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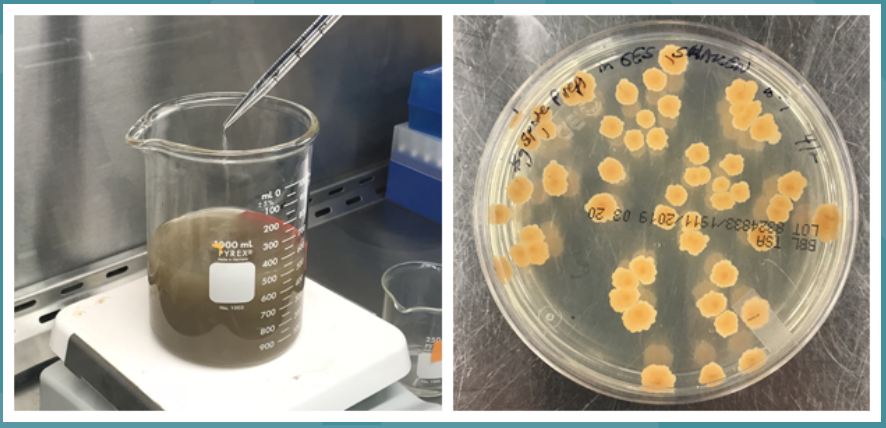
A Bench-Scale Procedure for Evaluating Chlorine Bleach Inactivation of *Bacillus* Spores in Wash Water from a Cleanup of a Site with Biothreat Agents



Office of Research and Development
Homeland Security Research Program

A Bench-Scale Procedure for Evaluating Chlorine Bleach Inactivation of *Bacillus* Spores in Wash Water from a Cleanup of a Site with Biothreat Agents

National Homeland Security Research Center
United States Environmental Protection Agency
Cincinnati, Ohio 45268

DISCLAIMER

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EXECUTIVE SUMMARY

This document describes a bench-scale procedure for testing the efficacy of chlorine bleach for the inactivation of *Bacillus* spores in wash water generated during a cleanup of a site contaminated with *B. anthracis* spores, the causative agent of anthrax. Full scale procedures exist for the on-site treatment of such wash water with chlorine bleach (US NRT, 2012), and these procedures have been tested and the results published (Mohammad et al., 2014). The published results were generated by testing the efficacy of chlorine bleach to inactivate spores using simulated wash waters. Although a wide variety of wash waters were created as part of the published research, one remaining concern is that wash water generated at a site cleanup is unique, different from the wash waters tested in the published research. Thus, one question that remains is how effective the full-scale inactivation procedures would be in the water generated at an actual cleanup site.

To help emergency management personnel address this last question, a streamlined bench scale procedure has been developed for testing the efficacy of chlorine bleach for the inactivation of *Bacillus* spores in wash water generated during a site cleanup. The procedure is meant to be a practical resource that site personnel can consult when faced with the challenge of dealing with water from this type of cleanup. This procedure is not official EPA guidance on what must be done but is one tool that can be used with other resources for the handling of wash water that could contain *B. anthracis* spores. It is anticipated that the bench scale procedure would be carried out first, and based on the results, a decision could then be made about full scale, on-site treatment of the wash water.

The procedure involves adding a known amount of *B. atrophaeus* subspecies *globigii* (*B. globigii*) to a known volume of wash water generated on-site. Concentrations of *B. globigii* are measured before and after chlorine bleach addition to the wash water. (*B. globigii* is a surrogate for *B. anthracis* and can be added at a high level to ensure detection. Any viable spores of *B. anthracis* will likely be at levels too low to allow for detection before or after chlorine bleach treatment.) The bench scale procedure was developed to allow measurement of 99.9999% (or 6 $\log_{10}$) inactivation of the spores. Recommendations are given for when samples of the inactivation reaction should be taken to yield useful data. Procedures for preparation of and analysis for *B. globigii* spores are included. Due to the potential that wash water generated on-site could contain low levels of *B. anthracis* spores, it is strongly recommended that the bench scale inactivation procedure outlined in this document takes place in a biosafety level 3 laboratory.

LIST OF ABBREVIATIONS AND ACRONYMS

5 log	When referring to inactivation, equivalent to 99.999% inactivation
6 log	When referring to inactivation, equivalent to 99.9999% inactivation
$[Bg]_{final}$	Final concentration of *B. globigii* spores
$[Bg]_{initial}$	Initial concentration of *B. globigii* spores
B. anthracis	*Bacillus anthracis*
B. globigii	*Bacillus atrophaeus* subspecies *globigii*
BSC	Biological safety cabinet
BSL	Biological safety level
CFU	Colony forming unit(s)
CT	The average chlorine concentration multiplied by the exposure time
EPA	U.S. Environmental Protection Agency
HOCl	Hypochlorous acid
LI	Log inactivation
Log_{10}	Common, or base 10, logarithm
NaOCl	Sodium hypochlorite
NB	Nutrient Broth
OCl^-	Hypochlorite ion
PBS	Phosphate buffered saline
PPE	Personal protective equipment
QRG	Quick Reference Guide
rcf	Relative centrifugal force
T_0	Initial sample, taken at time = 0
TNTC	Too numerous to count
TSA	Tryptic soy agar
US NRT	U.S. National Response Team
UV	Ultraviolet

1.0 DESCRIPTION AND BACKGROUND

This document describes a bench scale procedure for testing the efficacy of chlorine bleach for the inactivation of *Bacillus* spores in wash water generated during a cleanup of a site contaminated with *B. anthracis* spores, the causative agent of anthrax. The procedure is meant to be a practical resource that site personnel can consult when faced with the challenge of dealing with water from this type of cleanup. The procedure is not official EPA guidance on what must be done but is one tool that can be used with other resources (e.g., published research on inactivation, Quick Reference Guides (QRGs)) for the handling of wash water that could contain *B. anthracis* spores.

Note: In this document, when the term "chlorine" is used, unless otherwise specified, it refers to the aqueous forms of chlorine: hypochlorous acid (HOCl) or the hypochlorite ion (OCl^-).

In the last two decades, numerous buildings have been contaminated with *B. anthracis* spores and have subsequently required substantial remediation. During the remediation process, disinfectant solutions were often used to wash equipment and building surfaces that may have contained viable *B. anthracis* spores. In addition, remediation personnel were often washed down with disinfection/cleaning solutions prior to removing personal protective equipment (PPE). In typical operations, the wash water generated from these decontamination processes has been collected in containers prior to planned disposal at a wastewater treatment plant. However, in some of these remediation efforts, the treatment plant would not accept the wash water because of the concern that it contained viable *B. anthracis* spores (EPA 2002).

In response to this concern, the U.S. National Response Team (US NRT), a multi-agency team chaired by the U.S. Environmental Protection Agency (EPA), developed a quick reference guide for the on-site treatment of PPE wash water containing *B. anthracis* spores (US NRT, 2012). The recommended treatment was originally 1 part household bleach, 1 part vinegar, and 8 parts wash water (by volume) with an exposure time of 1 hour. This treatment resulted in a solution of approximately 6,000 mg as Cl_2/L. The recommended US NRT treatment was derived from

inactivation procedures developed for *B. anthracis* spores on solid surfaces and had not been specifically tested in water matrices.

Because of this lack of testing, the US EPA evaluated the inactivation of *Bacillus* spores in simulated wash water using different levels of bleach and vinegar (Muhammad et al., 2014). In this study a surrogate for *B. anthracis* spores was used: *B. atrophaeus* subspecies *globigii* (*B. globigii*). In a water environment, *B. globigii* spores have been observed to be more resistant to chlorine bleach inactivation than *B. anthracis* spores (Sivaganesan et al., 2006). Thus, it can reasonably be assumed that if 99.9999% inactivation of *B. globigii* can be achieved under a given set of conditions, then an even greater amount of inactivation of *B. anthracis* would be expected under the same conditions.

