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Decontamination Process Indicators: Biological Indicators

Assessment and Lessons Learned in the Development of Biological Indicators for Chlorine Dioxide Fumigation

Office of Research and Development
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Disclaimer

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List of Acronyms and Abbreviations

µm	Micrometer(s)
AAC	Amino Acid cocktail
ALG	Alginate (sodium salt)
ALU	6061 Aluminum
APPCD	Air Pollution Prevention and Control Division
ATCC	American Type Culture Collection
B.	*Bacillus*
BI	Biological Indicator
BSC	Biological Safety Cabinet
CAR	Carrageenan
CBD	Chipboard Discs
CEM	Cellulose Ester Membranes
CER	Ceramic Tile
CFU	Colony Forming Unit(s)
CLB	Cellobiose
ClO_2	Chlorine Dioxide
COC	Chain of custody
COTS	Commercial off-the-shelf
CRK	(Adhesive) Cork Dots
CSN	Casein
CT	Concentration-Time
CUP	C14500 Copper disc
DI	De-ionized (Water)
DAS	Data Acquisition System
DCMD	Decontamination and Consequence Management Division
DMS	Dimethyl Sulfoxide
DQI	Data Quality Indicator
DQO	Data Quality Objective
DTRL	Decontamination Technologies Research Laboratory
DTT	Dithiothreitol (Test E onward)
ECBC	Edgewood Chemical Biological Center
EMS	Environmental Monitoring System
EPA	U. S. Environmental Protection Agency
EtO	Ethylene oxide
FCL	Ferrous Chloride
FLT	(Adhesive) Felt Dots
g	Gram(s)
G.	*Geobacillus*
GEL	Gelatin
GLU	Glutathione
GS	*Geobacillus(G.) stearothermophilus*
HBB	High Bay Building
HCl	Hydrochloric Acid
HMA	Humic Acid (sodium salt)

HSRP	Homeland Security Research Program
ISO	International Organization for Standardization
kDa	KiloDalton
KI	Potassium Iodide
KIPB	Phosphate Buffer Solution Containing KI
L	Liter(s)
lpm	Liter(s) per minute
LR	Log reductions
mg	Milligram(s)
mm	Millimeter(s)
mM	Millimolar
mol	Mole
MOP	Miscellaneous Operating Procedure
mSM	Modified Standard Method
NA	Not applicable
NHSRC	National Homeland Security Research Center
NIST	National Institute of Standards and Technology
ORD	Office of Research and Development
pAB	pH-Adjusted bleach
PBST	Phosphate Buffered Saline with 0.05 % TWEEN®20
PCD	Process Challenge Device
PPE	Personal Protective Equipment
ppmv	Part(s) per million volume
QA	Quality Assurance
QAPP	Quality Assurance Project Plan
QC	Quality Control
RCT	Mesa Labs culture test kits
RH	Relative Humidity
RTP	Research Triangle Park
RUB	(Adhesive) Rubber Dots
SD	Standard Deviation
SM	Standard Method
SMC	Stumbo-Murphy-Cochran
SST	Stainless Steel
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UV	Ultraviolet (light)
WACOR	Work Assignment Contracting Officer Representative
WOD	Wooden Discs
XYZ	Porous Polypropyl

Executive Summary

The objective of the research presented in this report was to develop a "custom BI(s)" that could be used to indicate the efficacy of fumigation more accurately, when ClO_2 fumigation is used to decontaminate building interiors following a *B. anthracis* contamination incident. The custom BI(s) would be engineered to yield complete kill after exposure to 9000 ppm*hours of ClO_2 gas, while at the same time providing Growth results at fumigation conditions unlikely to deactivate *B. anthracis* spores. The resulting custom BI(s) would therefore have significant utility in 1) modeling decontamination and kill kinetics of building material-bound spores more accurately, 2) providing an easily deployed method to assess decontaminant effectiveness with laboratory and field applications, and 3) offering pertinent information to a "multiple lines of evidence" approach to building clearance, thereby potentially reducing the number of surface and air samples needed to be collected to build confidence in clearance decisions.

To achieve the objective, custom BIs were prepared by numerous approaches, resulting in an increased resistance of the indicator spores to ClO_2 gas. These approaches included using carrier materials other than stainless steel or paper as is used in commercial off the shelf (COTS) BIs; or combining the spore suspension with a protective chemical additive (i.e., burden material) prior to pipetting spores onto the carrier material. The BIs were then exposed to ClO_2 for between 1000 ppm*hours and 20000 ppm*hours, typically at 1000 ppm ClO_2. After exposure, the BIs were placed in growth media and incubated for seven days to test for viable spores.

While no BI modification tested achieved a precise BI deactivation point of 9000 ppm*hours exposure to ClO_2, results from this study suggest sources of BI kill point variability may be an important focus of future research in this area.

Burdens can have the effect of increasing survival rates of BIs. Burdens that seemed to increase BI survival to 7000 ppm*hours and yet not provide protection so that all BIs survived 9000 ppm*hours included cellobiose, dithiothreitol, carrageenan, gelatin, and casein, all of which could be evaluated further. Most promising was 1% casein as a burden on low inoculum (10^3 CFU) *B. atrophaeus* BIs. Results were variable, however, with large variations between batches and fumigations due to unidentified factors apparently related to production.

While coupon materials did affect the survival rates of BIs, none of the carriers showed promise, providing either too much or too little protection. Unlike burdens, carriers cannot be tested in different concentrations. Fumigated wooden carriers would not support growth of the target organism, and were therefore not a suitable carrier material. When rubber was used as the carrier material, 100% of BIs demonstrated growth following exposures, suggesting that rubber surfaces may be difficult to decontaminate with ClO_2.

Either semi-permeable barriers or lumens (open tubes) can be used as physical barriers, and types of both were demonstrated to extend the survival rates of spores. More research should be conducted on semi-permeable membranes, as incorporation of membranes into the BI manufacturing process would be easy to implement. BIs incorporating tortuous paths such as lumens should also be further investigated.

Various COTS BIs were investigated, including *B. atrophaeus* BIs from Apex Laboratories., Raven Laboratories, and three variations from Mesa Laboratories. Some were more hardy, and some were less hardy, than the target 9000 ppm*hour full kill. Moreover, the spore preparation was found to have an impact on spore survival rates. Because spore preparations exhibit this variability, the behavior of BIs can fluctuate

from batch to batch, though there is also variability between batches using the same spore preparation, which suggests some other production factor may be causing the variation in survival rates. Future research should focus on removing variation from spore preparations, or focus on species that are more easily destroyed, thus removing the significance of spore variation. Regardless, the apparent variability in kill points amongst COTS BIs suggests that BI variability may be unavoidable, and therefore to some degree acceptable.

This study reaffirmed previous reports that BI D-values are non-linear over the duration of the fumigation. Many BIs resilient enough to survive 7000 ppm*hours would also tend to survive 9000 ppm*hours due to a subpopulation of spores with higher resistance than the main population, due either to a protective location or inherent hardiness. While a BI with very hardy spores may predict the behavior of bacterial spores in an actual event, it may represent too high a benchmark. One possible explanation of this tailing effect was the protective bio-burden of clumping in high-inoculum BIs. To reduce the bio-burden, lower inoculum BIs were tested, and showed a lower tendency towards the long-surviving tail and an increasing resistance with increasing casein burden. These techniques may be used to tune a BI to better model the inactivation of any target organism. To produce a good model of *Bacillus anthracis* with ClO_2 fumigations, the authors would recommend side-by-side comparisons of *Bacillus anthracis* to BIs with low inoculum and 1% and 2% casein burden.

