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Inactivation of *Bacillus* Spores in Wash Waters Using Dilute Chlorine Bleach Solutions at Different Temperatures and pH Levels

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Abstract

Inactivation of *Bacillus globigii* spores in wash water was studied to simulate chlorine inactivation of *Bacillus anthracis* spores in water generated during biological cleanups. Eight waters were studied, with six containing detergent. Chlorine levels were approximately 3000 mg/L. Results across different waters showed decreasing inactivation with increasing pH. Inactivation did not appear to be influenced by chemical oxygen demand, suspended solids, turbidity, or dissolved solids. Inactivation efficacy was expressed as the time calculated to yield 6 log₁₀ inactivation at 3000 mg NaOCl/L. This time ranged from 5 to 51 minutes at ~21 °C and from 11 to 209 minutes at ~5 °C. For one wash water, inactivation was conducted when there was no pH adjustment, and when the pH was buffered at 7 and 8. Inactivation in these buffered waters was rapid, but inactivation decreased sharply at a pH above ~9.3.

Keywords

anthrax; *Bacillus*; spores; inactivation; wastewater; wash water; hypochlorite; bleach; chlorine

Introduction

In the last fifteen years a number of buildings have been intentionally or accidentally contaminated with *Bacillus anthracis* (*B. anthracis*) spores (Guh et al., 2010; Whitney et al., 2003). During remediation following these events, disinfectant solutions were often used to wash equipment and building surfaces that may have contained viable *B. anthracis* spores. In addition, personnel performing the remediation activities were often washed down with cleaning solutions prior to removing personal protective equipment (PPE). In typical operations, the wash water generated from these decontamination processes was collected in

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containers prior to disposal. Because of the possibility of this water containing *B. anthracis* spores, disposal often posed a challenge. For example, water generated during the cleanup of the Hart Senate building was transported to the U.S. Army Medical Research Institute for Infectious Diseases (USAMRIID and steam sterilized prior to disposal (U.S. EPA, 2002).

Because of disposal challenges, the U.S. National Response Team (U.S. NRT), a multi agency team chaired and vice chaired by the U.S. EPA and the U.S. Coast Guard, respectively, developed a quick reference guide for the on-site treatment of PPE wash water containing *B. anthracis* spores (U.S. NRT, 2012). The recommended treatment was originally 1 part household bleach, 1 part vinegar, and 10 parts wash water (by volume) with an exposure time of 1 hour. This resulted in a solution of approximately 5000 milligrams (mg) sodium hypochlorite (NaOCl) per liter (L), assuming 6% (w/v) NaOCl in household bleach. The recommended U.S. NRT treatment was derived from inactivation procedures developed for *B. anthracis* spores on solid surfaces and had not been specifically tested in water matrices. In light of this lack of testing, Muhammad et al. (2014) tested the inactivation of *Bacillus* spores in simulated wash water using different levels of bleach and vinegar. In the study, a surrogate for *B. anthracis* spores was used: *Bacillus atrophaeus* subspecies *globigii* (*B. globigii*). In a water matrix, *B. globigii* spores have been observed to be more resistant to chlorine than *B. anthracis* spores (Sivaganesan et al., 2006) and thus served as a conservative surrogate for this study. Muhammad et al. (2014) initially showed that the recommended dose (1 part bleach, 1 part vinegar, and 10 parts wash water) yielded 5 log₁₀ (log) or 99.999% inactivation in less than 1 minute. This finding prompted their investigation of lower strength bleach solutions (600 to 3000 mg NaOCl/L) with no vinegar. These changes had potential to simplify the process, lessen the amount of hazardous materials (i.e., bleach) needed on-site, and reduce the concentration of waste products from the inactivation process. They found that in the simulated wash waters, the rate of inactivation increased as the level of bleach increased from 600 to 3000 mg NaOCl/L and that 6 log inactivation (99.9999%) was achieved in 30 minutes or less at 20 °C using a 3000 mg NaOCl/L concentration. The lower NaOCl concentration and the elimination of vinegar did result in a less germicidal solution, compared to the U.S. NRT recommendations, but it also eliminated the danger of chlorine gas formation as a result of an excessive amount of acid addition, which could be problematic for on-site cleanup personnel during an actual anthrax cleanup. In addition, the less aggressive conditions allowed greater ability to see differences in inactivation because of differences in wash waters and other process conditions, which could help optimize the process.

The work described in this article expanded upon the studies reported in Muhammad et al. (2014). The current study evaluated a larger variety of wash waters at room temperature and at 4 °C. Given that wash water generated at a site cleanup would be unique to that site, there was interest in evaluating wash waters from a wider range of sources. The rationale was to increase the likelihood that one of the wash waters studied would be similar to the wash water that could be generated at an actual cleanup site of *B. anthracis* spores. For all tests the treatment target was 6 log inactivation. In addition, since treatment of wash water could occur in an outdoor environment where the temperature would not be controlled, there was also interest in evaluating bleach inactivation at colder temperatures. Given that wash water would likely contain a substantial amount of particulate matter, tests were conducted to

study the possibility of spores being shielded from chlorine if they adhered to other particles in the water. Lastly, as the testing described in this article progressed, it was seen that the treatment goal of 6 log removal was not always achieved, especially for the studies conducted at colder temperatures and at relatively high pH values (i.e., ~10). In response, studies were conducted where the pH of the bleach solution was lowered using a phosphate buffer to increase sporicidal activity. The phosphate buffer provided a safer way to lower the pH of a bleach solution than the addition of vinegar as recommended by the U.S. NRT guidance.

