated spores were produced using the same sporulation medium and centrifuged to remove debris and residual medium. The spore concentration did not change over the research period. The heat-shock treatment was performed in small vials to prevent local temperature variations. Spore concentration in the tailing portion was within an acceptable range of more than 10^2 CFU mL^{-1} (Table 1), and for lower counts numerous repetitions were performed as recommended by Cerf (1977). Given these controls, it was assumed that any tailing identified in the inactivation of the spores would be the result of spore aggregation or modification of the spore suspension during inactivation treatment and not because of other artefacts.

It was expected that selecting indigenous spores appearing at high u.v. fluence on the membrane filter would result in strains with higher u.v. resistance after sporulation and purification compared with those exposed to lower u.v. fluence. Previous research indicated that selected colonies resistant to heat were generated by choosing survivors of heat treatment (Davis and Williams 1948); however, choosing indigenous colonies surviving after high u.v. fluence

exposure did not result in selection of spores that were more resistant to u.v. when comparing the first order inactivation rate constants (Table 2).

The inactivation data for the isolated cultured spores were effectively modelled using a mathematical equation accounting for shoulder, first order linear and tailing region of inactivation (Fig. 4). The agreement between the data and the model indicates that eqn (2) can be used to describe the behaviour of isolated environmental spores or any other micro-organism with shoulder and tailing. Equation (2) is useful for validating a u.v. reactor with a wide fluence distribution, such as, for an organism that exhibits both a shoulder and tailing.

Comparison of laboratory and indigenous spore inactivation

Indigenous aerobic spores were found to be very resistant to u.v. disinfection compared with isolated laboratory-cultured spores (Fig. 3), where the extent of resistance to u.v. differed depending on the isolated strain cultured. The inactivation of indigenous natural aerobic spores followed first order kinetics without tailing or shoulder regions observed with the isolated spores. Moreover, indigenous spores from various water sources all followed first order inactivation kinetics throughout the entire u.v. fluence examined (Mamane-Gravetz and Linden 2004). Assuming linearity, a fluence of 2350 J m^{-2} will be needed to achieve 4 logs of the indigenous spores as illustrated in Fig. 3, which is not a practical fluence for u.v. reactors at water treatment plants. With chlorine disinfection, decreased resistance of purified *B. subtilis* spores relative to indigenous spores was attributed to the consistent nature of the purified spores preparation in contrast to the different stages of dormancy and metabolism that are characteristic of the indigenous spores (Rice *et al.* 1996). Another explanation may be attributed to association of indigenous spores with particles in the raw water, phenomenon known to protect microbes in wastewater from lethal u.v. fluence (Emerick *et al.* 2000; Loge *et al.* 2001).

In nature, bacterial spores develop long-term adaptations that allow them to survive for prolonged periods of exposure to solar u.v.A and u.v.B by protecting their DNA from radiation. The most typical induced u.v.C photoproduct of spores is 5-thymine-5,6-dihydrothymine adduct termed SP (Setlow 1992, 1995). The small acid-soluble DNA binding proteins (SASP) interact with DNA in the spores and alter u.v. photochemistry of spores to result in significant SP formation and reduced thymine dimer (TT) formation upon exposure to u.v. irradiation. SP is less lethal than TT as spores have an SP-specific repair system that repairs spore SP photoproduct during spore germination. SASP sequences are highly conserved; however, slight differences

can result in different resistance capabilities for different *Bacillus* spores (Setlow 1992, 1995). Resistance of spores to chlorine or u.v. disinfection was influenced by the sporulation medium (Yokoya and York 1965; Sommer and Cabaj 1993), by high concentrations of divalent cations and pH (Barbeau *et al.* 1999) and by reduced core water contents that slows down reactions that damage DNA, slowing water-catalysed reactions and promoting binding of SASP to DNA (Setlow 1992; Popham *et al.* 1995). Indigenous spores were found to be more resistant to u.v. irradiation because of the constant possibility of exposure to solar u.v. light, being a soil organism (Setlow 1992). In addition, u.v. resistance of spores in the soil environment includes mechanisms such as adhering tightly to soil particles and humic substances and shielding spores from solar u.v. (Nicholson and Law 1999).

Longer u.v. wavelengths can produce nonspore photoproducts and other non-DNA cellular damage. However types of repair pathways in addition to SP and excision repair can induce resistance of indigenous spores to solar u.v. Therefore, the solar u.v. system is complex and u.v.C photodamage and repair capabilities are not comparable (Slieman and Nicholson 2000). Thus, it appears that the resistance of indigenous aerobic spores in natural waters to solar u.v.A and u.v.B is because of shielding from u.v., natural selection as a result of constant exposure, different types of photo-damage and repair compared with u.v.C or the environment in which the sporulation process occurred, traits that may not necessarily be present in laboratory-cultured spores exposed to u.v.C irradiation. Future research will study the effect of pre-exposure of laboratory-cultured spores to solar u.v. on induction of solar repair processes and subsequent resistance to u.v.C inactivation.

The kinetics of tailing in isolated spores was statistically similar to overall kinetics of indigenous spores (Table 3). Tailing was presumably because of aggregation in the case of the isolated or natural spores or increased resistance of a subpopulation, more likely in the natural environmental sample. Therefore, investigation of the mechanisms behind spore-spore interactions, such as aggregation that affect disinfection of spores, is important.

