EPA
United States
Environmental Protection
Agency

EPA/600/R-15/146 | April 2016
www.epa.gov/homeland-security-research

Water Security Test Bed Experiments at the Idaho National Laboratory

Office of Research and Development
Homeland Security Research Program

April 2016
EPA/600/R-15/146

WATER SECURITY TEST BED EXPERIMENTS AT THE IDAHO NATIONAL LABORATORY

EPA Contract No. EP-C-14-012
Work Assignment No. 0-08
CB&I DN: 500204-QA-RP-000103

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Disclaimer

The U.S. Environmental Protection Agency through its Office of Research and Development funded the research described here under Interagency Agreement (IA) DW-89-92381801 with the Department of Energy and under contract EP-C-14-012 with CB&I Federal Services LLC. It has been subjected to the Agency's review and has been approved for publication. Note that approval does not signify that the contents necessarily reflect the views of the Agency. Mention of trade names, products, or services does not convey official EPA approval, endorsement, or recommendation.

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Acknowledgements

Contributions of the following organizations to the development of this document are acknowledged:

CB&I
Idaho National Laboratory

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Abbreviations

AC	Alternating Current
AWBERC	Andrew W. Breidenbach Environmental Research Center
BTEX	benzene, toluene, ethylbenzene and xylene
BWS	Bulk Water Sample
CB&I	CB&I Federal Services LLC
CFU/in^2	Colony Forming Units per square inch
CFU/mL	Colony Forming Units per milliliter
CP	Coupon
DC	Direct Current
EPA	U.S. Environmental Protection Agency
gpm	gallons per minute
HPC	Heterotrophic Plate Count
INL	Idaho National Laboratory
IP	Instrument Panel
MPN/mL	Most Probable Number per milliliter
MWS	Mobile Water System
NHSRC	National Homeland Security Research Center
PVC	Polyvinyl Chloride
T&E	Test and Evaluation
TOC	Total Organic Carbon
UV	Ultraviolet
WSC	Water Sample Concentrator
WSTB	Water Security Test Bed

Executive Summary

The EPA's National Homeland Security Research Center partnered with the Idaho National Laboratory (INL) to build the water security test bed (WSTB) at the INL facility in Idaho Falls, Idaho. The WSTB was built using an 8 inch (20 cm) diameter cement-lined drinking water pipe that had been previously taken out of service. The pipe was exhumed from the INL grounds and oriented in the shape of a 448 ft (137 m) long drinking water system (see Section 1.1 for a detailed description). Effluent from the pipe is captured in a lagoon. The WSTB can support drinking water distribution system research related to drinking water treatment including biofilms, water quality, sensors and homeland security related contaminants. Because the WSTB is constructed of real drinking water distribution system pipes, research can be conducted under conditions similar to those in a real drinking water system.

After the construction of the WSTB was completed in late September 2014, the following experiments were performed using the WSTB:

1. A dye test (tracer) to evaluate travel times and system flows
2. Dechlorination of the water in the WSTB and triggering of an automated hydrant-based flushing device
3. A contamination (using *Bacillus globigii*) and decontamination (using chlorine dioxide) test
4. Evaluation of the WaterStep mobile water system (MWS) for its ability to disinfect spores in the lagoon water that was flushed from the WSTB pipe

The following is a summary of conclusions based on the testing performed at the INL WSTB:

- The dye-testing confirmed the theoretical flow velocity and travel time of water flowing down the pipe. There was some visible mixing and dispersion/diffusion of the dye slug observed during testing.
- Sodium thiosulfate (a surrogate contaminate) removed free chlorine from the water and successfully triggered the hydrant-based flushing device. Some of the dispersed sodium thiosulfate was lodged in dead end pipes and was released with subsequent hydraulic changes.
- The test results indicated a 5 to 8 $\log_{10}$ reduction of the contaminant (*B. globigii*) in bulk water upon decontamination with an initial chlorine dioxide at 110 mg/L for 24 hours (the chlorine dioxide decayed to 35 mg/l over 7 hours). The testing results also indicate a less effective removal (~2 log reduction based on highest observed *B. globigii* density before decontamination) of the contaminant from the pipe surface. These data are different than decontamination results generated in a pilot-scale pipe system developed by EPA, where chlorine dioxide was much more effective.
- The WaterStep MWS was ineffective at treating water in the lagoon contaminated with *B. globigii* spores. However, the treatment unit was operated in manner different than its original design, which may have affected its performance.

1.0 Introduction

The EPA's National Homeland Security Research Center (NHSRC) partnered with the Idaho National Laboratory (INL) to build the water security test bed (WSTB) at the INL facility 50 miles west of Idaho Falls, Idaho. The WSTB was built using an 8 inch (20 cm) diameter cement-lined drinking water pipe that was previously taken out of service. The pipe was exhumed from the INL grounds and oriented in the shape of a small drinking water distribution system (see Section 1.1 for a detailed description). Effluent from the pipe is captured in a lagoon. The WSTB can support drinking water distribution system research on a variety of topics including biofilms, water quality, sensors and homeland security related contaminants. Because the WSTB is made of previously used drinking water distribution system pipes, research can be conducted under conditions that simulate those in a real drinking water system. This is important to note since the contamination/decontamination experiments described in this report had been previously conducted on the bench and pilot scale, and were being conducted again to verify their results in a real-world setting.

EPA/NHSRC led the experiments described in this study with technical support from CB&I Federal Services LLC (CB&I) under contract. Testing and analyses described in this report were conducted by CB&I. Under direction from EPA, CB&I personnel conducted a dechlorination-triggered flushing test, a contamination/decontamination experiment and assessed the effectiveness of a mobile water treatment device using the WSTB at INL in October 2014. *Bacillus globigii* spores, which are a surrogate for pathogenic *B. anthracis*, were used as the contaminant in the decontamination and treatment experiments. This report summarizes the data and results obtained from this testing.

1.1 WSTB Description

The WSTB consists primarily of an 8 inch (20 cm) diameter drinking water pipe oriented in the shape of a small drinking water distribution system. The WSTB contains ports for simulating water demands from service connections and a 15 ft (5 m) removable coupon (extracted samples) section designed to sample the pipe interior. Figure 1 schematically depicts the main features of the WSTB.

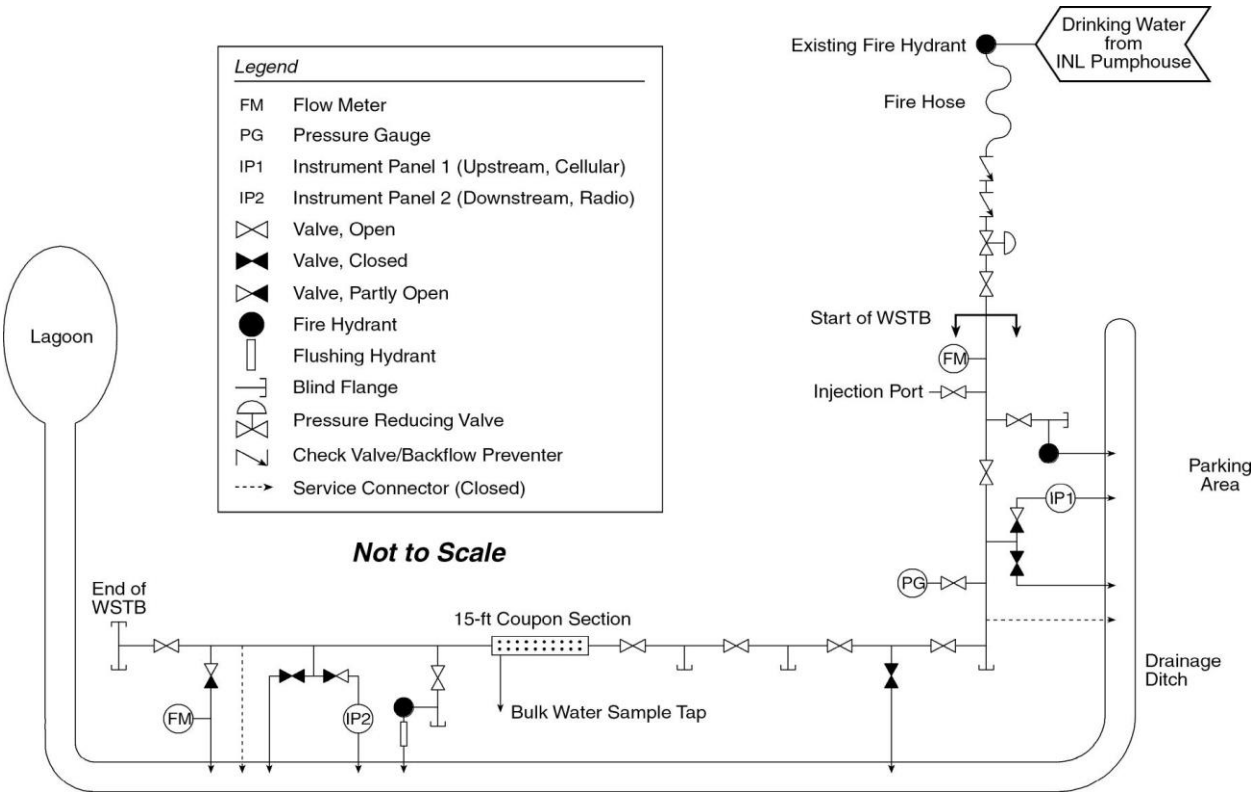

Figure 1. Schematic overview of the water security test bed.

Figure 2 shows the aerial view of the WSTB. The lower right corner shows the upstream and system inlet; the upper left corner shows the lagoon.

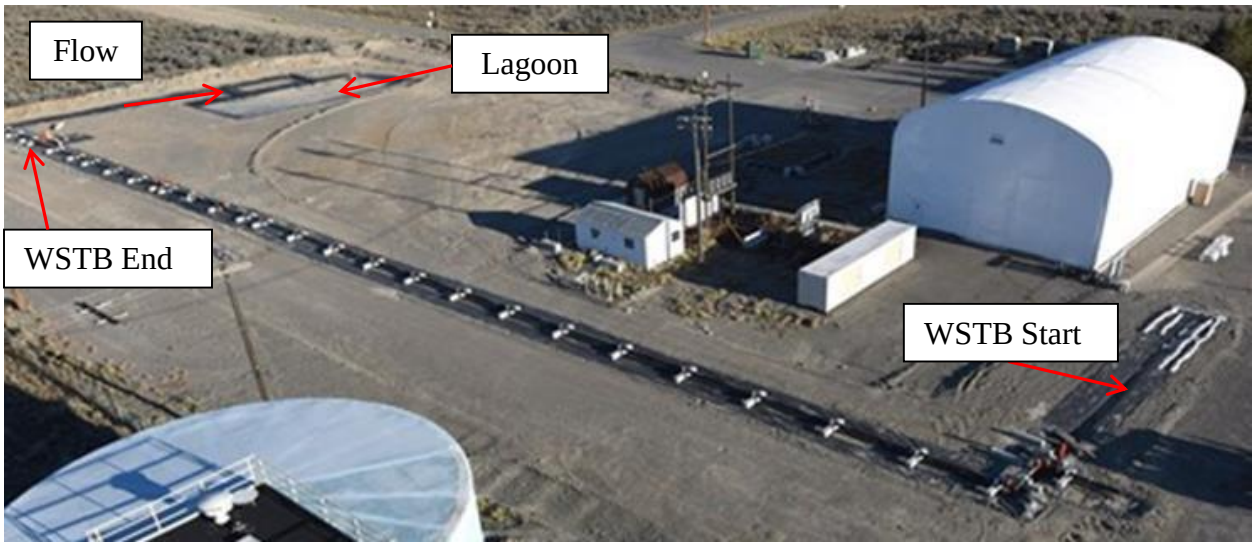

Figure 2. Aerial View of the water security test bed (WSTB).

As depicted in Figure 1, drinking water was supplied to the WSTB through an existing fire hydrant. Drinking water was chlorinated ground water that also supplied the surrounding INL facilities. The WSTB incorporates approximately 448 ft (137 m) of 8 inch (20 cm) diameter cement-lined pipe. The 8 inch (20 cm) pipe system is constructed directly over the lined drainage ditch for spill/ leak containment. The total volume of the WSTB is estimated to be ~1,150 gallons (4,353 L). A positive displacement pump was used to inject the target contaminant at the beginning (~10 ft (3 m) from the pipe start) of the 448 ft (137 m) WSTB system. Figure 3 shows the injection setup.

Figure 3. Injection setup for water security test bed.

A 15 ft (5 m) polyvinyl chloride (PVC) pipe-segment unique to this pipe system was designed and fabricated to contain 10 sets of duplicate removable coupons (totaling 20 coupons) made from the cement-lined pipe used to construct the rest of the WSTB. The coupons allow measurement of biofilm growth, contaminant persistence on pipe material, and the effectiveness of decontamination. Placement of the coupons on the top section of the pipe allowed for coupon removal without entirely draining the pipe. Figure 4 shows a portion of the coupon section.

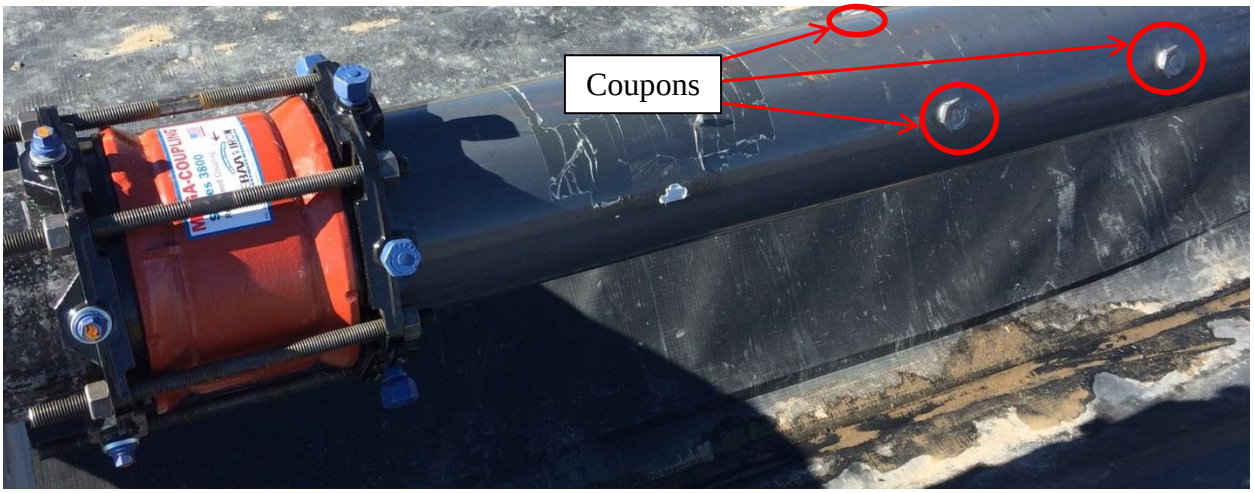

Figure 4. Removable PVC coupon section.

The pipe material for the 20 small coupons (22/32 of an inch (1.8 cm) in diameter and 0.371 square inches (2.4 square centimeters) in area) were cut from the cement mortar-lined iron pipe obtained from INL and set into threaded plugs that were inserted into the PVC-coupon section of the pipe. Figure 5 shows a picture of the threaded coupon that was inserted into the pipe main. The twenty coupons were individually numbered CP-0/CP-0D through CP-9/CP-9D in duplicate.

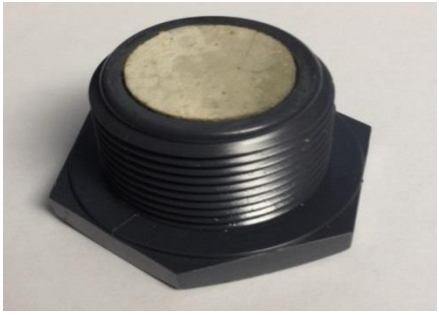

Figure 5. Pipe coupon.

Figure 6 shows a picture of the WSTB as-built (view from the upstream-end of the WSTB), with the upstream red fire hydrant and the instrument panel (IP1) in the view.

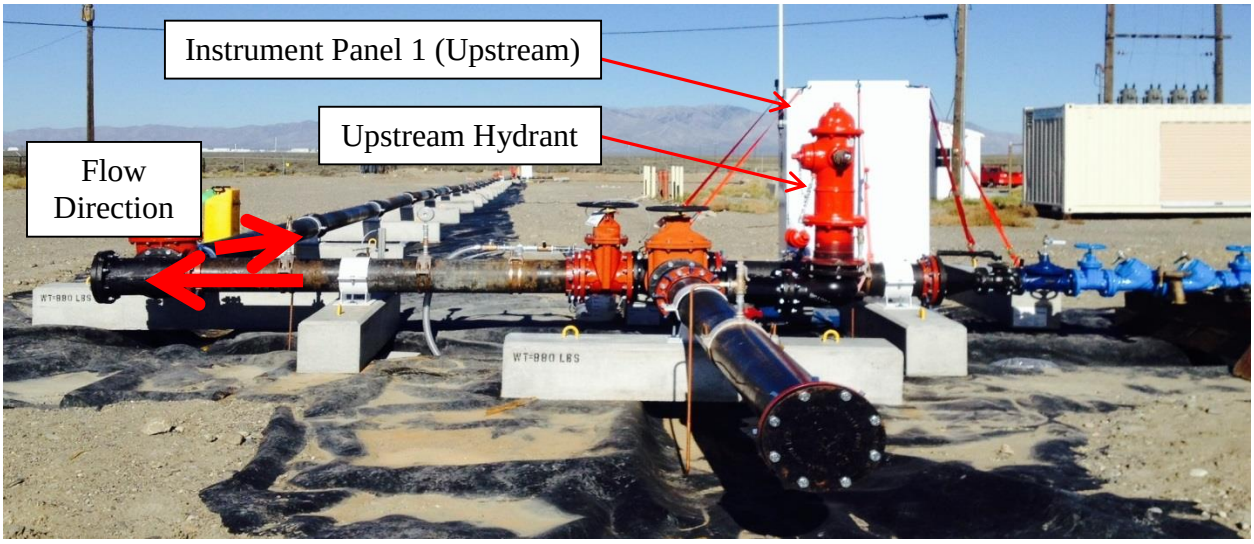

Figure 6. Water security test bed as-built (view from upstream-end).

The second yellow fire hydrant (downstream location) was installed and paired with the Hydro-Guard®[1] HG-6 hydrant-based automatic flushing system (the red box). The downstream hydrant setup and the associated instrument panel (IP2) are shown in Figure 7.

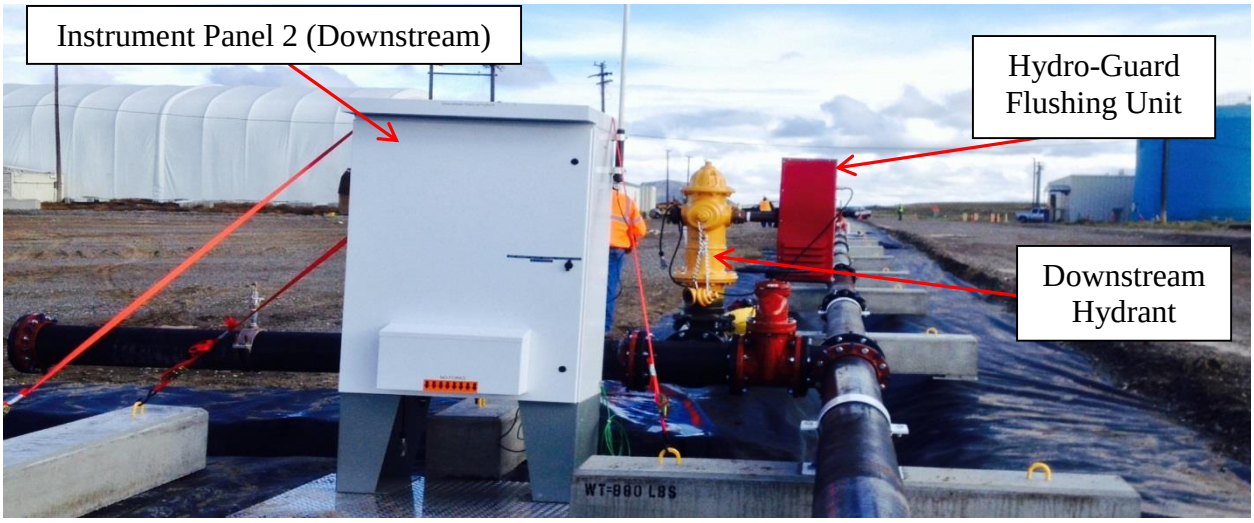

Figure 7. Flushing hydrant (view from downstream-end).

The WSTB instrumentation panels (IP1 and IP2) were equipped with sensors that continuously measure two basic water quality parameters: free chlorine and total organic carbon (TOC). One Hach® CL-17 chlorine analyzer (Hach Co., Loveland, CO) and one RealTech M4000 TOC analyzer (Real Tech Inc., Whitby, ON, Canada) were included in each of the instrumentation panels. The Hach CL-17 chlorine analyzer uses colorimetric DPD chemistry to monitor water continuously for free chlorine. The RealTech M4000 uses the ultraviolet (UV) 254 nanometer

[1] Mueller Co., 633 Chestnut Street, Suite 1200, Chattanooga, TN, USA

wavelength (i.e., UV254) for determining the TOC content. UV254 instruments are often used as an inexpensive indicator of TOC in water. UV254 measurements are known to have some bias towards aromatic organics; however, they are relatively inexpensive to maintain and to operate when compared to the traditional UV-persulfate based TOC analyzers. Figure 8 depicts the inside of one of the instrument panels.

Figure 8. Instrument panel water quality analyzers (inside view).

When active experimentation was not taking place, the WSTB system was operated at a low flow rate of around 2.5 gallons per minute (gpm) (10 L/min), resulting in a total of 25,200 gallons (95,382 L) discharged per week into the lagoon. The valve near the end of WSTB along with the flow meter (shown in Figure 9) was used to regulate and maintain flow.