In total, eight different types of wash waters were studied. Wash waters were generated by using aqueous solutions of detergent to wash floors, automobiles, or personnel wearing PPE. Cincinnati tap water originating from a surface water source was used in the testing. In order to simulate inactivation using tap water from a groundwater source, salts such as potassium chloride, sodium bicarbonate, and calcium sulfate were added to some of the wash waters for increased hardness. In addition, water containing a high amount of particulate iron was tested to evaluate whether chlorine demand from the iron decreased the amount of inactivation. Storm water was also collected and tested with no detergent addition. Lastly, a wash water with 19 mg/L motor oil was tested to simulate wash water from the runoff of ground surfaces containing petroleum chemicals.

Methodology

The procedures for preparing and enumerating *B. globigii* spores were conducted as described in Muhammad et al. (2014) and are briefly summarized below.

***B. globigii* Spore Preparation.**

Vegetative *B. globigii* cells were grown in generic spore medium (8 g nutrient broth, 40 mg MnSO_4 , and 100 mg CaCl_2 in 1 liter deionized water) for 5 days at 36 °C in a rotary shaker, and the resulting endospores were purified using gradient separation. A subsample of the purified spores was heat-shocked and analyzed to determine the spore concentration. The purified spores were stored in 40% (v/v) ethanol until use. Prior to a particular inactivation study, purified spores were reintroduced into generic spore media and again cultured for 5 days at 36 °C. The resulting spore suspension was heat-shocked at 80 °C for 10 minutes, analyzed for the exact concentration, and used in the inactivation experiments.

***B. globigii* Enumeration.**

The enumeration of *B. globigii* in non-chlorinated water samples included heat-shocking the sample to eliminate vegetative cells, filtering the sample through a 0.45 μm cellulose acetate membrane, transferring the membrane to Tryptic Soy Agar (TSA) plates, incubating the plates for 24 hours at 36 °C, and counting the number of colony forming units (CFU) on each plate. The detection limit for *B. globigii* was 5 CFUs/100 mL. For each sample, three volumes were membrane filtered: 0.1 mL, 1.0 mL, and 10 mL, each in duplicate. Spore samples taken during the inactivation studies were not heat-shocked since the chlorine level used was adequate to inactivate all vegetative cells.

Wash Water Generation.

Table 1 describes the eight wash waters studied and the specific methods used for generation. Except for Wash Water F (storm water), wash water was generated by conducting washing activities followed by the collection of the resulting runoff water. Unless otherwise noted, the water used for generation was Cincinnati tap water which was filtered through a point-of-use filter containing carbon media to remove residual chlorine prior to use. Detergents were added to six of the eight wash waters studied. The detergents used were either Alconox Powdered Precision Cleaner (Alconox) or Dawn Dishwashing Liquid (Dawn). The resulting concentration of Alconox and Dawn were both 1 % (w/v). After generation, the wash waters were stored in clean 5 gallon buckets at 4 °C until needed. After generation, the wash waters were initially characterized by analyzing for water quality parameters such as chemical oxygen demand (COD), turbidity, total suspended solids (TSS), and total dissolved solids (TDS). The results of this characterization are shown in Table 2.

Bleach Solution in Test Matrix.

The target NaOCl concentration in the bleach/wash water solution was 3000 mg NaOCl/L. Muhammad et al. (2014) showed that this concentration provided the desired amount of inactivation (6 log) in approximately 30 minutes for the wash waters tested. The NaOCl solution used in this current study was made from Clorox regular bleach that had either 6% NaOCl or 8.25% NaOCl. During the conduct of this study, household bleach manufacturers changed the concentration of NaOCl in bleach from 6% to 8.25% NaOCl. The volume of bleach was adjusted accordingly in order to maintain the target NaOCl concentration.

General Protocol for Inactivation Studies.

The inactivation experiments were conducted in a 5 liter glass reactor with a sampling port fitted at the bottom of the reactor. For each wash water and temperature combination, at least three inactivation trials were carried out. Two liters of wash water were transferred to the reactor and spiked with *B. globigii*, with the goal of achieving a target spore concentration of $10^7/100$ mL in the reactor. The reactor contents were stirred constantly at approximately 120 rpm using a magnetic stir bar for approximately 10 minutes prior to bleach addition. Samples were collected via the port located at the bottom of the reaction vessel. In the earlier experiments described in this study, samples for *B. globigii* were collected prior to the addition of bleach solution ($T = 0$) and then at 3, 5, 10, 15, 30 minutes after the addition of bleach solution. However, during the course of the testing, it was realized that 6 log inactivation was not always achieved in 30 minutes, and thus it was decided to increase the length of the inactivation experiments. For the later experiments additional samples were taken at 60, 180, and 240 minutes to help ensure documentation of the time required for 6 log inactivation for each of the waters. The concentration of *B. globigii* at $T = 0$ was the starting concentration used in the calculation of log inactivation. The samples taken after bleach addition were collected in 100 mL sample bottles, using sodium thiosulfate solution (9.7 ml of 11% (w/v) anhydrous salt solution) as a quenching agent to neutralize the sodium hypochlorite. The amount of quenching agent needed was based on the amount typically used to quench the chlorine residual in drinking water samples (APHA et al., 2005) and increased in an amount directly proportional to the level of chlorine in the inactivation

experiments. This level of the quenching agent required was also verified experimentally to ensure removal of residual chlorine prior to analysis. Matrix water samples were collected in 125 mL bottles without sodium thiosulfate at each sampling event and tested for pH and free chlorine concentration. Tests were conducted either at room temperature or at a target temperature of 4 °C. For these latter tests, the temperature was maintained by conducting the experiments in a refrigerator maintained at 4 °C and adding ice packs to the exterior of the reactor. For the room temperature tests, the wash water temperature varied from 19.2 °C to 24.3 °C with an average temperature of 21.5 °C. Cold water test temperatures varied from 2.8 °C to 7.9 °C with an average temperature of 4.6 °C.

