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Persistence of Non-pathogenic *Bacillus* Spores on Sewer Infrastructure and Assessment of Decontamination Using Chlorine

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Disclaimer

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The contractor role did not include establishing Agency policy.

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Acronyms

B. globigii	*Bacillus atrophaeus* subspecies *globigii*
CFU	colony forming units
cm	centimeter
DPD	N,N-diethyl-p-phenylenediamine
EPA	U.S. Environmental Protection Agency
gal	gallon
gpm	gallons per minute
HDPE	high-density polyethylene
hr	hour
L	liter
L/m	liter per minute
min	minute
mL	milliliter
MSDGC	Metropolitan Sewer District of Greater Cincinnati
PVC	polyvinyl chloride
rpm	revolutions per minute
sec	second
SETBC	Secondary Effluent Test Bed Channels
T&E	Test and Evaluation

Executive Summary

The objective of this study was to examine the persistence of *Bacillus atrophaeus* subsp. *globigii* (*B. globigii*) spores (a surrogate for pathogenic *B. anthracis* spores) on the surface materials that make up common sewer systems. To achieve this goal, the U.S. Environmental Protection Agency (EPA) has built a set of six identical pilot-scale Secondary Effluent Test Bed Channels (SETBC) at the EPA Test and Evaluation (T&E) Facility in Cincinnati, Ohio. The SETBC system consists of six 6-inch diameter polyvinyl chloride (PVC) pipes. Each pipe has been fabricated with two open grids to mount and hold infrastructure test material coupons in the effluent flow. The SETBC system has service connections that deliver a total flow of 280 gallons per minute (gpm) of unfiltered secondary effluent from the Metropolitan Sewer District of Greater Cincinnati (MSDGC) (a maximum of approximately 47 gpm per pipe).

Coupons (excised samples) of various sewer infrastructure materials were randomly placed in the SETBC and conditioned to grow biofilms for two months by exposing them to MSDGC unfiltered secondary effluent before introduction of the *B. globigii* spores. The persistence of the spores on the various infrastructure materials (high density polyethylene [HDPE], brick, rubber, concrete, iron, clay, PVC) was examined for up to 42 days. In a subsequent experiment, the efficacy of chlorine disinfection on infrastructure-adhered *B. globigii* was examined by introducing chlorine bleach into the flow. The results of the pilot-scale study are presented in this report along with SETBC structure, system operating conditions, and possible future research.

A summary of the results from the experiments conducted in the six channel SETBC system are as follows:

- The data suggest that shear forces from water flow only (no disinfection) are capable of achieving 2 to 4 log spore removal on all materials tested except for iron. Spore removal of 3.2 and 3.4 log were observed from shear forces at 14 days for HDPE and concrete. Shear forces from normal flow removed 1.9, 2.3 and 2.7 log of the adhered spores from PVC, rubber and vitrified clay at 14 days of exposure.
- Few spores adhered to brick above the background levels, and adhered spore levels dropped below the background concentration by 4 hours after spore injection
- At 42 days of exposure to secondary effluent flow, log removals of 3.7, 3.8 and >4.0 were observed for PVC, clay and rubber.
- For PVC, rubber, clay, HDPE and concrete, the number of spores detected at the end of the experiment were below the background levels. This may be due to variations in spore

adherence to coupons due to different levels of biofilm and organic matter accumulation on the coupons, variations in the secondary effluent concentrations, or variations in flow patterns in the test bed channels.

- Spores were observed on iron above background levels up to 42 days after injection. Log removal due to flow was 1.2 at day 14 and 1.5 at day 42. Persistence on iron might be due to corrosion products on the surface of the iron.
- Secondary effluent may represent a dilute raw wastewater, but it still exerted a large free chlorine demand. When enough free chlorine was added to initially achieve 10 and 25 mg/L in the secondary effluent, no chlorine was detected in the flow. When enough chlorine was added to achieve 50 mg/L in the secondary effluent, 27 mg/L was detected. This demand is due to the organic compounds in the secondary effluent and will likely be more pronounced in raw wastewater.
- Adding chlorine into the flowing wastewater to disinfect *B. globigii* spores adhered to wastewater infrastructure coupons was ineffective. Log removals of 1.5 were observed on both brick and clay after the 50 mg/L chlorine injection compared to 0.7 and 1.0 log removal for brick and clay, respectively, after 4 hours in secondary flow only (in Phase 1). All other materials had more spore removal after 4 hours in secondary effluent flow only compared to chlorination. However, it is unknown if the spores removed during chlorination were the same fraction of spores removed due to shear forces from the flow.

1.0 Introduction

1.1 Background and Project Description

The U.S. Environmental Protection Agency's (EPA's) Homeland Security Research Program (HSRP) conducts research to protect infrastructure, and to detect, respond to, and recover from terrorist attacks on the nation's water and wastewater infrastructure. The potential for biological contamination of sewer system infrastructure is one area of concern. In the event of a drinking water distribution system contamination incident involving a biological agent, contamination of the sewer system infrastructure could result from flushing of the drinking water distribution system to remove the contaminant. In addition, in the event of a biological contamination incident over a wide outdoor area or a building exterior, contamination of the sewer system could result from wash down activities or rain releasing biological agents into a sewer system. An open question is whether biological agents like pathogenic *Bacillus* spores, which are hardy and resistant to inactivation in the environment, will persist on wastewater infrastructure and if they can be removed via flushing or disinfection.

