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Development and Testing of Methods to Decontaminate a Building's Plumbing System Impacted by a Water Contamination Event:

DECONTAMINATION OF BACILLUS SPORES

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Development and Testing of Methods to Decontaminate a Building's Plumbing System Impacted by a Water Contamination Event: Decontamination of *Bacillus* Spores

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Disclaimer

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Foreword

Following events of September 11, 2001, U.S. Environmental Protection Agency's (EPA's) mission was expanded to account for critical needs related to homeland security. Presidential Directives identified EPA as the primary federal agency responsible for the country's water supplies and for decontamination following a chemical, biological, and/or radiological (CBR) attack. To provide scientific and technical support to help EPA meet this expanded role, EPA's National Homeland Security Research Center (NHSRC) was established. The NHSRC research program is focused on conducting research and delivering products that improve the capability of the Agency to carry out its homeland security responsibilities.

As part of this mission, NHSRC conducts research on contaminants that could be intentionally injected into a community's water supply and water distribution system. The possibility of such intentional contamination raised questions that had been heretofore largely unasked: what is the fate of the contaminant in the water system? and how can the contaminant be removed? The approach to answering these and other related questions depends greatly on where the contaminant is located: the water treatment system, the water distribution system, or in a building's plumbing system. For example, the remediation strategy for a large underground water main that had been impacted by intentional contamination would most likely be different than a remediation strategy for a similarly impacted plumbing system in a typical house.

The work summarized in this report addresses contamination of plumbing systems in houses and other buildings. Specifically, the objective of the work was to develop and test of methods to decontaminate a building's plumbing system impacted by a water contamination event. The contaminants studied were *Bacillus* spores, which had proven challenging to decontaminate. A follow on report will address other aspects of chemical and biological decontamination of plumbing systems.

Acronyms and Abbreviations

ANOVA	analysis of variance
BA	*Bacillus anthracis* Sterne
BT	Bonide Thuricide™ Bacillus Thuringiensis (BT) Concentrate (Bonide Products, Inc., Oriskany, New York)
CBR	CDC Biofilm Reactor (BioSurface Technologies, Bozeman, Montana)
C/C_o	concentration divided by initial concentration
CDC	Centers for Disease Control
CFU	colony forming unit
cm	centimeter
Ct	concentration-time (concentration multiplied by time)
DAPI	4',6-diamidino-2-phenylindole
DPA	dipicolinic acid
DPD	N,N-diethyl-p-phenylenediamine
EDTA	ethylenediamine tetraacetic acid
EPA	U.S. Environmental Protection Agency
FISH	fluorescent in situ hybridization
g	grams
g	acceleration due to gravity, 9.81 m/s^2
h	hour
L	liter
LB	Luria-Bertani agar
µg	microgram
M	molar (moles/liter)
mg	milligram
mL	milliliter
mm	millimeter
mM	millimolar
m/s	meters/second
min	minute
MQ	Milli-Q®
NaCl	sodium chloride
NIST	National Institute of Standards and Technology
PBS	phospate buffered saline
PFA	paraformaldehyde
PVC	polyvinyl chloride
RO	reverse osmosis
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TOC	Total Organic Carbon
Tris	tris (hydroxymethyl)aminomethane
v/v	volume to volume

Acknowledgements

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Executive Summary

This report describes the work on decontamination of bacillus spores in building water systems done at the National Institute of Standards and Technology (NIST) (Gaithersburg, Maryland). Specifically *Bacillus anthracis* Sterne (BA) spores and a simulant, *Bacillus thuringiensis* (BT) were the subject of this group of studies. Bacillus spores have proven to be very challenging organisms to inactivate in a water environment. Compared to vegetative bacteria, the bacterial endospores BA and BT are more resistant to disinfectants such as chlorine. In addition, spores can adhere to water system biofilms which may provide protection from disinfectants. The work with bacillus spores is summarized in this section, and divided into three separate topics:

1. Decontamination of Bacillus Spores Associated with Water System Biofilms
2. Enhanced Decontamination of Bacillus Spores in a Simulated Drinking Water System
3. The Effect of High Flow on the Adhesion and Disinfection of BT Spores in Pipe Loop Experiments

In addition, gaps in research on biological threats were identified and studies have been suggested for further investigation. These are given in Chapter 4 of this report.

Decontamination of Bacillus Spores Associated with Water System Biofilms

The objective of this work was to elucidate the disinfectant susceptibility and fate of BA and a commercial preparation of BT spores associated with (i.e., attached to) a water system biofilm. A native water system biofilm was accumulated on copper and polyvinyl chloride (PVC) pipe material surfaces in a low-flow pipe loop and in a uniformly mixed tank reactor.

Spores were exposed to two commonly used disinfectants (free chlorine and monochloramine) in planktonic phase (i.e., spores unattached to the biofilm) and after association with biofilm-conditioned pipe materials. Biofilm associated spores required 5 to 10 fold higher disinfectant concentrations to observe the same 2- to 4-$\log_{10}$ reduction of viable spores as observed in the planktonic phase. In a synthetic tap water, Ct values (where C is the concentration of disinfectant in mg/L and *t* is exposure time in minutes) for a 2-$\log_{10}$ reduction of BA and a commercial preparation of BT (planktonic phase) were 290 mg·min/L and 620 mg·min/L, respectively, for chlorine and 1200 mg·min/L and 880 mg·min/L, respectively, for monochloramine. Shear during spore contact with biofilm-conditioned surfaces had a profound effect on spore attachment. Application of a distributed, low-shear (60 revolutions per minute (rpm), CDC biofilm reactor (CBR)) during spore contact with the biofilm-conditioned surfaces resulted in a 1.0 and 1.6 $\log_{10}$ increase in the number of BT spores associating with copper and PVC surfaces, respectively, compared to uniform mixing alone (i.e., no applied shear). High disinfectant concentrations (103 mg/L free chlorine and 49 mg/L monochloramine) and contact time (60 minutes) yielded less than a 2-$\log_{10}$ reduction of viable BT and BA spores associated with the biofilm under low shear. Both spore preparations showed a similar susceptibility to the disinfectants when associated with the biofilm-conditioned surfaces.

Enhanced Decontamination of *Bacillus* Spores in a Simulated Drinking Water System

Contact with germinant solutions, followed by commonly used disinfectants was investigated as a way to enhance decontamination of BA and BT. PVC and copper pipe materials were used in a continuously stirred tank reactor that permitted for controlled shear. Simulated water system biofilms were accumulated on pipe material surfaces with synthetic tap water containing humic

acids as a carbon source. Once the biofilms were established, BT subspecies *kurstaki* or BA spores were added to the water system. Pipe surfaces were examined for biofilm accumulation, spore adhesion, and disinfectant (chlorine and monochloramine) susceptibility before and after germination with 1 mM inosine and 8 mM L-alanine. Biofilm associated spores required 5 to 10 fold higher disinfectant concentrations to observe the same 1 $\log_{10}$ to 4 $\log_{10}$ reduction of viable spores as observed in the planktonic phase. Applied shear increased spore attachment (0.4 $\log_{10}$ to 1.6 $\log_{10}$ increase) to biofilm-conditioned pipe surfaces. High disinfectant concentrations (103 mg/L free chlorine and 49 mg/L monochloramine) yielded less than a 2 $\log_{10}$ reduction in biofilm-associated viable spores after 60 minutes. A 4 $\log_{10}$ reduction in the associated spores was observed when coupons were in contact with germinants (24 hours) prior to sampling. When germinant contact was followed by heat (50 ºC, 20 minutes) or disinfectant contact, a greater than 4 $\log_{10}$ reduction in the associated viable spores was observed. Contact with germinants appeared to dramatically enhance the susceptibility of surface-associated spores to elevated water temperature and disinfectants.

The Effect of High Flow on the Adhesion and Disinfection of BT Spores in Pipe Loop Experiments

In this study, a pipe loop with intermittent high flow rate was used to measure the effects of shear flow rate on the biofilm growth, the adhesion of BT spores, and the disinfection process. It was seen that the high flow rate had a large effect on the disinfection process by chlorine on BT spores. For high flow conditions, moderate concentrations of chlorine (approximately 10 mg/L) effectively disinfected the spores associated with the biofilm-conditioned pipe loops. This is in contrast to the results we obtained with the static experiments in the laboratory in which much higher levels of chlorine (100 mg/L) were required to achieve significant levels of disinfection.

1. 0 Decontamination of *Bacillus* Spores Associated with Water System Biofilms

Introduction

The fate and persistence of potential bioterrorist agents such as *Bacillus* spores in water distribution systems is a current concern for homeland security. Several recent studies have defined Ct (disinfectant concentration multiplied by exposure time) values for bioterrorist agents, including *Bacillus* spores, by studying disinfectant efficacy in simple buffer solutions (Rice, et al. 2005; Rose, et al. 2005; Rose, et al. 2007). *Bacillus* spores were found to be more resistant to commonly used disinfectants (monochloramine and free chlorine) than vegetative bacteria (Rice, et al. 2005; Rose, et al. 2005; Rose, et al. 2007). However, little work has focused on disinfectant efficacy for *Bacillus* spores in water systems (Gibbs, et al. 2004; Szabo, et al. 2007).

Contaminant organisms delivered to water distributions systems under varied shear conditions encounter complex solution chemistries (Rose, et al. 2005; Dow, et al. 2006), as well as different native biofilms on numerous pipe material surfaces (Ridgway and Olson, 1981; LeChevallier, et al. 1993; Manz, et al. 1993; Schwartz, et al. 2003). Water quality parameters such as pH, dissolved organic carbon and temperature result in different Ct values for contaminant inactivation (Dow, et al. 2006). Association with biofilm matrices further increases bacterial resistance to disinfection (LeChevallier, et al. 1988; Cochran, et al. 2000; Donlan, et al. 2002; Gibbs, et al. 2004; Szabo, et al. 2006; Szabo, et al. 2007). Substratum material and integrity can impact biofilm formation and contaminant survival in water systems (Domek, et al. 1984; LeChevallier, et al. 1987; Gagnon, et al. 2004; Szabo, et al. 2006; Szabo, et al. 2007). Microorganisms have been found associated with corrosion-induced pits and tubercles where there are localized differences in chemistry and hydrodynamic conditions (LeChevallier, et al. 1988; De Beer, et al. 1994). Hydrodynamic conditions vary greatly in complex water distribution systems, yet few studies have addressed the role of fluid shear on bacterial biofilm populations and pathogen association with substrata (Rickard, et al. 2004; Azevedo, et al. 2006).

Disinfectant efficacy on biofilms and contaminant organisms harbored by biofilms is largely dependent on the chemical reactivity and the ability of the disinfectant to penetrate the biofilm matrix. Highly reactive oxidants such as free chlorine, as well as the less reactive chloramines, have been shown to be consumed by the top layers of the biofilm matrix, resulting in a retarded penetration of the biofilm (De Beer, et al. 1994; Huang, et al. 1995; Chen and Stewart, 1996; Stewart, et al. 2001). Due to its slower reaction kinetics, monochloramine has been shown to be more effective at penetrating polysaccharide layers that make up the biofilm (LeChevallier, et al. 1990; Samrakandi, et al. 1997; Turetgen, 2004). Additionally, strong oxidants are more corrosive on metal pipe materials and are more susceptible to reaction with corrosion byproducts, limiting their disinfectant ability when compared to less corrosive biocides (e.g., monochloramine) (LeChevallier, et al. 1993). In another study, ***B. atrophaeus*** spores associated with biofilms on corroded iron pipe surfaces were resistant to high levels of chlorine (10, 25 and 70 mg/L) for extended periods of time (Szabo, et al. 2007).

The objective of this work was to elucidate the association and disinfection sensitivity of *B. anthracis* Sterne (BA) and a commercial preparation of *B. thuringiensis* (BT) spores when contacted with biofilm-conditioned pipe material surfaces in a simulated treated water system. We measured the ability of commonly used disinfectants (free chlorine and monochloramine) to inactivate BA and BT spores in synthetic tap water and when associated with biofilm-conditioned surfaces (copper and polyvinyl chloride (PVC)). Two different reactor systems − a low-flow pipe loop and a uniformly mixed tank reactor, the CDC biofilm reactor (CBR) (BioSurface Technologies Corp., Bozeman, Montana) − were utilized to grow biofilms similar to those found in water distribution systems. The impact of fluid shear on spores associated with biofilm-conditioned surfaces and on subsequent decontamination was evaluated for two contact conditions: the uniformly mixed pipe loop reactor and the low shear, uniformly mixed tank reactor.

Materials and Methods

Spore Preparations and Growth Conditions

Pure suspensions of BA were compared to a commercially available BT preparation (Bonide Thuricide™ Bacillus Thuringiensis (BT) Concentrate, Bonide Products, Inc., Oriskany, New York), for the ability to associate with water system biofilms and to determine disinfectant susceptibility. BT was chosen as a simulant for BA due to the similar exosporium composition (Matz, et al. 1970). The commercial BT preparation was washed to yield a concentrated spore preparation (≈$1x10^9$ (colony forming units) CFU/mL) by the following procedure: aliquots of the BT spore suspension were centrifuged at 16,000 x *g* for 6 minutes, supernatant was removed and discarded and the spores were re-suspended in phosphate buffered saline (PBS) containing 0.01 % (v/v) Triton™ X-100. PBS (0.01 % Triton™ X-100) was prepared by dissolving 8 g of NaCl, 0.2 g of potassium phosphate, 1.15 g of sodium phosphate and 0.2 g of potassium chloride in 1 L of MQ water (Milli-Q® , Millipore Corp., Billerica, Massachusetts), resistivity 18 MΩ), pH 7.4, and autoclaved for 15 minutes, 121 °C. To minimize spore aggregation, 1 mL of 10 % (v/v) Triton™ X-100 was added to the PBS after it had cooled to room temperature. The spore pellet was washed 4 times in PBS (0.01 % (v/v) Triton™ X-100) by centrifugation at 16 000 x *g* for 6 minutes, pipetting and vortexing to re-suspend the sample, then rinsed one additional time and stored in 20% (v/v) ethanol at 4 ºC. BA spore suspensions from the U.S. Army's Dugway Proving Ground, Dugway Proving Ground, Utah, characterized and noted as "Lot 3" in a previous publication (Almeida, et al. 2008) ($3x10^8$ CFU/ml) were stored in sterile Milli-Q® (MQ) water at 4 ºC. Spore concentrations were determined by the spread plate method on Luria-Bertani (LB) agar and incubating at 35 °C (see Appendix A, Protocol 8). PBS (0.01 % Triton™ X-100) was used as a dilution buffer for biofilm and spore enumeration to enhance dispersal of biofilm bacteria and biofilm associated spores. Spore preparations contained > 95 % phase bright spores as determined by phase microscopy.

Pipe Material Preparation

PVC and copper pipe surfaces were rinsed with reverse osmosis (RO) water prior to connecting to the reactor tubing. (PVC and copper coupons were purchased from BioSurface Technologies Corp., Bozeman, Montana)). PVC coupons were rinsed briefly in 10 % (v/v) bleach solution followed by RO water prior to inserting into the reactor holder. Copper coupons were briefly buffed in a circular motion with a 3M Scotch-Brite™ pad and then soaked in 5 %

(v/v) nitric acid for 30 minutes and rinsed with RO water prior to inserting into the reactor holder. For the pipe loop reactor, all pipe surfaces had been conditioned by several years of laboratory use.

Biofilm Colonization of Pipe Materials

Simulated water system biofilms were accumulated by contacting pipe surfaces in synthetic tap water with the following formulation: 1.2 mM $NaHCO_3$, 0.54 mM $MgSO_4 \cdot 7H_2O$, 0.2 mM $CaSO_4 \cdot 2H_2O$, 0.004 mM K_2HPO_4, 0.002 mM KH_2PO_4, 0.08 mM $(NH_4)_2SO_4$, 0.17 mM NaCl, 36×10^{-6} mM $FeSO_4 \cdot 7H_2O$, 0.011 mM $NaNO_3$, 0.2 mM $CaCO_3$, pH = 8.2 ± 0.2

(LeChevallier, 2005, personal communication providing aforementioned synthetic tap water formulation.) made from the laboratory RO water supply. Growth of organisms indigenous to the RO water supply system at the National Institute of Standards and Technology (NIST) was stimulated by adding 24 mg/L humic acid sodium salt to the synthetic tap water for two weeks. A final carbon deprivation period of 3 to 5 days, achieved by omitting humic acids, was utilized to produce viable plate counts of biofilm organisms consistent with literature reports for water distribution systems (Schwartz, et al. 2003). Biofilm organisms were enumerated by spread plating on R2A agar and incubating at room temperature for up to 7 days (Schwartz, et al. 2003). Biofilms were grown using two reactor system types: the pipe loop reactor and the CBR.

Pipe Loop Reactor Operation

The pipe loop reactor system consisted of a maximum of eighteen, two inch long (51 mm), ¾ inch nominal diameter (19 mm), alternating PVC schedule 80 pipe and copper pipe sections connected by silicone tubing (50 mm long) (Figure 1.1). Synthetic tap water was delivered from a 20 L carboy with Norprene® Food Process Tubing (6402-14) tubing at 1 mL/min (a fluid velocity of 5.8×10^{-6} m/s and hydraulic retention time of 15 to 30 hours (depending on reactor length) with a peristaltic pump. Midway through the biofilm accumulation period (7 days), the pipe sections were rotated from influent to effluent to obtain uniform biofilm growth along the length of the reactor. After 14 days, the reactor was switched to synthetic tap water without humic acids, and flow was continued for an additional 3 to 5 days. Pipe sections were removed from the reactor and placed in sterile synthetic tap water. Biofilm accumulation was quantified by scraping the inside surface of the pipe with a sterile cell scraper (23 cm, Nunc™). The inside of the pipe section was then rinsed several times with 10 mL of dilution buffer (0.0425 g/L KH_2PO_4, 0.405 g/L $MgCl_2$ $6H_2O$) solution into a sterile, 50 mL conical tube. The tube was vigorously vortexed three times at 10 second intervals.

Pipe sections were contacted with bacterial spores by placing individual sections vertically on a perforated, plastic rack in a 4 L beaker with 1.5 L of sterile synthetic tap water with 5×10^6 CFU/mL spores. To reduce settling of the spores during batch contact, the spore solution was gently stirred with a DuPont™ Teflon® coated stir bar under the perforated rack. After a given contact time, pipe sections were removed from the spore solution with sterile forceps and gently rinsed by placing in sterile synthetic tap water for several minutes using gentle agitation to wash away the unbound spores. Rinsed pipe sections were then either directly sampled by scraping to

determine initial spore association or placed in a sterile 250 mL beaker containing 150 mL of the disinfectant solution. Initial association experiments (performed for two to 48 hours spore contact times) indicated that steady state spore association was reached after 24 hours in batch contact conditions (data not shown). All inactivation studies were performed with 24 hours spore contact time.