One result of this study showed that the dose recommended by the US NRT (2012) yielded > 5 $\log_{10}$ (log) or 99.999% inactivation in less than 1 minute (Muhammad et al., 2014). This finding prompted investigation of lower strength bleach solutions (1,000 to 3,000 mg as Cl_2/L) with no vinegar, the results of which are also found in Muhammad et al., (2014). These changes had potential to simplify the process, lessen the amount of hazardous materials needed on-site, simplify quenching of residual chlorine prior to analysis or disposal, and reduce the concentration of waste products from the inactivation process. These latter results showed that 6 log inactivation (99.9999%) was achieved in 30 minutes or less at 20 °C using a ~3,000 mg as Cl_2/L concentration, equivalent to a ~5% diluted bleach solution (5 parts bleach, 100 parts water, by volume) using the bleach available at the time of testing. The lower chlorine concentration and the elimination of vinegar did result in a less germicidal solution but also eliminated the potential of chlorine gas formation due to an excessive amount of acid addition, which could be problematic for on-site personnel during an actual cleanup of biothreat agents, especially if large volumes (e.g., 1,000 gallons or more) are treated on-site.[1]

[1] It is thermodynamically unlikely that an overdose of household vinegar, which is a dilute form of acetic acid, would lower the pH enough to cause hazardous levels of chlorine gas to form. However if a more concentrated form of acetic acid is inadvertently used, this could lead to chlorine gas formation. There have been hospital incidents where glacial acetic acid was mistakenly used instead of vinegar for patient care and led to harmful results (Grissinger, 2009). In addition, swimming pools bathers have been hospitalized due to exposure to chlorine gas that originated from accidental mixing of acid with calcium

As a followup from the studies by Muhammad et al. (2014), additional studies were done that tested a larger variety of wash waters at room temperature as well as colder temperatures using bleach without acid addition (Gallardo et al., 2018). These latter studies used the same chlorine concentration (3,000 mg as Cl_2/L) as Muhammad et al. (2014) and showed effective inactivation. However, longer contact times were sometimes needed to achieve 99.9999% inactivation depending on the temperature and the wash water tested. As expected, colder temperatures required longer contact time for inactivation. In a majority of this latter testing, complete inactivation was achieved as evidenced by the fact that no bacterial colonies were detected in the samples taken at the end of the inactivation experiment.

It was envisioned that one of the two methods mentioned above, either the method described in US NRT (2012) or else the method described in Muhammad et al. (2014), could be used in the on-site treatment of wash water generated during a cleanup of a wide area contaminated with *B. anthracis* spores. However, one remaining concern is that wash water generated at a site cleanup is unique, different from the wash water tested in the above cited references. Thus, even though the results of the cited studies showed the methods were effective in inactivating spores in simulated wash waters, one question that would remain is how effective the methods would be in the water generated at an actual cleanup site. To help emergency management personnel address this last question, a streamlined procedure has been developed for testing the efficacy of chlorine bleach for the inactivation of *Bacillus* spores in wash water generated during a site cleanup. The procedure is based on the method used in Muhammad et al. (2014) for 3,000 mg as Cl_2/L but has been streamlined so that results can be produced in a more-timely manner.

This procedure is *not* for treating the large volumes of wash water generated during a

hypochlorite, a pool disinfectant similar to household chlorine bleach (CDC, 2017). Hospitalizations due to mishandling of pool chemicals is not an uncommon occurrence, and approximately 4000 trips to emergency rooms result from such accidents, on an annual basis (CDC, 2009). Thus there are some safety advantages if bleach without acid addition can be used to treat wash water from a cleanup of a biothreat agent.

remediation but for conducting bench-scale testing of chlorine bleach inactivation of *B. globigii* spores in samples of the generated wash water. The results of this testing can then inform emergency management personnel as to the conditions (i.e., chlorine bleach concentration and contact time) needed to properly treat the large volumes of wash water on-site that may contain *B. anthracis* spores.

Water from a *B. anthracis* cleanup may indeed contain virulent spores. However, the levels would likely be too low for detection. Consequently, it would not be possible to test the efficacy of chlorine inactivation by analyzing for *B. anthracis* spores before and after chlorination. Using a surrogate such as *B. globigii* spores is a practical way of testing for disinfection with the assumption that if 6 log inactivation of *B. globigii* spores is accomplished, then a greater log inactivation of *B. anthracis* spores should be accomplished since *B. anthracis* spores are less resistant to chlorine than *B. globigii* spores (Sivaganesan et al., 2006).

If the more aggressive method (US NRT, 2012) is desired for on-site treatment, the bench scale procedure can still be used for inactivation testing, the assumption being that if complete inactivation can be shown using the less aggressive bench-scale procedure, then significantly greater inactivation can be expected using the US NRT (2012) method for on-site treatment. The rapid kinetics and low pH of this latter method would lead to a greater degree of difficulty if bench-scale testing were to be conducted to evaluate inactivation efficacy under these more aggressive conditions. For example, the kinetics would require very rapid sampling and quenching, which would likely be impractical if it is desired to take an intermediate sample that would still have some viable spores. Section 4.0 discusses the importance of this intermediate sample.

Note: A confounding factor in using this procedure is that the wash water may already have high levels of chlorine bleach. Historically, wash solutions for decontamination of personnel included only detergent and water (EPA, 2013) due to the concern of chlorine bleach attacking the seams of the PPE suits and causing a breach in the suit during decontamination. However,

the current practice for making wash solutions calls for using the same ratio as the US NRT method for treating wash water: 1 part bleach, 1 part vinegar, 8 parts wash water. (EPA, 2018). It is not envisioned that inactivation can be accurately measured for spores added to this type of wash water due to the fast kinetics that would likely characterize the reaction.

2.0 SUMMARY OF THE PROCEDURE

The procedure is broken down into sections 3 – 5 of this report: 3.0. Preparation of *B. globigii* spores; 4.0. Inactivation studies of the prepared spores in samples of wash water; 5.0. Analysis of samples from the inactivation studies. These sections are summarized below.

Preparation of B. *globigii spores* (Section 3)

Preparation of the spores is the most challenging part of the procedure and requires capabilities and equipment not always found in a microbiological laboratory. The process entails taking a sample of *B. globigii* spores, causing them to germinate into vegetative cells, and then controlling conditions so that the vegetative *Bacilli* form spores again. The resulting suspension of spores is then washed and centrifuged numerous times to produce a pure suspension of spores. Users of this procedure may want to consider using a research laboratory (e.g., US EPA's National Homeland Security Research Center or a university) for a source of *B. globigii* to be used in the inactivation studies.

