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Determining the Efficacy of Liquids and Fumigants in Systematic Decontamination Studies for *Bacillus anthracis* Using Multiple Test Methods

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National Homeland Security Research Center

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U.S Environmental Protection Agency
Research Triangle Park, NC 27711

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Executive Summary

The U.S. Environmental Protection Agency (EPA) Office of Research and Development is helping to protect human health and the environment from adverse impacts resulting from acts of terror by carrying out performance tests (i.e., efficacy assessments) on homeland security technologies. In this study, EPA compared three laboratory methods for evaluating the decontamination efficacy of sporicidal products and the use of relatively safe- to-handle surrogates. The three methods were used in a side-by-side fashion, to the extent logistically feasible, to evaluate the performance of various fumigant and liquid technologies for inactivating *Bacillus anthracis* Ames spores and surrogate (i.e., *Bacillus subtilis*) spores applied to test coupons/carriers. Evaluations were run at a range of efficacy levels for the various fumigant and liquid technologies.

The efficacies were evaluated using three methods acceptable for the registration of sporicidal decontaminants through EPA's Office of Pesticide Programs (OPP). The efficacy results were compared. Each decontamination technology was evaluated using three different test methods:

- AOAC International (formerly the Association of Official Analytical Chemists or simply AOAC) Official Method 966.04, Sporicidal Activity of Disinfectants Test, Alternate Method[1], referenced herein as "AOAC 966.04". AOAC 966.04 used porcelain penicylinder and silk suture loop carriers, and was generally conducted with *B. anthracis* and *B. subtilis* spores. In the actual test, 60 penicylinder carriers were inoculated with approximately 1×10^5 - 1×10^6 spores. The inoculated carriers were then exposed to sporicidal treatment. After the treatment the carriers were transferred, individually, into tubes containing nutrient medium, capped, and incubated. Only if no turbidity was observed (turbidity being indicative of the presence of at least one viable spore) in any of the 60 carriers after 21 days of incubation did the sporicidal treatment pass the test. Here, we used 30 carriers (rather than 60 carriers) of each type. The test was assumed to be passed for a given material if no turbidity was observed (negative for growth) in the culture of any of the 30 carriers of each type (penicylinders and silk suture loops). Method AOAC 966.04 is considered a qualitative test because the number of surviving spores is not determined by the test method. However, AOAC 966.4 is quite sensitive because a single viable spore may cause growth to be observed in tubes containing nutrient medium. The published method is available from AOAC.
- AOAC First Action Official Method 2008.05, "Determination of Efficacy of Liquid Sporicides against Spores of *Bacillus subtilis* on a Hard Nonporous Surface Using the Quantitative Three Step Method (TSM)", referenced herein as "AOAC 2008.05". AOAC 2008.05 used glass carriers (three per test) and was conducted with *B. anthracis* and *B. subtilis* spores. Each glass carrier was inoculated with 5×10^6 - 5×10^7 spores. AOAC 2008.05 is considered a quantitative test because the method determines the number of surviving spores that are extracted from each carrier after the sporicidal treatment. In test method AOAC 2008.05, the sporicidal treatment passes the test if a mean log reduction in spores recovered from the three small glass carriers is $\geq$6.0. From a sensitivity perspective, if extraction is less than 100% efficient, it is possible for viable spores on a coupon not to be counted. However, when no spores are observed, five spores are assumed to be present for calculating the log reduction. AOAC 2008.05 is a published method available from AOAC.
- The third method, referenced herein as the internal standard operating procedure, "iSOP", was developed previously by EPA at Battelle for determining efficacy of decontamination against *B. anthracis* Ames spores on building materials. For iSOP, carpet (liquid technologies only), ceiling tile (fumigants only), galvanized metal, and glass coupons were used and testing was conducted with *B. anthracis* spores. Five test coupons of each material type were included in each test. From a sensitivity perspective, if extraction is less than

AOAC Official Method 966.04, Sporicidal Activity of Disinfectants (Method 1) received First Action status in 1966, Final Action status in 1967, and was revised in 2002. The AOAC Official Method 966.04, Sporicidal Activity of Disinfectants (Alternate Method) received First Action status in 2006.

100% efficient, viable spores on a coupon may not be counted. However, the extracted coupon was placed into a tube of nutrient medium and incubated to perform a qualitative test for viable spores. In practice, when no spores are detected by the quantitative portion of this test, with rare exceptions, there are no spores detected in the qualitative test. AOAC 2008.05 is documented in the test/QA plan and amendments and will be posted on the EPA NHSRC website.

A qualitative evaluation of biological indicators (BIs) to detect the presence of viable spores after fumigation was run in parallel with the different test methods. BIs have often been used to indicate the level of control and distribution of the decontamination agent. While use of BIs is not a sporicidal test method, for purposes of this report the sporicide was assumed to pass a BI test if no viable spores were detected using a qualitative method.

A goal of EPA's systematic decontamination investigations is to generate data that can be used to assess the efficacy of technologies for an array of building material types and, in the process, generate data that can inform and support crisis exemptions for use of these technologies in building decontaminations for *B. anthracis*. The EPA Office of Pesticide Programs (OPP) is currently considering alternative test methods and data, other than the AOAC 966.04 method, for the registration process. The comparison of a product's efficacy as determined by multiple methods is needed to advance OPP knowledge of performance standards to support changes to the registration process. The intent of this study was not to verify or test manufacturers' claims or registration, but to evaluate the sensitivity of the test methods when the decontamination technologies were used at efficacy levels that resulted in viable spores being recovered after the treatment.

An additional goal was to evaluate the extent to which *B. subtilis* may be useful as a safer alternative to *B. anthracis* for efficacy testing. Would the surrogate provide conservative results relative to *B. anthracis* Ames? The ability to use safer surrogate spore to evaluate decontaminants intended for use against *B. anthracis* spores would significantly lower the cost of testing, enable testing in a much wider range of laboratories, and lower barriers to registration of sporicides with claims for use against *B. anthracis* spores.

The personnel who performed the testing using these three methods were located in two separate Battelle Biosafety Level III laboratories and were trained and approved to work with virulent *B. anthracis* Ames spores. The personnel had experience performing the iSOP method. The personnel were trained by the EPA OPP staff in the performance of the AOAC 966.04 and AOAC 2008.05 methods. Each laboratory had experience performing the AOAC 2008.05 in a previous project for the EPA OPP. Each laboratory performed a "practice" run of the AOAC 966.04 method prior to beginning the test matrix.

The spores used for all test methods were prepared in the same manner and approximately the same numbers of spores were included on each carrier or coupon in each test method. Per each method, spores were applied to the carriers/coupons by soaking or inoculation, dried, and then exposed to the decontamination technologies for various contact times under specified environmental conditions. Decontamination efficacy treatments (i.e., presumed high, medium, and low efficacy) were selected to generate a range of efficacy results to enable the sensitivity of the test methods to be assessed and compared. The presumed lower efficacy treatments were included to provide a likelihood of differences between tests; if complete kill is observed across all test methods, there is no basis for evaluating the relative sensitivity of the tests. The high efficacy treatments followed the respective label recommendations, followed manufacturers' recommendations, or used concentrations and contact times previously demonstrated to be efficacious against *B. anthracis* spores. Medium and low efficacy treatments were selected to obtain incomplete decontamination. The medium and low treatments involved using relatively lower concentrations of decontaminants or relatively shorter contact times. In some cases the lower efficacy level treatment still resulted in complete decontamination as determined by one or more of the test methods. All treatments and tests were conducted in triplicate for fumigants and in duplicate for liquids with replication performed in the same laboratory to minimize variability.

The measurement of interest was residual viable spores on coupons/carriers after specified treatments with various decontamination technologies. Efficacy was generally calculated from the observation or measurement of residual viable spores on coupons/carriers following decontamination (test coupons compared to the spores on the positive control carrier/coupons).

Three fumigation technologies were used: Sabre Technical Services (Sabre) chlorine dioxide (ClO_2), STERIS Corporation (STERIS) hydrogen peroxide (H_2O_2, "HP"), and methyl bromide (CH_3Br, "MeBr"). Four liquid technologies were used: pH-amended bleach (sodium hypochlorite solution [NaOCl]), Exterm liquid ClO_2, Oxonia Active® disinfectant (HP and

peroxyacetic acid [CH_3CO_3H]), Spor-Klenz® Ready-to-Use disinfectant (HP and peroxyacetic acid), and Virkon® S disinfectant (potassium peroxymonosulfate [$KHSO_5$] and sulfamic acid [H_3NSO_3]). For clarity, the commercial liquid technologies are referenced herein by their respective trademarks.

Summaries of the test results are provided in the following sections by technology. Results are discussed in terms of whether or not the efficacy results would indicate a "pass" or "fail" of the corresponding test method if used for registration purposes. Because BIs are not a registration method, in order to "pass," no viable spores could remain on any replicate BI.

In this report, "stringency" is used to describe the relative difficulty of passing a specific test method. Thus, if in a given decontamination test the iSOP method was passed, but the AOAC 966.04 was not passed, the AOAC 966.04 method, for the specific decontamination method, would be rated as "more stringent" than the iSOP method.

Findings are summarized as follows:

- The three methods do not give identical efficacy results. Generally the AOAC 966.04 is the most stringent and conservative method.
- Carriers impact the efficacy outcomes: glass or galvanized metal as the carrier with B. anthracis spores as the organism is generally less stringent than use of ceiling tile, suture loops, or penicylinders.
- BIs consisting of B. atrophaeus on steel in Tyvek® envelopes were as stringent as or more stringent than AOAC 2008.05 or iSOP, and generally as stringent as AOAC 966.04 for the three fumigation methods tested.

Sabre ClO_2

- High efficacy treatment: 3 hr at 3000 pmv
- Moderate efficacy treatment: 3 hr at 300 ppm
- Low efficacy treatment: 3 hr at 150 ppm

When using Sabre ClO_2, the least stringent test methods (i.e., indicative of effective sporicides when other methods are not) were iSOP (with galvanized metal and glass coupons only) and AOAC 2008.05 (Table ES-1); 100% for iSOP (with galvanized metal and glass) and AOAC 2008.05 passed the test methods at all efficacy levels. When AOAC 966.04 was used, iSOP (with ceiling tile) and BI, none of the replicate tests passed at the moderate or low efficacy levels, but 100% of the replicate tests passed for all test methods when conducted at the high efficacy treatment of 3 hr at 3000 ppmv ClO_2.

Use of *B. subtilis* (Table ES-2) and BIs yielded results very similar and at least as stringent as those observed for *B. anthracis* (Table ES-1).

Table ES-1. Sabre Efficacy Results against *B. anthracis* by Test Method and Material

Contact Time, Concentration and Temperature as Applicable	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*				
	AOAC 966.04 *(B. anthracis)*		AOAC 2008.05 *(B. anthracis)*	iSOP *(B. anthracis)*	
	Porcelain Penicylinder	Suture Loop	Glass	Ceiling Tile	Galvanized Metal and Glass
	Sabre ClO_2				
3 hr at 3000 ppmv	100%	100%	100%	100%	100%
3 hr at 300 ppmv	0%	0%	100%	0%	100%†
3 hr at 150 ppmv	0%	0%	100%	0%	100%†

* AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 and iSOP require six log reductions in order to be considered effective (i.e., passing).

† One of the three replicate tests at the moderate and low efficacy levels had a log reduction of only ≥5.8, even though no viable spores were recovered. Replicates with no viable spores recovered but with log reductions <6.0 were excluded from the calculation of the percent passing the iSOP test. The results shown are based on two replicates of the galvanized metal and three replicates of the glass, each, for the 3 hr at 300 ppmv and the 3 hr at 150 ppmv treatments.

Table ES-2. Sabre Efficacy Results against *Bacillus* Surrogates by Test Method and Material

Contact Time and Concentration	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*			
	AOAC 966.04 *(B. subtilis)*		AOAC 2008.05 *(B. subtilis)*	BI *(B. atrophaeus)*
	Porcelain Penicylinder	Suture Loop	Glass	Stainless Steel in Tyvek® Packaging
	Sabre ClO_2			
3 hr at 3000 ppmv	100%	100%	100%	100%
3 hr at 300 ppmv	0%	0%	100%	0%
3 hr at 150 ppmv	0%	0%	67%	0%

* AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 and iSOP require six log reductions to be considered effective (i.e., passing).

STERIS HP

- High efficacy treatment: gassing phase + 1.5 hr at 250 ppmv
- Moderate efficacy treatment: gassing phase + 0.5 hr at 250 ppmv
- Low efficacy treatment: gassing phase followed by aeration (no dwell time; gassing phase is roughly estimated to have a concentration x time (CT) of 140 ppmv-hr.

At the low STERIS HP efficacy treatment and the moderate efficacy treatment, partial efficacy was generally observed with AOAC 2008.05 and iSOP and some of the replicate tests were passed, but none of the replicates passed the efficacy test associated with AOAC 966.04 and BI (Table ES-3). At the high efficacy level, 100% of the iSOP and AOAC 2008.05 replicate tests were passed, 67% of the AOAC 966.04 replicate tests were passed, but none of the BI replicate tests were passed.

Table ES-3. STERIS HP Efficacy Results against *B. anthracis* by Test Method and Material

Contact Time and Concentration	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*				
	AOAC 966.04 *(B. anthracis)*		AOAC 2008.05 *(B. anthracis)*	iSOP *(B. anthracis)*	
	Porcelain Penicylinder	Suture Loop	Glass	Ceiling Tile	Galvanized Metal and Glass
STERIS HP					
Gassing phase up to 250 ppmv + 1.5 hr at 250 ppmv	67%	67%	100%	100%	100%
Gassing phase up to 250 ppmv + 0.5 hr at 250 ppmv	0%	0%	0%†	67%	100%†
Gassing phase up to 250 ppmv followed by aeration (no dwell time)	0%	0%	67%	0%	83%

*AOAC 966.04 and BI require 100% kill and AOAC 2008.05 and iSOP require six log reductions to be considered effective (i.e., passing).

†One of the three replicate tests at the moderate efficacy level had a log reduction of only ≥5.9, even though no viable spores were recovered. Tests with log reductions <6.0 were excluded from the calculation of the percent passing the test, even though no viable spores were recovered. The results shown are based on two replicates for the moderate efficacy level treatment.

Use of *B. subtilis* yielded results that were generally similar, but in some cases less stringent, compared to the results observed for *B. anthracis* (Table ES-4).

Table ES-4. STERIS HP Efficacy Results against *Bacillus* Surrogates by Test Method and Material

Contact Time and Concentration	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*			
	AOAC 966.04 *(B. subtilis)*		AOAC 2008.05 *(B. subtilis)*	BI *(B. atrophaeus)*
	Porcelain Penicylinder	Suture Loop	Glass	Stainless Steel in Tyvek® Packaging
STERIS HP				
Gassing phase up to 250 ppmv + 1.5 hr at 250 ppmv	100%	67%	100%	0%
Gassing phase up to 250 ppmv + 0.5 hr at 250 ppmv	0%	0%	33%	0%
Gassing phase up to 250 ppmv followed by aeration (no dwell time)	0%	0%	67%	0%

*AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 requires six log reductions to be considered effective (i.e., passing).

MeBr

- High efficacy treatment: 18 hr at 211mg/L and 37 °C
- Moderate efficacy treatment: 9 hr at 211mg/L and 37 °C
- Low efficacy treatment: 9 hr at 211mg/L and 23 °C.

With the high efficacy treatment (18 hr at 211 mg/L and 37 °C), 67% of the replicate tests were passed in the AOAC 966.04 (with suture loop) and AOAC 2008.05, and 100% of the replicate tests passed the iSOP with all materials. None of the replicates with the high efficacy treatment passed the AOAC 966.04 (with porcelain penicylinder) or BI.

Against *B. anthracis* spores, MeBr with the low and moderate efficacy treatments (9 hr at 211 mg/L and 25 °C and 9 hr at 211 mg/L and 37 °C) did not pass any of the test methods (Table ES-5).

Use of *B. subtilis* yielded results that were very different from those observed for *B. anthracis*. MeBr exhibited little efficacy against *B. subtilis*, even with the high efficacy treatment at which the iSOP test was showing complete kill of the *B. anthracis* spores (Table ES-6). These results suggest that use of *B. subtilis* would be more stringent and highly conservative in testing for MeBr efficacy against *B. anthracis* spores.

Table ES-5. MeBr Efficacy Results against *B. anthracis* by Test Method and Material

Contact Time, Concentration and Temperature as Applicable	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*				
	AOAC 966.04 *(B. anthracis)*		AOAC 2008.05 *(B. anthracis)*	iSOP *(B. anthracis)*	
	Porcelain Penicylinder	Suture Loop	Glass	Ceiling Tile	Galvanized Metal and Glass
MeBr					
18 hr at 211mg/L and 37 °C	0%	67%	67%	100%	100%
9 hr at 211mg/L and 37 °C	0%	0%	0%	0%	0%
9 hr at 211mg/L and 23 °C	0%	0%	0%	0%	0%

* AOAC 966.04 and BI require 100% kill and AOAC 2008.05 and iSOP require six log reductions to be considered effective (i.e., passing).

Table ES-6. MeBr Efficacy Results against *Bacillus* Surrogates by Test Method and Material

Contact Time, Concentration and Temperature	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*			
	AOAC 966.04 *(B. subtilis)*		AOAC 2008.05 *(B. subtilis)*	BI *(B. atrophaeus)*
	Porcelain Penicylinder	Suture Loop	Glass	Stainless Steel in Tyvek® Packaging
MeBr				
18 hr at 211mg/L and 37 °C	0%	0%	0%	0%
9 hr at 211mg/L and 37 °C	0%	0%	0%	0%
9 hr at 211mg/L and 23 °C	0%	0%	0%	0%

* AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 requires six log reductions to be considered effective (i.e., passing).

pH-Amended Bleach

- High efficacy treatment: 60 min
- Low efficacy treatment: 10 min.

For pH-amended bleach used against *B. anthracis* spores, test methods using porous materials (AOAC 966.04 [suture loop] and iSOP [carpet]) resulted in no replicate tests being passed (Table ES-7). AOAC 966.04 (porcelain penicylinder, but not suture loops) results indicated an increasing number of replicate tests passing with increasing contact time to the pH-amended bleach (50% of the replicate tests passed with a 10-min contact time and 100% of the replicate tests passed with a 60-min contact time). AOAC 2008.05 resulted in 100% of the replicate tests passing at both contact times, and iSOP (galvanized metal and glass) resulted in 100% of the replicate tests passing at both contact times.

Use of *B. subtilis* yielded results that were generally similar to but less stringent than the AOAC 966.04 test using penicylinders and more stringent than the AOAC 2008.05 test compared to results observed for *B. anthracis* (Table ES-8).

Table ES-7. pH-Amended Bleach Efficacy Results against *B. anthracis* by Test Method and Material

Contact Time	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*				
	AOAC 966.04 *(B. anthracis)*		AOAC 2008.05 *(B. anthracis)*	iSOP *(B. anthracis)*	
	Porcelain Penicylinder	Suture Loop	Glass	Carpet	Galvanized Metal and Glass
pH-Amended Bleach					
60 min	100%	0%	100%†	0%	100%
10 min	50%‡	0%‡	100%†	0%	100%

* AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 and iSOP require six log reductions to be considered effective (i.e., passing).

† Replicates with no viable spores recovered but log reductions <6.0 (≥5.9 log reduction in both cases) were excluded from the calculation of the percent passing the test. The results shown are based on one replicate for each treatment.

‡Only 29, rather than 30, carriers were included in this test.

Table ES-8. pH-Amended Bleach Efficacy Results against *Bacillus subtilis* by Test Method and Material

Contact Time, Concentration and Temperature	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*		
	AOAC 966.04 *(B. subtilis)*		AOAC 2008.05 *(B. subtilis)*
	Porcelain Penicylinder	Suture Loop	Glass
pH-Amended Bleach			
60 min	100%	0%	50%
10 min	100%	0%	50%

* AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 requires six log reductions to be considered effective (i.e., passing).

Exterm Liquid ClO_2

- High efficacy treatment: 60 min
- Low efficacy treatment: 10 min.

Exterm liquid ClO_2 yielded results indicating high efficacy against *B. anthracis* spores when using iSOP and AOAC 966.04 (porcelain penicylinder); 100% of the replicates passed these test methods at the 60-min and 10-min contact times (Table ES-9). However, none of the replicate tests passed AOAC 966.04 (suture loop). Using AOAC 2008.05 with *B. anthracis* and *B. subtilis* spores, 50% of the replicate tests passed with a 10-min contact time and 100% of the replicate tests passed at the 60-min contact time

Use of *B. subtilis* in the AOAC 2008.05 method yielded results that were the same as results observed for *B. anthracis* (Table ES-10).

Table ES-9. Exterm Liquid ClO_2 Efficacy Results against *B. anthracis* by Test Method and Material

Contact Time	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*				
	AOAC 966.04 *(B. anthracis)*		AOAC 2008.05 *(B. anthracis)*	iSOP *(B. anthracis)*	
	Porcelain Penicylinder	Suture Loop	Glass	Carpet	Galvanized Metal and Glass
	Exterm Liquid ClO_2				
60 min	100%	0%	100%	100%	100%
10 min	100%	0%	50%	100%	100%

*AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 and iSOP require six log reductions to be considered effective (i.e., passing).

Table ES-10. Exterm Liquid ClO_2 Efficacy Results against *Bacillus* Surrogates by Test Method and Material

Contact Time	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*
	AOAC 2008.05 *(B. subtilis)*
	Glass
	Exterm Liquid ClO_2
60 min	100%
10 min	50%

*AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 requires six log reductions to be considered effective (i.e., passing).

Oxonia Active®

- High efficacy treatment: 60 min
- Low efficacy treatment: 10 min.

Oxonia Active® yielded results indicating high efficacy against *B. anthracis* spores when evaluated using the iSOP test: 100% of the replicate tests passed the test method at the 60-min and 10-min contact times (Table ES-11). In comparison, when using AOAC 2008.05, none of the replicate tests passed the efficacy test against *B. anthracis* spores. None of the replicate tests at the 10-min contact time passed AOAC 966.04 (porcelain penicylinder and suture loop) with *B. anthracis* spores, but 50% of the replicate tests passed per material with the 60-min contact time.

Use of *B. subtilis* in the AOAC 2008.05 method yielded results that were not the same as results observed for *B. anthracis* (Table ES-12).

Table ES-11. Oxonia Active® Efficacy Results against *Bacillus anthracis* by Test Method and Material

Contact Time	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*				
	AOAC 966.04 *(B. anthracis)*		AOAC 2008.05 *(B. anthracis)*	iSOP *(B. anthracis)*	
	Porcelain Penicylinder	Suture Loop	Glass	Carpet	Galvanized Metal and Glass
	Oxonia Active®				
60 min	50%	50%	0%	100%	100%
10 min	0%	0%	0%	100%	100%

*AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 and iSOP require six log reductions to be considered effective (i.e., passing).

Table ES-12. AOAC 2008.05 Results for Oxonia Active® against *Bacillus subtilis*

Contact Time	% of Replicate Tests Passing AOAC 2008.05 (*B. subtilis*)
	Glass
	Oxonia Active®
60 min	100%
10 min	100%

* AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 requires six log reductions to be considered effective (i.e., passing).

Spor-Klenz® Ready-to-Use

- High efficacy treatment: 30 min
- Low efficacy treatment: 10 min.

Spor-Klenz® Ready-to-Use was found to be completely effective (100% of the replicate tests passed) only when tested against *B. anthracis* spores using iSOP (carpet and glass) (Table ES-13). None of the replicate tests passed iSOP (galvanized metal). For AOAC 2008.05, 100% of the replicate tests with *B. subtilis* passed, but none of the replicate tests with *B. anthracis* passed although all log reductions were rather high (≥5.1). AOAC 966.04 was also conducted with *B. anthracis* spores. When porcelain penicylinders were used, 25% of the replicate tests passed with a 10-min contact time and 75% of the replicate tests passed with a 30-min contact time. None of replicate tests passed when using AOAC 966.04 (suture loop).

B. subtilis in the AOAC 966.04 (porcelain penicylinders only) and AOAC 2008.05 methods was less stringent than *B. anthracis* (Table ES-14).

Table ES-13. Spor-Klenz® Ready-to-Use Efficacy Results against *Bacillus anthracis* by Test Method and Material

Contact Time	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*					
	AOAC 966.04 (*B. anthracis*)		AOAC 2008.05 (*B. anthracis*)	iSOP (*B. anthracis*)		
	Porcelain Penicylinder	Suture Loop	Glass	Carpet	Galvanized Metal	Glass
			Spor-Klenz®			
30 min	50%	0%	0%†	100%	0%	100%
10 min	0%	0%	0%†	100%	0%	100%

*AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 and iSOP require six log reductions to be considered effective (i.e., passing).

†Log reductions ranged from 5.1 to ≥5.9; the replicate with no recovered spores and an efficacy of ≥5.9 was excluded from the calculation of percent passing the test.

Table ES-14. AOAC 2008.05 Results for Spor-Klenz® Ready-to-Use against *Bacillus subtilis*

Contact Time	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*		
	AOAC 966.04 (*B. anthracis*)		AOAC 2008.05 (*B. anthracis*)
	Porcelain Penicylinder	Suture Loop	Glass
		Spor-Klenz®	
30 min	100%	0%	100%
10 min	50%	0%	100%

* AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 requires six log reductions to be considered effective (i.e., passing).

Virkon® S

Virkon® S was not carried through the entire evaluation because poor sporicidal properties were observed during method demonstration testing.

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Abbreviations/Acronyms

AOAC	AOAC International (formerly the Association of Official Analytical Chemists)
BI	biological indicator
BSC	biological safety cabinet
°C	degrees Celsius
CFU	colony-forming unit(s)
ClO_2	chlorine dioxide
cm	centimeter
CT	concentration x time
EPA	U.S. Environmental Protection Agency
HP	hydrogen peroxide
hr	hour
iSOP	internal standard operating procedure
L	liter
LB	Luria-Bertani
MeBr	methyl bromide
min	minute
mL	milliliter
mm	millimeter
N	Normality
ppm	parts per million
ppmv	parts per million by volume
QA	quality assurance
QC	quality control
QMP	quality management plan
RH	relative humidity
TSA	technical systems audit
TSB	tryptic soy broth
µg	microgram
µL	microliter
µm	micrometer
v/v	volume to volume

1.0 Introduction

The U.S. Environmental Protection Agency (EPA) Office of Research and Development (ORD) is helping to protect human health and the environment from adverse impacts resulting from intentional acts of terror. With an emphasis on decontamination and consequence management, water infrastructure protection, and threat and consequence assessment, EPA is working to develop tools and information that will help detect the intentional introduction of chemical or biological contaminants into buildings or water systems, contain these contaminants, decontaminate buildings and/or water systems, and dispose of material resulting from cleanups.

Within the ORD, the National Homeland Security Research Center is conducting technology evaluations for the decontamination of spores (e.g., *Bacillus anthracis* Ames spores and potential surrogates) on building materials. In developing guidance based on lessons learned from past decontamination events, one area that has been identified to aid in the restoration response to a contamination with *B. anthracis* Ames spores is the registration of technologies for use in building decontaminations. The EPA's Office of Pesticide Programs (OPP) currently requires that registration of a product for use as a sporicide pass AOAC 966.04 using *B. anthracis* Ames. A goal of the systematic decontamination process has been to generate data that can be used to assess the efficacy of technologies for an array of building material types and, in the process, generate data that can be used for a successful product registration for use in building decontaminations for *B. anthracis* Ames. OPP is currently considering the use of alternative test methods and data, other than the AOAC 966.04 method, for the registration process. The comparison of a products' efficacies as determined by multiple methods is needed to advance OPP's knowledge of performance standards to support changes to the registration process. ORD and OPP collaborated on the test/QA plan for this investigation to ensure that the results, presented in this report, meet the needs of both organizations.

The purpose of the investigation is to determine a technology's efficacy for the decontamination of *B. anthracis* Ames spores by three methods. Determination of efficacy will aid in providing baseline data determined by different approaches or standard methods. The results of the testing will inform EPA decisions with respect to determination of acceptable data for use in registering technologies for application in building decontaminations of *B. anthracis* Ames spores. The first method, AOAC 966.04, requires testing a prospective sporicide with *B. anthracis* Ames spores on porcelain penicylinders and silk suture loops as standardized nonporous and porous materials, respectively, for a claim against that particular organism. The second method, AOAC 2008.05, requires testing with *B. anthracis* Ames spores on standardized 5 x 5 mm glass coupons. AOAC 2008.5 has been extensively tested using *B. subtilis* spores as a surrogate for *B. anthracis* Ames spores. The third method, referenced herein as iSOP, is a method developed by EPA at Battelle, in which efficacy is determined by enumeration of viable *B. anthracis* Ames spores remaining on coupons of an array of porous and nonporous building materials before and after application of decontamination technologies. The three methods, AOAC 966.04, AOAC 2008.5, and iSOP, have been peer reviewed, have a clear test design and template, and are all acceptable for sporicidal registration through EPA's Office of Pesticide Programs.

In addition, biological indicators (BIs) were included in the fumigation tests. BIs are used in the field to indicate effective fumigation of a contaminated facility.

The results of the efficacy tests with various technologies were compared across the various test methods to evaluate the extent to which the test methods provide comparable efficacy results and sensitivity. The intent of the efficacy testing was not to challenge or test manufacturers' claims, but to evaluate the sensitivity of the various test methods when the decontamination technologies were used at lower efficacy levels than label recommendations.