Effect of physicochemical parameters on spore aggregation

In the study of u.v. inactivation of microbes, it is often assumed that the log-linear kinetics region describes microbes that are mostly dispersed while the tailing region is formed mainly because of microbes that are associated with particles (Loge *et al.* 2001). As indicated in Fig. 3, isolated cultured spores exhibited tailing at high u.v. fluence, hypothesized to be caused by aggregation of spores, while the log-linear phase is further hypothesized to result from inactivation of the portion of spores that are 'dispersed'.

Aggregation of spores is likely to be the result of surface factors. micro-organisms can possess hydrophobic or charged areas on their surface. As hydrophobicity is the tendency for apolar molecules to associate more with other apolar molecules than to water (Doyle 2000), it may lead nonpolar species to interact by cell-cell or cell-surface association. Numerous researchers have also indicated electrostatic interactions, which influence bacterial adhesion to surfaces (Mozes *et al.* 1986; Husmark and Ronner 1990). Therefore, the tendency of spores to aggregate was investigated by measuring the surface charge, hydrophobicity, surface area and particle sizes of each isolated spore type (Table 4). The zeta potential of isolated spores are comparable with the zeta potential of *B. subtilis* spores spiked into filtered lake water ranging between -16 and -20 mV at pH values of 5-10.5 (Rice *et al.* 1996). Zeta potential for *Cryptosporidium* oocysts decreased slowly as pH increased starting at -35 mV for alkaline pH and reaching the isoelectric point for optimum adhesion at acidic pH (*ca* 2.5) (Drozd and Schwartzbrod 1996). The isoelectric point for optimum attachment of isolated spores could be reached at acidic pH values that are not typical of natural or treated waters.

ENV 1 and ENV 3 are least hydrophobic, with *ca* 7 and 10% adherence, respectively, and ENV 2 is the most hydrophobic isolated spore tested with *ca* 70% adherence. Larger surface area or smaller particle size can theoretically increase sites for attachment or aggregation of spores. A suspension of ENV 2 spores had a lower specific surface area and a larger particle size than suspensions of ENV 1 and ENV 3; however, ENV 2 is the most hydrophobic spore suggesting that difference in specific surface area of the spores and the mean spore size is not a factor that governs aggregation when comparing between the isolated spores.

Aggregate formation may also be monitored by PSD measurements for the different spore isolates (Fig. 6). The presence of particles in the larger size range (>3 μm) for ENV 2 (insert of Fig. 6) supports the hypothesis of aggregation in ENV 2 samples, which correlates also with the increased hydrophobicity of ENV 2. Figure 7 provides additional indication of ENV 2 aggregates larger than 3 μm , as the sample was filtered through a 3 μm filter, essentially to differentiate between the dispersed spores (size of 1 μm ; Fig. 7c) to the aggregates (Fig. 7b,d). Mature spores are released to the surrounding by lysis of the vegetative mother cell. The lysed mother cell remains in the solution and may aggregate or shield spores (possibly together with other spores) resulting in reduced u.v. radiation reaching the target spore.

ENV 1 and ENV 3 reach tailing phase after 4.65 and 3.30 log inactivation (with hydrophobicity of 6 and 10%, respectively), whereas ENV 2 reaches tailing after only 2 log inactivation (with hydrophobicity of 70%), suggesting

less aggregates formed with ENV 1 and ENV 3 when compared with ENV 2. The data from the log inactivation results and the clear differences in hydrophobicity, PSD and volume distribution support the hypothesis that increased hydrophobicity is accountable for enhancing aggregation and as a result, the survival of aggregated ENV 2 spores exposed to u.v. irradiation and the level of log reduction are achieved before tailing. Based on the similarity of the electrical potentials for isolated spores, it could not be determined whether the surface charge was a dominant factor affecting u.v. inactivation kinetics among the spores. *Bacillus subtilis* spores cultured by fermentation and surface technique showed the same trend of correlation between hydrophobicity and tailing. Surface spores with very low hydrophobicity did not show tailing while fermented spores with higher hydrophobicity showed tailing, which strengthens the interpretation of the previous findings with isolated spores.

Hydrophobicity could be the dominant characteristic in controlling adhesion of microbes to surfaces irrespective of the microbe surface charge (van Loosdrecht *et al.* 1987), as aggregation of certain *Bacillus* spore strains can be associated with higher hydrophobicity measurements (Ronner *et al.* 1990). Ronner *et al.* (1990) attributed adhesion to hydrophobic proteins that contribute to overcoming electrostatic repulsion. It was not possible to provide evidence of hydrophobicity of indigenous spores as the source water contains organic and inorganic particles and other organisms such that this measurement would not relate solely to the properties of indigenous spores. In future research, it is suggested to study the effect of adding dispersants, used to minimize aggregate formation, on surface modification of spores and contrast the effect of aggregated vs nonaggregated spores on u.v. inactivation. The spores isolated were randomly chosen and identified as *B. subtilis*; therefore, it is also suggested to study other types of *Bacillus* spores in order to generalize these findings to other species.

To summarize, the extent of log inactivation prior to tailing was lowest for the isolate with the highest measured hydrophobicity, suggesting that spore hydrophobicity is correlated with increased aggregation and can be identified via particle size and volume distribution measurements. Five dissimilar spores that differed in their colony morphology or culturing method supported the hypothesis of correlation between hydrophobicity, aggregation and tailing in the u.v. fluence-response curve. Tailing implies a residual of spores present in the water system even at very high u.v. fluence, which could result in a public health concern. Therefore, the impact of cell-cell or cell-particle association on disinfection of hydrophobic micro-organisms needs to be recognized and could potentially be monitored using PSD.

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