The efficacy of chlorine inactivation was calculated assuming Chick-Watson kinetics, which proposes that the inactivation rate is first-order with respect to the concentration of microorganisms (James M. Montgomery Inc., 1985). For this type of kinetics, when the logarithmic value of the amount of inactivation, or log inactivation (LI), is plotted versus the multiplication product of the disinfectant concentration (C) and exposure time (T), or CT, a straight line is produced. The slope of this line can then be used to estimate the required CT for a given amount of inactivation.

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

$$LI = \log[Bg]_{\text{initial}} - \log[Bg]_T \quad (1)$$

where $[Bg]_{\text{initial}}$ is the initial ($T = 0$) concentration of *B. globigii*, $[Bg]_T$ is the concentration of *B. globigii* at a given time, T. CT was calculated by multiplying the applied dose of NaOCl in mg/L and the exposure time in minutes. Inactivation data was plotted and a linear regression was performed assuming the regression line passed through the origin. The exact value of the dose varied depending on the volume removed from the reactor vessel for sampling prior to bleach addition, and the volume of buffer added, but was approximately 3000 mg NaOCl/L. The slope of the linear regression was then used to estimate the time needed for 6 log removal, or $T_{6\log}$. $T_{6\log}$ was the main parameter used to compare inactivation rates among the different experiments.

Inactivation Tests after Prolonged Contact of Spores with Wash Water.

Inactivation tests were also conducted where bleach addition to the spore laden wash water was delayed. Floor wash water with 1% Alconox (Wash Water A) was generated for this set of tests. For one aliquot of the wash water, inactivation experiments were conducted as described above. For two other aliquots, after spore addition and subsequent mixing for 10 minutes, the mixer was turned off and the wash water was allowed to sit for 48 hours and 96 hours, respectively, before the inactivation experiments commenced. Tests were conducted both at ambient temperature and at 4 °C. For the 4 °C tests, the entire test (i.e., spore addition, prolonged contact, and inactivation) occurred at 4 °C.

Inactivation Tests at Controlled pH.

A series of tests were conducted in Wash Water E (car wash water with 1% Dawn) to compare inactivation rates in wash water without pH adjustment with inactivation rates when the pH of the wash water was adjusted to 7 and 8 using a phosphate buffer.

Inactivation tests proceeded as described earlier, except that for the buffered tests, after adding spores and bleach, 10 % (w/v) K_2PO_4 solution was added to reach the target pH.

Analytical Procedures for Water Quality Parameters.

Turbidity, free chlorine, and COD were analyzed by Hach methods (HACH, 1997; HACH, 1999; HACH, 2007). Alkalinity (Method 2320B), TSS (Method 2540-D), and TDS (Method 2540-C) were analyzed by Standard Methods (APHA et al., 2005).

Results and Discussion

General Results.

Table 3 presents the results of inactivation studies where there was no prolonged contact of spores with wash water and where the pH of the wash water was not adjusted. The parameter T_{6log} (the estimated time for 6 log removal) was useful for comparing different data sets, however there were challenges in the calculation of this parameter. For some wash waters, no colonies were detected on the TSA plates for samples collected after 5 minutes of reaction time, signifying complete inactivation. Thus, in these cases, there was only one data point to estimate T_{6log} . While this showed rapid inactivation, it may also have led to inaccuracies in estimating T_{6log} . For other plots, there was a high degree of scatter in the data resulting in low coefficients of correlation (R^2). These low R^2 values may have been because, in part, a small variation in the CFUs on different plates led to a large variation in the level of log inactivation. Lastly, for some tests, there was an initial time lag where there was no inactivation below a certain CT value, but beyond that value, the inactivation appeared to be linear.

For the individual plots of log inactivation versus CT, R^2 values from the linear regressions are shown in Table 3 to give an indication of data variability. The number of data points (other than the origin) used in the linear regression are also shown in Table 3. Figure 1 shows examples of some of the data sets mentioned above, as well as results of a test that showed a high coefficient of correlation.

In addition to the calculated estimate for T_{6log} ; the maximum log inactivation (LI_{max}) achieved and the associated sample time (T_{max}), when LI_{max} was first observed during each test, are also shown in Table 3. Typically, there were enough spores in the reaction vessel and sufficient exposure time to allow a measurement of at least 7 log inactivation. For the majority of the cases, LI_{max} is expressed as ∞ values because complete inactivation was reached, that is, there was an absence of visible colonies on all plates associated with a given sample. For these latter cases, since LI_{max} was usually within 1.5 logs of the target log inactivation (i.e., 6 log), the associated sample time, T_{max} , can be used as an approximate check for the calculated value of T_{6log} . (When LI_{max} reflected a ∞ value, the data from this sample point was not used in the T_{6log} calculation.) In most of these cases, T_{6log} and T_{max} typically differed by a factor of approximately two or less. For example, for Wash Water E, (car wash with Dawn), the calculated time for 6 log removal (T_{6log}) was 28 minutes, whereas the samples taken at 60 minutes showed greater than 7 log removal. In a number of cases, the opposite was true: T_{6log} was greater than T_{max} .