Inactivation studies of the prepared spores in samples of wash water (Section 4)

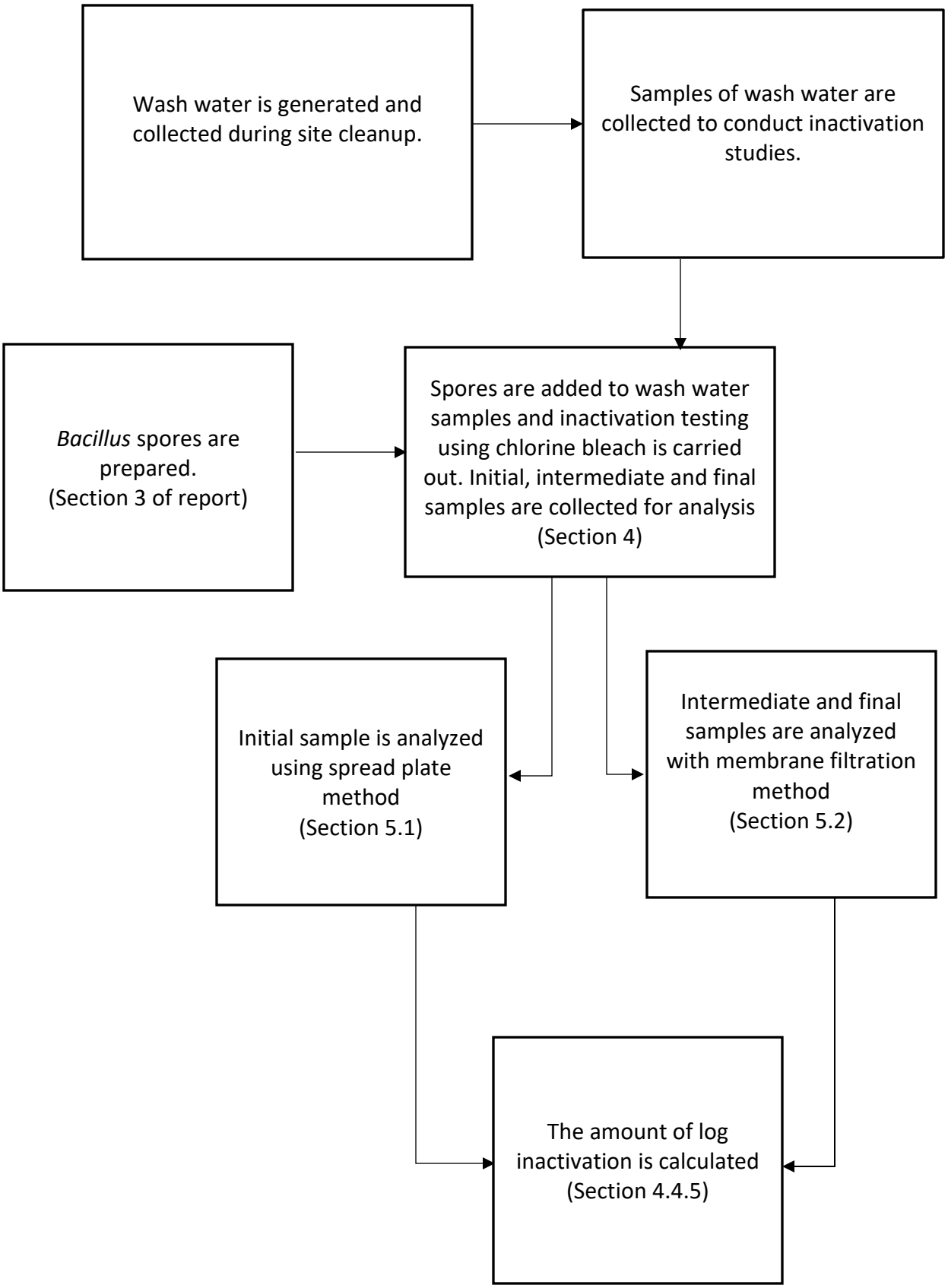
In this inactivation procedure, a known volume of a *B. globigii* spore suspension is spiked into a volume of the wash water to be tested. A sample of the resulting mixture is taken for later analysis to determine the starting spore concentration. After initial sampling, a known volume of chlorine bleach is added to the spiked wash water and allowed to react for a predetermined amount of time with the goal of achieving at least 6 log inactivation of the *Bacillus* spores. Two samples are collected during this period of inactivation, an intermediate point and a final point.

Analysis of wash water samples from inactivation studies (Section 5)

Two analytical methods for spores are presented: a spread plate method for high levels (e.g., initial sample prior to chlorine addition) and a membrane filtration method for low levels (i.e., intermediate and final samples).

Figure 1 contains a flow chart that summarizes the above procedure.

Figure 1. Flow Chart of Inactivation Procedure.



3.0 PREPARATION OF *BACILLUS* SPORES

3.1 Purpose

This procedure describes the preparation of a suspension of *B. globigii* spores. The starting material is an aliquot of *B. globigii* spores. In this procedure, *B. globigii* spores are plated out on tryptic soy agar (TSA) plates and incubated so that the spores germinate and form vegetative cells of *B. globigii*. These cells are then transferred to a medium that facilitates vegetative cell growth and then, as the carbon source and nutrients are exhausted, the bacteria form spores due to the adverse conditions. A series of centrifuging and washing steps are then carried out to form a suspension of pure *B. globigii* spores.

3.2 Equipment and Materials

3.2.1 Equipment

- Biological Safety Cabinet
- Autoclave
- Stir plate
- Stir bar
- Microscope with phase contrast capability, suitable for inspection of vegetative cells and endospores
- Incubator Shaker (New Brunswick Scientific, Innova 42 or equivalent)
- Vortex mixer
- Sorvall Evolution *RC* Centrifuge with fixed angle rotor (Sorvall SA-600) and swinging bucket rotor (Sorvall HB-6), or equivalent
- 2 L Erlenmeyer Flask

- Five 500 mL Erlenmeyer flasks
- 250 mL beaker
- Graduated cylinder, 1 liter
- Sterile 10 mL polystyrene serological pipettes
- Sterile 25 mL polystyrene serological pipettes
- 15 mL conical centrifuge tubes (Fisher Scientific Cat. #. 05-527-90 or equivalent)
- Spatula
- Weigh boats
- Microscope slides
- Slide coverslips
- 30 mL round bottomed Oak Ridge® centrifuge tubes, (Fisher Scientific Cat. # 05-529B or equivalent)
- Bleach Wipes – Dispatch® Hospital Cleaner Disinfectant Towels with Bleach, or equivalent
- PPE – laboratory coat, nitrile gloves, safety glasses

3.2.2 Materials

- Viable stock of *B. globigii* spores
- Nutrient Broth (NB) – Sigma N7519 or equivalent
- Manganese Sulfate ($MnSO_4$)– Fisher M118-3 or equivalent
- Calcium Chloride ($CaCl_2$) – Sigma C1016 or equivalent
- Tryptic Soy Agar (TSA) plates (Fisher Scientific, Cat. # B21283X or equivalent)
- Hypaque™ (RenoCal-76) - Bracco Diagnostics 086050
- Ethanol – 40 %
- Purified Type 1 water, e.g., SuperQ® or Milli-Q® water
- Purified Type 1 water, cold, sterilized
- Chlorine bleach with 8.25% (w/v) NaOCl
- Ethanol – 70%

- Fingernail polish
- Immersion oil

3.3 Procedure

Note: Always wear appropriate PPE (disposable laboratory coat or gown, doubled nitrile gloves, safety glasses) when performing this laboratory work.

3.3.1 Prepare 1000 mL of generic spore medium

1. Place a stir bar into a 2 L Erlenmeyer Flask and add approximately 900 mL of purified Type 1 water to the beaker.
2. Weigh the respective amounts of Nutrient Broth, $MnSO_4$ and $CaCl_2$ and add them to the beaker.
    - Nutrient Broth, 8.0 g
    - $MnSO_4$, 40 mg
    - $CaCl_2$,100 mg
3. Stir until chemicals are dissolved or approximately 30 minutes.
4. Adjust the volume of the mixed reagents to 1,000 mL with purified Type 1 water. (Use this step to wash down the sides of the flask to capture any residual ingredients remaining on the wall of the beaker.)
5. Dispense 200 mL aliquots into each of five 500 mL Erlenmeyer flasks.
6. Autoclave flasks of medium at 121°C for 15 minutes being sure to use the liquids cycle.
7. Store medium at room temperature.

