Environmental Technology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tent20

Persistence of non-native spore forming bacteria in drinking water biofilm and evaluation of decontamination methods

William T. Shane [a], Jeffrey G. Szabo [b] & Paul L. Bishop [a]

[a] Department of Civil and Environmental Engineering, University of Cincinnati, Cincinnati, Ohio, 45221, USA

[b] United States Environmental Protection Agency (EPA), National Homeland Security Research Center, Water Infrastructure Protection Division, Cincinnati, Ohio, 45268, USA

Available online: 17 Jun 2011

To cite this article: William T. Shane, Jeffrey G. Szabo & Paul L. Bishop (2011): Persistence of non-native spore forming bacteria in drinking water biofilm and evaluation of decontamination methods, Environmental Technology, 32:8, 847-855

To link to this article: http://dx.doi.org/10.1080/09593330.2010.515038

Environmental Technology
Vol. 32, No. 8, June 2011, 847–855

Taylor & Francis
Taylor & Francis Group

Persistence of non-native spore forming bacteria in drinking water biofilm and evaluation of decontamination methods

William T. Shane[a]*, Jeffrey G. Szabo[b] and Paul L. Bishop[a]

[a]*Department of Civil and Environmental Engineering, University of Cincinnati, Cincinnati, Ohio 45221, USA;* [b]*United States Environmental Protection Agency (EPA), National Homeland Security Research Center, Water Infrastructure Protection Division, Cincinnati, Ohio, 45268, USA*

(*Received 5 May 2010; Accepted 6 August 2010*)

Persistence of *Bacillus globigii* spores, a surrogate for *Bacillus anthracis*, was studied on biofouled concrete-lined slides in drinking water using biofilm annular reactors. Reactors were inoculated with *B. globigii* spores and persistence was monitored in the bulk and biofilm phases, first in dechlorinated water and later with free chlorine concentrations of 1 and 5 mg/L. In the dechlorinated study, a steady state population of spores developed on the slides. The addition of free chlorine at 5 mg/L decreased the adhered spore density by 2-logs within 4 hours and spores were not detected after 67 and 49 hours in the presence of 1 and 5 mg/L free chlorine, respectively. This suggests that adhered spores can persist in non-chlorinated conditions, but detach and/or are inactivated upon addition of free chlorine. When injected into a chlorinated reactor, adhered spore density continually decreased and spores were either undetectable or unquantifiable by 48 hours for both 1 and 5 mg/L chlorine concentrations. Results from these experiments suggest that the presence of a free chlorine residual limits adherence of viable spores to biofouled concrete-lined pipe walls by inactivating spores before they have attached. Both free chlorine concentrations (1 and 5 mg/L) were equally effective at inactivating spores in terms of log reduction, but the higher concentrations yielded faster rates of log reduction.

Keywords: spores; biofilm; concrete; chlorine; decontamination

Introduction

Introduction of pathogenic microorganisms into water distribution systems and their interaction with biofouled pipe walls is a security concern to drinking water producers and users. Because contaminated drinking water distribution networks can spread epidemic disease, a strategic attack with microbiological agents on a vulnerable system could sicken a large number of people [1]. The ultimate goals of this research are to understand the persistence of microbiological agents on distribution system infrastructure after a contamination event and to aid in the decontamination decision-making process if a contamination event were to occur.

Bacillus anthracis has emerged as a commonly scrutinized bioterror agent [1–6]. In this research, a surrogate for *B. anthracis* spores (*B. globigii* spores) was used [2,5]. Past research has focused on the inactivation of planktonic *B. anthracis* and *B. globigii* spores by free available chlorine, monochloramine and chlorine dioxide [2–7]. Other research has studied the persistence of *B. globigii* spores on ductile iron coupons, polyvinylchloride (PVC) and copper under non-chlorinated and chlorinated conditions [8,9]. Additionally, biofilm growth has been studied on pipe materials, such as ductile-iron, PVC, and concrete [10–11]. However, the authors are not aware of data in the peer-reviewed literature describing the persistence of pathogenic spore-forming microorganisms on the walls of biofouled concrete-lined water distribution pipe, which is a common drinking water infrastructure material.

Several important factors affect the accumulation of biofilm in drinking water distribution systems. They include temperature, flow velocity, concentration of biodegradable organic matter, disinfectant residual, disinfectant type, and pipe material [12]. The use of a biofilm annular reactor allowed manipulation of some of these factors in order to simulate a drinking water pipe surface. In this research, persistence of *B. globigii* spores was studied on biofouled concrete-lined substrata in biofilm annular reactors under non-chlorinated and chlorinated conditions.

*Corresponding author. Email: william.shane@ky.gov

ISSN 0959-3330 print/ISSN 1479-487X online

DOI: 10.1080/09593330.2010.515038
http://www.informaworld.com

Materials and methods

Biofilm annular reactor

The conditions in a concrete or concrete-lined drinking water pipe were approximated in biofilm annular reactors (BioSurface Technologies Corporation, Bozeman, MT). The reactors consist of an outer glass cylinder and a rotating inner polycarbonate cylinder with 20 flush mounted concrete-coated 15 x 1.5 cm polycarbonate slides (BioSurface Technologies Corporation, Bozeman, MT). The fluid volume between the inner and outer cylinders is approximately 1 L. Shear stress was applied to the slide surfaces by rotating the inner cylinder at 100 RPM. Assuming a Hazen-Williams coefficient of 130, this produced shear similar to a flow of 34.3 cm/sec (1.125 ft/sec) in a 20.3 cm (8 in) pipe. Reactors were cleaned with soap and water and sterilized in an autoclave for 25 minutes at 121°C before use. Two identical reactors hereafter referred to as reactors 1 and 2 were used for all experimentation.