Information on adhesion of microorganisms to drinking water infrastructure and their survival in the presence of chlorine is abundant (LeChevallier *et al*. 1988; De Beer *et al*. 1994; Chu *et al*. 2003; Emtiazi *et al*. 2004; Szabo *et al*. 2007; Miller *et al*. 2015). Previous studies on drinking water infrastructure materials have noted the increased resistance of *Bacillus atrophaeus* subspecies *globigii* (*B. globigii*) to free chlorine disinfection while they are associated with biofilms (Miller *et al*. 2015). (In infrastructure studies related to biological agents, nonpathogenic *Bacillus* spp., such as *B. globigii*, are used a surrogate for pathogenic *Bacillus anthracis*.) However, information on persistence of microbiological agents and/or their survival ability on sewer pipe materials is limited. Additionally, the literature cited above focuses mostly on drinking water biofilms grown on limited numbers of materials with smooth surfaces, which do not fully represent the composition of the infrastructure materials in sewer systems.

To address the absence of data on biological contaminant persistence on sewer infrastructure, the EPA's HSRP has built six identical pilot-scale Secondary Effluent Test Bed Channels (SETBC) at the EPA Test and Evaluation (T&E) Facility in Cincinnati, Ohio. The SETBC system consists of six 6-inch* diameter polyvinyl chloride (PVC) pipes; each pipe has been fabricated with two open grids to mount and hold wastewater infrastructure test material coupons (excised samples) in the secondary effluent flow. The SETBC system is plumbed to deliver a total of 280 gallons

*Note that English units are used in this text when they are the industry standard in the U.S.

per minute (gpm) of unfiltered secondary effluent from the Metropolitan Sewer District of Greater Cincinnati (MSDGC). *B. globigii* spores were released into the flow and their persistence on wastewater infrastructure coupons was examined over time. These studies also examined the effect of adding chlorine to the wastewater flow on the persistence of *B. globigii* spores.

1.2 Project Objective

The main purpose of this pilot scale study was to evaluate the persistence of *B. globigii* on various conditioned (biofilm covered) sewer infrastructure materials in flowing, unfiltered secondary effluent, which was used as a simulant for dilute raw wastewater. The secondary objective of this study was to determine the effect of adding chlorine bleach on the persistence of *B. globigii* adhered to various sewer infrastructure materials.

2.0 Description of Experimental Protocol

This experiment had two phases. Phase 1 focused on the persistence of *B. globigii*, a surrogate for *B. anthracis* spores, on sewer infrastructure materials after conditioning (growing biofilm) the infrastructure for two months using secondary effluent. The persistence of *B. globigii* was conducted initially for three 1-inch diameter coupons representing sewer infrastructure materials (brick, concrete and high density polyethylene [HDPE]) followed by testing on four additional materials (vitrified clay [clay], iron, polyvinyl chloride [PVC] and rubber). Phase 2 focused on persistence of *B. globigii* on sewer infrastructure materials after introduction of chlorine into the wastewater flow. All seven types of infrastructure material coupons were tested together during Phase 2.

At the conclusion of an experiment, the SETBC system was drained and its wetted surfaces were disinfected with 70% methanol to eliminate residual *B. globigii* from the previous test. Similarly, used coupons were decontaminated with undiluted regular bleach (Clorox) followed by 70% methanol and tested for residual *B. globigii* prior to use in the next experiment. Additional details on the test protocol and time-line are presented later in Table 1.

2.1 Description of the SETBC Apparatus

The Test and Evaluation (T&E) Facility SETBC system consists of six 6-inch diameter PVC pipes, six secondary effluent flow control valves, six injection ports, one secondary effluent flow open/close and flow control valve, one secondary effluent drain port, six sets of flow sensors (Greyline AVFM 5.0 velocity area flow meters, Greyline Instruments Inc., Ontario, Canada)

(Greyline, 2017) connected to a flow rate display panel, two sets of pH and temperature monitoring sensors (not shown) (Hach® GLI online temperature/ORP meter; Hach Company, Loveland, CO) (Hach, 2005), a data logger (not shown) and two sections (per each pipe) of fabricated coupon-holding grids (Figure 1). Temperature and pH sensors were calibrated and maintained according to the manufacturer's instructions.

Figure 2 shows the SETBC apparatus. The top of the figure shows sections A and B where coupon-holding grids were located (also, see the labeled grids in Figure 1). The bottom portion of the figure shows the flow sensors (white sections of pipe with cords coming out). The SETBC system was directly connected to the MSDGC secondary effluent service pipe line via an effluent valve. This effluent valve allowed secondary effluent to flow into the SETBC setup. Additionally, each of the six pipes has a globe valve that can be turned to refine the flow in each pipe. These valves appear as circular handles on each pipe and are visible at the top of Figure 2. One-inch diameter coupons, prepared from various infrastructure materials, were secured to a metal rod which was mounted on the grid, allowing exposure of the coupon surfaces to flowing secondary effluent (Figure 3). Each coupon was mounted on the grid ensuring that the surface of the coupon was parallel with the water flow. During testing, the secondary effluent flow rates in each of the six SETBC pipes was adjusted, via the control valve, to 45 gallons per minute (gpm) (Figure 3).

