Influence of bacterial biofilms on *Bacillus globigii* spore viability in model chlorinated water distribution systems

C. M. Arnett, A. M. Beckman, M. D. Ginsberg and V. F. Hock

ABSTRACT

Viability of *Bacillus globigii* spores in chlorinated polyvinyl chloride (PVC) pipe loop systems were examined under oligotrophic conditions. Three 2.5 cm × 10 m pipe loops having poised free chlorine concentrations of 0.0, 0.5, and 1.0 mg/L were seeded with 3.0×10^8 *B. globigii* spores each and viability was assessed over a 21 day period in both the recirculating waters and within the biofilms associated with pipe wall surfaces. After 10 min of exposure, viable spores were found to be associated within the pipe biofilms. In the untreated pipe loop spore counts remained statistically consistent in both the bulk water and biofilm until 1.0 mg/L free chlorine was introduced, then spores were completely inactivated in less than seven days. Spores within the pipe loop poised at 0.5 mg/L free chlorine showed a 7.6-$\log_{10}$ inactivation in the bulk water phase, but only a 2.7-$\log_{10}$ inactivation was observed within the biofilm after 14 days of treatment. Complete inactivation was observed in the 1.0 mg/L free chlorine system in both the biofilm and the bulk water phase in less than 10 min. These data demonstrated that *B. globigii* spores were readily incorporated into PVC pipe biofilms, which decreased spore inactivation nearly five orders of magnitude under moderate free chlorine concentrations.

Key words | *Bacillus globigii*, biofilm, chlorinated, PVC, spore inactivation, water distribution system

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INTRODUCTION

The recent intentional dissemination of *Bacillus anthracis* spores by means of the U.S. Postal Service has heightened concerns regarding the safety of many government and municipal services including potable water distribution systems (Copeland & Cody 2006). The American Water Works Association estimates that there is nearly one million miles of distribution pipe containing roughly 15 million valves, 24 thousand large capacity storage tanks, and seven million fire hydrants within the U.S. (American Water Works Association 2007). Due to vulnerabilities associated with such an expansive water network, deliberate contamination is of considerable concern for homeland security. Bacterial spores are of particular interest due to their inherent resistance to both physical and chemical inactivation. Even if contaminant concentrations are insufficient to cause disease, infrastructure disruption and reclaiming public trust after a release would be costly. According to the Centers for Disease Control and Prevention (CDC), the release of pathogenic spores in a heavily populated area could result in economic impacts ranging from $478 million to $26 billion per 100 thousand persons exposed (Kaufmann *et al.* 1997). In order to minimize human exposure and reduce impacts on infrastructure, it is imperative to understand spore fate within urban environments including water distribution systems.

Currently within the U.S. chlorination is the most common method for treating water for human consumption (American Water Works Association 2000). However, many bacteria are resistant to chlorine inactivation particularly if in spore form (Rose *et al.* 2005, 2007). Bacterial spores differ

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from vegetative cells in that they contain a cortex, spore coat, and exosporium outside the cell wall, all of which provide protection from chemical oxidation, ultraviolet light, and heat inactivation (Driks 2002). Spores belonging to the genus *Bacillus* are known to be extremely resistant to adverse environmental conditions and can remain dormant in hostile environments indefinitely (Nicholson *et al.* 2000). In fact, *Bacillus* spores are believed to be the oldest living organisms in existence today, dating millions of years old (Vreeland *et al.* 2000).

In addition to environmental persistence, several species within the *Bacillus* genus express pathogenic traits in humans, making them potential biological weapons (Beeching *et al.* 2002). One of the first documented modern occurrences of bacterial spores being used as a weapon was during the First World War when German forces attempted to infect livestock with *B. anthracis* (Redmond *et al.* 1998). More recently, *B. anthracis* spores were disseminated through the U.S. Postal Service resulting in the contamination of both government and private-sector buildings (Centers for Disease Control and Prevention 2001). Because *Bacillus* spore cultivation is a relatively simple process (Zhao *et al.* 2008), future attacks are believed to be highly probable (Salem 2003).