The reactors were operated for one month prior to spore addition so that a biofilm could establish. Dechlorinated Cincinnati tap water was pumped into the reactors from a 50 L tank at a rate of 0.5 L/hr, resulting in a residence time of 2 hours. This flow rate minimized the water used, but provided enough flow to maintain the influent water quality in the reactor bulk phase [8]. Dechlorination was performed by adding sterile 10% sodium thiosulfate solution to the tank feeding the reactors. Average pH in the reactors was 8.5, which is typical of Cincinnati tap water. Water temperature in the reactor was 23±2°C, which was the laboratory air temperature. Initially, the dechlorinated tap water feeding Reactor 1 was supplemented with 500 μg/L of sodium acetate as a microbial growth media since it was not known whether a detectable biofilm would establish on the concrete slides. Reactor 2 was not provided with any additional substrate.

Sampling and analysis of biofilm material

Concrete coated polycarbonate slides were aseptically removed from the reactor using a sterile slide removal tool (BioSurface Technologies Corporation, Bozeman, MT) and one-third of the slide surface was scraped with a sterile scalpel. Slides were re-inserted into the reactor in case more samples were needed. Unscraped slides were sampled before previously scraped slides. Much of the concrete lining was removed during the scraping process and it was assumed that all of the adhered spores were removed with it. The scrapings were placed into 9 mL of dilution water (0.05 M sterile KH_2PO_4 buffer) and the combined solution was thoroughly mixed using a tissue homogenizer (Teledyne Tekmar, Mason, OH). The mixture was serially diluted, and heterotrophic place count (HPC) was determined by plating on pre-prepared R2A in triplicate (Becton, Dickinson and Company, Franklin Lakes, NJ), and incubating for 7 days at 20°C (Standard Method 9125 C) [13]. One slide per day was removed for the first 10 days of growth, after which slides were removed every two days.

Culturing, inoculation, and analysis of spores

Spores of *Bacillus atrophaeus* subsp. *globigii* (*B. globigii*) were prepared as reported previously [8,14]. Purified spores were suspended in a 40% ethanol solution and stored at 4°C. Before use, *B. globigii* viability was verified by spread plating the stock solution on Trypticase™ Soy Agar (TSA) (BD Diagnostic Systems, Sparks, MD) and incubating at 35°C for 48 hours [13]. The reactors were inoculated with spores by pulse injecting volumes of the stock suspension necessary to achieve the desired spore density in each reactor. Injections were performed while water was flowing through the reactor.

Spore density was enumerated in the biofilm and bulk liquid by spread plating in triplicate (TSA, 35°C, 48 hours) [13]. Ten millilitres of reactor water were removed for bulk phase samples. The coupon sampling method was identical to the procedure for sampling biofilm for HPC analysis. However, if free chlorine was present in the reactor, 0.1 mL of sterile 10% sodium thiosulfate was added to the buffer and the bulk phase sample. Before dilution, heat shock was performed to inactivate background biofilm organisms by placing the sample vials in a water bath (Fisher Scientific, Pittsburgh, PA) at 80°C for 12 minutes. Assuming time zero to be the injection of the spores, the following bulk phase and biofilm sampling times were targeted: 10 minutes, 1.5 hours, 4 hours, and 8 hours. Thereafter, samples were removed daily from each reactor and until no spores were detected, slides were exhausted or until decontamination started, at which time samples were taken as deemed necessary.

Free chlorine was pumped into the reactors using a KDS220 multiple syringe infusion pump (KD Scientific Inc., Holliston, MA). The target disinfectant concentrations were 1 and 5 mg/L, which represent typical chlorine residual (1 mg/L) and a decontamination free chlorine concentration (5/mg/L). The disinfectant solution was prepared by mixing Clorox® bleach and distilled water. The free chlorine in each reactor was measured and recorded during each sampling event using a Hach Method 8021 with a DR/2010 Spectrophotometer and DPD (N, N-diethyl-*p*-phenylenediamine) powder pillows (Hach Co, Loveland, CO).

Spore persistence and decontamination experiments

Experiments were conducted in three phases. During Phase 1 the reactors were inoculated with 0.1 ml of the stock spore suspension. This volume achieved an initial bulk phase spore density of $1x10^5$ CFU/ml when calculated by dilution. Chlorine was not added to the reactors until 389 hours after inoculation in order to provide time for spore attachment to the biofilm and to monitor persistence under the most favorable attachment scenario (complete dechlorination). During Phase 2, 1 mL of spore suspension was pulse injected into each reactor at time zero. This volume achieved an initial bulk phase spore density of $1x10^6$ CFU/ml when calculated by dilution. Addition of chlorine to the reactors began at time zero. Phase 3 was a repeat of Phase 2 with 1 mL of spore suspension pulse injected into each reactor at time zero and addition of chlorine to the reactors begun at time zero. During all phases, chlorine was added to the reactor influent at the appropriate time, and flow through the reactor was maintained at 0.5 L/hr.