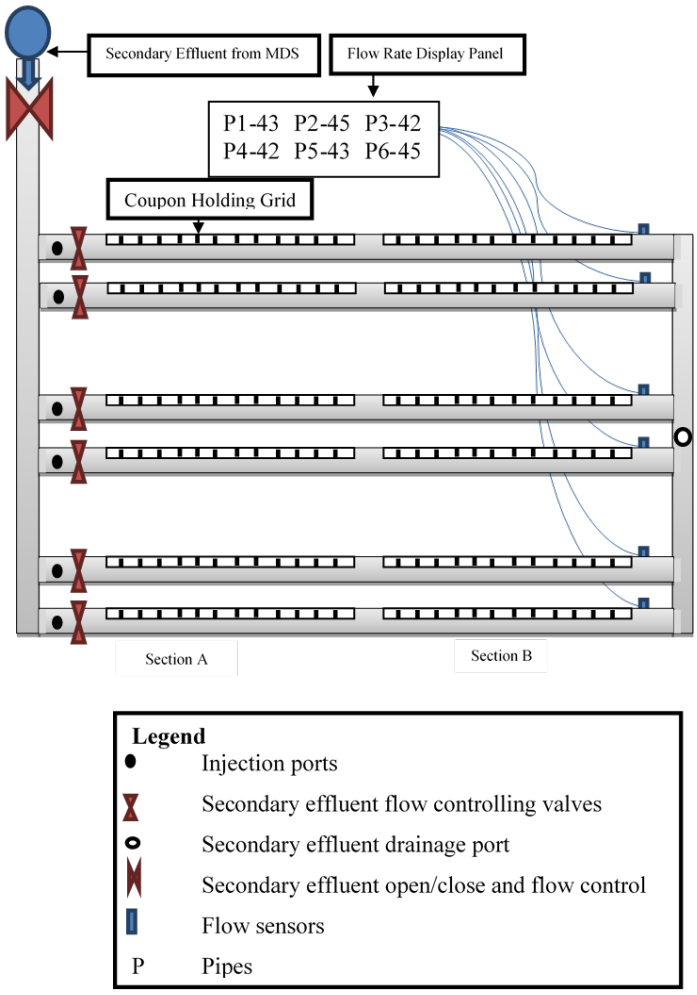

Figure 1. Schematic overview of the Secondary Effluent Test Bed Channel setup.

Figure 2. View of the Secondary Effluent Test Bed Channel system.

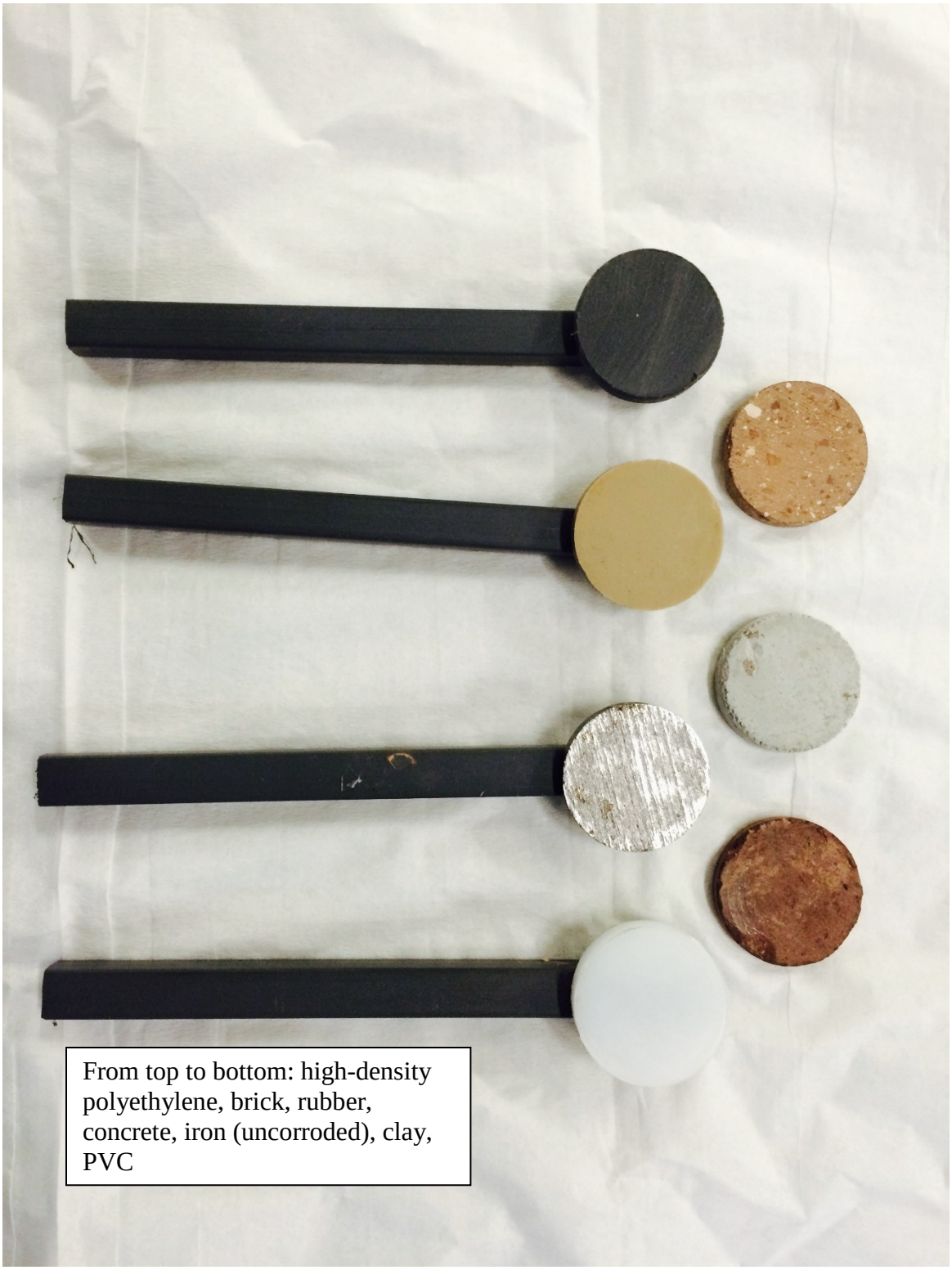

Figure 3. Sewer infrastructure coupons.