1 Introduction

This project supports the mission of the U.S. Environmental Protection Agency's (EPA) Office of Research and Development (ORD), Homeland Security Research Program (HSRP). The EPA's National Homeland Security Research Center (NHSRC), conducts high-quality research to support the HSRP, by providing information pertinent to the decontamination of contaminated areas, such as those resulting from an act of terrorism. Previously, biological indicators (BIs) from Apex Laboratories (Sanford, NC, USA), consisting of *Bacillus (B.) atrophaeus* spores on stainless steel coupons, were used in laboratory decontamination studies of building materials as the standard surrogates for spores of *B. anthracis*. However; recent systematic decontamination studies conducted jointly by EPA and Edgewood Chemical Biological Center (ECBC) within the US Army, using chlorine dioxide (ClO_2) as the fumigant of choice, showed that *B. anthracis* spores on certain building materials (such as bare pine wood, painted wall wallboards, painted I-beam steel, and concrete cinder blocks) are more resilient to decontamination than *B. atrophaeus* BIs and required a considerably higher Concentration-Time (CT) to achieve zero viable *B. anthracis* spores in samples recovered from building material [1]. Subsequently, EPA's HSRP initiated a study to develop and evaluate a BI designed specifically for Homeland Security decontamination applications.

The objective of the research presented in this report was to develop a "custom BI(s)" that could be used to indicate the efficacy of ClO_2 fumigation more accurately, when ClO_2 fumigation is used to decontaminate building interiors following a *B. anthracis* contamination incident. The developed BI would be engineered to yield complete kill after exposure to 9000 ppm*hour of ClO_2 gas. This target kill point was selected based upon previous laboratory data and target exposure criteria for fumigations following the 2001 anthrax incidents [2] [3]. The resulting custom BI(s) would therefore have significant utility in 1) modeling decontamination and kill kinetics of building material-bound spores more accurately, 2) providing an easily deployed method to assess decontaminant effectiveness with laboratory and field applications, and 3) offering pertinent information to a "multiple lines of evidence" approach to building clearance (http://www.epa.gov/osweroe1/docs/misc/cdc-epa-interim-clearance-strategy.pdf), thereby potentially reducing the number of surface and air samples needing to be collected to build confidence in clearance decisions.

To achieve the objective, custom BIs were prepared by numerous approaches, resulting in an increased resistance of the indicator spores to ClO_2 gas. These approaches included using carrier materials to construct the BIs, rather than using stainless steel or paper as is used in commercial off the shelf (COTS) BIs; or combining the spore suspension with a protective chemical additive (i.e., burden material) prior to pipetting spores onto the carrier material. As used here, burden material refers to any chemical added to a BI that will act to partially shield (chemically or physically) the biological portion of the BI (i.e., *B. atrophaeus or Geobacillus (G.) stearothermophilus* spores). This latter approach proved more promising, as the volumes and concentrations of the burden materials could be varied to achieve a target inactivation point, once a dose-dependent relationship of the burden and spore survival was established. The procedures and data in this report document the recent efforts to develop the above-described custom BI.

1.1 Process

Numerous custom BIs were designed, procured, and subsequently subjected to bench-scale fumigations with ClO_2, under highly-controlled environmental conditions. BIs were evaluated for their viability following

the fumigation. An opaque chamber (830 series glove box, Plas-Labs, Inc., Lansing, MI, USA) was used to maintain and control a leak-free fumigation atmosphere and allow for the periodic addition and removal of BIs during fumigation. ClO_2 was generated by a ClorDiSys-GMP (ClorDiSys, Inc., Lebanon, NJ, USA), which passes 2 % chlorine in nitrogen through sodium chlorite cartridges. The generator includes real-time feedback control of ClO_2 concentration in the chamber atmosphere via an internal photometric monitor. A second photometric monitor, was controlled by an Environmental Monitoring System (EMS) (ClorDiSys, Inc., Lebanon, NJ, USA), and used to assess the accuracy of the primary monitor.

Modified Standard Method (mSM)-4500 samples (see Section 2.4.4.2) were collected a minimum of every 60 minutes to confirm the concentration of ClO_2 in the test chamber. A fan inside the chamber provided internal mixing. Pressure relief valves and check valves prevented over-pressurization of the chamber.

Humidity within the chamber was controlled by a custom-built data acquisition system (DAS). A relative humidity (RH)/temperature sensor (Vaisala, Vantaa, Finland) was used in a feedback loop to control RH. When the Vaisala RH sensor read lower than the RH setpoint, solenoid valves were opened to inject humid air from a gas humidity bottle into the chamber. The gas humidity bottle (Fuel Cell Technologies, Albuquerque, NM, USA), heated to 60 °C, passes compressed air through Nafion® tubes surrounded by deionized water, creating a warm air stream saturated with water vapor. Temperature was controlled by circulation of cooling water through radiators. Figure 1-1 shows the schematic of the configuration used for the tests. HOBO RH sensor/loggers (Onset Computer Corporation, Bourne, MA, USA) were placed throughout the chamber to assess RH spatial variability within the chamber.

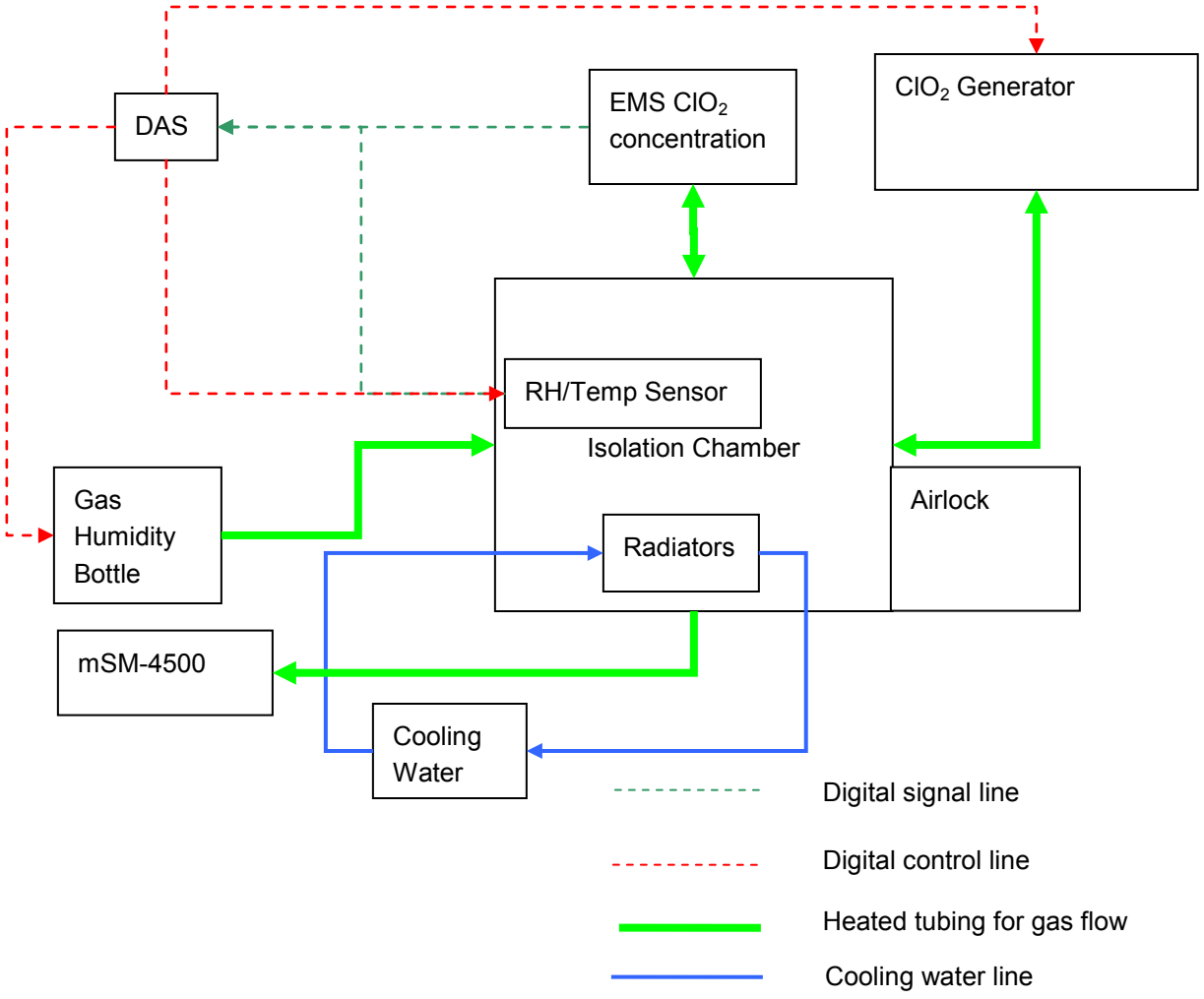

Figure 1-1. Conceptual Diagram of the Fumigation System

1.2 Project Objectives

The objective of this project was to develop a custom BI that would provide reliable results of No Growth after fumigation with 9000 ppm*hours ClO_2, while at the same time providing Growth results at fumigation conditions unlikely to deactivate *B. anthracis* spores.