Results and discussion

Formation of biofilm on concrete-coated slides

Figure 1 shows the change in biofilm density over time on concrete-coated slides in both reactors over approximately one month. Since the reactors were initially sterile, biofilm is presumably formed from the microorganisms in Cincinnati tap water. Biofilm density in both reactors reached a level density after 30 days. The addition of substrate encouraged more growth of biofilm. After one month, HPC was 7.0×10^4 CFU/cm^2 on the slides in Reactor 1 (supplemented with acetate) and 6.0×10^3 CFU/cm^2 on the slides in Reactor 2 (no acetate). However, since biofilm did form in Reactor 2, feed water was not supplemented with acetate before any of the spore persistence experiments. Biofilm data is summarized in Table S1 (Supplementary Material, available online).

HPC values for both reactors are comparable to previously reported values. Ollos et al. reported that HPC values ranged from 3.7×10^4 to 6.0×10^5 CFU/cm^2 on polycarbonate slides under dechlorinated conditions [12]. Niquette *et al.* found that, when comparing bacterial densities on different pipe materials, values on plastic-based materials were similar to values on concrete-based materials [15]. It should be noted that the HPC values reported in this study might underestimate actual biofilm density, both due to concrete particles in the biofilm scrapings causing clumping of the heterotrophs and to variation in scraping between coupons.

Spore viability

Before spiking the reactors with *B. globigii* spores, the stock suspension was enumerated. The stock suspension

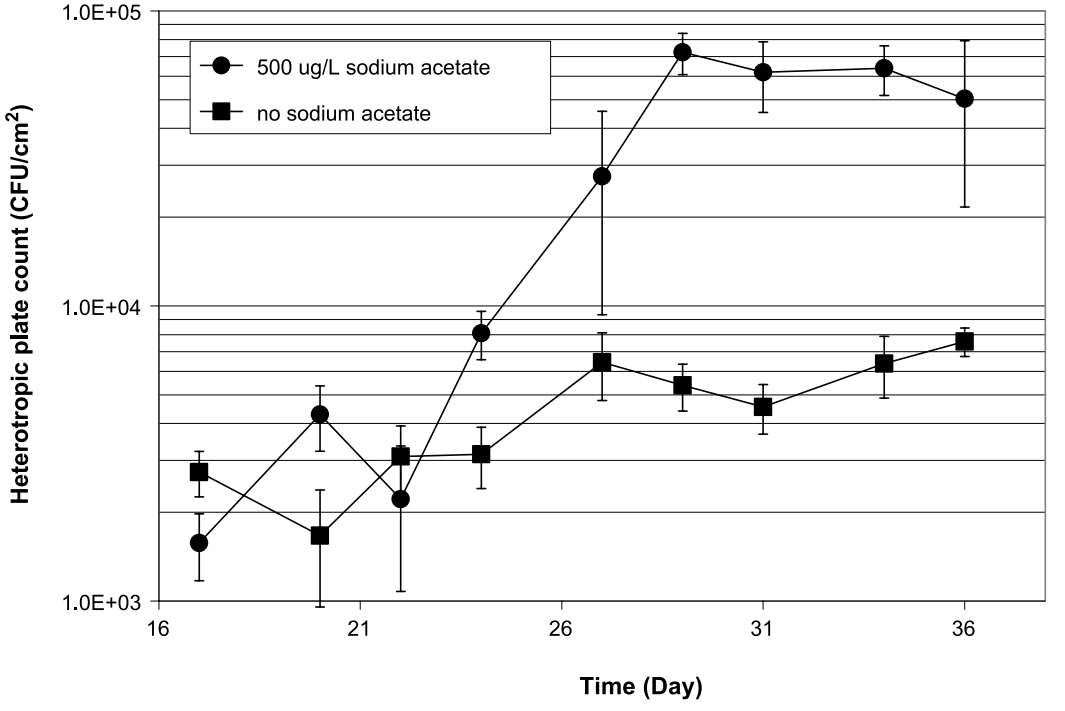

Figure 1. Development of biofilm on concrete coated slides. A sodium acetate solution was continuously fed to Reactor 1, but not to Reactor 2. At the end of one month, the slides in Reactor 1 showed approximately 1 log higher population of biofilm microorganisms per unit area of surface. [●] = 500 μg/L sodium acetate, [■] = no sodium acetate.