2.2 Description of *Bacillus globigii* Preparation Procedure

A stock of *B. globigii* cells was grown in generic spore medium (8 g nutrient broth, 40 mg $MnSO_4$ and 100 mg $CaCl_2$ in 1 L deionized water) for 5 days at 35°C in a shaking (145 rpm) incubator. The concentration of spores in the resulting suspension was determined by spread plating. A sub-sample of the purified spores was heat-shocked at 80° C for 10 min and analyzed to determine the stock spore concentration (Coroller et al. 2001; Szabo et al. 2007). The concentration of *B. globigii* in the injection solution was approximately 10^8 colony forming units (CFU)/mL. The *B. globigii* stock solution was injected into each SETBC pipe separately at 170 mL/min for one minute using a pre-calibrated peristaltic pump to achieve a target *B. globigii* concentration of approximately 10^5 CFU/mL.

Table 1. List of Experimental Activities

Phase and Number of Tests	Testing Date	Sampling Time	Activities
Phase 1 Test 1	May 24th 2016		The brick, concrete and HDPE infrastructure material coupons were placed in the pilot SETBC system for conditioning after setting the secondary effluent flow to each pipe of the pilot unit at 45 ± 5 gpm for the duration of the experiment. The system was operated 24 hours per day during the experiment.
	Jul. 18th 2016	0 hour	A pair of coupons from each material was collected prior to *Bacillus globigii* spiking to determine the background *B. globigii* concentration.
	Jul. 18th 2016		*Bacillus globigii* was injected for 1 minute
	Jul. 18th 2016	1 hour	A pair of coupons from each material was collected
	Jul. 18th 2016	4 hour	A pair of coupons from each material was collected
	Jul. 19th 2016	Day 1	A pair of coupons from each material was collected
	Jul. 20th 2016	Day 2	A pair of coupons from each material was collected
	Jul. 21st 2016	Day 3	A pair of coupons from each material was collected
	Jul. 22td 2016	Day 4	A pair of coupons from each material was collected
	Jul. 25th 2016	Day 7	A pair of coupons from each material was collected
	Jul. 28th 2016	Day 10	A pair of coupons from each material was collected
	Aug. 1st 2016	Day 14	A pair of coupons from each material was collected
Phase 1 Test 2	Aug. 29th 2016		The clay, iron, PVC and rubber infrastructure material coupons were placed in the pilot SETBC system for conditioning after setting the secondary effluent flow to each pipe of the pilot unit at 45 ± 5 gpm. The system was operated 24 hours per day during the experiment.
	Oct. 24th 2016	0 hour	A pair of coupons from each material was collected prior to *B. globigii* spiking to determine the background *B. globigii* concentration.
	Oct. 24th 2016		*B. globigii* was injected for 1 minute
	Oct. 24th 2016	1 hour	A pair of coupons from each material was collected
	Oct. 24th 2016	4 hour	A pair of coupons from each material was collected
	Oct. 25th 2016	Day 1	A pair of coupons from each material was collected

Phase and Number of Tests	Testing Date	Sampling Time	Activities
	Oct. 26th 2016	Day 2	A pair of coupons from each material was collected
	Oct. 27th 2016	Day 3	A pair of coupons from each material was collected
	Oct. 28th 2016	Day 4	A pair of coupons from each material was collected
	Oct. 31st 2016	Day 7	A pair of coupons from each material was collected
	Nov. 03rd 2016	Day 10	A pair of coupons from each material was collected
	Nov. 07th 2016	Day 14	A pair of coupons from each material was collected
	Nov. 14th 2016	Day 21	A pair of coupons from each material was collected
	Nov. 21st 2016	Day 28	A pair of coupons from each material was collected
	Nov. 28th 2016	Day 35	A pair of coupons from each material was collected
	Dec.05th 2016	Day 42	A pair of coupons from each material was collected
Phase 2 Test 1	Feb. 1st 2017		The brick, clay, concrete, HDPE, iron, PVC and rubber infrastructure material coupons were placed in the pilot SETBC system for conditioning after setting the secondary effluent flow to each pipe of the pilot unit at 45 ± 5 gpm for the duration of the experiment. The system was operated 24 hours per day during the experiment.
	Apr. 11th 2017	0 hour	A pair of coupons from each material was collected prior to *B. globigii* spiking to determine the background *B. globigii* concentration.
	Apr. 11th 2017		*B. globigii* was injected for 1 minute
	Apr. 11th 2017	1 hour	A pair of coupons from each material was collected
	Apr. 11th 2017	1.5 hour	Chlorine 10 mg/L was injected for 5 minutes
	Apr. 11th 2017	1.5 hour	A pair of coupons from each material was collected (after chlorine exposure)
	Apr. 11th 2017	2.0 hour	Chlorine 25 mg/L was injected for 5 minutes
	Apr. 11th 2017	2.0 hour	A pair of coupons from each material was collected (after chlorine exposure)
	Apr. 11th 2017	2.5 hour	Chlorine 50 mg/L was injected for 5 minutes
	Apr. 11th 2017	2.5 hour	A pair of coupons from each material was collected (after chlorine exposure)

gpm, gallons per minute; HDPE, high-density polyethylene; SETBC, secondary effluent test bed channels

During the Phase 1 of the experiment, 1-inch diameter coupons made from seven different infrastructure materials were placed in both Sections A and B. Coupon materials included brick, clay, concrete, HDPE, iron, PVC and rubber. Due to the nature of the Phase 2 of the experiment only Section A was used. Regardless of the phase, test material coupons were conditioned for two months in flowing MSDGC unfiltered secondary effluent. Two months of conditioning were chosen based on the time available for experimentation. The purpose of this two-month conditioning period was to grow biofilm on the coupons. At the end of two-month conditioning period, coupons from each infrastructure material were collected in pairs prior to and after *B. globigii* injection (Table 1). The specific pair of coupon sampling was based on a complete randomization technique as outlined in the Table 2.