3.3.2 Vegetative growth and spore production

1. From a working stock or freezer stock of *B. globigii,* streak onto a TSA plate and incubate at 37 °C for 16 – 24 hours. The B. *globigii* colonies will have a distinct orange tint on TSA.

2. After 16 - 24 hours of incubation, pick either a single large isolated colony or several smaller isolated colonies and suspend in approximately 8 mL of spore medium in a sterile 15 mL centrifuge tube. Cap tube and vortex for 30 seconds.

3. Transfer 2 mL of the inoculated spore medium into each of the 500 mL flasks containing 200 mL Generic Spore Medium. Alternatively, picked colonies can be added directly to the flasks of medium.

   Note: If making a fresh spore preparation to replenish freezer stocks, then several isolated colonies should be picked and added to each 500-mL flask.

4. Cap the flasks and wipe them with bleach wipes followed by 70% ethanol. Use laboratory tape to hold the slightly loosened flask caps in place. Incubate with continuous gentle shaking at 37 °C for at least five days.

5. After five full days of incubation, check each flask for the presence of spores by viewing wet mount slide preparations using phase contrast microscopy with the 40× and 100× objectives. Vegetative cells will appear as rod shaped organisms. Spores will appear as phase bright ovals.

6. Repeat step 5 to view spore suspension. An adequate spore suspension typically requires 5-7 days of incubation and is characterized by very few intact vegetative cells and many phase bright oval spores. When an adequate spore suspension has been achieved, proceed with spore purification (Section 3.3.3)

3.3.3 Spore purification

1. Aseptically transfer the contents of each flask into sterile 30-mL round-bottomed centrifuge tubes (12 tubes).

2. Balance the tubes and centrifuge at approximately 5,900 rcf at 4 °C for 20 minutes, using a fixed-angle rotor with acceleration and deceleration set at 3 or lower if using the Sorvall Evolution *RC* Centrifuge and the SA-600 rotor (or equivalent). Using a 25-mL pipette, draw up supernatant and transfer to a discard beaker (or use emptied culture flasks). The same pipette can be used after each centrifugation step if it is not contaminated between uses. (Note: If an increased titer is desired, this step can be repeated using the remaining contents of the spore preparation flasks.)

3. Add 30 mL of cold, sterile purified Type 1 water to each tube using a 25-mL pipette. Replace the pipette in its packaging sleeve and reuse if sterility is maintained.

4. Vortex tubes until spores are completely resuspended in the water (Wash A-1) (approximately 30 seconds).

5. Centrifuge the tubes again at approximately 5,900 rcf at 4 °C for 10 minutes.

6. Carefully pipette off the supernatant and resuspend pellet in 30 mL of cold, sterile purified Type 1 water per tube (Wash A-2).

7. Centrifuge for another 10 minutes as before and discard the supernatant.

8. Combine the contents of the tubes into multiples that will allow ease of centrifugation: For example, for this setup, combine and resuspend the spores into six centrifuge tubes with a final volume of 20 mL in cold sterile purified Type 1 water.

9. Aseptically combine 58 mL of Hypaque with 42 mL of sterile purified Type 1 water in a sterile 250 mL beaker. Mix well.

10. Add 12 mL of Hypaque solution to a clean, sterile 30-mL round-bottom centrifuge tubes. Carefully add the solution to the bottoms of tubes; do not splash the sides of the tubes.

11. Using a sterile 10-mL pipette, carefully layer the aqueous spore suspension on top of the Hypaque™ solution. Start very slowly to ensure a good boundary between the solutions.

12. Centrifuge at approximately 5,900 rcf at 4 °C for 30 minutes using a swinging bucket rotor. If using the Sorvall Evolution *RC* with the HB-6 rotor, the centrifuge acceleration

rate should be set at 1 or 2 while the deceleration rate should be set at 0 for this buoyancy centrifugation using the swinging bucket rotor. Slow acceleration and deceleration is required to maintain the boundary at the interface of the two solutions.

13. Carefully pipette off and discard the supernatant. From this point forward, the spore pellets will be soft and easily disturbed, which can result in the loss of spores with the supernatant.

14. Add 30 mL of cold sterile purified Type 1 water to the pellet in each tube and resuspend the spores (Wash B-1). For the remaining washes, acceleration can be set at 3 and deceleration can be set at 1 or 2 using the Sorvall Evolution *RC* Centrifuge with the fixed angle SA-600 rotor.

    Note: for remaining centrifuge steps, because the rotor weight is significantly heavier than the centrifuge tubes, precise balancing of tubes is not as necessary.

15. Centrifuge at 5,900 rcf at 4 °C for 15 minutes using the fixed-angle SA-600 rotor.

16. Carefully pipette off the supernatant and resuspend the spores in 30 mL of cold sterile purified Type 1 water (Wash B-2).

17. Centrifuge at 5,900 rcf and wash the spores by resuspending them two more times (Washes B-3 and B-4).

18. Centrifuge again at 5900 rcf to pellet the spores.

19. Discard the supernatant and resuspend the spores in a 40 % (v/v) ethanol solution. Each of the six spore pellets should be resuspended in 20 to 40 mL of 40 % ethanol. The final volumes can be altered if a higher or lower concentration of spores is desired.

20. Check each of the six spore suspensions again microscopically by phase contrast using the 40× and the 100× objectives; virtually no cell debris should be present. (Typically if 90 % of the microbial material in the microscope's field of view are spores, then little cell debris is present. If there is less than 90%, then the spore prep procedure should be started over.) The coverslip should be sealed to the slide with fingernail polish to prevent excessive evaporation of the wet mount.

21. Store the purified *B. globigii* spore preparation at 4 °C.

3.3.4 Assessing the Spore Concentration in the *B. globigii* Spore Preparation

1. Prepare serial dilutions starting with 100 µL of the spore preparation.

2. Spread plate 100 µL of each dilution between 10^{-5} and 10^{-8} onto 3 to 5 TSA plates. More concentrated dilutions can be plated if a low yield is anticipated.

3. Plate 3 negative control plates with 100 µL of dilution buffer.

4. Invert and incubate the plates at 37 °C overnight.

5. Count the number of colonies on each plate of the dilution series that produces 30 - 300 colonies per plate. Calculate the average colony forming units (CFU) for the three plates, and determine the CFU/mL. This is the total CFU/mL in the spore preparation.

6. Spore density (CFU) per mL of suspension is calculated using the following formula:

$$\textbf{Number of CFU/mL} = \frac{\textit{Average CFU per plate}}{\textit{Volume plated (mL)}} \times \frac{1}{\textit{tube dilution factor}}$$

For example:

Tube Dilution	Volume Plated	Replicate	CFU per plate
10^{-5}	0.1 mL	1	150
10^{-5}	0.1 mL	2	250
10^{-5}	0.1 mL	3	200
		Average =	200

$$\frac{200\ CFU}{0.1\ mL}\ X\ \frac{1}{10^{-5}} = 2.0x10^{8} CFU/mL$$

4.0 INACTIVATION OF *BACILLUS* SPORES IN WASH WATER

In this inactivation procedure, a known volume of a *B. globigii* spore suspension is spiked into a volume of the wash water to be tested. A sample of the resulting mixture is taken for later analysis to determine starting spore concentration. After initial sampling, a known volume of chlorine bleach is added to the spiked wash water and allowed to react for a predetermined amount of time with the goal of achieving at least 6 log inactivation of the *Bacillus* spores. The time needed to achieve this amount of inactivation is largely dependent on the buffering capacity of the wash water and is discussed further in section 4.1.