If *Bacillus* spores were released in to a potable water distribution system, they would likely represent a long-term threat due to their intrinsic ability to withstand unfavorable physiological conditions. Compounding this threat is the fact pipe surfaces within water networks, regardless of construction material, easily form polysaccharide coatings known as biofilms (Tsvetanova 2006). Bacteria associated with biofilms are generally less susceptible to chemical disinfection than associated planktonic forms (De Beer *et al.* 1994; Chen & Stewart 1996; Morin *et al.* 1996; Szabo *et al.* 2006). Therefore, biofilms could serve as a reservoir for spores protecting them from inactivation.

Although many studies have evaluated vegetative bacterial disinfection in biofilms (Murga *et al.* 2001; Park *et al.* 2001; Steed & Falkinham 2006: Szabo *et al.* 2006; DeQueiroz & Day 2007; Bressler *et al.* 2009), few have evaluated the effects of biofilms on spore susceptibility to disinfection. Szabo *et al.* (2007) studied the persistence of *B. atrophaeus* subsp. *globigii* spores on corroded iron and established spores readily adhered to the corroded surface and were found to remain viable for months even in the presence of chlorine concentrations greater than 10 mg/L. Similarly, Morrow *et al.* (2008) evaluated the susceptibility of *B. anthracis* Sterne and *B. thuringiensis* spores to chlorine disinfection on both copper and PVC surfaces and found spores associated with biofilm required 5- to 10-fold increase in disinfectant to achieve the equivalent $\log_{10}$ inactivation of planktonic spores. Additionally, Ryu & Beuchat (2005) investigated *B. cereus* inactivation on stainless steel surfaces and establish that both vegetative cells and spores embedded within biofilms were protected from chlorine, chlorine dioxide, and peroxyacetic acid-based sanitizers.

While each of the fore mentioned studies has contributed greatly to our understanding of biofilm/spore interactions, each used annular reactors and coupons except Morrow *et al.* (2008), which used PVC pipe segments. Even though annular reactors have historically functioned as model water systems, discrepancies in spore assimilation into biofilms and inactivation from actual pipe surfaces may exist (Griebe & Flemming 2000; Lawrence *et al.* 2000). Although, Morrow *et al.* (2008) did use pipe segments in their study, spore exposures and pipe surface disinfections were performed separately from the reactor used to establish the biofilms, which may have affected spore assimilation and inactivation. Here we studied the influence of bacterial biofilms on the viability of *B. globigii* spores in three continuous-flow water loops constructed completely of PVC pipe in an effort to better understand the role biofilms play on spore viability at various chlorine concentrations. This information is intended to help determine proper PVC pipe surface decontamination procedures, which could help minimize infrastructure disruption and preserve human health in the event of an attack with bacterial spores.

METHODS

Microorganism

Non-weaponized *Bacillus globigii* spores were obtained from Dugway Proving Ground (Dugway, UT). Initial viable spore counts were obtained by serial diluting 1 mg of dry

spores in sterile water from 10^{-1} to 10^{-7}, heat-shocking at 80°C for 10 min, and plating 100 μL of each dilution series onto tryptic soy agar (TSA) (Fluka, Milwaukee, WI). All dilutions were plated in triplicate and distinctive orange colony forming units (CFUs) were enumerated after 48 hours incubation at 35°C. The plates in a dilution series that yielded 50 to 200 CFUs were counted and viable spores were calculated.

Experimental drinking water system

Planktonic and sessile *B. globigii* spore viability was evaluated in three separate PVC loop systems operated at two different free chlorine concentrations. Each pipe loop was fabricated out of standard 2.5 cm, schedule 40, PVC pipe having a length of 10 m and a total volumetric capacity of 17 L, including the bulk water reservoir (Figure 1). Each loop consisted of a total of five removable 20 cm pipe segments for sampling of biofilm associated with pipe wall surfaces. PVC ball valves and threaded unions were placed flanking each segment to allow for easy removal. Water flow in each loop system was powered by a magnetic drive centrifugal pump at a continual linear flow rate of 30 cm/sec. Flow was monitored using an in line flow meter. Clear PVC sight glasses measuring 30 cm in length were installed in front of each pump head to visually observe biofilm formation on the pipe surface. In addition, each loop had a 20 L polycarbonate carboy for recirculating bulk water. Carboys were covered with black plastic in order to minimized phototrophic growth. Each loop system was operated at ambient room temperate, approximately 25°C. Flow rates, temperature, pH, and free chlorine in each pipe loop were monitored daily.