Figure 9. Water security test bed system flow regulator.

During experiments, the system was operated at a higher flow rate (~15 gpm or 57 L/min)) to reduce travel time and manage sampling activities. The flow had to be regulated because of the limited discharge capacity of the lagoon. The lagoon (Figure 10) has a water storage capacity of 28,000 gallons (105,980 L). The water and any contaminant or decontaminating agent used during experimentation were conveyed via the drainage ditch and discharged to the lagoon. The discharged water was trucked out for disposal on an as-needed basis.

Figure 10. Water security test bed discharge lagoon.

2.0 Description of Experiments

2.1 Dye Test

Prior to contamination/decontamination tests, a simple dye tracer study (using non-toxic biodegradable dye from Bright Dyes – www.brightdyes.com) was performed to visually confirm the theoretical calculations of tracer travel times and system flows. This dye testing was performed on October 1, 2014 at 9:00 AM. A 5-minute injection of red dye was performed. The dye mixture was prepared by dissolving 3 tablets of the red-colored Bright Dye in 2 gallons (8 L) of water using the injection setup (previously shown in Figure 3). The dye mixture injection rate was 1,500 mL/min. At 10:12 AM, the dye was visibly noticed at the outlet flow control rotameter. The theoretical calculated travel time (based on plug flow travel, total pipe length of 429 ft (131m) from the injection port to end, and a flow rate of 15 gpm (57 L/min)) is 1 hour and 15 minutes. The exit flow rotameter is located slightly upstream of the end-of-pipe and the actual travel time was 1 hour and 12 minutes. The testing confirmed the theoretical flow velocity and travel time calculations. As expected, there was some visible mixing, dispersion and diffusion of the dye slug observed during testing because the dye color was visible for a period of 10 minutes in the flow meter, whereas the injection only lasted 5 minutes. Figure 11 shows the injected dye mix (left) and diluted dye exiting the WSTB system flow regulator (right).

Figure 11. Injected dye mix (left) and diluted dye exiting the water security test bed system flow regulator (right).

2.2 Dechlorination and Triggered Flushing

The purpose of the dechlorination and flushing experiments was to demonstrate the feasibility of using online water quality sensors in concert with an automated flushing hydrant to intelligently divert and remove contaminants from water distribution systems. Sodium thiosulfate was used to dechlorinate the drinking water in the WSTB pipe, and the resulting loss of chlorine level was used

to trip the automated flushing device. Ten grams of thiosulfate was mixed with 2 gallons (8 L) of water for a 5-minute injection (similar to the dye tracer study). The mixture was injected into the WSTB on October 1, 2014, at 12:48 PM for 5 minutes. Figure 12 shows the online chlorine data (mg/L) over time from the upstream and downstream instrument locations.

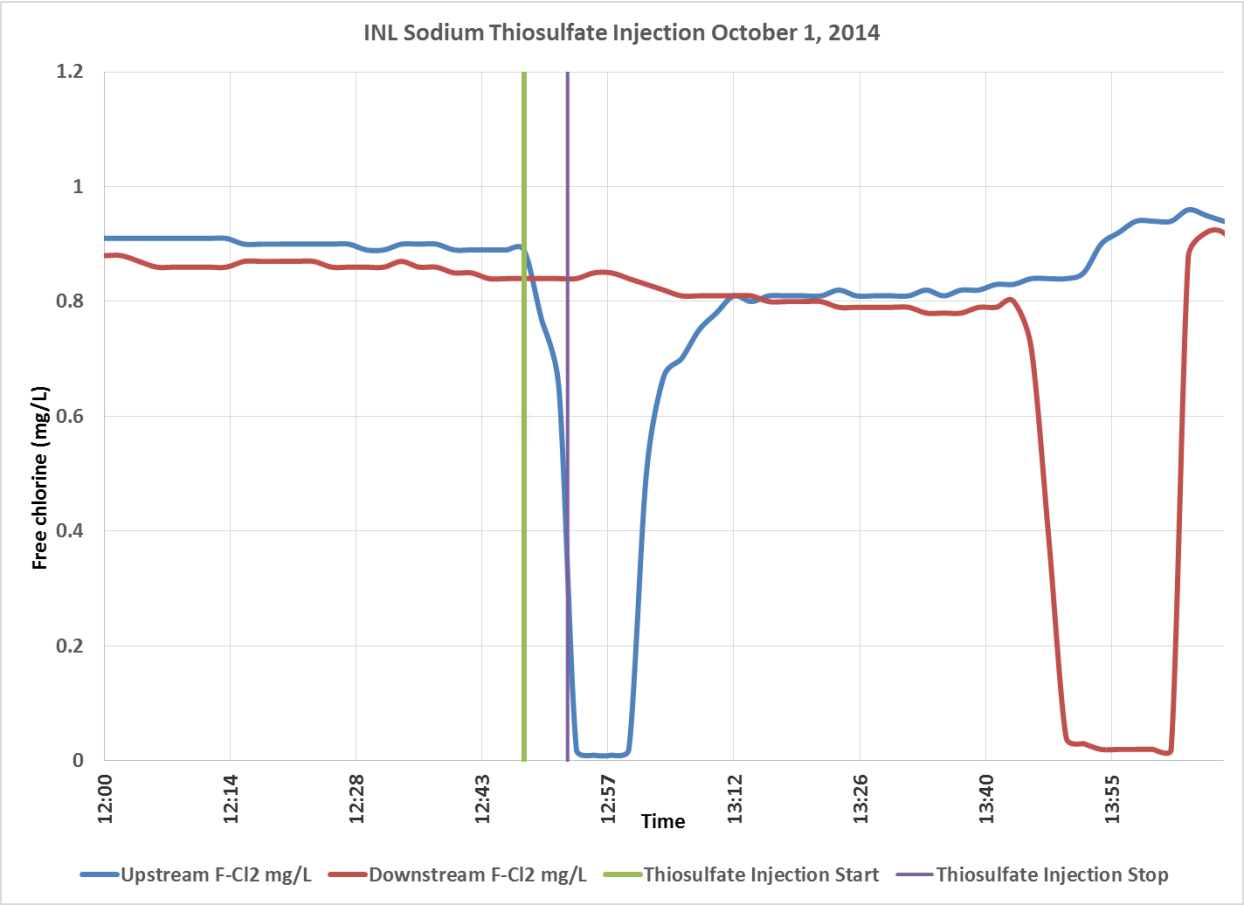

Figure 12. Online monitoring of free chlorine (denoted F-Cl_2 in mg/L) during testing.

Based on the data, the slug appears to reach the downstream instrument panel in 1 hour and 2 minutes. The shape of the response on the downstream chlorine monitor shows that the 5-minute injected slug dispersed to a 12-minute slug by the time it reached the downstream location. From the chlorine level signals, it is clear that they can be used to trigger a flushing device.

Figure 13 shows the flushing hydrant in operation during the testing. The signal from the downstream Hach CL-17 chlorine monitor was used to trigger the flushing hydrant to open and flush. When the chlorine level measured by the Cl-17 dropped below 0.05 mg/L, the flushing hydrant valve triggered open (Figure 14). After the chlorine value recovers (i.e., > 0.05 mg/L), the flushing valve was set to automatically trigger to a closed position.

Figure 13. Flushing hydrant in operation.

The flushing hydrant tripped open at 13:50 and stayed open till 14:05. The grab sample collected at 13:50 from the downstream system flow regulator confirmed that the chlorine level had dropped below the trigger point of 0.05 mg/L. Figure 14 shows the downstream chlorine levels in conjunction with the automated flushing operation. The 15-minute flushing hydrant operation period corresponds to the 12-minute spread of the slug (based on the dye-tracer study) and a 2.5 to 3.5 minute delay between sensor measurement and the triggered response. The data plot indicates that the automated flushing hydrant operated as designed.

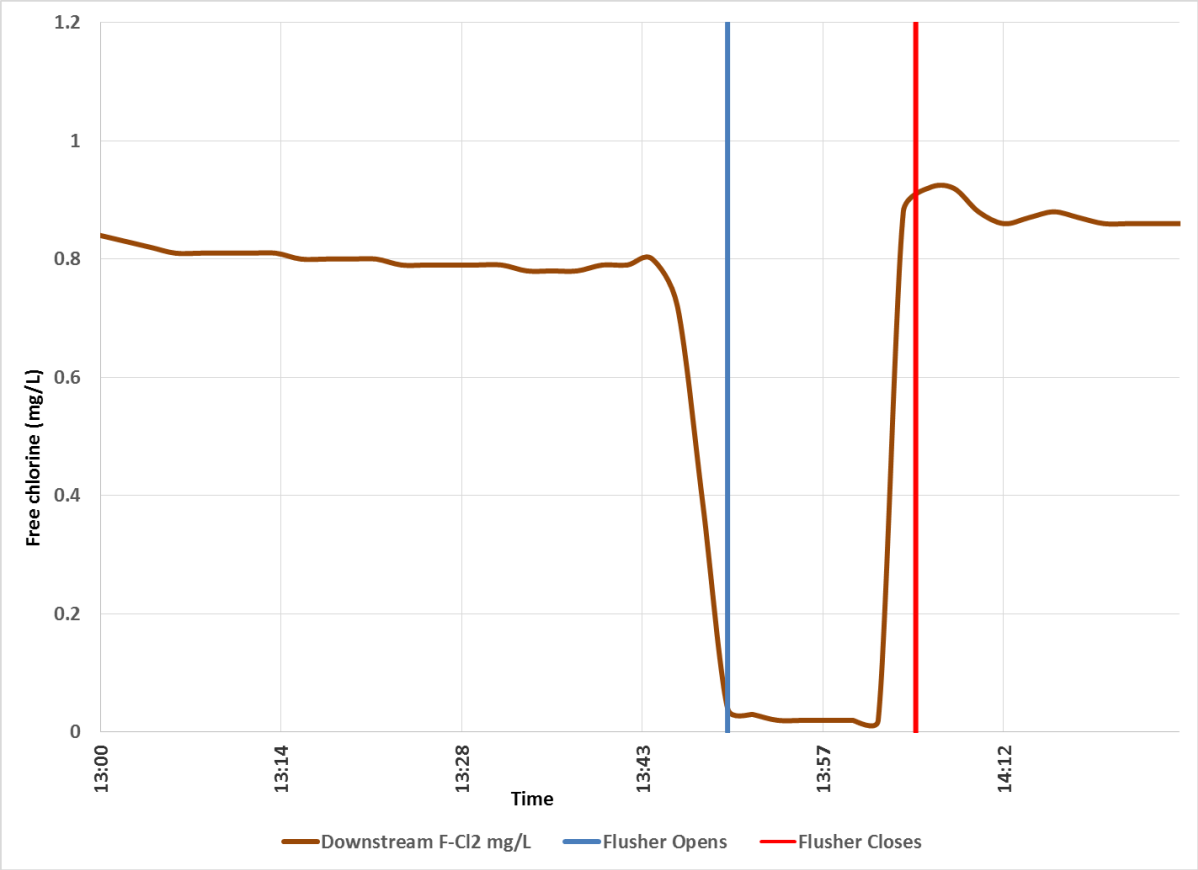

Figure 14. Downstream free chlorine free chlorine (denoted F-Cl_2 in mg/L) response and flushing hydrant operation.

Figure 15 shows the real time instrumentation from both TOC (as measured by UV absorbance) and chlorine instruments at the downstream location. The data show the first dip at 13:50 that caused the flusher to open and a second dip in chlorine level at 14:42. The chlorine level dips are also matched by a peak in RealTech UV TOC response at the downstream location. The peak in TOC is likely due to absorbance exhibited by thiosulfate in the UV 254 wavelength. At 14:40, the system flow was stopped (near the downstream location) for several minutes to collect a coupon sample. This change in hydraulic condition appears to have caused a release of some residual thiosulfate that remained in the system (possibly at pipe dead ends) and was not flushed out during the automated flushing operations.

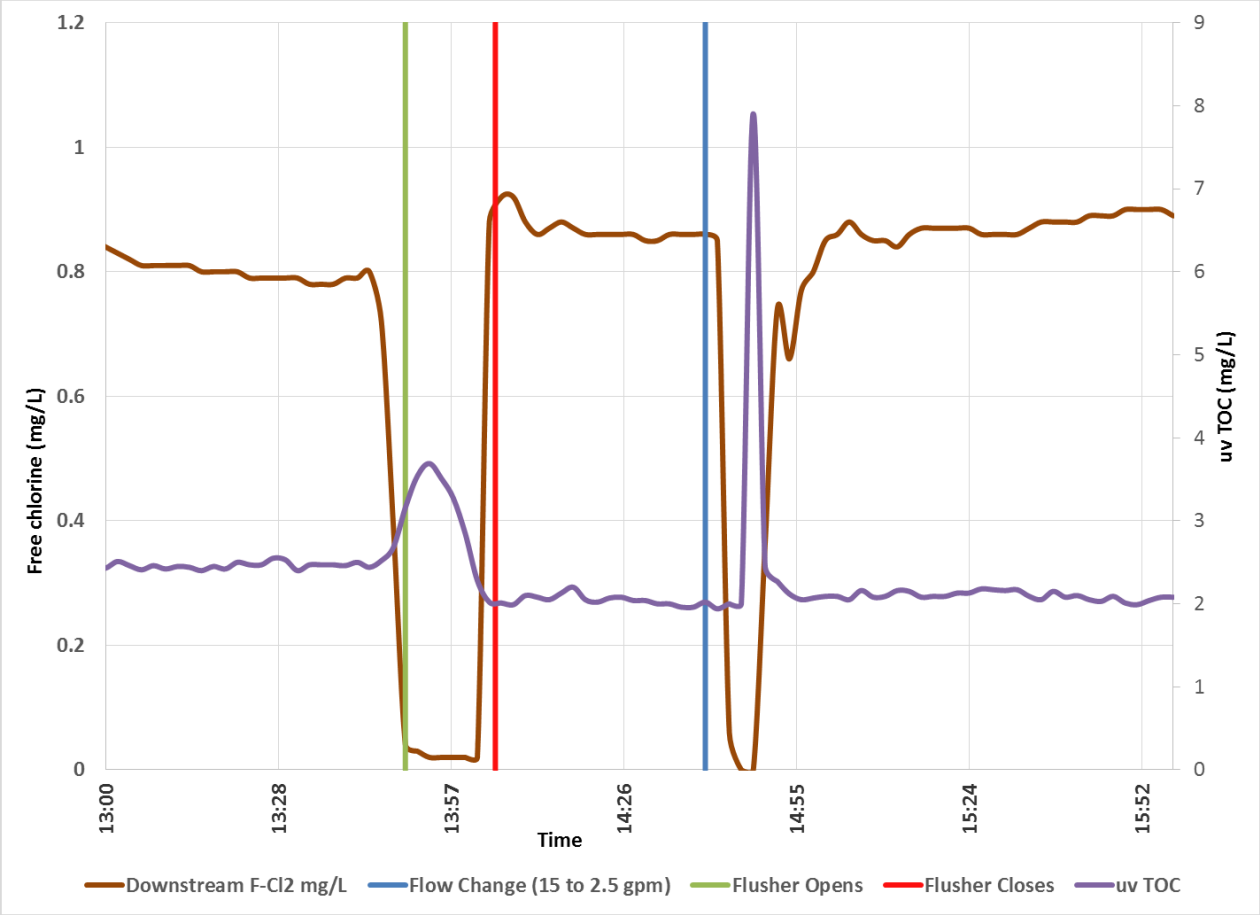

Figure 15. Free chlorine (denoted F-Cl_2 in mg/L) response and UV-determined total organic carbon (TOC) to change in hydraulic condition (downstream).

A dip in chlorine similar to the downstream sensor was noted in the upstream location (Figure 16) when the flusher was opened and closed. The change in hydraulic condition appears to have caused a release of some residual thiosulfate that remained in the system (possibly at dead ends).

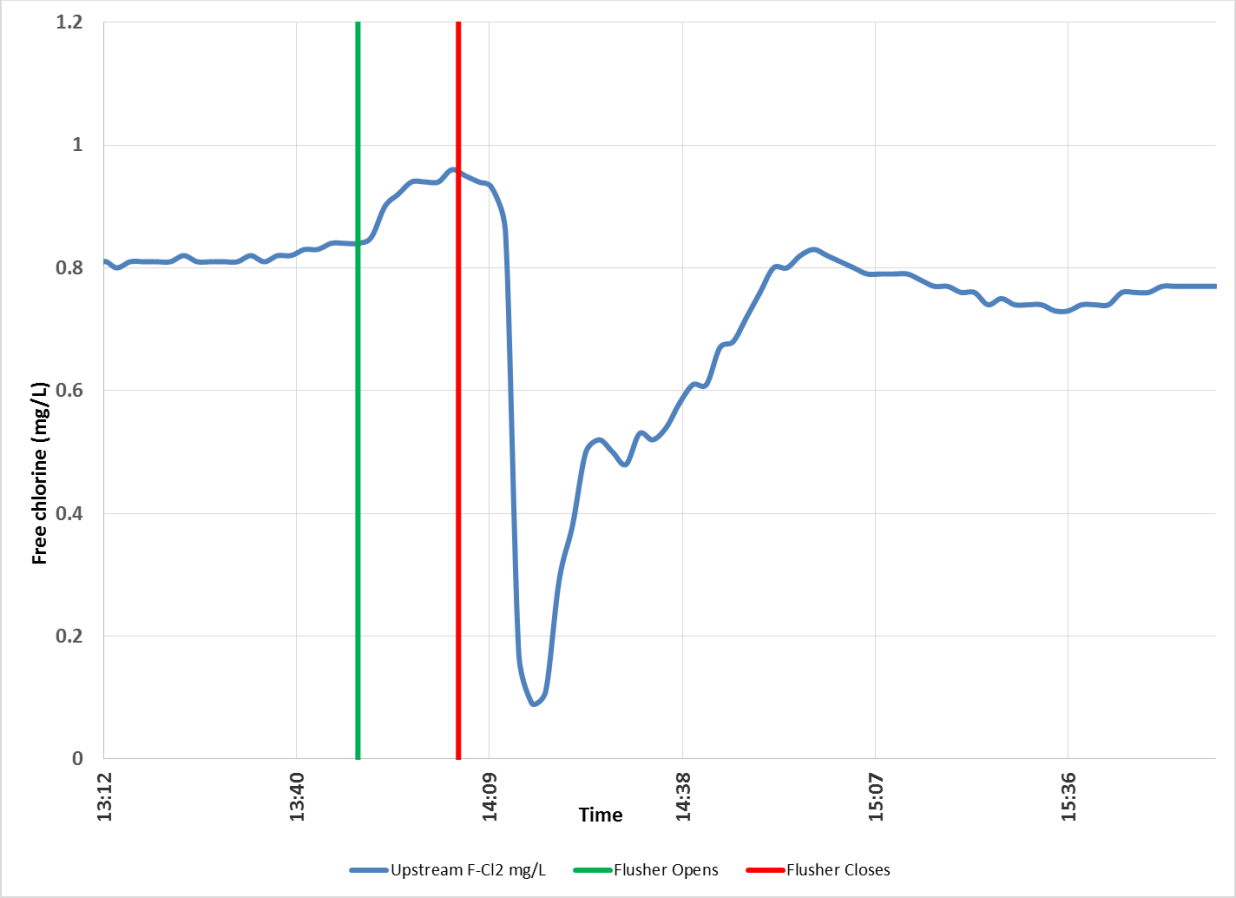

Figure 16. Free chlorine (denoted F-Cl_2 in mg/L) response to change in hydraulic condition (upstream).

These data appear to indicate that while automated flushing operations remove the bulk of the injected test contaminants, some of the dispersed contaminants lodged in the dead-ends are released later on when the system hydraulic conditions change. Therefore, any automated flushing operations should take into account any dead-end zones trapping the dispersed/diffused contaminants that may be re-suspended due to hydraulic changes in the system. Also a stop and restart protocol during the flushing operation warrants further consideration and testing.

2.3 Contamination/Decontamination Tests

These experiments involved contamination of the WSTB using *B. globigii* spores and the subsequent decontamination using chlorine dioxide as the decontaminating agent. The contamination/decontamination experiment consisted of the following main steps:

1. Injection of contaminant (addition of *B. globigii* spores to WSTB)
2. Decontamination (addition of chlorine dioxide for a specified contact time)
3. Post-decontamination flushing and monitoring

On October 6, 2014, at 8:15 AM prior to contamination/decontamination testing, the online instrumentation data was reviewed for the presence of stable free chlorine concentration recorded

as 0.69 mg/L at the upstream instrument panel location and as 0.5 mg/L at the downstream instrument panel location of the WSTB. The stable value served as an indication of stabilization of pipe wall chlorine demand. Biofilm formation was confirmed by collecting baseline bulk water sample (BWS-0) and duplicate coupon (CP-0/CP-0D) samples. The water and coupon samples were analyzed in the laboratory for the presence of heterotrophic plate count (HPC). In addition, BWS-B was collected during the sampling event as a quality assurance check. The BWS-B sample consisted of a sterile buffer bottle that was opened and placed in the vicinity of the coupon and bulk water sampling area. Any contamination that was in the air or aerosolized by bulk water or coupon sampling would be collected by BWS-B.

The coupon sampling event was performed by isolating the coupon section of the main pipe by closing the upstream flanking gate valve (previously shown in Figure 1), removing the duplicate coupon plugs (CP-0 and CP-0D) and replacing the coupon plugs with blank plugs. The detailed sampling procedures, sample containers, sample preservation, sample labeling, sample shipping and analytical methods are described in Appendix A. The specific sampling activities are described in Table 1.