While a receiving entity such as a wastewater treatment plant may have specific (and higher) treatment criteria, 6 log inactivation is seen as a practical limit for the purposes of this bench scale procedure. Higher log inactivation can be measured but this requires a higher starting spore concentration. For example, to show a 7 log or 8 log inactivation would require a tenfold or 100-fold increase in the starting spore concentration, respectively, assuming all other parameters (e.g., the volume that is membrane-filtered) remain the same.

Samples for spore analysis are taken at two additional points: an intermediate point and a final point. After these latter samples are collected, they are then quenched to halt chlorine inactivation and later analyzed for *B. globigii* spores. The sample taken at an intermediate time serves as an important check for quality control. Analysis of this sample will help show whether proper technique was being used that allows detection of viable spores after chlorine inactivation of spores has started but has likely not resulted in complete inactivation. The final sample is to test whether 6 log inactivation has been achieved. It is possible that the analysis of the final sample may yield complete inactivation, i.e., no viable colonies on any of plates for the final sample. Thus, having an intermediate chlorinated sample that shows viable colonies gives assurance that a non-detect final sample is valid.

If results of the final sample show complete inactivation, log inactivation will then be expressed as a “greater than” value.

Depending on the spore concentration expected in the sample, the samples are analyzed using one of two methods. For high levels of spores (i.e., initial sample), serial dilution followed by spread plating and standard plate counting is used (see Section 5.1). For lower levels, i.e., intermediate and final samples, membrane filtration followed by filter plating is used (see Section 5.2).

The goal of the procedure is to show whether 6 log inactivation of *B. globigii* spores can be achieved at a specific contact time and specific chlorine concentration. Published data on inactivation are generally much more comprehensive than what this method provides. Typically, published data for inactivation include a correlation between log inactivation and CT, generated from multiple data points allowing the calculation of kinetic reaction rate constants, which are useful for scale-up efforts.

Generating this type of correlation is beyond the scope of this procedure, since multiple trials could be needed to ensure sampling events are within the linear range and yield finite amounts of inactivation. In addition, depending on the wash water (e.g., water with a high buffering capacity), the samples may need to be taken every minute for the first 5 minutes to sample in this linear range; this level of effort is considered too intensive for a streamlined procedure.

Data Interpretation

The resulting data from the bench scale procedure will show whether 6 log or greater inactivation was achieved. If this level of inactivation is achieved, then the bench scale conditions can be scaled up to treat wash water on-site. If not achieved, the experiment could be repeated, and a longer contact time and/or higher bleach concentration used. Or else, instead of repeating the procedure, in scaling up, one could add a safety factor based on the results of the bench scale procedure. For example, if 4 log inactivation was achieved in 90 minutes, full scale treatment could be carried out at the same concentration of bleach and a contact time of 180 minutes, i.e., twice as long as the time used in the bench scale procedure.

Assuming the chlorine concentration does not decrease substantially, this increased CT should yield a log inactivation of approximately 8 log.

4.1 Factors that Affect Inactivation

Previous testing has shown that the pH at which inactivation takes place strongly affects the amount of inactivation achieved. Bleach is alkaline and, when added to wash water, will typically cause the pH to rise. However, the amount of the pH rise is dependent on the buffering capacity of the bleach/wash water mixture. This buffering capacity is significant since the higher the resulting pH of the bleach solution, the less germicidal it is. In bleach, two aqueous species of chlorine are at equilibrium with each other. The more germicidal species, hypochlorous acid (HClO), is more prevalent at lower pH, while the less germicidal species, the hypochlorite ion (OCl^-), is more prevalent at higher pH. Thus, a good way to estimate the amount of time needed to achieve 6 log inactivation is to measure the pH of the wash water after bleach addition. The first steps in the following procedure call for adding 2 mL of bleach to 50 mL of wash water and then measuring the pH. In essence, this step is a titration in which bleach is the titrant and gives an estimate of how germicidal the bleach will be when the inactivation test is carried out.

Based on previous results (Muhammad et al., 2014; Gallardo et al., 2018), it is estimated that if the resulting pH is below 9 after bleach addition, then 20 minutes at room temperature is likely to be long enough to achieve greater than 6 log inactivation. If the pH after bleach addition is between 9 and 10, then a contact time of 60 minutes is recommended, and if the pH is above 10, then 120 minutes is recommended. The above is simply a rough approximation to help the user choose sampling times that will provide useable results. Actual times for 6 log inactivation may be influenced by other constituents in the wash water. However, other parameters such as pH *prior* to chlorination, chemical oxygen demand, solids concentration, and turbidity were not seen to have a noticeable effect on inactivation at the levels of chlorine studied (Mohammad et al., 2014; Gallardo et al., 2018).

For the intermediate sample, suggested sampling times are as follows: 5 minutes for a pH less than 9, 12 minutes for a pH between 9 and 10, and 30 minutes for a pH above 10. These times are not expected half way points but times that should be long enough to show partial inactivation, and thus contain viable spores that should be detectable using the methods referenced in this procedure.

4.2 Definitions and Abbreviations

B. globigii – *Bacillus atrophaeus* subspecies *globigii* (ATCC® 49760™)

BSC – Biological safety cabinet

Biosafety Level (BSL) – 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 biological agents used within the laboratory.

CFU – Colony Forming Unit(s)

CT – The average chlorine concentration multiplied by the exposure time.

Heat Treatment – The effect of subjecting an organism cell to a higher temperature than that of the ideal body temperature of the organism from which the cell line was derived. This technique is used to inactivate vegetative bacteria while leaving bacterial spores intact.

Log inactivation (LI) – A measure of the effectiveness of *B. globigii* inactivation equal to the common logarithm of the initial *B. globigii* concentration minus the common logarithm of the *B. globigii* concentration at time t.

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.

PBS – Phosphate Buffered Saline (PBS), pH 7.4 ± 0.2

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

Serial Dilutions – The stepwise dilution of a substance in suspension.

Spore – a protective form that bacteria may assume when the organism is exposed to stressful conditions. Spores are more difficult to destroy than the active vegetative form of the bacteria.

TSA – Tryptic Soy Agar. Medium used to grow *B. globigii* spores

4.3 Equipment and Supplies

4.3.1 Equipment

- Magnetic stir plate
- Magnetic stir bar
- 1000 mL Pyrex® Glass beakers, or equivalent (reaction vessels)
- 1000 mL graduated cylinder
- Motorized pipette pump, Drummond XP Pipet-Aid® (cat. # 4-000-101) or equivalent
- 50 mL graduated cylinder
- Pipettor, 200 µL capacity
- Pipettor, 1000 µL capacity
- Analytical balance
- Timer
- pH Meter (Accumet® Basic AB 15) or equivalent
- pH Electrode (Accumet, Fisher cat. # 13-620-111) or equivalent
- Vortex mixer

4.3.2 Media and Reagents

- Dilution buffer, 1X PBS with 0.02% Tween® 80 buffer; Teknova No. P3875 or equivalent
- Chlorine bleach with 8.25% NaOCl, Clorox Concentrated® Bleach or equivalent
- 10% (w/v) Sodium thiosulfate ($Na_2S_2O_3$) aqueous solution – Fisher Scientific 7867-632 or equivalent

- 70% ethanol – Fisher Scientific 2546701 or equivalent
- Wash water from on-site generation.