1.3 Experimental Approach Overview

The experimental approaches that were used to meet the objectives of this project are:

- Fumigation using a glovebox chamber.
- Use of burdens (chemical additives) in BI spore inoculums. Burdens were added to alter spore survival on the BI carrier, either through physical or chemical mechanisms.

- Use of BI carrier materials that are more difficult to decontaminate. Such materials may alter BI spore survival by providing physical protection, or by catalytically reducing fumigant concentrations within close proximity of the material-bound spores.
- Use of material barriers or lumens to physically delay or lessen spore exposure to the fumigant.
- Use of multiple fumigation time-points to evaluate spore kill as a function of time and exposure (concentration x time, CT).
- All fumigation testing was conducted at EPA's Research Triangle Park (RTP), NC campus, within the High Bay Building (HBB). The general test method for the fumigation tests was as follows:

1. Design and order (from BI vendor) custom BIs.
2. Receive BIs. Label and group by type and exposure duration (time).
3. Establish the target temperature and RH for the trial in the fumigation chamber.
4. Charge the chamber with ClO_2 to achieve the target concentration.
5. Through the chamber airlock, place the appropriate BIs for the trial in the chamber. The BIs were present for ClO_2 ramp-up for some tests (Step 4).
6. Maintain the target concentration, temperature, and RH for the specified time (Note: Time zero was defined as the time at which the target concentration was achieved in the chamber).
7. Use the airlock to remove BIs at desired time points during fumigation.
8. After the final exposure time, aerate the chamber for a defined length of time and until a safe ClO_2 concentration was achieved in the chamber.
9. Process and analyze BIs in HBB Room H130A or in the NHSRC-RTP Microbiology Laboratory, (E390).

This report presents the developmental tests conducted toward the creation of a custom BI and evolved from two primary tasks: **Task 1,** Testing of Candidate Burden Materials, and **Task 2,** Testing of Coupon Materials. This work spanned five years and was performed under an approved Quality Assurance Project Plans (QAPP). The methods approved and reported within the QAPP are summarized in sufficient detail in this report. During the project period, while many tests evolved from these two primary tasks, a number of others were conducted to characterize BI stability, reproducibility and comparability to commercial off-the-shelf (COTS) BIs. The following sections describe the sequence and reasoning behind each of these tests.

1.4 Data Treatment

Survivability was calculated by a simple percentage of BI replicates showing growth out of the total number of replicates. The perfect BI formulation would have high survivability at 7000 ppm*hours and 0 % survivability at 9000 (± 500) ppm*hours. The BI formulations in Task 1 and Task 2 closest to this perfect survivability rate were chosen for additional tests. Maximum BI survival rates were propagated to all earlier time points; i.e., if 20 % of BIs survive at 9000 ppm*hours, then the BI survival rate at 7000 ppm*hours must be at least 20 % theoretically. This information is listed in Tables as "Maximum Survival Rate".

Figure 1-2 shows the classifications of BIs identified and discussed in this study.

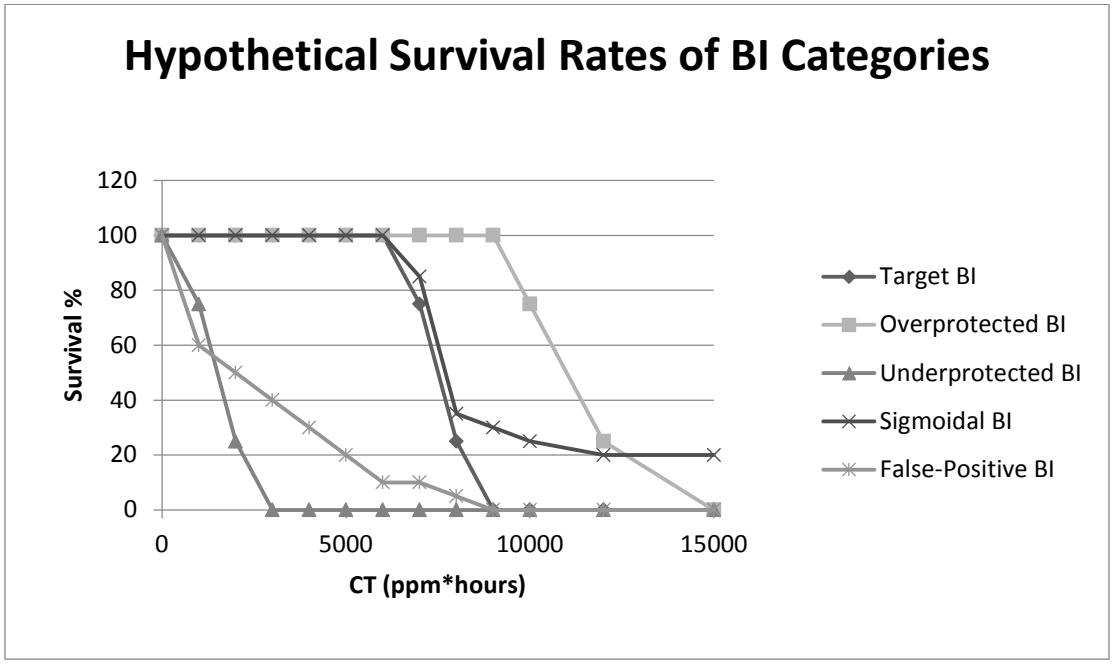

Figure 1-2. Theoretical Survival Curve Types of BIs

The optimal BI for this effort would have a time lag before survival rates are affected, giving the target BI a high probability of surviving 7000 ppm*hour ClO_2 fumigations, but would be completely inactivated at 9000 ppm*hours. Three of the survival curve categories show this time lag or shoulder. An overprotected BI would survive at exposures equal to or greater than 9000 ppm*hours, and would falsely indicate an inadequate fumigation. The under-protected BI is inactivated much faster than *B. anthracis*, and so is not indicative of adequate fumigation (typical of current COTS BIs). The sigmoidal BI has a tailing as well as a shoulder. The sigmoidal BI may show survival rates similar to the target BI at exposures less than 9000 ppm*hours, but then may prove very difficult to achieve complete deactivation. This type of BI would be more likely to falsely indicate an inadequate fumigation for the 9000 ppm*hours target CT required for full kill of *B. anthracis* spores. Finally, the false positive BI is linear and has a deactivation at the target 9000 ppm*hours, but has a higher likelihood of falsely indicating a successful fumigation at exposures less than 9000 ppm*hours.

D-value (or decimal reduction time) is a measure of the time a deactivation technique requires to effect a 90% (1 Log_{10} reduction) reduction in population. This measurement assumes a first order reaction, so that if it takes one hour to reduce the population of a BI from 1×10^5 colony forming units (CFU) to 1×10^4 CFU, then after five hours there would be less than one CFU present. Put another way, the time it takes to reduce the population from 100,000 to 10,000 CFU is the same it would take to reduce the population from <10 CFU to <1 CFU. D-values were calculated according to the two methods described in Section 1.4.1 and 1.4.2. D-values were used even in cases where there was an obvious time lag. However, it is common practice to characterize BIs by D-values, even though their kill kinetics are often known to be other than first order.

1.4.1 Stumbo-Murphy-Cochran (SMC) Method

The SMC method can be used for qualitative results under any conditions that produce a fractal survival set; i.e., for all time points that had both some deactivated BIs and some BIs showing growth. The D-value equation is shown in Equation 1.

$$D_t = \frac{U_i}{\left(\log N_o - \log N_{ui}\right)} \qquad \text{(Eqn. 1)}$$

where

D_t = D-value at time point *i*,

U_i = fumigation time,

N_o = the original population (CFU) of the BI before fumigation, and

N_{ui} = the Most Probable Number, calculated by $\ln(n_i/r_i)$, where

n_i = total number of replicate BIs at time point i, and

r_i = number of BIs negative for growth at time point i.