Loops were flushed with tap water periodically for a two week period prior to experimental use to remove residual organics present from the pipe manufacturing process and the cement used in construction. After the initial flushing, each loop was completely drained and filled with dechlorinated tap water. Chlorine was removed by the addition of sodium thiosulfate to a final concentration of 8 mg/L. All three loop system reservoirs were temporarily connected to each other with a diverter valve to allow water to flow freely between each system in order to promote homogeneous biomass formation. Visual inspections of

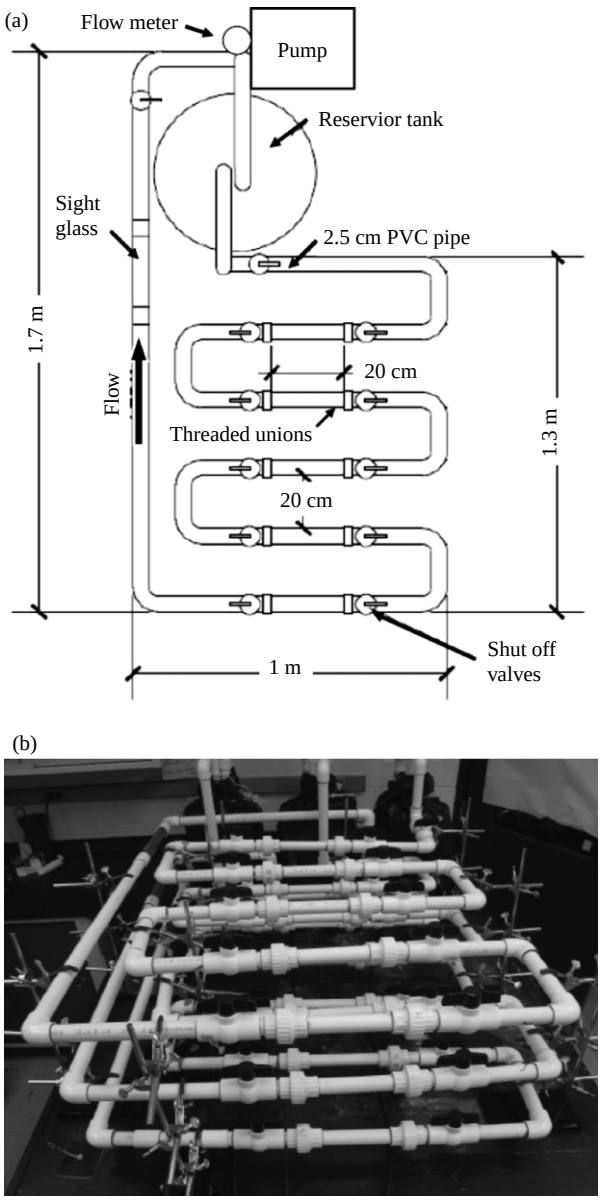

Figure 1 | Simulated PVC water distribution system: (A) Illustration of pipe loop system constructed with standard 2.5 cm PVC pipe and fittings. Three separate systems were constructed each having a total length of 10 m, bulk phase volume of 17 L, operation temperature of approximately 25°C, and a flow rate of 30 cm/sec; (B) Photograph of the loop systems in operation. Each was allowed to acclimate for 63 days as one unit then individually poised at 0.0, 0.5, and 1.0 mg/L free chlorine with sodium hypochlorite three days prior to the addition of *B. globigii* spores.

biofilm formation were made periodically through the sight glasses and optical densities at 600 nm were taken from the recirculating tanks as a measurement of planktonic growth. After 28 days of circulation, a synthetic tap water was used to stimulate biofilm growth containing the following per

L: $NaHCO_3$, 100 mg; $MgSO_4{\cdot}7H_2O$, 13.4 mg; $CaSO_4$, 27.0 mg; K_2HPO_4, 0.7 mg; KH_2PO_4, 0.3 mg; $(NH_4)_2SO_4$, 0.01 mg; NaCl, 0.01 mg; $FeSO_4$, 0.001 mg; humic acid, 1.0 mg; and $NaNO_3$, 1.0 mg. On a weekly basis, the pipe loops were amended with 50 mg of technical grade humic acid (Sigma-Aldrich, St. Louis, MO) and the pH was adjusted with NaOH to approximately 7.5 as needed. After an additional 28 days of continual operation, the pipe loops were drained and flushed with dechlorinated tap water to remove residual nutrients and excess planktonic bacteria. Each pipe loop was then segregated from one another and two of the three loops were amended with a 10% sodium hypochlorite solution to final free chlorine concentrations of 0.5 and 1.0 mg/L. After three additional days of circulation, each loop system was inoculated with *B. globigii* to a final viable density of approximately 1.8×10^4 CFUs/mL.