This report addresses the following objectives:

- Compare the performance of decontamination technologies against *B. anthracis* Ames spores as determined by three methods: AOAC 966.04, AOAC 2008.05, and iSOP: do the three test methods for determining efficacy give the same results with the same sensitivity?
- Compare the efficacy results for various decontamination technologies against *B. anthracis* Ames and *B. subtilis* (surrogate) spores using AOAC 966.04 and AOAC 2008.05: is it at least as difficult to pass the tests using *B. subtilis* spores as with *B. anthracis* Ames spores?

- Evaluate whether qualitative analysis of biological indicators (BIs) is consistent with the efficacy results from the three test methods during fumigation decontamination technology.

This evaluation was conducted according to a peer-reviewed test/QA plan[1] that was developed according to the requirements of the quality management plan (QMP).[2]

2.0
Technology Descriptions

Replicate efficacy results from the use of three test methods were compared using three fumigation technologies: 1) Sabre ClO_2, 2) STERIS HP, and 3) MeBr. Efficacy results from the three test methods were also compared using multiple liquid technologies: 1) pH-amended bleach, 2) Exterm liquid ClO_2, 3) Oxonia Active®, and 4) Spor-Klenz® Ready-to-Use. Virkon® S, which was originally included in the testing, was dropped because low efficacy was observed during the screening phase. Technology descriptions are provided in the following sections.

2.1 Sabre ClO_2

The Sabre ClO_2 gas generator was provided by the vendor (Sabre Technical Services, Slingerlands, NY). The Sabre ClO_2 gas generator included a 20.3 cm x 20.3 cm base onto which a sparging column (15.2 cm x 15.2 cm, 91.4 cm high) was mounted (Figure 2-1).

A solution was prepared on-site for each testing day in a 19 L container. The ClO_2-generating solution (3 L) was prepared according to Sabre instructions by mixing household Clorox® bleach (The Clorox® Company, Oakland, CA) (5%-6% sodium hypochlorite), 6 N hydrochloric acid (Fisher Scientific, Pittsburgh, PA), 25% sodium chlorite (SabreClor 25, Sabre Technical Services, NY), and distilled water. Following mixing, this solution typically generated a ClO_2 concentration of 3000 parts per million (ppm) and a chlorite concentration (ppm) that was at least half of the ClO_2 concentration. The ClO_2-generating solution was pumped into the top of the sparging column using a peristaltic pump integral to the Sabre system, and air from the test chamber was pumped as a counter-current against the flow of liquid in the sparging column. This air flow strips ClO_2 from the liquid into the air stream that was then pumped into the test chamber to establish the desired gaseous ClO_2 concentration.

Based on prior experience working with the Sabre ClO_2 gas generator, liquid was introduced from the reservoir of ClO_2-generating solution to the sparging column initially at the rate of 60 milliliters per min (mL/min); when the desired ClO_2 concentration in the test chamber was achieved, the liquid introduction into the sparging column was stopped. When needed, the ClO_2 concentration was increased in the test chamber by introducing more ClO_2-generating liquid into the sparging column and stripping the ClO_2 from the liquid with the counter-current air stream. The air from the chamber was recirculated into and out of the sparging column in a closed-loop fashion. The spent liquid exiting the sparging column was collected in a reservoir containing 10% sodium hydroxide. Following decontamination, the ClO_2 in the test chamber was vented through activated carbon.

The desired humidity level in the test chamber was established and maintained using a custom-designed ultrasonic fogging chamber. No chemical neutralization was required for the ClO_2 as ventilation and off-gassing serve to neutralize the test coupons/carriers.

Figure 2-1. Sabre bench-scale ClO_2 gas generator.

The three efficacy treatment levels used with Sabre ClO_2 were:

- High efficacy treatment: 3 hr at 3000 ppmv ± 10%
- Moderate efficacy treatment: 3 hr at 300 ppmv ± 10%
- Low efficacy treatment: 3 hr at 150 ppmv ± 10%.

The high efficacy level of 3000 ppmv for 3 hr achieves a concentration x time (CT) of 9000 ppm-hr that was used by Sabre in the decontamination of facilities contaminated with *B. anthracis* spores.

Temperature for the decontamination was maintained in the range of 22 °C ± 2 °C, and the relative humidity (RH) was maintained at 75% ± 5%. The fumigant concentration was required to be within 10% of the target concentration. (See Appendix A for concentration, temperature and RH data.) For the results of the test to be accepted, the temperature, RH, and fumigant concentration were required to be within the specified ranges.

2.2 STERIS HP

The STERIS VHP® Generator Series 1000ED (STERIS Corporation, Mentor, OH) was used to introduce and control the HP vapor. Because HP vapor is not stable as a compressed gas, HP vapor had to be produced on site by vaporization of concentrated aqueous solutions of HP. Thus, this technology included the equipment and chemicals for on-site generation, dispersion, and neutralization of the HP vapor.

The HP fumigation technology was operated at ambient temperature and atmospheric pressure in a closed loop configuration. As depicted in Figure 2-2 (from STERIS; http://www.technomartinc.com/steris/VHP%20100%20catalog.pdf) and Figure 2-3 (actual results from three cycles), the testing chamber was subjected to four phases: 1) dehumidification, 2) condition (introduce HP up to the target level), 3) sterilization (or decontamination), and 4) aeration. During dehumidification the RH was reduced by the HP fumigation technology by re-circulating the air through a reusable desiccant cartridge. Once the desired RH was reached, HP vapor was injected at the rate set to achieve the desired concentration of HP inside the chamber. The system then maintained the set concentration for the desired contact period for decontamination. Once the decontamination phase was complete, the enclosure air was re-circulated through the HP fumigation technology to reduce the HP vapor concentration to the desired level. No chemical neutralization of the test coupons/carriers was required for the HP vapor as the STERIS VHP® system catalyzes the breakdown of the HP to remove the HP from the decontaminated space. The cycle used was:

- Dehumidification: 15 min @ 20 cubic feet per min
- Conditioning: 20 min @ 3.8 g of HP/min
- Sterilization: 90 min (high efficacy treatment); 30 min (moderate efficacy treatment): or 0 min (low efficacy treatment)
- Aeration: 30 min @ 20 cubic feet per min

The coupons were removed from the test chamber through a transfer port during the aeration phase.

The STERIS system controlled the 250 ppmv decontamination cycle that was used for each efficacy level. Differences among the efficacy levels were based on the contact time. The three efficacy treatment levels used with STERIS HP were:

- High efficacy treatment: gassing phase ("conditioning" in Figure 2-2) + 1.5 hr at nominally 250 ppmv
- Moderate efficacy treatment: gassing phase + 0.5 hr at nominally 250 ppmv
- Low efficacy treatment: gassing phase to nominally 250 ppmv followed by aeration (no dwell time).

The high efficacy treatment of HP at 250 ppmv for 1.5 hr is the treatment specified on the STERIS Vaprox® HP sterilant package insert for sporicidal efficacy (sterilization) for pre-cleaned enclosures. The VHP® process was followed as specified on the package insert.

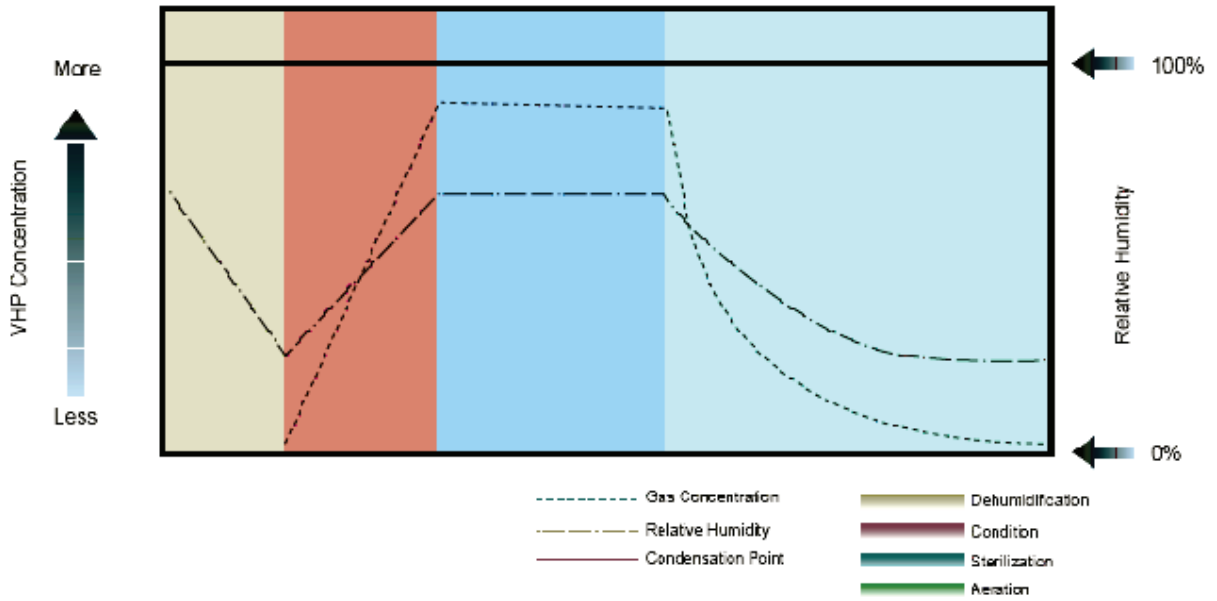

Figure 2-2. STERIS VHP® biodecontamination cycle.[7]

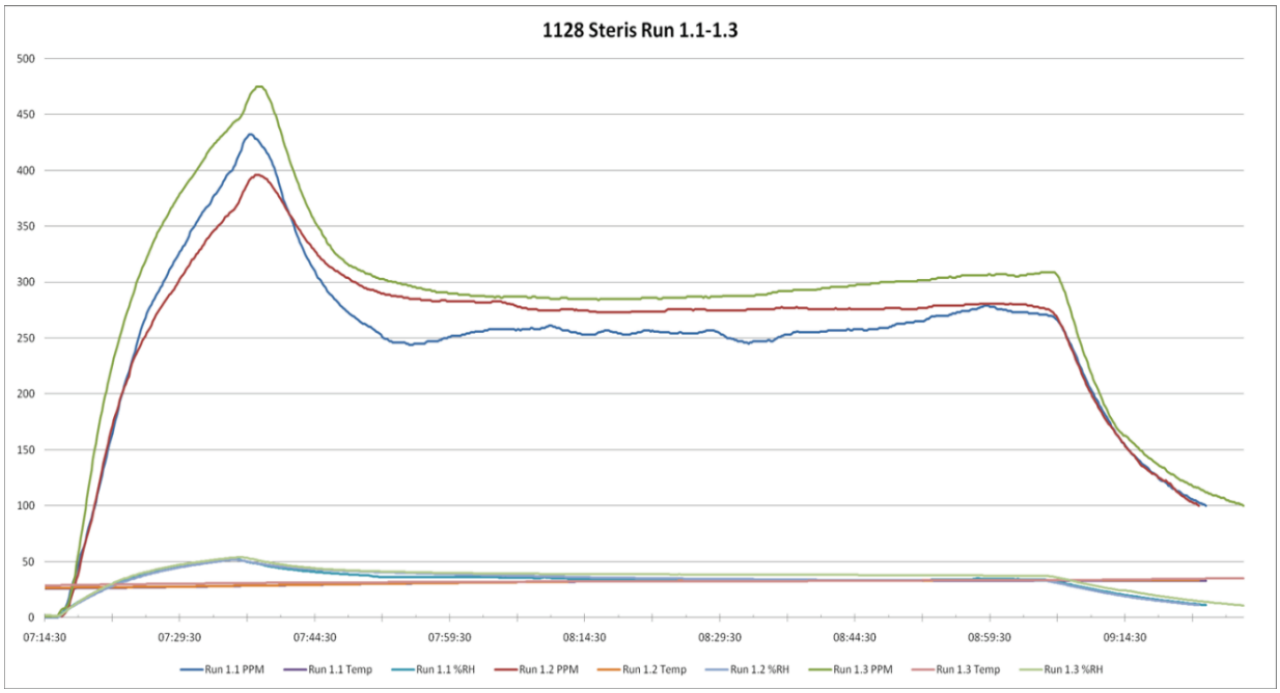

Figure 2-3. Actual HP concentrations (top curves) in three STERIS HP cycles.

For the results of the test to be accepted, the initial temperature had to be in the range of 23 °C ± 3 °C and the STERIS system had to complete the selected automated decontamination cycle. The concentration of the HP, the RH, and (passively) the temperature during fumigation were determined by the STERIS automated decontamination cycle. (See Appendix A for concentration, temperature and RH data.)

2.3 Methyl Bromide

MeBr has been registered by EPA for soil fumigation (injected into the soil before a crop was planted to effectively sterilize the soil), commodity treatment (used for post-harvest pest control), structural pest control (used to fumigate buildings for termites, and warehouses and food processing facilities for insects and rodents), and quarantine uses (used to treat imported commodities).[12] Testing with MeBr was conducted in a test chamber and containment system as shown in Figure 2-4 and did not require a specific vendor technology. The 23 L test chamber (approximately 29 cm x 29 cm x 29 cm) was glass and a second chamber of the same size made of Lexan® polycarbonate sheets (American Plastic Distributors, Columbus, OH) (not shown) served as the control chamber. The chambers were insulated to prevent condensation on the inside of cool chamber walls. The test chamber was static. Use of a static chamber made it relatively simple to establish and maintain stable gas concentrations.

The high toxicity and penetrability of MeBr required a primary and secondary containment chamber for protection of laboratory personnel. A biological safety cabinet (BSC) class III (SG603, Baker, Sanford, ME) (Figure 2-4) provided secondary containment. Air from the BSC III was exhausted through HEPA filters. MeBr concentrations in the test chamber were monitored using the method described below. The MeBr from the test chamber was inactivated by scrubbing through potassium hydroxide traps prior to release into the secondary containment.

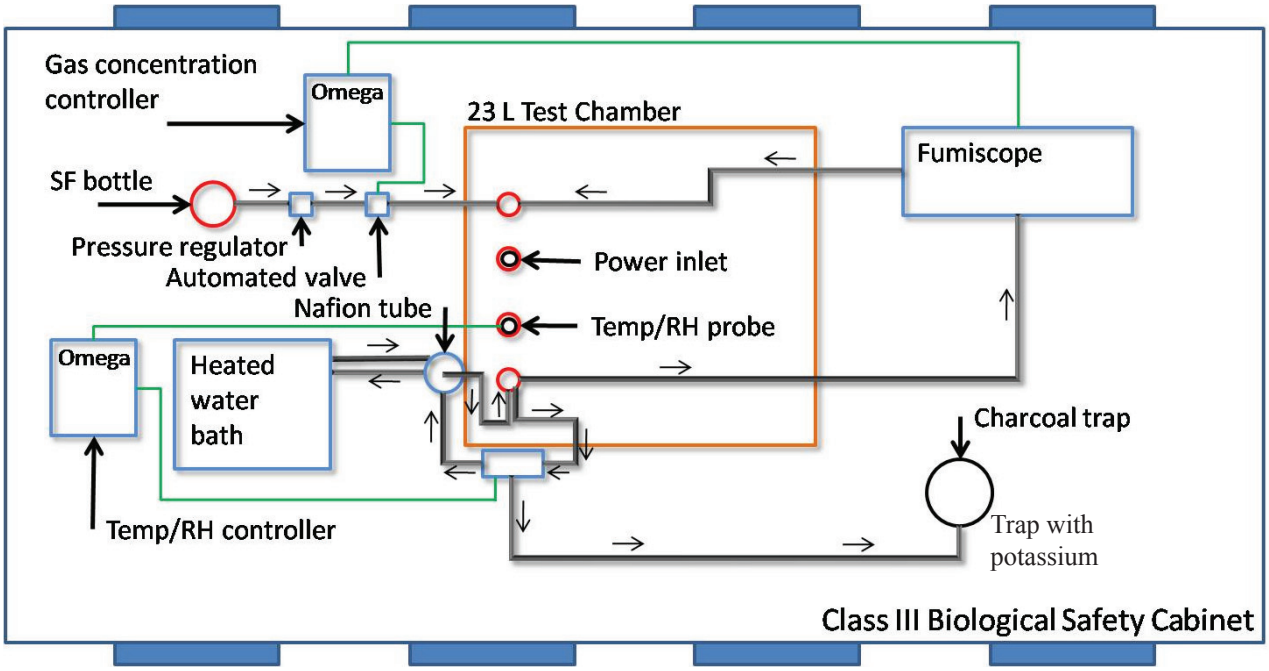

Figure 2-4. Schematic of MeBr decontamination testing.

The target MeBr concentration (211 mg/L ±10%) in the test chamber was achieved by introducing into the chamber a known volume of nominally 99.5% pure MeBr (Matheson Tri-Gas, Montgomeryville, PA), with approximately 0.5% chloropicrin (as a sensory warning) (Sigma-Aldrich, St. Louis, MO). The required volume of MeBr was introduced using a pressure regulator (Speedaire Model # 4ZM16, Grainger, Worthington, OH) in conjunction with an automated valve (Model #SV51C19T34, Valcor Engineering Corporation, Springfield, NJ). The MeBr concentration was measured using a Fumiscope Version 5.0 meter (Key Chemical and Equipment Company, Clearwater, FL), which was calibrated by the manufacturer for quantifying MeBr in air. Temperature of the test chamber was raised to the desired level using a thermostatically-controlled heating pad. A typical MeBr concentration profile during a decontamination run is shown in Figure 2-5.

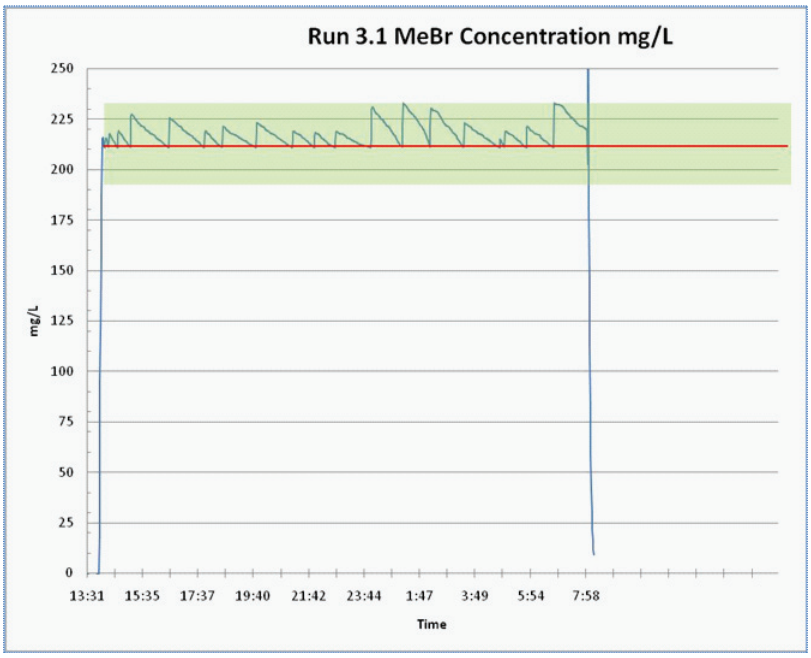

Figure 2-5. Typical MeBr concentration profile during a decontamination run.

The high efficacy treatment was 18 hr at 211 mg/L and 37 °C, based upon the results of previous testing.[19] Moderate and low efficacy treatments were achieved by lowering the time to 9 hr (moderate and low) and lowering the temperature to 23 °C (low). The RH was 75% ± 5%. The three efficacy treatment levels used with MeBr were:

- High efficacy treatment: 18 hr at 211 mg/L ± 10% and 37 °C ± 2 °C
- Moderate efficacy treatment: 9 hr at 211 mg/L ± 10% and 37 °C ± 2 °C
- Low efficacy treatment: 9 hr at 211 mg/L ± 10% and 23 °C ± 2 °C.

For the results of the test to be accepted, the temperature, RH, and MeBr concentration in the test chamber were required to be within the specified ranges. (See Appendix A for concentration, temperature and RH data.)

2.4 pH-Amended Bleach

Clorox® Regular Bleach (The Clorox® Company, Oakland, CA) (5%-6% sodium hypochlorite; EPA registration number 777-66) was pH-amended by adding acetic acid (SA36-1, Fisher Scientific, Pittsburgh, PA) and water. The solution was prepared using 9.4 parts deionized water, 1 part bleach, and 1 part 5% acetic acid to yield a solution having a pH of approximately 7 and a total chlorine content of approximately 6,000 ppm. The pH of the amended bleach was monitored and the amended bleach was used within 3 hr of preparation. The decontamination was performed in a water bath with the temperature controlled at 20 °C± 2 °C.

The high and low efficacy treatment levels were based on the contact time with the amended bleach:

- High efficacy treatment: 60 min
- Low efficacy treatment: 10 min.

2.5 Exterm Liquid ClO_2

Exterm-6 (ClorDiSys Solutions, Inc., Lebanon, NJ, EPA registration number 70060-19) disinfection tablets were a formulation of sodium chlorite that rapidly produced ClO_2 when mixed with water.[8] Working solutions containing 1,000 ppm of free ClO_2 were prepared fresh each day of use following vendor instructions (e.g., one 6 gram Exterm-6 tablet was used per 500 mL of water prepared and the tablet was allowed to dissolve before use).[8]

The high efficacy treatment for the Exterm used a solution containing 1,000 ppm of free ClO2 prepared according to manufacturer's instructions with a 60-min contact time. The decontamination was performed in a water bath with the temperature controlled at 20 °C± 2 °C. The high and low efficacy treatment levels were based on the contact time with the Exterm solution:

- High efficacy treatment: 60 min
- Low efficacy treatment: 10 min.

2.6 Oxonia Active®

Based on information provided by the vendor (Ecolab USA Inc., St. Paul, MN; EPA registration number 1677-129), Oxonia Active® is a liquid sanitizer for food processing equipment in dairies, breweries, wineries, beverage and food processing plants. The active ingredients are HP (27.5%) and peroxyacetic acid (5.8%). Oxonia Active® was noted as being a strong oxidizing agent that was corrosive. For sterilization, the Oxonia Active® label recommended adding 50 mL of Oxonia Active® concentrate per liter of water (5% v/v) with a 6 hr contact time at 20 °C.

In previous testing for EPA, the vendor recommended a 7% concentration of Oxonia Active® be used.The 7% concentration of Oxonia Active® was therefore selected for use in this investigation. The decontamination was performed in a water bath with the temperature controlled at 20 °C± 2 °C.

The two levels of efficacy used for Oxonia Active®, prepared as a 7% solution, were based on contact time:

- High efficacy treatment: 60 min
- Low efficacy treatment: 10 min.

2.7 Spor-Klenz® Ready-to-Use

As indicated by the vendor (STERIS Corporation, Mentor, OH, EPA registration number 52252-7), Spor-Klenz® Ready-to-Use is specifically formulated for use in the sterilization and disinfection of hard surfaces. This product is a stabilized blend of peracetic acid (0.08%), HP (1.0%), and acetic acid (<10%).

Spor-Klenz® Ready-to-Use required no mixing or activation. In accordance with the label instructions for sporicidal use, the high efficacy treatment was a 30-min contact time with the undiluted solution. The decontamination was performed in a water bath with the temperature controlled at 20 °C± 2 °C. The two levels of efficacy used for Spor-Klenz® Ready-to-Use were based on contact time:

- High efficacy treatment: 30 min
- Low efficacy treatment: 10 min.

2.8 Virkon® S

Virkon® S is a broad spectrum viricidal veterinary disinfectant product from DuPont (E. I. du Pont de Nemours and Company, Wilmington, DE, EPA registration number 71654-6). Virkon® S achieves deactivation and/or destruction of the target organism through general oxidative disruption of key structures and compounds vital to normal activity (e.g. proteins and lipids). Ingredients include potassium peroxymonosulfate, sulfamic acid, and sodium chloride.

For routine disinfection of surfaces, earth, wood, and concrete, the recommended dilution rate was 1:100 (10 grams of Virkon® S per 1 L of water); a 1:100 solution of Virkon® S was used for testing. While Virkon® S has been considered to be a technology potentially useful against *B. anthracis* spores, the label makes no claims for efficacy against *B. anthracis*, or for use as a sporicide, or for use as a sterilant.

3.0
Test Procedures

Comparative efficacy testing was conducted using three methodologies that are briefly described in the following sections:

- AOAC International (formerly the Association of Official Analytical Chemists or simply AOAC) Official Method 966.04, Sporicidal Activity of Disinfectants Test, Alternate Method, referenced herein as "AOAC 966.04" [3]
- AOAC First Action Official Method 2008.05, "Determination of Efficacy of Liquid Sporicides Against Spores of *Bacillus subtilis* on a Hard Nonporous Surface Using the Quantitative Three Step Method (TSM)", referenced herein as "AOAC 2008.05" [4]
- A method developed previously by EPA through Battelle for determining efficacy of decontamination against *B. anthracis* Ames spores on building materials, referenced herein as the internal standard operating procedure "iSOP". [5]

All three methods have been peer reviewed, have a clear test design and template, and are all acceptable for sporicidal registration through EPA's Office of Pesticide Programs. In some cases modifications were made to the methods to improve comparability or to accommodate testing with fumigants.

A qualitative evaluation of the ability of biological indicators (BIs) that were *B. atrophaeus* spores (approximately 106 on stainless steel in Tyvek® packaging) to detect the presence of viable spores after a decontamination treatment was run in parallel with the different test methods. BIs have often been used to indicate that sterility or complete kill of microorganisms has been achieved. Consistent with the practice in the field, BIs were used with the fumigation technologies, but not the liquid technologies. A brief discussion is also provided on the BIs used with each fumigation test.

The three efficacy test methods were compared by using each method to evaluate the performance of various fumigant and liquid technologies for inactivating *Bacillus anthracis* Ames spores and surrogate (i.e., *Bacillus subtilis*) spores applied to test coupons/carriers. Evaluations were run under both high efficacy and lower efficacy levels for the various fumigant and liquid technologies.

All *B. anthracis* Ames and *B. subtilis* spores used in all three methods were prepared according to the AOAC 966.04 Section C(b), culturing on amended nutrient agar (nutrient agar with 5µg/mL manganese sulfate), to yield a stock spore suspension of approximately 1 x 10^9 CFU/mL. The AOAC 966.04 spore preparation is consistent with the AOAC 2008.05. Use of the AOAC 966.04 spore preparation method for iSOP is a change from the spore preparation method that has been used in previous testing by EPA performed by Battelle. The target spore densities on all carriers/coupons across all methods was 1 x 10^7, except that 1 x 10^8 spores were applied to ceiling tile to offset low recoveries from this material.

The decontamination technologies included in the comparison of the test methods incorporated three fumigants: Sabre chlorine dioxide (ClO_2), STERIS hydrogen peroxide (HP), and methyl bromide (MeBr). Four liquid technologies were carried through the evaluation including pH-amended bleach, Exterm liquid ClO_2, Oxonia Active® HP/ peroxyacetic acid, and Spor-Klenz® Ready-to-Use peroxyacetic acid. Virkon® S potassium peroxymonosulfate and sulfamic acid was dropped from the testing because poor sporicidal properties were observed during method demonstration tests.

No neutralizers were used for fumigant testing. For liquid decontamination testing, neutralizers were included as indicated by the respective test methods. Table 3-1 provides a matrix of the decontamination technologies, test methods and neutralization method that were used in the liquid decontamination technology testing.

For a given decontamination technology, the treatment of test coupons/carriers was consistent across all three test methods (AOAC 966.04, AOAC 2008.05, and iSOP) for concentration, method for determining the start and end of contact time, and neutralization methods. The methods for extraction or analysis prescribed in the respective test methods began immediately after the decontamination treatment was completed. Fumigation tests of all three test methods were run side-by-side and simultaneously in the test chamber. Liquid decontamination comparisons of the three test methods were run on separate days.

Table 3-1. Matrix of the Decontamination Technologies, Test Methods, and Applied Neutralization Methods

Liquid Decontamination Technology	Method	Neutralization
Amended Bleach	AOAC 966.04	Neutralizer: 0.09 g sodium thiosulfate per 100 mL phosphate buffered saline solution (PBS)
Amended Bleach	AOAC 2008.05	Neutralizer: 0.1g sodium thiosulfate /100 mL Luria-Bertani (LB) Broth
Amended Bleach	iSOP	Extraction solution with neutralizer: 0.09 g sodium thiosulfate per 100 mL PBS (with 100 uL Triton)
Chlorine Dioxide (1000 ppm)	AOAC 966.04	0.04 g sodium thiosulfate per 100 mL PBS
Chlorine Dioxide (1000 ppm)	AOAC 2008.05	0.04g sodium thiosulfate /100 mL LB Broth
Chlorine Dioxide (1000 ppm)	iSOP	0.04g STS/100 mL per 100 mL PBS (with 100 uL Triton)
Oxonia Active®	AOAC 966.04	1 mL catalase per 100 mL Dey Engley Broth
Oxonia Active®	AOAC 2008.05	1 mL catalase per 99 mL Dey Engley Broth
Oxonia Active®	iSOP	1 mL catalase per 99 mL Dey Engley Broth
Spor-Klenz® Ready-to-Use	AOAC 966.04	1 mL catalase per 99 mL Dey Engley Broth
Spor-Klenz® Ready-to-Use	AOAC 2008.05	1 mL catalase per 99 mL Dey Engley Broth
Spor-Klenz® Ready-to-Use	iSOP	1 mL catalase per 100 mL PBS (with 100 uL Triton)
Virkon® S	Solution Test Only	1 g STS per 100 mL sterile water

3.1 AOAC 966.04

The efficacy achieved by the use of decontamination technologies against spores on porcelain penicylinders and suture loops was determined following the AOAC 966.04[3] methodology. In the methodology, 60 carriers are exposed to the liquid disinfectant for a specified contact time and transferred into a nutrient broth. The product passes if no growth of viable spores, i.e., no turbidity, is observed in any of the 60 replicate broth cultures. This test is highly sensitive; a single viable spore on any carrier will result in turbidity that will result in a failed test. (Note that in the testing reported here only 30 carriers were exposed to the disinfectant in a given test. If no growth was observed for all 30 carriers in a given test, the result was reported as a "pass".) Both the porcelain penicylinders and the suture loop carriers were inoculated as described for the porcelain penicylinder carriers in the 966.04 Alternative Method. Inoculated carriers had spore counts in the range of 2.9×10^5 - 3.9×10^6 colony-forming units (CFU).