All experiments in Phase 1 were originally planned to span 42 days. However, based on the low levels of *B. globigii* detected on the coupons, Test 1 of the Phase 1 experiments was terminated after two weeks (Table 1).

Table 2. Randomized Coupons Sample Pattern

Phase 1 of the Experiment		
Time of Coupon Collection	Section A Coupon positions	Section B Coupon positions
0 hour	23	01
1 hour	07	29
4 hour	01	07
Day 1	29	03
Day 2	09	11
Day 3	27	25
Day 4	13	19
Day 7	03	27
Day 10	21	09
Day 14	11	05
Day 21	15	17
Day 28	25	21
Day 35	05	23
Day 42	19	13
Phase 2 of the Experiment		
0 hour	04 and 07	Not required
1 hour	01 and 03	
1.5 hour	08 and 13	
2.0 hour	09 and 10	
2.5 hour	05 and 11	

2.3 Determination of *Bacillus globigii* Adhered to Infrastructure Materials

After coupons were removed from the SETBCs, their surfaces were sampled to determine the density of surface-adhered *B. globigii*. The method used to sample coupon surfaces varied depending upon the type of coupon material. Concrete, iron, brick and clay pipe coupons had their surfaces scraped with sterile scalpels until a visible layer of the surface had been removed. Surface deposits from those coupons were scraped into a sterile coliform sample bottle. The coupon surface and scalpel were then rinsed with sterile dilution buffer (Hardy Diagnostics, Springboro, OH) into the respective coliform sample bottle. Large pieces of coupon material that peeled off during surface scraping were crushed using sterile metal rods and each rod was rinsed into the respective sample bottle using sterile dilution buffer.

Each of the other infrastructure material (HDPE, PVC and rubber) coupons were sampled using sterile swabs stored in 10mL Butterfield's buffer (Copan Diagnostics, Murrieta, CA). After swabbing, each swab was shaken for 30 sec in 10 mL of Butterfield's buffer. After shaking, the content in Butterfield's buffer was added to 90mL of sterile dilution buffer in a coliform sample bottle.

Samples were then heat-shocked to remove vegetative cells from the secondary effluent, and analyzed using membrane filtration. Three different volumes (10 mL, 1.0 mL, and 0.1 mL) were filtered, in duplicate, from each sample. Filters were placed on tryptic soy agar plates and incubated at 35°C for 24 hours.

2.4 Preparation of Chlorine Solutions Using Sodium Hypochlorite

Chlorination was performed by adding three successive injections of free chlorine into the secondary effluent flow for 5 minutes each. If no chlorine demand were present, the first injection would have achieved 10 mg/L in the flow. The subsequent injections would have produced 25 and 50 mg/L in the flow. Each injection was separated by 30 min. Stock solutions of chlorine were prepared by diluting sodium hypochlorite (Clorox® bleach [The Chlorox Company, Oakland, CA]) and chlorine-free granular activated carbon filtered water. Prior to preparing the stock solutions, the bleach was diluted in granular activated carbon filtered water, and the concentration of the bleach was determined by using colorimetric DPD (N,N-diethyl-p-phenylenediamine) Hach method 8021 (Hach, 2014). In order to achieve the target in-pipe chlorine concentrations (10, 25 and 50 mg/L), 10.2 L stock solutions were prepared with varying amounts of bleach, and then metered into the flow. Table 3 shows how each chlorine solution was prepared.

Table 3. Concentrations and Volumes of Stock Chlorine Concentrations

Waste water Flow Rate (L/min)	Chlorine Application Rate (mL/min)	Chlorine Concentration in Source Bleach (mg/L)	Stock Solution Chlorine Concentration (mg/L)	Volume of Source Bleach Required (mL)	Total time of Stock application to each SETBC (min)	∗Total Stock Volume prepared (L)
170.3	170.3	101,000	10,000	1,010	5	10.2
170.3	170.3	101,000	25,000	2,525	5	10.2
170.3	170.3	101,000	50,000	5,050	5	10.2

∗Total stock volume was calculated based on 10 minute application time for 6 pipes. SETBC, Secondary Effluent Test Bed Channels

In addition to measuring the chlorine concentration of the source bleach and stock solutions, grab samples from the SETBC system were analyzed prior to and after spiking with chlorine. The measured chlorine concentration in the SETBC system prior to chlorine spiking was non-detectable. The average chlorine concentrations in the SETBC system after spiking at the 10 mg/L and 25 mg/L levels were also non-detectable. The average chlorine concentration in the SETBC after spiking at the 50 mg/L level was 27 mg/L. All the grab samples were obtained 30-45 cm from the injection port at 2.5 minutes after the chlorine injection.

3.0 Analysis of Test Results

3.1 Evaluation of *B. globigii* Adhesion to Infrastructure Materials

The data in Table 4 shows that, for each material tested, *B. globigii* spores were detected on the coupons before the injection of *B. globigii*. The spores could be coming from the secondary effluent, or from external contamination in the T&E facility. As this was an unexpected result, analysis of *B. globigii* spores did not occur during the coupon conditioning stage. The data in Table 4 also shows that *B. globigii* adhered to the coupons at concentrations ranging from 8.2×10^3 to 1.9×10^4 CFU/in^2 on all the materials except brick at one hour after the injection. There was no apparent persistence of *B. globigii* above the background levels on the brick coupons at the 4-hours after injection sample point and beyond. For the other materials, the level of *B. globigii* adhesion at 1 and 4 hours appears to be an increase beyond the background levels of adhered spores.