Table 1. Background Sampling Activity

Sample ID	Sample Description	Date/Time and System Flow
BWS-0 (Control)	Bulk water sample collected from the pipe prior to injection of *B. globigii*	October 6, 2014 – 8:50 AM, Flow at 2.5 gpm (10 L/min)
BWS-B (Background)	Background control collected at the same time as BWS-0	October 6, 2014 – 8:50 AM, Flow at 2.5 gpm (10 L/min)
CP-0 and CP-0D	Coupon sample collected at the same time as BWS-0	October 6, 2014 – 8:50 AM, Flow at 2.5 gpm (10 L/min)

CP, coupon; BWS, bulk water sample; gpm, gallons per minute; L/min, liters per minute; 0, sample taken before injection; B, background sample (an open bottle of sterile water sitting beside the pipe to detect any aerosolized BG); D, duplicate

The resulting HPC values from the background and baseline samples collected are reported as most probable number per milliliter (MPN/mL) in Table 2. The same background samples were also analyzed for the absence of *B. globigii* spores which are reported separately along with other experimental data. The CP-0 and CP-OD values indicate that a biofilm was established on the pipe section prior to the contaminant injection.

Table 2. Background Heterotrophic Plate Count (HPC) Data

Sample ID	HPC Counts
BWS-0	299 MPN/mL
BWS-B	0 MPN/mL
CP-0	6.87E+03 MPN/in^2
CP-0D	5.12E+03 MPN/in^2

BWS, bulk water sample; CP, coupon; MPN, most probable number; 0, sample taken before injection; B, background sample (an open bottle of sterile water sitting beside the pipe to detect any aerosolized BG); D, duplicate; Note: to determine the microbial density on the coupons in MPN/cm^2, divide by 6.45.

2.3.1 Injection of Contaminant (addition of B. globigii spores to WSTB)

Offsite Preparation of the Contaminant Stock - The *B. globigii* spores for this study were obtained from NHSRC's laboratory at the Andrew W. Breidenbach Environmental Research Center (AWBERC) in Cincinnati, Ohio. A culture of *B. globigii* vegetative cells were mixed with generic sporulation media and incubated by gentle shaking at 35°C for 7 days at the EPA Test and Evaluation (T&E) Facility in Cincinnati, OH. The concentration of *B. globigii* stock was determined following the method described by Rice *et al*. (1994). The *B. globigii* stock was heat-shocked to remove any remaining vegetative cells and analyzed using the spread plate method and membrane filtration as described in Appendix A. Thirty-eight liters of the spore stock was prepared over several weeks and shipped in individual one-liter containers (preserved at 4±2°C) to the site.

Onsite Preparation and Injection of the Contaminant Stock - On October 6, 2014, the above referenced *B. globigii* stock suspensions were mixed in three five gallon (19 L) buckets and prepared for injection. At 10:00 AM, the mix was introduced into the WSTB using a positive displacement pump (injection setup shown previously in Figure 3) to achieve a target bulk water concentration between 10^5 and 10^6 Colony Forming Units/milliliter (CFU/mL) in the pipe. During the injection period, the WSTB was operated at 15 gpm (57 L/min) to allow for a minimum contact time of approximately 1 hour (and to accommodate for travel time through the system). The injection was stopped at 11:01 AM so that there was at least 1 hour of contact time and to ensure the bolus of *B. globigii* suspension had reached past the coupon section of the pipe.

Sample Collection – Bulk Water Samples (BWS) and coupon (CP) samples were collected at 11:10 AM, which allowed for the initial pass-through of the contaminant through the coupon section. Coupon samples (CP-1, CP-1D, CP-2 and CP-2D) were extracted (scraped) immediately after removal as described in Appendix A (Specific Sampling Procedures). Another BWS/CP sampling event was performed at 11:30 AM. During this sampling period, one sample was collected and analyzed onsite to confirm that chlorine dioxide was not detectable in the background. Chlorine dioxide was measured onsite using the Hach DR/890 pocket colorimeter as described in Appendix A (Specific Sampling Procedures). The ClO_2-0 sample was measured as 0 mg/L which confirmed the absence of chlorine dioxide in the WSTB prior to the introduction of chlorine dioxide in the decontamination step. The contamination step-related sampling activities are summarized in Table 3.

Table 3. Contamination Sampling Activity

Sample IDs	Sample Description	Date/Time and System Flow
BWS-1, CP-1 and CP-1D	Collected after the injection of *B. globigii* reaches the coupon section and prior to the introduction of chlorine dioxide	October 6, 2014, 11:10 AM Flow at 15 gpm (57 L/min)
BWS-2, BWS-B2, CP-2, CP-2D, and ClO_2-0	Collected after the injection of *B. globigii* and prior to the introduction of chlorine dioxide	October 6, 2014, 11:30 AM Flow at 15 gpm (57 L/min)

BWS, bulk water sample; CP, coupon; gpm, gallons per minute; L/min, liters per minute; B, background sample (an open bottle of sterile water sitting beside the pipe to detect any aerosolized BG); D, duplicate; ClO2, chlorine dioxide sample; 1, 2, etc., sequential sample number

2.3.2 Decontamination (addition of chlorine dioxide for a specified contact time)

Preparation of Decontaminant Agent Stock – Prior to injection, chlorine dioxide decontaminant stock solution was prepared onsite at INL using the GO2™ kit. Components A and B from the GO2™ (GO2 International, 6700 Caballero Blvd., Buena Park, CA 90620, USA) kit were mixed in 25 L of tap water (~0.5 mg/L free chlorine) in a covered carboy in an outside ventilated area. The stock concentration yield was expected to be around 4,000 mg/L and the targeted in-pipe concentration of chlorine dioxide was a minimum of 25 mg/L (based on previous pilot-scale testing performed at the EPA Test & Evaluation Facility in Cincinnati, Ohio). The stock concentration can be variable depending upon the water temperature and reaction time of the two kit components.

Decontamination Test Protocol – Prior to injection of the decontaminant, the flow to instrument panels was cut-off to protect the instruments from the potentially detrimental effect of chlorine dioxide. Subsequently, on October 6, 2014 at 12:00 PM, the chlorine dioxide stock solution (as prepared above) was injected into the WSTB to achieve a minimum target in-pipe bulk water chlorine dioxide concentration of 25 mg/L. At 12:45 PM, a chlorine dioxide sample was collected from the pipe (first air release valve near inlet) and reported a value of 150 mg/L; another sample collected at 12:57 PM at a downstream location reported a value of 105 mg/L. At 12:57 PM, the chlorine dioxide injection was completed. All of the injectable prepared stock was used, except for a small amount in the bottom of the 25-L carboy. At 1:12 PM, the flow through the WSTB was stopped to hold the decontaminant in the pipe for the next 24 hours. The sampling activities for the decontamination step are summarized in Table 4.

Table 4. Decontamination Sampling Activity

Sample ID	Sample Description	Date/Time and System Flow
BWS-3, CP-3, CP-3D, and ClO_2-1	• Collected after 80 minutes of injection/~20 minute contact time.	October 6, 2014, 1:20 PM Flow at 0 gpm (0 L/min)
BWS-4 and ClO_2-2	• Collected after 140 minutes of the introduction of chlorine dioxide and 80 minute contact time.	October 6, 2014, 2:20 PM Flow at 0 gpm (0 L/min)
BWS-5 and ClO_2-3	• Collected after 200 minutes of the introduction of chlorine dioxide and 140 minute contact time.	October 6, 2014, 3:20 PM Flow at 0 gpm (0 L/min)
CP-4 and CP-4D ClO_2-4	• Collected after 260 minutes of the introduction of chlorine dioxide and 200 minute contact time.	October 6, 2014, 4:20 PM Flow at 0 gpm (0 L/min)
BWS-6 and ClO_2-5	• Collected after 320 minutes of the introduction of chlorine dioxide and 260 minute contact time.	October 6, 2014, 5:20 PM Flow at 0 gpm (0 L/min)
BWS-7, CP-5, CP-5D, and ClO_2-6	• Collected after 1400 minutes of the introduction of chlorine dioxide and 1340 minute (~22 hours) contact time.	October 7, 2014, 11:20 AM Flow at 0 gpm (0 L/min)

BWS, bulk water sample; CP, coupon; gpm, gallons per minute; L/min, liters per minute; D, duplicate; ClO2, chlorine dioxide sample; 1, 2, 3, etc., sequential sample number

2.3.3 Post-decontamination flushing and monitoring

At 12:00 PM on October 7, 2014, following collection of the last sample shown in Table 4, the flow through the WSTB was resumed at 15 gpm (57 L/min) for the purposes of flushing the decontaminant from the WSTB. The system was flushed with fresh water at 15 gpm (57 L/min) for 2 hours to clear the chlorine dioxide. The flow was then reduced to 5 gpm (19 L/min) at 2:00 PM. Bulk water samples, chlorine dioxide, and coupon samples were collected following the procedures described in Appendix A (Specific Sampling Procedures).

In addition to these samples, large volume samples (20 L Cubitainers® [Hedwin Division of Zacros America, Inc., Baltimore, MD]) of water were also collected for analysis using the water sample concentrator (WSC). Briefly, the WSC is a device that uses ultrafiltration membranes to concentrate low numbers of biological agents from large volumes of water into a smaller volume of water. This increases the chance that a biological agent can be detected in a large volume water sample and lowers the detection limit of the analytical method used for enumeration (spread plating). A description of the WSC, how it is operated and enumeration methods are included in Appendix A. Additional large WSC-related samples were taken at 7 and 14 days after the initial flushing by INL personnel. The post flushing sampling activities are summarized in Table 5.

Table 5. Flushing Sampling Activity

Sample ID	Sample Description	Date/Time and System Flow
BWS-8, CP-6, CP-6D and ClO_2-7	• Collected 180 minutes after the start of flushing	October 7, 2014, 3:00 PM Flow at 5 gpm (19 L/min)
BWS-9, CP-7, CP-7D and ClO_2-8	• Collected 1,440 minutes after the start of flushing	October 8, 2014, 12:00 PM Flow at 5 gpm (19 L/min)
BWS-10, CP-8, CP-8D and WSC-1	• Collected 1,440 minutes after the start of reconditioning • Collected WSC sample of 20 Liters 1440 minutes after the start of reconditioning	October 9, 2014, 12:00 PM Flow at 2.5 gpm (10 L/min)
BWS-11, CP-9, CP-9D and WSC-2	• Collected 1,440 minutes after the start of reconditioning • Collected 20 Liters 1,440 minutes after the start of reconditioning	October 10, 2014, 12:00 PM Flow at 2.5 gpm (10 L/min)
WSC-3	• INL collected 20 Liters 7 days after the start of reconditioning	October 15, 2014, 12:00 PM Flow at 2.5 gpm (10 L/min)
WSC-4	• INL collected 20 Liters 14 days after the start of reconditioning	October 22, 2014, 12:00 PM Flow at 2.5 gpm (10 L/min)

BWS, bulk water sample; CP, coupon; D, duplicate; ClO2, chlorine dioxide sample; 1, 2, 3, etc., sequential sample number; gpm, gallons per minute; L/min, liters per minute; INL, Idaho National Laboratory; WSC, water sample concentrator

The results from the samples collected are presented in Section 3.0.

2.4 The Waterstep Mobile Water System

The WaterStep ((WaterStep, Louisville, KY)) emergency management mobile water system (MWS) was used to assess disinfection capability of a mobile treatment unit. The self-contained WaterStep MWS ships in a pallet/skid for easy deployment. The WaterStep MWS is contained in one locking, rolling storage cart with the following components:

1. The WaterStep M-100 chlorinator (an onsite chlorine generator)
2. Pumps: Circulating pump (12V DC), distribution pump (120V AC) and a hand pump.
3. Electrical Components: connectors and cords for equipment needing a power supply including: ground fault interrupter, one 12V DC, deep cycle battery, storage case, a solar panel, one 10/2/50 ampere automatic battery charger.
4. Plumbing Components: tubing and quick-connect cam-lock fittings for all water connections.
5. Filter: Disc Filter (100 and a 25 micron – not used).
6. Storage: Collapsible potable water bladders with protective ground cover (not used)

2.4.1 WaterStep M-100 Water Treatment Experiments

The WaterStep M-100 chlorinator uses salt (sodium chloride) and the process of electrolysis using direct current from a 12 volt (car) battery to produce chlorine gas and sodium hydroxide (WaterStep, 2013). Table salt purchased from a grocery store was used in this experiment. The system runs an electrical current between the two electrodes, separated by a membrane, in a solution of sodium chloride. Electrolysis breaks up the salt molecules and frees chlorine gas from the brine. The chlorine gas is used as the disinfectant. Only a small amount sodium hydroxide is generated which needs to be disposed separately.

The chlorine gas is introduced into the water stream using a venturi connected to the M-100. A pressure pump (a shallow well pump with bladder tank and a pressure switch) is used to draw water from the source and circulate it through the venturi using a garden hose. As the water passes through the venturi, it creates a vacuum which draws the chlorine gas out of the M-100. As the water is mixed with the chlorine gas, it flows through and returns to the source or bladder tank for storage and disinfection contact time. This process is typically continued until the free chlorine concentration in the water reaches the desired level.

The WaterStep MWS has the capability to pump water into 10,000 gallon (37,850 L) portable bladders, where the contaminated water is temporarily stored to provide contact time for disinfection and then treated water is disposed. These bladders were not used during tests at INL. Instead, the WaterStep MWS was set-up to pump contaminated water directly from the lagoon through the chlorinator and then recirculated back into the lagoon for storage/disinfection. During planning of the water treatment experiments, it was felt that pumping water from the lagoon directly into the WaterStep unit (and bypassing the bladders) would be a more accurate representation of how the unit would be deployed during an emergency water treatment scenario. The enclosed lagoon was expected to provide the necessary contact time and storage. Figure 17 shows the WaterStep M-100 chlorinator in operation at WSTB.

Figure 17. WaterStep M-100 chlorinator.

Operationally, water was drawn from near the lagoon inlet (the presumed point of highest contamination in the lagoon) into the WaterStep MWS. The chlorinated effluent from the WaterStep was pumped back into the far end of the lagoon, away from the inlet near the WSTB piping. It was hoped that this configuration would increase or promote mixing within the lagoon which is not mechanically mixed. Figure 18 shows the operational setup of the WaterStep system.

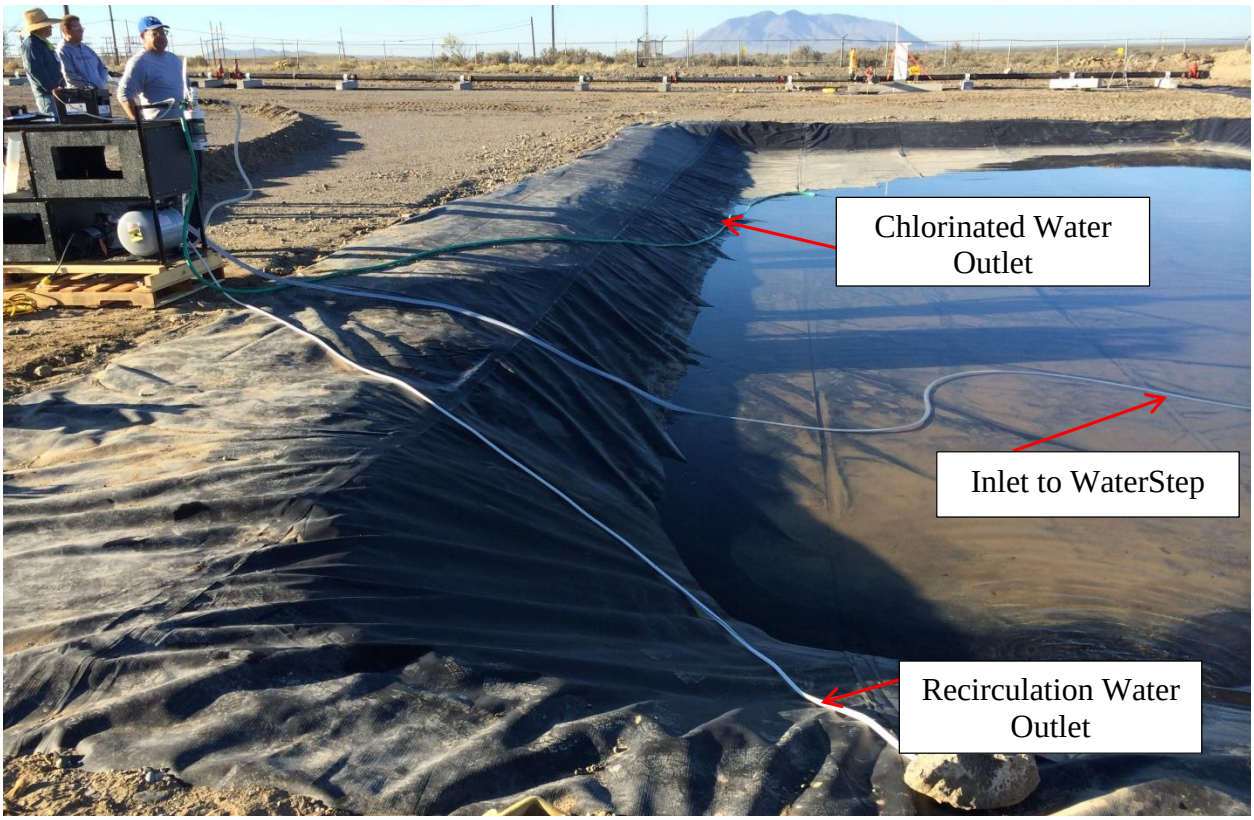

Figure 18. WaterStep setup at the water security test bed lagoon.

On October 6, 2014, at 12:50 PM, the WaterStep M100 unit was put in operation to disinfect the water present in the lagoon. At 4:00 PM, additional salt (a "handful" or approximately 50 to 100 grams) was added to replenish the chlorinator. Subsequently, at 5:30 PM, the chlorinator was shut off (~280 minutes of operation). The unit operated for 4 hours and 40 minutes. Throughout this period, samples from the chlorinated water outlet were collected and analyzed for free chlorine using a pool-kit. The numbers reported were consistently above 5 ppm (the kit can only report values up to 5 ppm). Field dilution was not performed because this was simply a check to determine if chlorine was being generated by the system. Grab samples were collected from the lagoon to evaluate the chlorine levels and submitted offsite for analysis of *B. globigii* to determine if disinfection was being accomplished. The lagoon sampling activities are summarized in Table 6.

Table 6. Lagoon Sampling Activity

Sample ID	Sample Description	Date/Time and System Flow
LG-1, LG-1D	• Lagoon samples for *Bacillus globigii* • Onsite sample for free chlorine, pH paper, and temperature	10/6/2014 2:20 PM Flow at 0 gpm (0 L/min)
LG-2, LG-2D	• Sample collected 150 minutes after the WaterStep was turned on. • Lagoon samples for *B. globigii* • Onsite sample for free chlorine, pH paper, and temperature	10/6/2014 3:20 PM Flow at 0 gpm (0 L/min)
LG-3, LG-3D	• Sample collected 245 minutes after the WaterStep was turned on. The WaterStep unit was turned off at 5:30 PM (~35 minutes after this sample was collected). • Lagoon samples for *B. globigii* • Onsite sample for free chlorine, pH paper, and temperature	10/6/2014 4:55 PM Flow at 0 gpm (0 L/min)
LG-4, LG-4D	• Lagoon samples for *B. globigii* • Onsite sample for free chlorine, pH paper, and temperature • Sample collected 1,230 minutes after the WaterStep was turned on and 950 minutes after the unit was turned off.	**10/7/2014 (Tuesday)** 9:20 AM Flow at 0 gpm (0 L/min)
LG-5, LG-5D	• Sample collected 1,350 minutes after the WaterStep was turned on and 1070 minutes after the unit was turned off. • Lagoon samples for *B. globigii* • Onsite sample for free chlorien, pH paper, and temperature	**10/7/2014 (Tuesday)** 11:20 AM Flow at 0 gpm (0 L/min)

gpm, gallons per minute; L/min, liters per minute; LG, water sample from the lagoon; D, duplicate; 1, 2, 3, etc., sequential sample number

3.0 Analysis of Decontamination and Water Treatment Results

3.1 Contamination and Decontamination Experiment

The field samples were packaged, shipped and analyzed as described in Appendix A. The experimental phase-specific bulk water and water sample concentrator sampling results for *B. globigii* are summarized in Table 7 and color categorized according to the experimental phase. In Table 7, log reduction of spore in the water is calculated as LR = -Log(N/No) where LR = log reduction, N = number of surviving spores at a particular time point, No = initial number of spores. The initial number of spores in the water was the sample taken at 11:30 am as this was the highest number observed.

Table 7. Bulk Water and Water Sample Concentrator Sampling Results

Sample Time	Elapsed Time After Spore Injection Start (min)	Elapsed Time After ClO_2 Injection Start (min)	Coupon contact time with ClO_2 (min)	Sample ID	*B. globigii* spore density in water (CFU/100 mL)	Log Reduct-ion in spore density	Experiment Phase
10/6/14 8:50 AM	0	-	-	BWS 0	2.7E+00	-	Pre-injection Baseline
10/6/14 11:10 AM	70	-	-	BWS 1	7.0E+07	-	*B. globigii* injection
10/6/14 11:30 AM	90	-	-	BWS 2	1.0E+08	-	
10/6/14 1:20 PM	200	80	20	BWS 3	2.0E+03	4.70	Decontamination with chlorine dioxide
10/6/14 2:20 PM	260	140	80	BWS 4	3.3E+01	6.48	
10/6/14 3:20 PM	320	200	140	BWS 5	1.0E+02	6.00	
10/6/14 4:25 PM	385	265	205	BWS 5A	1.4E+00	7.85	
10/6/14 5:20 PM	440	320	260	BWS 6	1.7E+00	7.78	
10/7/14 11:20 AM	1,520	1,400	1340	BWS 7	5.0E+00	7.30	
10/7/14 3:00 PM	1,740	1,620	N/A	BWS 8	5.0E+00	7.30	Flush WSTB
10/8/14 11:50 AM	2,990	2,870	N/A	BWS 9	5.0E+00	7.30	
10/9/14 12:00 PM	4,440	4,320	N/A	BWS 10	ND	>8	Return to Baseline
10/10/14 12:00 PM	5,880	5,760	N/A	BWS 11	5.0E+02	5.30	
10/9/14 12:00 PM	4,440	4,320	N/A	WSC-1	2.1E+00	7.68	
10/10/14 12:00 PM	5,880	5,760	N/A	WSC-2	7.9E-01	8.10	
10/15/14 12:00 PM	13,080	12,960	N/A	WSC-3	3.2E-01	8.50	
10/22/14 10:00 PM	23,760	23,640	N/A	WSC-4	1.7E+00	7.76	

BWS, bulk water sample; cfu, colony forming units; WSC, water sample concentrator; WSTB, water security test bed; ND, none detected

The experimental phase-specific average bulk water *B. globigii* values shown in Table 7 along with the associated chlorine dioxide concentrations in their color category are plotted in Figure 19.