The revised AOAC 966.04 method applies only to liquid technologies and was modified to accommodate fumigant technologies as follows. After the test chamber was stable at the desired temperature and RH for the testing, the test carriers were placed onto sterile cellulose nitrate filter paper (0.2 µm pore size, 90 mm diameter) (Fisher Scientific, Pittsburgh, PA) in the bottom of sterile 100 mm Petri dishes (Fisher Scientific, Pittsburgh, PA), which were covered and transferred into the test chamber. The porcelain penicylinders were oriented with the open beveled end down, in contact with the filter paper. Suture loops were placed flat on the filter paper. Petri dishes, each containing 15 carriers of a single type (porcelain penicylinders or suture loops), were placed as far as practical from the fumigant inlet and the adjacent mixing fan. The covers were removed from the Petri dishes and the carriers were allowed to equilibrate with the fumigant chamber conditions (temperature and RH) for 1hr before fumigation (introduction of ClO_2, HP, or MeBr).

Within 1 hr of fumigation, the carriers were transferred individually using a sterile wire hook (fabricated by Battelle using handles [50816-009, VWR, West Chester, PA] and nichrome wire [No. 20 B & S gauge, 66258-088, VWR, West Chester, PA]) or forceps (10-300, Fisher Scientific, Pittsburgh, PA) into a vial (14-961-33, Fisher Scientific, Pittsburgh, PA) containing 10 mL of appropriate recovery medium (culture tubes), e.g., tryptic soy broth (TSB) (Remel Inc. Lenexa, KS or Becton Dickinson and Company, Franklin Lakes, NJ) and capped. The racks of tubes were gently shaken after all of the carriers were transferred and then incubated for 21 days at 36 °C ± 1 °C. For suture loops only, if no growth was observed after 21 days, the tubes were heat shocked at 80 °C and re-incubated for 72 hr at 36 °C ± 1 °C. Results were reported as growth (+) or no growth (-). A positive result (+) was recorded when the culture medium appeared turbid indicating bacterial growth, while a negative result (-) was recorded when the culture medium was clear and without turbidity indicating no bacterial growth. Each tube was shaken prior to recording results to determine the presence or absence of growth/turbidity. Identification confirmation was performed on a minimum of three positive culture tubes

(as available) per test, using Gram stain (S71297, Fisher Scientific, Pittsburgh, PA) and/or plating on tryptic soy agar (R01917, Remel, Lenexa, KA).

For each carrier type in each AOAC 966.04 test, three sterile (negative control) carriers and one positive control carrier (inoculated with spores, but not decontaminated) were placed into culture medium and incubated, along with the test coupons, as described in the preceding paragraph. These carriers control for medium and system sterility (negative controls) and adequacy of the system to support spore growth (i.e., adequate quantity of spores on carrier, and quality of culture medium and incubation system).

The relative efficacy achieved by the use of decontamination technologies against spores on porcelain penicylinders and suture loops was determined with AOAC 966.04 as the fraction of broth cultures exhibiting growth of viable spores.

Additionally, a method described in Tomasino and Hamilton (2006)[6] was also used to estimate the log reduction in viable spores, given the AOAC 966.04 qualitative results. The conversion of the qualitative growth/no growth data into log reduction used the following formula:

Equation 3-1.

$$\text{Log reduction} = C - \log_{10}[-\ln([G + ½]/[M_t + 1])]$$

where:

$C = \log_{10}$ spores per carrier (6 – 7 log inoculation used here)

G = number of treated carriers experiencing no growth

M_t = total number of treated carriers (30 used here).

For example, if no growth was observed in the nutrient broth assays for all of the carriers (G = 30, M = 30 total carriers) and if the inoculation (mean carrier load) was 1 × 106 spores, i.e., $C = \log_{10}(1 \times 10^6) = 6$:

Equation 3-2.

$$\text{Log reduction} = 6 - \log_{10}[-\ln([30 + ½]/[30 + 1])] = 6 - (-1.8) = 7.8$$

Log reduction values calculated by this method can be higher than the 'log density' for the carrier; however, the method used for calculating log reduction values for 2008.05 or iSOP cannot be higher than the 'log density'.

A liquid or fumigant technology was considered to be effective via AOAC 966.04 (i.e., pass AOAC 966.04) if 100% kill (no growth in any of the 30 replicate culture tubes for a given carrier type) was achieved.

3.2 AOAC 2008.05

The efficacy achieved by the use of decontamination technologies against spores on (5 mm × 5 mm) glass carriers was determined with AOAC 2008.05 as log reduction in viable spores. Spore preparation methods followed those described for AOAC 966.04. Spores were applied to each carrier in a single 10 µL drop providing approximately 1×10^7 ($5 \times 10^6 – 5 \times 10^7$) spores per each inoculated carrier. Carriers were allowed to dry for a minimum of 1 hr before using them in a test. Each test included six inoculated carriers: 3 carriers that were placed in contact with the liquid technology or fumigant technology and 3 carriers that were positive controls. For fumigation testing, the positive controls were prepared, handled, extracted, and analyzed identically to the test carriers, except that the positive controls were not exposed to the fumigant. For liquid technology testing, the positive controls were prepared, handled, extracted, and analyzed identically to the test carriers, except that the positive controls were placed into sterile water rather than into liquid technology during the decontamination contact time. The positive controls demonstrate the adequacy of the system to extract, grow, and quantify viable spores (i.e., adequate quality of spores on carrier, extraction efficiency, and quality of culture medium and incubation system).

The AOAC 2008.05 method applies only to liquid technologies and was modified for application to fumigation technologies. For application to fumigation technologies, the test chamber was allowed to stabilize to the desired temperature and RH for the testing. Inoculated carriers were placed flat onto sterile cellulose nitrate filter paper (0.2 µm pore size, 90 mm diameter), with the inoculated surface facing up, in the bottom of sterile 100 mm Petri dishes. The Petri dishes were covered and transferred into the test chamber as far as practical from the fumigant inlet and the adjacent mixing fan. The covers were removed from the Petri dishes and the carriers were allowed to equilibrate with the test chamber conditions (temperature and RH) for 1 hr prior to fumigating.

After fumigation, forceps were used to transfer each carrier into a microcentrifuge tube (THUM-200, Diversified Biotech, Boston, MA) labeled Fraction A. A 1.0 mL volume of ice-cold sterile Luria-Bertani (LB) broth (46050CM, MediaTech, Manassas, VA) was added to each fumigation Fraction A tube, and the tubes were slightly agitated. Each test carrier was transferred using sterile forceps (10-300, Fisher Scientific, Pittsburgh, PA) from the Fraction A tube to a corresponding Fraction B tube (which contained 400 µL of ice-cold sterile water [W3500, Sigma, St. Louis, MO]). Fraction B tubes were sonicated (8510 Sonicator, Branson, Danbury, CT) 5 min ± 30 seconds using a floating Styrofoam microcentrifuge tube holder. After the sonication was complete, 600

µL of ice-cold LB broth was added to the Fraction B tubes, which were then vortexed (Vortexer, VWR, West Chester, PA) for approximately 1 min. Each carrier was transferred using sterile forceps from Fraction B tubes to corresponding Fraction C tubes, which contained 400 µL ice-cold LB broth. Fraction C tubes were placed in a hematology rotator (415110Q, Barnstead, Dubuque, IA) inside an incubator at 36 °C ± 1 °C. The Fraction C tubes were removed from the incubator after 30 ± 2 min of rotation/incubation. A 600 µL volume of ice-cold LB broth was added to each Fraction C tube. Each microcentrifuge tube was mixed by vortexing prior to making dilutions. Fractions A, B, and C were serially diluted with sterile water and 100 µL of appropriate dilutions were plated onto tryptic soy agar (R01917, Remel, Lenexa, KS) to achieve 30-300 CFU/plate. The plates were incubated a minimum of 24 ± 2 hr at 36 °C ± 1 °C.

Counts falling within 0-300 CFU/plate were used for calculations. The number of CFU/carrier was determined by multiplying the average number of colonies per plate by the reciprocal of the dilution and accounting for the volume plated. The total number of spores per carrier was obtained by adding the total number of viable spores of Fractions A, B, and C. Log density of the total number of viable spores per carrier was determined by taking $\log_{10}$ (total number of spores per carrier). Log reductions were determined by subtracting the log density of the test carriers from the log density of the control carriers. The log of 0 is mathematically undefined. Therefore, when no (zero) CFU were recovered from the A, B, and C fractions for all replicate carriers in a given test treatment, per the method, 0.5 was substituted at the first dilution of Fraction A. This minimum efficacy convention conservatively assumes 5 CFU on each carrier in the efficacy calculation. If spores in all dilutions of a fraction (A, B, or C) were too numerous to count, 300 was substituted at the last dilution for that fraction. See the referenced AOAC 2008-05 method for calculation details.

A liquid or fumigant decontamination technology was considered to be effective via AOAC 2008.05 (i.e., pass AOAC 2008.05) if a ≥6-log reduction in viable spores was achieved. In cases where the mean recovered spores from positive control coupons was slightly low (less than 6 log), the log reduction, even with no recovered spores, will be less than 6 log and not meet the requirement to pass the AOAC 2008.05 test. Those cases (log reduction <6, but no viable spores recovered from the test coupons) are noted in the efficacy results and the tests are excluded from the calculation of the percent of replicate tests that passed.

3.3 iSOP

For iSOP, the test coupons were glass (5 mm × 5 mm, as specified in AOAC 2008.05), along with one porous material (ceiling tile or carpet, 1.9 cm × 7.5 cm) and one other nonporous material (galvanized metal, 1.9 cm × 7.5 cm). Spore preparation methods followed those described for AOAC 966.04. Spores were applied to each coupon in a single 10 µL drop so that there were $5 \times 10^6 - 5 \times 10^7$ spores per each inoculated coupon, except that ten 10 µL drops were applied to ceiling tile to achieve spore recovery comparable to other coupon types.

For each coupon type for each replicate test, five test coupons (inoculated and decontaminated), five positive control coupons (inoculated, but not decontaminated), and one procedural blank coupon (not inoculated, but decontaminated) were included. All coupons were prepared in the same batch and all were extracted and analyzed in a single batch. These coupons control for medium and system sterility (negative controls) and the adequacy of the system to extract, grow, and quantify viable spores (i.e., adequate quality of spores on carrier, extraction efficiency, and quality of culture medium and incubation system). For fumigant testing, the test coupons were exposed to the fumigant inside of a test chamber for the specified contact time and at specified temperature and RH. For liquid decontamination testing the test coupons were immersed.

The efficacy achieved by the use of liquid and fumigant decontamination technologies against spores on various test materials was determined via iSOP as the log reduction in viable spores. The first step in the calculation of overall efficacy was the separate calculation of efficacy for each individual coupon in a given set of coupons. Efficacy was defined as the extent (by log reduction) by which the viable spores extracted from the coupons after the treatment with the decontamination technology were less than the number of spores extracted from the positive control (not exposed to the decontamination technology) maintained at ambient laboratory conditions after being inoculated with the same amount and type of biological agent as the test coupons. Efficacy was calculated for each test coupon within each combination of contact time (i) and test material (j) as:

Equation 3-3.

$$\text{Efficacy}_{ij} = \log_{10}\left(\overline{C}_{ij}\right) - \log_{10}\left(N_{ijk}\right)$$

Where:

$\overline{C}_{ij}$ = mean number of viable spores (CFU) recovered from the positive controls at the i^{th} contact time from the j^{th} material.

N_{ijk} = number of viable organisms recovered from the k^{th} test coupon at the i^{th} contact time and j^{th} test material.

In some cases, no (zero) CFU were observed from all test coupons at a given time point. Minimum efficacy was conservatively estimated in such cases by assuming one CFU on each coupon in the efficacy calculation. Because the log of 1 (the assumed mean) is zero, the efficacy when no spores were recovered from any coupon is numerically equal to the log of the mean of the CFU/coupon of the corresponding positive control coupons.

For purposes of comparison, a liquid or fumigant technology was considered to pass the iSOP test if a 6-log reduction in viable spores was achieved. This is consistent with the minimum requirement that may be accepted for registration purposes. In cases where the mean recovered spores from positive control coupons was less than 6 log, the log reduction, even with no recovered spores, will be less than 6 log and not meet the requirement to pass the iSOP test. Those cases (log reduction <6, but no viable spores recovered from the test coupons) are noted in the efficacy results and the tests are excluded from the calculation of the percent of replicate tests that passed.

After spores are extracted from the test coupons, the test coupons are place into tryptic soy broth and incubated and results analyzed in the same manner as the BIs described in Section 3.4. This qualitative test is highly sensitive: turbidity, indicative of growth of bacteria, will be observed if a single viable and culturable spore remains on a coupon. Negative growth in this qualitative evaluation is a check for residual spores on coupons when there are no extracted spores observed in the quantitative test.

3.4 Biological Indicators

BIs have often been used to indicate that sterility or complete kill of microorganisms was achieved. The experimental design included BIs in the test chamber along with the test coupons during fumigant testing in order to correlate observations of no growth of spores from the BIs with the respective efficacies as measured by the three methods. The BIs (Apex Laboratories, Apex, NC) that were used consisted of *B. atrophaeus* spores (approximately 1 × 106 on stainless steel disks in Tyvek® packaging). After each fumigation treatment, five BIs that received the treatment along with the various test coupons and carriers were aseptically removed from the packaging. The BIs were transferred into individual 50 mL conical vials (Fisher Scientific, Pittsburgh, PA) containing tryptic (trypticase) soy broth culture medium (Remel Inc., Lenexa, KS or Becton Dickinson and Company, Franklin Lakes, NJ) at a level sufficient to cover the BI; the vials were then capped. The vials were cultured at 37 °C ± 2 °C to encourage viable spore germination and subsequent proliferation of vegetative bacteria. At one and seven days post-decontamination, the vials were visually assessed for cloudiness. A cloudy culture medium indicated "growth" of viable spores. Clear culture medium was indicative of "no growth". Data were expressed as "growth" (+) or "no growth" (-).

A fumigant technology was considered to be effective via BIs (i.e., pass BI test) if a 100% kill was achieved, i.e., no growth was observed from any of the five BIs under a given treatment.

3.5 Test Overview

Decontamination treatments were selected to obtain a range of efficacy results to enable the sensitivity of the test methods to be compared. The high efficacy treatment selected by the EPA for each technology followed the label recommendations, followed manufacturers' recommendations, or, absent such guidance, was at concentrations and contact times previously demonstrated to be efficacious against *B. anthracis* spores. Medium and low efficacy treatments were selected to obtain incomplete decontamination thereby providing treatments necessary to compare all three methods used in this evaluation. High efficacy treatments, where all test methods result in a complete kill of spores, would not enable a comparison of the sensitivity of the respective tests. By using less efficacious treatments in which spores were expected to survive, a method may be shown to be equivalent, or more or less stringent, than the other methods. The medium and low treatments involved using relatively lower concentration of decontaminant, relatively shorter contact times, or using less effective treatments, i.e., lower temperature. In some cases the medium and low decontamination treatments still resulted in complete decontamination.

3.5.1 Test Materials

Testing was conducted with porous materials (e.g., silk suture loop, ceiling tile, or carpet) and nonporous materials (e.g., porcelain penicylinder, glass, and

galvanized metal), which are described in Table 3-2. Generally, the coupons were cut from the interior of a large piece of test material to the widths and lengths shown in Table 3-2. Edges and damaged areas were avoided in cutting test coupons. The suture loops, penicylinders, and glass coupons were used as received (not cut). The test coupons were prepared (cleaned/sterilized) as described in Table 3-2. Test coupons were visually inspected prior to being inoculated with the biological agents, and coupons with anomalies on the test (application) surface were rejected from use.

On each day of testing, each coupon was assigned and marked with a unique identifier code for traceability. To prevent contamination of test surfaces, sterile technique, following Battelle policies and guidelines[9-11] was exercised during all phases of handling the coupons.

Table 3-2. Material Characteristics

Material	Lot, Batch, ASTM No., or Observation	Manufacturer or Supplier	Approximate Coupon Size, width × length (thickness)	Material Preparation	Test Method Using the Material
Porcelain Penicylinder	Catalog number: #PP	Presque Isle Cultures (Erie, PA)	0.8 cm outside diameter, 0.6 cm inside diameter, 1 cm long	180 °C air oven	AOAC 966.04
Suture Loop	Catalog number: SS 6.0 metric silk, black, braided	Presque Isle Cultures (Erie, PA)	6.5 cm of suture in 2 loop coil	Chloroform and hydrochloric acid	AOAC 966.04
Glass	Custom order part number EPA-1101	Erie Scientific Company (Portsmouth, NH)	0.5 cm × 0.5 cm (0.1 cm)	Autoclaved	AOAC 2008.05 and iSOP
Carpet (industrial grade)	ShawTek, EcoTek 6, Color: mottled gray, dark brown, and black	Shaw Industries, Inc. (Dalton, GA)	1.9 cm × 7.5 cm (0.7 cm)	Gamma irradiated	iSOP
Ceiling Tile	Style M7978, color # 910	Carpet Corporation of America (Rome, GA)	1.9 cm × 7.5 cm (0.3-0.4 cm)	Autoclaved	iSOP
Galvanized Metal	ASTM A653 G90 industry HVAC standard 24 gauge galvanized steel ductwork	Accurate Fabrication (Columbus, OH)	1.9 cm × 7.5 cm (0.1 cm)	Cleaned with acetone; autoclaved	iSOP

3.5.2 Spores

The biological agents used in the testing include:

- *B. anthracis* Ames spores (United States Army Medical Research Institute for Infectious Diseases M-BAA202) were used with all three test methods (AOAC 996.04, AOAC 2008.05, and iSOP) and with all decontamination technologies
- *B. subtilis* spores (ATCC® 19659) were used with AOAC 996.04 (except when testing Exterm liquid ClO2 and Oxonia Active®) and AOAC 2008.05
- *B. atrophaeus* spores were used as the BI (Apex Laboratories, Apex, NC) on stainless steel disks in Tyvek® packaging; BIs were included in the test chamber during all fumigation tests only.

Test matrices in Section 3.5.3 provide details of decontamination technologies, test methods and microbes used.

In general, spore counts and spore preparation methods were the same for all test methods. *B. anthracis* and *B. subtilis* spores were prepared according to the AOAC 996.04 Section C(b) and cultured on amended nutrient agar plates (prepared by Battelle; nutrient agar powder [213000, Becton Dickinson, Franklin Lakes, NJ] with 5 µg/mL manganese sulfate [M8828, Fisher Scientific, Pittsburgh, PA]) to yield a stock spore suspension of approximately 1×10^9 CFU/mL. The AOAC 966.04 spore preparation was consistent with requirements of AOAC 2008.05. Use of the AOAC 966.04 spore preparation method for iSOP was a change from the spore preparation method that was used in previous iSOP testing by EPA performed by Battelle.

The carrier inoculations used for AOAC 966.04 met the requirements of AOAC 966.04: “Average spore counts

should be in the range of 1.0×10^5 and approximately 1.0×10^6 spores/carrier."[3] The inoculation of suture loops, not discussed in AOAC 966.04, followed the method specified for the porcelain penicylinders in AOAC 966.04. Spore enumeration (carrier counts) and hydrochloric acid resistance tests were performed as specified in AOAC 966.04 for both porcelain penicylinders and suture loops. Inoculated carriers were stored in vacuum desiccators (420200000, Bel-Art Products, Pequannock, NJ) as specified in AOAC 966.04 Section C(d).

The carriers used for AOAC 966.04 in fumigation testing had actual spores/carrier values between 2.9×10^5 and 9.4×10^5. The carriers used for AOAC 966.04 in liquid testing had spores/carrier values between 5.6×10^5 and 3.9×10^6.

For AOAC 2008.05 and iSOP, coupons/carriers were inoculated with spores as specified in the respective test methods. The same spore loading (target range of $5 \times 10^6 – 5 \times 10^7$ spores/coupon) per carrier/coupon was used except that $5 \times 10^7 – 5 \times 10^8$ spores were applied to ceiling tile. The higher inoculation level for ceiling tile was intended to compensate for the low recoveries expected based on prior experience.

An exception regarding spore counts inadvertently occurred with some of the testing associated with iSOP as other coupons, in addition to ceiling tile, were inoculated with approximately 1×10^8 CFU rather than 1×10^7 CFU. These exceptions are documented as deviations in Section 4.4. Tests with higher levels of spores applied to the coupons include the galvanized metal coupons in the STERIS HP fumigation testing (actual application range: $8.8 \times 10^7 – 9.8 \times 10^7$) and all of the liquid decontamination testing (actual application range: ($1.2 \times 10^7 – 6.4 \times 10^8$). These higher levels of spores/coupon reflect the levels that are routinely used in the practice of iSOP. As described in Section 4.4, the difference in higher inoculated amounts did not appear to impact the results.

3.5.3 Test Matrices

The fumigant (ClO_2, HP, and MeBr) test matrix is provided in Table 3-3. Testing associated with each decontamination technology was conducted in triplicate at each contact time and fumigant concentration. In the test matrix (Table 3-3) this replication is indicated by trials numbered X.Y, where X is the trial number and Y is the replication, e.g., 1.1, 1.2, and 1.3 are replications 1, 2, and 3 of Trial 1. The liquid decontamination test matrix is provided in Table 3-4. All three methods (AOAC 966.04, AOAC 2008.05, and iSOP) were replicated twice. Liquid decontaminations were all conducted at 20 °C.

3.5.4 Statistical Comparisons

To make comparisons between the efficacies for *B. anthracis* Ames spores and for *B. subtilis* spores within each combination of decontaminant, contact time, concentration and test method, a similar nonparametric approach is taken. The Kolmogorov-Smirnov test is performed through PROC NPAR1WAY in SAS v 9.1. If a p-value of 0.1 or less is found the overall distribution of the two spores is different. Stated simply, for a p-value greater than 0.1, in the decontaminants and methods tested, there are no significant differences between the results obtained using *B. anthracis* Ames spores or *B. subtilis*spores.. The p-value of 0.1 was selected to specify significant differences because of the low power of this analysis, given the small sample size.

Analyses were performed to evaluate whether the three methodologies yielded the same efficacies (expressed as log reductions) for each treatment. A nonparametric approach was taken, since the normality assumption for a parametric approach was not valid in these cases. The non-parametric Kruskal-Wallis test was performed through PROC NPAR1WAY in SAS v 9.1. If a p-value of 0.05 or less was found, the overall distribution of the three methods was different.

Table 3-3. Fumigant Test Matrix

Fumigant	Contact Time, Target Concentration	Trial*	Method	Material‡	Spore
ClO_2	3 hr, 3000 ppm	1.1, 1.2, 1.3	AOAC 966.04	PP, SL	*B. anthracis, B. subtilis*
		2.1, 2.2, 2.3	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		3.1, 3.2, 3.3	iSOP	C. Tile, GM, Glass	*B. anthracis*
		1.1 - 3.3	BI	Steel	*B. atrophaeus*
ClO_2	3 hr, 300 ppm	4.1, 4.2, 4.3	AOAC 966.04	PP, SL	*B. anthracis, B. subtilis*
		5.1, 5.2, 5.3	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		6.1, 6.2, 6.3	iSOP	C. Tile, GM, Glass	*B. anthracis*
		4.1 - 6.3	BI	Steel	*B. atrophaeus*
ClO_2	3 hr, 150 ppm	7.1, 7.2, 7.3	AOAC 966.04	PP, SL	*B. anthracis, B. subtilis*
		8.1, 8.2, 8.3	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		9.1, 9.2, 9.3	iSOP	C. Tile, GM, Glass	*B. anthracis*
		7.1 - 9.3	BI	Steel	*B. atrophaeus*
HP	90 min, 250 parts per million by volume (ppmv)	10.1, 10.2, 10.3	AOAC 966.04	PP, SL	*B. anthracis, B. subtilis*
		11.1, 11.2, 11.3	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		12.1, 12.2, 12.3	iSOP	C. Tile, GM, Glass	*B. anthracis*
		10.1 - 12.3	BI	Steel	*B. atrophaeus*
HP	30 min, 250 ppmv	13.1, 13.2, 13.3	AOAC 966.04	PP, SL	*B. anthracis, B. subtilis*
		14.1, 14.2, 14.3	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		15.1, 15.2, 15.3	iSOP	C. Tile, GM, Glass	*B. anthracis*
		13.1 - 15.3	BI	Steel	*B. atrophaeus*
HP	0 min, 250 ppmv†	16.1, 16.2, 16.3	AOAC 966.04	PP, SL	*B. anthracis, B. subtilis*
		17.1, 17.2, 17.3	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		18.1, 18.2, 18.3	iSOP	C. Tile, GM, Glass	*B. anthracis*
		16.1 - 18.3	BI	Steel	*B. atrophaeus*
MeBr	18 hr, 211 mg/L (at 37 °C)	22.1, 22.2, 22.3	AOAC 966.04	PP, SL	*B. anthracis, B. subtilis*
		23.1, 23.2, 23.3	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		24.1, 24.2, 24.3	iSOP	C. Tile, GM, Glass	*B. anthracis*
		22.1 - 24.3	BI	Steel	*B. atrophaeus*
MeBr	9 hr, 211 mg/L (at 37 °C)	25.1, 25.2, 25.3	AOAC 966.04	PP, SL	*B. anthracis, B. subtilis*
		26.1, 26.2, 26.3	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		27.1, 27.2, 27.3	iSOP	C. Tile, GM, Glass	*B. anthracis*
		25.1 - 27.3	BI	Steel	*B. atrophaeus*
MeBr	9 hr, 211 mg/L (at 25 °C)	19.1, 19.2, 19.3	AOAC 966.04	PP, SL	*B. anthracis, B. subtilis*
		20.1, 20.2, 20.3	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		21.1, 21.2, 21.3	iSOP	C. Tile, GM, Glass	*B. anthracis*
		19.1 - 21.3	BI	Steel	*B. atrophaeus*

* Each trial was conducted in triplicate, designated as X.1, X.2, and X.3, where X represents the trial number. All three tests were run simultaneously. The test coupons, procedural control coupons, and BIs in a given trial (X) were in the test chamber and fumigated at the same time in this side-by-side comparison.

† Test carriers were exposed to HP during conditioning (addition of HP into the test chamber up to 250 ppmv). Once the target HP concentration was attained the "contact time" was ended.

‡ Material type key:

C. Tile = ceiling tile
PP = porcelain penicylinder
Steel = stainless steel in Tyvek® packaging
GM = galvanized metal
SL = suture loop

Table 3-4. Liquid Decontamination Test Matrix

Liquid	Contact Time	Trial*	Method	Material‡	Spores
pH-Amended Bleach	10 min	1.1, 1.2, 1.3	AOAC 966.04	PP, SL	*B. anthracis, B. subtilis*
		29.1, 29.2	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		30.1, 30.2	iSOP	Carpet, GM, Glass	*B. anthracis*
pH-Amended Bleach	60 min	31.1, 31.2	AOAC 966.04	PP, SL	*B. anthracis, B. subtilis*
		32.1, 32.2	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		33.1, 33.2	iSOP	Carpet, GM, Glass	*B. anthracis*
Exterm Liquid ClO_2	10 min	34.1, 34.2	AOAC 966.04	PP, SL	*B. anthracis*
		35.1, 35.2	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		36.1, 36.2	iSOP	Carpet, GM, Glass	*B. anthracis*
Exterm Liquid ClO_2	60 min	37.1, 37.2	AOAC 966.04	PP, SL	*B. anthracis*
		38.1, 38.2	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		39.1, 39.2	iSOP	Carpet, GM, Glass	*B. anthracis*
Oxonia Active®	10 min	40.1, 40.2	AOAC 966.04	PP, SL	*B. anthracis*
		41.1, 41.2	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		42.1, 42.2	iSOP	Carpet, GM, Glass	*B. anthracis*
Oxonia Active®	60 min	43.1, 43.2	AOAC 966.04	PP, SL	*B. anthracis*
		44.1, 44.2	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		45.1, 45.2	iSOP	Carpet, GM, Glass	*B. anthracis*
Spor-Klenz® Ready-to-Use	10 min	46.1, 46.2	AOAC 966.04	PP, SL	*B. anthracis, B. subtilis*
		47.1, 47.2	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		48.1, 48.2	iSOP	Carpet, GM, Glass	*B. anthracis*
Spor-Klenz® Ready-to-Use	30 min	49.1, 49.2	AOAC 966.04	PP, SL	*B. anthracis, B. subtilis*
		50.1, 50.2	AOAC 2008.05	Glass	*B. anthracis, B. subtilis*
		51.1, 51.2	iSOP	Carpet, GM, Glass	*B. anthracis*

*Each trial was replicated twice, designated as X.1 and X.2, where X represents the trial number. Various test methods were performed on separate days.