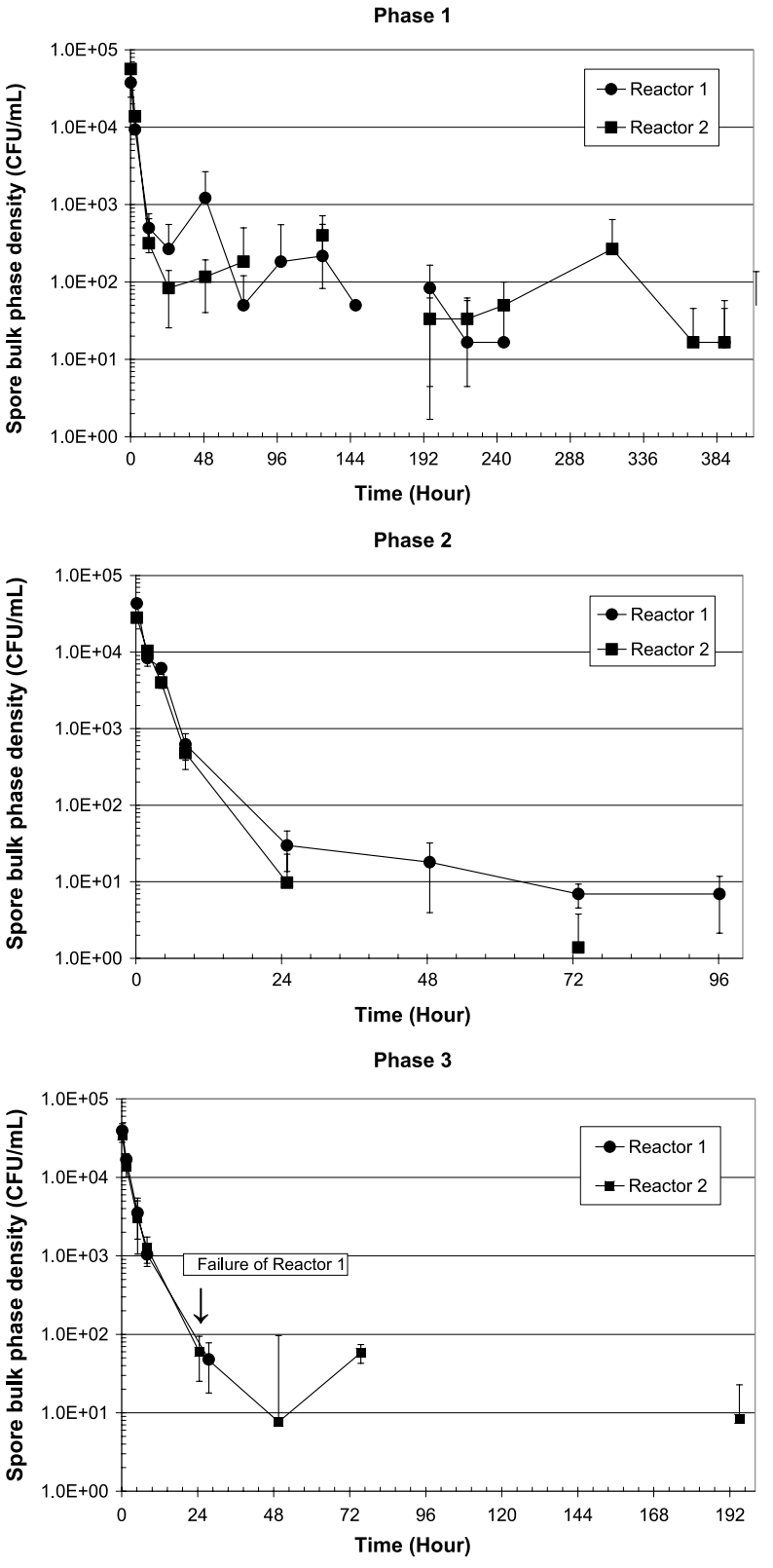

Figure 2. Spore persistence in the bulk water for Phases 1, 2, and 3. No chlorine was added to the reactors during Phase 1 until approximately 400 hours. Target chlorine concentrations: [●] = 1 mg/L (Reactor 1), [■] = 5 mg/L (Reactor 2). Mechanical problems caused biofilm reactor 1 to stop working at hour 24.