Please note that the pre-injection baseline phase is a single point and does not appear color-coded in Figure 19.

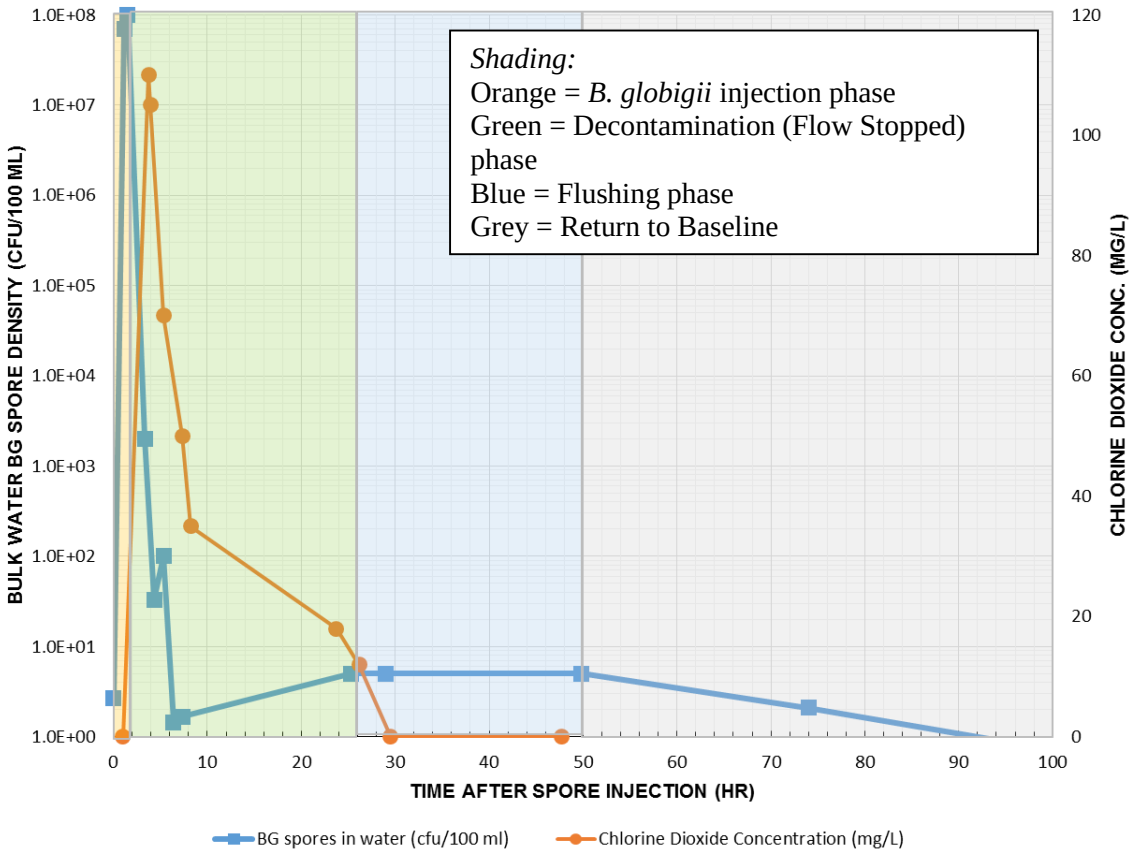

Figure 19. Bulk water *B. globigii* (BG) and chlorine dioxide results over time after spore injection (hr).

The trends in Figure 19 indicate that the chlorine dioxide level drops within minutes; correspondingly, the *B. globigii* values also drop in the bulk water phase. A minimum of 5-log reduction was observed throughout the decontamination phase. However, some *B. globigii* appears during the flushing phase and the return to baseline phase of the experiment. The results also indicate that the WSTB flushing phase is successful in removing chlorine dioxide from the system.

The corresponding experimental phase-specific coupon sampling results for *B. globigii* are summarized in Table 8 and color categorized according to the experimental phase.

Table 8. Coupon Sampling Results

Sample Day/Time	Sample ID	Average *B. globigii* (CFU/in^2) attached to Coupon Surface	Range between coupons (CFU/in^2)	Log Reduction	Experiment Phase
10/6/14 8:50 AM	CP 0	6.7E+00	1.3E+01	NA	Pre injection Baseline
	CP 0D				
10/6/14 11:10 AM	CP 1	9.5E+03	1.5E+04	NA	*B. globigii* injection
	CP 1D				
10/6/14 11:30 AM	CP 2	1.3E+06	1.7E+06	0	
	CP 2D				
10/6/14 1:20 PM	CP 3	1.3E+04	2.7E+03	2.0	Decontamination with chlorine dioxide
	CP 3D				
10/6/14 4:30 PM	CP 4	1.8E+04	1.5E+04	1.8	
	CP 4D				
10/7/14 11:20 AM	CP 5	3.4E+04	1.6E+04	1.6	
	CP 5D				
10/7/14 3:00 PM	CP 6	9.4E+03	1.1E+04	2.1	Flush WSTB
	CP 6D				
10/8/14 12:00 PM	CP 7	1.6E+04	2.8E+04	1.9	
	CP 7D				
10/9/14 12:00 PM	CP 8	7.7E+03	1.0E+04	2.2	Return to Baseline water quality
	CP 8D				
10/10/14 12:00 PM	CP 9	2.6E+03	2.9E+03	2.7	
	CP 9D				

CFU, colony forming units; CP, coupon; WSTB, water security test bed; NA, not applicable (pre-injection); Note: to determine the microbial density on the coupons in CFU/cm^2, divide by 6.45

The experimental phase-specific average coupon *B. globigii* values shown in Table 8 along with the associated chlorine dioxide concentrations in their color category are plotted in Figure 20. Please note that the pre-injection baseline phase is a single point and does not appear color-coded in Figure 20. The range between the duplicate coupons samples that make up the average number is also displayed. In most cases, there is variability between the duplicate samples, which indicates that spore adhesion was spatially heterogeneous.

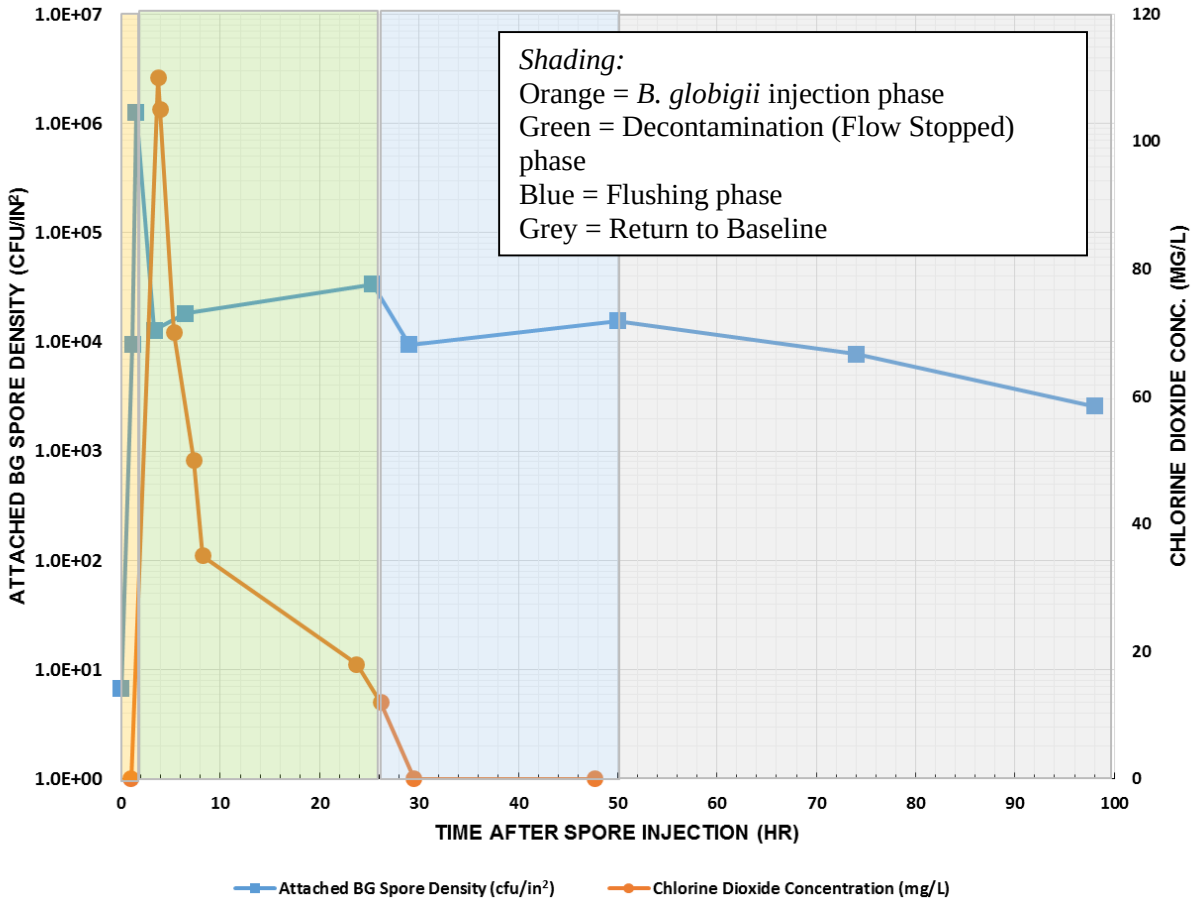

Figure 20. Coupon *B. globigii* (CFU/in^2) and bulk water chlorine dioxide (mg/L) results over time after spore injection (hr). Note: to determine the microbial density on the coupons in CFU/cm2, divide by 6.45

The trends in Figure 20 indicate that the chlorine dioxide level drops within minutes; however, the coupon *B. globigii* values stabilize (~ order of magnitude 10^4 CFU/in^2) after an initial ~ 2 log reduction indicating that the chlorine dioxide decontamination procedure was not as effective in removing *B. globigii* from the pipe wall. The limited effectiveness of the chlorine dioxide may be due to demand from the pipe walls or surface adhesion and layering effects. Although the chlorine dioxide concentration was high in the bulk phase, demand from the pipe wall may have limited the penetration of the chlorine dioxide into the cement-mortar matrix and thus contact with the attached spores. *B. globigii* from the pipe wall or those trapped in dead end sections of pipe appear to release into the bulk water during the flushing phase and return to baseline phase of the experiment as shown previously in Figure 19 and the extended timeline plot in Figure 21.

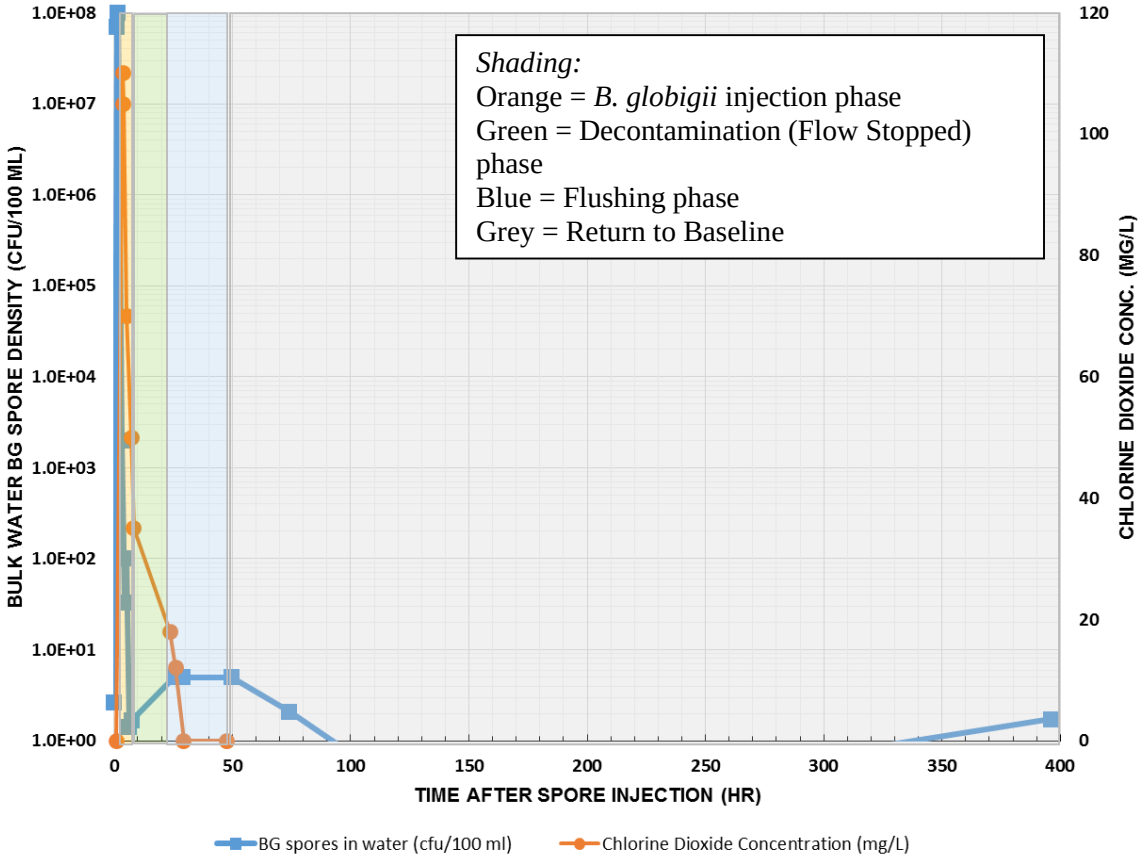

Figure 21. Extended timeline bulk water *B. globigii* (BG) and chlorine dioxide (mg/L) results over time after spore injection (hr).

One of the key benefits of using the WSTB to conduct research is the ability to generate data on a realistic field scale. This data can then be compared to other data generated using smaller bench or pilot scale systems. Comparing bench and/or pilot scale data with data obtained from the WSTB will allow EPA to understand whether using a bench or pilot scale system for research yields results similar to those obtained from a realistic field scenario. Alternatively, if data generated from the WSTB is different from data obtained from a smaller research scale, it is likely due to the fact that the WSTB simulates real world drinking water system attributes that cannot be simulated on a smaller scale.

The data show in Figures 22 and 23 compare data on contamination and decontamination of drinking water infrastructure obtained from a pilot-scale research system at EPA's T&E facility (Figure 22) and the WSTB (Figure 23). Data in both figures comes from experiments where *B. globigii* spores were injected into an experimental pipe system and allowed to come into contact with cement-mortar drinking water infrastructure surfaces. The surfaces with adhered spores were then decontaminated with chlorine dioxide. The pilot scale system at the T&E facility system used a six inch (15 cm) diameter pipe loop with cement-mortar coupons installed in the same manner as the WSTB and Cincinnati tap water flowing at a velocity of 1 ft/sec (0.3 m/sec). A detailed description of the pilot scale system at the T&E facility can be found in Szabo et al., 2012.

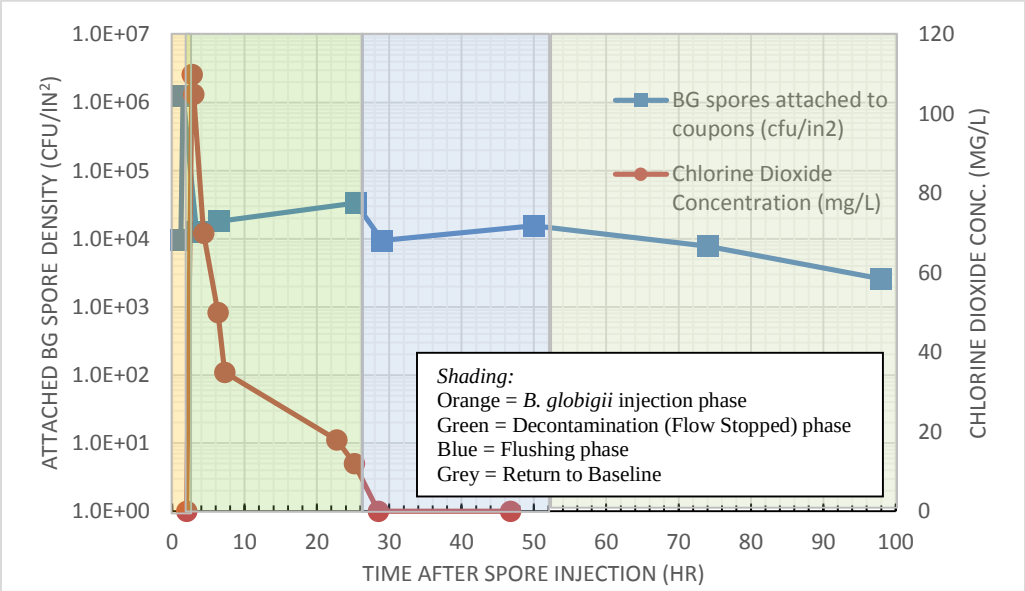

Figure 22. *B. globigii* (BG) spore contamination (CFU/in^2) of the water security test bed and decontamination with chlorine dioxide over time after spore injection (hr). Note: to determine the microbial density on the coupons in CFU/cm^2, divide by 6.45

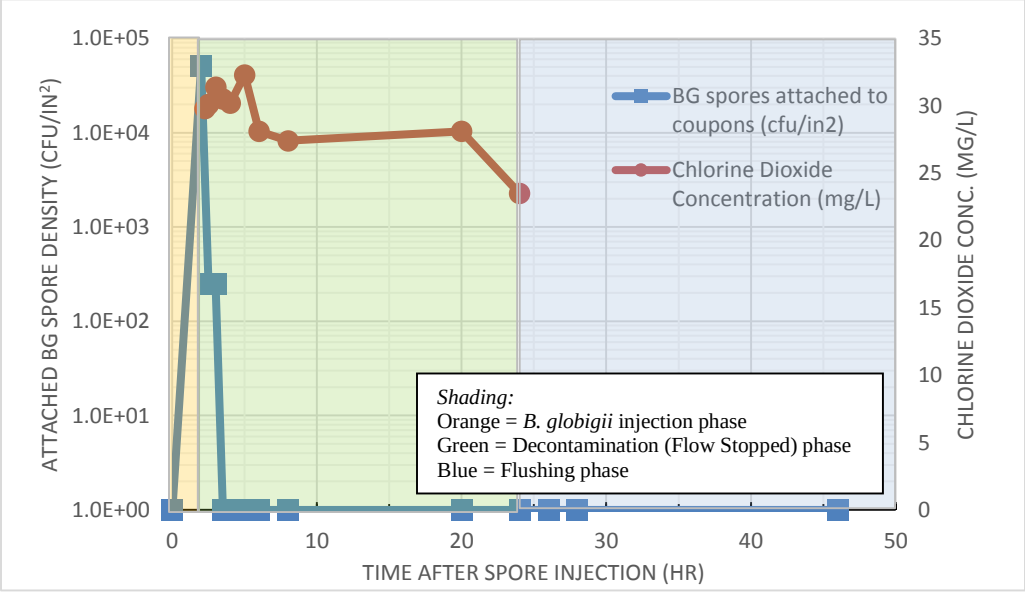

Figure 23. *Bacillus globigii* (BG) spore contamination (CFU/in^2) of the T&E pilot scale drinking water pipe system and decontamination with chlorine dioxide (mg/L) over time after spore injection (hr). Note: to determine the microbial density on the coupons in CFU/cm^2, divide by 6.45

There are two key differences in the decontamination data obtained from the field scale WSTB and the pilot scale pipe system at the T&E facility. First, in the pilot scale system, chlorine dioxide at 25 mg/L achieved a greater than 4 $\log_{10}$ reduction of the adhered spores within 2 hours of contact time. The number of spore dropped below the detection limit of the analytical method. In the WSTB, 110 mg/L of chlorine dioxide was initially achieved, and this concentration remained above 50 mg/L for the first six hours of decontamination. However, only 2 $\log_{10}$ reduction was achieved in the WSTB, and the spores were still easily detectable on the coupons.

The second key difference between the experiments is the chlorine dioxide concentration. In the pilot-scale test at the T&E facility, a chlorine dioxide concentration of 25 to 30 mg/L was easily achieved over 24 hours. In the WSTB experiment and initial chlorine dioxide concentration of 110 mg/L dropped to 70 mg/L at 4 hours after decontamination began, 35 mg/L at 8 hours and 12 mg/L after 24 hours of contact time. This suggests that the pipe material exerted a significant disinfectant demand. If the pipe exerted significant demand, is possible that the chlorine dioxide was not effectively penetrating into the cement-mortar matrix where the spores were adhered. The presence of spores remaining after the chlorine dioxide decontamination phase suggests that pipe demand was a factor in their persistence.

The key message from the decontamination experiment in the WSTB is that drinking water infrastructure decontamination is more challenging in a real world field environment. Future decontamination research efforts will require a higher chlorine dioxide concentration or a different decontamination approach altogether (for example, physical removal through pigging). However, these results underscore the importance of conducting research at a real world experimental facility like the WSTB, which can yield results more relevant to the real world than a bench or pilot scale system.