Bulk water and biofilm sampling

Bulk water and biofilm samples were taken from each pipe loop after 10 min, 3, 7, 14, and 21 days of circulation. One hundred mL liquid samples were collected from each reservoir tank using sterile 50 mL pipettes. Biofilm was sampled at each predetermined time interval by removing one sampling port per loop in a sequential order. Segments were replaced with sterile PVC segments of the same dimension. Pipes were gently rinsed with sterile ultra-pure water to remove residual planktonic bacteria and three 2 cm section were cut from the center of each sample port using a PVC pipe-cutter sterilized with 90% ethanol. The internal surface of each segment was scraped with a sterile micro-spatula and resuspended into 1 mL of sterile ultra-pure water. Bulk water samples, biofilm scrapings, and remaining sample port segments were immediately frozen at − 20°C until further analysis.

Bacterial enumerations

Heterotrophic plate counts (HPCs) were conducted with both bulk water and biofilm scrapings by serially diluting samples 10^{-1} to 10^{-7} in sterile ultra-pure water and plating 100 μL of each dilution in triplicate onto plate count agar (Fluka). Plates were allowed to incubate for 48 hr at 35°C and CFUs were counted on plates within a given dilution series having 50 to 200 colonies. Viable *B. globigii* were enumerated using the heat-shock plating method described previously. Levels of inactivation were determined by plotting the $\log_{10}$ ratio of survivors against exposure time (days). Values were expressed as the total number of heterotrophs or spores in each pipe loop system.

Biofilm characterization

Bulk water free chlorine concentrations were monitored using U.S. Environmental Protection Agency (USEPA) approved *N*,*N*-diethyl-*p*-phenylenediamine (DPD) photometric method (American Public Health Association 1995) using a DR/2000 spectrophotometer (Hach, Loveland, CO). Total polysaccharide content of the biofilm scrapings was determined with a modified colorimetric phenol-sulfuric acid method (Dubois *et al.* 1956). Briefly, 0.5 mL of sample and 0.5 mL of 5% phenol in 0.1 M hydrochloric acid were added to 2.5 mL concentrated sulfuric acid and incubated at room temperature for 10 min. Absorbance was read at 490 nm and unknown polysaccharide concentrations were determined with a glucose standard curve. Total extra-cellular enzyme content was evaluated using a Coomassie Plus Better Bradford Protein Assay Kit (Pierce, Rockford, IL) according to the manufactures micro test tube protocol. Free nucleic acids (ssDNA, dsDNA, and RNA) present were quantified using a Quant-iT^{TM} ssDNA Assay Kit (Invitrogen, Eugene, OR) according to the manufactures protocol. Bulk water and biofilm data were reported as the means ± standard deviation (SD) where $n = 3$.

Microscopy

Nonviable spores within biofilms were qualitatively assessed by light microscopy using a Nikon Eclipse E400 light microscope (Melville, NY) equipped with a Diagnostic Instruments, Inc. Insight digital camera and Spot imaging software (Sterling Heights, MI). Spores were differentially stained using a malachite green method as described by Schaeffer & Fulton (1993).

RESULTS AND DISCUSSION

Free chlorine, temperature, and pH were measured on a daily basis and values are reported for the days bacterial enumerations took place in Table 1. Free chlorine measured in the form of hypochlorous acid for each of the three pipe loop systems was found to remain relatively stable throughout the study. No free chlorine was detected in the untreated pipe loop until after the addition of sodium hypochlorite on day 14. Concentrations in the 0.5 mg/L and the 1.0 mg/L amended loops varied throughout the study 0.1 and 0.3 mg/L, respectively. Temperatures fluctuated less than 0.5°C between each of the three pipe loops on a given day. Slight variations in pH were observed among the pipe loops due to periodic sodium hypochlorite amendments; however, readings remain well within the range of a typical potable water distribution system (World Health Organization 2007). Due to the tremendous physiochemical tolerances associated with *Bacillus* spores (Nicholson *et al.* 2000), the slight variability in these physiochemical parameters were not believed to have a significant effect on *B. globigii* viability within any given pipe loop over the course of the study.