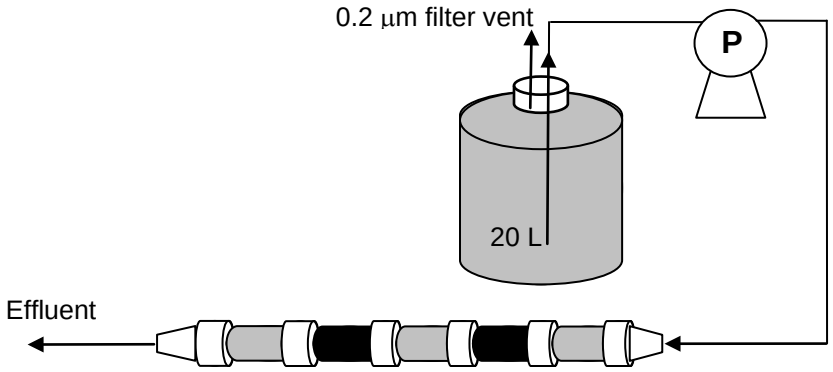

Figure 1.1. Pipe Reactor Schematic. Synthetic water was pumped at 1 mL/min from a 20 L carboy with a peristaltic pump (P) through alternating PVC and copper pipe sections connected by silicon tubing.

CDC Biofilm Reactor (CBR) Operation
The impact of distributed shear on spore association with the biofilm surface was determined with the CBR. The CDC biofilm reactor was chosen due to its history as a model potable water system (Donlan, et al. 2002) and for the ability to control the applied fluid shear flow during biofilm development and spore contact conditions (Goeres, et al. 2005). The reactor was assembled and vented as directed by the manufacturer.

Synthetic tap water was supplied from a 20 L feed carboy to the CBR by a peristaltic pump using Masterflex® tubing (Norprene® Food Tubing 6402-14). Prepared coupons were inserted into the coupon holders and the reactor was filled with 400 mL of synthetic tap water and autoclaved (121 ºC for 15 minutes). Reactors were allowed to cool and then placed on digital stir plates and connected to the peristaltic pump. A flow rate of 0.3 mL/min (hydraulic retention time of 29 hours) supplied the reactors with synthetic tap water containing 24 mg/L humic acids for 14 days at room temperature. After 7 days, mixing was introduced by turning on the digital stir plate to begin baffle rotation at 120 ± 5 rpm (confirmed by a tachometer) while fluid flow was continued. After 2 weeks of synthetic tap water with humic acids, reactors were switched to synthetic tap water without humic acids, applied at the same flow rate for 3 to 5 days.

Contact with spores was performed by filling the reactor beaker to the 700 mL mark with synthetic tap water and adding the appropriate amount of a concentrated spore stock suspension resulting in a concentration of 1.4 (0.8) x 10^7 CFU/mL of spores. Spores were contacted for 24 hours under uniform mixing in batch (baffle was removed using only the stir bar rotating at

60 rpm) and with an applied shear (baffled stir bar with 60 rpm rotation) in the reactor. Steady state spore association with the biofilm-conditioned surfaces was found to occur after 24 hours when tested for adhesion for 2, 12, 24, 36 and 48 hours (data not shown). The coupon holders were removed, rinsed in sterile synthetic tap water and sampled or exposed to disinfectants.

The CBR was sampled by removing coupon holders and placing them on a sterile piece of aluminum foil. Coupon surfaces were scraped in a circular fashion, collecting the biofilm in the center of the coupon while still positioned in the coupon holder (Zelver, et al. 1999; Zelver, et al. 2001). The method by Zelver, et al. 2001 was adapted by using a sterile piece of DuPont™ Teflon® cut to resemble a spatula. The method had originally called for the use of a cotton swab, but the aforementioned spatula was seen as superior. Coupons were removed from the holder with a pair of sterile forceps, positioned over a 15 mL BD Falcon™ polypropylene centrifuge tube and rinsed by dispensing 5 mL of sterile synthetic tap water directly on the coupon surface. The rinsing procedure was repeated 2 times using the same 5 mL of synthetic tap water to minimize sample volume. Biofilm samples were vortexed for 30 seconds at the highest speed to disperse the cells and associated spores. Biofilm and biofilms post-spore contact were diluted and enumerated on R2A and LB agar, respectively. R2A plates were enumerated after 7 days at room temperature. Spore samples were counted after storing the LB plates at 35 ºC for 16 hours. Since it was relatively straightforward to distinguish between BA or BT spores and the native bacteria in the biofilm, it was not necessary to heat shock the spore samples prior to spread plating onto the LB agar. The biofilm bacteria grew slowly (i.e., about 1 week before colonies were visible), whereas the spores germinated and grew rapidly overnight on the plates and could be easily recognized by the morphologies of the colonies.

Preparation of Disinfectants

Disinfectant solutions consisted of 150 mL of synthetic tap water containing either free chlorine at average concentrations of 11 mg/L (ranged from 10 mg/L to 12 mg/L) and 103 mg/L (ranged from 98 mg/L to 108 mg/L) or monochloramine at average concentrations of 13 mg/L and 49 mg/L (ranged from 8 mg/L to 18 mg/L and 47 mg/L to 57 mg/L, respectively). Free chlorine was derived from a stock solution of sodium hypochlorite (Clorox®, bleach). Free chlorine concentrations were determined using N,N-diethyl-p-phenylenediamine (DPD) reagent and chlorine standards (Hach Method 8021, Hach Company, Loveland, Colorado). Monochloramine solutions were prepared as described by Camper et. al. (Camper, et al. 2003). Monochloramine concentrations were determined by DPD and the indophenol method (Hach Methods 8167, 8021, and 10171, Hach Company, Loveland, Colorado). Concentrations of all disinfectant solutions were determined after 30 minutes of stirring the post-disinfectant addition to synthetic tap water to eliminate chlorine demand contributions to effective disinfectant determinations.

Spore Suspension Disinfection

BA and BT spores were diluted to approximately 1 x 10^6 CFU/mL in sterile glass vials that contained either a disinfectant in sterile synthetic tap water or sterile synthetic tap water as a control. Gentle stirring with small stir bars was used to prevent settling of spore suspensions during contact. Viability after contact was determined at various time points by

sampling 100 µl from the vial and diluting 10-fold in 0.3 mM sodium thiosulfate in PBS (0.01% (v/v) Triton™ X-100).

Pipe Surface Disinfection

Pipe surfaces (coupons in coupon holders or pipe sections) were contacted with disinfectant solutions (150 mL) in 250 mL beakers of the disinfectant solution. A small stir bar was used to uniformly mix the disinfectant solution during batch contact of the CBR coupons. Beakers with pipe sections were gently swirled every 10 minutes of contact time to ensure adequate mixing. After the given contact time, pipe surfaces were immediately removed and placed in 150 mL of sterile synthetic tap water containing 7.5 mM sodium thiosulfate to neutralize residual disinfectant prior to sampling.

Estimation of Ct Value

Ct values were estimated using the Chick-Watson Law assuming first-order kinetics (Weavers and Wickramanayake, 2001). $Log_{10}(C/Co)$ for a given concentration multiplied by the contact time (Ct, mg·min/L) of spore inactivation was plotted and linear regression analysis was performed to determine the Ct value for a 2-log_{10} reduction in the viable spore fraction (Le Dantec, et al. 2002).

Fluorescent In Situ Hybridization of Biofilm Organisms

Probes and hybridization conditions for fluorescent *in situ* hybridization (FISH) were used as described by Manz, et. al. (1993) for drinking water biofilm organism identification. Oligonucleotide probes for the β- and γ-proteobacteria labeled with fluorescent dyes were purchased from Invitrogen Corp. (Carlsbad, California). Alexa Fluor 546 and Alexa Fluor 647 were added to the 5′ end of the β-42a and γ-42a probes, respectively, as defined by Manz, et al. (1993). The hybridization protocol described by Manz, et. al. (1993) was modified to probe biofilm samples removed from the pipe material surfaces as described in the following sampling procedure: 1 mL of the 5 mL sample collected was centrifuged and re-suspended in 1 part PBS buffer and 3 parts 4% paraformaldehyde (PFA). The sample was vortexed and fixed for 3 hours at 4°C. Biofilm samples were washed twice with PBS and re-suspended in 1 part PBS and 1 part ethanol (95 %). Twenty µL samples were immobilized by spotting on poly-L-lysine slides and allowed to air-dry. The samples were dehydrated by dipping the slides in an ethanol series (50 %, 80 %, 95 %; 3 minutes each) and the slides were air dried in a vertical position. Twenty-five µL hybridization buffer (20 mM Tris-HCl pH 7.2, 0.9 M NaCl, 39% formamide, and 0.01 % sodium dodecyl sulfate (SDS)) containing 80 ng of probe was added to the sample surface. Two milliliters of hybridization buffer was poured into a 50 mL centrifuge tube that contained a Kimtech Science Kimwipes® (Kimberly-Clark Corp., Dallas, Texas) wiper. The slide was sealed in the 50 mL tube and incubated in a horizontal position at 50 °C for 16 hours. Slides were rinsed with preheated (50 °C) washing buffer (20 mM Tris-HCl pH 7.2, 40 mM NaCl, 5 mM ethylenediamine tetraacectic acid (EDTA), and 0.01 % SDS) and incubated at 50 °C for 15 minutes. The slides were rinsed with water, air-dried and imaged with an epifluorescence microscope (Olympus® AX70) with the suited filter sets. Image analysis was performed with Image J software, a public domain, open source software (Rasband, 1997-2007)

Statistical Comparisons
Dunnett's multiple comparison procedure was used for treatments versus controls. A one-way analysis of variance (ANOVA) was performed to test the hypothesis that the average mean value across categories of the groups were equal. In the presence of significance for the omnibus ANOVA test, a Newman-Keuls multiple comparison test is used to perform pairwise comparisons. Statistical decisions were made at $\alpha = 0.05$. Statistics were performed using WINKS SDA Software (TexaSoft, Cedar Hill, Texas.)

Results
Solution Phase Spore Disinfection
Significant reductions in the viable spore count were observed for both spore suspensions after 60 minutes of contact with both free chlorine and monochloramine (Figure 1.2). However, free chlorine was more effective than monochloramine at reducing the viable spore fraction for both preparations. BA spores were more susceptible to free chlorine than the commercial preparation of BT were. A four $\log_{10}$ reduction in the viable fraction of BA spores was noted after 60 minutes of contact with approximately 11 mg/L of free chlorine. A 2.5 $\log_{10}$ reduction in viable spores was observed after 60 minutes of contact with the same concentration of free chlorine for the commercial preparation of BT spores. Ct values for a 2-$\log_{10}$ reduction in the viable spore fraction when contacted

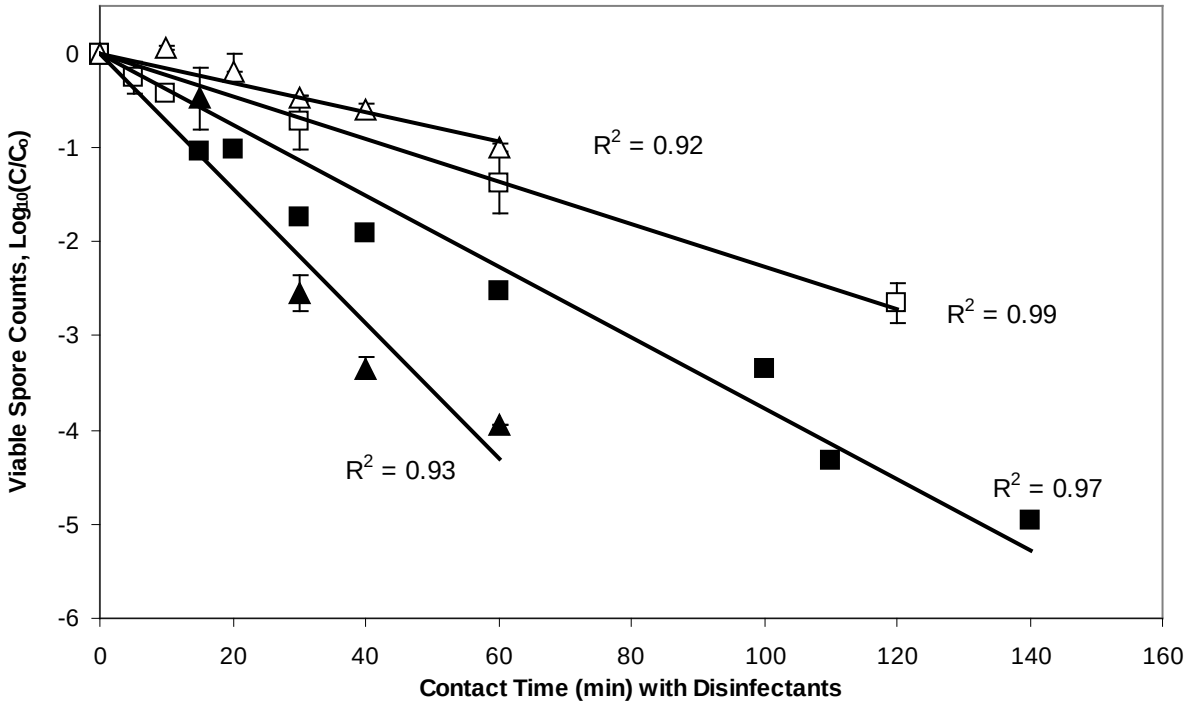

Figure 1.2. Solution Phase Disinfection of Spore Suspensions and Contact Time with Disinfectants. BA spores, ▲, and the commercial preparation of BT, ■, were contacted with free chlorine (average concentration of 11 mg/L) shown as solid symbols, and monochloramine (average concentration of 13 mg/L) shown as open symbols, in sterile synthetic water, pH = 8.1, at 23°C for the given contact time in minutes, error bars indicate the observed range of duplicate experiments, linear regression analysis R^2 values are shown next to the trend lines. C/Co indicates final concentration divided by the initial concentration of spores in suspension.

with free chlorine were 290 and 620 mg·min/L for BA and BT spores, respectively. Monochloramine was less effective at killing free spores in solution yielding only a 1.0 to 1.4 $\log_{10}$ reduction after 60 minutes of contact with the BA and the commercial preparation of BT, respectively. Ct values for a 2-$\log_{10}$ reduction in the viable spore fraction due to monochloramine contact were 1200 and 880 mg·min/L for BA and BT spores, respectively.

Biofilm Accumulation

Heterotrophic plate counts on R2A media were utilized to quantify the biofilm community associated with the pipe material surface. Typically, six colony morphologies were apparent on R2A plates. Average heterotrophic plate counts of the biofilm bacteria were not significantly different for the CDC reactor compared to the pipe reactor ($\alpha = 0.05$, $p = 0.105$ and 0.069, for PVC and copper, respectively) (Table 1.1). In all cases, our heterotrophic plate counts were quantitatively consistent at 1.1-2.5 x 10^5 CFU/cm^2 (when converted to similar units) with those reported in the literature for mature water distribution systems (ranges of 0.23 to 8.4 x 10^5 CFU/cm^2) (Schwartz, et al. 2003).

Table 1.1: Biofilm Surface Coverage in CFU/cm2 for the Different Reactor Types

	Material	Pipe Reactor	CDC Reactor
Biofilm	PVC	2.5 (1.7) x 10^5	1.7 (0.8) x 10^5
	Cu	1.0 (0.8) x 10^5	1.8 (1.4) x 10^5

Values presented are averages with 1 standard deviation shown in parentheses, $n \geq 5$.

Epifluorescent microscopy was utilized to determine biofilm morphology and the results of FISH probing. Imaging of biofilms indicated sparsely distributed aggregates (10 to 70 μm in diameter) of bacteria with a more uniform layer of extracellular polysaccharides. FISH results indicated 91 % and 94 % of the total bacterial population as stained by 4',6-diamidino-2-phenylindole (DAPI) were positive for either β or γ-proteobacteria, respectively. All culturable colony morphologies were positive for either the β or γ-proteobacteria.

Spore Association with Biofilm-Conditioned Pipe Materials

The number of BT spores associating with the biofilm-conditioned surfaces was largely dependent on spore contacting conditions. Significantly more spores were associated with the pipe material surfaces when the reactor was uniformly mixed and a low shear was uniformly applied over the coupon surfaces (60 rpm with the baffle present in the CBR) during the 24 hours contact time compared to uniform mixing alone (pipe reactor and no baffle in the CDC reactor, Table 1.2). Uniformly distributing the fluid shear over the coupon surfaces in the CDC reactor with the baffle present resulted in more spores on average associating with the biofilm compared to results when the baffle was removed. The impact of uniform shear application was consistent for the spore preparations; both spore preparations had similar surface coverage of associated spores on the different pipe material surfaces (Table 1.2).

Table 1.2: Initial Spore Surface Coverage in CFU/cm2 for the Different Reactor Types and Contact Conditions

		Pipe Reactor	CDC Reactor	
Spore Association, CFU/cm^2		Uniformly mixed (no baffle)	Uniformly mixed (no baffle)	Uniformly mixed, Low-shear (baffle)
BT	PVC	1.1 (1.0) x 10^3 **	1.8 (0.8) x 10^3 *	1.7 (1.3) x 10^4
	Cu	0.8 (0.6) x 10^3 **	3.2 (1.4) x 10^3	2.5 (1.6) x 10^4
BA	PVC	ND	ND	5.6 (5.4) x 10^4
	Cu	ND	ND	5.7 (5.3) x 10^4

Values presented are averages with 1 standard deviation shown in parentheses, $n \geq 3$.
* indicates values are significantly lower than the CDC reactor with the baffle in place ($\alpha = 0.05$, $p \leq 0.04$),
** indicates values are significantly lower than the CDC reactor with and without the baffle ($\alpha = 0.05$, $p \leq 0.009$),
ND = not determined

Decontamination of Surface Associated Spores

Spores associated with pipe materials conditioned with synthetic tap water system biofilms were more difficult to disinfect with chlorine and monochloramine than spores in suspension. In order to see the same 2 to 4 $\log_{10}$ reductions observed for free spores in solution, biofilm associated spores required 5 times the monochloramine concentration and nearly 10 times the free chlorine concentration (Figure 1.3). Contrary to what was observed in suspension, monochloramine was more effective than free chlorine at reducing the viable spore fraction when the spores were associated with the biofilm-conditioned pipe materials. Disinfection of the commercial preparation of BT spores was tested for both reactor configurations with free chlorine and monochloramine (shown in Figure 1.3). Depending on the pipe material surface and contact conditions, a 1 to 4 $\log_{10}$ reduction in the viable spore fraction was observed after contact with monochloramine at ≈49 mg/L. Spores associated with low-shear contact conditions were more difficult to disinfect resulting in a slightly lower reduction in the viable spore fraction for the CDC reactor (baffle attached) than just uniformly mixed contact conditions (pipe reactor). There were no significant differences in the reduction of BA or BT spores associated with the biofilm-conditioned pipe surfaces following monochloramine contact.