3.2 Water Treatment Experiment

The results from the treatment of *B. globigii* spore contaminated water flushed into the lagoon using the WaterStep treatment unit are tabulated in Table 9.

Table 9. Lagoon Sampling Results

Date/Time	Sample ID	*B. globigii* (CFU/mL)	Average *B. globigii (CFU/mL)*
10/6/14 1:40 PM	LG-0	2.2E+05	2.2E+05
10/6/14 1:40 PM	LG-0D	2.1E+05	
10/6/14 2:20 PM	LG-1	3.0E+05	2.8E+05
10/6/14 2:20 PM	LG-1D	2.7E+05	
10/6/14 3:30 PM	LG-2	2.5E+05	2.5E+05
10/6/14 3:30 PM	LG-2D	2.6E+05	
10/6/14 4:30 PM	LG-2A	1.9E+05	1.5E+05
10/6/14 4:30 PM	LG-2AD	1.2E+05	
10/6/14 5:20 PM	LG-3	8.9E+04	1.0E+05
10/6/14 5:20 PM	LG-3D	1.2E+05	
10/7/14 9:20 AM	LG-4	1.1E+05	1.1E+05
10/7/14 9:20 AM	LG-4D	9.9E+04	

CFU, colony forming units; LG, water sample from the lagoon; D, duplicate; 1, 2, 3, etc., sequential sample number

The results in Table 9 indicate that each of the average *B. globigii* values reported from the lagoon samples were greater than 10^5 CFU/mL. The field data (previously reported in Section 3.1, reporting chlorine values consistently above 5 ppm) proved that the WaterStep unit was successful in producing chlorine as designed. However, field methodology for delivering the chlorine disinfectant to the lagoon without the bladders was ineffective. The highest free chlorine residual detected in the lagoon was 0.03 mg/L, but the highest total chlorine residual detected was 1.71 mg/L. This indicated that the free chlorine being generated by the WaterStep unit was being transformed into total (or combined) chlorine once it entered the lagoon. The large exposed surface area of the lagoon, in combination with shallow depth, and intense sunlight, may all have contributed to the rapid degradation of the chlorine delivered to the lagoon. Other confounding factors include: high organic load from the dusty lined lagoon and the presence of growth media carried over from flushing.

4.0 Conclusions and Future Work

The following is a summary of conclusions based on the testing performed at the INL WSTB:

- The dye-testing confirmed the theoretical flow velocity and travel time calculations (~ 1 hour travel time). As expected, there was some visible mixing and dispersion/diffusion of the dye slug observed during testing.
- Sodium thiosulfate (a surrogate contaminant) removed free chlorine from the water and successfully triggered the hydrant-based flushing device. Some of the dispersed sodium thiosulfate was lodged in dead end pipes and was released with subsequent hydraulic changes. The experiment showed that changes in water quality resulting from contamination can trigger a flushing hydrant and remove contaminated water from a distribution pipe.
- The decontaminant (chlorine dioxide) targeted in-pipe concentration of >25 mg/L was also achieved. The highest observed chlorine dioxide concentration was 110 mg/L, but the chlorine dioxide decayed to 35 mg/l over the next 7 hours.
- The contaminant (*B. globigii*) targeted in-pipe bulk water concentration (10^7 and 10^8 CFU/100 mL) was achieved. The water sampling results indicated a 5 to 8 log reduction of the contaminant in bulk water over the course of 24 hours (chlorine dioxide ranging from 110 mg/L down to 18 mg/L. The sampling results also indicate a less effective removal (~2 log reduction based on highest observed *B. globigii* density before decontamination) of the contaminant from the coupon surface over the same 24 hour period.
- Comparison of the decontamination results from the WSTB and those from pilot scale decontamination research studies performed in EPA facilities suggests that decontamination of biological agents in a real world field setting is more challenging than the data from the pilot scale studies had indicated. Certain aspects of a real water distribution system that could influence the effectiveness of a decontamination method, such as pipe wall disinfectant demand and dead end spaces, are difficult to simulate on the pilot scale. Therefore, future decontamination research should ideally be performed at the field scale instead of the bench or pilot scale, because a realistic setting will provide a truer picture of decontamination effectiveness.
- The lagoon/WaterStep decontamination procedure was ineffective as performed. In the future, the temporary storage bladders will need to be used to provide sufficient contact time, reduce surface area, remove the adverse effects of sunlight on the disinfection process, and reduce the impact of the organic load from the lagoon. Operationally, the unit could not run much over 3 hours without replenishing the sodium chloride solution in the generator. Because the membrane will burn out if the salt solution gets too low, this system is not suitable for an unmanned operation.

Overall, the WSTB was operated without issues and enabled EPA NHSRC to perform the study. Specifically, the following operational observations were made during the performance of the EPA study:

- The actual pressure drop matched the theoretical pressure gradient drop of ~3 psi across the system.
- The WSTB pipe system (linear pipe length of 448 ft (137 m)) operated leak-free during the testing.

- The WSTB maintained pressure throughout the testing, including the flushing event where large amounts of water were withdrawn from the system.
- A stable free chlorine residual was maintained through the length of the pipe when active testing was not occurring at a baseline flow of 2.5 gpm (10 L/min).
- The flow monitoring and flow control device (i.e., rotameter) operated successfully.
- The lagoon volume was sufficient to perform a complete test.
- The instrument panels were installed and continuous monitoring data was successfully telemetered via cellular/radio back to the EPA Test and Evaluation Facility in Cincinnati, Ohio.
- The injection system successfully delivered a controlled volume of tracer/contaminant/ decontaminant.
- The automated flushing hydrant functioned as expected.
- The PVC pipe coupon section functioned as expected. The isolation valves allowed for the removal and replacement of coupons during the experiment without draining the WSTB.
- The lined trench under the pipe directed the flow to the lagoon as intended.

Future research using the WSTB will focus on answering some of the remaining questions and filling data gaps in this report, as well as addressing other outstanding EPA National Homeland Security Research Center needs.

- Decontamination with chlorine dioxide will be reattempted in the spring of 2015. The results from experiments described in this report indicate that the WSTB was not thoroughly decontaminated, and *Bacillus* spores remain attached to the pipe. The WSTB will be decontaminated again using chlorine dioxide. An increased contact time with the pipe wall will be implemented and the dead end portion of the WSTB will be thoroughly flushed by adding flow ports to the dead-end portions of the pipe.

- Treatment of water in the lagoon contaminated with *Bacillus* spores will be attempted with additional commercially available water treatment units. The goal will be gathering data on the field performance of commercially available water treatment units.

- Crude oil will be injected into the WSTB and the persistence of constituents in the crude oil such as benzene, toluene, ethylbenzene and xylene (BTEX) will be assessed. Decontamination approaches such as flushing or adding surfactants will be studied. This study is warranted because crude oil spilled into a water body that feeds a drinking water treatment plant could make it through the treatment works and into the distribution system. Should this happen, first responders will need to know if the BTEX constituents persist on the distribution system infrastructure and the effectiveness of decontamination methods.

5.0 References

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Appendix A – Detailed Experimental Protocols and Quality Assurance Criteria

Contamination/Decontamination Tests

These experiments involve contamination of the water security test bed (WSTB) using *Bacillus globigii* spores and the subsequent decontamination of WSTB using chlorine dioxide as the decontaminant. Each contamination/decontamination experiment consists of the following main steps:
Step 1 - Pipe Conditioning (cultivation of biofilm)
Step 2 - Instrumentation Panel/Injection Setup and Dye Tracer Study
Step 3 - Injection of Contaminant (addition of *B. globigii* spores to WSTB)
Step 4 - Decontamination (chlorine dioxide/flushing)
Step 5 - Post-Decontamination Flushing, Reconditioning and Monitoring

Step 1 - Pipe Conditioning (Cultivation of Biofilm)
To effectively study the adsorption of contaminants on pipe walls, it is essential to ensure that there is a viable biofilm. The biofilm could influence adsorption of the contaminant on the pipe wall in addition to metabolism, biodegradation, or detoxification of the contaminant.

Previously under EPA Contract EP-C-09-041, CB&I Federal Services LLC (CB&I) performed a literature review of biofilm cultivation and identified four primary techniques that could potentially be used for cultivating biofilm within the WSTB:

1. Sequential batch fermentation and introduction into the WSTB
2. Using the WSTB as a reactor by passing water with low concentrations of carbon, nitrogen, and salts
3. Use of an external annular reactor
4. Natural biofilm cultivation by passing water through the WSTB

The fourth option, natural cultivation of biofilm, has been chosen as the cultivation procedure for testing of the WSTB. This will be accomplished by passing Idaho National Laboratory (INL) tap water through the WSTB continuously over a period of time (estimated to be 4 weeks – starting early to mid September 2014). After initial flushing to remove any debris, the flow rate will be set at 2.5 gallons per minute (gpm) (10 L/min) with a total discharge of 25,200 gpm (95,382 L) to the lagoon which allows for weekly trucking and disposal of the accumulated discharge.

Step 2 - Instrumentation Panel/Injection Setup and Dye Tracer Study
Late September 2014, the CB&I team will arrive at INL to install the instrument panel and injection pump. A simple dye tracer study (using non-toxic biodegradable dye such as Bright Dyes®, Kingscote Chemicals, Miamisburg, OH; www.brightdyes.com) will be performed to visually confirm the theoretical calculations of travel times and system flows.

The presence of stable free chlorine concentration and temperature at the downstream instrument panel location of the WSTB will indicate stabilization of pipe wall chlorine demand and biofilm formation. If the measured free chlorine levels are stable, a coupon in duplicate and a bulk water sample (BWS) will be collected. The heterotrophic plate count (HPC) concentration on the coupon surface will be measured to determine the presence of viable biofilm in the WSTB. The heterotrophic colony count (HPC) coupon and BWS sample can be collected anytime between one day and 1 week before injection of contaminant (Step 3).

This HPC sampling will be performed by isolating the coupon section of the main pipe by closing the upstream flanking gate valve, removing the duplicate coupon plugs (CP-0 and CP-0D) and replacing the coupon plugs with blank plugs. The biofilm sample will be collected from the duplicate coupon plugs as described in Sampling Procedures section (later in this Appendix) to determine the formation of biofilm on the coupons and measure HPC concentration. At the same time, a BWS background sample as described in the Sampling Procedures section (later in this Appendix) will be collected to serve as the background control. The sampling activities are described in Table A1.

Table A1. Background Sampling Activity

Sample ID	Sample Description	Estimated Timeline & System Flow
BWS-0 (Control)	Collect at ~1 week prior to injection of *B. globigii*	Late September 2014 Flow at 2.5 gpm (10 L/min)
BWS-B (Background)	Background control collected at the same time as BWS-0	Late September 2014 Flow at 2.5 gpm (10 L/min)
CP-0 and CP-0D	Collect at the same time as BWS-0	Late September 2014 Flow at 2.5 gpm (10 L/min)

BWS, bulk water sample; CP, coupon; gpm, gallons per minute; L/min, liters per minute

Step 3 Injection of Contaminant (Addition of *B. globigii* Spores to WSTB)

Preparation of Spores (Contaminant Stock) - The *B. globigii* spores for this study were originally obtained from EPA NHSRC at the Andrew W. Breidenbach Environmental Research Center (AWBERC) in Cincinnati, Ohio. A culture of *B. globigii* vegetative cells will be mixed with generic sporulation media and incubated by gentle shaking at 35°C for 7 days at the EPA T&E Facility. The concentration of *B. globigii* stock will be determined following the method described by Rice *et al*. (1994). The *B. globigii* spores will be heat-shocked and analyzed using the spread plate method and membrane filtration. After the stock is ready, 40 liters of prepared stock will be shipped in separate 1 liter containers (preserved at 4±2°C) to the site.

Injection of Contaminant Test Protocol – In early October, the *B. globigii* suspension will be introduced into the WSTB using a positive displacement pump to achieve a target bulk water concentration between 10^5 and 10^6 CFU (Colony Forming Units)/mL in the pipe. The WSTB will be operated at 15 gpm (57 L/min) under this condition with a minimum contact time of approximately 1 hour (to accommodate for travel time). Injection duration is also estimated to be 1 hour so that there is a contact of 1-hour after the bolus of *B. globigii* suspension reaches the coupon section of the pipe.

BWS and coupon samples will be collected at 5 and 60 minutes after initial pass-through of the contaminant at the coupon section. A sample for chlorine dioxide will also be collected at 60 minutes after initial pass-through of the contaminant at the coupon section. Coupon samples (CP-1, CP-1D, CP-2 and CP-2D) will be extracted (scraped) immediately after removal as described in the Sampling Procedures section (later in this Appendix). The ClO_2-0 sample serves as background for chlorine dioxide (prior to the introduction of chlorine dioxide in the decontamination step). The WSTB operations will be continued at the same rate until the

contaminant bolus has passed through the system (estimated to be ~ 1 hour and 5 minutes based on theoretical calculations and the dye tracer travel time confirmation). This is necessary to remove the contaminant from the bulk phase of water. A BWS will be collected after the bolus is estimated to have passed through the system. The sampling activities are described in Table A2.

Table A2. Contamination Sampling Activity

Sample IDs	Sample Description	Estimated Timeline & System Flow
BWS-1, CP-1 and CP-1D	Collect after 5 minutes of the injection of *B. globigii* reaches the coupon section and prior to the introduction of chlorine dioxide	Early October 2014 Flow at 15 gpm (57 L/min)
BWS-2, CP-2, CP-2D and ClO_2-0	Collect after 60 minutes of the injection of *B. globigii* and prior to the introduction of chlorine dioxide	Early October 2014 Flow at 15 gpm (57 L/min)

BWS, bulk water sample; CP, coupon; gpm, gallons per minute; L/min, liters per minute

Step 4 - Decontamination (Chlorine dioxide/flushing)

Preparation of Decontaminant Agent Stock – Chlorine dioxide decontaminant stock solution will be prepared in advance onsite at INL using the GO2™ kit (GO2 International, Buena Park, CA). Components A and B from the GO2™ will be mixed in 25 L of deionized water using two buckets, covered, and kept static for 3 hours in an outside ventilated area. Chlorine dioxide stock concentration will be measured using the Hach® DR/890 pocket colorimeter (Hach Inc., Loveland, CO). The expected stock concentration yield is about 4,000 mg/L.

Decontamination Test Protocol – Approximately 5 minutes following the 60 minute sample collection (shown in Table A2), the prepared chlorine dioxide stock solution will be injected into the WSTB to achieve a target bulk concentration of approximately 25 mg/L. Concentration of the chlorine dioxide stock will be verified before injection. Injection of chlorine dioxide will continue until the chlorine dioxide has reached the end of the pipe (estimated to be approximately 1 hour and 5 minutes based on theoretical calculations and the dye tracer travel time confirmation). Injection will be stopped, online instrumentation will be stopped, and water flow out of the WSTB will be stopped for 18-24 hours so that the water containing disinfectant will be stagnant in the pipe to perform disinfection. Duplicate coupon samples will be collected at 0 min, 180 min and 1,200-1,440 minutes (20-24 hours) after the decontaminant injection is shut down and disinfection is occurring. Each coupon will be extracted (scraped) immediately after removal (see Sampling Procedures section later in this Appendix) and the resulting suspension stored in a cooler at 4°C. BWS and ClO_2 samples will be collected at the same time coupons are removed, and analyzed for *B. globigii* and chlorine dioxide, respectively. Additional chlorine dioxide samples will be collected at 60 min, 120 min and 240 min after decontamination injection is shut down. The sampling activities are described in Table A3.

Table A3. Decontamination Sampling Activity

Sample ID	Sample Description	Estimated Timeline & System Flow
BWS-3, CP-3, CP-3D, and	Collect after 0 minutes of the introduction of chlorine dioxide.	Early October 2014 Flow at 15 gpm (57 L/min)

ClO_2-1		
	Allow chlorine dioxide to reach the end of the pipe – estimate 65 minutes. Stop flow.	Early October 2014 Flow at 15 gpm (57 L/min)
BWS-4 and ClO_2-2	Collect after 60 minutes of the introduction of chlorine dioxide	Early October 2014 Flow at 0 gpm (0 L/min)
BWS-5 and ClO_2-3	Collect after 120 minutes of the introduction of chlorine dioxide	Early October 2014 Flow at 0 gpm (0 L/min)
CP-4 and CP-4D	Collect after 180 minutes of the introduction of chlorine dioxide	Early October 2014 Flow at 0 gpm (0 L/min)
BWS-6 and ClO_2-4	Collect after 240 minutes of the introduction of chlorine dioxide	Early October 2014 Flow at 0 gpm (0 L/min)
BWS-7, CP-5, CP-5D, and ClO_2-5	Collect after 1,200 – 1,440 minutes (20 – 24 hrs.) of the introduction of chlorine dioxide	Early October 2014 Flow at 0 gpm (0 L/min)

BWS, bulk water sample; CP, coupon; gpm, gallons per minute; L/min, liters per minute

Step 5 - Post-Decontamination Flushing, Reconditioning, and Monitoring

Following collection of the 1,440 minutes samples (shown in Table A3), the WSTB will be flushed with fresh water for approximately 1 hour at 15 gpm (57 L/min) to clear the chlorine dioxide. The flow will then be reduced to 5 gpm (19 L/min). BWS, chlorine dioxide and coupon samples will be collected following the procedures described in the Sampling Procedures section (later in this Appendix) at 180 min and 1,200-1,440 min (20-24 hours) after the start of flushing. The sampling activities are described in Table A4.

Table A4. Flushing Sampling Activity

Sample ID	Sample Description	Estimated Timeline & System Flow
BWS-8, CP-6, CP-6D and ClO_2-6	Collect after at 180 minutes from the start of flushing	Early to mid October 2014 Flow at 5 gpm (19 L/min)
BWS-9, CP-7, CP-7D and ClO_2-7	Collect after at 1,200 – 1,440 minutes from the start of flushing	Early to mid October 2014 Flow at reset at 2.5 gpm (10 L/min)

BWS, bulk water sample; CP, coupon; gpm, gallons per minute; L/min, liters per minute

Large volume samples of 20 L for water sample concentrator (WSC), BWS, and coupons will be removed at 1 and 2 days after flushing is initiated. Additional large volume 20 L WSC samples will be taken at 7 and 14 days after flushing stops by INL personnel. The sampling activities are described in Table A5.

Table A5. Return to Baseline Sampling Activity

Sample ID	Sample Description	Estimated Timeline & System Flow
BWS-10, CP-8, CP-8D and WSC-1	Collect after 1,440 minutes of the start of reconditioning Collect WSC sample 20 Liters after 1440	Mid October 2014 Flow at 2.5 gpm (10 L/min)

	minutes of the start of reconditioning	
BWS-11, CP-9, CP-9D and WSC-2	Collect after 1,440 minutes of the start of reconditioning Collect 20 Liters after 1,440 minutes of the start of reconditioning	Mid October 2014 Flow at 2.5 gpm (10 L/min)
WSC-3	INL will collect 20 Liters after 7 days after the start of reconditioning	Mid October 2014 Flow at 2.5 gpm (10 L/min)
WSC-4	INL will collect 20 Liters after 14 days after the start of reconditioning	Late October 2014 Flow at 2.5 gpm (10 L/min)

BWS, bulk water sample; CP, coupon; gpm, gallons per minute; L/min, liters per minute; WSC, water sample concentrator

New coupons will be inserted into the WSTB and flow will resume through the WSTB at 2.5 gpm (10 L/min) to restart the biofilm cultivation process (Step 1) for the next test. If an experiment is the last test before the WSTB is shut down for the winter, blank coupons will be inserted and the WSTB will be drained. Given the project schedule, it is anticipated that only one test condition will be completed using the cement mortar-lined iron pipe from INL (test material), *B. globigii* (contaminant), and chlorine dioxide (decontaminant) in 2014.

Dechlorination/Flushing Experiments

The purpose of these experiments is to demonstrate the feasibility of using online water sensors in concert with flushing hydrants to intelligently divert and remove contaminants from water distribution systems. For these experiments, the Hach CL-17 and the Real Tech instruments (Real Tech Inc., Whitby, ON, Canada) will be used to signal a flushing hydrant to open and flush the injected contaminant from the WSTB. For this purpose, a test will be performed using sodium thiosulfate as the "injected contaminant" to de-chlorinate the system for approximately 30 minutes. A set point or trigger value of measured chlorine level (e.g., 0.05 mg/L or lower) using the Hach CL-17 at the upstream location will be used for opening the flushing hydrant valve. After the chlorine value at the upstream recovers (e.g., > 0.5 mg/L) to the background value, the valve will be automatically triggered to close. Grab samples will be collected and analyzed for free chlorine residuals at a downstream location from the flushing hydrant. The purpose of this grab sample will be to determine if dechlorinated water is able to "jump" across the "tee" to the flushing hydrant and proceed downstream. Free chlorine levels for these grab samples will be measured using the Hach DR/890 pocket colorimeter (same instrument used to measure chlorine dioxide). The triggered flushing experiment will be independent of the contamination/ decontamination experiment and will occur after the contamination/decontamination experiment if time and weather permits.

For the second objective, the Hach CL-17 will be used to trigger a flushing event based on chlorine concentrations, as described above. The RealTech M4000 TOC instrument's ability to trigger events based on organic concentrations will be evaluated at a later date.

Disinfection of Large Water Volumes

This experiment will assess the ability of a portable disinfection unit to disinfect a large volume of water containing *Bacillus* spores. During the contamination and decontamination experiment described above, there will be an 18-24 hour time period where water is not flowing into the lagoon (see steps 3 and 4). After contamination, the contaminant bolus will be allowed to flow

out of the WSTB and into the lagoon. Chlorine dioxide will then be added to the WSTB. Once the WSTB is full of chlorine dioxide, flow will be stopped for 18-24 hours to allow disinfection to occur. This 18-24 period of no flow will be ideal for testing a field portable disinfection device since the contaminant bolus will be contained in the lagoon, but flow from the WSTB will not be diluting it.