When multiple time points produce fractal values, the D-value for each time point was averaged to provide a D-value for the BI under that fumigation condition.

1.4.2 Quantitative Method

When quantitative populations of BIs are available, then the D-value may be calculated directly per Equation 1, but where:

N_o = the original population (CFU) of the BI before fumigation, and

N_{ui} = the population quantitatively determined at time point *i*.

2 Materials and Methods

2.1 Experimental Approach and Test Matrices

2.1.1 Task 1: Testing of Candidate Burden Materials

In Task 1, several burden materials were experimentally tested with one carrier material (stainless steel) to determine both compatibility with the surrogate organisms and the ability, if any, to increase the CT required for BI inactivation.

The growth and production of spores, preparation of burden-amended inocula, and inoculation of BI carriers with (1×10^6 spores) *B. atrophaeus* or *G. stearothermophilus* spores was performed by Yakibou (formerly Apex Laboratories of Sanford, NC, now Yakibou, Inc., Holly Springs, NC, http://ivdesignhouse.com/yak/). Apex laboratories was acquired by Mesa Laboratories (Lakewood, CO) during the timeframe of this testing. Ten candidate burden materials at two concentrations (and one "no burden" control) were identified and tested under this task. Burden materials were chosen based upon their water solubility, shelf stability, and having a chemically reduced oxidation state (able to be oxidized). The resulting custom BIs were challenged by fumigation with 1,000 parts per million volume (ppmv) gaseous ClO_2 at 75% RH and 24 °C in the Decontamination Technologies Research Laboratory (DTRL) located in HBB Room H224. The test samples were collected at 5, 7, and 9 hour exposure times (5,000, 7,000, and 9,000 ppm*hours).

Each fumigation test included positive and negative control BIs. The negative control BIs were the same stainless steel discs used by Yakibou for BIs, not inoculated, yet packaged in Tyvek® envelopes in the same manner as test BIs. Since BIs are produced to be used in sterilization environments and not produced for scientific study, the BI vendor did not guarantee the sterility of non-inoculated BIs. An additional set of laboratory blank BIs were generated by autoclaving negative control BIs upon arrival. These laboratory blank BIs were used to assess the aseptic technique of the handling laboratories. The positive control BIs consisted of standard BIs of each organism that were not fumigated. The spores were inoculated onto stainless steel discs and packaged in Tyvek® envelopes by Yakibou. Performance control BIs were also included for the longest exposure time in each test. These BIs included the burden but no spores on the BI carrier. After fumigation, the BIs were placed in a bacterial growth medium, which was then spiked with the test organism (~ 1×10^3 CFU), as a control to demonstrate that the fumigated coupon would not inhibit growth of the test organism. The number of replicates per test and control BI type is shown in Table 2-1.

Table 2-2 is a summary of the burdens used during testing, and Table 2-3 summarizes the tests conducted; each one of the burden tests was developed based upon the results of previous tests. For ease in presentation, each test is given an alphabetical designation in the order that they were conducted. *B. atrophaeus* and *G. stearothermophilus* are referred to as BG and GS, respectively.

Table 2-1. Numbers of Replicates per Experimental and Control BI Type for Task 1

Sample	Time Point (hours)	Replicates	Total	Test/Control	BI Inoculated	Burden Spiked	Media Spiked	Fumigated
Performance Baseline	0	3	3	C	No	Yes	Yes	No
Positive Control	0	3	3	C	Yes	Yes	No	No
Performance Control	5,7,9	3	9	C	No	Yes	Yes	Yes
Experimental	5,7,9	5	15	T	Yes	Yes	No	Yes
			30					

Note: 30 BIs * 11 burden materials (including no burden control) * 2 burden concentrations * 2 organisms = 1,320 Custom BIs, + 10 positive controls and 3 negative controls per fumigation.

Table 2-2. Burden Additives for Custom BIs

#	Chemical Name	Burden Code	Sigma Catalog #	Formula Wt.	Low Concentration	High Concentration
1	Humic Acid (sodium salt)	HMA	H16752	2-500 kDa	1.25%	5%
2	Amino Acid cocktail	AAC				
	Cysteine		W326305	121.16 g/mol	15 mM	59 mM
	Methionine		M9625	149.21 g/mol		
	Glutamine		G3126	146.14 g/mol		
3	Ferrous Chloride	FCL	44939	198.81 g/mol	63 mM	250 mM
4	Glutathione	GLU	G6529	307.32 g/mol	25 mM	100 mM
5	Dithiothreitol	DTT	43816	154.25 g/mol	63 mM	250 mM
6	Gelatin	GEL	G7765	~60 kDa	2.5 %	10 %
7	Alginate (sodium salt)	ALG	180947	10-600 kDa	0.5 %	2 %
8	Carrageenan	CAR	C1013	Variable	0.25 %	1 %
9	Dimethyl Sulfoxide	DMS	D8418	78.13 g/mol	10 %	40 %
10	Cellobiose	CLB	22150	342.3 g/mol	83 mM	333 mM

kDa = kiloDalton
g/mol = grams per mole
mM = millimolar

Table 2-3. Summary of Test Variables during Task 1 Burden Tests

Test	Date	Burdens*	Burden Concentrations	Spores	Time Points	Purpose
A	1/20/2010	10 kinds (see Table 2-2)	(see Table 2-2)	BG, GS	5, 7, and 9 hours	Primary identification of effective burdens
C	4/14/2010	CAR	0.063% (GS) and 0.125% (BG)	BG, GS	4, 8, 8.5, 9, 9.5, 10, and 10.5 hours	Follow-up investigation of promising burdens
		GLU	5 mM			
		DTT	5 mM			
D	5/5/2010	GEL	0.1%, 1.0%	BG, GS	4, 8, 8.5, 9, 9.5, 10, and 10.5 hours	Follow-up investigation of promising burdens
		CLB	0.17%			
E	7/21/2010	GEL ,	0.25%, 0.50%, 0.75%, 1.00%, 1.25%, 1.50%, 2.00%	BG	1, 5, 7 and 9 hours	Follow-up investigation of promising burdens
		CAR	0.01%, 0.03%, 0.05%, 0.1%, 0.25%			
		CLB	0.10%, 0.25%, 0.50%, 0.75%, 1.00%, 1.50%, 2.00%, 4.00%			
		DTT	10 mM, 20 mM, 40 mM, 50 mM			
		new burden CSN	0.1%, 1.0%			
		CAR	0.05%, 0.075%, 0.100%, 0.125%, 0.250%	GS	1, 5, 7 and 9 hours	Follow-up investigation of promising burdens
		CLB	0.10%, 0.25%, 0.50%, 0.75%, 1.00%, 1.50%, 2.00%, 4.00%			
F	9/15/2010	GEL	1.6%, 1.7%, 1.8%, 1.9%, 2.0%	BG	1, 5, 7 and 9 hours	Follow-up investigation of promising burdens
		CLB	0.005%, 0.010%, 0.050%			
		DTT	10 mM, 12 mM, 14 mM, 16 mM			
		CSN	0.10%, 0.25%, 0.50%, 0.75%, 1.00%, 1.20%,			
G	11/10/2010	GEL	1.0%. 1.5%, 1.6%, 1.7%	BG	1, 5, 7, 8 and 9 hours	Follow-up investigation of promising burdens
		CLB	0.050%, 0.060%, 0.070%, 0.10%			

Test	Date	Burdens*	Burden Concentrations	Spores	Time Points	Purpose
		CSN	0.8%, 0.9%, 1.0%, 1.1%			
H	2/22/2011	GEL	0.8%, 0.9%, 1.0%	BG	1, 5, 7, 8 and 9 hours	Follow-up investigation of promising burdens
		CSN	0.90%, 1.00%, 1.05%, 1.10%, 1.15%, 1.20%			
I	3/8/2011	GEL	0.8%, 0.9%, 1.0%	BG	1, 5, 7, 8 and 9 hours	Follow-up investigation of promising burdens
		CSN	0.90%, 1.00%, 1.05%, 1.10%, 1.15%, 1.20%			
O	2/28/2012	CSN	0.85%, 0.90%, 0.95%, 1.00%, 1.05%, 1.10%,	Yakibou prepared and Apex purchased spore inocula	5 hours only; replication 1	Investigated the differences in spore preparations (vendors) on burden BIs
P	3/12/2012	CSN	0.85%, 0.90%, 0.95%, 1.00%, 1.05%, 1.10%,	Same as Test O	5, 7, and 9 hours; replication 2	
Q	3/20/2012	CSN	0.85%, 0.90%, 0.95%, 1.00%, 1.05%, 1.10%,	Same as Test O	5, 7, and 9 hours; replication 3	
R	7/24/2012	CSN	0.5%, 1.0%		0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 hours	Investigated D-value with quantitative and qualitative analysis

* Burden abbreviations are defined in Table 2-2.