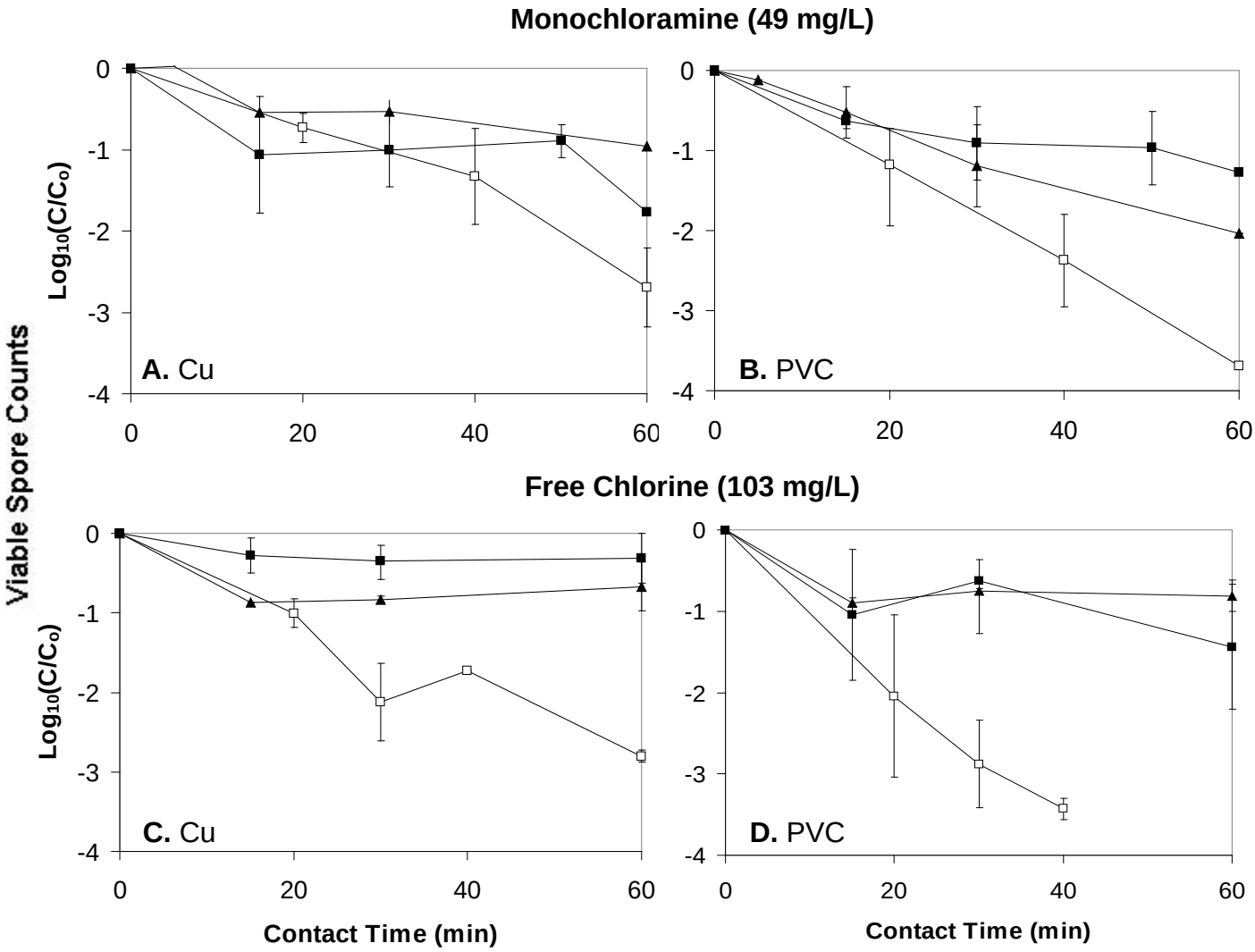

Figure 1.3. Impact of Disinfectant and Contact Time on Spores Associated with Biofilm-conditioned Cu or PVC Pipe Material Surfaces. Inactivation curves, presented as $\log_{10}$ of the fraction remaining on the surface are depicted for the commercial preparation of BT, ■, and BA, ▲, after contact with monochloramine (49 mg/L), panels A and B, and free chlorine (103 mg/L), panels C and D. Contact conditions for spore association were uniformly mixed, pipe reactor (open symbols), and uniformly mixed under low-shear, CDC reactor (solid symbols). Temperature was 22 °C to 23 °C and pH 8.1. Averages of duplicate experiments are shown, bars indicate the observed range of data.

Free chlorine was not as effective as monochloramine at inactivating spores from the biofilm-conditioned surfaces. Free chlorine contact at an average concentration of 103 mg/L was required to yield a 1 to 4 $\log_{10}$ reduction in viable BT spores associated with the biofilm-conditioned pipe surfaces. When spores were continuously stirred with a baffle present to distribute the shear across the coupon surface (CDC reactor data, Figure 1.3), significant tailing was observed for both pipe material surfaces after 15 minutes of contact with free chlorine at ≈103 mg/L. The tailing resulted in single $\log_{10}$ reductions after 30 and 60 minutes of contact that were the same as reductions observed after 15 minutes of contact. Tailing behavior was consistent for both spore preparations resulting in insignificant differences in measured $\log_{10}$ reductions for BA and BT spores ($p \leq 0.4$) after 30 minutes of contact with free chlorine. Tailing was not due to depletion of disinfectant over the course of the study. Residual disinfectant concentrations, post biofilm contact, were not significantly reduced during contact with biofilms (up to 120 minutes).

Biofilm Disinfection

Water system biofilms accumulated on pipe surfaces were tested for vulnerability to two disinfectants, free chlorine and monochloramine, in the CDC reactor. Both free chlorine and monochloramine at the concentrations tested resulted in a significant reduction in biofilm surface coverage ($p = 0.02$-0.04, $\alpha = 0.05$). As with the spores associated with biofilm-conditioned pipe materials, the biofilm organisms themselves were more effectively removed when contacted with monochloramine compared to free chlorine. A lower number of viable bacteria remained on the copper and PVC coupons after 30 minutes of contact with monocholoramine (≈13 mg/L) than chlorine at ≈11 and 103 mg/L (Figure 1.4). Interestingly, a near 10 fold increase in the free chlorine concentration did not result in a significant decrease in the viable cell fraction of the biofilm for either substratum ($p = 0.22$ and 0.27 for PVC and copper, respectively, $\alpha = 0.05$).

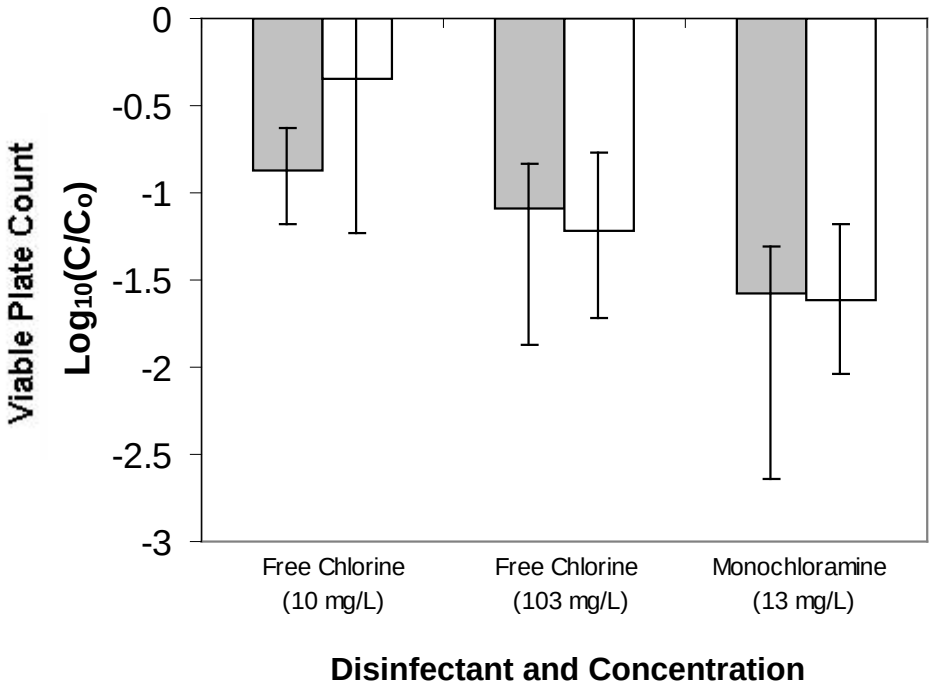

Figure 1.4. Biofilm Susceptibility to Disinfection. Heterotrophic plate count $\log_{10}$ reductions of biofilm organisms accumulated on pipe material surfaces in the CDC reactor, copper (solid) and PVC (open), after contact with average concentrations with 11 mg/L and 103 mg/L free chlorine and 13 mg/L monochloramine for 30 minutes. Temperature was 22 °C to 23 °C and pH 8.1. Experimental averages are shown ($n \geq 2$), experimental ranges are indicated by bars.

Discussion of Disinfection of Spores in Solution and in Contact With Biofilms

Biofilms have been implicated as reservoirs for their ability to harbor and protect bacterial contaminants in water distribution systems (LeChevallier, et al. 1987; Storey and Ashbolt, 2003). This work sought to examine the role of biofilm conditioning of pipe materials on the fate and disinfection susceptibility of spores in water systems.

Free chlorine and monochloramine are proven disinfectants for the removal of planktonic bacteria from the water column. The inherent resistance of spores to disinfection is due to the complex nature of the spore coat and the inability of biocides to penetrate and inactivate the spore core (Cortezzo, et al. 2004). Typical chlorine Ct values are 2-3 orders of magnitude higher for spores than vegetative pathogens (Rose, et al. 2005) and Ct values vary by an order of magnitude among spores from different *Bacillus* species (Rice, et al. 2005). The Ct value reported here for BA inactivation by free chlorine in synthetic tap water (290 mg·min/L) is higher than literature reported values of 60 mg·min/L and 127 mg·min/L for a 2-$\log_{10}$ reduction in the same strain when disinfection was performed at equivalent pH and temperatures (Rice, et al. 2005; Rose, et al. 2005). Unlike the results reported here where synthetic tap water was utilized to simulate a real water distribution system, the studies by Rice et. al. (1) and Rose et. al. (2) were performed in a simple potassium phosphate buffer. Differences in calculated Ct values observed between various studies have been attributed to minor differences in water quality parameters (pH, temperature) and different environements during the spore preparation process, (Rose, et al. 2005; Dow, et al. 2006). Although chlorine demand by water components was accounted for by contacting the water with free chlorine for 30 minutes prior to determining the chlorine and monochloramine concentrations, water constituents may still contribute to an enhanced spore stability and disinfectant resistance. Oxidizing agents are known to damage the inner membrane of spores (Cortezzo, et al. 2004). Synthetic tap water constituents such as magnesium and phosphorus may enhance membrane stability (Sowers and Gunsalus, 1988; Yethon and Whitfield, 2001; Juhna, et al. 2007) and repair oxidative damage resulting in larger Ct values. Additionally, strain differences and spore preparation procedures may be responsible for the increased resistance of BT spores to free chlorine observed here (Ct = 620 mg·min/L for a 2-$\log_{10}$ reduction) in comparison to the BA Sterne preparation and a literature reported value of 246 mg·min/L for another strain of *B. thuringiensis* (Rice, et al. 2005).

Monochloramine was less effective than free chlorine at disinfecting the spores in solution resulting in lower $\log_{10}$ reductions and higher Ct values. As previously reported, monochloramine was less effective than free chlorine at removing planktonic cells from the water column (Gagnon, et al. 2004) and free spores in suspension (Rose, et al. 2007). A Ct value of 1,442 mg·min/L was reported to achieve a 2-$\log_{10}$ reduction of *B. anthracis* Sterne spores with monochloramine at 25 ºC and a pH of 8.0 in a monopotassium phosphate solution (Rose, et al. 2007), which is consistent with values reported here (1200 and 880 mg·min/L, for BA and BT respectively).

Association with biofilm-conditioned pipe surfaces increased the resistance of spores to the two disinfectants tested. Once associated with the biofilm-conditioned surfaces, nearly 5 to 10 times the disinfectant was required to inactivate the associated spores when compared to

concentrations needed to see the same $\log_{10}$ reductions in solution (a 16 to 46 fold increase in estimated Ct values). No significant difference in disinfectant susceptibility was noted for the different spore preparations (BA and the commercial preparation of BT spores). Ct values for free chlorine disinfection (10,000 and 28,570 mg·min/L, for BT spores associated with PVC and copper pipe surfaces, respectively) were comparable to the Ct value of 31,680 mg·min/L reported by Szabo et. al for a 2-$\log_{10}$ reduction of *B. atrophaeus* spores associated with biofilms on corroded iron coupons (Szabo, et al. 2007).

Disinfectant efficacy was largely dependent on the spore contact conditions. Significantly lower BT spore association was observed when diffusion alone was the means of transport compared to the number attached when a low shear was applied (60 rpm in the CDC reactor, Figure 1.3) and the number that associated by diffusion were easier to decontaminate (Figure 1.3). The increase in the number of spores associating with the biofilm-conditioned pipes is likely due to an increase in the number of spores coming in contact with the biofilm in the CDC reactor where uniform mixing was employed. However, the increase in resistance to disinfectants when mixing is uniform may be due to an increase in the ability of the spores to penetrate the biofilm matrix or change in biofilm morphology as a result of the enhanced shear environment. The biofilm morphology may be significantly altered when mixing is increased during spore contact in the CDC reactor (60 rpm with the baffle). Altered biofilm surface morphology can occur when fluid shear is increased, initiating biofilm sloughing events (Stoodley, et al. 1999; Telgmann, et al. 2004), and contact with biocides has been shown to alter biofilm stability (Daly, et al. 1998; Chaw, et al. 2005). Therefore, biofilm sloughing events that occur during spore contact may stabilize the biofilm remaining on the pipe material surfaces, resulting in a decrease in subsequent vulnerability to sloughing from additional disinfectant contact. Szabo, et. al. observed an initial drop with a subsequent stabilization and persistence of surface associated spores at large disinfectant residuals (Szabo, et al. 2007). A stable biofilm during disinfectant contact may explain the decreased sensitivity to the disinfectants observed here for spores associated under uniform mixing conditions.

The ability of the disinfectant to penetrate the biofilm may also explain the differences in disinfection observed for biofilm and biofilm associated spores by free chlorine and monochloramine. Monochloramine was more effective than chlorine at reducing the viable cell fraction in biofilms and biofilm associated spores, but less effective than free chlorine in suspension. In both Figures 1.3 and 1.4, the significant advantage of using monochloramine to reduce the viable fraction of associated spores and biofilm bacteria, respectively, over free chlorine is demonstrated. The observed enhanced effectiveness of monochloramine compared to free chlorine at biofilm disinfection has been previously reported for biofilm bacteria (LeChevallier, et al. 1990; Samrakandi, et al. 1997; Turetgen 2004). Monochloramine is more stable and has a lower reactivity toward inorganic and organic constituents in the biofilm matrix resulting in a greater disinfection ability (LeChevallier, et al. 1990; Samrakandi, et al. 1997). Chlorine penetration of the biofilm matrix is the limiting factor in biofilm disinfection (Chen and Stewart, 1996). Chlorine has been shown to be less effective than monochloramine at disinfecting biofilm organisms where high quantities of extracellular polysaccharide are found, and

the organic and inorganic constituents of biofilms with high polysaccharide levels consume the more reactive chlorine species (Samrakandi, et al. 1997; Stewart, et al. 2001). Association with extracellular matrices in natural water distribution systems provides a survival niche for microorganisms when disinfectant is present (Ridgway and Olson, 1981; LeChevallier, et al. 1987). Biofilm bacteria and spores associated with the pipe surfaces might be encased in a polysaccharide matrix or shielded from the disinfectants by extracellular components including the inorganic material from pipe corrosion.

Bacterial attachment to biofilm-conditioned surfaces is known to drastically increase the inherent resistance to disinfection (LeChevallier, et al. 1988) and cells associated with biofilms are more resistant to disinfection than planktonic cells (Cochran, et al. 2000). Therefore, it is not surprising that the association of spores with the biofilm-conditioned pipe surfaces resulted in an increased resistance to disinfection. However, the degree to which the spores are resistant and the potential for continued sloughing and leaching of biofilm associated spores into the water column makes traditional chemical disinfection with monochloramine and chlorine a less than ideal strategy for decontamination of *Bacillus* spores from treated water systems. More research is needed to determine alternate disinfectants and disinfection strategies for the reduction of spores in water systems in the case of an accidental or intentional contamination of a water distribution facility. Additionally, future work should include investigating enhanced disinfection due to increases in fluid shear and examining the role of localized alterations in hydrodynamic and chemical differences due to corrosion as both have been found to contribute to disinfectant efficacy (De Beer, et al. 1994). Finally, this work demonstrates the use of simulant organisms is justified for large-scale disinfection studies as BT spore disinfection was either more difficult or similar to BA inactivation in the simulated water system reported here.

2.0 Enhanced Decontamination of *Bacillus* Spores in a Simulated Drinking Water System

Introduction

The fate of potential bioterror agents (e.g., *Bacillus anthracis* spores) in water distribution systems is a homeland security concern. As discussed in the previous chapter, as well as in several recent studies, biofilm-associated spores are dramatically more resistant to commonly used disinfectants as compared to their planktonic counterparts (Ryu and Beuchat, 2005; Morrow, et al. 2008; Szabo, et al. 2007). *Bacillus* spores are more resistant to commonly used disinfectants (monochloramine and free chlorine) than vegetative bacteria (Rice, et al. 2005; Rose, et al. 2005; Rose, et al. 2007). Disinfectant concentrations 5 to 10 times the levels needed to reduce the viable spore fraction in solution are required when spores are associated with biofilm-conditioned surfaces (Morrow, et al. 2008) and the spores can persist up to a month at high (70 mg/L) chlorine concentrations (Szabo, et al. 2007). Spore resistance to commonly used decontamination strategies is a concern for the disinfection of the public water systems in the event of a bioterrorist attack or an unintentional release.