Turbidity was monitored prior to the start of the study as a measurement of biological activity. Only after the addition of synthetic tap water was an increase in optical density observed. Each of the pipe loop systems increased in absorbance from 0.01 to 0.03 at 600 nm after three weeks of circulation in the presence of humic acid (data not shown). Based on these measurements, it was believed each pipe loop contained equal biomass. This was confirmed by enumerating the bulk water heterotrophs, which revealed each system contained approximately 3.4 x 10^5 CFUs/mL prior to flushing the system. In order to conserve sampling port segments, heterotrophs were not enumerated within the biofilm prior to the addition of spores. However, the untreated pipe loop harbored 1.4 × 10^4 CFUs/cm^2 at the start of the study, which was indicative of a mature water distribution system (Schwartz *et al.* 2003). These data demonstrated the conditions in each of the pipe loops were similar at the onset of the study and each of the systems remained stable throughout the 21 day circulation period. Therefore, deviations in *B. globigii* spore viabilities were attributed directly to chlorine treatments.

Table 1 | PVC pipe loop physiochemical and chemical conditions

	Time (days)*				
	0	3	7	14	21
0.0 mg/L free chlorine†					
Free Cl^- (mg/L)	0.0	0.0	0.0	0.0	0.9
Temperature (°C)	23.3	25.4	22.8	26.4	23.2
pH	7.5	7.2	7.4	7.4	7.6
0.5 mg/L free chlorine†					
Free Cl^- (mg/L)	0.5	0.4	0.4	0.5	1.0
Temperature (°C)	23.6	25.9	23.1	26.9	23.4
pH	7.7	7.4	7.6	7.6	7.7
1.0 mg/L free chlorine					
Free Cl^- (mg/L)	1.0	1.1	0.9	1.1	1.2
Temperature (°C)	23.2	25.5	23.0	26.8	23.3
pH	7.7	7.8	7.9	7.9	7.9

*Days after the addition of 3.0 × 10^8 *B. globigii* spores to each PVC pipe loop system (Time 0 = 10 min).
†Pipe loop systems were amended to a target concentration of 1.0 mg/L free chlorine on day 14.

Total polysaccharide, free protein, and extracellular nucleic acid concentrations were determined within the biofilm of each pipe loop system as a measurement of biological activity and possible biofilm decay (Table 2). In the chlorine untreated pipe loop, the polysaccharide concentrations increased steadily over the course of the study to a maximum concentration of 3.6 μg/cm^2 after 14 days of circulation. Free protein and nucleic acids also accumulated to maximum concentrations of 0.3 μg/cm^2 and 2.4 μg/cm^2 on day 14, respectively. Poising the system at 1.0 mg/L free chlorine decreased the polysaccharide concentration to 1.6 μg/cm^2 and completely oxidized both extracellular proteins and free nucleic acids after seven additional days of circulation. The biofilms within the chlorine treated pipe loops remained intact and no statistical variation in polysaccharide concentrations were observed over the course of the study. This was consistent with studies that have demonstrated that free chlorine concentrations less than 5 mg/L have little effect on biofilm removal (Chen & Stewart 2000) and biofilm sloughing can be inhibited by chlorine treatment (Daly *et al.* 1989). The nearly 2-fold decrease in the polysaccharide composition observed in the untreated pipe loop after the addition of sodium hypochlorite was likely do to detachment caused by environmental stress (Garny *et al.* 2009). Free proteins

Table 2 | PVC pipe loop biofilm biochemical compositions

	Time (days)*				
	0	3	7	14	21
0.0 mg/L free chlorine†,‡					
Polysaccharide ($\mu g/cm^2$)	0.6 ± 0.1	1.2 ± 0.4	1.4 ± 0.5	3.6 ± 0.5	1.6 ± 0.1
Protein ($\mu g/cm^2$)	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.0	0.0 ± 0.0
Nucleic acids (ng/cm^2)	0.8 ± 0.4	1.7 ± 0.7	1.1 ± 0.4	2.4 ± 1.2	0.0 ± 0.0
0.5 mg/L free chlorine†,‡					
Polysaccharide ($\mu g/cm^2$)	0.6 ± 0.1	0.6 ± 0.2	0.8 ± 0.2	0.4 ± 0.2	0.4 ± 0.2
Protein ($\mu g/cm^2$)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
Nucleic acids (ng/cm^2)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1.0 mg/L free chlorine†					
Polysaccharide ($\mu g/cm^2$)	0.4 ± 0.1	0.4 ± 0.2	0.6 ± 0.3	0.8 ± 0.2	0.6 ± 0.2
Protein ($\mu g/cm^2$)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Nucleic acids (ng/cm^2)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