Additional tests conducted to characterize stability, reproducibility comparability of custom BIs to COTS BIs are listed in Table 2-4. During Test K, physical barriers instead of burdens were tested to determine if they could be used to provide protection to spores. Three barriers were used: one layer of Breathe Easy (Breath Easy membranes, Diversified Biotech, USA Scientific Part# 9123-6100), two layers of the Breath Easy membrane, and 1 layer of NuFab (DuPont, Wilmington, DE, USA discontinued product). For the physical barrier tests, spore suspensions were evaporated to dryness in the wells of micro-titer plates; barrier membranes were then affixed to the tops of the plates to seal the wells.

Table 2-4. Ancillary Tests for Characterization of BIs

Test	Fumigation Date	Purpose	Details
J	4/26/2011	Tested age of BI (stability over time) and vortexing vs. not vortexing	The burden on some BIs resulted in encapsulated growth (in a bubble) during analysis, resulting in a false-positive result due to the lack of turbid media. This test attempted methods to liberate surviving spores from the BI carrier prior to incubation to circumvent this issue

Test	Fumigation Date	Purpose	Details
K	5/24/2011	Tested D-value	Tested at 0, 1.5, 2.5, 3, 3.5, 4, 4.5 and 5 hours
		Tested Breath Easy film on well plates	Tested at 2, 6, and 9 hours with no filter, 1 layer of filter, and 2 layers of filter
		Tested Nufab on well plates	Tested at 2, 6, and 9 hours
L	8/1/2011	Tested Apex vs. Raven BIs	Investigated effect of proximity with compact (dense) and loose packing of BIs during exposure.
M	9/21/2011	Tested Apex vs. Raven BIs	1000 ppmv ClO_2 fumigation
N	11/1/2011	Tested Apex vs. Raven BIs and RCT culture test kits (see Section 2.2.6.6)	250 ppmv ClO_2 fumigation
S	10/9/2012	0% and 1.0% CSN concentrations to measure D-value and kill point.	Triplicate identical BIs/test conditions. Tested at 0,1,4,8,12,16, and 20 hours
T	10/30/2012	Repeat of Test S	Triplicate identical BIs/test conditions. Tested at 0,1,4,8,12,16, and 20 hours
U	1/22/13	Repeat of Test S, adding Mesa COTS ethylene oxide (EtO) BI	Triplicate identical BIs/test conditions. Tested at 0,1,4,8,12,16, and 20 hours Included COTS BIs
V	3/13/13	Repeat of Test S, adding three Mesa COTS BIs	Tested at 0,1,4,8,12,16, and 20 hours Included three COTS BIs: • Mesa Laboratories Releasat® for Chlorine Dioxide Sterilization, 10^6 *B. atrophaeus* (reorder no. RCD/50) • Mesa Laboratories MesaStrip for Low Temperature Steam Formaldehyde Sterilization, 10^6 *G. stearothermophilus* (reorder no. SGMLF/6). • Mesa Laboratories MesaStrip for Steam Sterilization, 10^6 *B. atrophaeus* (reorder no. SGMG/6)
W	8/20/13	Low Inoculum Test on unburdened vs. 1.0%, 2.0%, and 5.0% CSN burdened BIs. Tested at 0,1,2,4,6, and 9 hours	Included ProLine Process Challenge Device (PCD) COTS BIs (see Section 2.2.6.7) with and without lumens
X	8/27/13	Low Inoculum Test on unburdened vs 1.0%, 2.0%, and 5.0% CSN burdened BIs. Tested at 0,2,4,6, 9, and 12 hours	Included ProLine PCD COTS BIs without lumens, and modified to have foreshortened inlet (not reported)
Y	9/9/13	Low Inoculum Test on unburdened vs 1.0%, 2.0%, and 5.0% CSN burdened BIs. Tested at 0,6, 9, and 12 hours	
Z	01/28/14	Low Inoculum Test on unburdened vs 1.0% and 2.0% CSN burdened BIs. Tested at 0, 6000, 9000, and 12000 ppm* hours. Included inoculated coupons of carpet, wood, and aluminum	Fumigated with 2000 ppm ClO_2
AA	03/05/14		Fumigated with 1000 ppm ClO_2
AB	03/20/14		Fumigated with 500 ppm ClO_2

2.1.2 Task 2: Testing of BI Carrier Materials

In this task, several carrier materials, unrelated to those used in Task 1, were evaluated for their compatibility with surrogate organisms and their ability to increase the CT required for BI inactivation. The number of replicates per material type and indicator organism is shown in Table 2-5. Under this task, carriers of 11 material types were used for the custom BIs (Table 2-6). Criteria for choosing carrier materials included being commercially available with relatively uniform size, shape, porosity; being of a size amendable to packaging within a Tyvek® envelope; and being of a material type that is easily sterilized.

Carrier materials were inoculated with 1 x 10^6 spores (either *B. atrophaeus* or *G. stearothermophilus*), and placed into Tyvek® envelopes. The resulting custom BI test samples were challenged by fumigation with 1,000 ppmv gaseous ClO_2 at 75% RH and 24 °C in the DTRL. The test samples and controls (negative, positive, and performance controls, discussed further in Section 2.1.3) were collected at 5, 7, and 9 hour exposure times.

Table 2-5. Number of Replicates per Experimental and Control BI Type for Task 2

Sample	Time Point (hours)	Replicates	Total	Test/Control	BI Inoculated	Media Spiked	Fumigated
Performance Baseline	0	3	3	C	No	Yes	No
Positive Control	0	3	3	C	Yes	No	No
Performance Control	5,7,9	3	9	C	No	Yes	Yes
Experimental	5,7,9	5	15	T	Yes	No	Yes

Note: 30 BIs * 10 carrier materials (including stainless steel) * 2 organisms = 600 Custom BIs, + 3 negative control BIs.

Table 2-6. Material Test

Test	Date	Materials	Spores	Time Points
B	2/3/2010	10 types (see Table 2-7)	BG, GS	5, 7, and 9 hours

2.2 Test Materials and Deposition

2.2.1 BI Preparation

The prepared BIs were stored and transported to EPA as individually packaged BIs in a Tyvek® envelope. Table 2-2 shows the burden additives and concentrations initially prepared for Task 1, Test A. Additional burdens at several concentrations were investigated for successive tests as discussed in Section 3. Casein, burden code CSN, was sourced from Sigma (P/N C7078, Sigma-Aldrich Corporation, St. Louis, MO, USA) and added starting at Test E. Table 2-7 shows the materials tested for Task 2. Figure 2-1 shows the mixture of burden and inoculum dried on the stainless steel carrier.