† Material type key:

GM = galvanized metal
PP = porcelain penicylinder
SL = suture loop

4.0
Quality Assurance/Quality Control

Quality assurance/quality control (QC) procedures were performed in accordance with the program QMP[2] and the test/QA plan[1], including Amendments 1 through 9, for this evaluation. QA/QC procedures are summarized below.

4.1 Equipment Calibration

All equipment (e.g., pipettes, thermometers, incubators, biological safety cabinets) used was verified as being certified, calibrated, or validated at the time of use in the evaluations.

4.2 Audits

4.2.1 Performance Evaluation Audit

No performance evaluation audit was performed for biological agents and surrogates because quantitative standards for these biological materials do not exist. The spores were prepared and tested for quality in accordance with AOAC 966.04 to ensure (1) the percentage of spores versus vegetative cells was at least 95% and (2) spores were able to pass the hydrochloric acid test (with viable spores remaining after two min exposure to 2.5 molar hydrochloric acid). The confirmation procedure, controls, blanks, and method validation efforts support the biological evaluation results.

Performance evaluation audits were conducted by the respective laboratory personnel to assess the quality of the results obtained during these experiments. The controls, blanks, and method validation efforts support the biological evaluation results. Table 4-1 summarizes the performance evaluation audits that were performed.

4.2.2 Technical Systems Audit

Battelle QA staff conducted technical systems audits (TSAs) on 10/1/08, 4/29/09, 5/12/09, and 1/12-13/10 to ensure that the evaluation was being conducted in accordance with the test/QA plan[1] and the QMP.[2] As part of the TSA, test procedures were compared to those specified in the test/QA plan; and data acquisition and handling procedures were reviewed. Observations and findings from the TSA were documented and submitted to the Battelle Task Order Leader for response. None of the findings of the TSA required corrective action. TSA records were permanently stored with the Battelle QA Manager.

EPA staff performed a TSA on October 1- 3, and October, 8, 2008. Three members of the EPA performed the audit: Ms. Eletha Brady-Roberts, NHSRC Director of Quality; Dr. Frank Schaefer, microbiology technical expert; and Ms. Ramona Sherman, QA Auditor. EPA staff viewed project documentation, sample collection, analysis, and storage; records; equipment maintenance, and calibration; and data management. Several items were noted, mostly pertaining to documentation and overcrowding of workspace. An exit debrief was held on October 8, 2008, and all issues were relayed to the Battelle staff. A copy of the draft EPA/QA report is stored with the Battelle QA Manager.

Table 4-1. Performance Evaluation Audits

Measurement	Audit Procedure	Allowable Tolerance	Actual Tolerance
Temperature	Compared to independent thermometer value	± 1 °C	<1 °C for 9 of 9 instances
Water bath temperature	Measure temperature at the beginning and end of one hour with calibrated thermometer	± 1 °C	<1 °C for 2 of 2 instances
Time (stopwatch)	Compare against NIST official U.S. time at http://nist.time.gov/timezone.cgi?Eastern/d/-5/java	± 1 second/min	0 sec/min for 10 of 10 measurements
RH	Compared to independent hygrometer value	± 5%	<5% for 4 of 4 instances
ClO_2 concentration	Titration of standard solution	± 10%	<10%
HP concentration	Hach HYP-1 HP test kit	± 10%	<10%
MeBr concentration*	Measured with a calibrated VIG M-20 total hydrocarbon analyzer	± 10%	2% average of 2 measurements

*Average of two measurements. Prior to use, the Fumiscope meter was returned to the manufacturer, Key Chemical and Equipment Company (Clearwater, FL), for calibration. The Fumiscope measurements were compared to measurements with a calibrated VIG M-20 total hydrocarbon analyzer (VIG Industries, Inc., Anaheim, CA). Differences between measurements made with the total hydrocarbon analyzer and the Fumiscope meter varied up to about 20% depending on concentration of MeBr. Fumiscope readings were reported as specified in the test/QA plan to reflect readings that would be expected in field use.

4.2.3 Data Quality Audit

All of the data transferred from raw data to spreadsheets were 100% peer reviewed. At least 10% of the data acquired during the evaluation were audited. A Battelle QA auditor traced the data from the initial acquisition, through reduction and statistical analysis, to final reporting to ensure the integrity of the reported results. All calculations performed on the data undergoing the audit were checked.

4.2.4 Control Coupons

Positive and negative controls were included in all tests. In all cases the positive controls were positive. In all cases negative controls were negative except for a single laboratory blank (negative control) that showed contamination noted in the deviations below. No other contamination was observed.

4.3 QA/QC Reporting

Each audit was documented in accordance with the QMP.[2] The results of the TSA and audit of data quality were submitted to the EPA.

4.4 Deviations

Deviations from the test/QA plan were documented, in compliance with the QMP.[2] Two types of deviations were noted: (1) methodological deviations and (2) replicate tests that were not rejected when:

- The amounts of biological agent inoculated onto the coupons were outside the acceptance criteria;
- Positive control spore recoveries were outside of the acceptance criteria (≤20% - ≥120%) of the spores applied; or
- Contamination of blank coupons occurred.

Methodological deviations included the following:

- The Grubbs' test for outliers was not used to exclude outliers because of inadequate knowledge of the sample distribution (a Gaussian distribution is required for application of the Grubbs' test)) and the decision that no data would be excluded unless a cause for the variation could be identified.
- Silk suture loops were inoculated on 4/28/2009 using 5 mL of inoculum per tube for 10 suture loops, instead of 10 mL of inoculum as written in an internal SOP that is different from the AOAC 966.04 language. Due to the low volume of spore suspension adsorbed onto the suture loops compared to the total volume of inoculum, no impact on testing was expected from this deviation.
- On 5/5/09, only four replicate *B. anthracis* Ames test coupons (rather than five replicate test coupons) were included in the decontamination test. The impact of the deviation was expected to be minimal; the deviation will reduce the sample size, thereby increasing the uncertainty in the measurement.
- In Trial 28.1 (Porcelain Penicylinders with pH-Amended Bleach), 29 carriers (rather the specified 30 carriers) were evaluated for the 60-min contact time. The impact of the deviation was expected to be minimal; the deviation will reduce the sample size, thereby increasing the uncertainty in the measurement.
- In Trial 28.1 (Suture Loops with pH-Amended Bleach), 29 carriers (rather the specified 30 carriers) were evaluated for the 10-min contact time. The impact of the deviation was expected to be minimal; the deviation will reduce the sample size, thereby increasing the uncertainty in the measurement.
- In Trial 35.1, the time of the third centrifugation was 5 min and not the required 6 min ± 30 seconds for Fraction A for the AOAC 2008-05 with Exterm chlorine dioxide solution as the decontaminant. The impact of the deviation was expected to be minimal; the results of the growth on the plates were as expected.
- In Trials 48.2 (iSOP assay using Spor-Klenz® on glass coupons, second replicate) and 45.2 (iSOP assay using 7% Oxonia Active® on glass coupons, second replicate), the coupons were extracted for 20 min instead of 15 min per the iSOP method. No impact on results was expected from the additional extraction time. In all decontamination tests within these trials, a mean log reduction of at least 6.7 was observed. (Viable spores were found in only one of forty test coupon extracts from these two trials. The coupon extract containing spores was extracted at the correct period of 15 min, so finding viable spores cannot be attributed to a longer extraction time.)

Trials were not rejected in those cases where the amounts of biological agent on the coupons/carriers were outside of the acceptance criteria; positive control recoveries of applied spores were outside the acceptance criteria (acceptable recovery range: ≥20% - ≤120%); or a blank coupon exhibited contamination. The specific instances of deviation are:

1. Spore applications exceeded the acceptance criteria as follows (the trials were not rejected):

   iSOP liquid decontamination testing:

   Target application range: $5 \times 10^6 – 5 \times 10^7$

   Actual Range (liquid testing):
   $1.2 \times 10^7 – 6.4 \times 10^8$ (Wrong target range inadvertently used.)

iSOP STERIS HP fumigation testing, galvanized metal coupons:

Target application range: $5 \times 10^6 – 5 \times 10^7$

Actual Range: $8.8 \times 10^7 – 9.8 \times 10^7$ (Wrong volume inadvertently used: 100 µL rather than 10 µL.)

The carrier counts for routine iSOP testing have a target application of $1.0 \times 10^7 – 1.0 \times 10^8$ spores per coupon; that target was inadvertently used for all of the liquid decontamination testing and, in one instance, for STERIS HP fumigation.

The inadvertent inclusion of tests with higher spore levels reflecting the level routinely used in the iSOP procedure does provide a comparison of the standard iSOP with the other methods. The higher numbers of spores per coupon for the iSOP tests did not appear to have an impact on the results, except that when no spores were recovered, the estimated log reduction was higher.

AOAC 966.04 decontamination testing as specified in Amendment 5 of the test/QA plan:

Carrier count target range: $5 \times 10^6 – 5 \times 10^7$

Range of actual carrier counts (fumigation testing): 2.9×10^5 - 9.4×10^5

Range of actual carrier counts (liquid testing): 5.6×10^5 - 3.9×10^6

(Wrong target range inadvertently used.)

The carrier count target for AOAC 966.04 specifies a "range of 1.0×10^5 – and approximately 1.0×10^6 spores/carrier."[3] The carrier count requirements of the AOAC 966.04 protocol were inadvertently followed, rather than targeting the higher spore count/carrier specified in Amendment 5 to the test/QA plan. The lower number of spores on carriers in the AOAC 966.04 method compared to the number of spores in the iSOP method introduced an unintended difference between the test methods.

The impact of this deviation was minimal. This deviation introduced a confound in making comparisons across test methods. Because the carrier counts were lower than the acceptable range, the calculated log reduction values if no growth was observed in the actual 30-carrier AOAC 966.04 tests was lower than the target log reduction value in a 30-carrier test within the range (minimum log reduction within range is 6.08).

By using the carrier counts specified in the AOAC 966.05 method, rather than the specified higher carrier counts, a conclusion can be drawn that AOAC 966.04 as practiced, even with lower spores/carrier than either iSOP or AOAC 2008-05, is generally as difficult as the other test methods, or possibly more difficult to pass.

2. Positive control recoveries for certain materials in some of the iSOP Trials were outside the acceptance criteria (acceptable recovery range: ≥20% - ≤120%) of the spores applied, but the test results were not rejected:

   - Trials 42.1 and 45.1 [same positive controls] with Oxonia Active® showed 18% recovery from the glass positive controls and 17% recovery from the metal positive controls.
   - Trials 48.1 and 51.1 [same positive controls] with Spor-Klenz® showed 18% recovery from the glass positive controls and 17% recovery from the metal positive controls.
   - Trials 36.1 and 39.1 [same positive controls] with Exterm liquid ClO2 showed 19% recovery from the carpet positive controls; showed 206% recovery from the metal positive controls (however, no spores were recovered from any of test coupons after decontamination; positive controls were highly consistent).
   - Trial 36.2 and 39.2 [same positive controls] with Exterm liquid ClO2 showed 10% recovery from the metal positive controls.
   - Trial 30.1 and 33.1 [same positive controls] with pH-amended bleach showed 10% recovery from the glass positive controls and 244% recovery from the metal positive controls.

   These data are included in this report but the deviations are noted in the tables. In the liquid three method comparison testing, the low recoveries noted above have minimal or no impact on the conclusions of the tests. In all cases (except a single test coupon) where no CFU were observed using the quantitative method, no residual culturable spores were recovered from the test coupon

or decontamination solution using the very sensitive qualitative testing. Therefore, even assuming that recoveries from test coupons were correspondingly low, high efficacy results in the quantitative method, e.g., no culturable spores recovered from the coupon, is confirmed by the qualitative tests that show that no culturable spores remained on the coupon.

In the liquid three method comparison testing (Trials 30.1 [metal], 33.1 [metal], 36.1 [metal], and 39.1 [metal]), the high recoveries have minimal impact on the conclusions of the test. In both cases, the results of the duplicate test were similar to the results of the test in which the spore recoveries were higher than the target as a percentage of spores applied. In Trials 36.1 [metal] and 39.1 [metal] (high recoveries) and Trials 36.2 and 39.2 (replicate with low recoveries), the absolute numbers of spores recovered were very similar in the replicate trials; mean log reductions were 7.3 or higher for both replicates. In Trials 30.1 [metal] and 33.1 [metal] (high recoveries) and Trials 30.2 [metal] and 33.2 [metal] (replicate with recoveries in the target range), no viable spores were recovered from any coupon after decontamination and mean log reductions were >6.7.

Ceiling tile in the iSOP testing (fumigation only): recovered spores from ceiling tile were below the target range of 20% in all trials but one; low recoveries ranged from 4% to 18%, typically about 10%. Low recoveries were expected. In the fumigant testing, the test/QA plan included application of ten times more spores on ceiling tile coupons than were applied on the glass and galvanized metal coupons to compensate for the expected lower absolute recoveries. The low recoveries from ceiling tile have minimal impact on the results of the testing.

There was one case in which the extract of the laboratory blank was positive, showing a low level of CFU for the target organism. The trial was not rejected. In this case, a laboratory blank tested positive for contamination with low levels of *Bacillus anthracis* Ames on carpet during decontamination testing with Spor-Klenz® (4/30/09) [Reference Trials 48.1 and 51.1]. There is no impact on the conclusions: the trial was replicated in Trials 48.1 and 51.1 and the results between trials are consistent.

5.0
Test Results

The decontamination evaluations for *B. anthracis* and surrogate spores using AOAC 966.04, AOAC 2008.05, iSOP, and BI (where applicable) are presented below by decontamination technology. The results are discussed as differences in efficacy as measured by the three test methods. The absolute efficacy may or may not be the same for the various test methods. The comparisons of efficacy results from the various test methods should be understood to be a comparison of the measurement of efficacy by the various methods and not the absolute efficacy of the decontamination.

5.1 Sabre ClO_2

All Sabre ClO_2 tests were conducted for 3 hr; the concentration of ClO_2 was varied to produce a range of efficacies.

- **AOAC 966.04:** Test results associated with AOAC 996.04 are presented in Table 5-1. The number of growth-positive tubes generally decreased with increasing ClO_2 concentrations. The number of culture tubes positive for *B. anthracis* on porcelain penicylinder and suture loop was 0 (complete kill) for all culture tubes associated with 3000 ppmv ClO_2, ranged from 1-15 at 300 ppmv ClO_2, and ranged from 14-30 (out of a total of 30 per treatment replicate) at 150 ppmv ClO_2. A similar trend was observed for *B. subtilis* spores on porcelain penicylinder and suture loop with the number of positive culture tubes being 0 (complete kill) at 3000 ppmv ClO_2, ranging from 8-19 at 300 ppmv ClO_2, and ranging from 17-30 at 150 ppmv ClO_2.
- **AOAC 2008.05:** *B. anthracis* and *B. subtilis* spores were tested on glass using AOAC 2008.05 (Table 5-2). Relatively high log reductions (≥6) except for one replicate at 5.0 were observed for all replicate tests at all three ClO_2 concentrations. Complete kill (no viable spores recovered) occurred during all tests with the exception of the 3 hr 150 ppmv ClO_2 test in which *B. anthracis* spores were recovered from one of the trials and viable *B. subtilis* spores were recovered from two trials.
- **iSOP:** The iSOP test results for the ClO_2 fumigation are presented in Table 5-3. *B. anthracis* spores were tested on ceiling tile, galvanized metal, and glass. Complete kill was observed on galvanized metal and glass under all three ClO_2 treatments. Complete kills were observed on ceiling tile after 3 hr of contact time with 3000 ppmv ClO_2; during the150 and 300 ppmv ClO_2 tests, log reductions in recovered *B. anthracis* spores ranged from 2.3 to 4.8 on ceiling tile.
- **BI:** BI results associated with the ClO_2 fumigation indicated that complete kill was obtained only at the 3000 ppmv ClO_2 condition (Table 5-4).

Table 5-1. Decontamination Efficacy of Sabre ClO_2 Using AOAC 966.04

Material / Contact Time, Concentration	Spore / Trial	Mean Carrier Count (log_{10} CFU)*	Culture Tubes Positive for Growth / Total	Estimated Log Reduction*
Porcelain	***B. anthracis***			
Penicylinder	1.1	5.97	0/30	≥7.8
3 hr at	1.2	5.97	0/30	≥7.8
3000 ppmv	1.3	5.97	0/30	≥7.8
	B. subtilis			
	1.1	5.81	0/30	≥7.6
	1.2	5.81	0/30	≥7.6
	1.3	5.81	0/30	≥7.6
Porcelain	***B. anthracis***			
Penicylinder	4.1	5.97	2/30†	7.0
3 hr at	4.2	5.97	7/30†	6.5
300 ppmv	4.3	5.97	9/30†	6.4
	B. subtilis			
	4.1	5.81	8/30‡	6.3
	4.2	5.81	15/30‡	6.0
	4.3	5.81	15/30‡	6.0

Material / Contact Time, Concentration	Spore / Trial	Mean Carrier Count (log_{10} CFU)˙	Culture Tubes Positive for Growth / Total	Estimated Log Reduction*
Porcelain	***B. anthracis***			
Penicylinder	7.1	5.97	30/30†	≤5.4
3 hr at	7.2	5.97	17/30†	6.1
150 ppmv	7.3	5.97	14/30†	6.2
	B. subtilis			
	7.1	5.81	30/30‡	≤5.2
	7.2	5.81	24/30‡	5.6
	7.3	5.81	19/30‡	5.8
Suture Loop	***B. anthracis***			
3 hr at	1.1	5.83	0/30	≥7.6
3000 ppmv	1.2	5.83	0/30	≥7.6
	1.3	5.83	0/30	≥7.6
	B. subtilis			
	1.1	5.75	0/30	≥7.5
	1.2	5.75	0/30	≥7.5
	1.3	5.75	0/30	≥7.5
Suture Loop	***B. anthracis***			
3 hr at	4.1	5.83	1/30†	7.1
300 ppmv	4.2	5.83	15/30†	6.0
	4.3	5.83	13/30†	6.1
	B. subtilis			
	4.1	5.75	20/30‡	5.7
	4.2	5.75	10/30‡	6.1
	4.3	5.75	14/30‡	6.0
Suture Loop	***B. anthracis***			
3 hr at	7.1	5.83	28/30†	5.4
150 ppmv	7.2	5.83	14/30†	6.0
	7.3	5.83	18/30†	5.9
	B. subtilis			
	7.1	5.75	28/30‡	5.4
	7.2	5.75	17/30‡	5.8
	7.3	5.75	18/30‡	5.8

* Log reductions were estimated using the method of Tomasino and Hamilton[6].

† Selected microbe characterizations indicated Gram positive rods with colony morphology of dull, opaque, irregular margin morphology that was consistent with the characterizations of *B. anthracis* positive controls.

‡ Selected microbe characterizations indicated Gram positive rods with colony morphology of round, shiny, raised middle morphology that was consistent with the characterizations of *B. subtilis* positive controls.

˙Spore counts are for a given lot of carriers and were determined at the time the carriers were prepared.

Table 5-2. Decontamination Efficacy of Sabre ClO_2 Using AOAC 2008.05

Material / Contact Time, Concentration	Spore / Trial	Recovered Log Density (CFU)*		Efficacy (Mean Log Reduction)
		Control Carriers †	Test Carriers †	
Glass	***B. anthracis***			
3 hr at	2.1	6.90 ± 0.04	0.70 ± 0.00§,#	≥6.2§
3000 ppmv	2.2	7.07 ± 0.06	0.70 ± 0.00§,#	≥6.4§
	2.3	7.16 ± 0.02	0.70 ± 0.00§,#	≥6.5§
	B. subtilis			
	2.1	6.82 ± 0.05	0.70 ± 0.00§,#	≥6.1§
	2.2	7.13 ± 0.03	0.70 ± 0.00§,#	≥6.4§
	2.3	7.01 ± 0.04	0.70 ± 0.00§,#	≥6.3§
Glass	***B. anthracis***			
3 hr at	5.1	7.55 ± 0.21	0.70 ± 0.00§,#	≥6.8§
300 ppmv	5.2	7.02 ± 0.13	0.70 ± 0.00§,#	≥6.3§
	5.3	7.05 ± 0.04	0.70 ± 0.00§,#	≥6.4§
	B. subtilis			
	5.1	7.04 ± 0.10	0.70 ± 0.00§,#	≥6.3§
	5.2	6.91 ± 0.01	0.70 ± 0.00§,#	≥6.2§
	5.3	7.05 ± 0.02	0.70 ± 0.00§,#	≥6.4§

Material / Contact Time, Concentration	Spore / Trial	Recovered Log Density (CFU)*		Efficacy (Mean Log Reduction)
		Control Carriers †	Test Carriers †	
Glass	***B. anthracis***			
3 hr at	8.1	7.92 ± 0.81	0.70 ± 0.00§,#	≥7.2§
150 ppmv	8.2	6.68 ± 0.06	0.70 ± 0.00§,#	≥6.0§
	8.3	7.02 ± 0.05	0.79 ± 0.15§	≥6.2§
	B. subtilis			
	8.1	7.73 ± 0.22	0.70 ± 0.00§,#	≥7.0§
	8.2	6.27 ± 0.05	1.23 ± 0.44	5.0
	8.3	7.08 ± 0.12	0.97 ± 0.28§	6.1§

* Data are expressed as mean ± standard deviation of three replicates.

† Control carriers are inoculated carriers that did not undergo decontamination.

‡ Test carriers are inoculated carriers that underwent decontamination.

§ Surviving spores were not recovered from one or more carriers; a value of 5 CFU per carrier was used for determining log density and log reduction.

No surviving spores were recovered.

Table 5-3. Decontamination Efficacy of Sabre ClO_2 Using iSOP

Material / Contact Time, Concentration	Spore / Trial	Recovered Log Density (CFU)*		Mean Log Reduction*
		Control Coupons †	Test Coupons †	
Ceiling Tile	***B. anthracis***			
3 hr at	3.1	$6.60 \pm 0.69 \times 10^6$	0.00 ± 0.00§	≥6.8 ± 0.0§
3000 ppmv	3.2	$9.61 \pm 0.75 \times 10^6$	0.00 ± 0.00§	≥7.0 ± 0.0§
	3.3	$3.26 \pm 0.28 \times 10^6$	0.00 ± 0.00§	≥6.5 ± 0.0§
Ceiling Tile	***B. anthracis***			
3 hr at	6.1	$6.88 \pm 1.12 \times 10^6$	$1.96 \pm 1.21 \times 10^3$	3.6 ± 0.2
300 ppmv	6.2	$5.53 \pm 0.49 \times 10^6$	$2.00 \pm 0.88 \times 10^3$	3.5 ± 0.2
	6.3	$4.44 \pm 1.09 \times 10^6$	$8.73 \pm 7.92 \times 10^2$	3.9 ± 0.5
Ceiling Tile	***B. anthracis***			
3 hr at	9.1	$6.35 \pm 1.13 \times 10^6$	$3.12 \pm 0.17 \times 10^4$	2.3 ± 0.0
150 ppmv	9.2	$2.52 \pm 0.85 \times 10^7$	$3.35 \pm 0.53 \times 10^4$	2.9 ± 0.1
	9.3	$1.43 \pm 2.16 \times 10^7$	$3.80 \pm 3.62 \times 10^2$	4.8 ± 0.5
Galvanized Metal	***B. anthracis***			
3 hr at	3.1	$3.42 \pm 0.59 \times 10^6$	0.00 ± 0.00§	≥6.5 ± 0.0§
3000 ppmv	3.2	$3.83 \pm 1.04 \times 10^6$	0.00 ± 0.00§	≥6.6 ± 0.0§
	3.3	$4.03 \pm 1.52 \times 10^6$	0.00 ± 0.00§	≥6.6 ± 0.0§
Galvanized Metal	***B. anthracis***			
3 hr at	6.1	$6.87 \pm 0.36 \times 10^6$	0.00 ± 0.00§	≥5.8 ± 0.0§
300 ppmv	6.2	$3.37 \pm 0.68 \times 10^6$	0.00 ± 0.00§	≥6.5 ± 0.0§
	6.3	$4.23 \pm 0.68 \times 10^6$	0.00 ± 0.00§	≥6.6 ± 0.0§
Galvanized Metal	***B. anthracis***			
3 hr at	9.1	$7.07 \pm 0.96 \times 10^5$	0.00 ± 0.00§	≥5.8 ± 0.0§
150 ppmv	9.2	$3.79 \pm 0.77 \times 10^6$	0.00 ± 0.00§	≥6.6 ± 0.0§
	9.3	$3.42 \pm 0.47 \times 10^6$	0.00 ± 0.00§	≥6.5 ± 0.0§
Glass	***B. anthracis***			
3 hr at	3.1	$3.13 \pm 0.38 \times 10^6$	0.00 ± 0.00§	≥6.5 ± 0.0§
3000 ppmv	3.2	$3.35 \pm 0.47 \times 10^6$	0.00 ± 0.00§	≥6.5 ± 0.0§
	3.3	$3.46 \pm 0.46 \times 10^6$	0.00 ± 0.00§	≥6.5 ± 0.0§
Glass	***B. anthracis***			
3 hr at	6.1	$4.14 \pm 1.15 \times 10^6$	0.00 ± 0.00§	≥6.6 ± 0.0§
300 ppmv	6.2	$2.77 \pm 0.18 \times 10^6$	0.00 ± 0.00§	≥6.4 ± 0.0§
	6.3	$3.37 \pm 0.56 \times 10^6$	0.00 ± 0.00§	≥6.5 ± 0.0§
Glass	***B. anthracis***			
3 hr at	9.1	$3.39 \pm 0.35 \times 10^6$	0.00 ± 0.00§	≥6.5 ± 0.0§
150 ppmv	9.2	$7.06 \pm 1.40 \times 10^6$	0.00 ± 0.00§	≥6.8 ± 0.0§
	9.3	$5.19 \pm 0.29 \times 10^6$	0.00 ± 0.00§	≥6.7 ± 0.0§

* Data are expressed as mean ± standard deviation of five replicates.

† Control coupons are inoculated coupons that did not undergo decontamination.

‡ Test coupons are inoculated coupons that underwent decontamination.

§ Surviving spores were not recovered from one or more coupons; the associated log reductions were based on the $\log_{10}$ of the mean control recovery.

Table 5-4. BI Results Associated with Sabre ClO_2 Decontamination

Material / Contact Time, Concentration	Spore / Trial	Mean Log Reduction*
	B. atrophaeus	
Stainless Steel in Tyvek® Packaging 3 hr at 3000 ppmv	*1.1 – 3.1*	0/5
	1.2 – 3.2	0/5
	1.3 – 3.3	0/5
	B. atrophaeus	
Stainless Steel in Tyvek® Packaging 3 hr at 300 ppmv	*1.1 – 3.1*	4/5
	1.2 – 3.2	2/5
	1.3 – 3.3	3/5
	B. atrophaeus	
Stainless Steel in Tyvek® Packaging 3 hr at 150 ppmv	*1.1 – 3.1*	4/5
	1.2 – 3.2	5/5
	1.3 – 3.3	5/5

The sensitivities of the three test methods are compared based on the percentage of replicate tests using *B. anthracis* spores passing each test method by coupon/carrier material and Sabre ClO_2 decontamination treatment (Table 5-5). The methods most readily passed were iSOP (with galvanized metal and glass) and AOAC 2008.05. Each Sabre ClO_2 decontamination treatment passed the iSOP test with galvanized metal and glass. With AOAC 2008.05, all of the *B. anthracis* replicates passed the test. AOAC 966.04 and iSOP (ceiling tile) had no replicate tests passing the low and moderate efficacy Sabre ClO_2 treatments, but 100% of the replicate tests passed at the high efficacy treatment (3000 ppmv ClO_2).

Table 5-5. Sabre Efficacy Results against *B. anthracis* by Test Method and Material

Contact Time and Concentration	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*				
	AOAC 966.04 *(B. anthracis)*		AOAC 2008.05 *(B. anthracis)*	iSOP *(B. anthracis)*	
	Porcelain Penicylinder	Suture Loop	Glass	Ceiling Tile	Galvanized Metal and Glass
	Sabre ClO_2				
3 hr at 3000 ppmv	100%	100%	100%	100%	100%
3 hr at 300 ppmv	0%	0%	100%	0%	100%
3 hr at 150 ppmv	0%	0%	100%	0%	100%†

* AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 and iSOP require six log reductions to be considered effective (i.e., passing).

† One of the three replicate tests at the moderate and low efficacy levels had a log reduction of only ≥5.8, even though no viable spores were recovered. Replicates with no viable spores recovered, but with log reductions <6.0, were excluded from the calculation of the percent passing the iSOP test. The results shown are based on two replicates of the galvanized metal and three replicates of the glass, each, for the 3 hr at 300 ppmv and the 3 hr at 150 ppmv treatments.

Shown in Table 5-6, use of *B. subtilis* yielded results that were generally similar, but for AOAC 2008.05, more stringent compared to those observed for *B. anthracis*.

The BI results are conservative, matching the most stringent tests with *B. anthracis*: AOAC 966.04 and iSOP with ceiling tile.