Based on the one-hour *B. globigii* adhesion concentration, HDPE and concrete had the highest log removal of *B. globigii* (3.2 to 3.4) within fourteen days, which is attributed to the shear force of the flowing wastewater (Table 4 and Figure 4). At day 14, the water shear force resulted in 1.2, 1.9, 2.3 and 2.7 log removal of *B. globigii* for iron, PVC, rubber and clay material coupons, respectively (Table 4 and Figure 4). The highest overall log removal of *B. globigii* was observed for clay (3.8), PVC (3.7) and rubber (>4.0) at forty-two days (Table 4 and Figure 4). However, the amount of adhered *B. globigii* changed little on the iron coupons after the first two days. Through day 42, the level of *B. globigii* detected on the iron coupons was above the background levels, and 1.5 log removal was observed. Corrosion on the surface of the iron may be a possible reason for this result.

With the exception of iron, the level of *B. globigii* detected on the coupon dropped below the background levels by day 14 or 42. In the day 28 clay sample, no spores were detected on the sampled coupons. It is difficult to explain why the level of spores dropped below the background levels. It could be that there was wide variability in the adhesion of spores to

individual coupon materials due to different amounts of adhered biofilm and organic matter. It is also possible that downstream water swirling and spiraling patterns in the wastewater flow caused adhesion variability. Also, the constant fluctuation of secondary effluent composition may also have contributed to the variability of the test results. The average pH and temperature in the unfiltered secondary effluent flow are summarized in Table 5. Note that temperature during the Phase 1 Test 1 period was considerably higher than the other two tests, although it is unclear if this influenced the experimental results. In summary, although there is variability in the results, the data suggests that most *B. globigii* spores injected into the pipes are not persistent on the coupon material over 14 or 42 days. The only exception is iron, where spores remained above background levels up to 42 days.

Table 4. Average *Bacillus globigii* Concentrations Adhered to Infrastructure Materials

Time after *B. globigii* injection	Phase 1; Average *B. globigii* Concentration (CFU/in^2)						
	Test 1			Test 2			
	Brick	Concrete	HDPE	Clay	Iron	PVC	Rubber
0 (pre-injection)	5.2E+02	1.2E+02	2.0E+02	1.3E+02	2.5E+01	2.5E+02	1.3E+02
1 hr	5.8E+02	1.2E+04	8.2E+03	1.9E+04	8.1E+03	1.2E+04	1.2E+04
4 hr	1.2E+02	7.0E+02	1.3E+03	2.0E+03	3.7E+03	2.1E+03	2.0E+03
Day 1	1.4E+02	7.4E+02	2.3E+02	5.8E+02	5.0E+02	3.5E+02	2.0E+03
Day 2	2.8E+01	1.8E+02	3.3E+02	3.4E+02	1.1E+03	6.4E+02	9.8E+02
Day 3	2.8E+01	1.3E+02	1.9E+01	1.7E+02	2.5E+02	2.1E+02	3.6E+02
Day 4	8.7E+01	4.0E+02	3.6E+01	9.8E+02	1.0E+02	7.1E+02	5.9E+02
Day 7	2.5E+02	5.0E+01	1.0E+01	1.6E+02	6.5E+02	2.6E+02	9.4E+01
Day 10	2.8E+01	5.0E+01	1.6E+01	0.0E+00	1.5E+02	1.3E+02	2.4E+01
Day 14	2.5E+02	5.0E+00	5.0E+00	3.5E+01	4.6E+02	1.6E+02	6.5E+01
Day 21				2.5E+01	3.0E+02	6.0E+01	3.5E+01
Day 28				0.0E+00	5.6E+02	3.8E+01	1.6E+02
Day 35				1.5E+01	1.6E+02	3.0E+01	2.8E+01
Day 42				2.5E+00	2.5E+02	2.5E+00	0.0E+00

HDPE, high density polyethylene

Table 5. Water Quality Measured via Online Sensors

Phase and Number of Tests	Testing Date	Average pH (pH units)	Average Temperature (°C)
Phase 1 Test 1	Jul. 18th -Aug. 01st 2016	6.9	26
Phase 1 Test 2	Oct. 24th -Dec. 01st 2016	7.0	19
Phase 2 Test 1	Apr. 11th 2017	7.2	21