*Days after the addition of 4.0×10^8 *B. globigii* spores to each PVC pipe loop system (Time 0 = 10 min).
†Data are means ± SD ($n = 3$).
‡Pipe loop systems were amended to a target concentration of 1.0 mg/L free chlorine on day 14.

remained steady at 0.1 $\mu g/cm^2$ in the 0.5 mg/L free chlorine amended pipe loop until the chlorine concentration was increased to 1.0 mg/L on day 14, then proteins were partially oxidized until no longer detectible by the method applied. No proteins or nucleic acids were detected in the 1.0 mg/L free chlorine amended pipe loop throughout the study. Collectively, the biofilm physiochemical conditions clearly demonstrated the ability of the PVC pipe biofilm to grow under oligotrophic conditions. Furthermore, free chlorine concentrations of 0.5 mg/L were sufficient to penetrate and suppress polysaccharide formation in addition to oxidize nucleic acids; however, free chlorine concentrations greater than 0.5 mg/L were required to partially oxidize biofilm proteins to levels below detection.

HPCs were conducted on the pipe loop bulk water prior to the addition of sodium hypochlorite and approximately 3.4×10^3 CFUs/mL were observed in each system after flushing with dechlorinated tap water. Heterotrophs remained stable in the untreated system until the addition of 1.0 mg/L free chlorine (Figure 2). After treatment on day 14, a 6.8-$\log_{10}$ reduction in the total number of heterotrophs was observed within 7 days, representing complete inactivation. In the 0.5 mg/L amended pipe loop, heterotrophs decreased 1.6-$\log_{10}$ within the 3 day stabilization period prior to spore inoculation. Three days after the addition of spores to the pipe loop a 6.2-$\log_{10}$ reduction was observed. No viable HPCs were detected in the 1.0 mg/L free chlorine treated pipe loop throughout the 21 day study indicating complete inactivation prior to the addition of *B. globigii* spores.

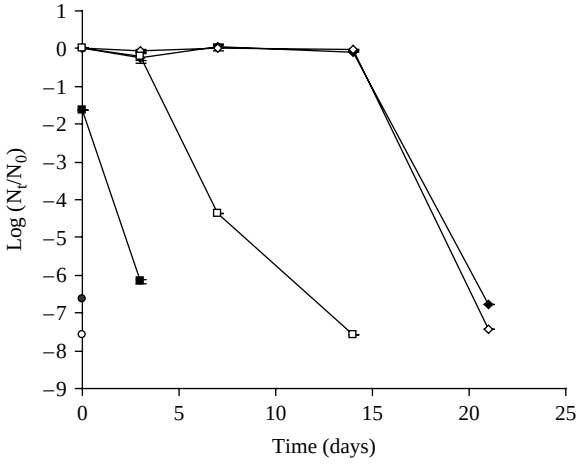

Figure 2 | Susceptibility of heterotrophic bacteria and *B. globigii* spores to free chlorine in PVC pipe loop bulk water, presented as the $\log_{10}$ of the total surviving fraction. Approximately 3.0×10^8 viable *B. globigii* spores were added to each pipe loop system on day zero. Free chlorine in all systems was poised at 1.0 mg/L on day 14. Symbols: ◆, heterotrophs, chlorine untreated; ◇, *B. globigii*, chlorine untreated; ■, heterotrophs, 0.5 mg/L free chlorine; □, *B. globigii*, 0.5 mg/L free chlorine; ●, heterotrophs, 1.0 mg/L free chlorine; and ○, *B. globigii*, 1.0 mg/L free chlorine. Data are means ± SD ($n = 3$), some error bars are not visible due to size.