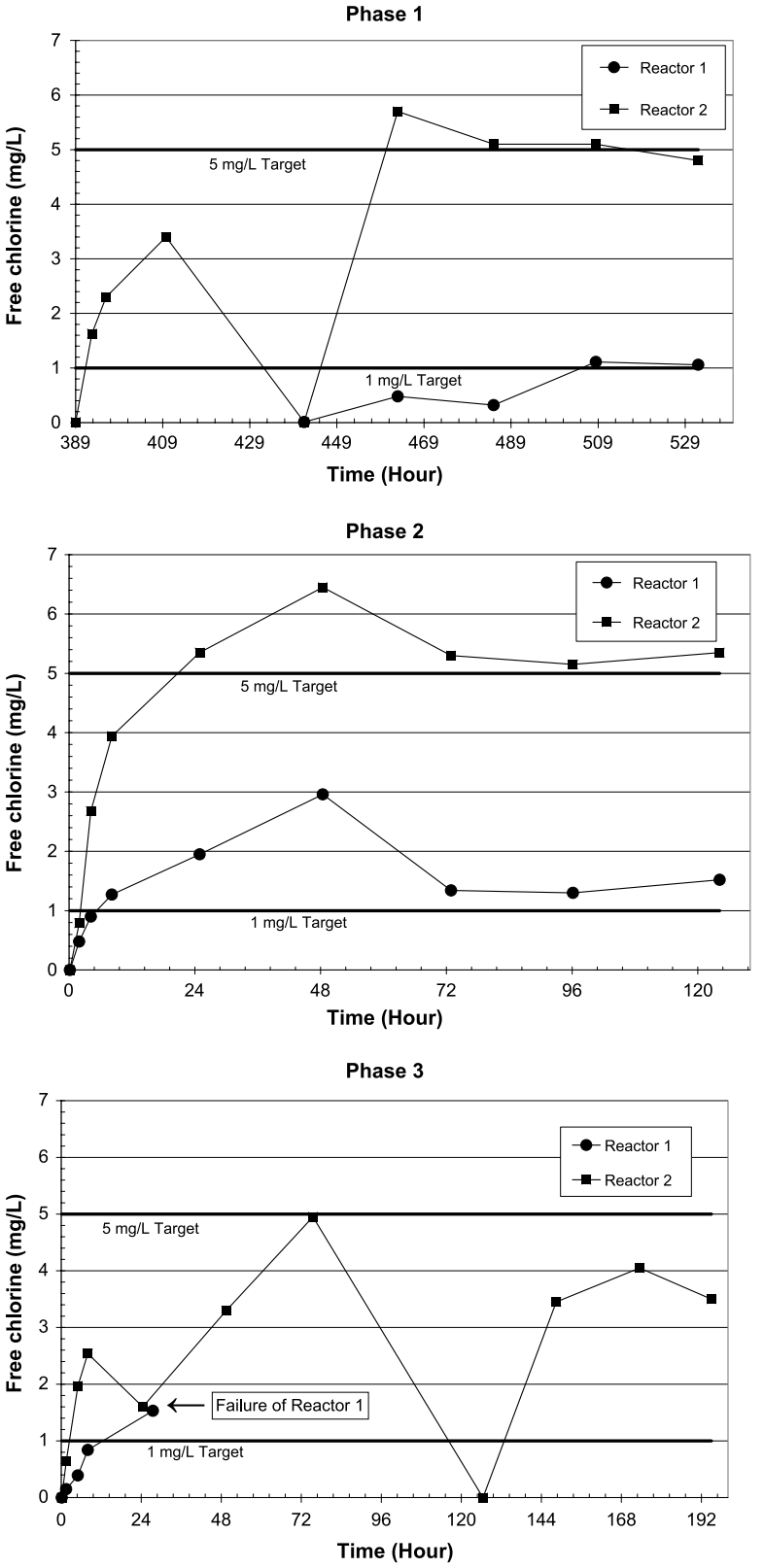

Figure 3. Measured free chlorine concentrations in the reactors for Phases 1, 2, and 3. Target chlorine concentrations: [●] = 1 mg/L (Reactor 1), [■] = 5 mg/L (Reactor 2). Mechanical problems caused biofilm reactor 1 to stop working at hour 24.