Many species of *Bacillus* and *Clostridium* bacteria form spores in response to external stress including nutrient starvation and desiccation. The spore has a cell wall that is similar in structure to vegetative cells (Popham, et al. 1996) but spores also have an additional series of membranes and a cortex that protect the spores internal components from heat, desiccation, UV and oxidative damage (Hashimoto and Conti, 1971). Spores outgrow to vegetative cells under favorable conditions initiated by small molecules (amino acids and nucleosides) in a process termed germination (Hashimoto and Conti, 1971). Germination results in degradation of the spore coat thereby exposing the peptidoglycan layer of the germ cell wall (Hashimoto and Conti, 1971). Outgrowth to the vegetative cell does not occur, however, unless sufficient nutrients are available for restoration of full metabolic activity (Setlow, 2003). During germination spore components are released and lost to the surrounding media (Kort, et al. 2005). Such components, including dipicolinic acid (DPA) protect the spore DNA by facilitating internal desiccation. Upon DPA release, sensitivity to heat inactivation increases (Kort, et al. 2005). Spore resistance to oxidizing agents (such as chlorine) is primarily due to a decreased permeability and inherently low water content of the spore, a property that increases the resistance of spores to heat inactivation as well (Setlow, 1995). Oxidizing agents such as chlorine dioxide, hydrogen peroxide and hypochlorite damage the inner membrane of spores making them more vulnerable to heat treatment (Cortezzo and Koziol-Dube, 2004).

Disinfectant efficacy on biofilms and contaminant organisms harbored by biofilms is largely dependent on the chemical reactivity and the ability of the disinfectant to penetrate the biofilm matrix. Highly reactive oxidants such as free chlorine have been shown to be consumed by the top layers of the biofilm matrix resulting in a retarded penetration of the biofilm (Huang, et al. 1995; Stewart, et al. 2001; De Beer, et al. 1994; Chen and Stewart, 1996). Monochloramine has been shown to be more effective at penetrating polysaccharide layers due to its slower reaction kinetics and thus longer half-life (Samrakandi, et al. 1997; Turetgen, 2004; LeChevallier, et al.

1990). Additionally, strong oxidants are more corrosive on metal pipe materials and are more susceptible to reaction with corrosion byproducts, limiting their disinfectant ability compared to less corrosive biocides (e.g., monochloramine) (LeChevallier, et al. 1993). Spore germination has been studied as a means to increase spore susceptibility to heat inactivation and other forms of disinfection (Kort, et al. 2005; Hornstra, et al. 2007), but has not been applied to spores in contact with biofilms.

Due to the unique resistance properties of spores, improved technologies must be developed to improve decontamination strategies (Whitney, et al. 2003). The objective of this work was to elucidate the potential of combining germinant addition with disinfection to increase the sensitivity of *Bacillus anthracis* Sterne (BA) and a commercial preparation of *B. thuringiensis* (BT) spores associated with biofilm-conditioned pipe material surfaces to decontamination procedures. We measured the ability of commonly used disinfectants (free chlorine and monochloramine), elevating the contact temperature (50 ºC) and then evaluating the impact of disinfection and heat in combination with germinant exposure to inactivate and remove BA and BT spores from biofilm-conditioned surfaces (copper and PVC).

Materials and Methods

Spore Preparations and Growth Conditions

Pure suspensions of BA were compared to a commercially available BT preparation, for the ability to associate with water system biofilms and to determine their disinfectant susceptibility. The commercial BT preparation was washed to yield a concentrated spore preparation at ≈$1x10^9$ CFU/mL (colony forming units/mL) by the following procedure: aliquots of the BT spore suspension were centrifuged at 16 000 x *g* for 6 minutes, supernatant was removed and discarded and the spores were re-suspended in phosphate buffered saline (PBS) containing 0.01% (v/v) Triton™ X-100 by pipetting several times and vortexing. PBS (0.01% (v/v) Triton™ X-100) was prepared by dissolving 8 g of NaCl, 0.2 g of potassium phosphate, 1.15 g of sodium phosphate and 0.2 g of potassium chloride in 1 L of water, pH = 7.4, and autoclaved for 15 minutes, 121 °C. 1 mL of 10 % Triton™ X-100 was added after to the PBS after it had cooled to room temperature. The spore pellet was washed 4 times in PBS (0.01 % (v/v) Triton™ X-100) by centrifuging at 16,000 x *g* for 6 minutes, pipetting and vortexing to re-suspend the sample and rinsed one additional time then stored in 20 % ethanol at 4 ºC to prevent aggregation from residual dispersant materials. Concentrated BA spore preparations (Dugway Proving Ground, Dugway, Utah) at ≈$3x10^8$ CFU/mL were stored in sterile MQ water (Milli-Q® , Millipore Corp., Billerica, Massachusetts resistivity 18 MΩ) at 4 °C. Spore suspensions were subsequently diluted in synthetic tap water (LeChavillier, 2005) for disinfection studies as described in the following sections. Spore concentrations were determined by plating on Luria-Bertani (LB) agar and incubating at 35 °C. PBS (0.01 % (v/v) Triton™ X-100) was used as a dilution buffer for biofilm and spore enumeration. Spore preparations contained greater than 95 % phase bright spores as determined by phase contrast microscopy. Spore germination was detected by plating on LB agar before and after heat treatment at 65 ºC for 25 minutes. The number of germinated spores (vulnerable to an elevated water temperature) was determined by the difference in plate counts before and after heat contact.

Preparation of Disinfectants
Disinfectant solutions consisted of 150 mL of synthetic tap water containing either free chlorine at average concentrations of 11 mg/L (ranged 10 mg/L to 12 mg/L) and 103 mg/L (ranged from 98 mg/L to 108 mg/L) or monochloramine at average concentrations of 13 mg/L and 49 mg/L (ranged from 8 mg/L to 18 mg/L and 47 mg/L to 57 mg/L, respectively). Free chlorine was derived from a stock solution of sodium hypochlorite (bleach). Free chlorine concentrations were determined using N,N-diethyl-p-phenylenediamine (DPD) reagent and chlorine standards (Method #8021, Hach Company, Loveland, Colorado). Monochloramine solutions were prepared as described by Camper, et. al.2003. Monochloramine concentrations were determined by DPD and the indophenol method (Methods #8167, #8021, and #10171, Hach Company, Loveland, Colorado). Concentrations of all disinfectant solutions were determined after 30 minutes of stirring post-disinfectant addition to synthetic tap water to eliminate chlorine demand contributions.

Coupon Preparation and Biofilm Colonization of Pipe Materials
Polyvinyl chloride (PVC) and copper pipe sections were rinsed with RO water prior to connecting to the reactor tubing. PVC and copper coupons were purchased from BioSurface Technologies Corp (Bozeman, Montana). PVC coupons were rinsed in 10 % (v/v) bleach and then RO water prior to inserting into the reactor holder. Copper coupons were buffed in a circular motion with a 3M Scotch-Brite™ pad and then soaked in 5 % (v/v) nitric acid for 30 minutes and rinsed with RO water prior to inserting into the reactor holder.

Simulated water system biofilms were accumulated by contacting pipe surfaces in synthetic tap water (1.2 mM $NaHCO_3$, 0.54 mM $MgSO_4 \cdot 7H_2O$, 0.2 mM $CaSO_4 \cdot 2H_2O$, 0.004 mM K_2HPO_4, 0.002 mM KH_2PO_4, 0.08 mM $(NH_4)_2SO_4$, 0.17 mM NaCl, 36 nM $FeSO_4 \cdot 7H_2O$, 0.011 mM $NaNO_3$, 0.2 mM $CaCO_3$, pH = 8.2 ± 0.2 (LeChevallier, M. Personal communication, 2005)) made from the laboratory RO water supply. Growth of organisms indigenous to the RO water supply system of NIST was stimulated by adding 24 mg/L humic acid sodium salt (Aldrich H16752-100G) to the synthetic tap water for two weeks. A final low-carbon adaptation period of 3 d to 5 d, achieved by omitting humic acids, was utilized to produce viable plate counts of biofilm organisms consistent with literature reports for water distribution systems (Schwartz, et al. 2003). Biofilm organisms were enumerated by plating on R2A media and incubating at room temperature for up to 7 days.

CDC Biofilm Reactor Operation
The impact of a distributed shear on spore association with the biofilm surface was determined with a biofilm reactor designed by the Centers for Disease Control and Prevention (CDC). The CDC biofilm reactor was chosen due to its history as a model potable water system (Donlan et al, 2002) and for the ability to control the applied fluid shear during biofilm development and spore contact conditions (Goeres, et al. 2005). The reactor was assembled and vented as directed by the manufacturer (BioSurface Technologies Corp., Bozeman, Montana) and synthetic tap water was supplied from a 20 L feed carboy to the CDC reactor by a peristaltic pump via Masterflex® tubing (Norprene® Food Tubing 6402-14). Prepared coupons were inserted into the coupon holders and the reactor was filled with 400 mL of synthetic tap water and autoclaved (121 ºC for 15 minutes). Reactors were allowed to cool

and then placed on digital stir plates and connected to the peristaltic pump. A flow rate of 0.3 mL/min (hydraulic retention time of 29 hours) supplied the reactors with synthetic tap water containing 24 mg/L humic acids for 14 days at room temperature. After 7 days, mixing was introduced by turning on the digital stir plate to begin baffle rotation at 120 ± 5 rpm (confirmed by a tachometer) while fluid flow was continued. After 2 weeks of synthetic tap water plus humic acids, reactors were switched to synthetic tap water without humic acids, applied at the same flow rate for 3 to 5 days.

Contacting with spores was performed by filling the reactor vessel to the 700 mL mark with synthetic tap water and adding the appropriate amount of a concentrated spore stock resulting in a concentration of $1.4 \pm 0.8 \times 10^7$ CFU/mL of spores. Spores were contacted for 24 hours under uniform mixing in batch with an applied shear (baffled stir bar with 180 rpm rotation) in the CDC reactor. After this 24 hour period, coupons mounted in holders were removed, rinsed in sterile synthetic tap water and either sampled directly, contacted with disinfectants or germinants and subsequently contacted with disinfectants.

The CDC reactor was sampled by removing coupon holders and placing them on a sterile piece of aluminum foil. Coupon surfaces were scraped in a circular fashion collecting the biofilm in the center of the coupon while still positioned in the coupon holder (Zelver and Hamilton, 2001; Zelver and Hamilton 1999). The method by Zelver and Hamilton, 1999 was adapted by using a spatula (made from a sterile piece of DuPont™ Teflon® cut to resemble a spatula) rather than a cotton swab for scraping. Coupons were removed from the holder with a pair of sterile forceps, positioned over a 15 mL BD Falcon™ tube and rinsed by dispensing 5 mL of sterile synthetic tap water directly on the coupon surface. The rinsing procedure was repeated two times using the same 5 mL of synthetic tap water to minimize sample volume. Biofilm samples were vortexed for 30 s at the highest speed to disperse the cells and associated spores and were diluted and enumerated under the described conditions.

Statistical Comparisons

Dunnett's multiple comparison procedure was used for treatments versus controls. A one-way Analysis of Variance was performed to test the hypothesis that the average mean value across categories of the groups were equal. In the presence of significance for the omnibus ANOVA test, a Newman-Keuls multiple comparison test is used to perform pairwise comparisons. Statistical decisions were made at $\alpha = 0.05$. Statistics were performed using WINKS SDA Software (TexaSoft, Cedar Hill, Texas.)

Decontamination Results

Once the biofilm was accumulated and spore contact had occurred, coupons were subjected to several different decontamination strategies as shown in Figure 2.1. Decontamination methods included direct disinfection, germinant addition, disinfection post germinant contact, and heat inactivation before and after germinant contact. In the decontamination studies, pipe material coupons in coupon holders were contacted with disinfectant solutions (150 mL) in 250 mL beakers of the disinfectant solution. A small stir bar was used to uniformly mix the disinfectant solution during batch contact of the CDC reactor coupons. After 30 minutes contact times, coupons were immediately removed and placed in 150 mL of sterile synthetic tap water containing 7.5 mM sodium thiosulfate to neutralize residual

disinfectant prior to sampling. Concentrated stocks of 10 mM inosine and 80 mM L-alanine were prepared by dissolving germinants in MQ water and filtering through 0.2 µm filters and stored at 4 ºC prior to use. The germinant stock solutions were diluted directly in synthetic tap water (pH 8.1) to a final concentration of 1 mM inosine and 8 mM L-alanine (Barlass and Houston, et al. 2002). Germination was performed at room temperature (22 °C to 23 °C) by contacting coupons in holders in the CDC reactor (containing 500 mL of germinant solution) for 24 hours with mixing (60 rpm). Heat treatment consisted of placing coupon holders in preheated, synthetic tap water in 50 mL conical tubes (sealed with Parafilm®) at 50 ºC for 25 minutes, cooling at room temperature, followed by sampling as described above.

Spore Suspension Disinfection

The sensitivity of BA and BT spores that detached from the coupon surface during contact with germinant solutions was determined for both free chlorine and monochloramine. Germinant contact solutions containing detached spores were added to glass beakers containing synthetic water with disinfectants (final concentrations of 2 mg/mL and 10 mg/mL). Gentle stirring with small stir bars was used to prevent settling of spore suspensions during contact. Viability after 30 minutes contact was determined by sampling 100 µl from the beaker and serially diluting in 0.3 mM sodium thiosulfate in PBS (0.01% Triton™ X-100). All two-sample

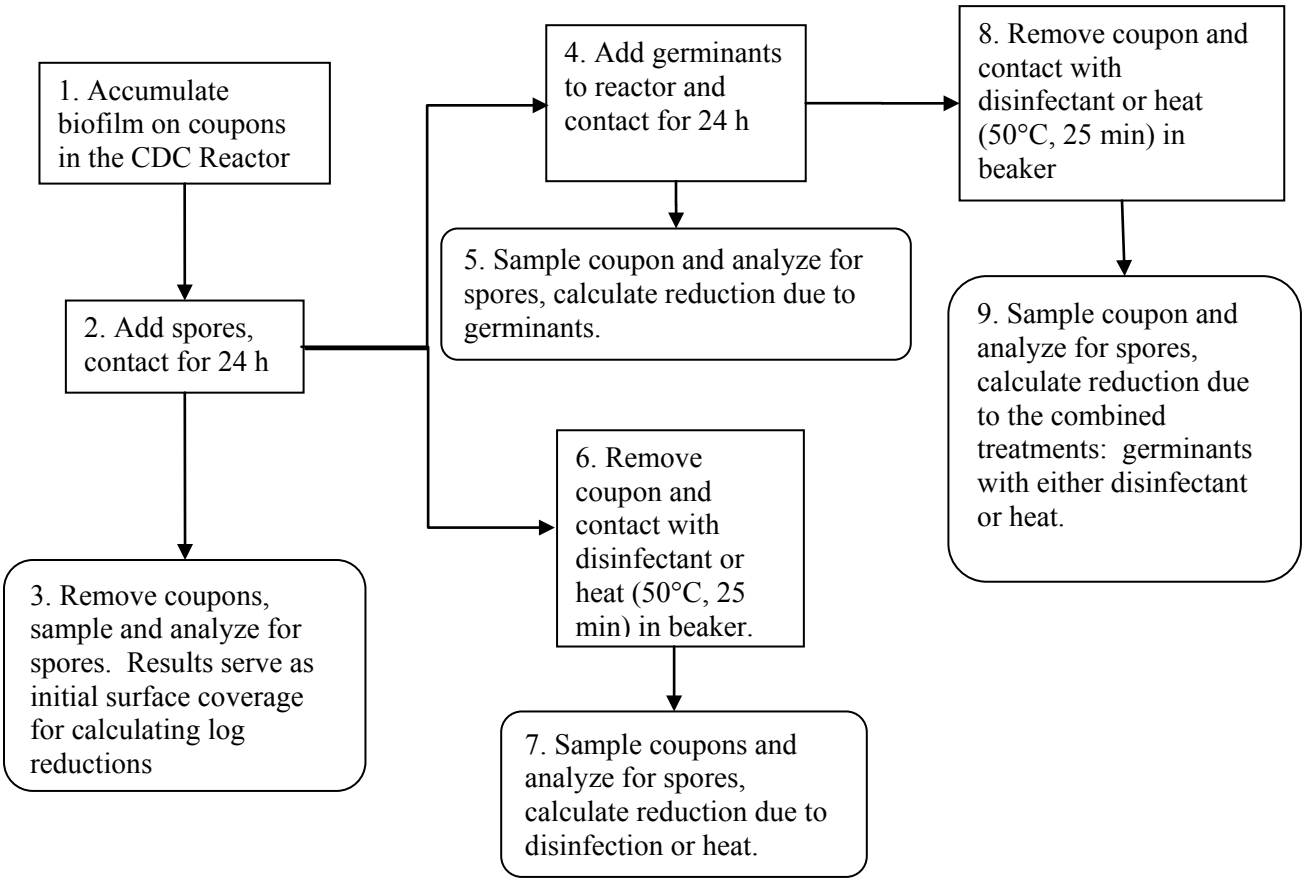

Figure 2.1. Flow Chart of Experimental Approach. The experimental procedure for processing coupons is shown.

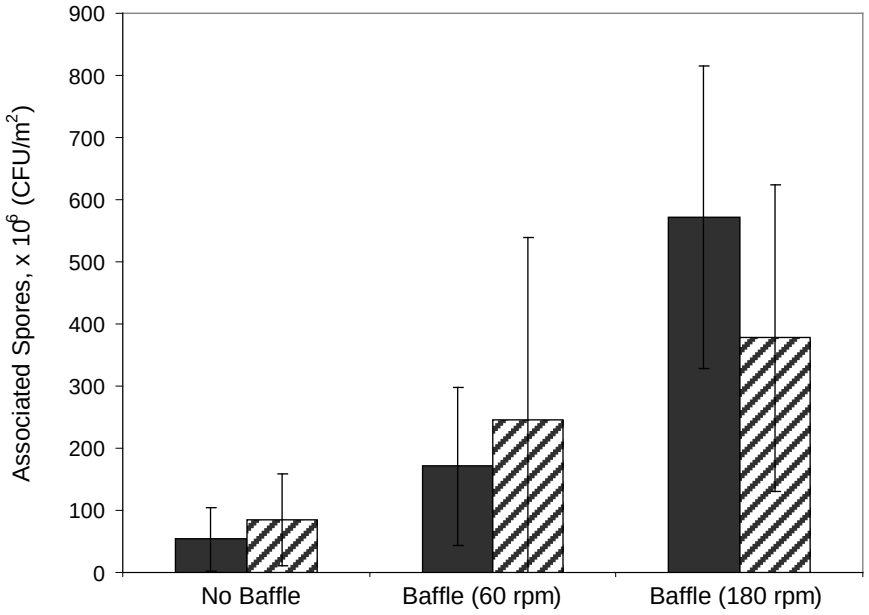

Figure 2.2. Impact of Shear on BT Spore Association with Biofilm-Conditioned Pipe Surfaces. Data for PVC surfaces, solid bars, and Cu, hatched bars, $n \geq 3$, error bars = 1 standard deviation.

comparisons employed Student's t-test to calculate the t statistics assuming unequal variances at $\alpha = 0.05$. The p-values for falsely rejecting the null hypothesis of no difference in compared means are reported.