Within the bulk water of the untreated pipe loop the viable *B. globigii* spore concentration was $1.5 \times 10^4 \pm 1.2 \times 10^3$ CFUs/mL and remained statistically consistent until the introduction of 1.0 mg/L free chlorine on day 14 (Figure 2). After 7 days of chlorine treatment, a 7.4-$\log_{10}$ reduction in spore viability was observed. The spore concentration in the 0.5 mg/L pipe loop was $2.3 \times 10^4 \pm 1.2 \times 10^3$ CFUs/mL after 10 min of circulation. No statistical variation in spore numbers were observed between the untreated and 0.5 mg/L free chlorine amended pipe loops, indicating no inactivation had occurred within the first 10 min of circulation prior to sampling. Subsequently, a 4.4-$\log_{10}$ reduction in spore numbers was observed after 7 days circulation and a 7.6-$\log_{10}$ reduction was observed after 14 days within the 0.5 mg/L free chlorine system. No viable spores were detected within the bulk water of the 1.0 mg/L free chlorine amended pipe loop throughout the study indicating an 8.0-$\log_{10}$ reduction within 10 min of contact time.

Biofilm HPCs in the chlorine unamended pipe loop were $1.4 \times 10^3 \pm 2.2 \times 10^2$ CFUs/cm^2 at the start of the study and remained stable until chlorine treatment (Figure 3). On day 14, after the addition of 1.0 mg/L free chlorine, a 7.1-$\log_{10}$ reduction in HPCs were observed within 7 days, which represented 100% of the sessile heterotrophic population. In the 0.5 mg/L free chlorine treated pipe loop, a 1.0-$\log_{10}$ reduction in heterotrophs was observed after 3 days circulation and complete inactivation (7.1-$\log_{10}$) was achieved after 7 days of incubation. Consistent with published studies (Murga *et al.* 2001; Park *et al.* 2001; Steed & Falkinham 2006; Szabo *et al.* 2006; DeQueiroz & Day 2007; Bressler *et al.* 2009), this clearly demonstrated that sessile heterotrophs were more resistant (nearly 3-times) to chlorine inactivation than planktonic forms. As expected, elevated free chlorine concentrations increased the rate of heterotroph disinfection. As seen with the bulk water, no CFUs were detected within the biofilm of the pipe loop amended with 1.0 mg/L free chlorine throughout the 21 day study, indicating 100% inactivation within the 3 days prior to spore inoculation.

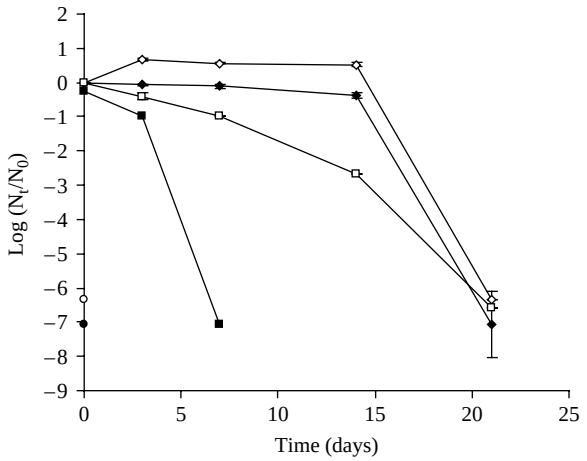

Figure 3 | Susceptibility of heterotrophic bacteria and *B. globigii* spores to free chlorine in PVC pipe biofilm, presented as the $\log_{10}$ of the total surviving fraction. Approximately 3.0×10^8 *B. globigii* spores were added to each pipe loop system on day zero. Free chlorine in all systems was poised at 1.0 mg/L on day 14. Symbols: ◆, heterotrophs, chlorine untreated; ◇, *B. globigii*, chlorine untreated; ■, heterotrophs, 0.5 mg/L free chlorine; □, *B. globigii*, 0.5 mg/L free chlorine; ●, heterotrophs, 1.0 mg/L free chlorine; and ○, *B. globigii*, 1.0 mg/L free chlorine. Data are means ± SD ($n = 3$), some error bars are not visible due to size.