The WaterStep mobile water system (MWS) will be used to assess disinfection capability of a mobile treatment unit. The MSW has the capability to pump water into 10,000 gallon (37,850 L) bladder where it is disinfected and then dispensed once it has been disinfected. These bladders will not be used during tests at INL. Instead, water will be pumped from the lagoon into the disinfection unit and then dispensed back into the lagoon after disinfection. This process will occur during the 18-24 hour period of no flow from the WSTB. Samples will be removed directly from the lagoon when water from the WSTB is shut off (time zero). Once the disinfection unit starts working, samples will be removed from the lagoon at 1, 2, 4, 18, 20 and 24 hours (if applicable). Samples will be removed in duplicate from the lagoon at each sampling time.

SAMPLING PROCEDURES

Site-Specific Factors

Contamination/decontamination and flushing experiments will be conducted at INL. Samples will be shipped to the EPA T&E Facility for analysis. A summary of the experimental sampling strategy (including the number of samples) is presented in Table A6.

Table A6. Summary of Experimental Sampling Strategy

Sample/ Sampling Location	Matrix	Measurement	Measurement Location	Sampling Frequency	Total No. of Samples
Contamination - Decontamination Tests/ WSTB	Biofilm	HPC	T&E Facility	1 sample in duplicate	2
	Biofilm	*B. globigii*	T&E Facility	9 sample in duplicate	18
	Water	*B. globigii*	T&E Facility	18 100 mL samples in duplicate Four 20 L samples in duplicate	36 (100 ml) 4 (20L)
	Water	Chlorine Dioxide	Field Site	7 samples	7
	Water	Free Chlorine	Field Site	~2 samples	2

HPC, heterotrophic plate count; WSTB, water security test bed

Specific Sampling Procedures

Extraction of Biofilm and Spores from Coupon Surface for HPC and B. globigii analyses

The coupons will be collected from the WSTB carefully without touching the surface that was exposed to WSTB water. The biofilm and spores will be scraped from the surface using a disposable sterile surgical scalpel. The extracted material will be collected in a sterile sample bottle with a sodium thiosulfate tablet and 100 mL of pre-filled carbon-filtered water. The extracted sample will be transferred to a cooler at 4±2°C. The samples will be shipped overnight to the EPA T&E Facility and analyzed upon receipt.

Samples for HPC Concentration Measurement

The BWS for HPC concentrations (BWS-0) will be collected using the grab sampling technique in 100-mL sterile sample bottles with a sodium thiosulfate tablet. The BWS sampling port will be opened and the water will be drained for 15 seconds prior to collection of 100 ml of water from the WSTB. The extraction of biofilm from the coupon surface (CP-0/CP-0D) will be conducted as described in the previous paragraph. The samples will be transferred to a cooler at 4±2°C. The samples will be shipped overnight to the EPA T&E Facility and analyzed upon receipt.

BWS for B. globigii spores

The BWS for *B. globigii* concentrations (BWS-1 through BWS-9) will be collected using the grab sampling technique in 100 mL sterile sample bottles with a sodium thiosulfate tablet. The BWS sampling port will be opened and the water will be drained for 15 seconds prior to collection of 100 ml of water from the WSTB. For larger samples, 20 L will be collected flexible plastic bladders (Cubitainers®, Hedwin Division of Zacros America, Inc., Baltimore, MD) with sodium thiosulfate tablets (0.01% w/v). A sample of water will be removed from the Cubitainers to ensure that no free chlorine residual is present. The samples will be transferred to a cooler at 4±2°C. The samples will be shipped overnight to the EPA T&E Facility and analyzed upon receipt.

To estimate the BWS background (BWS-B), a sample bottle containing sterile buffer solution will be exposed to background air while the actual BWS is being collected to serve as the background control.

Samples for Chlorine Dioxide – Field Measurement

During the decontamination step, with every bulk water sample collected using the grab sampling technique and a laboratory beaker (ClO_2-0 through ClO_2-7) will be used to draw a sample for chlorine dioxide measurement. The sample will be immediately processed for measurement using the Hach Method 10126 (pocket Colorimeter) in the field.

Samples for Free Chlorine – Field Measurement

During the dechlorination/flushing experiments, grab samples will be collected from a downstream location of the flushing hydrant using the grab sampling technique and a laboratory beaker and analyzed for free chlorine. The sample will be immediately processed for measurement using the Hach Method 10102 (pocket colorimeter) in the field.

Water Sample Concentrator

Once received at the EPA T&E Facility, the 20 L water samples (labeled WSC) will be subjected to concentration using the water sample concentrator. The water sample concentrator will be operated according to EPA NHSRC's Water Sample Concentrator Standard Operating Procedure (SOP) 030 (Automated Concentrator Ultrafiltration Protocol), which is in Appendix D of this report. The resulting concentrated sample will be placed into sterile 100 mL sample bottles and analyzed in the same manner as all other *B. globigii* BWS.

WaterStep Mobile Water System

B. globigii water samples will be removed from the lagoon to assess the disinfection capability of

the MSW. A sampling position in the lagoon will be chosen so that is away from the MSW effluent entering the lagoon. This will allow for some mixing in the lagoon to occur. Samples will be collected using the grab sampling technique in 100 mL sterile sample bottles with a sodium thiosulfate tablet. Samples will be removed from the lagoon with a sterile pipette, and the contents of the pipette dispensed into the bottle. The pipette tip will be lowered 4 to 6 inches (10 to 15 cm) below the water surface when collecting a sample. The samples will be transferred to a cooler at 4±2°C. The samples will be shipped overnight to the EPA T&E Facility and analyzed upon receipt.

Sampling Containers and Quantities

Sample containers and quantities are shown in Table A7.

Sample Preservation and Holding Times

Sample preservation and holding times are shown in Table A7.

Sample Labeling

Sample identification is summarized below.

Samples collected for analysis will be identified by type (BWS, CP, or Grab), collection interval (-0, -1, -2, etc.), analysis (*B. globigii*, HPC, chlorine dioxide (ClO_2), free chlorine), and date collected. Duplicate coupons will be identified using a "D" after the collection interval.

Table A7. Grab Sampling and Analytical Procedures

Measurement	Sampling Method	Analysis Method	Sample Container/ Quantity of Sample	Preservation/ storage	Holding times
Chlorine Dioxide	See Specific Sampling Procedures	Hach Method 10126	Glass beaker (~50 mL)	None	Immediate
Free Chlorine	See Specific Sampling Procedures	Hach Method 10102	Glass beaker (~50 mL)	None	Immediate
B. globigii spore	See Specific Sampling Procedures	CB&I T&E SOP 309 (Appendix C)	100 mL sterile sample bottles 20 L Cubitainers[1]	The bottles contains sodium thiosulfate tablet. Cool 4 ± 2°C	Analyze upon receipt at the EPA T&E Facility.
HPC	See Specific Sampling Procedures	CB&I T&E SOP 304 (Appendix B)	100 mL sterile sample bottles	The bottles contain sodium thiosulfate tablets. Cool 4 ± 2°C	48 hours

HPC, heterotrophic plate count; SOP, standard operating procedure; T&E, Test and Evaluation
[1]The 20 L Cubitainer samples will be concentrated via the water sample concentrator and placed into the 100 mL sterile sample bottles for analysis.

Sample Packaging and Shipping

The biofilm samples, BWS, and WSC samples will be preserved in coolers with ice and shipped to the EPA T&E Facility overnight. Chain of custody forms will be completed and shipped with the samples.

MEASUREMENT PROCEDURES

ANALYTICAL METHODS

The analysis methods are shown in Table A7. The microbiological methods are further discussed below.

HPC determinations will follow T&E SOP 304, *Heterotrophic Plate Count (HPC) Analysis Using IDEXX SimPlate Method*. This method is based on multiple enzyme technology which detects viable bacteria in water by testing for the presence of key enzymes known to be present in these organisms. It uses multiple enzyme substrates that produce a blue fluorescence when metabolized by bacteria. The sample and media are added to a SimPlate® plate (Idexx Laboratories, Inc., Westbrook, ME), incubated, and then examined for fluorescent wells. The number of fluorescing wells corresponds to a most probable number (MPN) of total bacteria in the original sample. This method is included as Appendix B in this document.

Preparation and analysis of *B. globigii* will follow T&E SOP 309, *Preparation and Enumeration of B. globigii Endospores*. *B. globigii* is an aerobic spore-forming bacteria used as a surrogate for evaluating the performance of water treatment systems for removal of bacterial endospores. In analyzing spores, the indigenous vegetative cells are inactivated by heat treatment. The surviving bacterial spores in the sample are analyzed by culturing that permits the spores to germinate and produce bacterial cells. Tryptic soy agar will be used for culturing *B. globigii*. This method is included as Appendix C in this document.

The samples are diluted, as necessary, depending on the expected concentration of cells/spores in the sample. For example, the expected initial concentration of spores in this study is 10^6 spores/mL. The initial samples will be diluted up to 10^5 fold. Duplicate plates using 0.1 mL of the 10^4 and 10^5 fold diluted samples will be analyzed using the spread plate method. If the number of colonies is too many to count in more than one plate, the sample will be diluted and re-analyzed. If the number of colonies is too many to count for one measurement, the remaining plates will be considered for enumeration of spore concentration for the sample.

CALIBRATION PROCEDURES

The calibration procedures, linearity checks, and continuing calibration checks are included in the T&E SOPs or the instrument manuals for the analysis methods referenced in Table A7.

QUALITY METRICS (QA/QC CHECKS)

QC Checks

Instruments/equipment will be maintained in accordance with the SOPs and analysis methods listed in Table A7, and for field instruments, in accordance with the manufacturer's instructions. Table A8 presents the QA/QC checks to be implemented for the measurement of the specific parameters.

Table A8 lists the QA/QC checks that will be used to verify the validity of the analyses conducted on grab samples conducted during this study. Table A9 summarizes the QA/QC

requirements for the optical devices used in this study.

The RPD is calculated for duplicate analyses based on the following:

$$RPD = \frac{(C_1 - C_2)}{0.5(C_1 + C_2)} \times 100\%$$

where:
RPD = Relative Percent Difference
C1 = Larger of two values
C2 = Smaller of two values

If calculated from three or more replicates, the relative standard deviation (RSD) will be used according to the following equation:

$$RSD = 100\% \ \frac{s}{y_{ave}}$$

where:
RSD = relative standard deviation (%)
s = standard deviation
y_{ave} = mean of the replicate analyses

Standard deviation is defined as follows:

$$s = \sqrt{\sum_{i=1}^{n} \frac{(y_i - y_{ave})^2}{n-1}}$$

where:
s = standard deviation
y_i = measured value of the ith replicate
y_{ave} = mean of the replicate measurements
n = number of replicates

Table A8. QA/QC Checks for Grab Samples

Measurement	QA/QC Check	Frequency	Acceptance Criteria	Corrective Action
B. globigii	Positive control using stock	Once per experiment	±10 fold of the spiking suspension	Investigate laboratory technique. Change stock organisms and use new set of media plates. Re-analyze the spiking suspension and change it if necessary.
B. globigii	Negative Control using sterile buffer	Once per experiment	0 CFU/plate	Investigate laboratory technique. Use a new lot. Re-analyze.

B. globigii	Negative control for heat shock	Once per experiment	0 CFU of vegetative cell/plate	Investigate the hot water bath. Heat samples for longer period.
B. globigii	Duplicate	Once per experiment	≤20% variation	Consider other dilutions. Reanalyze.
B. globigii	Field blank (an open bottle of sterile water in the vicinity of the BWS location)	Every 5 BWS	0 CFU/plate	Determine if background values impact results.
HPC	Negative Control	Before every set of measurements	No fluorescent wells	Re-analyze sterile buffer and change it if necessary.
HPC	Positive Control	Once per experiment	Fluorescent wells	Investigate laboratory technique. Re-analyze.
HPC	Duplicate	Once per experiment	Duplicate plates much agree within 5%	Investigate laboratory technique. Re-analyze.
Chlorine Dioxide/ Free Chlorine	Manufacturer DPD color standards kit	Once per experiment	As specified by the color standards kit	Clean the colorimeter measuring cell. Clean the DPD standards vials and recheck.

BWS, bulk water sample; CFU, Colony Forming Unit; HPC, heterotrophic plate count

Table A9. Quality Assurance/Quality Control (QC) Checks for Online Equipment

Instrument/ Measurement	Calibration/QC Alternative	Frequency	Acceptance Criteria	Corrective Action
RealTech UV254/ TOC	Custom Zero using deionized water	One time per quarter according to instrument O/M manual	N/A	Clean the quartz windows using 5% bleach solution.
Hach CL-17/ Free Chlorine	**Factory calibration – do not change**. Perform a one-point check against a DPD colorimetric method calibration based on DPD method	Quarterly	±10%	Clean colorimeter and check the instrument flow.

TOC, total organic carbon; UV, ultraviolet; N/A, not applicable

DATA ANALYSIS, INTERPRETATION, AND MANAGEMENT

Data Reporting Requirements

All data generated during the study will be presented in tabular/spreadsheet format. Table A10 identifies the reporting units for the various parameters.

Table A10. Reporting Units for Measurements

Measurement	Units

Bacillus globigii	CFU/ mL
HPC (heterotrophic plate count)	MPN/ mL
Chlorine Dioxide	mg/L
Free Chlorine	mg/L
TOC (total organic carbon)	mg/L

CFU, colony forming unit; MPN, most probable number

Data Validation Procedures

Calculations will be carried out on a computer and will be checked initially by the analyst for gross error and miscalculation. The calculations and data entered into computer spreadsheets will be checked by a peer reviewer for accuracy, and checking the calculation by hand or checking entries of data from the original. Detected errors will be corrected and other data in the same set investigated before it is released to the EPA work assignment contract officer's representative (WACOR).

Data Summary

All sample data will be presented by CB&I in tabular/spreadsheet format and submitted to the EPA WACOR for evaluation. Tabular data summaries will be included in the main discussion of the reports and raw data will be included as appendices.

Data Storage

Laboratory records will be maintained in accordance with Section 13.2, *Paper Laboratory Records*, of the Office of Research and Development (ORD) Policies and Procedures Manual. Controlled access facilities that provide a suitable environment to minimize deterioration, tampering, damage, and loss will be used for the storage of records. Whenever possible, electronic records will be maintained on a secure network server that is backed up on a routine basis. Electronic records that are not maintained on a secure network server will be periodically backed up to a secure second source storage media, transferred to an archive media (e.g., compact discs, optical discs, magnetic tape, or equivalent), or printed. Electronic records that are to be transferred for retention will be transferred to an archive media or printed, as directed by EPA.

Appendix B: T&E SOP 304, Heterotrophic Plate Count (HPC) Analysis Using IDEXX SimPlate® Method.

Shaw Environmental & Infrastructure, Inc.

EPA T&E Contract Technical Standard Operating Procedure

Heterotrophic Plate Count (HPC) Analysis Using IDEXX SimPlate Method

T&E SOP 304

Revision Number: 1
Revision Date: 02/08/2012

SOP Approval

1. E. Radha Krishnan, P.E.
Program Manager

Signature — Date: 02/10/2012

2. Steven Jones, ASQ CQA/CQE
Quality Assurance Manager

Signature — Date: 02/10/2012

Revision Summary

Revision	Name	Date	Description of Change
0	Nur Muhammad	01/31/2006	Developed SOP.
1	Nancy Shaw/ Steven Jones	01/25/2012	Revised Sections 1, 2, 4, 6, 7, 8, 9.2, 10 and 12. Added Attachments A and B.

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1.0 Scope and Applicability

The method described in this standard operating procedure (SOP) is applicable to the enumeration of heterotrophic bacteria, generally known as heterotrophic plate counts (HPC), in water and wastewater samples.

2.0 Summary of Method

IDEXX SimPlate method for quantification of HPC is based on multiple enzyme technology which detects viable bacteria in water by testing for the presence of key enzymes known to be present in these organisms. It uses multiple enzyme substrates that produce a blue fluorescence when metabolized by bacteria. The sample and media are added to a SimPlate plate, incubated and then examined for fluorescing wells. The number of fluorescing wells corresponds to a Most Probable Number (MPN) of total bacteria in the original sample.

3.0 Definitions

3.1 HPC – Heterotrophic Plate Count

3.2 IDEXX – Biological system and reagent developing company.

3.3 SimPlate – Registered trademark of BioControl Systems Inc., and is used by IDEXX under license from BioControl System Inc.

4.0 Health and Safety Warnings

4.1 Standard laboratory personal protective equipment (i.e., laboratory coat, gloves, and safety glasses) is required. In addition, any chemical-specific or project-specific protective gear required will be described in the project-specific Health and Safety Plan (HASP).

4.2 If using an ultraviolet (UV) light system without a viewing chamber, wear UV protective safety glasses and direct light away from eyes.

4.3 Special precautions, such as wearing heat-resistant gloves, are required for autoclaving.

5.0 Cautions

Samples collected for analysis in accordance with this Standard Operating Procedure (SOP) shall be preserved at 4±2 ^{o}C after collection and processed preferably within 48 hours after sample collection.

6.0 Interferences

6.1 Contamination during analysis affects the results. Aseptic technique should be followed during analysis.

6.2 Chlorinated samples should be treated with sodium thiosulfate prior to testing.

7.0 Personnel Qualifications

The techniques of a first time analyst shall be reviewed by an experienced analyst prior to initiating this SOP alone. During this review, the new analysts will be expected to demonstrate their capability to perform this analysis.

8.0 Equipment and Supplies

8.1 IDEXX multi dose sterile media

8.2 IDEXX sterile SimPlate plates with lids

8.3 10 ml sterile disposable pipettes

8.4 Sterile dilution buffer (90 ml vials) from Hardy Diagnostics (www.hardydiagnostics.com; Cat # D690)

8.5 UV light set (6 watt, 365 nm) with viewing chamber

8.6 Incubator capable of maintaining a temperature of 35±0.5 ^{0}C

8.7 SimPlate® For HPC Most Probable Number (MPN) Table (supplied with the IDEXX media and plates)

8.8 100 ml sampling bottles with sodium thiosulfate (0.01% w/v) (Fisher Scientific, Cat..No. 09 730 91)

8.9 Autoclave capable of sterilizing with fast, liquid, and dry cycles

9.0 Procedure

9.1 Sample Collection, Handling, and Analysis

9.1.1 Use 100 ml sampling bottles containing sodium thiosulfate for sample collection.

9.1.2 Samples should be transported to the laboratory immediately and stored at 4±2 ^{0}C until processed.

9.1.3 Samples should be processed within 48 hours of sample collection.

9.2 Media Preparation and Sample Analysis

9.2.1 Open the IDEXX multi dose media vessel and add 100 ml sterile dilution buffer. Re-cap the vessel and shake to dissolve the media properly.

9.2.2 Prepare serial dilutions of the sample if necessary.

9.2.3 Pipette 1 ml sample and then 9 ml of the re-hydrated IDEXX multi dose media onto the center of an IDEXX SimPlate plate base.

9.2.4 Cover the SimPlate plate with lid and gently swirl to distribute the sample into all the wells.

9.2.5 Tip the plate 90 – 120^{0} to drain excess sample into the absorbent pad.

9.2.6 Invert the plate, and incubate for 45 - 72 hours at 35±0.5 ^{0}C.

9.2.7 Remove cover and put the plate in the UV system viewing chamber. Turn the UV light (Section 8.5) on 5 inches above the plate, and count the number of fluorescent wells.

9.2.8 Refer to the SimPlate® For HPC Most Probable Number (MPN) Table (see Attachment A) to determine the MPN of heterotrophic plate count bacteria in the original sample. Report the MPN to reflect the dilution used. For example, if 1 mL of a 1:10 dilution of the original sample was tested, then the reported MPN is the table number multiplied by 10 and the result is reported as MPN per 10 mL.

9.2.9 Record the analysis date, dilutions, number of fluorescence wells and heterotrophic bacterial counts on Attachment B, *Datasheet for Heterotrophic Plate Count Analysis*.

9.2.10 Autoclave the plates to sterilize, and dispose of the plates.

9.2.11 Refrigerate any unused rehydrated media and discard after 5 days if not used.

9.2.12 Store dehydrated media in the dark at room temperature.

10.0 Data and Records Management

10.1 All original analysis documentation generated and prepared for the U.S. Environmental Protection Agency (EPA) shall be controlled in accordance with Shaw T&E SOP 101, *Central Files*.

10.2 All data packages shall be assembled and reviewed per Shaw T&E SOP 102, *Data Review and Verification*.

11.0 Quality Control and Quality Assurance

11.1 Negative Control - test a negative control following the test procedure using 10 ml re-hydrated media before every set of measurements. No wells should fluorescence after incubation. In case of failure, use a new media vessel and dilution buffer.

11.2 Positive Control – test a positive control following the test procedure using 10 mL dechlorinated tap water to rehydrate the media. An acceptable positive control should yield 10 – 30 fluorescent wells (21 – 74 MPN) or more. To dechlorinate, add tap water to 100 ml sampling bottle containing sodium thiosulfate (Section 8.8).

11.3 Duplicate - for verification purposes, perform tests in duplicate per sample dilution and for each positive control. Counts from duplicate plates must agree within 5%.

12.0 References

12.1 IDEXX. *Instructional Manual for SimPlate for HPC Multi Dose*, Maine, USA.

12.2 Shaw Environmental & Infrastructure, Inc., 2011. EPA T&E Contract Administrative SOP 101, *Central Files*.

12.3 Shaw Environmental & Infrastructure, Inc., 2011. EPA T&E Contract Administrative SOP 102, *Data Review and Verification*.

12.4 Standard Methods for the Examination of Water and Wastewater, 20th edition, 1998. Method 9215 A, Heterotrophic Plate Count. American Public Health Association.