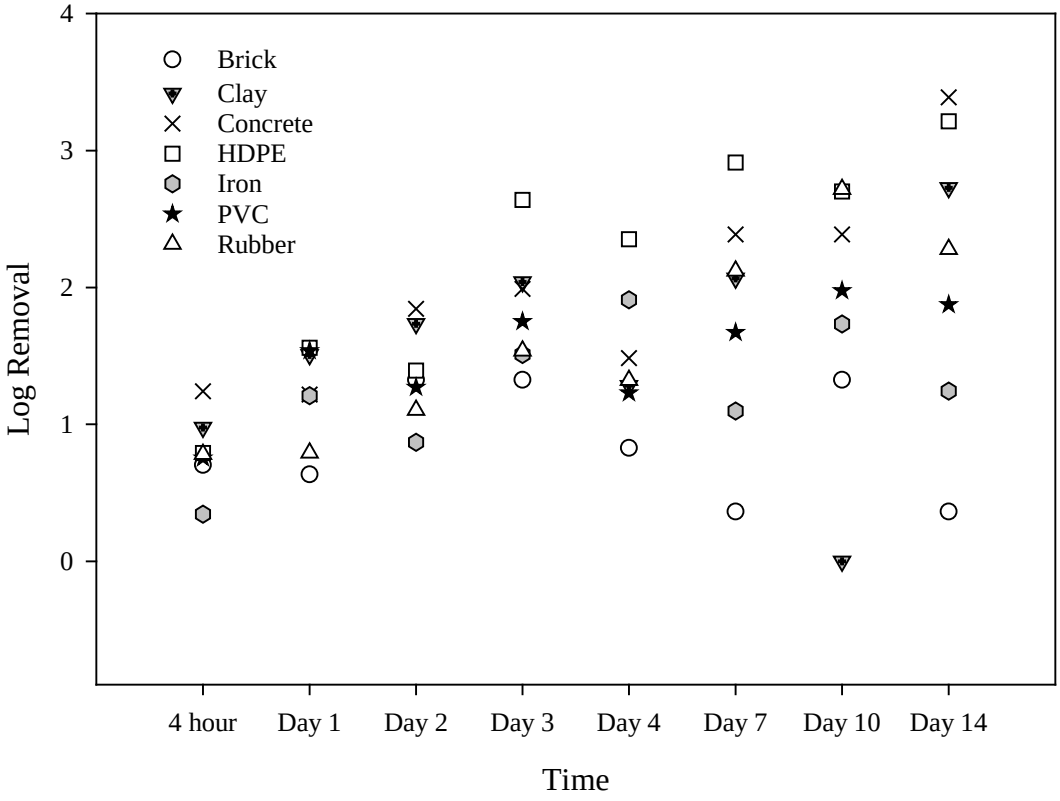

Figure 4. *Bacillus globigii* removal from infrastructure materials over time.

3.2 Impact of Chlorine on Infrastructure Material-Adhered *B. globigii*

In contrast to the Phase 1 *B. globigii* adhesion to brick (5.2×10^2 CFU/mL), Phase 2 coupons exhibited 3.2×10^4 CFU/mL adhered to the brick one hour after injection, which was similar to the *B. globigii* levels detected on clay (2.8×10^4 CFU/mL). *B. globigii* adhesion varied from 10^2 to 10^3 CFU/mL on other materials (Table 6), which was similar to Phase 1. Overall, the effectiveness of adding chlorine to decontaminate spores adhered to each of the pipe materials was either small or negligible. When chlorine was added to the flow at concentrations that would have resulted in 10 or 25 mg/L by dilution, no chlorine was detected in the flow. This is due to the chlorine demand from organic materials in the secondary effluent. When free chlorine was added at levels that would have produced 50 mg/L, only 27 mg/L was detected in the flow. It should be noted that the secondary effluent might represent a dilute raw wastewater, similar to what would occur in a combined sewer after a rain event mixed rainwater with the sewage. There will likely be more chlorine demand in undiluted raw wastewater, although wastewater characteristics vary widely. It should be noted that the authors are not aware that adding chlorine to sewers for disinfection is typical, and the levels used in this report are not based on standard

procedures used by wastewater utilities.

With respect to chlorine disinfection of adhered *B. globigii*, brick and clay were the only materials that had more *B. globigii* removal at the 50 mg/L chlorine level compared to the removal observed after 4 hours in secondary effluent flow during Phase 1. After 4 hours of flow in Phase 1, spore removals of 0.7 and 1.0 log were observed for brick and clay, respectively (Figure 4). After chlorination at the 10 mg/L, then 25 mg/L, and finally 50 mg/L level, spore removals were 1.5 log for both materials (Figure 5). However, it is unknown if the spores removed during chlorination were the same fraction of spores removed due to shear forces from the flow.

For all other materials tested, more spore removal was observed at 4 hours with secondary effluent flow only, compared to the 50 mg/L level of chlorination (Table 4 and Table 6). Even though direct comparison of data from the current secondary effluent study to past drinking water studies was difficult, previous research has reported that biofilm adhered organisms had significantly increased resistance to chlorine disinfection (Miller *et al*. 2015). It is possible that this resistance carries over to wastewater environments.

It is difficult to explain the reason for the lower log inactivation of *B. globigii* in PVC at 50 mg/L chlorine than 25 mg/L chlorine. This could again be a result of lower adhesion of *B. globigii* to some coupons due to the water flow variations at the time of *B. globigii* injection, or the composition of the secondary effluent. Additionally, variation in the accumulated organic matter on the coupons surfaces may result in variable chlorine efficacy.

Table 6. Average *Bacillus globigii* Concentrations Before and After Chlorination

Coupon Collection Time / Calculated Chlorine Concentration / Measured Chlorine Concentration	**Phase 2; Average *B. globigii* Concentration (CFU/in^2)**						
	Brick	**Concrete**	**HDPE**	**Clay**	**Iron**	**PVC**	**Rubber**
0 hr/NA/ND	0.0E+00	2.5E+01	5.3E+02	1.3E+02	9.5E+02	5.0E+00	1.8E+02
1 hr/NA/ND	3.2E+04	2.1E+03	1.9E+03	2.8E+04	2.6E+03	8.6E+02	3.9E+03
1.5 hr/10 mg/L/ND	2.3E+04	8.4E+03	5.0E+03	4.1E+03	8.0E+03	3.1E+03	2.8E+03
2.0 hr/25 mg/L/ND	2.3E+03	2.9E+03	1.2E+03	1.8E+03	1.4E+03	6.3E+02	2.2E+03
2.5 hr/50 mg/L/27 mg/L	1.0E+03	1.6E+03	5.5E+02	9.0E+02	1.4E+03	1.4E+03	1.3E+03

∗CFU, colony forming units; HDPE, high-density polyethylene; NA, not applicable; ND, non detect