Table 5-6. Sabre Efficacy Results against *Bacillus* Surrogates by Test Method and Material

Contact Time and Concentration	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*			
	AOAC 966.04 *(B. subtilis)*		AOAC 2008.05 *(B. subtilis)*	BI *(B. atrophaeus)*
	Porcelain Penicylinder	Suture Loop	Glass	Stainless Steel in Tyvek® Packaging
	Sabre ClO_2			
3 hr at 3000 ppmv	100%	100%	100%	100%
3 hr at 300 ppmv	0%	0%	100%	0%
3 hr at 150 ppmv	0%	0%	67%	0%

*AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 requires six log reductions to be considered effective (i.e., passing).

Figure 5-1 provides a graphical comparison of the mean log reduction in *B. anthracis* spores surviving on test carriers/coupons from all three methods at the three efficacy levels. Except for iSOP with ceiling tile the results are very similar.

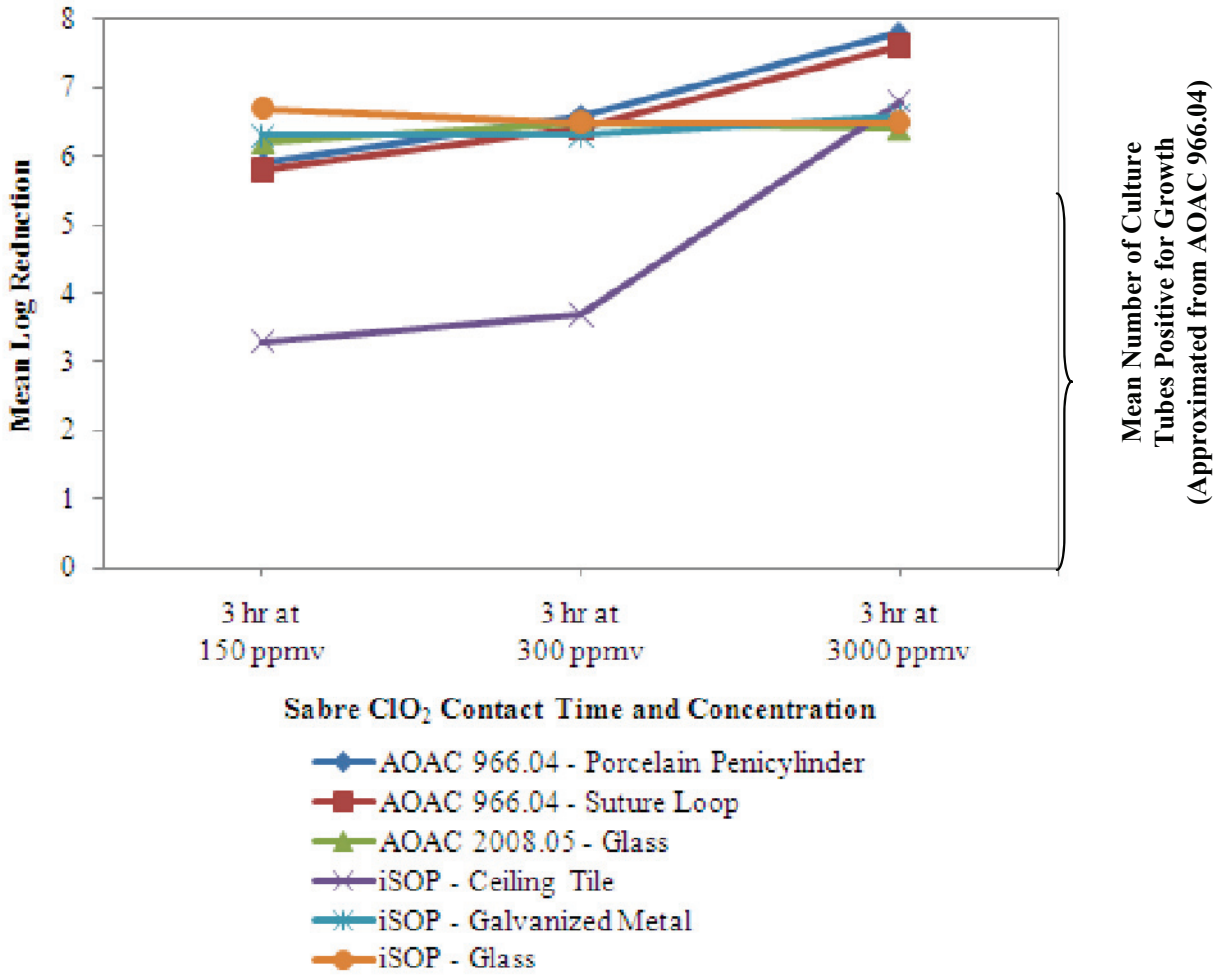

Figure 5-1. Summary of Sabre ClO_2 decontamination efficacy against *B. anthracis* spores.

Key observations:

- All methods, with all materials, showed complete kill of spores at the high treatment levels; with the AOAC 966.04 and iSOP (ceiling tile), lower levels of efficacy were observed with the moderate and low efficacy treatments; iSOP (galvanized metal and glass) and AOAC 2008.05 were not sensitive to the lower efficacy treatments
- AOAC 966.04 with both carriers was the most difficult of the methods to pass (even though only half the number of carriers required for the full test were used (30 carriers instead of 60 carriers of each material type)
- Moderate and low efficacy treatments would pass the AOAC 2008.05 and iSOP tests (get registration claim) for hard, non-porous for, but would not pass the more stringent AOAC 966.04
- Use of *B. subtilis* yielded results very similar to, and at least as stringent as, those observed for *B. anthracis* with the AOAC 966.04 or AOAC 2008.05 tests
- Use of *B. atrophaeus* BIs yielded results identical to AOAC 966.04 and iSOP with ceiling tile, and was more conservative than AOAC 2008.05 or iSOP with hard, nonporous coupons
- The choice of coupon materials selected for the iSOP method impacts the stringency of the test.

5.2 STERIS HP

All STERIS HP tests were conducted at an HP concentration of 250 ppmv; the contact time in the sterilization phase of the cycle was varied (90, 30, or 0 min) to produce a range of treatment levels.

- **AOAC 966.04:** Test results associated with AOAC 996.04 are presented in Table 5-7. The number of growth-positive tubes generally decreased with increasing contact time. The number of culture tubes positive for *B. anthracis* on porcelain penicylinder and suture loop ranged from 0 (complete kill)-1 (out of a total of 30 per trial replicate) for all culture tubes associated with a 90 min contact time; ranged from 6-14 at 30-min contact time, and 14-26 (out of a total of 30 per trial replicate) with no contact time after the target concentration was reached ("0 min"). A similar trend was observed for *B. subtilis* spores on porcelain penicylinder and suture loop with the number of positive culture tubes ranging from 0 (complete kill)-1 for all culture tubes associated with a 90 min contact time; ranged from 5-13 at 30-min contact time, and 13-30 (out of a total of 30 per trial replicate) with no contact time after the target concentration was reached ("0 min").
- **AOAC 2008.05:** *B. anthracis* and *B. subtilis* spores were tested on glass using AOAC 2008.05 (Table 5-8). High log reductions (≥6) were observed for all replicate tests with a 90-min contact time; log reductions ranged from 5.1- ≥6.2 for all replicate tests with a 30-min contact time; and log reductions ranged from 4.6- ≥6.4 for all replicate tests with a 0-min contact time (aeration phase begins immediately after target concentration level was reached). Efficacies for *B. anthracis* and *B. subtilis* spores were very similar across the range of treatments tested.
- **iSOP:** The iSOP test results of the ClO2 fumigation are presented in Table 5-9. *B. anthracis* spores were tested on ceiling tile, galvanized metal, and glass. Complete kill was observed on ceiling tile, galvanized metal, and glass with 90-min contact time. Complete kill was observed for both galvanized metal and glass with 30-min contact times; ceiling tile log reductions ranged from a 5.2 to ≥6.9 (complete kill) with 30-min contact time. With 0-min contact time (aeration phase begins immediately after target concentration level was reached), log reductions ranged from 4.4 to ≥7.8 (complete kill).
- **BI:** BI results associated with the STERIS HP fumigation indicated that complete kill did not occur, even following the 90-min contact time with 250 ppmv HP (Table 5-10).

Table 5-7. Decontamination Efficacy of STERIS HP Using AOAC 966.04

Material / Contact Time, Concentration	Spore / Trial	Mean Carrier Count (log_{10} CFU)[#]	Culture Tubes Positive for Growth / Total	Estimated Log Reduction*
Porcelain	***B. anthracis***			
Penicylinder	16.1	5.88	1/30	7.2
90 min at	16.2	5.88	0/30	≥7.7
250 ppmv	16.3	5.88	0/30	≥7.7
	B. subtilis			
	16.1	5.70	0/30	≥7.5
	16.2	5.70	0/30	≥7.5
	16.3	5.70	0/30	≥7.5
Porcelain	***B. anthracis***			
Penicylinder	13.1	5.88	14/30‡	6.1
30 min at	13.2	5.88	7/30‡	6.4
250 ppmv	13.3	5.88	7/30‡	6.4
	B. subtilis			
	13.1	5.70	13/30§	5.9
	13.2	5.70	6/30§	6.3
	13.3	5.70	5/30§	6.4
Porcelain	***B. anthracis***			
Penicylinder	10.1	5.88	18/30‡	5.9
0 min at	10.2	5.88	14/30‡	6.1
250 ppmv†	10.3	5.88	19/30‡	5.9
	B. subtilis			
	10.1	5.70	13/30§	5.9
	10.2	5.70	20/30§	5.7
	10.3	5.70	18/30§	5.7
Suture Loop	***B. anthracis***			
90 min at	10.1	5.64	1/30	6.9
250 ppmv	10.2	5.64	0/30	≥7.4
	10.3	5.64	0/30	≥7.4
	B. subtilis			
	10.1	5.51	1/30	6.8
	10.2	5.51	0/30	≥7.3
	10.3	5.51	0/30	≥7.3
Suture Loop	***B. anthracis***			
30 min at	13.1	5.64	10/30‡	6.0
250 ppmv	13.2	5.64	6/30‡	6.3
	13.3	5.64	9/30‡	6.1
	B. subtilis			
	13.1	5.51	9/30§	5.9
	13.2	5.51	9/30§	5.9
	13.3	5.51	11/30§	5.8
Suture Loop	***B. anthracis***			
0 min at	16.1	5.64	18/30‡	5.7
250 ppmv†	16.2	5.64	20/30‡	5.6
	16.3	5.64	26/30‡	5.4
	B. subtilis			
	16.1	5.51	30/30§	≤4.9
	16.2	5.51	28/30§	5.1
	16.3	5.51	29/30§	5.0

* Log reductions were estimated using the method of Tomasino and Hamilton[6].

† Test carriers were exposed to HP during conditioning. Once the target HP concentration was attained the "contact time" was ended.

‡ Selected microbe characterizations indicated Gram positive rods with colony morphology of dull, opaque, irregular margin morphology that were consistent with the characterizations of *B. anthracis* positive controls.

§ Selected microbe characterizations indicated Gram positive rods with colony morphology of round, shiny, raised middle morphology that were consistent with the characterizations of *B. subtilis* positive controls.

#Spore counts are for a given lot of carriers and were determined at the time the carriers were prepared.

Table 5-8. Decontamination Efficacy of STERIS HP Using AOAC 2008.05

Material / Contact Time, Concentration	Spore / Trial	Recovered Log Density (CFU)*		Mean Log Reduction
		Control Carriers†	Test Carriers‡	
Glass	***B. anthracis***			
90 min at	11.1	6.92 ± 0.07	0.70 ± 0.00#,¶	≥6.2#
250 ppmv	11.2	6.66 ± 0.03	0.70 ± 0.00#,¶	≥6.0#
	11.3	7.08 ± 0.07	0.70 ± 0.00#,¶	≥6.4#
	B. subtilis			
	11.1	7.22 ± 0.02	0.70 ± 0.00#,¶	≥6.5#
	11.2	6.70 ± 0.15	0.70 ± 0.00#,¶	≥6.0#
	11.3	7.00 ± 0.03	0.70 ± 0.00#,¶	≥6.3#
Glass	***B. anthracis***			
30 min at	14.1	6.59 ± 0.04	0.70 ± 0.00#,¶	≥5.9#
250 ppmv	14.2	6.66 ± 0.10	1.31 ± 0.60	5.4
	14.3	6.76 ± 0.12	0.96 ± 0.45#	5.8#
	B. subtilis			
	14.1	6.51 ± 0.22	1.36 ± 0.33	5.1
	14.2	6.69 ± 0.17	0.80 ± 0.17#	5.9#
	14.3	6.93 ± 0.18	0.70 ± 0.00#,¶	≥6.2#
Glass	***B. anthracis***			
0 min at	17.1	7.14 ± 0.05	0.70 ± 0.00#,¶	≥6.4#
250 ppmv§	17.2	7.04 ± 0.04	0.70 ± 0.00#,¶	≥6.3#
	17.3	6.93 ± 0.06	2.38 ± 0.13	4.6
	B. subtilis			
	17.1	7.16 ± 0.14	0.87 ± 0.15#	6.3#
	17.2	7.14 ± 0.02	0.70 ± 0.00#,¶	≥6.4#
	17.3	6.90 ± 0.06	2.08 ± 0.42	4.8

* Data are expressed as mean ± standard deviation of three replicates.

† Control carriers are inoculated carriers that did not undergo decontamination.

‡ Test carriers are inoculated carriers that underwent decontamination.

§ Test carriers were exposed to HP during conditioning. Once the target HP concentration was attained the "contact time" was ended.

Surviving spores were not recovered from one or more carriers; a value of 5 CFU per carrier was used for determining log density and log reduction.

¶ No surviving spores were recovered.

Table 5-9. Decontamination Efficacy of STERIS HP Using iSOP

Material / Contact Time, Concentration	Spore / Trial	Recovered Log Density (CFU)*		Efficacy (Mean Log Reduction)*
		Control Coupons†	Test Coupons‡	
Ceiling Tile	***B. anthracis***			
90 min at	12.1	$9.31 \pm 0.81 \times 10^6$	0.00 ± 0.00#	≥7.0 ± 0.0#
250 ppmv	12.2	$4.33 \pm 2.24 \times 10^7$	0.00 ± 0.00#	≥7.6 ± 0.0#
	12.3	$3.87 \pm 0.96 \times 10^6$	0.00 ± 0.00#	≥6.6 ± 0.0#
Ceiling Tile	***B. anthracis***			
30 min at	15.1	$8.33 \pm 1.77 \times 10^7$	0.00 ± 0.00#	≥6.9 ± 0.0#
250 ppmv	15.2	$9.30 \pm 0.70 \times 10^6$	$2.93 \pm 3.57 \times 10^{2}$#	5.2 ± 1.2#
	15.3	$8.07 \pm 1.64 \times 10^6$	0.00 ± 0.00#	≥6.9 ± 0.0#
Ceiling Tile	***B. anthracis***			
0 min at	18.1	$9.20 \pm 2.42 \times 10^6$	$2.33 \pm 1.51 \times 10^2$	4.7 ± 0.3
250 ppmv§	18.2	$8.85 \pm 0.98 \times 10^6$	$5.32 \pm 6.05 \times 10^{1}$#	5.8 ± 1.1#
	18.3	$8.20 \pm 0.75 \times 10^6$	$4.20 \pm 2.39 \times 10^2$	4.4 ± 0.3
Galvanized Metal¶	***B. anthracis***			
90 min at	12.1	$5.42 \pm 0.64 \times 10^{7}$¶	0.00 ± 0.00#	≥7.7 ± 0.0#
250 ppmv	12.2	$4.90 \pm 1.31 \times 10^{7}$¶	0.00 ± 0.00#	≥7.7 ± 0.0#
	12.3	$5.56 \pm 1.23 \times 10^{7}$¶	0.00 ± 0.00#	≥7.7 ± 0.0#

Material / Contact Time, Concentration	Spore / Trial	Recovered Log Density (CFU)*		Efficacy (Mean Log Reduction)*
		Control Coupons†	Test Coupons‡	
Galvanized Metal¶	***B. anthracis***			
30 min at	15.1	$4.36 \pm 1.25 \times 10^{7}$¶	0.00 ± 0.00#	≥7.6 ± 0.0#
250 ppmv	15.2	$6.62 \pm 2.03 \times 10^{7}$¶	0.00 ± 0.00#	≥7.8 ± 0.0#
	15.3	$4.26 \pm 1.79 \times 10^{7}$¶	0.00 ± 0.00#	≥7.6 ± 0.0#
Galvanized Metal¶	***B. anthracis***			
0 min at	18.1	$4.11 \pm 0.98 \times 10^{7}$¶	0.00 ± 0.00#	≥7.6 ± 0.0#
250 ppmv§	18.2	$4.74 \pm 1.03 \times 10^{7}$¶	0.00 ± 0.00#	≥7.7 ± 0.0#
	18.3	$4.76 \pm 0.88 \times 10^{7}$¶	$2.66 \pm 4.34 \times 10^{1}$#	7.0 ± 1.0#
Glass	***B. anthracis***			
90 min at	12.1	$6.89 \pm 2.71 \times 10^{6}$	0.00 ± 0.00#	≥6.8 ± 0.0#
250 ppmv	12.2	$2.89 \pm 1.10 \times 10^{6}$	0.00 ± 0.00#	≥6.5 ± 0.0#
	12.3	$5.26 \pm 1.74 \times 10^{6}$	0.00 ± 0.00#	≥6.7 ± 0.0#
Glass	***B. anthracis***			
30 min at	15.1	$7.03 \pm 1.66 \times 10^{6}$	0.00 ± 0.00#	≥6.8 ± 0.0#
250 ppmv	15.2	$6.13 \pm 1.90 \times 10^{6}$	0.00 ± 0.00#	≥6.8 ± 0.0#
	15.3	$4.56 \pm 2.44 \times 10^{6}$	0.00 ± 0.00#	≥6.7 ± 0.0#
Glass	***B. anthracis***			
0 min at	18.1	$3.57 \pm 0.61 \times 10^{6}$	$2.00 \pm 4.47 \times 10^{1}$#	6.2 ± 0.9#
250 ppmv§	18.2	$3.27 \pm 0.19 \times 10^{6}$	0.00 ± 0.00#	≥6.5 ± 0.0#
	18.3	$3.31 \pm 0.21 \times 10^{6}$	$6.66 \pm 5.27 \times 10^{1}$#	5.0 ± 0.9#

* Data are expressed as mean ± standard deviation of five replicates.

† Control carriers are inoculated carriers that did not undergo decontamination.

‡ Test carriers are inoculated carriers that underwent decontamination.

§ Test carriers were exposed to HP during conditioning. Once the target HP concentration was attained the "contact time" was ended.

Surviving spores were not recovered from one or more coupons; the associated log reductions were based on the log10 of the mean control recovery.

¶ The spores applied to the coupons were higher than the target range. Spores applied were in the range of 8.8×10^{7} - 9.8×10^{7}.

Table 5-10. BI Results Associated with STERIS HP Decontamination

Material / Contact Time, Concentration	Spore / Trial	BIs Positive for Growth / Total
Stainless Steel in Tyvek® Packaging	***B. atrophaeus***	
90 min at	10.1 -12.1	3/5
250 ppmv	10.2 -12.2	3/5
	10.3 -12.3	2/5
Stainless Steel in Tyvek® Packaging	***B. atrophaeus***	
30 min at	13.1 – 15.1	3/5
250 ppmv	13.2 – 15.2	3/5
	13.3 – 15.3	2/5
Stainless Steel in Tyvek® Packaging	***B. atrophaeus***	
0 min at	16.1 – 18.1	4/5
250 ppmv*	16.2 – 18.2	5/5
	16.3 – 18.3	4/5

* Test carriers were exposed to HP during conditioning. Once the target HP concentration was attained the "contact time" was ended.

The sensitivities of the three test methods (plus the BIs) were compared based on the percentage of replicate tests passing each test method by material at each STERIS HP decontamination treatment (Table 5-11 and Table 5-12). At the highest efficacy treatment (90-min contact time) using *B. anthracis* spores, 100% of the iSOP and AOAC 2008.05 replicate tests passed, 67% of the AOAC 966.04 replicate tests passed, and none of the BI replicate tests passed.

At the moderate efficacy treatment (30-min contact time) using *B. anthracis* spores, none of the AOAC 966.04 or AOAC 2008.05 passed (although one of the

AOAC 2008.05 replicate tests is [not included] had a ≥5.9 log reduction with no viable spores recovered). At the low efficacy treatment level, 67% of the AOAC 2008.05 replicate tests passed; none of the AOAC 966.04 replicate tests passed, and none of the BI replicate tests passed.

At the low STERIS HP efficacy treatment, partial efficacy was generally observed with AOAC 2008.05 and iSOP, but none of the replicate tests passed the efficacy test associated with AOAC 966.04.

The BI results are conservative, with no replicate trial passing at any of the treatments.

Use of *B. subtilis* yielded results that were not significantly different from those observed for *B. anthracis* at the moderate and low efficacy levels; no spores of either organism were recovered after the high efficacy treatment.

Table 5-11. STERIS HP Efficacy Results against *B. anthracis* by Test Method and Material

Contact Time and Concentration	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*				
	AOAC 966.04 *(B. anthracis)*		AOAC 2008.05 *(B. anthracis)*	iSOP *(B. anthracis)*	
	Porcelain Penicylinder	Suture Loop	Glass	Ceiling Tile	Galvanized Metal and Glass
STERIS HP					
Gassing phase up to 250 ppmv + 1.5 hr at 250 ppmv	67%	67%	100%	100%	100%
Gassing phase up to 250 ppmv + 0.5 hr at 250 ppmv	0%	0%	0%†	67%	100%
Gassing phase up to 250 ppmv followed by aeration (no dwell time)	0%	0%	67%	0%	83%‡

* AOAC 966.04 and BI require 100% kill and AOAC 2008.05 and iSOP require six log reductions to be considered effective (i.e., passing).

† One of the three replicate tests at the moderate efficacy level had a log reduction of only ≥5.9, even though no viable spores were recovered. Tests with log reductions <6.0 were excluded from the calculation of the percent passing the test, even though no viable spores were recovered. Results shown are based on two replicates for the moderate efficacy level treatment.

‡The single test that was not passed was glass which contained the correct spore application; one galvanized metal coupon that had a higher than target spore application also had a small number (27) of spores recovered, comparable to a glass coupon (Trial 18.1 with 20 spores recovered) that passed the test.

Table 5-12. STERIS HP Efficacy Results against *Bacillus* Surrogates by Test Method and Material

Contact Time and Concentration	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*			
	AOAC 966.04 *(B. subtilis)*		AOAC 2008.05 *(B. subtilis)*	BI *(B. atrophaeus)*
	Porcelain Penicylinder	Suture Loop	Glass	Stainless Steel in Tyvek® Packaging
STERIS HP				
Gassing phase up to 250 ppmv + 1.5 hr at 250 ppmv	100%	67%	100%	0%
Gassing phase up to 250 ppmv + 0.5 hr at 250 ppmv	0%	0%	33%	0%
Gassing phase up to 250 ppmv followed by aeration (no dwell time)	0%	0%	67%	0%

* AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 requires six log reductions to be considered effective (i.e., passing).

- Figure 5-2 provides a graphical comparison of the mean log reduction in *B. anthracis* spores surviving on test carriers/coupons from all three methods at the three efficacy levels. The results for the various test methods are very similar.

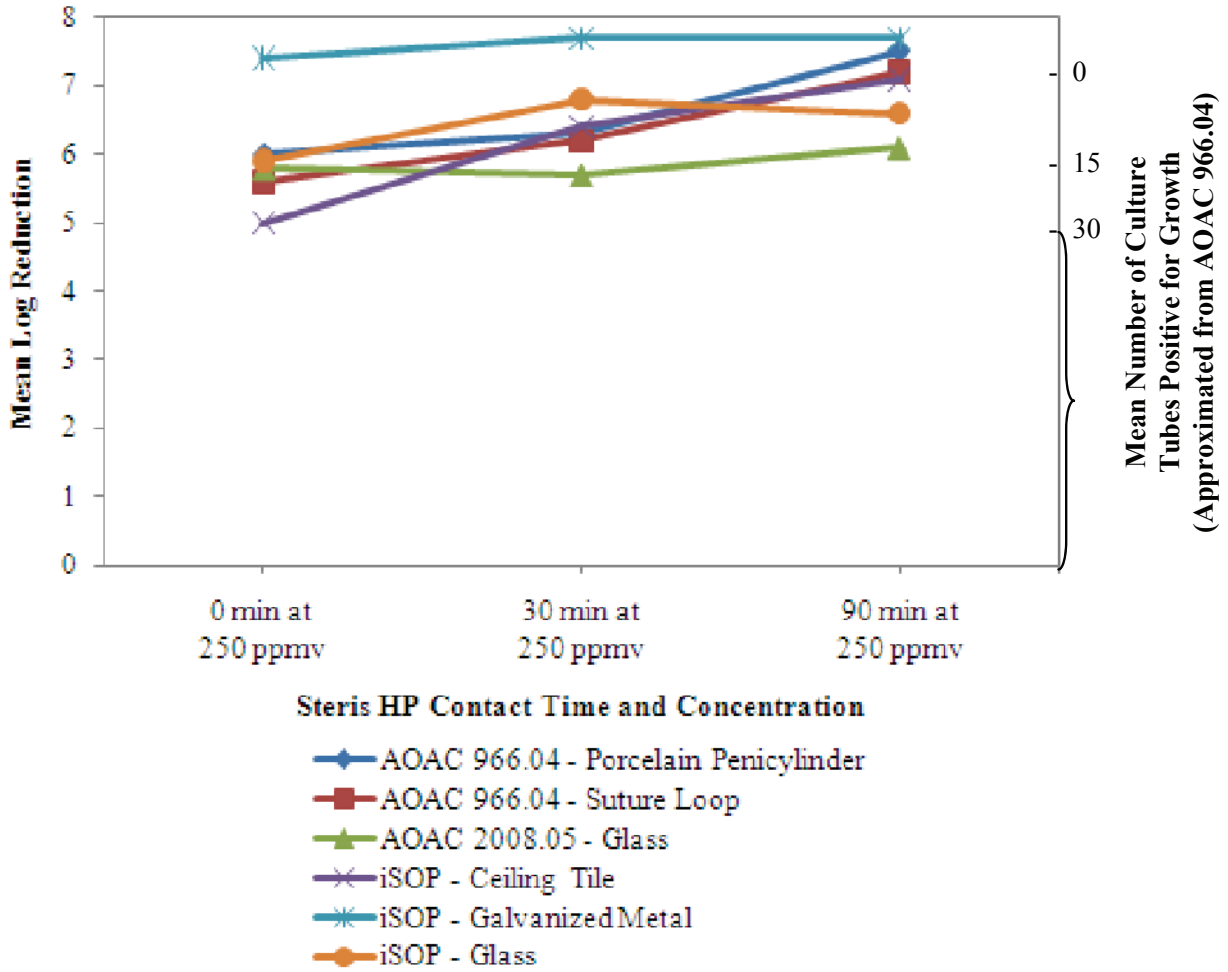

Figure 5-2. Summary of STERIS HP decontamination efficacy against *B. anthracis* spores.

Key observations:

- AOAC 966.04 was the most stringent of the test methods for hard, nonporous materials
- One or more replicates of the low efficacy treatments pass the AOAC 2008.05 or iSOP on hard, porous surfaces that would meet registration requirement; AOAC 966.04 did not meet the requirement for registration in any replicate test at either the moderate or low efficacy levels
- Use of *B. subtilis* yielded results not significantly different from those observed for *B. anthracis* with the AOAC 2008.05 tests
- *B. atrophaeus* BIs were more conservative, harder to pass, than all other methods used
- The choice of coupon materials selected for the iSOP method impacts the stringency of the test.

5.3 Methyl Bromide

The MeBr concentration used for all MeBr tests was 211 mg/L. The contact time was reduced from 18 hours to 9 hours for the moderate efficacy treatment, and the temperature was reduced from 37 °C to 25 °C (at 9 hr contact time) for the low efficacy treatment.

- **AOAC 966.04:** Test results associated with AOAC 996.04 are presented in Table **5-13**. MeBr was ineffective (30 of 30 culture tubes were positive for growth per in each moderate and low efficacy treatment) against *B. anthracis* and *B. subtilis* spores at every test condition except against *B. anthracis* spores at the 18-hr contact time at 211 mg/L and 37 °C (the highest decontamination treatment tested). For *B. anthracis*, the ratio of positive culture tubes at the high efficacy treatment ranged from 4/30 to

11/30 on porcelain penicylinder and 0/30 to 1/30 on suture loop after the 18-hr contact time. MeBr was ineffective against *B. subtilis* spores at the high efficacy treatment (30 of 30 culture tubes, in each replicate and both penicylinders and suture loops, were positive for growth).

- **AOAC 2008.05:** *B. anthracis* and *B. subtilis* spores were tested on glass using AOAC 2008.05 (Table 5-14). Relatively low log reductions (≤2.7) were observed for all tests, except for *B. anthracis* exposed for the 18-hr contact time at 211 mg/L and 37 °C, which had log reductions ≥5.9; with the high efficacy treatment *B. subtilis* spores showed a range of log reductions of 1.5 – 2.0.
- **iSOP:** The iSOP test results for the MeBr fumigation are presented in Table 5-15. *B. anthracis* spores were tested on ceiling tile, galvanized metal, and glass. Relatively low log reductions (≤3.0) were observed for all replicate tests at the moderate and low efficacy levels (9 hr at 211 mg/L and 37 °C and 9 hr at 211 mg/L and 25 °C). Log reduction ≥6.4 and complete kill (no viable spores recovered from any of the three materials) were observed against *B. anthracis* spores on all three materials with the 18-hr contact time at 211 mg/L and 37 °C.
- **BI:** BI results associated with the MeBr fumigation testing indicated that complete kill did not occur on any BI under any of the MeBr test conditions (Table 5-16).