4.3.3 Supplies

- Wash water from on-site generation.
- 2 mL polystyrene serological pipettes
- Sterile 10 mL polystyrene serological pipettes
- Sterile 25 mL polystyrene serological pipettes
- Sterile 200 and 1000 µL micropipette tips
- 15 mL centrifuge conical tubes (Fisher Scientific No. 05-527-90 or equivalent)
- Rack for 15 mL centrifuge conical tubes (Fisher Scientific No. 21-200-85 or equivalent.)
- 1 L or 500 mL Pyrex or equivalent autoclavable glass bottles for waste collection
- Bleach Wipes – Dispatch Hospital Cleaner Disinfectant Towels with Bleach, or equivalent
- Disposable cotton cloth, Wypall X60® or equivalent
- Absorbent production pads, Fisher Scientific No. 19-065-089 or equivalent
- Polypropylene autoclave bags, Fisher Scientific No. 01-814C or equivalent
- Nalgene autoclavable pans or equivalent

PPE as required by site-specific safety plan, but should include items such as disposable laboratory coat/gown, nitrile gloves, safety glasses, and closed-toe shoes.

4.3.4 Organism

B. atrophaeus subsp. *globigii* (ATCC® 49760™) spores prepared and titered as described in Section 3

4.4 Inactivation Procedure

4.4.1 Special Safety Precautions

For this procedure, a site-specific safety plan must be followed.
The paragraph below contains information relevant to such a plan.

The wash water used in this procedure may contain organisms other than *B. globigii* that pose a potential health risk, such as *B. anthracis* spores. Therefore biosafety level 3 (BSL 3) precautions must be considered. These precautions include wearing appropriate PPE (e.g., disposable laboratory coat or gown, nitrile gloves, safety glasses) and performing work within a biological safety cabinet (BSC) (Chosewood and Wilson, 2009). All materials removed from the BSC should be bleached for five minutes using bleach wipes followed by wiping the bleach off the material using 70% ethanol. Disposable items used in the BSC should be placed in an autoclave bag before being moved from the BSC. Plates to be incubated should be bagged before being transported to an incubator. The interior of the BSC itself should be bleached for five minutes using bleach wipes followed by wiping down with 70% ethanol to remove bleach residue. If the BSC is equipped with a germicidal ultraviolet (UV) lamp, it should be subsequently illuminated per the manufacturer's instructions to ensure decontamination of the cabinet. If wash water samples are shipped to a BSL 3 laboratory, the samples should be appropriately packaged, sealed in a cooler with ice and shipped for overnight delivery.

4.4.2 Inactivation Test

The inactivation test is conducted using 500 mL of wash water and 20 mL of concentrated bleach. The amount of NaOCl in this type of bleach is 8.25 % w/v and results in a chlorine concentration of 3,210 mg as Cl_2/L when mixed at the above ratio. This concentration is slightly higher than what was used in the cited studies (Muhammad et al., 2014; Gallardo et al., 2017) because of the choice of the volumes of water and bleach to use.

It is anticipated that during an actual site cleanup, treatment of generated wash water would occur at a chlorine concentration of the magnitude discussed above. The goal of this test is to check whether a 6 log reduction can be attained at this level of chlorine. All experiments are conducted at room temperature (23 ± 3 °C).

The following table contains the bench scale procedure for testing for 6 log inactivation of on-site wash water.

Table 1. Bench Scale Inactivation Procedure

Step #	Step
	Steps 1- 4 are for measuring pH after bleach addition to estimate inactivation time.
1	Add 50 mL of wash water to a 100 mL beaker using a 50-mL graduated cylinder.
2	Using a motorized pipette pump and a 2 mL pipette, add 2 mL of bleach into the beaker containing the wash water (The resulting mixture is at the same chlorine concentration as the procedure to be carried out.).
3	Mix for 1 minute.
4	Measure pH of the beaker contents.

The following is a conservative approximation based on past studies, meant to be an approximate guide to help the user of this procedure know how long to run the chlorine reaction to have viable spores at the intermediate point and to achieve a 6 log or greater amount of inactivation at the final sample point.

pH after bleach addition	*Time for intermediate sample (minutes)*	*Time for final sample (minutes)*
< 9	*5*	*30*
9 – 10	*12*	*60*
> 10	*30*	*120*

5	Prepare 15 mL tubes for *B. globigii* samples by labeling and adding 1.0 mL of 10 % sodium thiosulfate solution (dechlorinating agent) to each tube. The tubes are for initial, intermediate, and final samples.
6	Add 500 mL test wash water to a 1000 mL glass beaker and set the beaker on a magnetic stir plate.
7	Add a magnetic stir bar to the glass beaker and start the beaker mixing at approximately 120 rpm. Hereafter, this will be referred to as the reaction vessel.
8	Add a sufficient volume (see Section 3) of *B. globigii* stock solution to the reaction vessel to create a *B. globigii* concentration of 1 x 10^5/mL to 5 x 10^5/mL in the wash water. This volume will be referred to as "spiked" in the following steps.
9	Using a motorized pipette pump and a sterile 10 mL pipette, transfer 10 mL of the

	spiked sample for determining the initial *B. globigii* concentration into the corresponding 15 mL tube prepared in Step 5. Dechlorinated samples can be stored at 4 °C for up to 48 hours.
10	Using the motorized pipette pump and a sterile 25 mL pipette, add 20.0 mL bleach and start the timer. This amount of bleach results in a concentration of approximately 3,210 mg as Cl_2/L.
11	At the intermediate sampling time, pipette a 10-mL sample from the reaction vessel into a sterile 15 mL polypropylene tube containing 1 mL of the dechlorinating agent. Cap tube and vortex for 30 seconds. Label tube to denote the time sample was collected.
12	At the end of the test (when the estimated time for 6 log inactivation has been reached), pipette a 10 mL sample from the reaction vessel into a sterile 15 mL polypropylene tube containing 1 mL of the dechlorinating agent. Cap tube and vortex for 30 seconds. Label tube to denote the time sample was collected.
13	(Optional) If it is desired to quench the remaining chlorine in the wash water prior to disposal, add 50 mL of 10 % sodium thiosulfate solution. Mix for 1 minute.
14	Stop the mixer, remove the magnetic stir bar, and dispose of the wash water (return to the bulk storage if possible).
15	Analyze the initial sample (T_0) using the standard plate method (see Section 5.1 of this report). Analyze all subsequent samples obtained for spore concentration using the membrane filtration method (see Section 5.2 of this report). For these latter samples, the entire contents of each sample tube should be filtered, i.e., 11 mL, to allow measurement of 6 log inactivation. In most cases, only a single filter will be required.

4.5 Positive Controls

A positive control is prepared by spiking a known concentration of *B. globigii* spores into sterile dilution buffer followed by performing a standard plate count or using membrane filtration and incubation at 35 °C – 37 °C for 24 hours. The calculated spore concentration should be within ± 0.5 log of the expected spore concentration. If the actual spore concentration from the positive control is not within 0.5 log of the expected spore concentration, the entire test (controls plus unknown samples) should be repeated with new plates and controls. All positive controls need to be heat-treated to ensure that vegetative cells are not present.

The initial sample (T_0) can be used as a positive matrix control since the approximate concentration is known, and no bleach has been added at this point in the experiment. This sample also needs to be heat-treated because no bleach was added to the reactor when the sample was collected.

The intermediate test point serves as a qualitative positive control check. The recommended time for this sample should yield a 2-3 log inactivation, so there will likely be spores in the sample that have survived exposure to the chlorine bleach. If the subsequent quenching and analytical procedures are carried out correctly, then the surviving target organisms should be detected as CFUs when plated. If the final sample shows no CFU on any plates, then positive results from the intermediate sample lend credence to the results of the analysis of the final sample. Plate counts for the intermediate sample may yield CFU that are too numerous to count (TNTC). TNTC is not considered problematic, since even a qualitative TNTC result gives assurance that CFU can be detected in samples that have been chlorinated.