Chlorine efficacy varied among the different wash waters tested. For six of the eight wash waters tested at room temperature (21.5 °C on average), $T_{6\log}$ was estimated to be 30 minutes or less. For Wash Water G (rusty water) and for Wash Water F (storm water), the values of $T_{6\log}$ were 41 minutes and 43 minutes, respectively. Complete inactivation was not achieved at room temperature for the majority of trials with these two types of waters.

At colder temperatures (4.6 °C on average), the amount of inactivation decreased. Complete inactivation was not attained in rusty water, storm water, or Wash Water E, (car wash water with Dawn). In rusty water, there was less than 1 log inactivation after 30 minutes. In addition, the calculated values of $T_{6\log}$ were greater than those at room temperature. The increase in $T_{6\log}$ ranged from 1.2 to 5 times longer, depending on the wash water, with the highest estimated $T_{6\log}$ value being 3.5 hours. The colder temperature tests are an example of a worst case scenario and point to the advantages of conducting chlorine inactivation at warmer temperatures. For the rusty water at colder temperatures, there was also higher variability in the calculation of $T_{6\log}$. This is likely a result, in part, of the relatively short length of time of the inactivation reaction (30 minutes), and thus only a maximum of 1 log removal was observed. It is suspected that a longer exposure time, and thus a greater log removal, may have led to less variability in the values for $T_{6\log}$. As mentioned earlier, later experiments summarized in this article were carried out for longer periods of time to address the possibility of slower inactivation in other challenging wash waters.

Of the water quality parameters measured, COD, TSS, TDS, and turbidity did not appear to affect inactivation: when $T_{6\log}$ was plotted versus any of these water quality parameters, no trend was observed, for example, $T_{6\log}$ did not increase with increasing COD, TSS, TDS, or turbidity. The two parameters that appeared to have the most influence on inactivation were pH after bleach addition (shown in Table 3) and alkalinity of the wash water prior to bleach addition (shown in Table 2). These two parameters are related since the pH value after bleach addition is dependent on the amount of buffering capacity, that is, the alkalinity of the wash water. Adding bleach, which is alkaline (pH = 11.2), results in a smaller increase in pH for waters with a higher amount of alkalinity. The remainder of the discussion will focus on pH rather than alkalinity measurements, whereas alkalinity before bleach addition was typically measured once per batch of wash water and pH readings were taken multiple times during each inactivation test.

Figures 2 and 3 show the effect of pH (post bleach addition) on $T_{6\log}$ at room temperature and at 4°C, respectively, for the different wash waters presented in Table 3. The figures show that the time to achieve 6 log inactivation increases with increasing pH. This is consistent with the published literature, which shows that the germicidal effectiveness of chlorine bleach decreases as pH increases. (Rice et al., 2005; Sivaganesan et al., 2006). The decrease is a result of the thermodynamic shift from hypochlorous acid (HOCl) to hypochlorite ion (OCl⁻) with increasing pH (Black and Veatch Corporation, 2010; Hoff, 1986).

The two detergents used during the inactivation studies had different amounts of buffering capacity. Dawn dishwashing liquid (commonly used to decontaminate personnel wearing PPE) contained a negligible amount of buffering agents compared to Alconox. After bleach addition the pH of the wash waters using Alconox ranged from 8.9 to 9.7, which reflects an

increase, on average, of 0.1 pH units. For the waters containing Dawn, the resulting pH ranged from 9.8 to 10.1, which reflects an increase, on average, of 1.7 pH units, after bleach addition. The lower pH in the wash waters containing Alconox is likely the reason that the average $T_{6\log}$ was less than 30 minutes, even at colder temperatures (2.8 °C to 6.7 °C). Earlier work showed that Alconox by itself had no detectable germicidal effect on *Bacillus* spores (Muhammad et al., 2014).

Achieving 6 log inactivation was the most challenging for wash waters that had the highest pH after bleach addition, and thus the lowest ratio of HOCl to OCl^- . The three most challenging waters were rusty water (Wash Water G), storm water (Wash Water F), and car wash water (Wash Water E), which contained Dawn dish detergent and yielded a relatively high pH (9.8 to 10.1) after bleach addition, compared to the other wash waters studied.

As previously mentioned, at colder temperatures, the inactivation of *B. globigii* spores in rusty water (Wash Water G) was less than 1 log after 30 minutes. A wash water with a high amount of particulate iron was selected, with the assumption that it would exert a high chlorine demand. However, none of the waters tested showed a measurable decrease in chlorine over the length of the inactivation test. The chlorine level was likely at such a high level that any decrease, as a result of demand from wash water constituents, was not seen in subsequent chlorine analyses. One explanation for the low inactivation seen in rusty water was that the *B. globigii* spores may have adhered to the iron particles and were thus partially shielded from chlorine. A similar phenomenon is observed in distribution systems where *Bacillus* spores adhered to ductile iron pipe material, albeit with a biofilm, and showed significant resistance to chlorine inactivation (Szabo et al., 2007). As will be discussed later, tests were performed in which *B. globigii* spores were allowed to contact the wash water for up to 96 hours prior to chlorination to test this shielding hypothesis.

As can be seen by comparing the inactivation results in Table 3 with the water quality parameters in Table 2, there was no apparent correlation between $T_{6\log}$ and any of the following parameters: COD, total suspended solids, total dissolved solids, turbidity, and pH of wash water prior to bleach addition. The initial assumption had been that there would be a significant chlorine demand because of high levels of COD and particulate matter, which would lead to lower inactivation. Chlorine demand was inferred by noting the chlorine concentration over the length of a particular inactivation experiment. The chlorine concentration did vary during a particular experiment, likely a result of the fact that significant dilutions of the samples were performed prior to chlorine analysis. However, although there was variability, there was no obvious trend that showed chlorine decreasing over time. This observation is shown in Figure 4, which is a plot of chlorine concentration versus time for numerous experiments.