Results of Spore Association with Biofilm-Conditioned Pipe Materials

This work confirms our previous report (Morrow, et al. 2008) that increasing the fluid shear during spore contact increases the number of spores that associate with the biofilm-conditioned surfaces up to the maximum value tested of 180 rpm. Significantly more spores ($p \leq 0.01$) were associated with the pipe material surfaces when the reactor was uniformly mixed and a low shear was uniformly applied over the coupon surfaces (180 rpm with the baffle present in the CDC reactor) during the 24 hours contact time when compared to previously published results (Morrow, et al. 2008) with lower shear (60 rpm) or uniform mixing alone (Figure 2.2).

Spontaneous germination of spores associated with the biofilm was measured by comparing the number of associated spores before and after contact at 65 ºC for 25 minutes. Significant spontaneous germination as indicated by heat sensitivity was not detected. Plate counts of the associated spores were not significantly different before and after heat inactivation for either PVC ($p = 0.36$) or copper ($p = 0.26$) surfaces, respectively.

Decontamination of attached spores was attempted using disinfection (monochloramine and free chlorine) and elevated water temperature (50 °C). Results fo r for BT are given in Table 2.1; results for BA are given in Table 2.2 and Figure 2.3. The effect of germination before heat treatment or before treatment with the disinfectants was evaluated. Reduction of the viable spores associated with the biofilm-conditioned surfaces was significant when germinant addition preceded disinfection or heat treatment. Germinant

addition alone reduced the viable spore fraction by 1.5 to 3.0 logs compared to the number that were initially adhered to the biofilm-conditioned pipe surfaces (Tables 2.1 and 2.2). Of the remaining spores, 99.9% bound to the copper surfaces were heat sensitive (1.3×10^1 CFU/cm^2 were still viable after heat treatment) and no viable spores were detected on the PVC surfaces after heat treatment. Germination in combination with disinfection or heat treatment resulted in an even more dramatic reduction in the number of viable spores associated with the biofilm-conditioned pipe surface numbers. Free chlorine or monochloramine contact post-germination resulted in a 1.5 to 3.4 $\log_{10}$ or 2.3 to 3.4 $\log_{10}$ reduction of attached spores, respectively (Tables 2.1 and 2.2). Both BA and BT spores that had detached and germinated in the germinant solution were inactivated (below the limit of detection of the plate count method) by both disinfectants at 10 mg/L and were reduced by 1.0 and 1.2 $\log_{10}$ after 30 minutes of contact with 2 mg/L monochloramine and free chlorine, respectively.

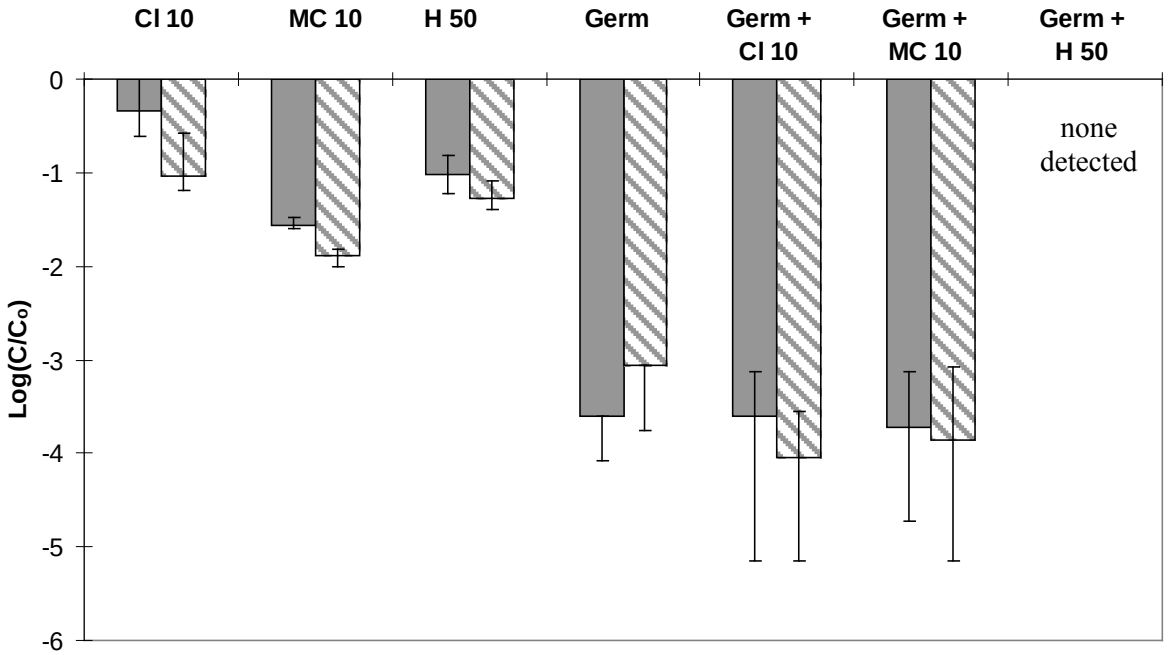

Figure 2.3. Impact of Germination on Decontamination of Adhered BA Sterne Spores. Log reductions in viable spores accumulated on biofilm-conditioned pipe material surfaces, copper (solid) and PVC (hatched), before and after germination (Germ) and after contact with 10 mg/L free chlorine (Cl 10) and monochloramine (MC 10) for 30 min or water temperature elevated to 50 ºC (H 50). Experimental averages are shown ($n \geq 3$), ranges are indicated by bars, $\log_{10}$ Co values were 4.1 for copper and 4.6 for PVC. See Table 2.2 for initial spore levels.

Interestingly, the most effective means of eliminating spores from biofilm-conditioned surfaces was completing a germination step and then elevating the contact temperature to 50ºC for 25 minutes (Tables 2.1 and 2.2. and Figure 2.3). No viable BA spores were detected in the solution or on the biofilm-conditioned surface post-contact at 50 ºC. Heat inactivation was more effective at the removal of viable BT spores from copper pipe surfaces compared with PVC (only a 2.6 log reduction was observed for PVC compared to below detection limits for copper). Additionally, it is important to note that BT spores were slightly more resistant to decontamination methods than BA, suggesting BT is a good simulant as it poses a greater challenge than BA Sterne to decontamination.

Germinant Addition Impact on Biofilm

Contact with inosine and L-alanine resulted in an insignificant ($p \leq 0.02$, $\alpha = 0.05$) change in the surface coverage of the biofilm (Table 2.3). Synthetic tap water biofilms accumulated on pipe surfaces were tested for vulnerability to free chlorine and monochloramine, prior to and after contact with germinant solutions. Monochloramine resulted in a significant reduction in biofilm surface coverage on both copper and PVC ($p \leq 0.03$) with and without germinant addition. Chlorine at ≈ 100 mg/L resulted in a significant decrease in the biofilm population pre and post germinant contact ($p \leq 0.03$) on both substrata. Chlorine at ≈ 10 mg/L resulted in a significant decrease in the biofilm population only on copper surfaces pre and post germinant contact ($p = 0.02$). Biofilms on PVC surfaces were resistant to ≈ 10 mg/L free chlorine prior to ($p = 0.13$) and post ($p = 0.12$) germinant addition. Elevated water temperature in the presence of germinant resulted in a significant reduction in the biofilm population on copper surfaces but not PVC.

Table 2.1. Impact of Treatment Strategy on BT Spore Surface Coverage

		Treatment								
	Initial	**Germ**	**Non-Germ Cl 100**	**Germ Cl 100**	**Non-Germ Cl 10**	**Germ Cl 10**	**Non-Germ MC 10**	**Germ MC 10**	**Non-Germ H 50**	**Germ H 50**
Copper Pipe Surface										
Γ, $x10^2$ CFU/cm^2	364 (221)	0.53 (0.52)*	93.0 (108)	ND		1.58 (1.42)*	401 (701)	0.40 (0.01)*	414 (269)	ND
Total LOG Reduction		**2.8**	**0.6**	**≈4.6**		**2.4**	**0.0**	**3.0**	**0.0**	**≈4.6**
PVC Pipe Surface										
Γ, $x10^2$ CFU/cm^2	556 (504)	16.9 (21.2)*	188 (106)	0.40 (0.39)*		16.2 (12.8)*	25.0 (16.4)*	2.70 (3.06)*	217 (134)	1.45 (1.14)*
Total LOG Reduction		**1.5**	**0.5**	**3.1**		**1.5**	**1.3**	**2.3**	**0.4**	**2.6**

Total LOG Reduction is the reduction of the initial spore surface coverage (Initial) due to the individual or combined treatments;
Treatment notation is Germ = 24 h contact with 1 mM inosine and 8 mM L-alanine, Non-Germ = no germinant contact, Cl = free chlorine with concentration, at either 10 mg/L or 100 mg/L, MC = monochloramine with concentration of 10 mg/L, H = heating to 50 ºC for 25 minutes, contact with free chlorine at 10 mg/L was not performed for Non-Germ samples due to observed limited reduction at 100 mg/L;
Values presented are averages with 1 standard deviation shown in parentheses, $n \geq 3$;
ND = none detected;
Consecutive treatments are shown with germination first and second treatment below;
*indicates significant reduction in surface coverage compared to initial value (determined using Dunnett's comparison test $\alpha = 0.05$ described in Statistical Comparison section).

Table 2.2. Impact of Treatment Strategy on BA Spore Surface Coverage

		Treatment						
	Initial	**Germ**	**Non-Germ**	**Germ**	**Non-Germ**	**Germ**	**Non-Germ**	**Germ**
			Cl 10	**Cl 10**	**MC 10**	**MC 10**	**H 50**	**H 50**
Copper Pipe Surface								
Γ, x10^2 CFU/cm^2	134 (123)	0.13 (0.20)*	241 (191)	0.13 (0.23)*	14.5 (2.28)*	0.10 (0.17)*	50.0 (25.4)*	ND
Total LOG Reduction		**3.0**	**0.0**	**3.0**	**1.0**	**3.1**	**0.4**	**≈4.1**
PVC Pipe Surface								
Γ, x10^2 CFU/cm^2	363 (459)	1.32 (2.28)*	134 (77.8)	0.13 (0.23)*	18.4 (4.56)	0.19 (0.48)	77.6 (25.7)	ND
Total LOG Reduction		**2.5**	**0.4**	**3.4**	**1.3**	**3.4**	**0.7**	**≈4.6**

Total LOG Reduction is the reduction of the initial spore surface coverage (Initial) due to the individual or combined treatments; Treatment notation is Germ = 24 h contact with 1 mM inosine and 8 mM L-alanine, Non-Germ = no germinant contact, Cl = free chlorine with concentration, at either 10 mg/L or 100 mg/L, MC = monochloramine with concentration of 10 mg/L, H = heating to 50 ºC for 25 minutes, contact with free chlorine at 100 mg/L for both Germ and Non-Germ was not performed for BA spores;
Values presented are averages with 1 standard deviation shown in parentheses, $n \geq 3$;
ND = none detected;
Consecutive treatments are shown with germination first and second treatment below;
*indicates significant reduction in surface coverage compared to initial (determined using Dunnett's comparison test $\alpha = 0.05$ described in Statistical Comparison section).

Table 2.3. Impact of Treatment Strategy on Biofilm Surface Coverage

		Treatment								
	Initial	**Germ**	**Non-Germ**	**Germ**	**Non-Germ**	**Germ**	**Non-Germ**	**Germ**	**Non-Germ**	**Germ**
			Cl 100	**Cl 100**	**Cl 10**	**Cl 10**	**MC 10**	**MC 10**	**H 50**	**H 50**
Copper Pipe Surface										
Γ, $x10^2$ CFU/cm^2	387 (217)	347 (235)	88 (120)*	165 (113)*	116 (45)*	145 (48)*	15 (20)*	35 (44)*		109 (9)*
Total LOG Reduction		**0.1**	**0.7**	**0.4**	**0.5**	**0.4**	**1.4**	**1.0**		**0.6**
PVC Pipe Surface										
Γ, $x10^2$ CFU/cm^2	715 (425)	560 (454)	183 (162)*	256 (87)*	299 (184)	273 (48)	22 (23)*	73 (79)*		271 (160)
Total LOG Reduction		**0.1**	**0.6**	**0.5**	**0.4**	**0.4**	**1.5**	**1.0**		**0.4**

Total LOG Reduction is the reduction of the initial spore surface coverage (Initial) due to the individual or combined treatments;
Treatment notation is Germ = 24 h contact with 1 mM inosine and 8 mM L-alanine, Non-Germ = no germinant contact, Cl = free chlorine with concentration, at either 10 mg/L or 100 mg/L, MC = monochloramine with concentration of 10 mg/L, H = heating to 50 ºC for 25 minutes, heat contact with Non-Germ samples was not performed;
Values presented are averages with 1 standard deviation shown in parentheses, $n \geq 3$;
ND = none detected;
Consecutive treatments are shown with germination first and second treatment below;
*indicates significant reduction in surface coverage compared to initial value (determined using Dunnett's comparison test $\alpha = 0.05$ described in Statistical Comparison section).

Discussion of Results

Biofilms have been implicated as reservoirs for their ability to harbor and protect bacterial contaminants in water distribution systems (Morrow, et al. 2008; Szabo et al. 2007; LeChevallier, et al. 1987; Szabo, et al. 2006) and matrix materials may protect contaminants from the disinfectants. This work confirms previous reports (Morrow, et al. 2008; Szabo et al. 2007) that indicate spores are exceptionally resistant to high levels of disinfectant when associated with biofilm matrices and examines the role of spore germination as a means to enhance disinfectant efficacy.

Germinant addition resulted in a significant reduction in the number of viable spores associated with the biofilm-conditioned surfaces, disinfectants and elevated water temperatures (Table 2.1 and Figure 2.3). The exact mechanism of the success of germinant addition is unknown; however, the observed vulnerability of germinated spores is believed to be due to a combination of physiological changes in the cell form and the inability to compete with the native biofilm bacteria for nutrients. Germinant addition resulted in a significant reduction in the viable spores associated with the biofilm-conditioned pipe surfaces. Ultrastructural changes occurring during germination result in a more vulnerable cell form as degradation of the spore coat exposes the underlying peptidoglycan layer of the germ cell wall to detrimental environmental effects (Hashimoto and Conti, 1971). Spore coat degradation and subsequent hydrolysis of DNA and cell proteins occurring during germination further enhance the spore's susceptibility to oxidants and heat that yield cell lethality through damage of those cell components (Setlow, 1995). Additionally, the depleted nutrient levels in the simulated water system are believed to limit the ability of germinated spores to outgrow and develop into vegetative cells as germination alone resulted in a significant decrease in the surface-bound viable spore fraction and spontaneous germination was not observed. Due in part to the depleted nutrient levels, it is likely that the germinated spores did not develop into fully vegetative cells, but that the spores began the germination process and became more susceptible to disinfection. In addition, any germinated spores remaining associated with the biofilm or in the water column are vulnerable to competition with the bacterial biofilm organisms for available nutrients.

The impact of germinant addition on the biofilm bacterial population was of concern as drinking water biofilm bacteria can readily utilize simple amino acids and chlorine demand has been shown to increase when biofilms are grown on amino acids (Butterfield, et al. 2002). Interestingly, germinant addition did not result in a significant increase in the biofilm bacterial population as biofilm surface coverage values were similar before and after germinant addition (Table 2.1). Furthermore, disinfectant efficacy on biofilm control was not significantly altered for either chlorine or monochloramine.

The disinfectant efficacy was dependent on the chemistry of the disinfectant when germinants were not added. The higher $\log_{10}$ reduction of biofilm-associated spores by monochloramine compared to free chlorine is consistent with previous reports that indicate its stability and lower reactivity toward inorganic and organic constituents results in a greater disinfection ability toward biofilms (Samrakandi, et al. 1997; LeChevallier, et al. 1990). Monochloramine has been shown to be more effective at reducing the viable cell fraction and adhered spores in some biofilms (Morrow, et al.

2008; (Samrakandi, et al. 1997; LeChevallier, et al.1990)). However, similar log reductions in the viable spore fraction were observed for both disinfectants for surface bound spores when germinants were added (Table 2.1). Finally, 2 mg/L of both free chlorine and monochloramine was effective at reducing the viable spore fraction that detached in the germinant solution by 1.0 to 1.2 $\log_{10}$, respectively, and no viable spores were detected in the germinant solution after contact with ≈ 10 mg/L of disinfectant. Disinfectant efficacy for germinated spores in the water column is consistent with values reported for the inactivation of other bioterrorist agents and vegetative cell forms of *Bacillus* (Rose et al. 2007; Rose et al. 2005; Beuchat, et al. 2005).

Contact at an elevated water temperature resulted in a significant decrease in the viable spores associated with the biofilm-conditioned surfaces. Heat sensitivity was enhanced with germinant addition resulting in no detectable viable spores on the biofilm-conditioned surfaces after contact at an elevated water temperature. The resistance of spores to heat is related to the dehydration state of the cell due to the presence of DPA (Kort, et al. 2005; Setlow, 1995). Spores are known to undergo a phase transition from glassy-like to a rubbery-like phase between 50 ºC and 60 ºC corresponding to the release of DPA (Alimova, et al. 2006). Such a physiological change may yield the observed vulnerability to the elevated water temperature (0.4 to 0.7 $\log_{10}$ reductions) observed here and DPA release is enhanced when germinants are added, stimulating spore coat degradation. Furthermore, oxidizing agents such as chlorine dioxide, hydrogen peroxide and hypochlorite damage the inner membrane of spores making them more vulnerable to sublethal heat levels and demonstrate an increased germination rate (Cortezzo, et al. 2004).

Attachment to biofilm-conditioned surfaces is known to increase the inherent resistance of spores to disinfection. This work demonstrates that adding germinants in combination with either a disinfectant or elevated water temperature drastically reduces or eliminates the need for high concentrations of reactive disinfectants to treat spore contamination of a treated water system.