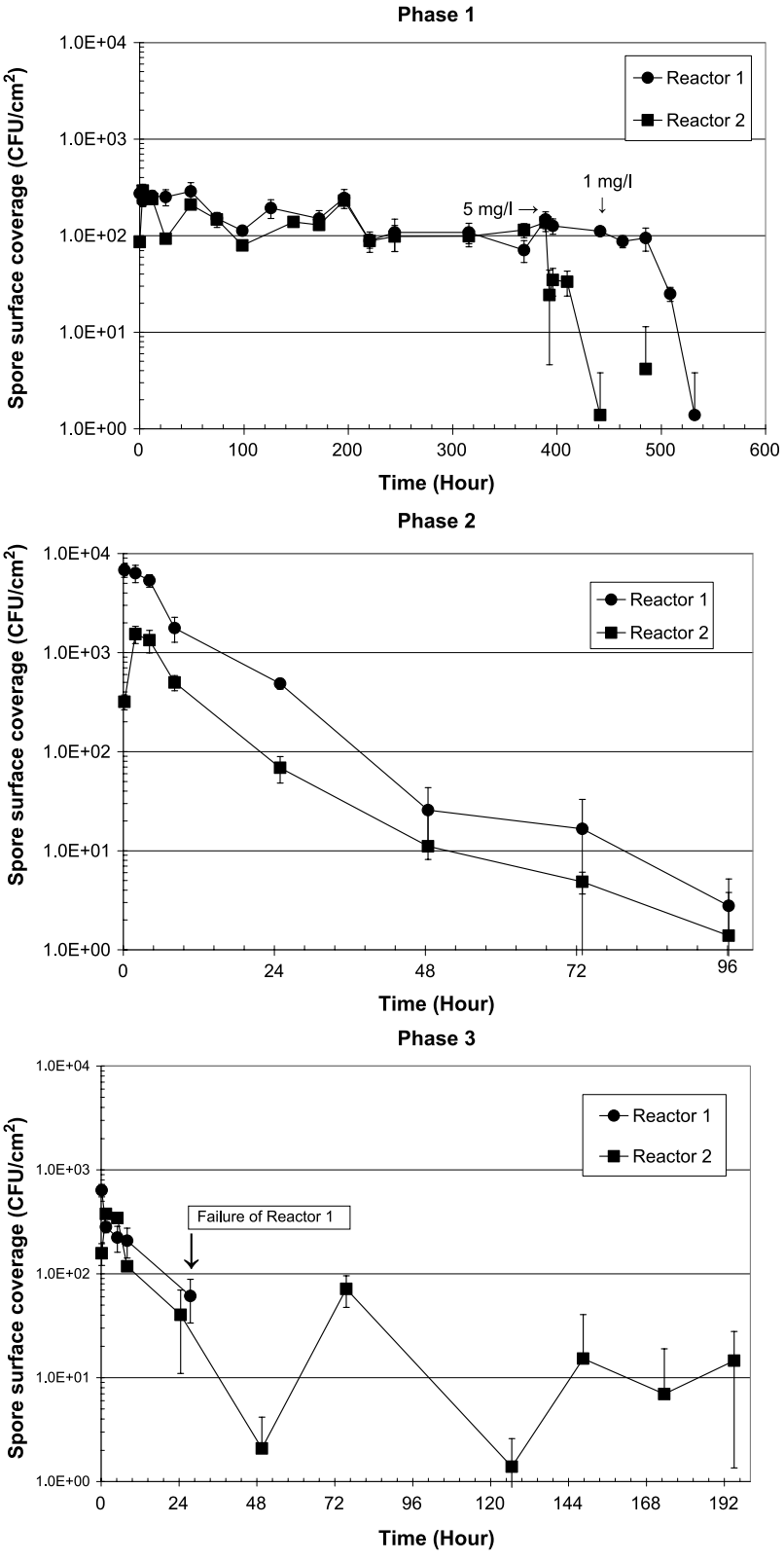

Figure 4. Spore persistence on concrete coated slides for Phases 1, 2, and 3. No chlorine was initially added to the reactors in Phase 1, but once a steady state existence of spores was reached, the chlorine disinfectant solution was added (arrows in Phase 1 indicate start of chlorine). Target chlorine concentrations: [●] = 1 mg/L (Reactor 1), [■] = 5 mg/L (Reactor 2). Mechanical problems surface. [●] = 500 μg/L sodium acetate, [■] = no sodium acetate caused biofilm reactor 1 to stop working at hour 24.

was found to contain 1×10^9 CFU/mL, and this density was verified using the spread plate method described earlier [8]. This ensured that the spore suspension had not degraded during storage at 4°C.

Spore persistence in the bulk water

Spore density in the bulk water phase was monitored over time under both non-chlorinated and chlorinated conditions. The results for each phase are shown in Figure 2. The free chlorine concentrations measured in each reactor during experimentation are shown in Figure 3. In Phase 1, spore concentrations of 3.7×10^4 and 5.6×10^4 CFU/mL were measured 10 minutes after spiking in reactors 1 and 2, respectively. After this, bulk liquid spore counts in both reactors dropped more than 2 logs within 12 hours. However, spores were detected in the bulk phase at 100–200 CFU/mL up to the point at which injection of chlorine began. If the reactor was modelled as an ideal continuously stirred tank reactor (CSTR) with no adsorption occurring, bulk phase spores would be completely washed out in 1.4 days. The data indicates that most of the spores were washed out of the reactors since no disinfectant was present to inactivate them. However, it is likely that the low level of spores detected from 12 to 120 hours after spiking resulted from sloughing of the biofilm or detachment of adhered spores.

During Phase 2, spore concentrations of 4.3×10^4 and 2.8×10^4 CFU/mL were measured after 10 minutes in Reactors 1 and 2, respectively. The spore density continuously decreased and no spores were detected after 25 hours in both reactors. Ct (disinfectant concentration x exposure time) values ranging from 40–400 mg-min/L indicate that the addition of chlorine to the reactors could have inactivated most of the planktonic spores after 4–6 hours of contact [3–9]. However, it is likely that the spores were also washed out of the system. During Phase 3, spore concentrations of 3.9×10^4 and 3.4×10^4 CFU/mL were measured after 10 minutes in Reactors 1 and 2, respectively. Spore density continuously decreased and became undetectable within 24 hours in both reactors.

Two observations can be made from data in these three experiments. The first is that the number of planktonic spores present at 10 minutes after injection was similar in Phases 1, 2 and 3 even though the spore density was 10 times higher in the second and third phases. This could be due to free chlorine inactivating some of spores before they could adhere to the biofilm matrix, or because more spores adhered to the coupons at the higher initial bulk phase density. Although free chlorine was likely the reason that spores remaining in the bulk phase (not washed out) were undetectable 24 hours after spiking in experiments 2 and 3, it is unlikely that a significant number of spores were inactivated after 10 minutes in contact with free chlorine [3–5]. This supports the second observation that, in a chlorinated biofilm annular reactor system, planktonic spores were not detectable in the bulk phase for longer than 24 hours.

Persistence of adhered spores

The persistence of adhered spores was monitored under both non-chlorinated and chlorinated conditions. The results for each phase are shown in Figure 4. The chlorine concentrations measured in each reactor during experimentation are shown in Figure 3. Prior to the start of Phase 1, biofilm HPC of 4.1×10^3 and 3.4×10^3 CFU/cm^2 were measured in Reactors 1 and 2, respectively. The first part of Phase 1 monitored persistence under non-chlorinated conditions, which allowed the spores to persist until chlorine was added. A decrease in adhered spore counts occurred in both reactors between spiking (hour 0) and hour 200, but a quasi-steady state spore density of approximately 1.0×10^2–1.5×10^2 CFU/cm^2 was measured on slides between hours 200 and chlorine addition. Since no chlorine was present in the system between hours 0 to 200, loss of adhered spores was likely due to sloughing of biofilm or detachment of spores caused by shear forces on the slides.

The addition of chlorine in Phase 1 contributed to the decrease of adhered spores to undetectable levels (arrows in Figure 4 [Phase 1] indicate start of chlorine). In Reactor 2, adhered spores decreased nearly one-log (83%) from hours 389 to 393 when the chlorine concentration was between 0 and 1.6 mg/L. Spores were detected at hour 410 (17 hours after chlorination started), and dropped to undetectable levels at hour 442 (49 hours after chlorination started). Spores were again detected at hour 475, but none were detected thereafter. The target free chlorine concentration in this reactor was 5 mg/L, but most spore density decrease had occurred by the time this concentration was reached. The drop in free chlorine at hour 440 was due to a mechanical problem with the syringe pump that delivered the free chlorine (this is also true for hour 125 in Phase 3). It should also be noted that since all pre and post chlorination samples were heat shocked prior to analysis, the decrease in adhered *Bacillus* is due to the action of free chlorine on spores and not vegetative *Bacillus* or heat sensitive cells in the process of germinating.