The large effect of pH on inactivation was unexpected at the pH range studied (8.8 to 10.9, postchlorination). In this range it was expected that the majority (95% or more) of the chlorine species would be OCl^- , which is less germicidal than HOCl, and that the different amounts of HOCl within this narrow pH range would not yield differences in inactivation. In published graphs (e.g., Black and Veatch Corporation, 2010) that show the relative percentages of OCl^- and HOCl as a function of pH, there is typically a sharp increase from

HOCl to OCl^- , with the inflection point (1:1 ratio of HOCl and OCl^-) at a pH of approximately 7.5. It is possible that in the wash waters studied, this inflection point shifts towards a higher pH, or the transition from HOCl to OCl^- occurs at a wider pH range. Either phenomenon could lead to a greater amount of HOCl present at a higher pH compared to a solution of only free chlorine species in water. In addition, published data that shows the inflection point at pH = 7.5 were generated at a much lower OCl^- concentration (e.g., 56 mg OCl^-/L in Nakagawara et al. (1998)) than in the current study (3000 mg OCl^-/L). Plots showing percent HOCl versus pH may display different characteristics at this higher concentration.

In the related work cited earlier (Muhammad et al., 2014), as the concentration of NaOCl increased from 600 mg/L to 3000 mg/L, the pH also increased, which in itself led to less germicidal conditions, however the net effect of increasing NaOCl was an increased inactivation. In this range of NaOCl concentrations, the effect of an increase in pH was more than offset by the effect of an increased level of the inactivating agent (OCl^-).

Prolonged Contact of Spores with Wash Water Constituents.

It is well known that source water containing a high amount of turbidity requires a higher CT for required disinfection compared to lower turbidity waters (LeChevallier et al., 1981). This is a result, in part, of microorganisms adhering to particulate matter and thus being less exposed to the disinfectant in the bulk water phase. In order to address whether this phenomenon is relevant to this work, spores were introduced to three separate aliquots of wash water generated by decontaminating floors with a 1% Alconox solution. Inactivation studies were immediately conducted on one of the aliquots and conducted on the other two aliquots after 48 and 96 hours, respectively. The inactivation results at the different contact times are shown in Table 4.

No shielding was observed in that there was no appreciable difference between inactivation conducted at 0 hours, 48 hours, or 96 hours for the wash water tested. It was originally hypothesized that if shielding was occurring, the more contact time there was between wash water particles and spores prior to chlorination, the more shielding (i.e., the less inactivation) would be seen. However, as inactivation data from the rusty water suggests, any shielding effect might depend on the type of particulate matter in the wash water, rather than the amount of time spores contacted other particulate matter prior to chlorination. For the rusty water experiments, spores were mixed with the rusty water for only ten minutes prior to chlorination, but evidence of shielding was suggested by the data. Thus, contact between spores and other particulate matter may not need to be for an extended time in order for processes to occur that lead to shielding (e.g., adherence). In addition, shielding is known to be influenced by other factors such as particle size (Winward et al., 2008).

Inactivation Studies at Different pH.

As was shown in Table 3, a lower pH during inactivation led to a greater log inactivation. This observation led to testing inactivation at controlled pH levels. One method of lowering the pH of a chlorine bleach solution is by the addition of an acid, such as vinegar, to the solution (U.S. NRT, 2012). However, because there is the potential of creating chlorine gas if

an excessive amount of acid is added, the pH was controlled using a phosphate buffer. For these studies, one of the waters that yielded a relatively low amount of inactivation was used: car wash water with 1% Dawn (Wash Water E).

Table 5 shows the results of the inactivation with no buffer, and with buffer added to maintain the pH levels at 7 and 8. As anticipated, inactivation at the lower (buffered) pH yielded more rapid inactivation, as shown by the lower values for T_{6log} . However, there was only a small difference in inactivation times for pH levels of 8 and 7. Similar to previously mentioned data, there was a relative lack of data for estimating T_{6log} : complete inactivation occurred in 5 minutes or less after bleach addition, for 3 out of the 5 tests at room temperature. Thus, there was only one data point to estimate T_{6log} . If samples had been collected every minute for the first five minutes, different inactivation rates may have been observed between the two buffered levels. For colder temperatures, there also was no clear difference in inactivation between the two buffered pH levels, but complete inactivation took slightly longer: T_{6log} ranged from 5 to 10 minutes.

For the nonbuffered wash waters, inactivation was slower than in the buffered wash waters. However, for nonbuffered wash waters where the pH only rose to 9.3 or less, inactivation was moderately fast, with an average T_{6log} of 20 minutes. Where pH postchlorination was 9.9 or above, the average inactivation was noticeably slower, with an average T_{6log} of 91 minutes. Inactivation data for both buffered and nonbuffered wash water at colder temperatures were combined and plotted in Figure 5. This plot shows that T_{6log} appears to rise sharply at a pH somewhere between 9.3 and 10. Although results with different wash waters may vary, these results suggest that improved inactivation can be realized if the pH of the chlorinated wash water is lowered to around 9 rather than 7. A pH of 9 would lead to a more stable $OCI^-/HOCl$ solution (Adam et al., 1992) as well as require less buffer or acid, and in the latter case, would allow for safer operation since there would be less risk associated with adding an excessive amount of acid. Results further suggest that detergents containing buffering agents would be a good choice for use in decontamination activities.