3.0 The Effect of High Flow on the Adhesion and Disinfection of BT Spores in Pipe Loop Experiments

Introduction

The aim of this study is to evaluate the effect of a high flow rate on the disinfection step of *Bacillus* spores using chlorine in a simulated drinking water system. In a previous study (Morrow, et al. 2008), we measured the disinfection of *B. thuringiensis* (BT) and *B. anthracis* (BA) spores in a simulated drinking water system using a commercial CDC biofilm reactor and a pipe-section loop bioreactor. Both of these reactors have been described in previous sections of this report. The reactors were used to establish a water system biofilm by contacting the plumbing materials with a synthetic formulation of tap water containing humic acids. The humic acids stimulated the growth of water system bacteria in a short period of time (three weeks) to levels similar to those found in mature water distribution systems (Schwartz, et al. 2003). A stirred paddle was used to establish a fluid shear in the CDC biofilm reactor; and a creeping flow (Reynolds number < 1) was used to grow the biofilm organisms in the pipe section reactor. An important feature of the experimental design in the earlier study was that the pipe section or coupons (CDC biofilm reactor) were removed from the bioreactors and the disinfection step was done by contacting the pipe materials with the chlorine solution without flow. We found that the spores adhered to the biofilm materials were resistant to disinfection to chlorine solutions when using these conditions (Morrow, et al. 2008).

In this study BT was chosen as a safe simulant for BA because the species are genetically closely related (Radnedge, et al. 2003) and the exosporium (outermost spore layer) composition of the two species is also similar (Matz and Beaman, et al. 2001). The disinfection of *Bacillus* spores by chlorine solutions is determined by the solution conditions used and the characteristics of the spore sample (Dychdala, 2001). Studies have indicated that the inactivation kinetics of BA and BT are similar (Rice, et al. 2005; Rose, et al. 2005) validating the use of BT as a simulant for BA, for chlorine disinfection studies. We also observed similar spore adhesion and disinfection kinetics for BT and BA (Sterne strain) in a previous study (Morrow, et al. 2008).

In this study, we focused on the effect of a relatively high flow (Reynolds number = 2,800) on the critical disinfection step using a pipe section bioreactor in a loop format using local tap water supplemented with humic acids to stimulate the biofilm growth. A high flow rate was used in all phases of the experiments including biofilm growth, spore contacting, flushing, and disinfection with chlorine. These changes were done with the pipe section reactor to better simulate the conditions that would be found in a building water system.

Materials and Methods

Pipe Section Loop Reaction Protocol

Water from a cold water faucet was collected for these experiments at the NIST facility in Gaithersburg, Maryland. The water obtained did not contain a significant amount of active chlorine (less than 0.04 mg/L) and was used without further treatment for the experiments. The pipe section bioreactor consisted of alternating PVC and copper pipe sections (19 mm inner

diameter and 51 mm long) connected by silicone tubing in a loop with a 20 L water container vented to the atmosphere. Silicone tubing (inside diameter 9.7 mm) and a peristaltic pump (operated at approximately 500 rpm) was used to circulate the water from the 20 L container through the pipe section loop and back to the container at a flow rate of 2.5 L/min (14.7 cm/s). The volume of the loop section containing the pipe sections was approximately 1 L with 36 pipe sections. During the entire biofilm accumulation stage, the peristaltic pump had a duty cycle of 2 hours of flow and 2 hours no flow, established by means of an electronic timer. Biofilm was established by contacting the pipe sections with 20 L of tap water containing humic acids (24 mg/L, sodium acid for a total of 21 days. The tap water was drained from the reactor and renewed with a fresh solution after 7 and 14 days (for a total of 21 days of biofilm growth with humic acids). The pipe loop was then flushed for 1 day with 20 L of fresh tap water (without humic acids) before contacting with the BT spores.

The pipe section biofilm reactor was then drained and contacted with 2 L of BT spores (in tap water containing a total of 2×10^9 CFU) for 24 hours with flow of 2.5 L/min (duty cycle 2 hours flow and 2 hours no flow). A commercial BT preparation was prepared as previously described (1). After spore contacting, the pipe-section bioreactor was drained, rinsed, and flushed for 2 hours with 2 L of fresh tap water. A 10 mg/L solution of free chlorine was prepared using a stock solution of sodium hypochlorite (commercial bleach) diluted in tap water. Free and total chlorine concentrations were measured using N, N-diethyl-p-phenylenediamine (DPD) reagent and chlorine standards (Method numbers #8167 and 8021, Hach Company, Loveland, Colorado). The chlorine concentration was measured on a spectrophotometer (Shimadzu UV160U) after 30 minutes of stirring. Chlorine disinfection was performed in the entire pipe section pipe loop at 2.5 L/min. The initial disinfection step involved introducing 2 L of chlorine solution (10 mg/L), which was replaced after 30 minutes by a fresh 2 L solution of chlorine (10 mg/L). After the disinfection steps duplicate pipe sections of both copper and PVC were removed and rinsed with 150 mL of tap water containing 7.5 mM thiosulfate. The biofilm was scraped off the pipe surfaces using sterile cell scrapers and rinsed with phosphate buffered saline (PBS, 0.01 M phosphate, 0.138 M NaCl, 0.0027 M KCl, pH 7.4) containing 0.01% (vol/vol) Triton™ X-100 and sampled as previously described (Morrow, et al. 2008). Spore counts were determined by plating on Luria-Bertani agar and biofilm organisms were counted on R2A agar plates (Morrow, et al. 2008). The stock spore samples and samples that had been exposed to disinfectant were concentrated by filtering half the sample (5 mL) on to a 0.45 micron filter (Nalgene #145-2045, Rochester, NY) that was applied directly to the nutrient plates for growth.

BT Spore Inactivation

To measure the disinfection of the BT spores used in the experiments we measured the inactivation of the spores in solution with a solution of chlorine. A solution of active chlorine (10 mg/L) was prepared in phosphate buffer (0.1 M pH 7.8). BT spores were suspended in the chlorine-phosphate buffer solution at a concentration of approximately 5×10^5 CFU/mL in a borosilicate glass vial. The glass vial was placed on a rocker to mix the contents during the inactivation process. Samples were taken at 10 minutes time intervals by vortexing the vial (5 sec) and removing a sample. The concentration of viable spores

was measured as described in the previous section.

Results and Discussion

The heterotrophic plate count of the biofilm organisms grown on the copper and PVC surfaces were 1.5 (1.8) x 10^9 CFU/$m^2$and 9.0 (6.5) x 10^8 CFU/m^2, respectively (means from 4 independent experiments, values in parenthesis are a standard error). These numbers were consistent with values in the literature for water distribution systems which range from 0.23 to 8.4 x 10^9 CFU/m^2 (Schwartz, et al. 2003).

The number of BT spores associated with the biofilm-conditioned pipes was 5.5 (6.0) x 10^8 CFU/m^2 and 2.4 (3.2) x 10^8 CFU/m^2 for copper and PVC pipe sections, respectively (means and standard deviations from 4 independent experiments). These values are comparable with the previous study using BT spores in the CDC bioreactor with a low shear contacting method that had 2.5 (1.6) x 10^8 CFU/m^2 and 1.7 (1.3) x 10^8 CFU/m^2 and for copper and PVC pipe sections, respectively (Morrow, et al. 2008). In this present study we used a flow rate of 2.5 L/min in 19 mm diameter pipe sections, resulting in a Reynolds number of approximately 2800, indicating the flow is transitioning to turbulent. The resulting flow velocity was 14.7 cm/s. The effect of a 2 hours flush with tap water on the removal of spores was reduction by approximately half a log (Figure 3.1).

Chlorine disinfection of the pipe loop was done in two stages because of depletion of the active chlorine by the large surface area of biofilm on the pipes and tubing of the reactor. The first chorine solution (2 L with concentration of 10 mg/L) was depleted to approximately half of the initial value after 30 minutes. A fresh chlorine solution was then used for the rest of the disinfection process. The second disinfection solution (2 L with concentration of 10 mg/L) was also reduced to approximately half of the initial value by the end of the second disinfection step (150 minutes). Figure 3.1 shows the effect of disinfection on BT spores associated with the biofilm in the pipe section loop. The first 30 minutes disinfection step resulted in a reduction of approximately 1.5 logs of the BT spores associated with the surface. The second disinfection step resulted in a further reduction of approximately 1.5 logs. The overall total reduction of BT spores was approximately 3 to 4 logs in the pipe section loop. Similar reductions were seen in either PVC or copper pipe sections.

Figure 3.1 also shows the effect of the chlorine disinfection on the removal of the biofilm organisms. The data shows that there is a significant decrease in the level of biofilm organisms that correlates with the removal of the BT spores. The biofilm organisms on copper and PVC pipe sections were reduced by approximately 2.5 logs using 10 mg/L chlorine at high flow rates, while in the previous study the reduction was less than 1 log reduction for copper and less than 0.5 log for PVC using 10 mg/L chlorine for 30 minutes without flow (Morrow, et al. 2008).

To make comparison to the disinfection effectiveness of chlorine, it is common to measure a CT value (the product of chlorine concentration times the exposure time) required to achieve a 2 or 3 log reduction in viability. The CT value to achieve a 2 log inactivation of BT spores in 0.1 M sodium phosphate buffer (pH 7.8) using 10 mg/L chlorine was 150 (60) min·mg/L (mean of 3 determinations and 1 standard deviation in parenthesis). This value for BT inactivation is lower than the value previously measured in the synthetic water formulation (Morrow,

et al. 2008), and comparable to values measured in a potassium phosphate buffer (pH 8) for BT (Rice, et al. 2005). The chlorine disinfection solutions used in pipe loop of this study were tap water with a pH of 8.0.

Conclusions

This study shows the importance of a high flow rate to increase the efficiency in disinfection of spores associated with biofilms on pipe surfaces. The high flow likely resulted in improved penetration of the disinfectant solution into the biofilm layer, which resulted in better removal of the biofilm layer and the entrapped spores. The calculated Reynolds number (value of 2800 based on velocity of 14.7 cm/s and19 mm diameter pipe) for the flow rate indicated transition to turbulent flow conditions in the pipe section reactor. The pipe section reactor was assembled with a number (up to 36) of short (51 mm) pipe sections joined by silicone tubing and several bends to achieve a loop configuration resulting in a number of changes in the diameter of the flow that would make laminar flow unlikely. Therefore, the flow was probably closer to turbulent conditions than the calculation suggested. The presence of turbulent flow would be expected to increase the delivery of fresh chlorine solution to the surface of the biofilm layer resulting in an improved mass transfer of active chlorine and a more effective disinfection process. Turbulent flow would also be expected to increase the sloughing of the biofilm also resulting in a more effective removal of the biofilm and the adhered spores.

We showed that an efficient reduction (approximately 3 logs) of spores from the pipe surfaces can be achieved in approximately 5 hours and using a moderate volume of disinfectant solution (approximately 5 volumes of the loop). Additional experiments are needed to determine the extent of reduction that was due to physical removal of the spores adhered to the biofilm due to the flushing and the extent due to inactivation of the spores adhered to the biofilm surfaces. An important consideration is that the chlorine will be consumed by the biomass in a short period of time under recirculation conditions, so it is important to monitor the active chlorine concentration and replenish it as necessary.

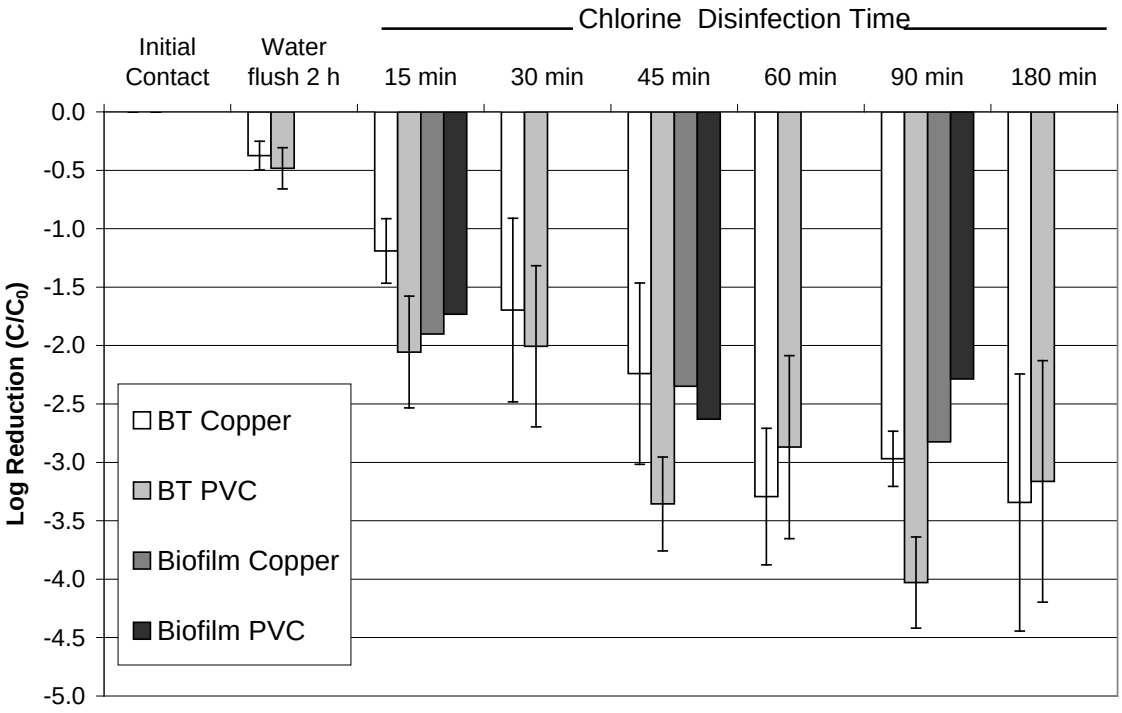

Figure 3.1. The Effect of Water Flushing and Chlorine Disinfection Time on the Reduction of BT Spores Associated with the Biofilm Conditioned Pipe Sections. Copper or PVC pipe sections were sampled at the indicated stage and the log (base 10) reduction calculated by dividing the measured BT concentration (C) by the BT concentration at the initial contact time (C_0). The conditions of the experiments were done in tap water pH 8.0 and 22 °C to 23 °C. The BT values are the means of 4 independent experiments (bars are 1 standard deviation of the mean) and the biofilm values (for 15, 45, and 90 minutes) are the means from two independent experiments.

4.0 Gaps in Research on Biological Threats to be Investigated in Additional Studies

This chapter summarizes research gaps that were identified during the course of this study and recommends additional research to help bridge these gaps. The objective of this research was to study the adherence and decontamination of biological contaminants. As the research progressed, knowledge gaps were identified that presented challenges in the development of decontamination strategies.

Recommended Research

The following is a summary of recommended research for bridging these knowledge gaps:

1. Long term persistence of bacteria and spores should be investigated in water systems. These experiments are technically difficult to do and will require significant investment in time and effort. One approach would be to investigate the fate of biological threats in water system using the fluorescent-tagged bacteria and spores that were developed in the third year of the project. The fate of *Bacillus* spores and *E. coli* in biofilms on a microscopic level using fluorescence confocal microscopy could be a subject of investigation.
2. Adhesion of other biological threats and disinfection of surfaces contaminated by them should be measured**.** Additional threat possibilities include *Yersinia pestis*, the bacterium that causes plague, and the single-stranded RNA virus, male-specific bacteriophage, MS2. The MS2 phage is a commonly used as a viral simulant in water systems.
3. Adhesion of biological threats and simulants to additional plumbing materials (e.g., stainless steel, rubber, and plastic surfaces) should be measured along with surface decontamination. The inclusion of used material with mineral deposits would be valuable addition to the existing data.
4. Transport and removal of bacteria and spores in a large-scale pipe system should be studied. The large-scale experiments will require significant planning and coordination to carry out the experiments in order to obtain meaningful results. The results of the bench level experiments completed in the current project and discussed in this report could be used as the basis of these experiments. The system would include additional water system appliances, and complex architecture. Experiments would involve injection of a sample of the simulant and measurement of the transport in the pipe system by sampling the water and coupons at various points. Development of sensors to detect the fluorescence signatures of the simulants would also be carried out. Classical microbiological techniques would be used to measure the bacterial and identification would be confirmed by the presence of the fluorescent tags.
5. Disinfection studies should be carried out on large-scale systems that have been contacted with the bacteria. The most promising conditions for disinfection of the biological stimulants (determined in

the previous years of the project) would be used on the large scale system to determine the efficiency of disinfection in a near to real life system. As in the contacting experiments, the sampling and analysis of these experiments would require significant planning and coordination of effort because of the large number and nature of the samples. Data on the efficiency of the disinfection process in the large scale systems would be valuable because of the relevance to real life systems.

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Appendix A: Protocols for Biological Threat Decontamination

List of Protocols

1. Protocol for Biosafety Level 2 Laboratory
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12. Protocol for Preparation of Synthetic Water
13. Protocol for Pipe Section CDC Biofilm Reactor Operation

1. Protocol for Biosafety Level 2 Laboratory

Purpose: To use proper safety techniques when in the biosafety level 2 laboratory .

Materials:
Laboratory coat
Latex gloves
Safety glasses
Class II, Type A2 laminar flow biological safety cabinet
Clorox® Bleach, diluted 1:10 (equivalent oto 0.5 – 0.6% sodium hypochlorite)
70% ethanol
Paper towels
NuAire Operation and Maintenance Manual for Class II Biological Safety Cabinet

Methods:

1. Enter key code on the key pad to enter room A212.
2. Gloves, safety glasses, and a laboratory coat should be worn at all times.
3. Turn on the blower in the biological safety cabinet. Spray the hood surface with 70% ethanol and wipe dry.
4. Place items to be used for the experiment (plates, dilution tubes, etc.) in the hood.
5. Allow the blower to run for 15 minutes before beginning work.
6. When working in the hood, do not make quick movements with your arms. This disrupts the air flow. Move arms slowly to shift items within the hood.
7. Do not place any items on the front grill or against the back wall of the hood as this disrupts air flow.
8. If a biological spill occurs, absorb the liquid using a paper towel and discard paper towel and gloves into the biohazardous waste. Replace gloves and apply 10% bleach to the location of the spill for 10 minutes. Wipe up the bleach with paper towel and place into biohazardous waste. Spray surface with 70% ethanol to remove any residual bleach and wipe clean.
9. A sterile working environment within the hood is located in the center of the hood (approximately 6" from the front grill). It is suggested to designate a "clean" and "dirty" side of the hood to avoid contamination.
10. Sharps are prohibited in the room (razor blades, syringe needles, scissors, etc.).
11. Design experiments to minimize aerosols whenever possible.
12. When work in the hood is completed, spray the hood surface with 70% ethanol and wipe dry with a paper towel. Discard paper towel in biohazardous waste.
13. Pull the sliding window all the way down and turn on the UV light for 15 minutes.
14. Turn off the UV light.
15. Remove laboratory coat and place gloves in biohazardous waste.
16. Wash hands thoroughly with soap and water before exiting the laboratory.

2 Protocol for Titration of Bacillus anthracis Sterne

Purpose: To determine concentration of *Bacillus anthracis* spores.