In Reactor 1, chlorine was introduced at hour 442, but a 73% decrease in spore counts was not recorded until hour 509 (67 hours). For the first 47 hours after chlorine introduction, the free chlorine concentration was approximately 0.5 mg/L and it did not reach

1 mg/L (the target concentration) until hour 509. No spores were detected on the coupons after hour 509. These data show that a free chlorine concentration of 1 mg/L caused a reduction in viable or culturable adhered spores.

In Phase 2, inoculation of the reactors and start of the chlorine addition occurred simultaneously. Prior to experimentation, biofilm densities of 3×10^4 and 4.5×10^4 CFU/cm^2 were measured in Reactors 1 and 2, respectively. In Reactor 1, a spore concentration of 6.8×10^3 CFU/cm^2 was measured on the slides after 10 minutes, while in Reactor 2, a spore concentration of 1.5×10^3 CFU/cm^2 was measured on the slides after 2 hours. Spore counts quickly dropped and were not detectable in either reactor after 48 hours. The increased volume of spore suspension (10×) injected into the reactors in Phase 2 was reflected in the number of spores adhered during this phase. In both reactors, approximately a 1 log increase in the number of initially attached spores was observed compared to in Phase 1.

Phase 3 of experimentation was a repeat of Phase 2 using the same procedures and materials. Prior to the start of Phase 3, biofilm densities of 3.0×10^4 and 9.8×10^4 CFU/cm^2 were measured on slides in Reactors 1 and 2, respectively. In Reactor 1, a spore density of 6.4×10^2 CFU/cm^2 was measured on the slides after 10 minutes, while in Reactor 2, a spore concentration of 3.8×10^2 CFU/cm^2 on the slides was measured after 1.5 hours. After seeing an initial spike in spore counts on the slides, spore counts in Reactor 2 quickly declined and were undetectable at 48 hours. However, spores were detected at 75 hours, and were sporadically present at levels too low to quantify at 144 and 192 hours. Due to mechanical problems with the reactor, experimentation in Reactor 1 was stopped after 24 hours, but the data show the same trend as Reactor 2.

Several important observations were noted from the three phases of data. First is the difference in initial adhered spore counts in Reactors 1 and 2. Whereas spore attachment trends were similar in both reactors during Phase 1, in Phase 2, 4.5 times more spore initially adhered to the coupons in Reactor 1 than in Reactor 2, even though the same volume of spore suspension was added to both reactors. In Phase 3, 1.7 times more spores adhered in Reactor 2 compared to reactor 1. Since Reactor 2 contained a higher concentration of free chlorine in both experiments, this suggests that elevated chlorine levels can prevent adherence to biofouled pipe walls by inactivating spores before they have attached. The second observation is that the number of adhered spores was reduced to undetectable levels by 48 hours after injection in the presence of free chlorine.

Spores disappeared from the concrete-lined coupons in all phases of experimentation when free chlorine was present. Szabo *et al.* [7] and Morrow *et al.* [9] found different results when using iron coupons and copper surfaces, respectively. The authors found that the spores could survive for long periods in the rust on the pipe surfaces, even in the presence of high concentrations of chlorine. Another observation is that the initial number of spores adhering to the coupons in Phase 3 for both reactors was one order of magnitude lower than in Phase 2. It is unclear why the number of attached spores was so low or why this only occurred for adhered spores. It is unlikely that the lower values were due to a problem with the viability of the spores or to experimental error. However, the biofilm reactor has glass, polycarbonate and concrete surfaces that the spores could potentially contact. It is difficult to quantify how many spores adhered to the glass or polycarbonate surfaces, but adhesion to these surfaces could have influenced disappearance of spores from the bulk phase. Glass is hydrophibic like the spores and this could have promoted adhesion, but all of the surfaces are negatively charged, which could inhibit the negatively charged spores. Also, the effect of chlorine on the biofilm adhesive properties was not quantified and this could influence the number of adhering spores. Future research on this subject is warranted. Finally, small spikes in spore counts occurred, even after all adhered spores had seemingly been inactivated. These spikes correlate to small values, but it is surprising that counts were still being measured. Future research might utilize different methods that could better quantify the low density of adhered spores.

Conclusions

The primary conclusion from this research is that *B. globigii* spores adhered to concrete-coated slides in drinking water are susceptible to free chlorine. In all phases of experimentation, the number of culturable adhered and planktonic spores was reduced to undetectable or unquantifiable levels after addition of free chlorine. It was found that an increased chlorine concentration (5 mg/L) was not critical for the reduction of attached viable or culturable *B. globigii* spores. Although an increased chlorine concentration quickened the reduction process, the lower chlorine concentration (1 mg/L) was just as effective over time at removal of the spores. Furthermore, it is possible that free chlorine levels greater than 5 mg/L could inactivate some of the spores that remained under the conditions of this study.

A second conclusion that can be drawn from the two studies is that pipe material plays a critical role in determining the effectiveness of chlorine decontamination of the spores. Under similar research conditions, adhered *B. globigii* spores could not be completely removed

from corroded iron slides, representing cast iron pipe, despite using free chlorine concentrations as high as 75 mg/L [7]. In general, chlorine is known to not penetrate biofilm effectively [16–18]. However, on a concrete coated slide, the results suggest that chlorine was effectively able to penetrate the biofilm/concrete matrix and reduce the number of viable or culturable spores.