CT Lag.

For spore inactivation, a CT lag (CT_{lag}) has been reported for numerous chlorine based disinfectants (Sivaganesan et al., 2006). For a given concentration of a disinfectant, it has been observed that a certain amount of time must elapse (T_{lag}) before any inactivation occurs. After times greater than T_{lag} , inactivation proceeds as expected, with a linear relationship between CT and log inactivation.

For the body of work presented in this article, a CT_{lag} was observed more often when inactivation was slower, especially at colder temperatures and at higher pH. Figure 6 shows an example of two sets of inactivation data from the same lot of car wash water. The nonbuffered wash water (pH = 10.06) shows a CT_{lag} of approximately 60 000 mg-min/L, which occurred at about 20 minutes, at the chlorine concentration used. For the pH 7 buffered wash water, there appears to be no lag. However, it is likely that in all cases there was a CT_{lag} , but this was only detected when inactivation was slow enough for the lag to be captured via sampling at the different times.

Young and Setlow (2003) proposed that the outer spore coat provides the majority of the resistance to chlorine inactivation. This hypothesis, combined with the well documented CT_{lag} , strongly suggests that the outer spore coat must be fully breached by the disinfectant before measurable inactivation can occur. This would explain why there is no evidence of inactivation at a CT less than CT_{lag} .

The fact that a CT_{lag} was not observed in this present study for faster inactivation tests (i.e., lower pH), may support the concept that HOCl is more effective in breaching the outer spore coat than OCl^- , although this study was not designed to test this concept. It has been hypothesized that HOCl is more germicidal than OCl^- , because the former is a smaller and uncharged molecule and can thus diffuse more rapidly into the cell interior, and also because it has a higher oxidation reduction potential compared to OCl^- , enabling it to react more rapidly with critical cell components (Black and Veatch Corporation, 2010). This latter feature of HOCl likely gives it a greater ability to destroy, or otherwise fully breach, the outer spore coat, which would explain the superior efficacy of chlorine at the lower pH values.

Conclusions

This study showed that the process conditions that significantly affected inactivation were temperature and pH after bleach addition. Other water quality parameters studied (pH of water prior to bleach addition, COD, TSS, TDS, and turbidity) did not appear to influence inactivation at the high chlorine concentrations studied. Chlorine demand from the wash water constituents was not observed to have a negative effect on inactivation. For a concentration of 3000 mg NaOCl/L, the time required for 6 log inactivation ranged from 5 to 51 minutes at room temperature and from 11 to 209 minutes at colder temperatures of around 4 °C.

Results show that decreasing the pH to a value of 9 or less (after bleach addition) would be advantageous for improvement in inactivation. A phosphate buffer was observed to be an effective way to lower the pH while avoiding the hazard of chlorine gas formation that could come about if vinegar is added in excess of published guidelines (U.S. NRT, 2014). During these experiments, it appears that the particulate matter in most of the wash waters did not shield microorganisms from chlorine, however, shielding may depend on the type of particulate matter in the water, rather than the amount of time spores contacted other particulate matter prior to chlorination, especially at such high levels of chlorine. A CT_{lag} was observed more often when inactivation was slower, especially at colder temperatures and higher pH. However, it is likely that in all cases there was a CT_{lag} , but the lag was only detected when inactivation was slow enough for it to be captured at the different sampling times.

Earlier work (Muhammad et al., 2014) showed that 30 minutes contact time at 3000 mg NaOCl/L was sufficient to yield 6 log of *B. globigii* inactivation in a number of different wash waters. However, as shown in this paper, low buffering capacity of the wash water, and low temperatures during inactivation can decrease the efficacy of chlorine bleach. This suggests that for successful treatment of wash water containing *Bacillus* spores, attention

should be given to these two parameters, and that lowering the pH of the reaction gives on-site personnel significant capability to lower the time needed for sufficient inactivation.

The calculated value, $T_{6\log}$, was helpful in comparing different data sets, although it posed some challenges when there was little data for the calculation, or when there was high variability in the data. In light of this variability and the resulting uncertainty, increasing the chlorine exposure time by a factor of two, compared to $T_{6\log}$, would help ensure sufficient inactivation at an actual cleanup site where chlorination of *B. anthracis* contaminated wash water is performed.

The data presented in this paper showed that chlorine levels did not noticeably decrease during the times studied (0 to 180 minutes), thus it is not anticipated that this suggested increase in exposure time would lead to a drop-off in chlorine levels and thus inactivation efficacy over the length of the reaction. Based on the data in this article, and assuming a safety factor of 2 and a 3000 mg NaOCl/L concentration, in order to achieve 6 log inactivation in actual wash water containing viable *B. anthracis* spores, chlorine exposure times of 100 minutes and 400 minutes are suggested for wash water temperatures of ~20 °C and ~4°C, respectively. If more aggressive inactivation is desired, the procedure developed by U.S. NRT (2012) could be used. This latter procedure could be particularly appropriate for smaller volumes (i.e., < 30 gallons) of wash water: the smaller the volume, the lower the total amount of chlorine gas that could be produced if excessive acid is added. However, whether these above suggested conditions would be acceptable at an actual site cleanup would be a decision for personnel such as on-scene coordinators and water resource recovery facility officials.

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Figure 1—
Examples of log inactivation vs. CT plots.

Figure 2—.

Time for 6 log inactivation vs. pH, after Cl_2 addition, room temperature.