Materials:
Bacillus anthracis Sterne (suspension in sterile distilled water, stored at 4 °C)
Sterile water
Dilution tubes
Luria-Bertani (LB) agar (Fisher Chemicals #BP1425-500)
Petri dishes
Class II Type A2 laminar flow biosafety cabinet
0.5-0.6% Clorox® bleach, diluted 1:10
70% ethanol
Latex gloves
Laboratory coat
Safety glasses
Ethanol lamp
Plate spinner
Bacterial spreaders
1 mL Pipetman® (RAININ #P-1000)
200µl Pipetman® (RAININ #P-200)
Aerosol barrier pipet tips - 200µl (RAININ #GP-200F)
Aerosol barrier pipet tips - 1000µl (RAININ #GP-1000F)

Methods:

1. Refer to SOP# A212-1 for the procedures used in the biosafety level 2 laboratory.
2. In the hood, label dilution tubes and add appropriate volume of diluent (sterile water, etc.) for dilutions.
3. Vortex vial containing *Bacillus anthracis* Sterne to evenly distribute the microbes.
4. Perform serial dilutions making sure to vortex between each dilution.
5. Light the ethanol lamp.
6. Add appropriate volume of dilution to LB agar plates (usually between 50-200µl).
7. Dip the end of the bacterial spreader in 70% ethanol and flame the end to burn the ethanol off for sterilization.
8. Spread the dilution around the plate using the sterile bacterial spreader (and plate spinner if needed).
9. Extinguish the flame in the ethanol lamp.
10. Allow plates to dry for 10 minutes.
11. Turn plates upside down and incubate at 37°C for 16 hours or until colonies are visible.
12. Count and record the number of colonies.
13. Place agar plates in the biohazardous waste.
14. Autoclave waste when it has sufficiently accumulated using a dry cycle of 40 minutes at 15-20 psi.

3 Protocol for Fluorescence In Situ Hybridization (FISH)

Materials:

Fluorescent labeled oligonucleotide probes labeled (Alexa Fluor 546 and Alexa Fluor 647) on 5' ends. Probe sequences as described in Manz, et al. (1993).
Beta 42a oligonucleotide probe labeled with Alexa Fluor 546 (GCC TTC CCA CTT CGT TT)
Gamma 42a probe labeled with Alexa Fluor 647 (GCC TTC CCA CAT CGT TT)
Pseudomonas probe labeled with Alexa Fluor 546 or 647 (GAT CCG GAC TAC GAT CGG TTT)
Phosphate buffered saline (0.01 M sodium phosphate, 0.138 M NaCl, 0.0027 M KCl, pH 7.4).
95% ethanol
Microscope slides
4',6-diamidino-2-phenylindole (DAPI)
Fluorescence microscope with appropriate filters
Hybridization buffer (39% formamide, 0.9 M NaCl, 0.01 % sodium dodecyl sulfate, 0.02 M tris(hydroxymethyl)aminomethane (Tris) pH 7.2)
Washing buffer (0.01% SDS, 0.04 M NaCl, 0.005 M (EDTA), 0.02 M Tris pH 7.2)

Methods:

Fixation for preparation of bacteria

1. Add a colony to 20μL 1X PBS, resuspend, and vortex 30 sec on high
2. Spin down cells,16,100 x *g*, for 0.5 minutes; remove supernatant
3. Add 60μL 4% PFA (PFA was at room temperature), vortex high for 30 seconds
4. Fix for 4 hours 5 minutes at 4°C
5. Spin down cells, 16,100 x *g*, for 0.5 minutes
6. Remove supernatant and wash with 80μL 1X PBS; vortex 15 sec
7. Wash twice with 1 X PBS
8. End fixation and storage in PBS/ethanol at -20°C
    a. 10 μL 1 X PBS + 10μL ethanol; vortex 5 seconds

Immobilization procedure

1. Spot 3 μL of sample on slide and air dry
2. Dehydrate samples by dipping the slides in an ethanol series (50-80-95%; 3 minutes each), let air dry in vertical position

Probe Procedure

1. Add 10μL hybridization buffer plus probe and mix carefully by pipetting
2. Pour remaining 2 mL hybridization buffer in 50 mL centrifuge tube
3. Place slide in prepared 50 ml tube, close the tube and incubate in horizontal position at 50°C for 16 hrs.
4. Preheat wash buffer at 50°C
5. Open hybridization tube in hood, rinse with 1 mL wash buffer and place slide in wash buffer and put the tube back at 50°C for 15 minutes
6. Rinse carefully with sterile water, let air dry

DAPI-staining:

- Per well, apply 10 μL DAPI, stain for 10 minutes on ice, rinse with water, let air dry

4. Protocol for CDC Biofilm Reactor Operation

Purpose: To grow and accumulate biofilm samples on pipe material coupons using the CDC biofilm reactor.

Materials:

CDC biofilm reactor (CBR)
Pipe surface coupons (RD128-CU-A101 - ASTM designation of C10100 and RD128-PVC)
20 L Nalgene® Carboy (Fisher Scientific # 2210-005)
Masterflex® tubing (6424-18 Precision C-Flex® and Norprene® Food Tubing 6402-14)
Cole-Parmer Masterflex® L/S® digital console pump (Model 7524-50)
Millipore Millex®-FG vent filter (Fisher Scientific # SLFG05010)
Corning® PC-410D stirrer
Latex gloves
Laboratory coat
Safety glasses
95 % ethanol
10% bleach solution
3M Scotch-Brite™ pads (cat. 7448)
15% nitric acid
Small hex head screw driver
20 L synthetic water with and without humic acids at 24 mg/L (SOP)

Method:

1. Assemble the CBR as directed by the manufacturer with the 20 L Nalgene Carboy with the Masterflex® tubing (Norprene® Food Tubing 6402-14) leading through the Masterflex® peristaltic pump to the CBR influent drip.
2. Vent the reactor using the Masterflex® tubing (6424-18) and the Millipore Millex®-FG vent filter.
3. Connect the Masterflex® tubing (6424-18) to the reactor effluent.
4. Pretreat coupons by removing any large corrosion products or debris on the copper coupons by sanding in a circular motion with 3M Scotch-Brite™ pads, to remove debris and large machine marks then soaked in 15% nitric acid for 30 minutes prior to inserting into the reactor holder. PVC coupons are rinsed briefly in 10 % bleach in-between experiments to remove any organic contaminants collected in the previous run. Note: copper and PVC coupons are both used continuously and age with the experimentation.
5. Insert the coupons into the coupon holder and tighten the holder screws with the small hex head screw driver.
6. Fill the reactor to the 400 mL mark with synthetic water without humic acids, add the coupons to the coupon holders, place them in the reactor submerged in the synthetic water and autoclave the whole system minus the feed carboy at 121 ºC for 15 minutes.

7. Remove from the autoclave, allow liquid and reactor to cool then place the reactors on the Corning PC-410D stir plate and connect the tubing to the Masterflex® pump.
8. Connect the tubing to the feed carboy and begin pumping synthetic water plus humics at 1 mL/min to each reactor for 5 days at room temperature.
9. After 5 days of shear free incubation, start shear by rotating baffle at 120 ± 5 rpm by adjusting the stir plate and confirming speed with the tachometer.
10. Continuously apply synthetic water plus humic acids for an additional 9 days (a total of 2 weeks with humic acid application).
11. After 2 weeks of synthetic water plus humic acids, switch to synthetic water without humic acids and apply at the same flow rate for 4 ± 1 days at room temperature.
12. Contact and sample according to appropriate SOPs.

5. Protocol for Sampling CDC Biofilm Reactor

Purpose: To obtain biofilm samples from pipe material coupons contacted in the CBR .

Materials:
CBR grown biofilms
Latex gloves
Laboratory coat
Safety glasses
Ethanol lamp
Plate spinner
Bacterial spreaders
70 % ethanol
95 % ethanol
10% Bleach Solution
3 test tube racks
Drummond electronic pipette
1mL Pipetman® (RAININ P-1000)
200µl Pipetman® (RAININ P-200)
Aluminum foil
Small hex screw driver provided with CDC reactor to loosen coupon holder screws.
Vortex Genie
Sterile Items (autoclave at 121 ºC or purchase sterile):
10 L Sterile synthetic water (SOP KC-1)
Nutrient agar plates (R2A (VWR #EM Science 1.00416.0500) for biofilm and LB (Luria-Bertani Miller agar plates, Fisher Scientific #BP1425-500) or selective media for pathogen simulants)
Dilution buffer (PBS, 0.01% Triton™ X-100)
1.7 mL Microcentrifuge tubes
15 mL BD Falcon™ polypropylene centrifuge tubes
5 mL Serological pipettes
Aerosol barrier pipette tips - 200µl
Aerosol barrier pipette tips - 1000µl
DuPont™ Teflon® scrapers
Kelly Forceps

Method:

1. Refer to SOP JBM-1 for the procedures used to grow and maintain the simulated drinking water biofilm with the CBR..
2. Prepare dilution buffer (1 x PBS with 0.01 % Triton™ X-100) by dissolving 8 grams of sodium chloride, 0.2 grams of potassium phosphate, 1.15 grams of sodium phosphate and 0.2 grams of potassium chloride in 1 L of reverse osmosis (RO) water, test pH is at 7.4, autoclave for 15 minutes, 121 °C. Add 1 mL of 10 % Triton™ X-100 in RO water and mix.
3. Dispense 900 µL of dilution buffer into 1.7 mL microfuge tubes as needed.

4. Dispense 5 mL synthetic water into 15 mL BD Falcon™ tubes with 5 mL serological pipette.
5. Remove the coupon holder from the CBR with gloved hands.
6. Place coupon holder on a 8 by 18 inch piece of aluminum foil wiped with 95 % ethanol.
7. Using the sterile DuPont™ Teflon® scraper, scrape the surface of the first of three coupons with the scraper in a circular motion collecting the biofilm in the center of the coupon.
8. Repeat the process to make sure all biofilm is loosened in the center of the coupon.
9. Fill the 5 mL serological pipette with sterile synthetic water from one BD Falcon™ tube.
10. Ethanol and flame the small hex screwdriver then loosen the coupon holder screw and using the forceps; gently apply pressure to the coupon to pop it out of the holder.
11. Pick up the coupon with the forceps and position over the BD Falcon™ tube and position the 5 mL pipette over the coupon and dispense the liquid directly on the coupon surface, rinsing the biofilm from the surface.
12. Repeat the rinsing procedure by pipetting the biofilm laden synthetic water and dispensing it directly on the coupon surface. Gently tap any residual liquid from the coupon surface into the BD Falcon™ tube.
13. Cap the BD Falcon™ tube and vortex the tube for 30 seconds at the highest speed.
14. Dilute biofilm samples to 10^{-2} dilution in dilution buffer.
15. Place 100µl of diluted biofilm sample on the surface of a labeled nutrient agar (R2A for biofilm heterotrophic plate count) plate. With flame sterilized bacterial spreaders (soak in the bleach solution for 10 minutes and blot dry prior to ethanol if working with spores), careful spin and distribute the liquid over the surface of the nutrient agar plate. Repeat according to diluted sample number.
16. Stack spread plates, then bag and store at room temperature for 7 days for biofilm and 37 ºC for 16 hours for pathogen simulants.
17. Count all visible colonies after incubation period.

6. Protocol for Contacting Pathogen Simulant in CDC Biofilm Reactor

Purpose: To monitor the association of a pathogen simulant to synthetic drinking water system biofilms in the biofilm reactor.

Materials:
CDC Reactor (BioSurface Technolgies, Corp. Bozeman, Montana)
run according to SOP
stir plate
Latex gloves
Laboratory coat
Safety glasses
95 % ethanol
Drummond electronic pipette
1mL Pipetman® (RAININ P-1000)
200µl Pipetman® (RAININ P-200)
10% bleach solution
Tubing clamp
Sterile Items (autoclave at 121 ºC or purchase sterile):
5 mL serological pipettes
Aerosol barrier pipette tips - 200µl
Aerosol barrier pipette tips - 1000µl
200 mL Nalgene low-form polypropylene beakers
3 L synthetic water without humic acids
Nutrient agar plates
Dilution buffer (PBS, 0.01% X-100 ™)
1.7 mL microcentrifuge tubes
1 L Kimax tall form beaker
1 L Kimax tall form beaker with a stir bar
LB, Miller agar plates (Fisher Scientific #BP1425-500)
LB, Lennox broth (Fisher Scientific #BP1427-500)

Organisms:
Escherichia coli O157:H7 , nontoxigenic, biosafety level 1
Bacillus thuringiensis kurstaki, Thuricide, Bonide Products Inc. Oriskany, New York, washed as described in Protocol 9 of this appendix, " Protocol for Preparation of *Bacillus thuringiensis* spores "

Method:

1. Accumulate the synthetic drinking water system biofilm and sample any background biofilm controls needed as described in SOP JBM-1 prior to starting the pathogen contact.
2. Use the tubing clamp to clamp off the reactor effluent tube.
3. Fill the reactor beaker to the 700 mL mark with synthetic water at a flow rate of 3 mL/min.

4. Prepare dilution buffer (1 x PBS with 0.01 % Triton™ X-100) by dissolving 8 grams of sodium chloride, 0.2 grams of potassium phosphate, 1.15 grams of sodium phosphate and 0.2 grams of potassium chloride in 1 L of RO water, test pH is at 7.4, autoclave for 15 minutes, 121 °C. Add 1 mL of 10 % Triton™ X-100 in RO water and mix.
5. Dispense 900 µL of dilution buffer into 1.7 mL microfuge tubes as needed.
6. Add the appropriate amount of the pathogen simulant to the beaker to get a final concentration of $3.2 \pm 1.6 \times 10^7$ cells/mL for *E. coli* O157:H7 and $1.4 \pm 0.8 \times 10^7$ CFU/mL for *B. thuringiensis* spores.
7. Mix at 125 rpm using the CBR baffled stir bar for 30 minutes then remove 1 mL from the supernatant, dilute and plate to determine initial concentration of simulant.
8. Start the stir plate set at either a high shear (180 rpm) or low shear (60 rpm) by adjusting stir plate digital setting.
9. Serially dilute the supernatant sample by transferring 100 µL of the sample into the 900 µL of dilution buffer in a 1.7 mL microfuge tube, vortexing and repeating in the next dilution tube.
10. Spread plate the dilutions by placing 100µl of diluted sample on the surface of a labeled LB agar plate. With flame sterilized bacterial spreaders (soak in the bleach solution for 10 minutes and blot dry prior to ethanol if working with spores), careful spin and distribute the liquid over the surface of the LB agar plate. Repeat according to diluted sample number.
11. Stack spread plates, then bag and store at 37 ºC for 16 hours for pathogen simulants.
12. Contact the CBR with the pathogen simulant for 24 hours at room temperature.
13. Sample the supernatant liquid as needed by removing a desired volume of liquid from the reactor with the 5 mL serological pipette.
14. Add 150 mL of the sterile synthetic water to a 200 mL Nalgene low-form polypropylene beakers; prepare two beakers per coupon holder rod.
15. After the contact time has ceased, turn off the stir plate and remove the coupon holders, placing them directly in the first 150 mL of sterile synthetic water.
16. Transfer the coupon holder to a new Nalgene low-form polypropylene beaker of sterile synthetic water.
17. Sample the coupons as described in SOP.
18. Once the samples are collected in the 15 mL BD Falcon™ tubes, spread plate as described in steps 9 and 10 on appropriate nutrient agar. For *Bacillus* spore contacted samples, place dilution tubes at 65 ºC for 25 minutes to inactivate any vegetative cells providing an estimate of *Bacillus* spore concentration, then plate on nutrient agar.

7. Procedure for Free and Total Chlorine Determination

Purpose: To determine the amount of free and total chlorine in a sample.

Quality Assurance:

1. Run a chlorine standard with each set of measurements, prepared as described below.
2. Run a standard curve as described below every month when doing measurements or repeat if standard is different than standard curve by more than 5%.

Materials:
SwifTest Bulk Dispenser, with DPD Total Chlorine Refill (Hach #2802400)
Total chlorine refill (Hach #2105660)
SwifTest Bulk Dispenser, with DPD Free Chlorine Refill (Hach #2802300)
Free Chlorine refill (Hach #2105560)
Chlorine Standard Solution, 50-75 mg/L as Cl2, pk/16 10 mL Ampules (Hach #1426810)

15mL conical tubes
1mL cuvettes

Methods:
Free chlorine

1. Place 10 mL of sample in tube or appropriate dilution in a 10 mL volume. Add 1 addition of DPD from Swiftest.
2. Cap tube and mix tube by inversion to dissolve powder. Start a timer for 1 minute.
3. Place sample in cuvette and read absorbance at 515 nm at 1 minutes.

Total chlorine

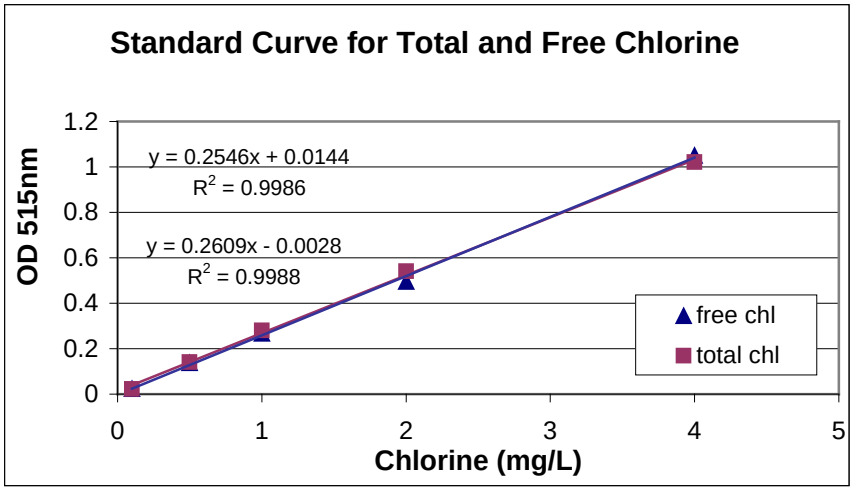
1. Place 10 mL of sample in tube or appropriate dilution in a 10 mL volume. Add 1 addition of DPD from Swiftest.
2. Cap tube and mix tube by inversion to dissolve powder. Start a timer for 3 minutes.
3. Place sample in cuvette and read absorbance at 515 nm at 3 minutes.

Preparation of Chlorine Standards by Serial Dilutions:
Should be linear from 0.02 to 2 mg/mL (maybe 4)
Stock solution is 66 mg/L.