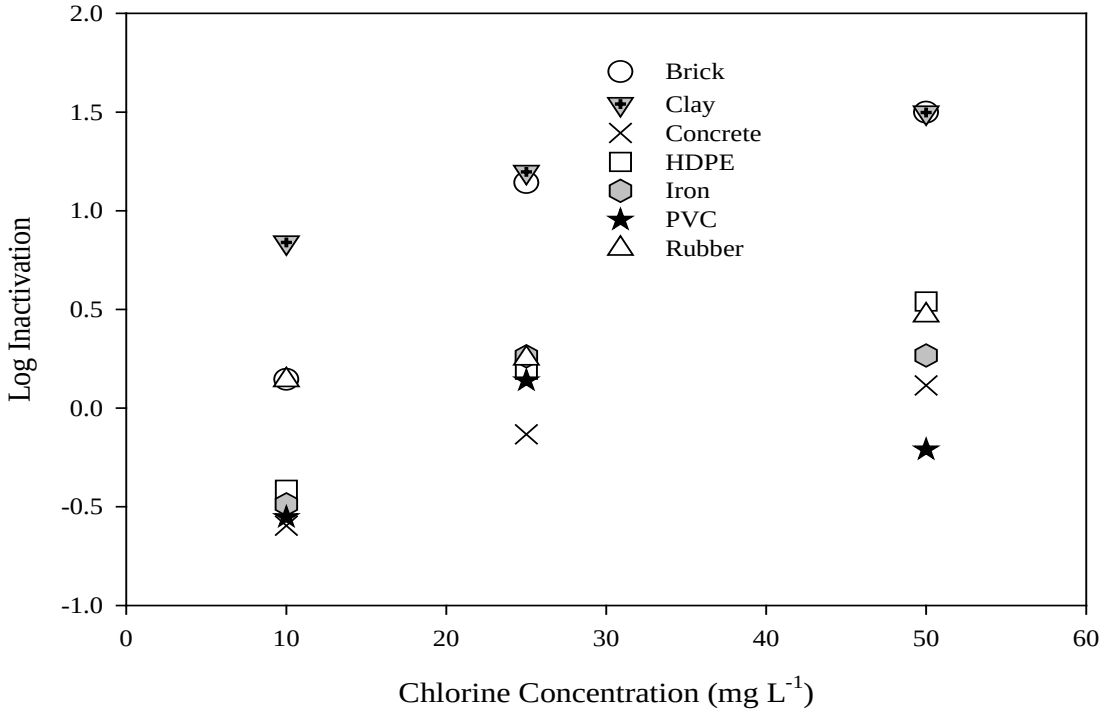

Figure 5. Impact of chlorine on *Bacillus globigii* adhered to infrastructure materials.

4.0 Conclusions

The following conclusions can be drawn from the data collected during Phase 1 of the experimentation, which observed spore persistence in the presence of continuous secondary effluent flow:

- The data suggest that shear force from water flow (45 gpm in a 6-inch diameter open channel) is capable of 2 to 4 log removal of spores on all materials tested except iron. Log removals of 3.2 and 3.4 via water flow were observed at 14 days for HDPE and concrete. Flow removed 1.9, 2.3 and 2.7 log of the adhered spores from PVC, rubber and clay, respectively, at 14 days of exposure.
- Few spores adhere to brick above the background levels, and adhered spore levels dropped below the background concentration by 4 hours after spore injection
- At 42 days of exposure to secondary effluent flow, log removals of 3.7, 3.8 and >4.0 were observed for PVC, clay and rubber.
- For PVC, rubber, clay, HDPE and concrete, the number of spores detected at the end of the experiment were below the background levels. This may be due to variations in spore adherence to coupons due to different levels of biofilm and organic matter accumulation on the coupons, variations in the secondary effluent concentration, or variations in flow

patterns in the test bed channels.

- Spores were observed to remain adhered to iron above background levels for at least 42 days after injection. Log removal due to shear forces from the flow was 1.2 at day 14 and 1.5 at day 42. Persistence on iron might be due to corrosion products on the surface of the iron.

The following conclusions can be drawn from the data collected during Phase 2 of the experimentation, which observed spore persistence in the presence of chlorine:

- Secondary effluent may represent a dilute raw wastewater, but it still exerted a large free chlorine demand. When enough free chlorine was added to achieve 10 and 25 mg/L in the secondary effluent, no chlorine was detected in the flow. When enough chlorine was added to achieve 50 mg/L in the secondary effluent, 27 mg/L was detected. This demand is due to the organic and inorganic compounds in the secondary effluent, and will likely be more pronounced in raw wastewater.
- Adding chlorine to disinfect *B. globigii* spores adhered to wastewater infrastructure coupons was ineffective. Log removals of 1.5 were observed on both brick and clay after the 50 mg/L chlorine injection compared to 0.7 and 1.0 log removal for brick and clay, respectively, after 4 hours in secondary flow only (in Phase 1). All other materials had more spore removal after 4 hours in secondary effluent flow only compared to chlorination. However, it is unknown if the spores removed during chlorination were the same fraction of spores removed due to shear forces from the flow.

Should *Bacillus* spore contamination flow into a sewer during a real contamination event, the data show that most of the spores will flow with the water. Spores that do adhere to infrastructure do so in a largely transient manner, and most are washed off of the infrastructure material in the days after the contamination event. There could be spores adhered to the infrastructure for at least 42 days on clay, PVC, rubber and especially on iron, where the spores were most persistent. It is possible that spores might persist longer, but times frames beyond 42 days were not addressed in this project. Adding chlorine to a wastewater system to decontaminate spores is largely ineffective. Future work should examine the efficacy of other disinfectants such as chloramines or peracetic acid, which may not degrade as quickly in wastewater as free chlorine. Alternatively, levels of free chlorine above 50 mg/L could also be tested, or the amount of contact time at 50 mg/L could be increased. However, users of this data must also consider that adding chlorine to wastewater in a sewer may have unintended consequences, such as formation of disinfection by products through reaction with organic matter, or trapped chlorine vapors in the collection system.

5.0 References

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