Finally, it should be noted that although biofilm growth in an annular reactor can approximate biofilm growth in a distribution system, actual conditions on the pipe wall will vary from conditions on the surface of the slides. For example, the concrete coating on the slides in this research was smooth, continuous and not eroded. An old concrete pipe or concrete-lined pipe surface might be more pitted, have exposed iron surfaces and could allow a safer harbour for the spores. Therefore the results and conclusions from this research might not apply to the surface of a corroded concrete pipe, a possibility which needs to be studied further. In spite of these limitations, the results of this and other research studies, along with advance planning and consideration, can help plan a decontamination strategy if such an event were to occur in a water distribution system.

Disclaimer

The U.S. Environmental Protection Agency (EPA) through its Office of Research and Development funded the research described herein under contract EP-C-05-056 to Pegasus Technical Services. It has been reviewed by the Agency but does not necessarily reflect the Agency's views. No official endorsement should be inferred. EPA does not endorse the purchase or sale of any commercial products or services.

References

[1] M.T. Madigan, J.M. Martinko, and J. Parker, *Brock Biology of Microorganisms,* Prentice Hall/Pearson Education, Upper Saddle River, NJ, 2002.

[2] E.W. Rice, *Use of surrogates for* Bacillus anthracis *in the study of chlorine inactivation of bacterial agents,* 2006. Available at <http://www.epa.gov/nhsrc/news/news 010306.html> (Accessed 9 April 2009).

[3] E.W. Rice, N.J. Adcock, M. Sivaganesan, and L.J. Rose, *Inactivation of spores of* Bacillus anthracis *Sterne,* Bacillus cereus*, and* Bacillus thuringiensis *subsp.* israelensis *by chlorination,* Appl. Environ. Microbiol. 71 (2005), pp. 5587–5589.

[4] L.J. Rose, E.W. Rice, B. Jensen, R. Murga, A. Peterson, R.M. Donlan, and M.J. Arduino, *Chlorine inactivation of bacterial bioterrorism agents,* Appl. Environ. Microbiol. 71 (2004), pp. 566–568.

[5] A.R. Brazis, J.E. Leslie, P.W. Kabler, and R.L. Woodward, *The inactivation of spores of Bacillus globigii and Bacillus anthracis by free available chlorine,* Appl. Environ. Microbiol. 6 (1958), pp. 338–342.

[6] L.J. Rose, E.W. Rice, L. Hodges, A. Peterson, and M.J. Arduino, *Monochloramine inactivation of bacterial select agents,* Appl. Environ. Microbiol., 73 (2007), pp. 3437–3439.

[7] A.A. Hosni, W.T. Shane, J.G. Szabo, and P.L. Bishop, *The disinfection efficacy of chlorine and chlorine dioxide as disinfectants of* Bacillus globigii*, a surrogate for* Bacillus anthracis*, in water networks: a comparative study,* Canadian J. Civil Engin. 36 (2009), pp. 732–737.

[8] J. Szabo, E. Rice, and P.L. Bishop, *Persistence and decontamination of* Bacillus atrophaeus *subsp.* globigii *spores on corroded iron in a model drinking water system,* Appl. Environ. Microbiol. 73 (2007), pp. 2451–2457.

[9] J.B. Morrow, J.L. Almeida, L.A. Fitzgerald, and K.D. Cole, *Association and decontamination of Bacillus spores in a simulated drinking water system,* Water Res. 42 (2008), pp. 5011–5021.

[10] A.K. Camper, K. Brastrup, A. Sandvig, J. Clement, C. Spencer, and A.J. Capuzzi, *Effect of distribution system materials on bacterial regrowth,* Journal AWWA 95 (2003), pp. 107–121.

[11] R.M. Donlan, W.O. Pipes, and T.L. Yohe, *Biofilm formation on cast iron substrata in water distribution systems,* Water Res. 28 (1994), pp. 1497–1503.

[12] P.J. Ollos, P.M. Huck, and R.M. Slawson, *Factors affecting biofilm accumulation in model distribution systems,* Journal AWWA 95 (2003), pp. 87–97.

[13] L.S. Clesceri, A.D. Eaton, and A.E. Greenberg, *Standard Methods for the Examination of Water and Wastewater,* American Public Health Association, American Water Works Association & Water Environment Federation, 1995.

[14] W.L. Nicholson and P. Setlow, *Sporulation, Germination, and Outgrowth. Molecular Biological Methods for* Bacillus*,* C.R. Harwood and S.M. Cutting, eds., John Wiley and Sons, Chichester, 1990, pp. 391–429.

[15] P. Niquette, P. Servais, and R. Savoir, *Impacts of pipe materials on densities of fixed bacterial biomass in a drinking water distribution system,* Water Res., 34 (2000), pp. 1952–1956.

[16] A. Jang, J. Szabo, A. Hosni, M. Coughlin, and P.L. Bishop, *Measurement of chlorine dioxide penetration in dairy process pipe biofilms during disinfection,* Appl. Microbiol. Biotech. 72 (2006), pp. 368–376.

[17] P.S. Stewart, J., Rayner, F. Roe, and W.M. Rees, *Biofilm penetration and disinfection efficacy of alkaline hypochlorite and chlorosulfamates,* J. Appl. Microbiol. 91 (2001), pp. 525–532.

[18] P.L. Bishop, A. Hosni, W. Shane, and J. Szabo, *Disinfection alternatives for water distribution systems following biological contamination,* 2008 AWWA Annual Conference, Atlanta, GA, 8–12 June 2008.