1. Add 0.606 mL of stock to 9.394 mL of water (4 mg/mL)
2. Add 0.303 mL of stock to 9.697 mL of water (2 mg/mL)
3. Add 0.152 mL of stock to 9.848 mL of water (1 mg/mL)
4. Add 0.076 mL of stock to 9.924 mL of water (0.5 mg/mL)
5. Add 0.015 mL of stock to 9.985 mL of water (0.1 mg/mL)

Plus blank (no stock)

Example of Values from Standard Curve


Standard Curve for Total and Free Chlorine
OD 515nm
1.2
1
0.8
0.6
0.4
0.2
0
y = 0.2546x + 0.0144
R2 = 0.9986
y = 0.2609x - 0.0028
R2 = 0.9988
free chl
total chl
0
1
2
3
4
5
Chlorine (mg/L)

8. Protocol for Titration of Bacillus Spores

Purpose: To determine concentration of *Bacillus* spores.

Materials:
Bacillus anthracis
Sterile water
Dilution tubes
Luria-Bertani agar (Fisher Chemicals #BP1425-500)
Petri dishes
Class II laminar flow biosafety cabinet
0.5-0.6% Bleach, diluted 1:10
70% Ethanol
Latex gloves
Lab coat
Safety glasses
Ethanol lamp
Plate spinner
Bacterial spreaders
1mL Pipetman® (RAININ P-1000)
200µl Pipetman® (RAININ P-200)
Aerosol barrier pipet tips - 200µl (RAININ #GP-200F)
Aerosol barrier pipet tips - 1000µl (RAININ #GP-1000F)

Methods:

1. Refer to SOP for the procedures used in the biosafety level 2 lab.
2. In the hood, label dilution tubes and add appropriate volume of diluent (sterile water, etc.) for dilutions.
3. Vortex vial containing *Bacillus* spores to evenly distribute the microbe.
4. Perform serial dilutions making sure to vortex between each dilution.
5. Light the ethanol lamp.
6. Add appropriate volume of dilution to agar plates (usually between 50-200µl).
7. Dip the end of the bacterial spreader in 70% ethanol and flame the end to burn the ethanol off for sterilization.
8. Spread the dilution around the plate using the sterile bacterial spreader (and plate spinner if needed).
9. Extinguish the flame in the ethanol lamp.
10. Allow plates to dry for 10 minutes.
11. Turn plates upside down and incubate at 37°C for 16 hours or until colonies are visible.
12. Count and record the number of colonies.
13. Place agar plates in the biohazardous waste.
14. Autoclave waste when it has sufficiently accumulated using a dry cycle of 40 minutes at 15-20 psi.

9. Protocol for preparation of Bacillus thuringiensis spores

Purpose: To prepare a working stock of *Bacillus thuringiensis* spores.

Materials:
Thuricide, Bonide Products Inc. *(*contains *B. thuringiensis kurstaki* spores*)*
Sterile PBS/0.01%Triton™ X-100
Sterile water
Sterile 1.5mL microfuge tubes
Luria-Bertani agar (Fisher Chemicals #BP1425-500)
Petri dishes
Microfuge (capable of 16,000x *g*)
20% Ethanol
Sterile 50 mL conical tube
Latex gloves
Lab coat
Safety glasses
1mL Pipetman® (RAININ #P-1000)
Aerosol barrier pipet tips - 1000µl (RAININ #GP-1000F)

Methods:

1. Shake the Bonide Thuricide bottle vigorously to mix the solution.
2. Aliquot 1mL of Bonide solution into ten microfuge tubes.
3. Spin down spores in microfuge tubes for 6 minutes at 16k xg.
4. Remove the supernatant.
5. Wash the spore pellet by resuspending the pellet in 1mL of sterile water by pipetting up and down.
6. Vortex all the tubes.
7. Repeat step 3.
8. Wash the pellet once more with water (repeat steps 4-7).
9. Wash pellet twice with PBS/0.01% Triton™ X-100.
10. Resuspend pellet in 20% ethanol and pool all suspensions into a sterile 50mL conical tube.
11. Store at 4°C.
12. Quantify the spore stock.

10. Preparation of Standard Solution of Monochloramine and Monochloramine Measurement

Purpose: To prepare a concentrated solution of monochloramine and measurement of mochloramine in solutions.

Quality Assurance:

1. Run a monochloramine with each set of measurements, prepared as described below.
2. Run a standard curve as described below every month when doing measurements or repeat if standard is different than standard curve by more than 5%.

Materials:
Potassium phosphate dibasic (Sigma #P8281)
Ammonium chloride (Riedel-de Haen #11209)
Bleach (Clorox®)
Buffer Powder Pillow, pH 8.3 (Hach #89868)
Chlorine Standard Solution, 50-75 mg/L as Cl2, pk/16 10 mL ampules (Hach #1426810)
monochloramine F pillows (Hach #28022-46)
Nitrogen, Ammonia Standard Solution, 100 mg/L (Hach #2406549)
Glass beakers, volumetric flasks, balance, pipettors, stir bars

Method:
To prepare 100 mL of monochloramine solution (approximately 300-250 mg/L)

1. Add 0.5g potassium phosphate to 1L RO water in a sterile glass beaker with stir bar. Stir solution to mix. Check the pH of the solution (should be in the range of 8.9-9.2). Adjust pH if necessary.
2. Add 0.11g ammonium chloride to 100 mL of phosphate solution in a glass beaker and stir using a stir bar.
3. Prepare a 4% bleach solution.
4. In a chemical hood, add 200µl per minute of the 4% bleach solution to the ammonium chloride solution for 5 minutes (total volume added is 1mL) while the solution is mixing. NOTE: Solution may get hot - do not add more than 200µl of the bleach solution per minute. Chlorine gas is released; solution must be made in the hood.
5. Allow solution to stir for 30 minutes in the hood. If making several preparations of 100 mL of monochloramine, after the 30 minutes stirring in the hood, the solutions can all be combined at this point before determining the concentration.

Procedure

1. Prepare the monochloramine standard fresh before use.
2. Add the contents of one Buffer Powder Pillow, pH 8.3 to about 50-mL of organic-free water in a clean 100-mL Class A volumetric flask. Swirl to dissolve the powder.
3. Using a Class A volumetric pipet, transfer 2.00 mL of Nitrogen, Ammonia Standard Solution, 100 mg/L as NH_3–N into the flask.
4. Dilute to volume with organic-free water, cap and mix thoroughly. This is a 2.00 mg/L buffered ammonia standard.
5. Pipet 50.00 mL of the buffered ammonia standard into a clean 100-mL beaker. Add a stir bar.
6. Obtain a recent lot of Chlorine Solution Ampules, 50–70 mg/L, and note the actual free chlorine concentration for this lot.
7. Calculate the amount of Chlorine Solution to be added to the ammonia standard using the following equation:
example
mL chlorine solution required = 455/ (free chlorine concentration)
our stock is 66 mg/L so 455/66 = 6.89 mL

8. Open an ampoule and, using a glass Mohr pipet, add the calculated amount of Chlorine Solution slowly to the ammonia standard, while mixing at medium speed on a stir-plate.
9. Allow the monochloramine solution to mix for 1 minute after all Chlorine Solution is added.
10. Quantitatively transfer the monochloramine solution to a clean 100-mL Class A volumetric flask. Dilute to the mark with organic-free water, cap, and mix thoroughly. This is a nominal 4.5 mg/L (as Cl_2) monochloramine standard. USE STANDARD WITHIN ONE HOUR OF PREPARATION

Indophenol Method of Monochloramine Assay (HACH 10172)

1. Turn on spectrophotometer to warm up and change wavelength to 655 nm.
2. Pipette 2 mL sample (or diluted sample range up to 10 mg/L) in cuvette place in instrument and press auto zero to blank.
3. Add contents of 1 pillow of monochlor F (Hach #28022-46) and seal cuvette with Parafilm®, start timer, and mix contents for about 20 seconds to dissolve.
4. After 5 minutes place in spectrophotometer and read absorbance.
5. Construct standard curve using standard

11. Procedure for disinfection of pipe materials using the contact method

Purpose: To disinfect pipes or pipe materials that have biofilms and other biologicals associated with them using various disinfectants.

Materials:
Monochloramine solution
Chlorine solution (dilute Clorox®)
0.1M Sodium thiosulfate (Sigma #S7026)
Potassium phosphate monobasic (Mallinckrodt #7100)
Magnesium chloride hexahydrate (Fluka #63068)
250 mL plastic beakers
Synthetic water
Stainless steel forceps
0.45µm bacterial plate filter (Nalgene #145-2045)
LB agar (Fisher Chemicals #BP1425-500)
R2A agar (EM Science #1.00416.0500)
Cell scraper (Nunc™ #179693)
DuPont™ Teflon® scraper
Petri dish
70% ethanol
10% bleach
Method:

1. Prepare the dilution buffered solution (0.0425g/L KH_2PO_4, 0.405g/L $MgCl_2 \cdot 6H_2O$). Autoclave the solution.
2. Prepare the disinfectants of a certain concentration.
3. Prepare 0.0075M sodium thiosulfate.
4. Transfer the pipe/pipe material to a beaker containing 150 mL of disinfectant (either monochloramine or chlorine).
5. Allow the pipe material to contact the disinfectant for a specified amount of time.
6. Remove the pipe material using sterilized forceps (70% ethanol and flame) and place it into a new 250 mL beaker containing 150 mL of synthetic water and 0.0075 M sodium thiosulfate (this concentration will stop the chlorine at 100 mg/L concentrations of MC and free chlorine).
7. Add 11.2 mL of 0.1M sodium thiosulfate to the 150 mL of disinfectant (0.0075 M concentration of sodium thiosulfate) that the pipe material was being contacted in to stop further action of the chlorine/chloramine. Filter the 150 mL using a Nalgene 0.45µm filter and place the filter onto a LB agar plate or a R2A agar plate using sterilized forceps.
8. Sample the pipe/pipe material by scraping the surface either with a DuPont™ Teflon® scraper that has been sterilized using 70% ethanol and a flame, or by using a sterile cell scraper, and rinse the scraping into a 50 mL conical tube with 10 mL of dilution buffered solution.

12. Protocol for Preparation of Synthetic Water

Purpose: To obtain water for biofilm growth.

Materials:
RO water
Sodium bicarbonate (Sigma #S-7277)
Magnesium sulfate heptahydrate (Sigma #M5921)
Calcium sulfate dehydrate (Sigma #C3771)
Potassium phosphate, dibasic (Sigma #P8281)
Potassium phosphate, monobasic (Mallinckrodt #7100)
Ammonium sulfate (Sigma #A4418)
Sodium chloride (J.T. Baker #3624-01)
Iron (II) sulfate heptahydrate (Sigma #F7002)
Humic acid sodium salt (Aldrich #H16752)
Sodium nitrate (Sigma #S-5022)
Calcium carbonate (Sigma #C6763)

Methods:

SYNTHETIC SOLUTION PREPARATION

Preparation of Synthetic Solution—10L are prepared at a time

	Chemicals needed	**F.W.**	**Conc mM**	**mg/10L**
1	$NaHCO_3$	84.0	1.2	1000
2	$MgSO_4.7H_2O$	246.5	0.054	134
3	$CaSO_4$-$2H_2O$ (corrected from anhydrous)	172	0.2	342
4	K_2HPO_4 (dibasic)	174.2	0.004	7.0
5	KH_2PO_4 (monobasic)	136.1	0.002	3.0
6	$(NH4)_2SO_4$	132.1	.00008	0.1*
7	NaCl	58.4	.00017	0.1*
8	$FeSO_4$- $72H_2O$ (corrected from anhydrous)	278.0	.000036	0.01**
9	Humic acid sodium salt			80-240
10	$NaNO_3$	84.99	0.011	10
11	$CaCO_3$	100.1	0.2	200

*Solutions prepared at 1 mg/mL add 0.1 mL to 10 L

** Solution prepared at 1.82 mg/mL add 0.01 mL to 10 L

Add powders to 10 L water and stir. Check pH and conductivity

This synthetic water, theoretically, should yield 2.0mg/L total organic carbon (TOC). If 4.0mg/L TOC is desired, then 16mg/L humic acid sodium salt will have to be added. The addition of humic acid to the synthetic water causes a visible color to form.

Synthetic water is prepared with 20mg/L $CaCO_3$. If 100mg/L $CaCO_3$ is required, add an additional 80mg/L $CaCO_3$ to synthetic water.
Measure synthetic water for the following:

pH	7.6 – 7.8
Conductivity	+/- 10% of nominal (TBD)
Turbidity	< 0.1 NTU (prior to addition of humic acid)
Chlorine	< 0.05 ppm
TOC	+/- 10% of nominal (2mg/L or 4mg/L)

The pH adjustment is done with 1N NaOH to get 8.5 pH and 1N HCl to lower pH to 7.2.
The prepared synthetic water does not have any storage conditions, no refrigeration necessary.

Temperature: room temperature, 4^{o}C
pH: 7.2, 8.5
Hardness: 100mg/L $NaHCO_3$/20 mg/L $CaCO_3$, 100mg/L $NaHCO_3$/100mg/L $CaCO_3$
TOC: 2mg/L, 4mg/L (8mg/L humic acid =2mg/L TOC, 16mg/L humic acid=4mg/L)
Incubation period: 7 days

Formula taken from: AwwaRF 2981, Standard Operating Procedures for Decontamination of Water Infrastructure, Phase 1a Report by Mark LeChevallier and Stacey Spangler

13. Protocol for Pipe Section CBR Operation

Purpose: The operation of a bioreactor designed to grow a biofilm on the inner surface of a pipe section. The methods for contacting the pipe sections with a biological agent, sampling the inner surface, and sampling the disinfectant solution are also included.

Materials:

Safety gloves
Lab coat
Safety glasses
Pipe sections made of standard plumbing materials (typically 0.5 inch to 0.75 inch in diameter by 2 inches long)

Silicone tubing (inside diameter to be slightly smaller than outside diameter of pipe sections being used)
Plastic connectors and diameter reducers
Plastic cell scrapers (Nunc™ #179693)
R2A agar plates (EM Science #1.00416.0500)
Synthetic tap water (referrer to SOP#KC-1 for formulation)
Humic acids sodium salt (technical grade Aldrich Chemical #H16752).
Sterile dilution buffer solution (0.405 g/L $MgCl \cdot 6H_2O$, 0.0425 g/L KH_2PO_4)
Sterile dilution tubes (plastic)
Rubber stoppers
Automatic pipette devices
Plastic pipits
Peristaltic pump with silicone tubing (flow rates range of 0.5 mL/min to 2 mL/min)
Plastic 20 L carboys
Volumetric cylinders of various sizes
Stopwatch
Monochloramine solution (refer to SOP# JLA-6)
Chlorine solution (dilute Clorox®)
Sodium thiosulfate (Sigma #S7026)
Potassium phosphate monobasic (Mallinckrodt #7100)
Magnesium chloride hexahydrate (Fluka #63068)
250 mL plastic beakers
Synthetic water
Stainless steel forceps
0.45µm bacterial plate filter (Nalgene #145-2045)
LB agar Fisher Chemicals #BP1425-500)
R2A agar (EM Science #1.00416.0500)
Cell scraper (Nunc™ #179693)
DuPont™ Teflon® scraper
Petri dish
70% Ethanol
10% Bleach

Methods:

1. The synthetic tap water was prepared in large 20 L plastic carboys. The water used was for the formulation of the synthetic tap water was reverse osmosis prepared in house prepared and the resulting solutions were not sterilized. The carboys have lids with a sterile vent filter (0.22 micron) that were connected to the peristaltic pump.
2. The pipe sections were assembled by the use of 5 cm lengths of silicone tubing. The silicone tubing inner diameter should be a snug fit around the outer diameter of the pipe section. It should be sufficiently snug to prevent leakage of the solution. Pipe sections were linked in chains up to 18 sections per fluid path. Different pipe section materials can be alternated in the chain. Additional chains can be placed in parallel using another fluid paths to obtain more pipe sections for large experiments.
3. Flow rate was established by pumping synthetic water containing humic acids (24 mg/L) for a period of 14 days. The fluid paths are kept free of bubbles by slightly raising the dripping end of the pipe section chain. Flow rate was determined by collection the fluid dripping out the end of a chain into a volumetric cylinder and measuring the time with a stopwatch. Typical flow rates were 1 mL/min for 0.75 inch inside diameter pipe sections.
4. At 7 days, midway through the growth period, the pump was stopped and the pipe sections were disconnected from the pump, rubber stoppers inserted into the ends, and the chain of pipe sections were reversed. The stoppers were removed and the pipe section chains were reconnected to the pump, so that the section that was nearest the pump is now furthest away from the pump. The pump was turned back on and the growth stage continued for another 7 days.
5. At the end of the 14-day growth period using synthetic tap water with humic acids, the flow was stopped. The plastic 20 L carboy is switched to a new carboy containing only synthetic tap water and the flow started again. This period of starvation was continued for 3 days.
6. At the end of this period, the flow was stopped and pipe sections were disassembled. Pipe sections were placed in sterile synthetic tap water until used for contacting with a biological threat agent or used to determine the level of biofilm bacteria on the pipe section.
7. To contact a pipe section with a biological agent (such as bacterial spores or vegetative cells) the pipe sections were placed in beaker containing the contacting solution. The solution was gently stirred by use of a DuPont™ Teflon® coated stir bar to prevent settling of the spores or bacterial cells. After contacting the pipe section was removed from the contacting solution and gently rinsed in sterile synthetic water for five minutes with gentle agitation of the solution to wash away the unbound biological agent.
8. The contacted pipe sections can be treated with a disinfectant solution to inactivate the biological agent. The rinsed pipe section was placed into a sterile 250 mL beaker containing 150 mL of the disinfectant solution (typically chlorine or monochloramine). After the desired time, the pipe section was removed using sterilized forceps and placed into a fresh 250 mL beaker containing 150 mL of synthetic water (for chlorine and monochloramine solutions the solution contained 7.5 mM sodium thiosulfate to stop the action of the chlorine and monochloramine). The surface of the pipe section may then be sampled.

9. To sample for the presence of viable biological agents in the disinfectant solution the disinfectant must be neutralized. A solution of thiosulfate (11.2 mL of 0.1M) was added to neutralize 150 mL solutions of chlorine or monochloramine (up to 100 mg active chlorine/L). A portion or the entire volume was vacuum filtered through a sterile 0.45µm filter. The filter was then placed on top of a nutrient agar plate (LB agar for bacteria and spores or a R2A agar for biofilm bacteria) using sterilized forceps. The plates with the filters were placed into an incubator (30 °C or 37 °C) for up to 6 days and checked daily for growth of bacterial colonies.
10. The pipe sections were sampled for biofilm bacteria or biological agent by material by scraping the surface either with a sterile cell scraper. The inside of the pipe section was rinsed with 10 mL of the dilution buffer solution into a 50 mL conical tube. The tube was vigorously vortexed several times for 10 sec. Dilutions were made and the solution was spread on nutrient agar plates (LB agar for bacteria and spores or a R2A agar for biofilm bacteria) in triplicate. The plates were placed into an incubator (30 °C or 37 °C) for up to 6 days and checked daily for growth of bacterial colonies.

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