Attachment A - MPN Tables (Page 1 of 2)

Unit-Dose

SimPlate® For HPC

Most Probable Number (MPN) Table

# Positive Wells	MPN	95% confidence limits	
		lower	upper
0	<0.2	<0.03	<1.4
1	0.2	0.03	1.4
2	0.4	0.1	1.6
3	0.6	0.2	1.9
4	0.8	0.3	2.2
5	1.0	0.4	2.5
6	1.2	0.6	2.7
7	1.5	0.7	3
8	1.7	0.8	3.3
9	1.9	1	3.6
10	2.1	1.1	3.9
11	2.3	1.3	4.2
12	2.6	1.5	4.5
13	2.8	1.6	4.8
14	3.0	1.8	5.1
15	3.3	2.0	5.4
16	3.5	2.2	5.8
17	3.8	2.3	6.1
18	4.0	2.5	6.4
19	4.3	2.7	6.7
20	4.5	2.9	7
21	4.8	3.1	7.4
22	5.1	3.3	7.7
23	5.3	3.5	8.0
24	5.6	3.8	8.4
25	5.9	4	8.7
26	6.2	4.2	9.1
27	6.5	4.4	9.4
28	6.8	4.7	9.8
29	7.1	4.9	10.2
30	7.4	5.1	10.6
31	7.7	5.4	10.9
32	8.0	5.6	11.3
33	8.3	5.9	11.7
34	8.6	6.2	12.1
35	9.0	6.4	12.6
36	9.3	6.7	13.0
37	9.7	7	13.4
38	10.0	7.3	13.9
39	10.4	7.6	14.3
40	10.8	7.9	14.8
41	11.2	8.2	15.2
42	11.6	8.5	15.7

# Positive Wells	MPN	95% confidence limits	
		lower	upper
43	12.0	8.8	16.2
44	12.4	9.1	16.7
45	12.8	9.5	17.3
46	13.2	9.8	17.8
47	13.7	10.2	18.3
48	14.1	10.6	18.9
49	14.6	10.9	19.5
50	15.1	11.3	20.1
51	15.6	11.7	20.7
52	16.1	12.1	21.3
53	16.6	12.5	22.0
54	17.1	13.0	22.7
55	17.7	13.4	23.4
56	18.3	13.9	24.1
57	18.9	14.4	24.9
58	19.5	14.9	25.7
59	20.2	15.4	26.5
60	20.9	15.9	27.3
61	21.6	16.5	28.2
62	22.3	17.1	29.2
63	23.1	17.7	30.2
64	23.9	18.3	31.2
65	24.8	19.0	32.3
66	25.7	19.7	33.5
67	26.6	20.4	34.7
68	27.6	21.2	36.1
69	28.7	22.0	37.5
70	29.9	22.9	39.0
71	31.1	23.8	40.7
72	32.4	24.8	42.5
73	33.9	25.8	44.4
74	35.5	27.0	46.6
75	37.2	28.2	49.1
76	39.2	29.6	51.9
77	41.4	31.1	55.1
78	44.0	32.8	58.9
79	47.0	34.8	63.6
80	50.7	37.1	69.5
81	55.5	39.8	77.5
82	62.3	43.2	89.9
83	73.8	47.6	114.6
84	>73.8	>47.6	>114.6

MPN is per ml of sample (pour-off is accounted for).

Attachment A — MPN Tables (Page 2 of 2)

Multi-Dose

SimPlate® For HPC

Most Probable Number (MPN) Table

# Positive Wells	MPN	95% confidence limits lower	upper
0	<2	<0.3	<14
1	2	0.3	14
2	4	1	16
3	6	2	19
4	8	3	22
5	10	4	25
6	12	6	27
7	15	7	30
8	17	8	33
9	19	10	36
10	21	11	39
11	23	13	42
12	26	15	45
13	28	16	48
14	30	18	51
15	33	20	54
16	35	22	58
17	38	23	61
18	40	25	64
19	43	27	67
20	45	29	70
21	48	31	74
22	51	33	77
23	53	35	80
24	56	38	84
25	59	40	87
26	62	42	91
27	65	44	94
28	68	47	98
29	71	49	102
30	74	51	106
31	77	54	109
32	80	56	113
33	83	59	117
34	86	62	121
35	90	64	126
36	93	67	130
37	97	70	134
38	100	73	139
39	104	76	143
40	108	79	148
41	112	82	152
42	116	85	157

# Positive Wells	AWN	95% confidence limits lower	upper
43	120	88	162
44	124	91	167
45	128	95	173
46	132	98	178
47	137	102	183
48	141	106	189
49	146	109	195
50	151	113	201
51	156	117	207
52	161	121	213
53	166	125	220
54	171	130	227
55	177	134	234
56	183	139	241
57	189	144	249
58	195	149	257
59	202	154	265
60	209	159	273
61	216	165	282
62	223	171	292
63	231	177	302
64	239	183	312
65	248	190	323
66	257	197	335
67	266	204	347
68	276	212	361
69	287	220	375
70	299	229	390
71	311	238	407
72	324	248	425
73	339	258	444
74	355	270	466
75	372	282	491
76	392	296	519
77	414	311	551
78	440	328	589
79	470	348	636
80	507	371	695
81	555	398	775
82	623	432	899
83	738	476	1146
84	>738	>476	>1146

MPN is per ml of sample (pour-off is accounted for).

Attachment B – Datasheet for Heterotrophic Plate Count Analysis

Analysis Date: _____ Work Assignment: _____

Sterile Dilution Buffer (for negative control) Lot #: _____ Exp. Date: _____

Sodium Thiosulfate Bottle (for positive control) Lot #: _____ Exp. Date: _____

Sample ID	Dilution Factor	# of Fluorescent Wells	Heterotrophic Bacteria (MPN / mL)	Heterotrophic Bacteria x dilution factor (MPN / mL)

Quality Control

Negative control buffer analyzed?	☐ Yes	☒ No
Negative control results acceptable (no yellow or fluorescent wells)?	☐ Yes	◉ No
Positive control results acceptable (5 – 30 fluorescent wells)?	☐ Yes	☐ No

Comments: _____

_____

_____

Analyst: _____ Date: _____

Reviewed by: _____ Date: _____

Appendix C: T&E SOP 309, Preparation and Enumeration of *Bacillus globigii* Endospores.

Shaw Environmental & Infrastructure, Inc.

EPA T&E Contract Technical Standard Operating Procedure

Preparation and Enumeration of *B. globigii* Endospores

T&E SOP 309

Revision Number: 2

Revision Date: 11/12/2012

SOP Approval

1. **E. Radha Krishnan, P.E.**
Program Manager

Signature	Date
	11/12/2012

2. **Steven Jones, ASQ CQA/CQE**
Quality Assurance Manager

Signature	Date
	11/12/2012

Revision Summary

Revision	Name	Date	Description of Change
0	Nur Muhammad	03/24/2010	Developed SOP.
1	Nancy Shaw / Steven Jones	07/27/2012	Revised Sections 4, 5, 6, 8, 9, 12 and Attachment A to update SOP.
2	Lee Heckman/ Gune Silva	11/12/2012	Revised Attachment A datasheet to incorporate columns for volume plated, average plate count, and reported results to provide additional information for peer review of the data.

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1.0 Scope and Applicability

The method described in this standard operating procedure is applicable to the preparation and purification of *Bacillus globigii (B. globigii)* endospores for use in research studies. This method is also applicable for the enumeration of stock and sample concentrations of *B. globigii*.

2.0 Summary of Method

B. globigii is an aerobic spore-forming bacteria used as a surrogate for *B. anthracis*. For the preparation of *B. globigii* stock, a culture of vegetative cells (stock) is mixed with generic sporulation media and incubated by gentle shaking (~150 rpm) at 35°C for five days. The presence of spores is confirmed using phase-contrast microscopy (<0.1% vegetative cells). The spores are purified using gradient separation (Section 9.5) and preserved in 40% ethanol in a refrigerator at 4 ± 2°C until use. The *B. globigii* stock is heat-shocked and analyzed using standard membrane filtration for determining the stock concentration.

3.0 Definitions

3.1 Biosafety Levels – The degree of containment (or the combinations of standard and special practices, safety equipment, and facility design criteria) appropriate for the operations performed and the biohazardous agents used within the laboratory.

3.2 CFU – Colony Forming Units

3.3 Negative Control – A sample that does not contain the desired analyte. It ensures that a test, its components, or the environment do not cause undesired effects, or produce incorrect test results.

3.4 Positive Control – A sample that contains a known concentration of the desired analyte. It ensures that a test and/or its components are working properly and producing expected results.

4.0 Health and Safety Warnings

4.1 Standard laboratory personal protective equipment (i.e., laboratory coat, gloves, safety glasses) is required. In addition, any project-specific protective gear is required as described in the project-specific Health and Safety Plan.

4.2 *B. globigii* are saprophytic organisms, whose principal habitat is soil. They do not pose a public health risk. Standard Biosafety Level 1 laboratory precautions apply for its microbiological analysis.

4.3 Special precautions, e.g., wearing heat-resistant gloves, are required for autoclaving the accessories.

5.0 Cautions

Spores prepared in accordance with this SOP require preservation in a refrigerator at 4±2°C immediately after preparation.

6.0 Interferences

Contamination interferes with accurate and precise enumeration and measurement. Preventive measures guarding against contamination include maintaining sterility throughout the processing procedure by using aseptic techniques and sterile containers when processing samples. In addition, the stock concentration may decline by germination if kept at room temperature. Declining concentration is prevented by transferring the stock to a refrigerator at 4±2°^{o}C immediately after use.

7.0 Personnel Qualifications

7.1 The techniques of a first-time scientist/ technician shall be reviewed by a senior scientist/ technician prior to initiating this SOP alone.

8.0 Equipment and Supplies

8.1 Non-consumable Equipment

8.1.1 Sterile sampling bottles (100 mL) with Sodium Thiosulfate (Corning Inc.)

8.1.2 Sterile Erlenmeyer flasks (250 mL) with screw-caps

8.1.3 Thermostatically controlled water bath with a 250 mL pilot flask and thermometer capable of registering temperature with a range of 37 to 90^{o}C

8.1.4 Incubator (35 – 37^{o}C)

8.1.5 Autoclave

8.1.6 UV Sterilizer

8.1.7 Ice bath

8.1.8 Hot plate

8.1.9 Colony counter

8.1.10 Tweezers

8.1.11 1-L glass bottles/flask

8.1.12 Fixed-angle rotor centrifuge capable of operating at 5,860 rcf

8.1.13 Swinging-bucket rotor centrifuge capable of operating at 5,860 rcf

8.1.14 50 mL Centrifuge tube with conical bottom

8.1.15 Phase Contrast Microscope

8.2 Consumable Equipment

8.2.1 Individually packed sterile plastic pipettes

8.2.2 60 × 15 mm Petri dishes with loose lids

8.2.3 47 mm, 0.45 µm porosity sterile membranes

8.2.4 Membrane filtration apparatus

8.2.5 Membrane filters with 47 mm diameter and a 0.45 micron pore size

8.3 Consumable Reagents

8.3.1 Sterile dilution phosphate buffer (90 mL bottles)

8.3.2 Peptone

8.3.3 Beef extract

8.3.4 Trypan blue dye

8.3.5 Agar

8.3.6 Nutrient broth

8.3.7 Manganese sulfate

8.3.8 Calcium chloride

8.3.9 Deionized water

8.3.10 *B. globigii* vegetative cells/stock

8.3.11 Either *Enterococcus faecium* (ATCC 35667), *Escherichia Coli* (ATCC13706) or *Pseudomonas aeroginosa* (ATCC27853)

8.3.12 Ethanol (95%)

8.3.13 Hypaque solution

9.0 Procedure

9.1 Preparation of Generic Sporulation Media

9.1.1 Measure 8 g nutrient broth, 40 mg manganese sulfate, and 100 mg calcium chloride into a 1 L glass bottle or flask, and add 1 L distilled water. Stir the contents until dissolved.

9.1.2 Aliquot 100 mL into ten 250 mL flasks.

9.1.3 Sterilize in the autoclave for 15 minutes at 15 psi and 121^{o}C.

9.1.4 Store the media at room temperature.

9.2 Preparation of Nutrient Agar

9.2.1 Measure and pour 5 grams (g) peptone, 3 g beef extract, 15 g agar and 0.015 g Trypan blue dye in a 1-liter (L) glass bottle and add 1-L distilled water. Heat to boiling using a hot plate and a stir bar for complete mixing.

9.2.2 Sterilize in the autoclave for 15 minutes at 15 psi and 121^{o}C.

9.2.3 After cooling to 25^{o}C, pour 5 to 6 mL of agar media in each Petri dish. Leave the plates covered at room temperature until solidified. Store the plates in a refrigerator at 4±2 ^{o}C.

9.2.4 Bring the required number of plates out of the refrigerator and warm them to room temperature for one hour before use.

9.2.5 If any type of growth other than the desired culture is observed, the plates are considered to be contaminated. If contamination is found, discard the plates and prepare new ones.

9.3 Controls

9.3.1 Positive control is prepared by spiking a known concentration of *B. globigii* into sterile water followed by membrane filtration and incubation at 35 – 37°C for 24 hours. Formation of colonies at the appropriate concentration is the acceptance criteria for a *B. globigii* positive control. If the acceptance criteria are not obtained, the result of the test is used as the true starting count of the stock and future positive controls are spiked with known concentrations based on this starting count. If the positive control is negative or contaminated with vegetative cells, the entire test (controls plus unknown samples) are repeated with new plates and controls.

9.3.2 Negative control for heat-shock is obtained by spiking one of the following into sterile water: *Enterococcus faecium* (ATCC 35667), *Escherichia Coli* (ATCC13706) or *Pseudomonas aeroginosa* (ATCC27853). The spiked sample is then heat-shocked and subjected to membrane filtration and incubation at 35 to 37°C for 24 hours. Formation of colonies indicates inadequate heat shock for killing the vegetative cells. The temperature of the heat shock water bath and the sterility capabilities of the autoclave will be monitored and the entire test (controls plus unknowns) will be repeated if the negative control produced growth (is positive).

9.3.3 Negative control for buffer is obtained by filtering 100 mL of sterile buffer and incubating the membrane at 37°C for 24 hours followed by membrane filtration and incubation at 35 to 37°C for 24 hours. Formation of colonies indicates contaminated dilution buffer and the entire test (controls plus unknowns) will be repeated using fresh buffer of a different lot.

9.4 Preparation of Endospores

9.4.1 Inoculate a flask of generic sporulation media (100 mL) with 1 mL of *B. globigii* vegetative cells/stock.

9.4.2 Incubate with continuous gentle shaking (~ 150 rpm) at 35 °C for at least five days (120 hours).

9.4.3 Check solution for the presence of spores with a wet mount slide preparation using phase contrast microscopy.

9.4.4 When the slide preparation reveals an adequate spore suspension (approximately 1 x 10^9 cfu/mL), proceed with the purification.

9.5 Purification of Endospores

9.5.1 Aseptically transfer the contents of each flask into sterile 35 mL centrifuge tubes. Balance the tubes and centrifuge at approximately 5,860 relative centrifugal force (rcf) for 20 minutes, using a fixed-angle rotor.

9.5.2 Pour off the supernatant into a discard beaker. Add 30 mL of cold, sterile deionized water to each tube. Vortex each tube until the spores are completely resuspended in the water. Centrifuge again at approximately 5,860 rcf for 10 minutes. Discard the supernatant and resuspend in 30 mL of cold, sterile deionized water per tube. Centrifuge for another 10 minutes as before and discard the supernatant. Autoclave the contents of the discarded supernatant and discard appropriately.

9.5.3 Combine the contents of the tubes into one tube or into multiples that will allow ease of centrifugation. Aseptically add 30mL of cold, sterile deionized water to each tube and resuspend the spores.

9.5.4 Combine 58 mL of Hypaque solution with 42 mL of sterile, deionized water. Mix well. Add 12 mL of the Hypaque solution to clean, sterile 35 mL centrifuge tubes. Pipette the spore suspension, carefully layering it on top of the Hypaque solution. Centrifuge at approximately 5,860 rcf for 30 minutes using a swinging bucket rotor.

9.5.5 Pour off and discard the supernatant. Add 30 mL of cold, sterile deionized water to the pellet in each tube and resuspend the spores. Centrifuge at 5,860 rcf for 15 minutes using a fixed-angle rotor.

9.5.6 Discard the supernatant and resuspend the spores in 30 mL of cold, sterile deionized water. Wash the spores by centrifuging at 5,860 rcf and resuspending twice more. Centrifuge again, discard the supernatant, and resuspend the spores in a 40% (v/v) ethanol solution. Store in a refrigerator at 4±2°C.

9.6 Enumeration of Stock/Sample Concentration

9.6.1 Prepare a 90°C water bath using a 50:50 mixture of deionized water and tap water. Add enough water to the bath so that when the samples are immersed, the water level meets the sample volume level.

9.6.2 Prepare serial dilutions of the stock/samples using sterile buffer and select at least two dilutions for analysis. Shake the diluted stocks/samples vigorously 25 times and transfer to appropriately labeled 250 mL Erlenmeyer flasks.

9.6.3 Place the samples and the pilot flask (containing distilled water) into the 90°C shaker water bath. Shake the samples at 60 to 80 rpm. Monitor the temperature in the pilot flask until it is 80°C.

9.6.4 Set a timer and incubate the samples and pilot flask for 10 minutes after the water reaches 80°C.

9.6.5 Quickly remove the samples and pilot flask from the water bath and place them into a slurry of ice and water to reduce temperature to approximately room temperature as soon as possible.

9.6.6 Use the **Membrane Filtration** apparatus and filter the samples according to the following procedure:

9.6.6.1 Turn on the Millipore vacuum system.

9.6.6.2 Wet a membrane support if necessary with 5 mL of sterile phosphate buffer.

9.6.6.3 Dip a pair of metal tweezers into methanol and flame them until the methanol is consumed. Using the sterilized tweezers, lay a membrane on to the support, avoiding any contamination by refraining from touching the membrane to any other surface.

9.6.6.4 Rinse the funnel with 10 mL sterile buffer and drain it.

9.6.6.5 Add 10 mL of sterile buffer to the funnel. Dispense 10 mL of sample with a sterile disposable pipette and filter.

9.6.6.6 Rinse and drain the funnel twice using 10 mL aliquots of sterile phosphate buffer.

9.6.6.7 Unscrew the funnel and place it in the UV sterilizer.

9.6.6.8 Carefully remove the membrane from the support, avoiding contamination, and place it on a labeled nutrient agar Petri plate.

9.6.6.9 Place the membrane supports and tweezers in the UV sterilizer.

9.6.6.10 Sterilize the whole assembly for at least five minutes.

9.6.6.11 Conduct duplicate analysis for each dilution of the stock/samples.

9.6.7 Incubate plates at 35 to 37°C for 24 hours.

9.6.8 The aim is to have 10 to 80 colonies per plate; adjust the dilution of the samples if necessary.

9.6.9 Count every colony as a spore forming organism using a colony counter, and report results of the analysis on Attachment A – Datasheet for *Bacillus globigii* Endospores.

10.0 Data and Records Management

All original laboratory data records shall be maintained in accordance with T&E SOP 101, *Central Files*.

11.0 Quality Control and Quality Assurance

11.1 It is necessary to carry out the positive and negative control tests prior to every set of analysis. If the positive control fails, change the stock organisms and use a new set of media plates. If the negative control fails, re-run the test, heating the sample for a longer period. In the case of negative control for buffer failure, use a new lot.

11.2 Duplicate analysis is required for each sample. A 20% variation is acceptable for duplicate samples. However, in case of failure of a duplicate sample, consider the other dilutions for enumeration of bacteria. If duplicates for all dilutions fail, discard the sample and re-run the experiment.

11.3 If the number of colonies counted is less than 30, filter a greater volume of sample; if the number of colonies count is greater than 80, further dilute the sample.

12.0 References

12.1 Shaw Environmental & Infrastructure, Inc. (2011). EPA Test and Evaluation Contract Administrative SOP 101: *Central Files*.

12.2 Shaw Environmental & Infrastructure, Inc. (2012). EPA Test and Evaluation Contract Technical SOP 301: *Enumeration of Bacillus Subtilis in Water Samples*.

ATTACHMENT A – DATASHEET FOR *BACILLUS GLOBIGII* ENDOSPORES

Analysis Date: _____ Work Assignment: _____

Stock Concentration: _____ Stock ID: _____

Dilution Buffer Lot No.: _____ Expiration Date: _____

Sample ID	Volume Plated	Colony Counts		Average Plate Count	Dilution Factor	*B. globigii* (CFU/ _____ mL)
		Plate #1	Plate #2			

Control Samples:

Positive control acceptable (colonies observed)? ☐ Yes ☐ No

Negative control for heat shock acceptable (no growth observed)? ☐ Yes ☐ No

Negative control buffer acceptable (no growth observed)? ☐ Yes ☐ No

All duplicate results within 20% RPD? ☐ Yes ☐ No

Comments: _____

_____

_____

_____

Analyst: _____ Date: _____

Appendix D: Operation of the Water Sample Concentrator

Introduction:

There is a need for detection of biological contaminants that could be used as weapons of terrorism against the nation's water supplies. Ultrafiltration is a method of concentrating a large volume of water to detect microorganisms. This procedure outlines the basic Ultrafiltration process. Changes have been made and are to be made to this process as research has been progressing.