Table 5-13. Decontamination Efficacy of MeBr Using AOAC 966.04

Material / Contact Time, Concentration	Spore / Trial	Mean Carrier Count (log_{10} CFU)[‘]	Culture Tubes Positive for Growth / Total	Estimated Log Reduction[*]
Porcelain	*B. anthracis*			
Penicylinder	22.1	5.95	4/30[†]	6.8
18 hr at	22.2	5.95	11/30[†]	6.3
211 mg/L, 37 °C	22.3	5.95	6/30[†]	6.6
	B. subtilis			
	22.1	5.73	30/30[‡]	≤5.1
	22.2	5.73	30/30[‡]	≤5.1
	22.3	5.73	30/30[‡]	≤5.1
Porcelain	*B. anthracis*			
Penicylinder	25.1	5.93	30/30[†]	≤5.3
9 hr at	25.2	5.93	30/30[†]	≤5.3
211 mg/L, 37 °C	25.3	5.93	30/30[†]	≤5.3
	B. subtilis			
	25.1	5.78	30/30[‡]	≤5.2
	25.2	5.78	30/30[‡]	≤5.2
	25.3	5.78	30/30[‡]	≤5.2
Porcelain	*B. anthracis*			
Penicylinder	19.1	5.93	30/30[†]	≤5.3
9 hr at	19.2	5.93	30/30[†]	≤5.3
211 mg/L, 25 °C	19.3	5.93	30/30[†]	≤5.3
	B. subtilis			
	19.1	5.78	30/30[‡]	≤5.2
	19.2	5.78	30/30[‡]	≤5.2
	19.3	5.78	30/30[‡]	≤5.2
Suture Loop	*B. anthracis*			
18 hr at	22.1	5.69	0/30	≥7.5
211 mg/L, 37 °C	22.2	5.69	0/30	≥7.5
	22.3	5.69	1/30[†]	7.0
	B. subtilis			
	22.1	5.56	30/30[‡]	≤5.0
	22.2	5.56	30/30[‡]	≤5.0
	22.3	5.56	30/30[‡]	≤5.0

Material / Contact Time, Concentration	Spore / Trial	Mean Carrier Count (log_{10} CFU)˙	Culture Tubes Positive for Growth / Total	Estimated Log Reduction*
Suture Loop	*B. anthracis*			
9 hr at	25.1	5.69	30/30†	≤5.1
211 mg/L, 37 °C	25.2	5.69	30/30†	≤5.1
	25.3	5.69	30/30†	≤5.1
	B. subtilis			
	25.1	5.54	30/30‡	≤4.9
	25.2	5.54	30/30‡	≤4.9
	25.3	5.54	30/30‡	≤4.9
Suture Loop	*B. anthracis*			
9 hr at	19.1	5.69	30/30†	≤5.1
211 mg/L, 25 °C	19.2	5.69	30/30†	≤5.1
	19.3	5.69	30/30†	≤5.1
	B. subtilis			
	19.1	5.54	30/30‡	≤4.9
	19.2	5.54	30/30‡	≤4.9
	19.3	5.54	30/30†	≤4.9

* Log reductions were estimated using the method of Tomasino and Hamilton[6].

† Selected microbe characterizations indicated Gram positive rods with colony morphology of dull, opaque, irregular margin morphology consistent with the characterizations of *B. anthracis* positive controls.

‡ Selected microbe characterizations indicated Gram positive rods with colony morphology of round, shiny, raised middle morphology that was consistent with the characterizations of *B. subtilis* positive controls.

˙Spore counts are for a given lot of carriers and were determined at the time the carriers were prepared.

Table 5-14. Decontamination Efficacy of MeBr Using AOAC 2008.05

Material / Contact Time, Concentration	Spore / Trial	Recovered Log Density (CFU)*		Mean Log Reduction
		Control Carriers†	Test Carriers‡	
Glass	***B. anthracis***			
18 hr at	23.1	7.18 ± 0.06	0.70 ± 0.00§, #	6.5§
211 mg/L, 37 °C	23.2	6.80 ± 0.11	0.70 ± 0.00§, #	6.1§
	23.3	6.87 ± 0.12	0.96 ± 0.45§	5.9§
	B. subtilis			
	23.1	6.98 ± 0.03	5.38 ± 0.02	1.6
	23.2	6.88 ± 0.20	4.87 ± 0.16	2.0
	23.3	6.74 ± 0.07	5.28 ± 0.14	1.5
Glass	***B. anthracis***			
9 hr at	26.1	6.96 ± 0.10	4.28 ± 0.02	2.7
211 mg/L, 37 °C	26.2	6.81 ± 0.07	4.11 ± 0.02	2.7
	26.3	6.73 ± 0.07	4.09 ± 0.04	2.6
	B. subtilis			
	26.1	6.94 ± 0.14	5.06 ± 0.07	1.9
	26.2	6.78 ± 0.10	4.75 ± 0.10	2.0
	26.3	6.91 ± 0.08	5.18 ± 0.04	1.7
G***lass***	***B. anthracis***			
9 hr at	20.1	6.97 ± 0.04	5.72 ± 0.03	1.3
211 mg/L, 25 °C	20.2	6.74 ± 0.06	6.58 ± 0.02	0.2
	20.3	6.88 ± 0.03	5.61 ± 0.03	1.3
	B. subtilis			
	20.1	7.27 ± 0.07	5.63 ± 0.03	1.6
	20.2	6.74 ± 0.10	6.77 ± 0.06	0.0
	20.3	6.84 ± 0.14	6.72 ± 0.12	0.1

* Data are expressed as mean ± standard deviation of three replicates.

† Control carriers are inoculated carriers that did not undergo decontamination.

‡ Test carriers are inoculated carriers that underwent decontamination.

§ Surviving spores were not recovered from one or more carriers; a value of 5 CFU per carrier was used for determining log density and log reduction.

No surviving spores were recovered.

Table 5-15. Decontamination Efficacy of MeBr Using iSOP

Material / Contact Time, Concentration	Spore / Trial	Recovered Spores (CFU)*		Efficacy (Mean Log Reduction)*
		Control Coupons†	Test Coupons‡	
Ceiling Tile	***B. anthracis***			
18 hr at	24.1	$1.63 \pm 0.72 \times 10^7$	0.00 ± 0.00§	≥7.2 ± 0.0§
211 mg/L, 37 °C	24.2	$2.34 \pm 0.99 \times 10^7$	0.00 ± 0.00§	≥7.4 ± 0.0§
	24.3	$2.45 \pm 1.12 \times 10^7$	0.00 ± 0.00§	≥7.4 ± 0.0§
Ceiling Tile	***B. anthracis***			
9 hr at	27.1	$2.87 \pm 0.20 \times 10^7$	$2.81 \pm 0.26 \times 10^4$	3.0 ± 0.0
211 mg/L, 37 °C	27.2	$3.00 \pm 0.32 \times 10^7$	$3.22 \pm 0.49 \times 10^4$	3.0 ± 0.1
	27.3	$3.06 \pm 1.40 \times 10^7$	$3.93 \pm 0.57 \times 10^4$	2.9 ± 0.1
Ceiling Tile	***B. anthracis***			
9 hr at	21.1	$3.14 \pm 0.57 \times 10^6$	$5.45 \pm 1.99 \times 10^6$	0.8 ± 0.2
211 mg/L, 25 °C	21.2	$3.18 \pm 0.29 \times 10^7$	$5.22 \pm 1.20 \times 10^6$	0.8 ± 0.1
	21.3	$7.23 \pm 0.90 \times 10^7$	$4.63 \pm 0.90 \times 10^6$	1.2 ± 0.1
Galvanized Metal	***B. anthracis***			
18 hr at	24.1	$5.29 \pm 2.63 \times 10^6$	0.00 ± 0.00§	≥6.7 ± 0.0§
211 mg/L, 37 °C	24.2	$6.48 \pm 1.79 \times 10^6$	0.00 ± 0.00§	≥6.8 ± 0.0§
	24.3	$5.97 \pm 2.28 \times 10^6$	0.00 ± 0.00§	≥6.8 ± 0.0§
Galvanized Metal	***B. anthracis***			
9 hr at	27.1	$5.75 \pm 0.55 \times 10^6$	$5.10 \pm 1.32 \times 10^4$	2.1 ± 0.1
211 mg/L, 37 °C	27.2	$6.79 \pm 1.00 \times 10^6$	$6.22 \pm 1.19 \times 10^4$	2.0 ± 0.1
	27.3	$5.20 \pm 0.80 \times 10^6$	$5.81 \pm 0.73 \times 10^4$	2.0 ± 0.1
Galvanized Metal	***B. anthracis***			
9 hr at	21.1	$8.83 \pm 0.87 \times 10^6$	$1.69 \pm 0.15 \times 10^6$	0.7 ± 0.0
211 mg/L, 25 °C	21.2	$7.13 \pm 0.73 \times 10^6$	$9.37 \pm 0.99 \times 10^5$	0.9 ± 0.0
	21.3	$6.32 \pm 1.37 \times 10^6$	$3.85 \pm 0.90 \times 10^6$	0.2 ± 0.1
Glass	***B. anthracis***			
18 hr at	24.1	$2.30 \pm 0.92 \times 10^6$	0.00 ± 0.00§	≥6.4 ± 0.0§
211 mg/L, 37 °C	24.2	$5.37 \pm 1.07 \times 10^6$	0.00 ± 0.00§	≥6.7 ± 0.0§
	24.3	$3.49 \pm 0.51 \times 10^6$	0.00 ± 0.00§	≥6.5 ± 0.0§
Glass	***B. anthracis***			
9 hr at	27.1	$6.59 \pm 0.39 \times 10^6$	$1.00 \pm 0.12 \times 10^4$	2.8 ± 0.0
211 mg/L, 37 °C	27.2	$7.42 \pm 0.43 \times 10^6$	$2.33 \pm 0.60 \times 10^4$	2.5 ± 0.1
	27.3	$6.93 \pm 1.51 \times 10^6$	$7.03 \pm 3.32 \times 10^3$	3.0 ± 0.2
Glass	***B. anthracis***			
9 hr at	21.1	$6.23 \pm 1.07 \times 10^6$	$6.97 \pm 0.80 \times 10^5$	1.0 ± 0.0
211 mg/L, 25 °C	21.2	$8.79 \pm 1.27 \times 10^6$	$6.43 \pm 0.37 \times 10^5$	1.1 ± 0.0
	21.3	$6.38 \pm 0.52 \times 10^6$	$2.72 \pm 0.87 \times 10^5$	1.4 ± 0.2

* Data are expressed as mean ± standard deviation of five replicates.

† Control carriers are inoculated carriers that did not undergo decontamination.

‡ Test carriers are inoculated carriers that underwent decontamination.

§ Surviving spores were not recovered from one or more coupons; the associated log reductions were based on the $\log_{10}$ of the mean control recovery.

Table 5-16. BI Results Associated with MeBr Decontamination

Material / Contact Time, Concentration	Spore / Trial	BIs Positive for Growth / Total
Stainless Steel in Tyvek® Packaging	B. atrophaeus	
18 hr at	22.1 – 24.1	5/5
211 mg/L, 37 °C	22.2 – 24.2	5/5
	22.3 – 24.3	5/5
Stainless Steel in Tyvek® Packaging	B. atrophaeus	
9 hr at	25.1 – 27.3	5/5
211 mg/L, 37 °C	25.2 – 27.3	5/5
	25.3 – 27.3	5/5
Stainless Steel in Tyvek® Packaging	B. atrophaeus	
9 hr at	19.1 – 21.1	5/5
211 mg/L, 25 °C	19.2 – 21.2	5/5
	19,3 – 21.3	5/5

The sensitivities of the three test methods (plus the BIs) were compared based on the percentage of replicate tests passing each test method by material and MeBr decontamination treatment (Table 5-17). Against *B. anthracis* spores, MeBr at the moderate (9 hr at 211 mg/L, 37 °C) and low (9 hr at 211 mg/L, 25 °C) efficacy levels did not pass any of the three test methods in any replicate test. At the high efficacy level (18 hr at 211 mg/L and 37 °C), 67% of the replicate tests passed AOAC 966.04 (with suture loop) and AOAC 2008.05, and 100% of the replicate tests passed iSOP with all materials. None of the replicate tests at the higher decontamination treatment passed AOAC 966.04 (with porcelain penicylinder).

Use of *B. subtilis* yielded the same results as *B. anthracis* at the moderate and low efficacy treatments, i.e., none of the three tests were passed in any replicate. However, very different results were observed for *B. subtilis* compared to *B. anthracis* at the high efficacy treatment. MeBr exhibited little efficacy against *B. subtilis* at the high efficacy level while the iSOP test was showing complete kill of the *B. anthracis* spores (Table 5-17 and Table 5-18). These results suggest that use of *B. subtilis* would be more stringent and conservative as a surrogate for testing for MeBr efficacy against *B. anthracis* spores.

BI results were conservative with no tests passing at any efficacy treatment level or replicate test.

Table 5-17. MeBr Efficacy Results against *Bacillus anthracis* by Test Method and Material

Contact Time and Concentration	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*				
	AOAC 966.04 *(B. anthracis)*		AOAC 2008.05 *(B. anthracis)*	iSOP *(B. anthracis)*	
	Porcelain Penicylinder	Suture Loop	Glass	Ceiling Tile	Galvanized Metal and Glass
MeBr					
18 hr at 211mg/L and 37 °C	0%	67%	67%	100%	100%
9 hr at 211 mg/L and 37 °C	0%	0%	0%†	0%	0%
9 hr at 211 mg/L and 23 °C	0%	0%	0%	0%	0%‡

*AOAC 966.04 and BI require 100% kill and AOAC 2008.05 and iSOP require six log reductions to be considered effective (i.e., passing).

†One of the three replicate tests at the moderate efficacy level had a log reduction of only ≥5.9, even though no viable spores were recovered. Tests with log reductions <6.0 were excluded from the calculation of the percent passing the test, even though no viable spores were recovered. Results shown are based on two replicates for the moderate efficacy level treatment.

Table 5-18. MeBr Efficacy Results against *Bacillus* Surrogates by Test Method and Material

Contact Time and Concentration	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*			
	AOAC 966.04 *(B. subtilis)*		AOAC 2008.05 *(B. subtilis)*	BI *(B. atrophaeus)*
	Porcelain Penicylinder	Suture Loop	Glass	Stainless Steel in Tyvek® Packaging
MeBr				
18 hr at 211mg/L and 37 °C	0%	0%	0%	0%
9 hr at 211mg/L and 37 °C	0%	0%	0%	0%
9 hr at 211mg/L and 23 °C	0%	0%	0%	0%

*AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 requires six log reductions to be considered effective (i.e., passing).

Figure 5-3 provides a graphical comparison of the mean log reduction in *B. anthracis* spores surviving on test carriers/coupons from all three methods at the three efficacy levels. The results are similar between the AOAC 2008.05 and iSOP. The AOAC 966.04 is difficult to compare at low efficacy levels because when all tubes show growth, the log reduction is about ≤5 (shown as about 5 on the graph).

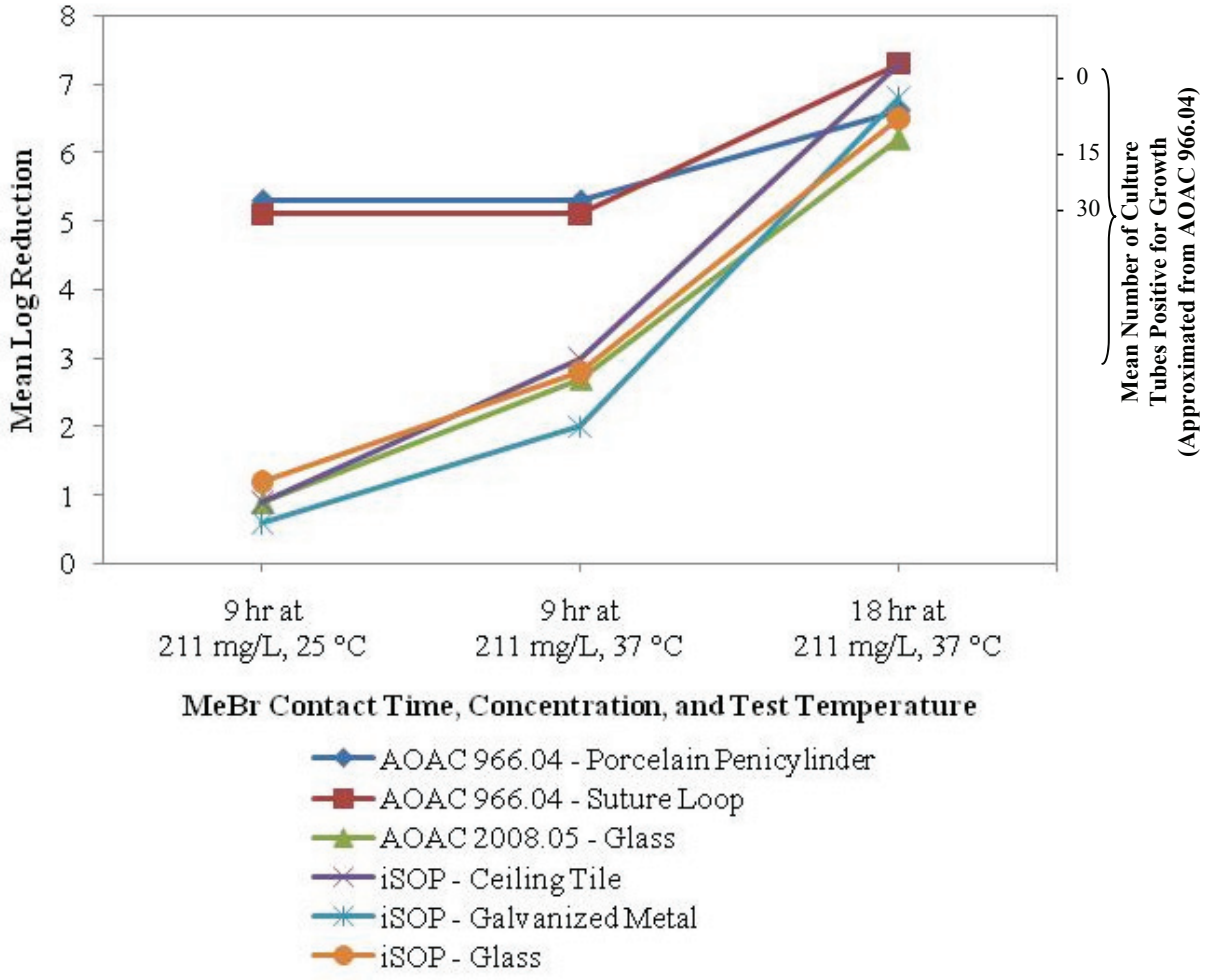

Figure 5-3. Summary of MeBr decontamination efficacy against *B. anthracis* spores.

Key observations:

- AOAC 966.04 was the most stringent of the test methods for hard, nonporous materials.
- Results from moderate and low efficacy treatments were in agreement across all test methods: no test was passed in any of the three test methods.
- Except for AOAC 966.04 with porcelain penicylinder, at the high efficacy treatment level the results from all three tests would meet the registration requirement in most or all replicates of the tests.
- Use of *B. subtilis* yielded results at least as stringent as those observed for *B. anthracis* with the AOAC 966.04 or AOAC 2008.05 tests; *B. subtilis* was much more difficult to kill with MeBr than *B. anthracis*.
- The AOAC 2008.05 results were more similar to 966.04 with suture loops than to the 966.04 with penicylinders, the hard, nonporous carrier.
- Use of *B. atrophaeus* BIs is conservative and as hard or harder to pass than the other three methods used for hard, nonporous surfaces.
- The iSOP test was the least stringent of the tests; the choice of coupon materials selected for the iSOP method did not impact the stringency of the test.

5.4 pH-Amended Bleach

The pH-amended bleach immersion was used with two levels of efficacy: the high efficacy treatment was a 60-min contact time and the low efficacy treatment was a 10-min contact time.

- **AOAC 966.04:** Test results associated with AOAC 996.04 are presented in Table 5-19. The pH-amended bleach was effective with a 60-min contact time (0 out of 29 or 30 culture tubes were positive for growth (complete kill) against *B. anthracis* and *B. subtilis* spores on penicylinders. The pH-amended bleach was less effective with a 30-min

contact time (3 out of 30 culture tubes were positive for growth against *B. anthracis* spores and a range of 6 – 16 out of 30 culture tubes were positive for growth against *B. subtilis* spores on suture loops). At the low efficacy treatment (10-min contact time) the pH-amended bleach exhibited complete or nearly complete kill (0 and 5 out of 30 culture tubes were positive for growth) against *B. anthracis* and complete kill (0 out of 30 culture tubes were positive for growth) in both replicate tests for *B. subtilis* spores on penicylinders.

- **AOAC 2008.05:** *B. anthracis* and *B. subtilis* spores were tested on glass using AOAC 2008.05 (Table 5-20). High log reductions (≥5.4) were observed for all tests. *B. anthracis* after a 60-min contact time showed log reductions of 6.5 and ≥5.9; at the ≥5.9 log reduction there were no viable spores remaining on the carriers. *B. anthracis* and *B. subtilis* spores were tested on glass using AOAC 2008.05 (Table 5-20). Relatively high log reductions (≥5.4) were observed for all trials with contact times of 10 min and 60 min. However, none of the treatments tested resulted in complete kill (no viable spores recovered) for both trial replicates of a spore and contact time combination. (Because of slightly low recoveries from control coupons only a ≥5.9 log reduction was possible in two of the replicates, even though no spores were recovered from the coupons. Where all spores were killed, but the criterion of ≥6 log reduction was not met, the results were not included in the calculation of the percentage passing the test.)
- **iSOP:** The iSOP test results associated with pH-amended bleach are presented in Table **5-21**. *B. anthracis* spores were tested on carpet, galvanized metal, and glass. For both the 60-min and the 10-min contact times, decontamination efficacies, as measured by log reductions, were lower when testing was conducted with carpet than with galvanized metal and glass. For both the high and low efficacy treatments, log reductions were ≤4.5 on carpet and ≥7.3 on galvanized metal and glass. Complete kill on both trial replicates occurred only with glass at the 60-min contact time.

Table 5-19. Decontamination Efficacy of pH-Amended Bleach Using AOAC 966.04

Material / Contact Time, Concentration	Spore / Trial	Mean Carrier Count (log_{10} CFU)[1]	Culture Tubes Positive for Growth / Total	Estimated Log Reduction*
Porcelain Penicylinder 60 min	***B. anthracis***			
	31.1	5.75	0/29†	≥7.5
	31.2	6.48	0/30	≥8.3
	B. subtilis			
	31.1	5.71	0/30	≥7.5
	31.2	6.56	0/30	≥8.4
Porcelain Penicylinder 10 min	***B. anthracis***			
	28.1	5.75	0/30	≥7.5
	28.2	6.48	5/30	7.2
	B. subtilis			
	28.1	5.71	0/30	≥7.5
	28.2	6.56	0/30	≥8.4
Suture Loop 60 min	***B. anthracis***			
	31.1	6.26	3/30	7.2
	31.2	6.59	3/30	7.5
	B. subtilis			
	31.1	6.56	16/30	6.7
	31.2	6.56	6/30	7.2
Suture Loop 10 min	***B. anthracis***			
	28.1	6.26	28/29†	5.7
	28.2	6.59	30/30	≤6.0
	B. subtilis			
	28.1	6.56	30/30	≤6.0
	28.2	6.56	29/30	6.1

* Log reductions were estimated using the method of Tomasino and Hamilton[6].

† Only 29 carriers (rather than 30) were included in the trial.

[1] Spore counts are for a given lot of carriers and were determined at the time the carriers were prepared.

Table 5-20. Decontamination Efficacy of pH-Amended Bleach Using AOAC 2008.05

Material / Contact Time, Concentration	Spore / Trial	Recovered Log Spores (CFU)*		Mean Log Reduction
		Control Coupons†	Test Coupons‡	
Glass	***B. anthracis***			
60 min	32.1	7.25 ± 0.08	0.79 ± 0.15§	6.5§
	32.2	6.58 ± 0.20	0.70 ± 0.00§, #	≥5.9§
	B. subtilis			
	32.1	6.43 ± 0.06	0.79 ± 0.15§	5.6§
	32.2	6.97 ± 0.01	0.70 ± 0.00§, #	≥6.3§
Glass	***B. anthracis***			
10 min	29.1	7.25 ± 0.08	0.97 ± 0.28§	6.3§
	29.2	6.58 ± 0.20	0.70 ± 0.00§, #	≥5.9§
	B. subtilis			
	29.1	6.43 ± 0.06	1.02 ± 0.55§	5.4§
	29.2	6.97 ± 0.01	0.70 ± 0.00§, #	≥6.3§

* Data are expressed as mean ± standard deviation of three replicates.

† Control carriers are inoculated carriers that did not undergo decontamination.

‡ Test carriers are inoculated carriers that underwent decontamination.

§ Surviving spores were not recovered from one or more carriers; a value of 5 CFU per carrier was used for determining log density and log reduction.

No surviving spores were recovered.

Table 5-21. Decontamination Efficacy of pH-Amended Bleach Using iSOP

Material / Contact Time, Concentration	Spore / Trial	Recovered Spores CFU)*		Efficacy (Mean Log Reduction)*
		Control Coupons†	Test Coupons‡	
Carpet	***B. anthracis***			
60 min	33.1§	$1.13 \pm 0.22 \times 10^{8}$§	$6.00 \pm 8.83 \times 10^{4}$§	4.0 ± 1.2§
	33.2	$1.12 \pm 0.25 \times 10^{8}$	$8.19 \pm 11.3 \times 10^{4}$#	4.5 ± 2.2#
Carpet	***B. anthracis***			
10 min	30.1§	$1.13 \pm 0.22 \times 10^{8}$§	$5.98 \pm 6.56 \times 10^{4}$§	3.9 ± 1.2§
	30.2	$1.12 \pm 0.25 \times 10^{8}$	$5.57 \pm 4.86 \times 10^{5}$	2.6 ± 0.7
Galvanized Metal	***B. anthracis***			
60 min	33.1	$1.00 \pm 0.14 \times 10^{8}$¶	$2.06 \pm 3.03 \times 10^{1}$#	7.3 ± 0.9#
	33.2	$9.44 \pm 1.16 \times 10^{7}$	0.00 ± 0.00#	≥8.0 ± 0.0#
Galvanized Metal	***B. anthracis***			
10 min	30.1	$1.00 \pm 0.14 \times 10^{8}$¶	$2.08 \pm 4.43 \times 10^{1}$#	7.6 ± 0.9#
	30.2	$9.44 \pm 1.16 \times 10^{7}$	$1.48 \pm 3.09 \times 10^{1}$#	7.6 ± 0.8#
Glass	***B. anthracis***			
60 min	33.1	$6.52 \pm 1.23 \times 10^{7}$¶	0.00 ± 0.00#	≥7.8 ± 0.0#
	33.2	$4.77 \pm 6.86 \times 10^{7}$	0.00 ± 0.00#	≥7.7 ± 0.0#
Glass	***B. anthracis***			
10 min	30.1	$6.52 \pm 1.23 \times 10^{7}$¶	$7.40 \pm 14.3 \times 10^{0}$#	7.5 ± 0.7#
	30.2	$4.77 \pm 6.86 \times 10^{7}$	0.00 ± 0.00#	≥7.7 ± 0.0#

* Data are expressed as mean ± standard deviation of five replicates.

† Control coupons are inoculated coupons that did not undergo decontamination.

‡ Test coupons are inoculated coupons that underwent decontamination.

§ Note: 5.50×10^{3} CFU were recovered from the associated laboratory blank (a non-inoculated coupon).

Surviving spores were not recovered or were recovered only from some coupons; determination of mean recovered spores (if any spores were recovered) and log reduction were based on CFU value(s) of 1.

¶ Note galvanized metal in Trial 1 had positive control recovery >120% (244%).

ǂ Note glass Trial 1 had positive control recovery <20%.

The three test methods were compared based on the percentage of replicate tests passing each test method by material and pH-amended bleach decontamination treatment, i.e., contact time (Table 5-22). The AOAC 966.04 (porcelain penicylinder), AOAC 2008.05, and iSOP (galvanized metal and glass) passed the replicate tests at 60 min; AOAC 2008.05, and iSOP (galvanized metal and glass) passed the replicate tests at 10 min contact time as well. For AOAC 966.04 (porcelain penicylinder), 50% of the replicate tests passed with the 10-min contact time. Porous materials presented a more stringent test than the hard, nonporous materials; none of the replicate tests passed the AOAC 966.04 (suture loop) or iSOP (carpet). Note that the high inoculum causes the log reductions to be correspondingly high. However, in terms of complete kill, the higher inoculum appears to have little or no effect on the results. Results for the iSOP (glass) at the lower inoculum were similar to iSOP (galvanized metal).

Use of *B. subtilis* (Table 5-23) yielded results that were generally similar, but less stringent in the AOAC 966.04 test using penicylinders and more stringent in the AOAC 2008.05 test compared to results observed for *B. anthracis*. A t-test of the number of *B. anthracis* and *B. subtilis* spores recovered from glass coupons in the 2008.05 test showed that there were no significant differences in results.