Figure 2-1. Stainless Steel Carrier with Burden and Inoculum

Table 2-7. Carrier Materials for Custom Biological Indicators

#	Material	Material Code	Supplier and Location	Cat #	Surface dimensions	Thickness	Inoculum location
1	Stainless Steel	SST	Yakibou* (Sanford, NC)	NA	10 mm diameter	0.19 mm	Surface
2	Wooden Discs	WOD	American Woodcrafters Supply Company (Riceville, IA, USA)	DIS-050	12.7 mm diameter	3.18 mm	Surface
3	Adhesive Felt Dots	FLT	Aetna Foot Care Products (Allentown, PA, USA)	½" Dots	12.7 mm diameter	1.6 mm	Surface
4	Adhesive Rubber Dots	RUB	Aetna Foot Care Products (Allentown, PA, USA)	016205	12.7 mm diameter	2.4 mm	Surface
5	Adhesive Cork Dots	CRK	Aetna Foot Care Products (Allentown, PA, USA)	004377	12.7 mm diameter	1.6 mm"	Surface
6	Ceramic Tile	CER	Mosaic Basics (Atlanta, GA, USA)	NA	9.5 mm x 9.5 mm	3.18 mm	un-glazed side
7	6061 Aluminum	ALU	McMaster Carr (Atlanta, GA, USA)	89015K 86	12.7 mm diameter	2 mm	Surface
8	Chipboard Discs	CBD	Wolter Pyro Tools (Montello, WI, USA)	CBD-58-16	15.9 mm" diameter	1.6 mm	Surface
9	0.2 µm pore-size Cellulose Ester	CEM	Whatman, GE Healthcare Bio-Sciences, (Pittsburgh, PA, USA)	104017 12	47 mm diameter	135 µm	Top Surface
10	C14500 Copper discs	CUP	Storm Copper Components (Decatur, TN, USA)	NA	12.7 mm diameter	2.5 mm	Surface
11	Porous Polypropyl	XYZ	Permaplas Corp. (Fayetteville, GA, USA)	20201	13.1 mm diameter	2.6 mm	Surface

* Then known as Apex Laboratories.
NA = Not applicable.

2.2.2 Spore Preparation

The different types of spore preparations used for this study are described below.

2.2.2.1 Performance Control Spore Preparation

An inoculum containing approximately 1 x 10^4 CFU mL^{-1} was used for all performance control spikes. This inoculum was a dilution of an original preparation of ATCC 9372 *B. atrophaeus* from Apex Laboratories, Lot 712691.

2.2.2.2 Burden and Material BI Spore Preparations

In general, custom BIs used an inoculum prepared and dispensed onto carriers by Yakibou. Table 2-8 shows the details of the inoculant used on burden and material BIs.

Table 2-8. Source and Lot of Inoculant used on BIs

Test	BI Batch Date	*B. atrophaeus* ATCC 9372			*G. Stearothermophilus* ATCC 12980		
		Provider	Lot	CFU Recovered	Provider	Lot	CFU Recovered
A	12/17/09	Yakibou*	2598GL	7.9×10^6	Yakibou*	0598ST	6.2×10^6
B	12/21/09	Yakibou*	2598GL	8.1×10^6	Yakibou*	0598ST	7.0×10^6
C	3/31/10	Yakibou*	2598GL	6.0×10^6	Yakibou*	0598ST	5.1×10^6
D	3/31/10	Yakibou*	2598GL	6.0×10^6	Yakibou*	0598ST	5.1×10^6
E	7/14/10	Yakibou*	2598GL	$7.6 \times 10^{6\dagger}$	Yakibou*	0598ST	4.6×10^6
F	8/30/10	Yakibou*	See note	1.0×10^7			
G	10/21/10	Yakibou*	See note	3.1×10^6			
H	12/30/10	Yakibou*	See note	4.6×10^6			
I	12/30/10	Yakibou*	See note	4.6×10^6			
J	Varies. See Section 3.9.7 for details						
O, P and Q	1/17/12	Yakibou*	2566GL	1.2×10^6			
		Mesa Labs	1073081	1.4×10^6			
R	6/6/12	Yakibou	2566GL	3.8×10^6			
S, T and U	9/25/12	Yakibou	2566GL	6.0×10^6			
V	2/21/13	Yakibou	2566GL	6.8×10^6 (1% CSN BI)			
W, X, and Y	8/14/13	Yakibou	2566GL	1.2×10^3 (1% CSN BI); 2.3×10^2 (unburdened BI) or 1.1×10^5 (1% CSN BI); 1.3×10^4 (unburdened BI) (see Section 3.9.6)			
Z, AA, and AB	1/6/14	Yakibou	2566GL	9.9×10^2 (unburdened BI), 1.4×10^3 (1% CSN BI); 1.3×10^3 (2% CSN BI)			

*Then known as Apex Laboratories.

† The dithiothreitol (DTT) samples alone were only 2×10^4 concentration following heat shock, suggesting the heat shock with the DTT either suppresses germination/growth or is lethal to the spores. With the heat shock eliminated, the concentration for the DTT samples was 3.5×10^6.

Note: The lot number was unavailable from the manufacturer, but the manufacturer did confirm that the source was American Type Culture Collection (ATCC) 9372.

The recovery (CFU) values listed are from BIs without burden unless otherwise noted. For Tests W, X, and Y, the recovery from BIs with burden was an order of magnitude higher than the recovery from BIs without burden, presumably due to more efficient spore dislodgement from the carrier during extraction.

2.2.2.3 Well Plate Spore Preparation

The spores for Test K were prepared from a serial dilution of ATCC 9372 *B. atrophaeus* spore stock solution obtained from Raven Laboratories, batch 304GB. Dilutions were made with 40% ethanol in sterile deionized water. Suspension recovery was 1.8 x 10^8 CFU/mL.

2.2.3 BI Carrier Inoculation

Custom BIs were typically inoculated by Yakibou with 20 µL of spore suspension. Carriers with burden were inoculated with 40 µL of a 50:50 mixture of burden and spore suspension. For performance control BIs, 20 µL aliquots of the most concentrated burden were pipetted onto the carriers. Carriers were typically dried 2.5 hours in a flowing air oven at 37 – 38 °C before packaging into uniquely labeled Tyvek® envelopes.

2.2.4 Control BIs

Each test included control BIs (Table 2-9) used to evaluate data quality. The negative control BIs were the same stainless steel discs used by Yakibou for BIs, not inoculated, and packaged in Tyvek® envelopes. These negative BIs were generally autoclaved before use and were not fumigated (laboratory blanks). Some negative BIs were simply BIs that were not inoculated, but not autoclaved and not guaranteed sterile from the BI vendor (field blanks). The positive control BIs were standard stainless steel BIs of each organism prepared by Yakibou. The spores were inoculated onto stainless steel discs and packaged in Tyvek® envelopes. The positive control BIs were not exposed to the fumigant. Burden tests included five replicate positive control BIs; material tests used three replicates as baseline controls, which were included in each fumigation. Turbidity control BIs included burden but no inoculum, and were incubated along with test BIs to determine if the presence of burden material could lead to a turbid result that could be misinterpreted as growth.

Performance control BIs (non-spore-inoculated carriers) were subjected to the longest fumigation duration for each test. The BIs were then aseptically placed into tryptic soy broth (TSB) and the medium was spiked (inoculated) with 0.1 mL of a ~5 x 10^3 CFU mL^{-1}solution of the target surrogate spores used during the test (either *B. atrophaeus* ATCC 9372 or *G. stearothermophilus* ATCC 19280). The inoculated tubes were then incubated for 7-9 days at the temperature most favorable for growth (35 °C ± 2 °C for *B. atrophaeus* and 55 °C ± 2° C for *G. stearothermophilus*) and afterwards visually inspected to confirm compatibility of the fumigated burden with viable spores (presence of turbid (cloudy) culture media, indicative of bacterial planktonic growth).

Table 2-9. Characteristics of Control BIs

Control BI	Information Provided	Inoculated	Fumigated	Burden
Negative Control BI (coupon or BI without biological agent)	Controls for sterility of materials and methods used in the procedure.	No	No	Yes
Positive control (BI or inoculated material not fumigated)	Shows incubation tubes ability to support and show growth	Yes	No	Yes
Turbidity Control (BI with material or burden, not inoculated but fumigated)	Provides information about the tendency towards false positives of candidate BI	No	Yes	Yes
Performance Controls	Confirms compatibility of the fumigated burden with growth of viable spores	After fumigation	Yes	Yes

2.2.5 Well-Plate Inoculation

The well plates used for Test K were sterile, polystyrene cell culture plates (Corning Incorporated P/N 3548, Corning, NY, USA). Each well was inoculated with *B. atrophaeus ranging* from 10^2 to 10^6 for the test samples or with 40% ethanol in DI water for the negative control samples and allowed to dry overnight. After fumigation, each well plate was charged with 725 µL of 3% Alamar Blue solution (P/N BUF012A, AbD Serotec, Oxford, UK).