Figure 3—.
Time for 6 log inactivation vs. pH, after Cl₂ addition, 4 °C.

Figure 4—.
Free chlorine concentration vs. time.

Figure 5—.

Time for 6 log kill vs. pH. combined results from buffered (pH = 7 and pH = 8) and non-buffered car wash water, 4 °C.

Figure 6—.
Log inactivation vs. CT, with and without an apparent CT lag.

Table 1—

Description of wash waters studied.

Wash Water	Description	Method of generation
A	Floor wash water w/1% Alconox	Floors at the U.S. EPA facility were mopped with a solution of 1% w/v Alconox. The water was then collected by wringing mops using a janitorial bucket equipped with a wringer.
B	Floor wash water w/1% Alconox with moderately hard reconstituted water (MHRW)	Same as above except MHRW was used instead of tap water. The water was produced by adding 1.45 g, 21.74 g, 34.78 g, and 21.74 g of KCl, MgSO ₄ , NaHCO ₃ , and CaSO ₄ ·2H ₂ O, respectively, to 329 liters of carbon filtered tap water.
C	PPE wash water w/1% Alconox	PPE-clad personnel were washed down with a solution of 1% (w/v) Alconox. Wash water was collected and stored as mentioned above.
D	PPE wash water w/1% Alconox in MHRW	Same as Wash Water, C, above except MHRW was used instead of tap water.
E	Car wash water with 1% Dawn	Vehicles were washed with a 1% solution of Dawn, and the water collected and stored.
F	Storm water from parking lot	Rain water run-off was collected at a storm sewer grate on the parking lot of the U.S. EPA facility. No other constituents were added.
G	Rusty water, high iron content	Tap water was sprayed through a heavily corroded ductile iron pipe and then collected at the other end of the pipe. The tap water did not pass through a point-of-use filter containing carbon media.
H	Floor wash water, w/1% Alconox and 19 mg/L motor oil	Same as Wash Water A. Prior to an individual inactivation test, 45 µL of motor oil was added to the starting volume (2,000 mL) of wash water. When bleach was added, this yielded a motor oil concentration of 19 mg/L.

Table 2—

Water quality parameters of wash waters.

Wash Water	COD (mg/L)	Turbidity (NTU)	Alkalinity ^I (mg CaCO ₃ /L)	TSS ^I (mg/L)	TDS ^I (mg/L)
A	3000	600	4473	540	NA
B	4260	650	4590	567	8758
C	3525	60	4030	96	4186
D	3070	13	4195	72	8410
E	6480	980	212	340	361
F	90	152	80	285	6185
G	191	283	NA	249	65
H	3569	471	NA	NA	NA

^I NA = not analyzed

Table 3—

General inactivation results. 3000 mg NaOCl/liter.

Wash Water	Temp °C	pH of water	pH after Cl ₂ addition	LI _{max} , maximum log inactivation observed	T _{max} , sample time for LI _{max} (min)	T _{6log} , time for 6 log kill, (min)	No. of points for T _{6log} calc.	R ²
A. Floor wash water	22.4	9.27	9.47	< 6.9	5	10	1	NA
w/1% Alconox	20.9	9.20	9.23	< 7.0	30	14	4	0.667
	19.9	9.26	9.30	< 7.1	10	8	2	0.975
Avg. (std. dev)	21.1	9.24	9.33			10 (2.3)		
	4.7	9.15	9.08	< 7.2	10	14	2	0.788
	4.5	9.31	9.53	5.1	60	49	5	0.548
	4.7	9.33	10.48	< 7.3	10	15	2	0.835
Avg. (std. dev)	4.6	9.26	9.69			26 (16.2)		
B. Floor wash water	22.5	8.98	8.88	< 6.1	5	6	1	NA
w/1% Alconox and	23.9	8.76	8.77	< 7.2	10	6	2	0.999
added hardness	23.7	8.64	8.69	< 7.2	10	6	2	0.927
Avg. (std. dev)	23.3	8.79	8.78			6 (0.2)		
	7.9	9.31	9.28	< 7.1	30	10	3	0.780
	6.7	9.34	9.34	< 7.1	15	11	3	0.904
	5.6	9.24	9.17	5.5	30	25	5	0.648
Avg. (std. dev)	6.7	9.30	9.26			15 (6.9)		
C. PPE wash water	20.2	9.27	9.29	< 7.5	15	10	3	0.870
w/1% Alconox	19.5	9.23	9.24	< 7.8	10	5	2	1.000
	20.2	9.35	9.35	< 6.6	10	12	2	0.841
Avg. (std. dev)	20.0	9.28	9.29			9 (2.9)		
	4.3	6.94	9.60	< 7.1	10	9	2	0.988
	3.7	9.52	9.53	< 7.3	15	12	3	0.949
	3.8	9.50	9.95	< 7.4	10	13	2	0.992
Avg. (std. dev)	3.9	8.65	9.69			11 (1.7)		
D. PPE wash water	22.7	8.85	8.71	< 7.1	5	4	1	NA
w/1% Alconox and	21.7	9.07	9.02	< 7.1	5	5	1	NA
added hardness	21.7	9.09	9.00	< 7.0	10	5	2	0.987
Avg. (std. dev)	22.0	9.00	8.91			5 (0.4)		
	4.9	9.25	9.21	< 7.1	30	13	4	0.934
	5.2	9.18	9.12	< 7.0	15	10	3	0.932
	5.7	9.30	9.25	< 7.2	15	9	3	0.938
Avg. (std. dev)	5.3	9.24	9.19			11 (1.3)		