Table 5-22. pH-Amended Bleach Efficacy Results against *Bacillus* Surrogates by Test Method and Material

Contact Time	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*				
	AOAC 966.04 *(B. anthracis)*		AOAC 2008.05 *(B. anthracis)*	iSOP *(B. anthracis)*	
	Porcelain Penicylinder	Suture Loop	Glass	Carpet	Galvanized Metal and Glass
	pH-Amended Bleach				
60 min	100%	67%	100%†	0%	100%
10 min	50%‡	0%‡	100%†	0%	100%

*AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 and iSOP require six log reductions to be considered effective (i.e., passing).

†Replicates with no viable spores recovered, but log reductions <6.0 (≥5.9 log reduction in both cases), were excluded from the calculation of the percent passing the test. The results shown are based on one replicate for each treatment.

‡Only 29, rather than 30, carriers were included in one of these tests.

Table 5-23. pH-Amended Bleach Efficacy Results against *Bacillus* Surrogates by Test Method and Material

Contact Time	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*		
	AOAC 966.04 *(B. subtilis)*		AOAC 2008.05 *(B. subtilis)*
	Porcelain Penicylinder	Suture Loop	Glass
	pH-Amended Bleach		
60 min	100%	0%	50%
10 min	100%	0%‡	50%

*AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 requires six log reductions to be considered effective (i.e., passing).

Figure 5-4 provides a graphical comparison of the mean log reduction in *B. anthracis* spores surviving on test carriers/coupons from all three methods at the three efficacy levels. The results are similar, except for iSOP (industrial carpet) which showed a lower log reduction at all treatment levels than the other methods and materials.

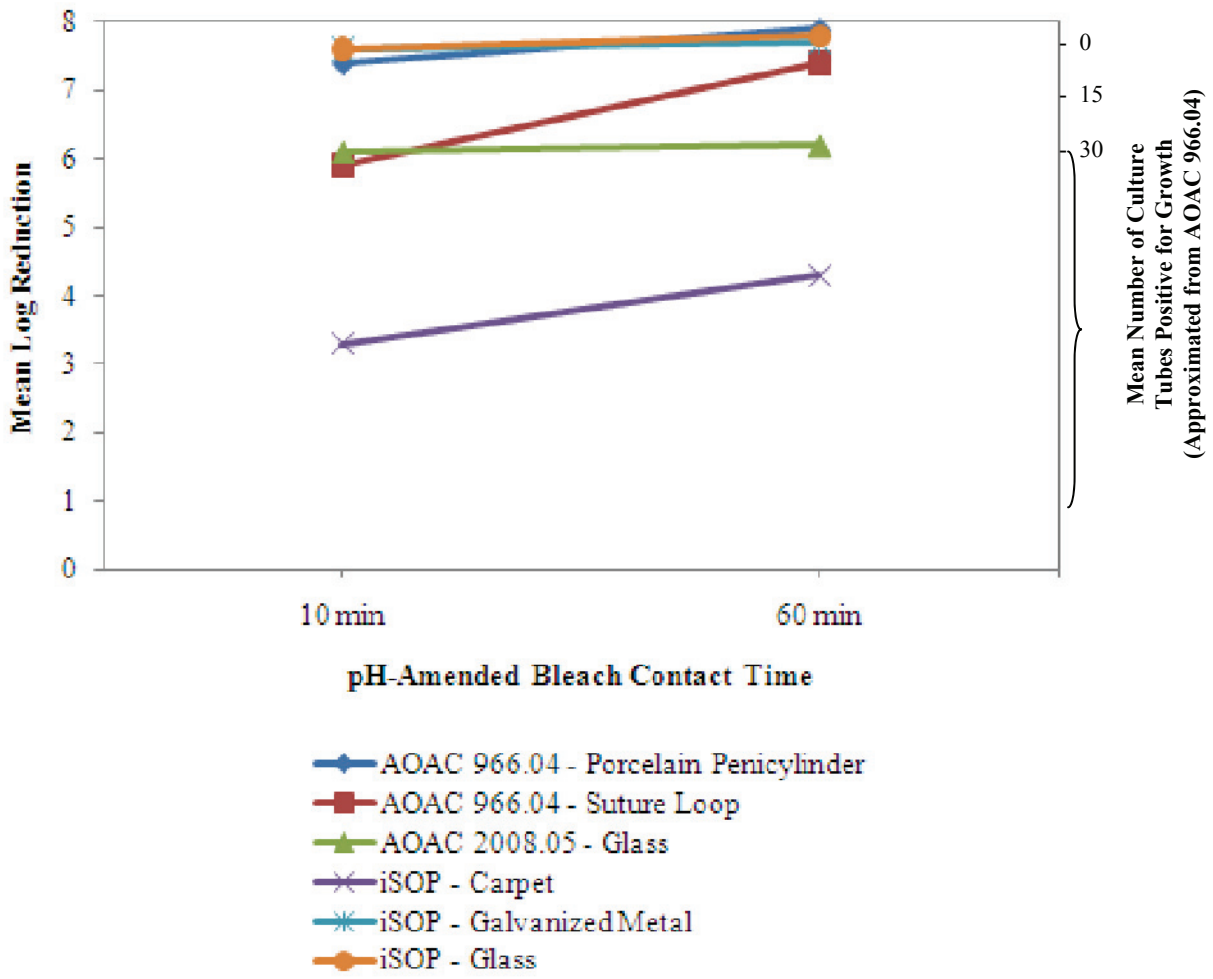

Figure 5-4. Summary of pH-amended bleach decontamination efficacy against *B. anthracis* spores.

Key observations:

- The most stringent of the tests were those that included suture loops and carpet; none of the AOAC 966.04 (suture loops) and iSOP (carpet) replicate tests passed.
- At the high efficacy treatment level, *B. anthracis* on hard, nonporous materials passed all three tests.
- At the low efficacy level, the AOAC 966.04 with porcelain peniclinder was the most stringent method for *B. anthracis* on hard, nonporous materials; both replicates of the AOAC 2008.05 (glass) and iSOP (galvanized metal and glass) passed the test in all replicates.
- Use of *B. subtilis* yielded results at least as stringent as those observed for *B. anthracis* with the AOAC 2008.05 tests but less stringent with the AOAC 966.04 (porcelain penicylinder) method

5.5 Exterm Liquid ClO_2

The Exterm liquid ClO_2 immersion test was used with two levels of efficacy: the high efficacy treatment was a 60-min contact time and the low efficacy treatment was a 10-min contact time.

- **AOAC 966.04:** Exterm liquid ClO_2 was completely effective at decontaminating *B. anthracis* spores applied to porcelain penicylinders as complete kill (0 tubes out of 30 culture tubes with positive growth) was observed for each trial replicate at both the 60-min and 10-min contact times (Table 5-24). Exterm liquid ClO_2 was less effective when *B. anthracis* spores were applied to suture loops; positive growth was observed in at least 13 of 30 culture tubes of every trial replicate. Decontamination efficacy of Exterm liquid ClO_2 on suture loops did increase with increasing contact time (the ratios of culture tubes positive for growth/

the total number of tubes were 18/30 and 13/30 with 60-min contact time and were 30/30 and 27/30 with 10-min contact time).

- **AOAC 2008.05:** *B. anthracis* and *B. subtilis* spores were tested on glass using AOAC 2008.05 (Table 5-25). Complete kill (no viable spores recovered) and log reductions ≥6.1were observed in all tests with *B. anthracis* and *B. subtilis* spores when exposed for 60-min contact time with Exterm liquid ClO2. At the 10-min contact time, log reductions were 5.0 and ≥6.5 for *B. anthracis* spores and 4.5 and ≥6.3 for *B. subtilis* spores.
- **iSOP:** The iSOP test results associated with Exterm liquid ClO_2 are presented in Table 5-26. *B. anthracis* spores were tested on carpet, galvanized metal, and glass. Mean log reductions in *B. anthracis* spores were ≥6.7 for all materials and contact times tested. Complete kill occurred on all tests with carpet and galvanized metal. Complete kill also occurred on half of the tests with glass; viable *B. anthracis* spores were recovered from one trial on glass at the 10-min contact time (8.06×10^1 CFU mean recovered spores) and from one trial on glass at the 60-min contact time (2.64×10^1 CFU mean recovered spores).

Table 5-24. Decontamination Efficacy of Exterm Liquid ClO_2 Using AOAC 966.04

Material / Contact Time	Spore / Trial	Mean Carrier Count (log_{10} CFU) [H]	Culture Tubes Positive for Growth / Total	Estimated Log Reduction*
Porcelain	***B. anthracis***			
Penicylinder	37.1	6.48	0/30	≥8.3
60 min	37.2	6.48	0/30	≥8.3
Porcelain	***B. anthracis***			
Penicylinder	34.1	6.48	0/30	≥8.3
10 min	34.2	6.48	0/30	≥8.3
Suture Loop	***B. anthracis***			
60 min	37.1	6.59	18/30	6.6
	37.2	6.59	13/30	6.8
Suture Loop	***B. anthracis***			
10 min	34.1	6.59	30/30	≤6.0
	34.2	6.59	27/30	6.3

[*] Log reductions were estimated using the method of Tomasino and Hamilton[6].

[H] Spore counts are for a given lot of carriers and were determined at the time the carriers were prepared.

Table 5-25. Decontamination Efficacy of Exterm Liquid ClO_2 Using AOAC 2008.05

Material / Contact Time	Spore / Trial	Recovered Spores CFU)*		Mean Log Reduction
		Control Carriers†	Test Carriers‡	
Glass	***B. anthracis***			
60 min	38.1	7.19 ± 0.02	0.70 ± 0.00§, #	≥6.5§
	38.2	6.79 ± 0.03	0.70 ± 0.00§, #	≥6.1§
	B. subtilis			
	38.1	6.98 ± 0.47	0.70 ± 0.00§, #	≥6.3§
	38.2	6.98 ± 0.02	0.70 ± 0.00§, #	≥6.3§
Glass	***B. anthracis***			
10 min	35.1	7.19 ± 0.02	0.70 ± 0.00§, #	≥6.5§
	35.2	6.79 ± 0.03	0.94 ± 0.43§	5.9§
	B. subtilis			
	35.1	6.98 ± 0.47	2.49 ± 1.55§	4.5§
	35.2	6.98 ± 0.02	0.70 ± 0.00§, #	≥6.3§

[*] Data are expressed as mean ± standard deviation of three replicates.

[†] Control carriers are inoculated carriers that did not undergo decontamination.

[‡] Test carriers are inoculated carriers that underwent decontamination.

[§] Surviving spores were not recovered from one or more carriers; a value of 5 CFU per carrier was used for determining log density and log reduction.

[#] No surviving spores were recovered.

Table 5-26. Decontamination Efficacy of Exterm Liquid ClO_2 Using iSOP

Material / Contact Time	Spore / Trial	Recovered Spores CFU)*		Efficacy (Mean Log Reduction)*
		Control Coupons†	Test Coupons‡	
Carpet	***B. anthracis***			
60 min	39.1	$1.22 \pm 0.08 \times 10^{8}$¶	0.00 ± 0.0^{0}§	≥8.1 ± 0.0§
	39.2	$1.81 \pm 0.27 \times 10^{7}$	0.00 ± 0.00§	≥7.3 ± 0.0§
Carpet	***B. anthracis***			
10 min	36.1	$1.22 \pm 0.08 \times 10^{8}$¶	0.00 ± 0.00§	≥8.1 ± 0.0§
	36.2	$1.81 \pm 0.27 \times 10^{7}$	0.00 ± 0.00§	≥7.3 ± 0.0§
Galvanized Metal	***B. anthracis***			
60 min	39.1	$1.34 \pm 0.09 \times 10^{8}$¶	0.00 ± 0.00§	≥8.1 ± 0.0§
	39.2	$5.22 \pm 1.24 \times 10^{6}$¶	0.00 ± 0.00§	≥6.7 ± 0.0§
Galvanized Metal	***B. anthracis***			
10 min	36.1	$1.34 \pm 0.09 \times 10^{8}$¶	0.00 ± 0.00§	≥8.1 ± 0.0§
	36.2	$5.22 \pm 1.24 \times 10^{6}$¶	0.00 ± 0.00§	≥6.7 ± 0.0§
Glass	***B. anthracis***			
60 min	39.1	$1.20 \pm 0.16 \times 10^{8}$	$2.64 \pm 2.84 \times 10^{1}$§	7.1 ± 0.9§
	39.2	$2.00 \pm 0.19 \times 10^{7}$	0.00 ± 0.00§	≥7.3 ± 0.0§
Glass	***B. anthracis***			
10 min	36.1	$1.20 \pm 0.16 \times 10^{8}$	$8.06 \pm 16.2 \times 10^{1}$§	7.3 ± 1.2§
	36.2	$2.00 \pm 0.19 \times 10^{7}$	0.00 ± 0.00§	≥7.3 ± 0.0§

* Data are expressed as mean ± standard deviation of five replicates.

† Control coupons are inoculated coupons that did not undergo decontamination.

‡ Test coupons are inoculated coupons that underwent decontamination.

§ Surviving spores were not recovered or were recovered only from some coupons; determination of mean recovered spores (if any spores were recovered) and log reduction were based on CFU value(s) of 1.

¶ Note carpet in Trial 1 and galvanized metal in Trial 2 had positive control recovery <20%.

Note galvanized metal in Trial 1 had positive control recovery >120% (206%).

The three test methods were compared based on the percentage of replicate tests passing each test method by material and Exterm liquid ClO_2 decontamination treatment, i.e., contact time (Table 5-27). All replicate tests at both 60-min and 10-min contact times passed AOAC 966.04 (porcelain penicylinder) and iSOP; none of the replicate AOAC 966.04 (suture loops) tests passed. Note that the high inoculum in the iSOP test causes the log reductions to be correspondingly high. However, in terms of complete kill, the higher inoculum appears to have little or no effect on the results. Results for the iSOP were similar to AOAC 966.04 (porcelain penicylinder). AOAC 2008.05 demonstrated an increase in the percentage of replicate tests passing with increasing contact time with the Exterm liquid ClO_2 (50% of the replicate tests passed with a 10-min contact time and 100% of the replicate tests passed with a 60-min contact time).

Table 5-28 shows the percentage of the AOAC 2008.05 replicate tests passing using Exterm liquid ClO_2 to decontaminate *B. subtilis*. The percentage of tests passing the test with *B. subtilis* were the same as observed with *B. anthracis*. A t-test of the number of *B. anthracis* and *B. subtilis* spores recovered from glass coupons in the 2008.05 test could not be performed because there were no spores recovered from any of the carriers in any replicate test.

Figure 5-6 provides a graphical comparison of the mean log reduction in *B. anthracis* spores surviving on test carriers/coupons from all three methods at the two efficacy levels. All mean log reductions were >6. The results were similar across treatment levels, test methods, and materials.

Key observations:

- The most stringent of the tests was the AOAC 966.04 (suture loops) in which no replicate at either the high efficacy or low efficacy treatment levels passed the test; the AOAC 966.04 (penicylinders) passed the test at both the 60-min and 10 min contact times.
- Only AOAC 2008.05 with *B. anthracis* spores showed the impact of contact time with half the tests passed at 10-min contact time and all tests passed at the 60-min contact time.
- The iSOP method was the least stringent for Exterm liquid ClO_2; the test was passed on all materials with a 10^7 initial spore density and with the higher 10^8

initial spore density; a small number of viable spores were recovered from glass with the initial 10^8 spore density after both the 60-min and 10-min contact times. Note that the high inoculum causes the log reductions to be correspondingly high, e.g., ≥8.1 at the high inoculum and ≥7.3 for low inoculum for carpet at 60 min. However, in terms of complete kill or passing the test, the higher inoculum appears to have little or no effect on the results.

- Use of *B. subtilis* yielded the same results as those observed for *B. anthracis* with the AOAC 2008.05 method.

Table 5-27. Exterm Liquid ClO_2 Efficacy Results against *Bacillus* Surrogates by Test Method and Material

Contact Time	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*				
	AOAC 966.04 *(B. anthracis)*		AOAC 2008.05 *(B. anthracis)*	iSOP *(B. anthracis)*	
	Porcelain Penicylinder	Suture Loop	Glass	Carpet	Galvanized Metal and Glass
	Exterm Liquid ClO_2				
60 min	100%	0%	100%†	100%	100%
10 min	100%‡	0%	50%†	100%	100%

* AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 and iSOP require six log reductions to be considered effective (i.e., passing).

Table 5-28. Exterm Liquid ClO2 Efficacy Results against *Bacillus* Surrogates by Test Method and Material

Contact Time	% of Replicate Tests Passing AOAC 2008.05
	AOAC 2008.05 *(B. anthracis)*
	Glass
	Exterm Liquid ClO_2
60 min	100%
10 min	50%

* AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 requires six log reductions to be considered effective (i.e., passing).

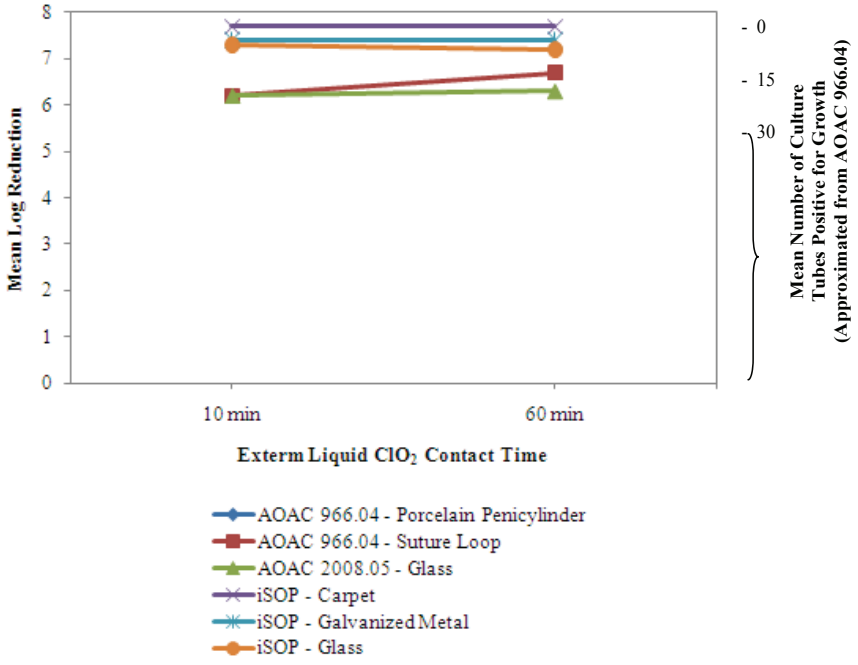

Figure 5-5. Summary of Exterm liquid ClO_2 decontamination efficacy against *B. anthracis* spores.

5.6 Oxonia Active®

The Oxonia Active® immersion test was used with two levels of efficacy: the high efficacy treatment was a 60-min contact time and the low efficacy treatment was a 10-min contact time.

- **AOAC 966.04:** Test results associated with AOAC 996.04 are presented in Table 5-29. Complete kill or nearly complete kill occurred during both replicate tests with Oxonia Active® on both materials (porelain penicylinders and suture loops) when the contact time was 60 min; one culture tube was positive for growth (out of 60 culture tubes for the replicate tests) during tests with porcelain penicylinders and one culture tube was positive for growth (out of 60 culture tubes for the replicate tests) during tests with suture loops at 60-min contact times. At the shorter contact time, the Oxonia Active® did not induce complete kill (0 tubes with positive growth) of B. anthracis spores on either the porcelain penicylinders or suture loops at a10-min contact time; positive growth was observed in 2/30 and 4/30 culture tubes associated with the porcelain penicylinder tests and all tubes (60 culture tubes for the replicate tests) associated with suture loops were positive for growth at the 10-min contact time.
- **AOAC 2008.05:** For Oxonia Active®, AOAC 2008.05 was the most stringent method with none of the replicate tests with *B. anthracis* spores at either the high or low efficacy levels passing the test (Table **5-30**). Complete kill was not observed with *B. anthracis* spores at the 60-min or 10-min contact times, and the log reductions were ≤5.7 (log reductions ranged from 4.2 to 5.7). With *B. subtilis* spores, all log reductions were ≥6.1, and complete kill (no recovery of viable spores) occurred with both replicate tests conducted at the 60-min contact time and with one replicate at the 10-min contact time.
- **iSOP:** The iSOP test results associated with Oxonia Active® are presented in Table 5-31. *B. anthracis* spores were tested on carpet, galvanized metal, and glass. Mean log reductions in recovered *B. anthracis* spores were ≥6.8 for all materials and contact times tested. Considering both the 60-min and the 10-min contact times, complete kill occurred in all replicate tests with carpet, three of four trials with galvanized metal, and three of four trials with glass. Viable *B. anthracis* spores were recovered from one trial with galvanized metal at the 60-min contact time (6.80×10^0 CFU mean recovered spores) and from one trial on glass at the 10-min contact time (2.68×10^1 CFU mean recovered spores). The recovery of a small number of spores from galvanized metal after a 60-min contact time is an unexplained anomaly.

Table 5-29. Decontamination Efficacy of Oxonia Active® Using AOAC 966.04

Material / Contact Time	Spore / Trial	Mean Carrier Count (log_{10} CFU) [H]	Culture Tubes Positive for Growth / Total	Estimated Log Reduction*
Porcelain	***B. anthracis***			
Penicylinder	43.1	6.59	1/30	7.9
60 min	43.2	6.65	0/30	≥8.4
Porcelain	***B. anthracis***			
Penicylinder	40.1	6.59	4/30	7.4
10 min	40.2	6.65	2/30	7.7
Suture Loop	***B. anthracis***			
60 min	43.1	6.59	0/30	≥8.4
	43.2	5.90	1/30	7.2
Suture Loop	***B. anthracis***			
10 min	40.1	6.59	30/30	≤6.0
	40.2	5.90	30/30	≤5.3

* Log reductions were estimated using the method of Tomasino and Hamilton[6].

[H] Spore counts are for a given lot of carriers and were determined at the time the carriers were prepared.

Table 5-30. Decontamination Efficacy of Oxonia Active® Using AOAC 2008.05

Material / Contact Time	Spore / Trial	Recovered Log Density (CFU)*		Mean Log Reduction
		Control Carriers†	Test Carriers‡	
Glass	***B. anthracis***			
60 min	44.1	6.54 ± 0.08	0.89 ± 0.32§	5.7§
	44.2	6.19 ± 0.35	1.13 ± 0.38§	5.1§
	B. subtilis			
	44.1	6.94 ± 0.05	0.70 ± 0.00§, #	≥6.2§
	44.2	7.00 ± 0.07	0.99 ± 0.50§, #	≥6.0§
Glass	***B. anthracis***			
10 min	41.1	6.54 ± 0.08	2.32 ± 0.11	4.2
	41.2	6.19 ± 0.35	0.89 ± 0.32§	5.3§
	B. subtilis			
	41.1	6.94 ± 0.05	0.70 ± 0.00§, #	≥6.2§
	41.2	7.00 ± 0.07	0.89 ± 0.32§	6.1§

* Data are expressed as mean ± standard deviation of three replicates.

† Control carriers are inoculated carriers that did not undergo decontamination.

‡ Test carriers are inoculated carriers that underwent decontamination.

§ Surviving spores were not recovered from one or more carriers; a value of 5 CFU per carrier was used for determining log density and log reduction.

No surviving spores were recovered.

Table 5-31. Decontamination Efficacy of Oxonia Active® Using iSOP

Material / Contact Time	Spore / Trial	Recovered Log Density (CFU)*		Efficacy (Mean Log Reduction)*
		Control Coupons†	Test Coupons‡	
Carpet	***B. anthracis***			
60 min	45.1	$1.12 \pm 0.11 \times 10^7$	0.00 ± 0.00§	≥7.1 ± 0.0§
	45.2	$1.14 \pm 0.09 \times 10^7$	0.00 ± 0.00§	≥7.1 ± 0.0§
Carpet	***B. anthracis***			
10 min	42.1	$1.12 \pm 0.11 \times 10^7$	0.00 ± 0.00§	≥7.1 ± 0.0§
	42.2	$1.14 \pm 0.09 \times 10^7$	0.00 ± 0.00§	≥7.1 ± 0.0§
Galvanized Metal	***B. anthracis***			
60 min	45.1	$1.20 \pm 0.43 \times 10^7$¶	$6.00 \pm 13.4 \times 10^0$§	6.8 ± 0.7§
	45.2	$8.70 \pm 1.29 \times 10^6$	0.00 ± 0.00§	≥6.9 ± 0.0§
Galvanized Metal	***B. anthracis***			
10 min	42.1	$1.20 \pm 0.43 \times 10^7$¶	0.00 ± 0.00§	≥7.1 ± 0.0§
	42.2	$8.70 \pm 1.29 \times 10^6$	0.00 ± 0.00§	≥6.9 ± 0.0§
Glass	***B. anthracis***			
60 min	45.1	$1.38 \pm 0.19 \times 10^7$¶	0.00 ± 0.00§	≥7.1 ± 0.0§
	45.2	$7.28 \pm 0.96 \times 10^6$	0.00 ± 0.00§	≥6.9 ± 0.0§
Glass	***B. anthracis***			
10 min	42.1	$1.38 \pm 0.19 \times 10^7$¶	$2.68 \pm 5.77 \times 10^1$§	6.7 ± 0.9§
	42.2	$7.28 \pm 0.96 \times 10^6$	0.00 ± 0.00§	≥6.9 ± 0.0§

* Data are expressed as mean ± standard deviation of five replicates.

† Control coupons are inoculated coupons that did not undergo decontamination.

‡ Test coupons are inoculated coupons that underwent decontamination.

§ Surviving spores were not recovered or were recovered only from some coupons; determination of mean recovered spores (if any spores were recovered) and log reduction were based on CFU value(s) of 1.

¶ Note galvanized metal in Trial 1 and glass in Trial 1 had positive control recovery <20%.

The three test methods were compared based on the percentage of replicate tests passing each test method by material and Oxonia Active® decontamination treatment i.e., contact time (Table 5-32). All replicate tests failed the AOAC 966.04

Table 5-33 shows that both AOAC 2008.05 replicate tests failed using Oxonia Active® to decontaminate *B. anthracis* for both 60-min and 10-min contact times. In contrast, *B. subtilis* passed the AOAC 2008.05 for all replicates at both contact times.

Table 5-32. Oxonia Active® Efficacy Results against *Bacillus anthracis* by Test Method and Material

Contact Time	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*				
	AOAC 966.04 *(B. anthracis)*		AOAC 2008.05 *(B. anthracis)*	iSOP *(B. anthracis)*	
	Porcelain Penicylinder	Suture Loop	Glass	Carpet	Galvanized Metal and Glass
	Oxonia Active®				
60 min	50%	50%	0%	100%	100%
10 min	0%	0%	0%	100%	100%

* AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 and iSOP require six log reductions to be considered effective (i.e., passing).

Table 5-33. AOAC 2008.05 Results for Oxonia Active® against *Bacillus subtilis*

Contact Time	% of Replicate Tests Passing AOAC 2008.05
	AOAC 2008.05 *(B. subtilis)* Glass
	Oxonia Active®
60 min	100%
10 min	100%

* AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 requires six log reductions to be considered effective (i.e., passing).

Figure 5-6 provides a graphical comparison of the mean log reduction in *B. anthracis* spores surviving on test carriers/coupons from all three methods at the two efficacy levels. AOAC 200.05 showed lower log reductions at the two treatment levels than the other methods.

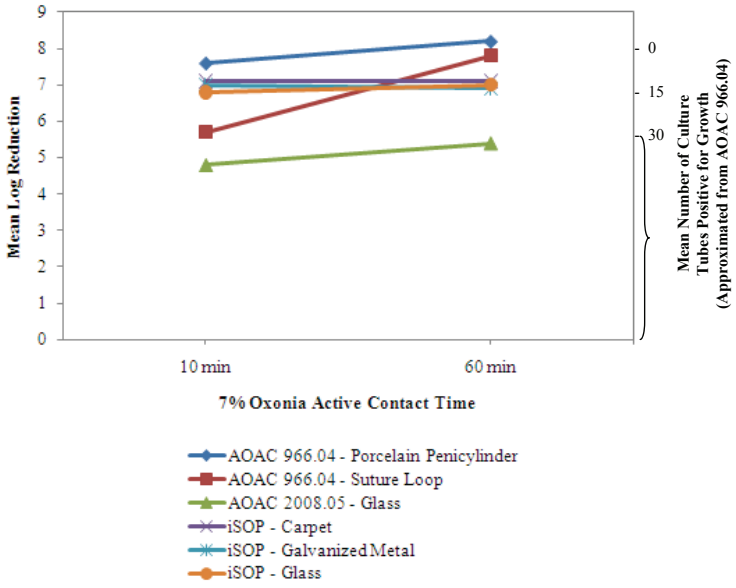

Figure 5-6. Summary of Oxonia Active® decontamination efficacy against *B. anthracis* spores.

Key observations:

- AOAC 2008.05 was the most stringent of the test methods for Oxonia Active® against *Bacillus anthracis* with no test being passed at either the high efficacy level or the low efficacy level; AOAC 966.04 was passed only half of the time at the high efficacy level and not at all at the low efficacy level (qualitative method is sensitive to effect of time on efficacy).
- iSOP was the least stringent of the test methods for Oxonia Active® against *Bacillus anthracis* with all tests being passed at both the high efficacy level and the low efficacy level.
- While than *B. anthracis* failed all of the AOAC 2008.05 tests and *B. subtilis* passed all of the AOAC 2008.05 tests at both the high efficacy level and the low efficacy level, the low levels of spores recovered from the coupons were very similar for the two *Bacillus* species. Slight differences in the number of spores recovered from the control coupons causes the difference between log reductions that result in all of the tests being passed and all of the tests being failed. The actual difference in efficacy is likely insignificant.