2.2.6 Off-The-Shelf BIs

COTS BIs were used throughout the test sequence as comparisons to the custom BIs. These COTS BIs are discussed below.

2.2.6.1 Mesa Ethylene Oxide (EtO) BIs

The EtO BIs used for Test U were COTS BIs recommended for ethylene oxide gas sterilization. This BI was an 8 mm x 12 mm stainless steel oblate disc inoculated with *B. atrophaeus* ATCC 9372 spores. Batch 301GB, used for Test U, had a nominal population of 2.8 x 10^6 spores. Batch 301GBN was identical to the Raven *B. atrophaeus* (P/N 1-6100-ST) BI.

2.2.6.2 Mesa Strips

Mesa Laboratories MesaStrip BIs consisted of a 6.4 mm x 38.1 mm strip of Schleicher & Schuell filter paper (#470) inoculated with bacterial spores and sealed in a glassine envelope. For this project, two varieties of MesaStrip were used: P/N SGMG/6 were inoculated with approximately 1 x 10^6 *B. atrophaeus* spores., and P/N SGMLF/6 were inoculated with approximately 1 x 10^6 *G. stearothermophilus* spores.

2.2.6.3 Mesa Laboratories Releasat® for Chlorine Dioxide Sterilization

The Releasat® BI (P/N RCD/50) used for Test V was a 19 mm x 6.3 mm paper carrier inoculated with approximately 1 x 10^6 *B. atrophaeus* spores, sealed in a glassine envelope. The kits included culture

tubes of specially formulated soybean casein digest culture medium containing a color indicator that turns yellow in the presence of bacterial growth.

Mesa strip

Proline PCD

Releasat®

Apex *B. atrophaeus* BI

Mesa EtO BI/Raven *B. atrophaeus* BI

Figure 2-2. Commercial Off-the-Shelf BIs

2.2.6.4 Apex *B. atrophaeus* BI

The Apex *B. atrophaeus* BI was used in tests L, M, and N, and was identical to the stainless steel Yakibou BI with no burden. The Apex *B. atrophaeus* BI was an COTS product made by Apex before the sale of Apex to Raven/Mesa Laboratories. It was a stainless steel disc inoculated with approximately 1 x 10^6 spores of *B. atrophaeus,* and sealed in a Tyvek® envelope.

2.2.6.5 Raven *B. atrophaeus* BI

The Raven *B. atrophaeus* (P/N 1-6100-ST) BI used for Tests L, M, and N was a stainless steel carrier inoculated with approximately 1 x 10^6 spores of *B. atrophaeus*, and sealed in a Tyvek® envelope.

2.2.6.6 Mesa Laboratories RCT

RCT kits were manufactured by the Raven Laboratories division of Mesa Laboratories. The kits included a 19 mm x 6.3 mm strip of paper inoculated with *B. atrophaeus* strain ATCC 9372, with a mean strip recovery of 2.1 x 10^6 CFU/strip. Batch 298GB (Lot number 1S62983) was used for Test N. The kits are designed specifically for use with ClO_2 sterilization. Each kit contained 25 spore strips (as previously described) individually wrapped and 25 culture media tubes. Following testing, each strip was aseptically placed into culture media tube and incubated for seven days, then evaluated based on color/ turbidity of tube. The tubes that retained the purple color and were not turbid were considered negative for growth, and tubes that exhibited a yellow color change and were turbid were considered positive for growth.

2.2.6.7 Mesa Laboratories ProLine PCD

Mesa Laboratories ProLine Process Challenge Device (PCD) BIs consisted of a 9 mm paper disc inoculated with *B. atrophaeus* spores inside a glassine envelope. These BIs are designed to go inside a lumen or other sterilizable tubing 1.6 mm to 14.3 mm ID. These BIs were tested with various lumen lengths from 0 cm to 122 cm of 1.6 mm ID tubing. The nozzle was completely removed from the BI for Test X.

2.2.7 Building Material Coupons

For tests Z, AA, and AB, wooden, carpet, and aluminum coupons were used in conjunction with the BIs (Figure 2-3). Decontamination kinetics of these coupons (surrogates for materials inside a building) were compared to the developed custom BIs. Coupons were made of wood or carpet affixed to an aluminum stub (P/N 16119 http://www.tedpella.com/SEM_html/SEMpinmount.htm, Ted Pella, Inc. Redding, CA, USA) using a carbon based adhesive, the third material being the aluminum stub itself. Wooden coupons were prepared from commercially available 19 mm oak stair plugs (http://www.craftparts.com/oak-stair-plugs-p-3943.html?cat_id=257, Woodworks. Ltd., Haltom City, TX, USA). The original planed but unfinished surface of the wood was used as the inoculation surface. The carpet coupons were made by punching an 18 mm core from a commercial carpet square (Ultimate Temptation model, color: allurement, carpet model number 85128/695, pile height 0.64cm, Sherwin-Williams, Cleveland, OH, USA), which was glued onto the aluminum stub. The adhesive was allowed to dry for 48 hours before undergoing sterilization by a steam autoclave on a gravity cycle following NHSRC RTP Microbiology Laboratory internal Miscellaneous Operating Procedure (MOP) 6570 (Appendix B). Each sterilization batch included all coupons for a single test. Three sample coupons were analyzed for growth/no growth from each sterilization batch as an indication of sterilization efficacy.

The liquid inoculum was obtained from Yakibou, Inc. (same inoculum as used for BIs), and the inoculation of the coupons was performed by the NHSRC RTP Microbiology Laboratory. Each coupon was inoculated with 0.1 mL of the spore suspension with mean population of 5.3 x 10^6 CFU/mL distributed across the coupon and allowed to dry overnight in a biological safety cabinet (BSC) before use.

Figure 2-3. 18 mm Coupons

2.3 Fumigation Methods

Fumigation conditions were established in a glovebox (P/N 830-ABC Glovebox, PlasLabs, Lansing, MI, USA) (Figure 2-4) that permitted removal of BIs during exposure. The atmosphere in the glovebox was brought to 75% RH by injection of hot, moist air generated by a Gas Humidity Bottle (P/N HF-HBA, Fuel Cell Technologies, Inc., Albuquerque, NM, USA). Injection of the hot moist air was regulated by a feedback loop from an RH sensor (P/N HMD40Y or P/N HMD53W, Vaisala, Vandaa, Finland). Once the RH conditions were met, a ClO_2 generator (P/N GMP or Minidox, ClorDiSys Solutions Inc., Lebanon, NJ, USA) supplied fumigant until the set-point was reached, as determined by an internal photometer. This phase is called the conditioning phase. Once the target concentration is reached, the exposure phase begins.