4.6 Negative Controls

A negative control is obtained by membrane-filtering 10 mL of sterile dilution buffer and

incubating the membrane on a TSA plate and incubating at 35 °C - 37 °C for 24 hours. Formation of colonies indicates contaminated dilution buffer, and the entire test (controls plus unknowns) needs to be repeated using fresh buffer of a different lot. It is not necessary to heat-treat this negative sample.

It is theoretically possible that *B. globigii* spores are present in the unspiked wash water, and a sample of this water can be taken and analyzed, after heat treatment, via membrane filtration following the procedure in Section 5.2. However, the starting concentration of *B. globigii* spores used for calculating the amount of inactivation will be determined from results of the analysis of the wash water after spiking. Thus, if there were *B. globigii* spores present in the unspiked wash water, these spores would be detected and would contribute to the value for the initial spore concentration in the equation below.

4.7 Calculations

Log Inactivation.

Log inactivation (LI) is calculated by the following equation.

$$LI = \log_{10}[Bg]_{initial} - \log_{10}[Bg]_{final}$$

where

$[Bg]_{initial}$ is the initial concentration of *B. globigii* spores in the spiked wash water prior to chlorine addition.

$[Bg]_{final}$ is the final concentration of *B. globigii* spores in the spiked wash water after chlorine treatment and subsequent quenching of residual chlorine.

If the final sample shows that there are no detectable *B. globigii* on any of the plates, then to calculate LI, assume a *B. globigii* count of 1 CFU, divide by the sample volume filtered, (i.e., 10 mL, which does not include the volume of sodium thiosulfate solution added for quenching) to calculate the final *B. globigii* concentration, and express LI as a greater than value.

Sample calculations

$[Bg]_{initial} = 1.5 \times 10^6$ CFU/mL;

$\log[Bg]_{initial} = 6.18$

$[Bg]_{final}$ = 11 CFU/10 mL = 1.1 CFU/mL

$\log[Bg]_{final} = 0.041$

This yields:

LI = 6.18 – 0.041

LI = 6.18

Chlorine concentration × time (CT)

CT = Chlorine concentration (mg/L) × time (minutes)

4.8 Volumes needed for full scale inactivation

If the results of the bench scale procedure show that 6 log or greater inactivation of *B. globigii*

spores was realized, then the ratio of bleach to wash water can be scaled up to treat the wash water generated on-site. Table 2 gives examples for different volumes for treatment of the wash water. (No sampling for organisms is necessary since *B. globigii* is not added in full scale inactivation and the amount of *B. anthracis* spores will likely be too low for detection.)

Table 2. Volumes of Bleach and Wash Water for On-site Treatment

Volume of wash water†	Volume of bleach† (8.25% NaOCl w/v)	Total volume
490 mL (procedure)	20 mL	510 mL
55 gallons	2.25 gallons	57.25 gallons
100 gallons	4.1 gallons	104.1 gallons
500 gallons	20.5 gallons	520.5 gallons

†The ratio between bleach and water is 20:490, rather than 20:500 because the procedure calls for a non-chlorinated 10 mL sample of the spiked water to be taken to measure the spore concentration at time 0. This initial sampling reduces the starting water volume from 500 to 490.

4.9 Quality Assurance

4.9.1 Dilution Buffers

Positive and negative control tests that use a dilution buffer must be carried out prior to every set of analyses. If the positive control fails, change the stock organisms and use a new set of media plates. In the case of buffer failure for negative controls, use a new lot of phosphate buffer.

4.9.2 Triplicate Analyses

Triplicate analyses for spread plating are advisable for each sample, i.e., three plates per sample. A 20% variation (relative percent difference) is acceptable for the sample results. However, in case of greater variation, consider the other dilutions for enumeration of bacteria. If all dilutions yield greater than 20% variation, discard the sample and re-run the experiment.

4.9.3 Countable Range

For spread plating, if the number of colonies counted is less than 30, plate a lesser dilution. If the number of colonies counted is greater than 300, further dilute the sample.

4.9.4 Replicate Experiments

If a greater amount of quality assurance is desired, the inactivation experiment outlined in Section 4.4 can be repeated in duplicate or triplicate.

5.0 ANALYSIS OF SAMPLES FROM THE INACTIVATION STUDIES USING SPREAD PLATING AND MEMBRANE FILTRATION

Spread plating should be used when a high level of spores are expected, e.g., for analysis for of the initial inactivation sample, prior to bleach addition. Membrane filtration should be used when a low number of spores are expected, e.g., samples taken at the intermediate and final time points in the inactivation experiments

5.1 Spread plating

In this procedure, a dilution series of the sample is carried out, and small aliquots (0.1 µL) of the relevant dilutions are placed on an agar plates, spread across the plate, and then incubated.

Equipment

- Class II biological safety cabinet
- Alcohol burner
- Vortex mixer
- Water bath set to 75 °C
- Incubator set at 35-37 °C
- Pipettor, 200 µL capacity

Reagents

- Phosphate buffered saline (PBS), sterile
- Dilution buffer, 1X PBS with 0.02% Tween 80 buffer; Teknova No. P3875 or equivalent
- Bleach Wipes – Dispatch Hospital Cleaner Disinfectant Towels with Bleach, or equivalent
- 70% ethanol – Fisher Scientific 2546701 or equivalent

Media

- Tryptic Soy Agar (TSA) plates (Fisher Scientific, Cat. # B21283X or equivalent)

Supplies

- Disposable pipette tips, 200 µL capacity, or equivalent
- Disposable pipette tips, 1 mL capacity, or equivalent
- 15 mL conical centrifuge tubes (Fisher Scientific, Cat. # 05-527-90 or equivalent)
- Sterile hockey stick spreaders (Fisher Scientific, Cat. # 14-665-231 or equivalent)
- Polypropylene autoclave bags (Fisher Scientific No. 01-814C or equivalent)

I. Heat treatment.

1. Submerse centrifuge tubes containing sample, t_0, in a pre-heated water bath that had been set at 75 °C. Let stand for hour to eliminate vegetative cells. (This immersion can be done in conjunction with the heat treatment for the samples for membrane filtration, Section 5.2)

II. Dilutions

1. Prepare tubes for serial dilution by adding 10 mL of dilution buffer to 15 mL centrifuge tubes.
2. Label tubes 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}.
3. Vortex tube containing heat treated sample, t_0 for 30 seconds.
4. Pipette 1 mL from the tube containing t0 into the 10^{-1} tube and cap.
5. Vortex tube for 30 seconds.
6. Repeat to make the 10^{-2}, 10^{-3} and 10^{-4} dilutions.

III. Spread plating.

1. Prepare three TSA plates per sample tube. Label plates to indicate volume of the amount spread plated and the dilution.

2. Pipette 0.1 mL from the 10^{-1} dilution sample onto the labeled TSA agar plate and spread using hockey stick spreader following a spread plating technique such as the one described in Method 9215 C. APHA et al., 2005. Repeat with two additional plates.

3. Repeat spread plating for the other serial dilutions.

4. Incubate plates (do not invert plates) at 35-37 °C for 18 to 24 hours.

5. Disinfect the biosafety cabinet using bleach wipes followed by 70% ethanol.

6. After the incubation period, count the bacterial colonies on the plates.

7. Using the serial dilution plates that yield CFU in the countable range (30 – 300 CFU), calculate the bacterial suspension density in the original (undiluted) sample.