5.7 Spor-Klenz® Ready-to-Use

The Spor-Klenz® Ready-to-Use immersion test was used with two levels of efficacy: the high efficacy treatment was a 30-min contact time (consistent with label instructions) and the low efficacy treatment was a 10-min contact time.

- **AOAC 966.04:** Test results associated with AOAC 996.04 are presented in Table 5-34. The results demonstrated sensitivity to contact time, with improved efficacy observed at the longer contact time of 30 min. Tests with porcelain penicylinders contaminated with *B. anthracis* spores resulted in 0 of 30 (passing test) and 3 of 30 culture tubes positive for growth after the 30-min contact time. In contrast, 5 of 30 and 15 of 30 culture tubes were positive for growth after the 10-min contact time. Spor-Klenz® Ready-to-Use was completely effective (i.e., no culture tubes were positive for growth) when decontaminating *B. subtilis* spores from porcelain penicylinder with a 30-min contact time. Spor-Klenz® Ready-to-Use showed no efficacy (30/30 culture tubes positive for growth) for all *B. anthracis* and *B. subtilis* replicates after the 10-min contact time on suture loops. Some marginal efficacy against *B. anthracis* and *B. subtilis* on suture loops was demonstrated with the longer contact time of 30 min: culture tubes with *B. anthracis* had positive growth ratios of 26/30 and 27/30 while *B. subtilis* culture tubes had positive ratios of 16/30 and 17/30.
- **AOAC 2008.05:** *B. anthracis* and *B. subtilis* spores were tested on glass using AOAC 2008.05 (Table **5-35**). Mean log reductions in CFU ranged from 5.1 to ≥6.3 for all spore and contact time combinations. No surviving *B. subtilis* spores were recovered following the 10-min contact time (both replicate tests) and one trial associated with the 30-min contact time. A complete kill was observed for one *B. anthracis* spore trial, which occurred at the 30-min contact time.
- **iSOP:** The iSOP immersion test results associated with Spor-Klenz® Ready-to-Use decontamination of *B. anthracis* spores on carpet, galvanized metal, and glass are presented in Table 5-36. Complete kill (no viable *B. anthracis* spores recovered) occurred with all tests using carpet and glass at both 30-min and 10-min contact times; the associated log reductions in *B. anthracis* spores were ≥6.9. Spor-Klenz® Ready-to-Use was ineffective against *B. anthracis* spores on galvanized metal as all associated mean log reductions in CFU were ≤3.6 and complete kill did not occur. Observations during the testing indicated that there was bubbling during the contact time, the galvanized metal appeared to become tarnished and changed color to a darker grey. A white precipitate was also observed in the tube. The level of tarnish and amount of precipitate appeared to increase with increased contact time. The interactions with Spor-Klenz® Ready-to-Use and the galvanized metal may have inhibited the sporicidal effects of Spor-Klenz®.

Table 5-34. Decontamination Efficacy of Spor-Klenz® Ready-to-Use Using AOAC 966.04

Material / Contact Time	Spore / Trial	Mean Carrier Count (log_{10} CFU) [H]	Culture Tubes Positive for Growth / Total	Estimated Log Reduction*
Porcelain	***B. anthracis***			
Penicylinder	49.1	6.08	3/30	7.0
30 min	49.2	6.11	0/30	≥7.9
	B. subtilis			
	49.1	5.71	0/30	≥7.5
	49.2	5.71	0/30	≥7.5
Porcelain	***B. anthracis***			
Penicylinder	49.1	6.08	5/30	6.8
10 min	49.2	6.11	15/30	6.3
	B. subtilis			
	46.1	5.71	0/30	≥7.5
	46.2	5.71	1/30	7.0
Suture Loop	***B. anthracis***			
30 min	49.1	6.11	26/30	5.8
	49.2	6.11	27/30	5.8
	B. subtilis			
	49.1	6.59	17/30	6.7
	49.2	6.59	16/30	6.7
Suture Loop	***B. anthracis***			
10 min	46.1	6.11	30/30	≤5.5
	46.2	6.11	30/30	≤5.5
	B. subtilis			
	46.1	6.59	30/30	≤6.0
	46.2	6.59	30/30	≤6.0

[*] Log reductions were estimated using the method of Tomasino and Hamilton[6].

[H] Spore counts are for a given lot of carriers and were determined at the time the carriers were prepared.

Table 5-35. Decontamination Efficacy of Spor-Klenz® Ready-to-Use Using AOAC 2008.05

Material / Contact Time	Spore / Trial	Recovered Log Density (CFU)[*]		Mean Log Reduction
		Control Carriers[†]	Test Carriers[‡]	
Glass	***B. anthracis***			
30 min	50.1	6.54 ± 0.08	0.70 ± 0.00[§,#]	≥5.9[§]
	50.2	6.19 ± 0.35	1.07 ± 0.44[§]	5.1[§]
	B. subtilis			
	50.1	6.94 ± 0.05	0.70 ± 0.00[§,#]	≥6.2[§]
	50.2	7.00 ± 0.07	0.89 ± 0.32[§]	6.1[§]
Glass	***B. anthracis***			
10 min	47.1	6.54 ± 0.08	0.87 ± 0.15[§]	5.7[§]
	47.2	6.19 ± 0.35	0.94 ± 0.43[§]	5.2[§]
	B. subtilis			
	47.1	6.94 ± 0.05	0.70 ± 0.00[§,#]	≥6.2[§]
	47.2	7.00 ± 0.07	0.70 ± 0.00[§,#]	≥6.3[§]

[*] Data are expressed as mean ± standard deviation of three replicates.

[†] Control carriers are inoculated carriers that did not undergo decontamination.

[‡] Test carriers are inoculated carriers that underwent decontamination.

[§] Surviving spores were not recovered from one or more carriers; a value of 5 CFU per carrier was used for determining log density and log reduction.

[#] No surviving spores were recovered.

Table 5-36. Decontamination Efficacy of Spor-Klenz® Ready-to-Use Using iSOP

Material / Contact Time	Spore / Trial	Recovered Spores (CFU)*		Efficacy (Mean Log Reduction)*
		Control Coupons†	Test Coupons‡	
Carpet	***B. anthracis***			
60 min	51.1#	$1.12 \pm 0.11 \times 10^7$	0.00 ± 0.00§	≥7.1 ± 0.0§
	51.2	$1.14 \pm 0.09 \times 10^7$	0.00 ± 0.00§	≥7.1 ± 0.0§
Carpet	***B. anthracis***			
10 min	48.1#	$1.12 \pm 0.11 \times 10^7$	0.00 ± 0.00§	≥7.1 ± 0.0§
	48.2	$1.14 \pm 0.09 \times 10^7$	0.00 ± 0.00§	≥7.1 ± 0.0§
Galvanized Metal	***B. anthracis***			
60 min	51.1	$1.20 \pm 0.43 \times 10^7$¶	$6.40 \pm 6.77 \times 10^5$	1.5 ± 0.5
	51.2	$8.60 \pm 0.84 \times 10^6$	$3.08 \pm 2.29 \times 10^3$	3.6 ± 0.4
Galvanized Metal	***B. anthracis***		$8.52 \pm 3.95 \times 10^6$	
10 min	48.1	$1.20 \pm 0.43 \times 10^7$¶	$2.96 \pm 5.63 \times 10^5$	0.2 ± 0.3
	48.2	$8.60 \pm 0.84 \times 10^6$		2.3 ± 1.0
Glass	***B. anthracis***			
60 min	51.1	$1.38 \pm 0.19 \times 10^7$¶	0.00 ± 0.00§	≥7.1 ± 0.0§
	51.2	$7.28 \pm 0.96 \times 10^6$	0.00 ± 0.00§	≥6.9 ± 0.0§
Glass	***B. anthracis***			
10 min	48.1	$1.38 \pm 0.19 \times 10^7$¶	0.00 ± 0.00§	≥7.1 ± 0.0§
	48.2	$7.28 \pm 0.96 \times 10^6$	0.00 ± 0.00§	≥6.9 ± 0.0§

* Data are expressed as mean ± standard deviation of five replicates.

† Control coupons are inoculated coupons that did not undergo decontamination.

‡ Test coupons are inoculated coupons that underwent decontamination.

§ Surviving spores were not recovered or were recovered only from some coupons; determination of mean recovered spores (if any spores were recovered) and log reduction were based on CFU value(s) of 1.

A laboratory blank coupon was positive for low-level contamination by *B. anthracis* (applies only to one replicate)

¶ Note galvanized metal in Trial 1 and glass in Trial 1 had positive control recovery <20%.

Spor-Klenz® Ready-to-Use was only found to be completely effective (100% of the replicate tests passed) when tested against *B. anthracis* spores using iSOP (carpet and glass) (Table 5-37). None of the replicate tests passed iSOP (galvanized metal).

B. subtilis in the AOAC 966.04 (porcelain penicylinders only) and AOAC 2008.05 methods was less stringent than using *B. anthracis* (Table 5-38). However, as noted above, the apparent difference in efficacy using the AOAC 2008.05 may be attributed to slight differences in spore recoveries from the positive control coupons and is not likely to be of practical importance.

AOAC 966.04 was also conducted with *B. anthracis* spores. When using porcelain penicylinder, 50% of the replicate tests passed with a 10-min contact time and 100% of the replicate tests passed with a 30-min contact time. None of replicate tests with either *B. anthracis* or *B. subtilis* passed when using AOAC 966.04 (suture loop).

Table 5-37. Spor-Klenz® Ready-to-Use Efficacy Results against *Bacillus anthracis* by Test Method and Material

Contact Time	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*					
	AOAC 966.04 *(B. anthracis)*		AOAC 2008.05 *(B. anthracis)*	iSOP *(B. anthracis)*		
	Porcelain Penicylinder	Suture Loop	Glass	Carpet	Galvanized Metal	Glass
	Spor-Klenz®					
30 min	50%	0%	0%†	100%	0%	100%
10 min	0%	0%	0%†	100%	0%	100%

*AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 and iSOP require six log reductions to be considered effective (i.e., passing).

†Log reductions ranged from 5.1 to ≥5.9; the replicate with no recovered spores and an efficacy of ≥5.9 was excluded from the calculation of percentage passing the test.

Table 5-38. AOAC 2008.05 Results for Spor-Klenz® Ready-to-Use against *Bacillus subtilis*

Contact Time, Concentration and Temperature as Applicable	% of Replicate Tests Passing Test Methods for Sporicidal Activity by Material*		
	AOAC 966.04 *(B. subtilis)*		AOAC 2008.05 *(B. subtilis)*
	Porcelain Penicylinder	Suture Loop	Glass
	Spor-Klenz®		
30 min	100%	0%	100%
10 min	50%	0%	100%

*AOAC 966.04 and BI require 100% kill on thirty or 5 carriers in the test, respectively; AOAC 2008.05 and iSOP require six log reductions to be considered effective (i.e., passing).

Figure 5-7 provides a graphical comparison of the mean log reduction in *B. anthracis* spores surviving on test carriers/coupons from all three methods at the two efficacy levels. Log reduction with iSOP (galvanized metal) was substantially lower that was observed on the other materials at both time points. All other tests and materials yielded generally similar results, with AOAC 966.04 (suture loops) and AOAC 2008.05 showing slightly lower log reductions than the other tests method or materials.

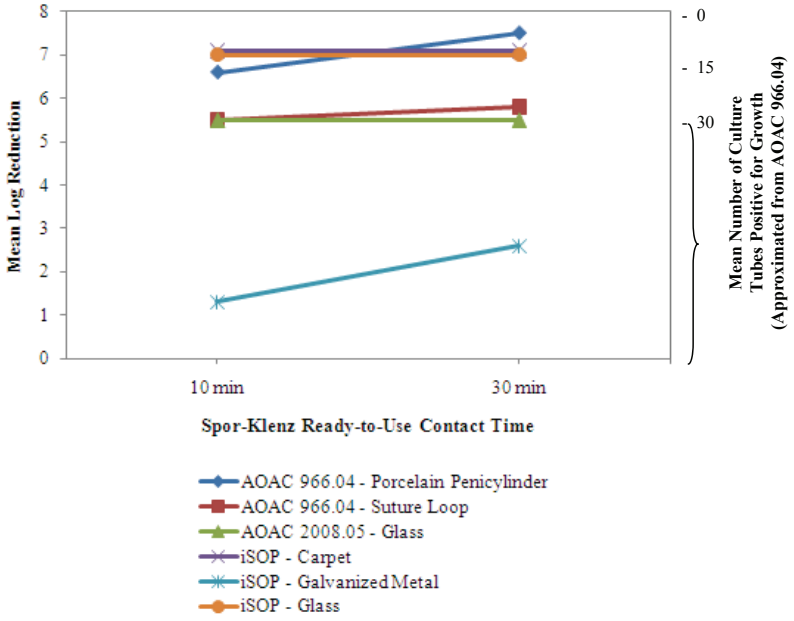

Figure 5-7. Summary of Spor-Klenz® Ready-to-Use decontamination efficacy against *B. anthracis* spores.

Key observations:

- It is not clear which test was most stringent with Spor-Klenz® Ready-to-Use against *Bacillus anthracis*. AOAC 2008.05 had no replicate pass the test, but all tests showed efficacy ranging from 5.1 to ≥5.9 (with no spores recovered, but not included in calculation of percent passing). AOAC 966.04 with porcelain penicylinder passed one of the replicates at 30 min and had only 3 of 30 cultures positive in the second test; these results are comparable to those obtained with the AOAC 2008.05. With iSOP, carpet and glass passed every replicate and treatment, making it seemingly less stringent than the other methods. However, virtually no efficacy was observed with iSOP on galvanized metal.
- In spite of differences in the passing rate with AOAC 2008.05 (100% of the *B. subtilis* replicate tests passed but none of the *B. anthracis* replicate tests passed), *B. subtilis* yielded log reduction results (all ≥5.1) that were very similar to those observed for *B. anthracis* (4.2 to ≥6.2)
- The qualitative AOAC 966.0.4 demonstrated the impact of time based on the relative number of tubes that exhibited growth (more positive at the shorter contact time).
- The materials selected for use in the iSOP impacted the test results, with the galvanized metal showing little or no efficacy when the other materials were exhibiting complete kill.

5.8 Virkon® S

Virkon® S[12] (and Virkon®[13]) include the following active ingredients: potassium peroxomonosulfate (CAS 70693-62-8; 50% or 40%-60%), sodium dodecylbenzene sulfonate (CAS 25155-30-0; 15% or 10%-20%), and sulfamic acid (CAS 5329-14-6; 5% or 1%-10%). DuPont Virkon® disinfectant was reported to have efficacy against spores.[14] Virkon® is sometimes recommended as a disinfectant for use against *B. anthracis* spores.[15] Efficacy determinations of Virkon® disinfectants against spores in solution tests indicated higher levels of efficacy in physiological solution than in distilled water.[16] Another study using suspension tests reported that a contact time of 1 hr in a 1% solution of Virkon® disinfectant was not sporicidal against *B. cereus*.[17]

In 2008, the Board of Scientific Counselors[18] suggested that the EPA test Virkon® S. In response to the Board of Scientific Counselors' recommendation, Virkon® S was included in the test matrix for this investigation. Therefore, Virkon S was selected for testing, even though there are no sporicidal label claims. However, preliminary method demonstration showed no efficacy against *B. anthracis* spores for a 1% Virkon® S solution in contact with the spores for 30 min. The method demonstration was therefore repeated with a 60-min contact time. The steps in the method demonstration were:

1. Add 400 µL of sterile water to microcentrifuge Tubes 1-6 and 400 µL of disinfectant to Tubes 7-12.
2. Allow tubes to equilibrate approximately 10 min at 20 ± 1 °C.
3. Add 600 µL neutralizer in ice-cold Luria-Bertanti (LB) broth to Tubes 4-6 (neutralizer controls).
4. Add 600 µL neutralizer in ice cold LB broth to Tubes 7-9 (ability of neutralizer to inactivate the disinfectant). Gently mix.
5. Add 10 µL of *B. subtilis* spore suspension (approximately 10^9 spores/mL) to each tube and vortex mix for approximately 15 sec. Incubate tubes for 30 min (or 60 min) ± 2 min at 20 ± 1 °C.
6. After incubation, add 600 µL ice-cold LB broth to Tubes 1-3 (survival controls). Add 600 µL ice-cold LB broth to Tubes 10-12 (disinfectant control).
7. Serially dilute each tube to achieve plate counts of 30-300 CFU/plate.
8. Prepare spread plates and incubate 24 ± 2 hr at 36 °C ± 1 °C.
9. Log densities in tubes 1-3 and 4-6 reflect the spore suspension titer and should be within one log of each other. If the differences in log densities between Tubes 1-3 and 4-6 are greater than one log, then the neutralizer has a sporicidal effect. If the disinfectant is highly effective, log densities in Tubes 10-12 should be approximately 6 logs lower than log densities in Tubes 1-6. To be an effective neutralizer, log densities in Tubes 7-9 should be within 1 log of the log densities in Tubes 1-6.

The method results, shown in Table 5-39, demonstrated that Virkon® S 1% was not effective against *B. anthracis* spores under the conditions tested. After contact times of both 30 min and 60 min, the log densities in Tubes 10-12 (decontamination without neutralization) were at best only 0.27 log lower than Tubes 1-6 (positive controls). The neutralizer (1% sodium thiosulfate solution [STS]/2% sodium bicarbonate [$NaHCO_3$]) did not exhibit any sporicidal effects. Because efficacy was not observed in the method demonstration, the Virkon® S disinfectant was dropped from further efficacy testing.

Table 5-39. Results of Virkon® S Decontamination Method Demonstration

Virkon® S	Time	Neutralizer	Average Log Density	Average Log Reduction
No	30 min	None (tubes 1-3)	6.83	n/a
No	30 min	1%STS/2% $NaHCO_3$ (4-6)	6.88	-0.05
Yes	**30 min**	**None (10-12)**	**6.97**	**-0.14**
Yes	30 min	1%STS/2% $NaHCO_3$ (7-9)	6.90	-0.07
No	60 min	None (tubes 1-3)	6.16	n/a
No	60 min	1%STS/2% $NaHCO_3$ (4-6)	6.25	-0.08
Yes	**60 min**	**None (10-12)**	**5.89**	**0.27**
Yes	60 min	1%STS/2% $NaHCO_3$ (7-9)	6.10	0.06

n/a indicates that "average log reduction" is not applicable.

5.9 Statistical Comparisons

The results of the statistical analysis in Table 5-40 and Table 5-41 show that there were no significant differences in efficacy using *B. anthracis* Ames spores or *B. subtilis* spores, except with MeBr. B. subtilis spores are more resistant and therefore a conservative surrogate for B. anthracis Ames with MeBr fumigation.

For the liquid and fumigant decontamination technologies evaluated, *B. subtilis* spores were at least as resistant to decontamination as *B. anthracis* spores with both the AOAC 966.04 and AOAC 2008.05 tests. These results support the potential use of *B. subtilis* as a surrogate for *B. anthracis* Ames in sporicidal tests for these sporicide formulations.

Table 5-40. Comparison between the Efficacies Determined for *B. anthracis* Ames and for *B. subtilis* for Fumigant Decontamination

Fumigant	Contact Time and Target Concentration	Method	p-Value*
ClO_2	3 hr at3000 ppm	AOAC 966.04	Complete Kill†
		AOAC 2008.05	1.0000
	3 hr at300 ppm	AOAC 966.04	0.1429
		AOAC 2008.05	Complete Kill†
	3 hr at 150 ppm	AOAC 966.04	0.1429
		AOAC 2008.05	1.0000
HP	90 min, 250 ppmv	AOAC 966.04	0.7662
		AOAC 2008.05	Complete Kill†
	30 min, 250 ppmv	AOAC 966.04	0.1429
		AOAC 2008.05	1.0000
	0 min, 250 ppmv	AOAC 966.04	0.4740
		AOAC 2008.05	1.0000
MeBr	18hr, 211 mg/L (at 37 °C)	AOAC 966.04	**0.0022**
		AOAC 2008.05	**0.1000**
	9hr, 211 mg/L (at 37 °C)	AOAC 966.04	0.3377
		AOAC 2008.05	**0.1000**
	9hr, 211 mg/L (at 25 °C)	AOAC 966.04	0.3377
		AOAC 2008.05	0.6000

*A p-value less than 0.05, shown in **bold**, indicates a significant difference between two spores.

†There was complete kill of all spores from all carriers for both *B. anthracis* and *B. subtilis*.

Table 5-41. Comparison between the Efficacies Determined for *B. anthracis* Ames and for *B. subtilis* for Liquid Decontamination

Fumigant	Contact Time and Target Concentration	Method	p-Value*
pH-Amended Bleach	AOAC 966.04	60 min	0.7714
		10 min	1.0000
	AOAC 2008.05	60 min	1.0000
		10 min	1.0000
Exterm Liquid ClO_2	AOAC 2008.05	60 min	Complete Kill†
		10 min	1.0000
Oxonia Active®	AOAC 2008.05	60 min	0.3333
		10 min	0.3333
Spor-Klenz® Ready-to-Use	AOAC 966.04	30 min	0.6571
		10 min	0.7714
	AOAC 2008.05	30 min	0.3333
		10 min	0.3333

*A p-value less than 0.05, shown in **bold**, indicates a significant difference between two spores.

†There was complete kill of all spores from all carriers for both *B. anthracis* and *B. subtilis*.

As shown in Table 5-42 and Table 5-43, there were significant differences in most cases among the three methods when testing fumigant technologies. Except for Oxonia Active® at the longest contact time, there were no significant differences among the efficacy determinations by the tests when applied to liquid technologies.

Table 5-42. Comparison between the Log Reductions Determined by Three Methods for Fumigant Decontamination

Fumigant	Treatment	p-Value*
ClO_2	3000 ppmv, 1 hr	Complete Kill†
	300 ppmv, 1 hr	0.3456
	150 ppmv, 1 hr	0.7250
HP	250 ppmv, 90 min	**0.0128**
	250 ppmv, 30 min	**0.0005**
	250 ppmv, 0 min	0.6277
MeBr	37 °C, 18 hr	**0.0260**
	37 °C, 9 hr	**0.0004**
	25 °C, 9 hr	**0.0003**

*A p-value less than 0.05, shown in **bold**, indicates a significant difference among three methods.

†There was complete kill of all spores from all carriers or coupons for both *B. anthracis* and *B. subtilis*.

Table 5-43. Comparison between the Log Reductions Determined by Three Methods for Liquid Decontamination

Liquid	Contact Time	p-Value*
pH-Amended Bleach	60 min	0.5224
	10 min	0.6387
Exterm Liquid ClO_2	60 min	0.0613
	10 min	0.1135
Oxonia Active®	60 min	**0.0007**
	10 min	0.0838
Spor-Klenz® Ready-to-Use	30 min	0.6789
	10 min	0.4347

*A p-value less than 0.05, shown in **bold**, indicates a significant difference among three methods.

6.0
Summary

Using iSOP, all seven technologies were tested at a minimum of one treatment that resulted in 100% of the replicate tests passing the test method. Using AOAC 2008.05, three technologies (Sabre ClO_2, STERIS HP, and Exterm liquid ClO_2) had 100% pass for at least one treatment. Using AOAC 966.04, Sabre ClO_2, pH-amended bleach (with porcelain penicylinder only) and Exterm liquid ClO_2 (with porcelain penicylinder only) had 100% pass for at least one treatment. Sabre ClO_2 was the only technology tested that had 100% pass with the BI, although the liquid technologies (pH-amended bleach, Exterm liquid ClO_2, Oxonia Active®, and Spor-Klenz® Ready-to-Use) were not tested with BI.

AOAC 2008.05 and iSOP (especially when using galvanized metal and glass) were more likely to show passing results than AOAC 966.04 and BI. Often the percent of replicate tests passing a test method was higher when nonporous materials (i.e., porcelain penicylinder, glass, and galvanized metal) were used compared to porous materials (i.e., suture loop, ceiling tile, and carpet). Wicking of the inoculated biological agents into the nonporous materials may have been a factor in cases where 0% of trials with porous materials passed but 100% of the trials with nonporous materials did pass. Wicking of the inoculum suspensions into the material was observed with both ceiling tile and carpet.

In general, the trend observed was for the quantitative and qualitative tests for hard, nonporous materials to yield similar results. The large number of replicates in AOAC 966.04 may increase the probability of a failure due to growth of one tube. In the full AOAC 966.04 test, twice as many culture tubes (60 of a given carrier type) are required as were used in this investigation (30 of a carrier type).

Most of the technologies tested passed some of the test methods but performed relatively poorly on other test methods. However, Sabre ClO_2 (tested for 3 hr at 3000 ppmv ClO_2) had 100% of the replicate tests passing each test method including the BI. None of the other fumigation technologies tested (STERIS HP and MeBr) passed the BI. None of the test methods were completely passed (100% of replicate tests passing all tested materials per method) for pH-amended bleach or Spor-Klenz® Read-to-Use. Exterm liquid ClO_2 passed all of the tests for each material in all cases except for AOAC 966.04 with suture loops, which failed to pass in each case. Oxonia Active® did pass all methods and all tested materials in at least one trial. Virkon® S was not efficacious in preliminary suspension testing and was, therefore, not included in the three method comparison testing.

Statistical analysis showed that there were significant differences in efficacy determinations among the tests when used to evaluate fumigants. Except with Oxonia Active®, there were not significant differences among the test methods when used to evaluate liquid decontamination technologies.

B. subtilis was generally a conservative surrogate for *B. anthracis*. Statistical analysis showed that in the AOAC 2008.05 and AOAC 266.04 testing, *B. subtilis* was either not significantly different from *B. anthracis*, or was significantly more resistant to decontamination that *B. anthracis* in all cases.

7.0
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8.0
Appendix A: Summary of Fumigation Conditions

Table A- 1. Mean (SD) Fumigant Concentrations, Temperature, and RH

Fumigant	Replicate 1	Replicate 2	Replicate 3
Sabre ClO_2, High Efficacy Treatments 3000 ppmv-hr			
Concentration, Mean (SD)	3101 (52.2)	3056 (193.7)	3117 (55.2)
Temperature, Mean (SD)	22 (0.7)	23 (0.1)	23 (0.2)
RH, Mean (SD)	77 (0.9)	77 (0.4)	74 (1.5)
Sabre ClO_2, Moderate Efficacy Treatments 300 ppmv-hr			
Concentration, ppmv, Mean (SD)	310 (12.7)	293 (12.3)	296 (4.7)
Temperature, °C, Mean (SD)	22 (0.4)	22 (0.4)	21 (0.2)
RH, Mean (SD)	76 (1.2)	76 (1.5)	75 (1.5)
Sabre ClO_2, Low Efficacy Treatments 150 ppmv-hr			
Concentration, ppmv, Mean (SD)	145 (9.3)	150 (12.0)	160 (6.2)
Temperature, °C, Mean (SD)	22 (0.2)	21 (0.3)	21 (0.2)
RH, Mean (SD)	77 (2.6)	77 (1.2)	76 (1.5)
STERIS HP, High Efficacy Treatments Condition, 90 min dwell			
Concentration, ppmv, Mean (SD)	265 (66)	277 (60)	303 (71)
Temperature, °C, Mean (SD)	31 (2.3)	31 (2.3)	32 (1.2)
RH, Mean (SD)	36 (7.1)	36 (7.2)	39 (7.3)
STERIS HP, Moderate Efficacy Treatments Condition, 30 min dwell			
Concentration, ppmv, Mean (SD)	249 (81)	221 (77)	269 (79)
Temperature, °C, Mean (SD)	28 (1.5)	28 (1.5)	27 (1.3)
RH, Mean (SD)	44 (9.6)	47 (10.1)	51 (9.8)
STERIS HP, Low Efficacy Treatments Condition, 0 min dwell			
Concentration, ppmv, Mean (SD)	271 (130)	247 (132)	258 (133)
Temperature, °C, Mean (SD)	27 (0.8)	27 (1.4)	27 (0.9)
RH, Mean (SD)	45 (12.5)	48 (12.9)	45 (12.0)
MeBr, High Efficacy Treatments 18 hr contact time			
Concentration, ppmv, Mean (SD)	218 (5.4)	213 (7.9)	212 (1.7)
Temperature, °C, Mean (SD)	37.4 (0.6)	37.3 (0.3)	37.2 (0.3)
RH, Mean (SD)	76.0 (1.0)	75.0 (0.1)	75.0 (0.1)
MeBr, Moderate Efficacy Treatments 9 hr contact time			
Concentration, ppmv, Mean (SD)	212 (3.6)	214 (3.3)	213 (2.3)
Temperature, °C, Mean (SD)	37.5 (0.5)	37.6 (0.7)	37.7 (0.9)
RH, Mean (SD)	75.0 (0.8)	75.0 (0.9)	74.8 (1.3)

Fumigant	Replicate 1	Replicate 2	Replicate 3
MeBr, Low Efficacy Treatments 9 hr contact time			
Concentration, ppmv, Mean (SD)	209 (11.1)	211 (12.0)	210 (10.5)
Temperature, °C, Mean (SD)	25.2 (0.3)	25.2 (0.3)	25.2 (0.3)
RH, Mean (SD)	74.0 (0.1)	74.0 (0.1)	74.0 (0.1)

* Calculated mean concentration includes concentrations during injection time.

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