B. globigii spores were found to associate rapidly with the pipe surface biofilm. After 10 min of circulation 285 ± 92 and 480 ± 6 viable spores/cm^2 of pipe were detected in the untreated pipe loop and 0.5 mg/L free chlorine loops, respectively (Figure 3). After 3 days of circulation, the number of spores associated with the biofilm in the untreated pipe loop increased to $1.3 \times 10^3 \pm 205$ spores/cm^2 (0.7-$\log_{10}$ increase), which represented roughly 3% of the total spores added to the system. Based on an inhalation critical dose of *B. anthracis*, an aqueous critical dose is estimated to be 171 spores/L at a consumption rate of 5 L/day for seven days (Burrows & Renner 1999). Studies have shown contingent on physical parameters, mainly nutritional state and hydraulic forces; more than 90% of a pipe biofilm can be sloughed from the surface (Garny *et al.* 2009). This suggested that spore concentrations within the PVC pipe biofilm were theoretically sufficient to cause infection in the event of biofilm detachment.

Spore numbers remained statistically consistent in the untreated pipe loop until the addition of 1.0 mg/L free chlorine to the system on day 14. After chlorine treatment *B. globigii* spores were not detected after 7 additional days of circulation, which represented a 7.1-$\log_{10}$ reduction in the total sessile spore population (Figure 3). In the 0.5 mg/L free chlorine loop system, only a 2.7-$\log_{10}$ reduction in sessile spores was observed after 14 days of circulation compared to a 7.6-$\log_{10}$ reduction observed in the bulk

water phase. This indicated a decrease in disinfection efficiency of nearly five orders of magnitude, which clearly demonstrated spores were protected form chlorine inactivation after being incorporated into the biofilm. Because free chlorine concentrations were kept balanced in each pipe loop throughout the study, it was believed limited diffusion was the most likely the mode of resistance (De Beer *et al.* 1994; Szabo *et al.* 2006).

Interestingly, both 0.5 mg/L and the 1.0 mg/L free chlorine pipe loops were found to be comprised of roughly equal concentrations of polysaccharides per cm^2, yet no viable spores were detected within the biofilm of 1.0 mg/L amended pipe loop throughout the study. Light microscopy revealed spores were embedded within the biofilm, but the lack of viable spores within the bulk water indicated spores were most likely inactivated prior to incorporation into the biofilm. In contrast, the 0.5 mg/L free chlorine pipe loop inactivation rate was decreased to such a degree as to allowed viable spores to integrate within the biofilm, which resulted in elevated persistence within the loop system.

Although 1.0 mg/L free chlorine was demonstrated to completely inactivate 1.8×10^4 *B. globigii* spores/mL within the PVC pipe systems, it is to be noted that these experiments were conducted at room temperature, and chlorine inactivation at typical water distribution temperatures is expected to reduce $\log_{10}$ inactivation ratios appreciably (Ndiongue *et al.* 2005). Rice *et al.* (2005) observed approximately a 3-fold increase in 2-$\log_{10}$ inactivation values for *B. anthracis* Sterne, and *B. cereus* when temperature was decreased from 23°C to 5°C in the presence of free chlorine. When treated with monochloramine, Rose *et al.* (2007) reported increased 2-$\log_{10}$ inactivation times of 8-fold for *B. anthracis* Ames and 7-fold for *B. anthracis* Sterne, when temperatures decreased from 25°C to 5°C. This suggests that spore inactivation intervals for a given $\log_{10}$ reduction within the pipe loop systems described in this study would increase significantly at lower water temperatures. Thus, we hypothesize that aggressive disinfection techniques requiring greater than 1.0 mg/L would be necessary to minimize exposure at typical water distribution system temperatures, this in addition to testing multiple pipe surfaces is the focus of future studies.

CONCLUSIONS

Based on the data presented here, the following conclusions can be drawn:

(1) *B. globigii* spores can rapidly associate within mature PVC bacterial biofilms and remain viable for weeks under moderate free chlorine concentrations.
(2) Incorporation of *B. globigii* spores into pipe biofilms can increase resistance to chlorine inactivation several orders of magnitude at free chlorine concentrations of 0.5 mg/L or less.
(3) Treatment with 1.0 mg/mL free chlorine can completely inactivate *B. globigii* spores prior to incorporation within a biofilm at room temperature. However, $\log_{10}$ reductions are expected to decrease significantly as temperatures are lowered. Therefore, aggressive decontamination procedures with free chlorine concentrations greater than 1.0 mg/L would likely be necessary eliminate exposure risks.

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