   Spore density (CFU) per mL of suspension is calculated using the following formula:

$$\frac{\boldsymbol{CFU\ on\ plate}}{\boldsymbol{Volume\ plated\ (mL)}} \times \frac{\mathbf{1}}{\boldsymbol{tube\ dilution}} = \text{Number of CFU/mL}$$

   For example:

$$\frac{\mathbf{200}\ \boldsymbol{CFU}}{\mathbf{0.1}\ \boldsymbol{mL}}\ \boldsymbol{X}\ \frac{1}{\mathbf{10^{-2}}} = \mathbf{2.0x10^5}\boldsymbol{CFU/mL}$$

Plate	CFU per plate	Volume Plated (mL)	Tube Dilution	CFU in initial sample, $[Bg]_{initial}$ (CFU/mL)	Log CFU in initial sample $[Bg]_{initial}$ (CFU/mL)	Average log CFU in initial sample $[Bg]_{initial}$ (CFU/mL)†
1	150	0.1	10^{-2}	1.5×10^5	6.18	6.29
2	250	0.1	10^{-2}	2.0×10^5	6.30	
3	200	0.1	10^{-2}	2.5×10^5	6.40	

†The average log CFU $[Bg]_{initial}$ is the value used for $\log_{10}[Bg]_{initial}$ in the equation in Section 4.4.5 for calculating log inactivation (LI).

5.2 Membrane Filtration

Membrane filtration (9215 D. APHA et al., 2005) allows for a larger volume to be analyzed compared to spread plating and is therefore the recommended method for samples with low levels of target organisms, e.g., samples taken at the intermediate and final time points in the inactivation experiments. If a sample contains significant quantities of suspended particles, the filter may be unable to pass all the liquid. The sample volume to be used per filter is 10 mL. Any organisms in the sample are concentrated on the surface of the membrane. The membrane is then placed on TSA plates where the passage of nutrients through the filter facilitates the growth of organisms on the upper surface of the membrane. The discrete colonies that form on the surface of the membrane during incubation are then enumerated after incubation.

Equipment and Supplies

Equipment

- Class II biological safety cabinet
- Stainless steel vacuum manifold
- Vacuum pump with a HEPA filter attached to the effluent port.
- Foot switch for the vacuum pump
- Motorized pipette pump, Drummond XP Pipet-Aid (cat. # 4-000-101) or equivalent
- Vortex mixer
- Water bath set to 75 °C
- Incubator set at 35-37 °C

Reagents

- Phosphate buffered saline (PBS), sterile

- Dilution buffer, 1X PBS with 0.02% Tween 80 buffer; Teknova No. P3875 or equivalent
- Bleach Wipes – Dispatch Hospital Cleaner Disinfectant Towels with Bleach, or equivalent
- 70% ethanol – Fisher Scientific 2546701 or equivalent
- Chlorine bleach with 8.25% NaOCl

Media

- Tryptic Soy Agar (TSA) plates (Fisher Scientific Cat. # B21283X or equivalent)

Supplies

- Glass vacuum flask (4 L size)
- Vacuum tubing for connecting the manifold to the flask and connecting the flask to the vacuum supply
- Rubber stopper fitted with a glass tube to connect the manifold to the vacuum flask
- Stainless steel forceps, pre-sterilized, one for each filter membrane
- Nalgene disposable analytical test filter funnels, 250 mL (Fisher # 09-740-30J or equivalent)
- Disposable sterile 10 mL serological pipettes
- Motorized pipette pump, Drummond XP Pipet-Aid (cat. # 4-000-101) or equivalent
- Disposable sterile polyethylene 50 mL conical tubes (Fisher # 02—683-173 or equivalent)
- Polypropylene autoclave bags (Fisher Scientific No. 01-814C or equivalent)

Special Safety Precautions

Because of the possibility of viable *B. anthracis* spores in the wash water samples, BSL 3 practices should be followed, which include wearing appropriate PPE (disposable laboratory

coat or gown, nitrile gloves, safety glasses) and performing work within a properly functioning BSC.

Procedure.

I. Heat treatment.

1. Submerse centrifuge tubes containing sample in in a pre-heated water bath that had been set at 75 °C. Let stand for hour to eliminate vegetative cells. (This immersion of tubes can be done in conjunction with the heat treatment of tubes from Section 5.1)

II. Set up Vacuum Manifold and Trap Flask

1. In a biological safety cabinet, place the vacuum manifold, trap flask, and vacuum tubing for assembly.

2. Add 100 mL bleach to the 4 L trap flask before assembling the filtering apparatus.

3. Once the vacuum manifold and trap flask are connected, connect the apparatus to a vacuum intake in the biological safety cabinet.

4. Fit three reusable filter funnel connection cones into rubber stoppers that will be placed on the vacuum manifold. If the holes in the rubber stopper(s) are not large enough, use a cork borer or drill to enlarge them. Never force anything through the stopper.

5. Set up the vacuum pump equipped with a HEPA filter on the exhaust port of the pump. Connect the inlet of the vacuum pump to the vacuum port on the outside of the biological safety cabinet.

6. Attach the foot switch to the power cord on the vacuum pump, so the pump can be powered on and off using the foot switch.

III. Sample Processing

1. With the vacuum turned on, aseptically transfer the entire sample from the centrifuge tube to the filter funnel.

2. After the sample has passed through the filter, wash the sides of the filter funnel with 10 mL PBS.

3. Turn the vacuum off once all the liquid has passed through the filter.

4. Using sterile forceps, aseptically transfer the filter to a labeled agar plate containing TSA. Care must be taken to ensure the filter is placed on the culture medium with the top remaining upright. Also place the filter on the medium without air pockets underneath, as air pockets will inhibit bacterial growth. If an air pocket is present, lift the filter with sterile forceps and carefully reposition the filter to eliminate the air pocket.

5. After removing the filter membrane, reattach the funnel to the pedestal. Disconnect the disposable filter unit from the vacuum manifold and discard it in the autoclave bag.

6. Incubate plates (do not invert plates) at 35-37 °C. The incubated plates should be checked after 18-24 hours of incubation and if colonies are not well developed, the plates should be examined and colonies counted again after 48 hours.

7. When all filtration has been completed, pour bleach into each manifold port and allow it to be aspirated into the trap flask.

8. After the bleach has drained out of the vacuum manifold, pour 70 % ethanol into each manifold port and allow it to be aspirated into the trap flask (If bleach is not used, let 70% ethanol set in the manifold for at least 15 minutes before opening ports and draining the manifold).

9. Disinfect the biosafety cabinet using bleach followed by 70% ethanol.

10. After the incubation period, count the bacterial colonies on the membrane.

11. Calculate the bacterial suspension density using the following equation:

$$\text{Number of CFU/mL} = \frac{\boldsymbol{CFU\ on\ membrane}}{\boldsymbol{Volume}\ filtered(\boldsymbol{mL})} \times \frac{\mathbf{1}}{\boldsymbol{tube\ diluiton}}$$

For example

Plate	CFU per plate	Volume Plated (mL)	Tube Dilution	CFU in original sample, $[Bg]_{final}$ (CFU/mL)	Average CFU in $[Bg]_{final}$ (CFU/mL)	Log of average CFU $[Bg]_{final}$ (CFU/mL)*
1	10	10	1	1	0	0.023
2	9	10	1	0.9	-0.046	
3	13	10	1	1.3	0.11	

*The Log of average CFU $[Bg]_{final}$ is the value used for $\log_{10}[Bg]_{final}$ in the equation in Section 4.4.5 to calculate log inactivation.

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