Wash Water	Temp °C	pH of water	pH after Cl ₂ addition	LI _{max} , maximum log inactivation observed	T _{max} , sample time for LI _{max} (min)	T _{6log} , time for 6 log kill, (min)	No. of points for T _{6log} calc.	R ²
E. Car wash water	21.3	8.21	9.84	< 7.0	60	29	5	0.885
w/1% Dawn	24.3	7.96	9.92	< 7.1	60	26	4	0.775
	21.7	8.12	9.76	< 7.0	60	29	5	0.965
Avg. (std. dev)	22.4	8.10	9.84			28 (1.2)		
	3.5	8.20	9.86	4.6	60	93	6	0.810
	4.1	8.58	10.27	4.8	60	90	6	0.785
	2.8	8.43	10.26	4.3	60	100	6	0.811
Avg. (std. dev)	3.5	8.40	10.13			94 (3.9)		
F. Storm water	20.9	7.46	10.02	5.0	60	55	6	0.605
from parking lot	19.2	7.76	9.93	5.7	60	50	6	0.801
	21.2	8.24	10.24	< 7.5	60	24	5	0.814
	19.4	7.85	10.07	6.3	60	41	5	0.443
Avg. (std. dev)	20.2	7.83	10.07			43 (11.8)		
	4.5	8.01	10.36	<7.5	120	65	5	0.925
	4.6	7.98	10.35	< 7.4	180	97	6	0.843
	5.1	7.51	10.09	< 8.4	120	53	5	0.993
Avg. (std. dev)	4.7	7.83	10.27			72 (18.9)		
G. Rusty water, high iron content	22.6	7.40	10.58	4.4	30	40	5	0.938
	23.2	7.45	10.76	4.3	30	40	5	0.958
	22.7	7.40	10.68	< 7.2	30	45	4	0.880
Avg. (std. dev)	22.8	7.42	10.67			42 (2.2)		
	4.6	7.57	11.00	1.1	30	162	5	0.955
	4.0	7.20	10.88	0.7	30	243	5	0.964
	4.0	7.10	10.88	0.8	30	223	5	0.952
Avg. (std. dev)	4.2	7.29	10.92			209 (34.5)		
H. Floor wash water,	22.4	8.96	9.07	< 7.4	10	23	1	NA
w/ 1% Alconox and 1.9 mg/l motor oil	22.1	9.13	9.10	< 7.6	10	20	1	NA
	19.9	9.14	9.14	< 7.3	10	26	1	NA
Avg. (std. dev)	21.5	9.08	9.10			23 (2.4)		
	4.3	9.13	9.16	< 7.4	30	19	3	0.893
	4.6	9.13	9.13	< 7.5	30	17	3	0.928
	4.9	9.20	9.07	< 7.4	30	17	3	0.956
Avg. (std. dev)	4.6	9.15	9.12			17 (1.1)		

Table 4—

Inactivation at different amounts of contact time before adding chlorine bleach.

Test	Time of Cl ₂ addition	Temp °C	pH of water	pH after Cl ₂ addition	LI _{max}	T _{max} sample time for LI _{max} (min)	T _{6log} time for 6 log kill, (min)	No. of points for T _{dog} calc.
Floor wash water	0 hours	19.9	9.2	9.21	< 7.1	10.0	6.4	2
w/1% Alconox	48 hours	21.7	9.1	9.05	< 8.6	5.0	4.3	1
21 °C	96 hours	19.9	9.3	9.34	< 8.0	5.0	5.6	1
Floor wash water	0 hours	5.5	9.2	9.18	< 7.3	15.0	13.1	3
w/1% Alconox	48 hours	4.6	9.3	9.26	< 7.9	10.0	12.2	2
4° C	96 hours	5.2	9.4	9.64	< 7.9	15.0	12.4	3

Table 5—

Inactivation results in nonbuffered and buffered car wash water w/1% Dawn.

Lot no. of Wash Water	Target pH of buffer	Temp °C	pH of water	pH after Cl ₂ addition	LI _{max}	T _{max} , sample time for LI _{max} (min)	T _{6log} , time for 6 log kill, (min)	No. of points for T _{6log} calc.
1	no buffer	4.5	8.33	9.97	< 7.3	120	85	5
	no buffer	3.5	6.76	9.25	< 7.6	60	32	4
	no buffer	4.6	6.67	9.24	< 7.3	15	22	2
	no buffer	4.1	6.42	9.24	< 7.5	60	26	4
2	no buffer	4.0	8.11	10.15	< 7.7	180	100	6
	no buffer	3.9	7.29	10.11	< 7.4	120	79	5
	8	4.3	7.61	8.08	< 7.9	5	< 5	0
	8	3.8	7.60	8.05	< 7.7	10	8	1
	8	4.0	7.19	8.03	< 7.4	20	9	1
	7	4.5	7.41	7.00	< 7.8	20	9	2
3	no buffer	4.0	9.12	10.21	< 7.7	120	101	5
	8	4.4	9.06	8.06	< 7.4	20	10	2
	7	5.0	9.03	7.01	< 7.8	10	7	1
4	no buffer	20.3	7.22	9.33	< 7.6	15	8	2
	8	20.1	6.92	8.05	< 7.5	10	10	1
	8	20.1	6.75	8.03	< 7.3	5	7	1
	7	20.9	6.74	7.04	< 7.7	5	6	1
5	no buffer	20.5	6.29	9.08	< 7.5	30	13	5
	8	19.9	6.30	7.99	< 7.6	5	5	1
	7	20.7	6.72	7.03	< 7.6	10	5	2