Materials:

Appropriate PPE

1. Disposable lab gown
2. N95 Particulate respirator
3. Disposable nitrile gloves
4. Safety glasses

Reagents

1. 2.5 % Bovine Serum Albumin – SOP NHSRC 001
2. Diluent – SOP NHSRC 002
3. Backwash – SOP NHSRC 003
4. Filter Block – SOP NHSRC 004
5. 10 % Thiosulfate – SOP NHSRC 005
6. Eluting Solution (0.001 % Tween 80) – SOP NHSRC 006
7. 10 % Bleach – SOP NHSRC 007
8. 70% Ethanol – SOP NHSRC 028

Equipment

1. Tube cutter (Cole Parmer catalog number EW-0638-10 or equivalent)
2. Sample carboy [For example, a 50-liter autoclavable polypropylene (pp) carboy with handles, with pp leak proof screw cap, without tubulation, Fisher Scientific catalog number 02-960 20B, Nalgene Nunc International No. 2250-0130; however, depending on the specific logistics or application, other carboys may be more appropriate.]
3. Filter, Rexeed 25S [Rexbrane Membrane High-Flux 2.5ml/2; manufactured by Asahi Kasei Medical America Inc. Henry Schein Item #6292966]
4. Tubing (all tubing is listed with US Plastics 2009 product number):
    a. Masterflex Tygon (R-3603 formulation) tubing, inside diameter ¼", outside diameter ½"; USP# 57111
    b. Masterflex Tygon (R-3603 formulation) tubing, inside diameter 3/8", outside diameter 5/8"; USP# 57117
    c. Tygon Sanitary Silicone (3350 formulation) tubing, inside diameter ¼", outside diameter 7/16"; USP# 57297
    d. Tygon Sanitary Silicone (3350 formulation) tubing, inside diameter ¼", outside

diameter 3/8"; USP# 57296
   e. Tygon Sanitary Silicone (3350 formulation) tubing, inside diameter 3/8", outside diameter 5/8"; USP# 57302
5. Retentate vessel: Heavy-duty bottles, pp, 1-liter capacity, with pp leak proof screw caps (Cole Parmer catalog number EW-06257-10 or equivalent)
6. Vented cap for retentate bottle: Filling/Venting cap, pp, size 53B (Cole Parmer catalog number EW-06258-10 or equivalent).
7. PendoTECH PressureMAT Single-Use Sensor, Luer Fitting, Polycarbonate [used as disposable water pressure transducer in the automator] Cole-Parmer19406-32.
8. Reusable tubing connectors, hose clamps, and filtered vent; disposable cableties.

NOTE *Alternative fitting combinations are possible. A functional fitting combination is given below. The fitting combination above represents the currently used fitting combination and seems to function better than the one thatfollows.*

   a. High density polyethylene Tee fitting with barbed ends; 3/8" x 3/8" x 3/8". USP# 62065
   b. Natural polypropylene reduction coupler 3/8"x ¼". USP# 64374
   c. Hose clamps; SNP-10 and SNP-3
   d. Check Valve with ¼" x 5/16" barb connectors by Smart Products (Part# 304305PS 0050S000-1402).
   e. DIN Adapter 1/4" barb and luer [connects tubing to filter].
   f. Polypropylene extra-flow coupling quick disconnects (inserts & bodies); USP# 60661 elbow insert with ¼" barb (3), 60653 body with ¼" barb (3), 60654 body with 3/8" barb (1), 60657 strait insert with ¼" barb (1).
   g. Whatman HEPA-Vent Filter; Fisher Scientific# 09-744-79
   h. Polypropylene Tee connector with ¼" barbs and luer lock for pressure transducer.

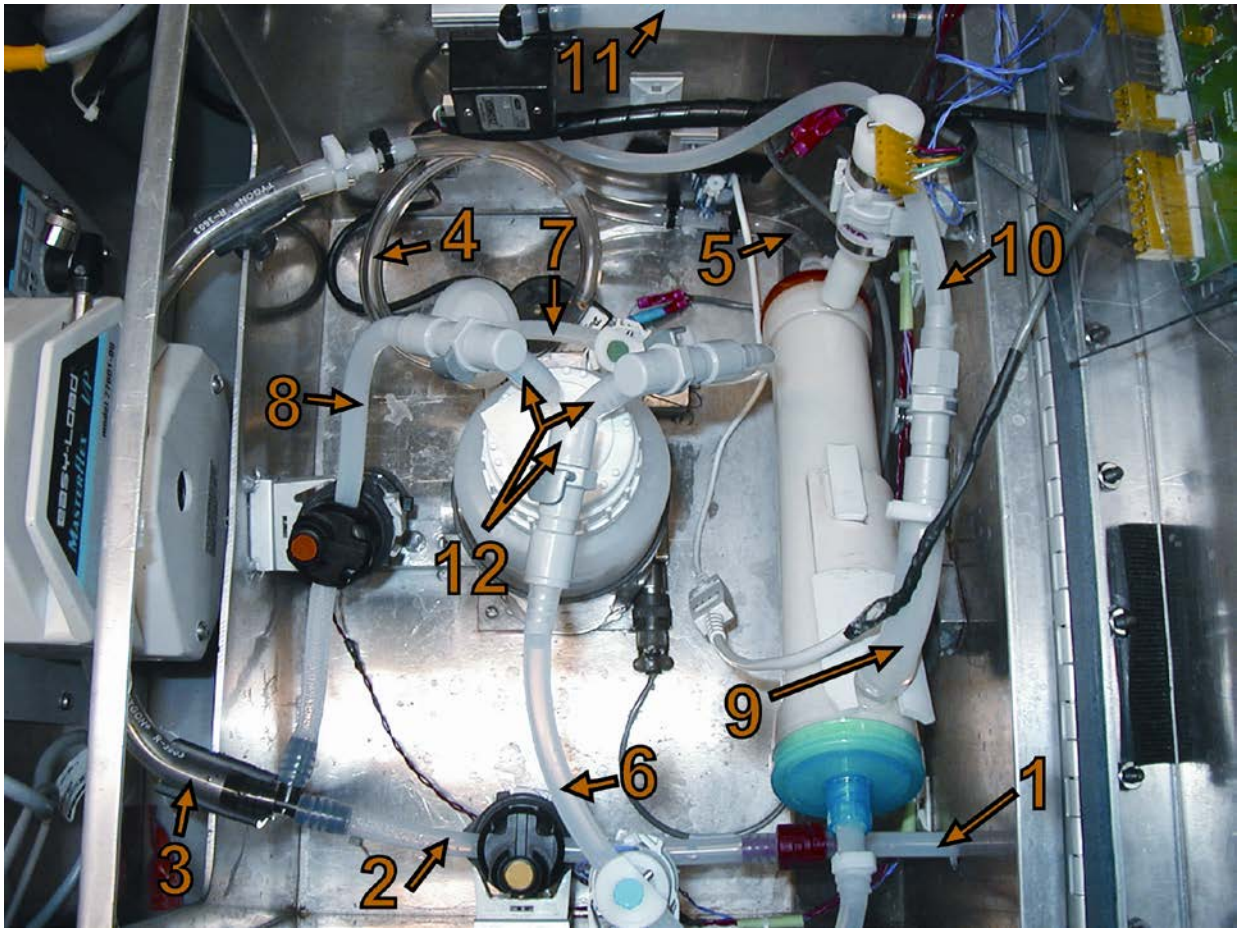

Figure A: An aerial few of the tubing set up inside the automated concentrator.

Key to Figure A:

1. Silicone tubing with ID ¼", OD 7/16". **Length varies according to sampling location**.
2. Silicone tubing with ID ¼", OD 7/16". **Section length: 8.5"**
3. Tygon R-3603 tubing with ID 3/8", OD 5/8". **Section length: 17.25"**
4. Tygon R-3603 tubing with ID ¼", OD ½". **Section length: 25" (wrapped into coil with 4.8-4.9" inner diameter.**
5. Tygon R-3603 tubing with ID ¼", OD ½". **Section length: 3.5"**
6. Silicone tubing with ID ¼", OD 3/8". **Section length 10.125"**
7. Silicone tubing with ID ¼", OD 3/8". **Section length 9.25"**
8. Silicone tubing with ID ¼", OD 7/16". **Section length 10.5"**
9. Silicone tubing with ID 3/8", OD 5/8". **Section length 3.25"**
10. Silicone tubing with ID ¼", OD 3/8". **Section length 14"**
11. Silicone tubing with ID 3/8", OD 5/8". **Section length 6.75"**
12. Silicone tubing with ID ¼", OD 7/16". **3X Section lengths 1.5" each**

Protocol:

A. Set up of tubing and filter apparatus within the automated concentrator; use Figure A photo as a reference when setting up:

1. Attach tube 2 to check valve (at 5/16" barb) and to the Tee connector with 3/8" barbs.
2. Attach tube 3 to the opposite barb of the Tee connector with 3/8" barbs, as well as the reducer, at the 3/8" barb. At the reducer, attach an SNP-10 hose clamp where the tubing connects just past the barb.
3. Attach tube 8 to the final barb of the tee connector with 3/8" barbs; (setup thus far is in a horizontal plane).
4. Wrap tube 4 into a loop (secure with cable ties), such that the inner diameter of the loop is between 4.8"- 4.9", leaving one open end slightly longer than the other (see Figure A).
5. Attach the loop in a vertical/perpendicular plane with the rest of the previously attached tubing, and attach to the ¼" barb of the reducer; secure tubing connection with a cable tie at the barb.
6. Attach the polypropylene tee connector with the luer lock and ¼" barbs to the free end of the loop, such that when the apparatus is in the concentrator, the luer is facing upward. Attach the pressure transducer to the luer lock, and attach the transducer adapter to the control panel connection cable. Secure the tubing connection with the barb using a cable tie.
7. On the filter, unscrew the small white cap attached to the red cap (bottom); attach DIN adapter by screwing in the luer. Observe the Figure A for the orientation of the filter in the concentrator, and attach tube 5 to the barb of the DIN adaptor such that the free end of the tube will be able to connect to the remaining barb from step six. Attach cable ties to secure the tubing at both connectors.
8. After making certain all solenoid valves are in the "open" position, place currently assembled apparatus into the automated concentrator as shown in Figure A, and clamp the pump head down on tube 3. Be sure tube 3 is correctly secured in the pump head.
9. Remove the small white cap on attached to the blue cap of the filter (top), and attach DIN adapter.
10. Roll one end of tube six back over itself, and attach this end to the DIN adapter; use a SNP-3 hose clamp to secure the tubing connection to the barb. Attach a quick disconnect body (female connection) with a ¼" barb to remaining end of the tube 6, and gently push tubing into its solenoid valve. Check that the tubing is not pinched on either side of the solenoid, such that water flow will not be obstructed.
11. Ensure that the vented 1L polypropylene bottle (retentate bottle) has a short length of silicone tubing with a small tee connector attached to the inside right barb, and a piece of Teflon tubing extending to nearly the bottom of the bottle on the inside left barb, with the inside of the down pointing vent having nothing attached to it.
12. Gently place the retentate bottle on the load cell, and attach tube 12 (three separate pieces) to each barb, and connect an quick disconnect elbow insert (male connector) on each tube.
13. Attach the quick disconnect body on tube 6 to the insert attached to the lower right barb (make sure there is a "click" when they connect).

14. Attach tube seven to another quick disconnect body with a ¼" barb, then attach a Whatman HEPA Vent filter to the opposite end, and gently push to the tube into the corresponding solenoid. Connect the quick disconnect body to the insert on the barb pointing towards the filter.
15. Attach another quick disconnect body with a ¼" barb to tube 8, and attach to the remaining quick disconnect insert on the top of the bottle.
16. On the filter, remove the large white cap on the upper side of the filter, and attach tube 9, with a quick disconnect body with a 3/8" barb on the opposite end.
17. Role back the end of tube 10 on itself (as was done with tube 6), attach to a quick disconnect strait insert with a ¼" barb, and then attach insert to the body on tube 9. Gently push tube 10 into the corresponding solenoid, and attach to the free end to the bottom of the flow meter.
18. Attach tube 11 to the top of the flow meter and to the quick disconnect leading to the effluent.
19. Push the end of tube 1 (influent line) into the automated concentrator and connect to the check valve.
20. Double check all tubing connections.

*Additional set up associated with the influent will vary depending on method and purpose of sampling. The length of the effluent line will also vary depending on sampling location.

B. Influent Options

Influent Option 1: Sampling from the distribution system or Seeding with BSL-1 organisms

For either sampling method, attach a disposable 10mL serological pipette (with cotton removed, and fine point tip broken off), to the influent line. During the concentrator run, this line will go directly into the sample reservoir.

Influent Option 2: Seeding a water sample with BSL-2 organisms using a syringe injection port from within a biological safety cabinet.

For seeding water with a BSL-2 organism that may require a biological safety cabinet for manipulation, run the influent line from the automated concentrator outside the BSC to a tee connector with a luer lock (the same size and type used for the pressure transducer) that is secured with a ring stand in the cabinet. Attach an additional length of the same tubing type used for the influent line to the other end of the tee connector, and run the back out of the biological safety cabinet, attach a 10 mL pipette (as described above), and use as needed.

Influent Option 3: Seeding a water sample with BSL-2 organisms a disposable cubitainer as an air gap from within a biological safety cabinet.

For seeding water with a BSL-2 organism that may require a biological safety cabinet for manipulation, attach a 10 mL pipette (as described above) to the influent line and run the influent line from the automated concentrator outside the BSC to a cubitainer inside the BSC. Take an additional length of tubing (length varies; use the same size used a tube 9 in the above setup), and place one end

into the cubitainer. Place the tubing into an additional pump head, and attach a 25mL serological pipette in the same fashion as the 10mL pipette described above. Place this new influent line into the water reservoir.

C. Running the Automated Concentrator, using Influent Option 3:

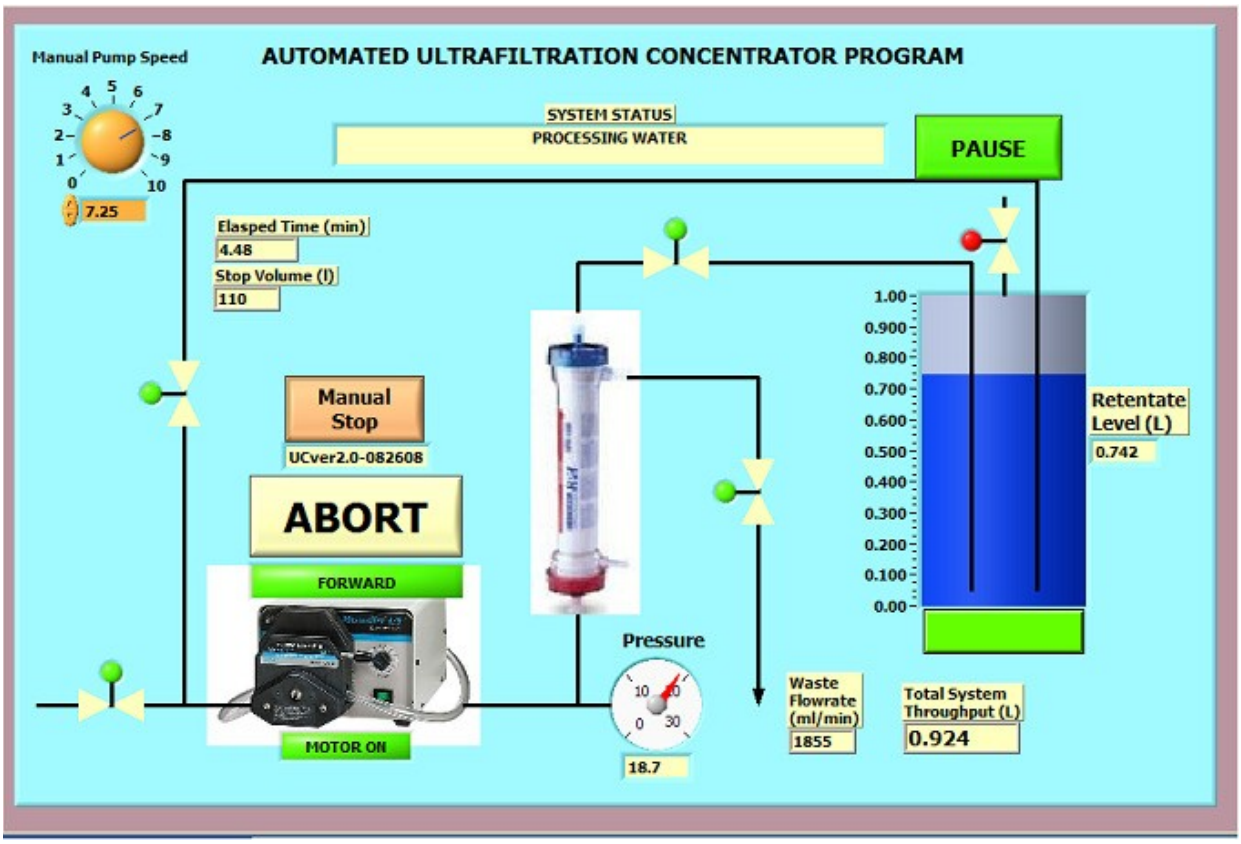

Figure B: View of the Automated Concentrator monitoring screen as seen during an ultrafiltration run.

1. Once the influent and effluent lines are attached and set up, remove the influent line with the 10 mL pipette from the cubitainer and place it in 1L of prepared filter block solution.
2. Using the pump attached to the large influent line, pump water into the cubitainer to the desired level to create a reservoir and air gap, and stop the pump.
3. Turn on the automated concentrator using the switch on the above right of pump head. It is imperative that this is done before opening the automated concentrator program.
4. On the computer, open the concentrator program.
5. When the program is pulled up, a prompt will ask if the retentate bottle is in place and empty inside the concentrator. If it is, click yes to proceed; if it is not, click no to abort the program and secure the bottle or empty it.

6. Click "Concentrate Water" to begin the ultrafiltration process. After this, the program can be stopped completely at any point throughout the run by clicking the "Abort" button on the monitoring screen.
7. Another series of prompts will ask if the influent line has been placed in 1L of filter block solution, and to click yes to start concentration.
8. The automated concentrator will then draw up an appropriate about of block, and go through a three minute re-circulation of the block solution through the UF system.
9. After the re-circulation, the concentrator will draw down the level of water in the retentate bottle and stop when it reaches its programmed level. At this time, remove the 10mL pipette influent from the bottle of filter block solution and place it in the cubitainer. A prompt from the program will tell the analyst to do this, and to click "OK" after it has been done.
10. Once the "OK" button has been clicked, the concentrator will fill the retentate bottle to a programmed level, and then begin the ultrafiltration of water. At this point, the program can also be paused by clicking the "Pause" button at any point if need be. The program monitoring screen will look like Figure B shown above.
11. At this point, monitor the cubitainer to keep a level of water as constant as possible by increasing or decreasing the speed of the pump delivering water to the container. It is imperative to constantly monitor the screen and the cubitainer because the speed and flow rate of water in the automated concentrator can vary, depending on the water conditions. Do not allow the cubitainer to over flow or run dry.
12. After allowing about 10L to pass through the system (the flow meter reading on the concentrator screen will provide the analyst with this information), seed the organisms being analyzed into the cubitainer using a sterile pipette.
13. Once the cubitainer has been seeded, the analyst should observe the screen throughout the run, monitoring the water level in the retentate bottle and the pressure readings. If there is a sudden large drop in pressure and water level in the retentate bottle, it could indicate a leak, at which point the program should be aborted.
14. Once the volume of water desired has been processed through the system, the analyst will click the "Manual Stop" button. At this point, the concentrator will stop drawing influent, and allow the water level in the retentate bottle to draw down to a programmed level.
15. A prompt will tell the analyst to place the influent line in 1L of elution solution. To do this, place a bottle of elution solution inside the BSC, and place the influent line with the 10mL pipette into the bottle. Click "OK" to start the elution procedure.
16. During the elution procedure, the concentrator will carry out four forward wash and draw down steps, then carry out a 3 minute recirculation. After the recirculation, the concentrator will fill the retentate bottle to programmed volume.
17. A final prompt will appear on the screen to allow the analyst to quit the program; however that analyst should first record the volume of retentate in the bottle before exiting the program.
18. At this point the program will close completely, and it will be safe to open the concentrator and remove the retentate bottle by disconnecting the quick disconnects. The analyst should wrap a 10% bleach wipe around the quick disconnects as they are being

disconnected to catch any contaminated water that may leak out. Place the retentate bottle in a BSC.

19. Remove the quick disconnects from the retentate bottle along with the short lengths of tubing (tube 12), and connect them to spare empty retentate bottle.
20. Place the spare bottle back in the concentrator, and reconnect the quick disconnects, and disconnect the effluent quick disconnect.

D. Disinfection of the Automated Concentrator

1. After the spare bottle has been connected to the concentrator system, open the concentrator program again. The same first prompt will come up; click yes.
2. Instead of entering the concentration mode, click "Test Mode."
3. The analyst will few a similar screen to that seen during the concentration procedure, however this screen will give the user freedom to manipulate the solenoid valves (green dot signifies "open", red dot signifies "closed"), pump on/off, and pump speed.
4. Open all solenoid valves; place the influent pipette in a 1L bottle of 10% bleach solution (made according to NHSRC SOP 007), and slowly manipulate the pump to draw up bleach into the system and fill the retentate bottle to 700mL. Quickly close the influent solenoid valve, and allow the bleach to re-circulate for 10 minutes. After the recirculation, stop the pump, and exit the Test Mode to allow the bleach to hold in the system for an additional 50 minutes.
5. Once the disinfection holding time is complete, reattach the effluent quick disconnect, but remove it from the current effluent line and connect it to a new effluent line that passes directly into a sink or drain. This step must be done so that bleach solution does not pass through the flow meter gauge.
6. Reopen the Test Mode, open all solenoid valves, but close the influent solenoid. Turn on the pump to remove the bleach from the system. When the tubing appears foamy or mostly full of air, turn of the pump, exit the Test Mode, and close the program.
7. Switch off the automated concentrator. Disassembly of the tubing may now begin.
8. Dispose of the tubing and the UF filter. Disinfect all connectors in 10% bleach for an additional half hour, then rinse with water, and allow to dry.

Refer to separate SOPs for sample processing.

References:

U.S. Environmental Protection Agency April 13, 2006. Quality Assurance Project Plan for Development of Sampling and Analytical Procedures for Detection of Targeted Biological Threat Agents in Tap Water Rev 1.2.a

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