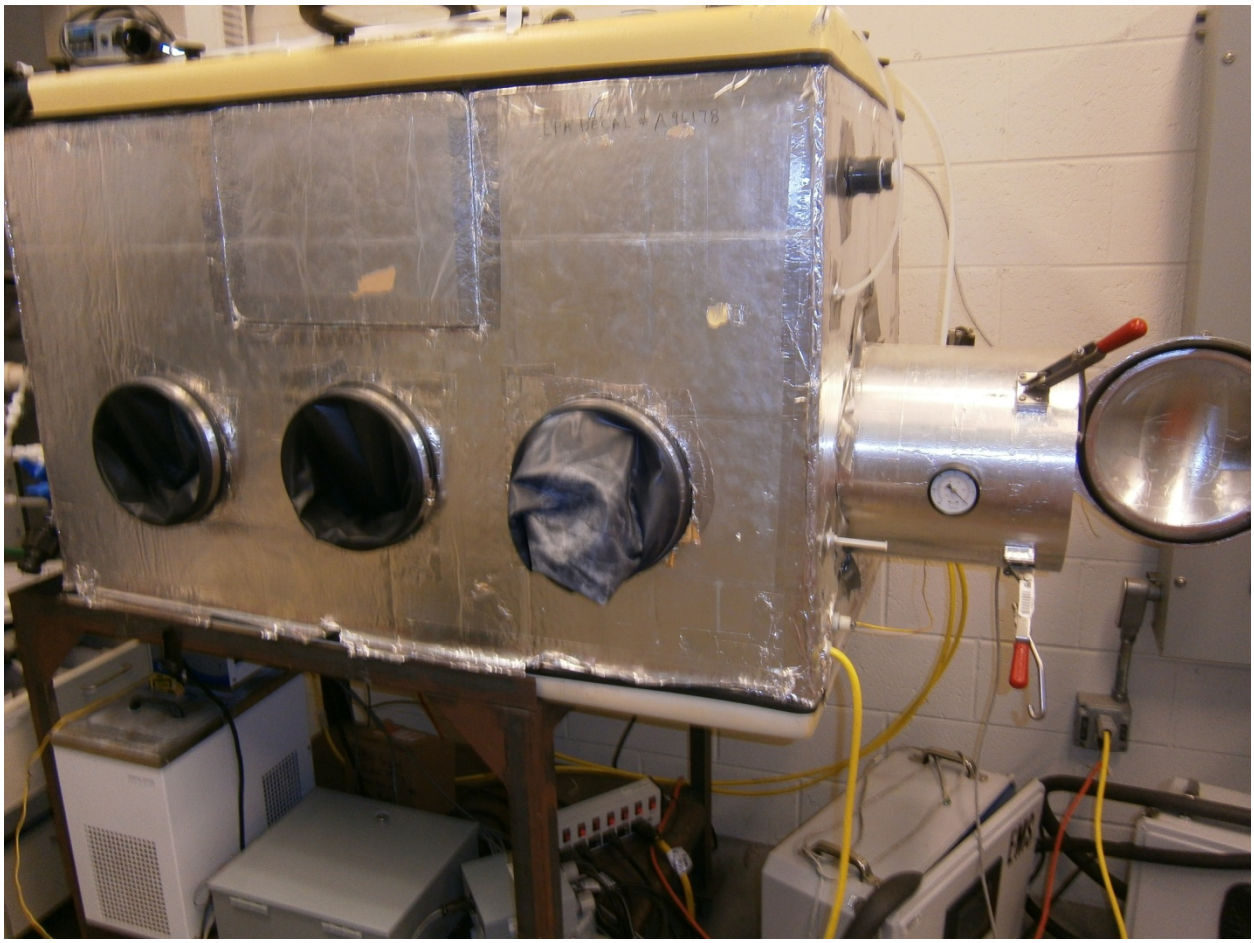

Figure 2-4. Glovebox Fumigation Chamber

For the initial tests, BIs were present in the glovebox for the conditioning phase as well as the exposure phase. For a two-hour exposure, the BIs would have been present for the ramp-up from ambient to target concentration, as well as the two-hour exposure at target concentration. Beginning with Test H and for all subsequent tests, BIs were placed in the glovebox once the target concentration was reached. This change in procedure was made to reduce the variability in exposures (exposure time, and total CT) between tests, and to achieve more precise exposures (CTs).

2.4 Sampling and Analytical Procedures

All materials needed to process the samples were prepared in the NHSRC RTP Microbiology Laboratory. These materials included, but were not limited to, growth media, culture broth, agar plates, and/or sterile liquids. Quality assurance (QA) checks (listed in Table 4-2) conducted on these materials in the NHSRC RTP Microbiology Laboratory verified that the materials were not contaminated with any organism and supported the growth of target organisms. All quality control (QC) records pertaining to these materials are retained in the NHSRC RTP Microbiology Laboratory. All personnel in the NHSRC RTP Microbiology Laboratory operate under an approved Facility Manual specific for the Lab, which contains MOPs that are relevant to this project.

2.4.1 Qualitative BI Analysis

MOPs 6560 and 6566 describe the qualitative BI analyses (all associated MOPs can be found in Appendix B). To analyze, the BIs were aseptically transferred in a Class II BSC, to 15 mL polypropylene culture tubes (P/N 169897, USA Scientific Inc., Ocala, FL, USA) containing 10 mL of tryptic soy broth (TSB). The BIs were either allowed to drop aseptically from the packaging into the TSB tubes or disposable sterile thumb forceps were used to transfer them. The tubes were incubated at the appropriate temperature for the target organism specified by manufacturer's instructions (e.g., all *B. atrophaeus* were incubated at 35 ± 2 °C, and all *G. stearothermophilus* were incubated at 55 ± 2 °C). The medium within the tubes was inspected visually for turbidity seven days later. To confirm the qualitative results and to verify that the turbidity was caused by the target organism, 10 % of the samples that were turbid were plated to confirm that the growth was from the target organism (by colony morphology). Additionally, all (100%) samples found to have No Growth were plated (0.1 mL) (to confirm that there indeed was no growth).

For tests A-I, the qualitative confirmation was completed using a sterile disposable 10 µL loop to remove a 10 µL aliquot from the TSB tube containing the BI and aseptically spread the aliquot onto a tryptic soy agar (TSA) medium plate. For Tests J-Y, the qualitative confirmation was completed by plating a 100 µL aliquot from the TSB tube containing the BI sample, directly onto TSA. All TSA plates were incubated at the proper temperature for the target surrogate, per manufacturer's instructions. Prior to removing either the 10 µL or 100 µL aliquot from the qualitative samples, some of the TSB tubes containing the BI samples (Tests J-Y), were homogenized by vortex mixing for a quick 2-5 second burst. Note that the vortexing of the samples did not have any effect on the growth of the BI samples based on data from Test J (see section 3.9.2). TSA plates were analyzed and results documented following18-24 hours of incubation at the appropriate temperature for the target organism.

2.4.2 Qualitative Well-plate Analysis

Well plates used in Test K were charged with 725 µL of TSB amended with 3 % Alamar blue solution and incubated for seven days at 35 °C ± 2 °C. The 3 % Alamar blue is a colorimetric indicator of bacterial growth, which turns pink when growth occurs or remains purple/blue in the absence of growth (Figure 2-5). The color change makes identification of growth-positive samples easier and more reliable. Alamar blue was used for the well-plate tests to aid in detecting growth, since well-plates could not be held in front of a light and inspected individually as done with culture tubes.

Figure 2-5. Well Plates with Alamar Blue Showing Growth (pink) and No-Growth (purple) Results

2.4.3 Quantitative BI Analysis

For quantitative analysis, BIs were placed in sterile 18 mm borosilicate glass tubes (Fisherbrand P/N 14-961-32, ThermoFisher Scientific, LLC. Waltham, MA, USA), containing 10 mL of Phosphate Buffered Saline with 0.05% TWEEN®20 (PBST) made according to MOP 6562 (Appendix B). BIs soaked in the PBST for a minimum of 15 minutes, as an initial procedural step. The tubes containing the BIs were then placed in an ultrasonic cleaner for three seven-minute intervals at 44 kHz ± 6 % (Bransonic Ultrasonic Cleaner, P/N 8510R-MT, Danbury, CT). The location of the tubes was changed between each seven-minute interval to increase uniform exposure of tubes. After sonication, 10 mL of PBST was removed and transferred to a 50 mL conical tube. The BI was discarded and the PBST extraction was treated with a heat shock per MOP 6576 (80 ± 2 °C for ten minutes). Following heat shock, the samples were re-homogenized by vortex. The liquid extracts were then tenfold serially diluted and spread plated according to MOP 6535a. Plates were incubated at 35 ± 2 °C for 18-24 hours.

2.4.4 Chlorine Dioxide Monitoring

ClO_2 measurements were conducted using two techniques; a ClorDiSys Solutions, Inc. photometric monitor and Standard Methods for the Examination of Water & Wastewater Method 4500-ClO_2 (mSM-4500). The first technique was used continuously for real-time control of the chamber fumigant concentration at 1,000 ppmv. The mSM-4500-ClO_2 was used periodically as a confirmation of the photometer. Because wavelengths of light in the visible and UV regions cause spontaneous breakdown of ClO_2, all sampling methods used opaque sample lines as a precaution.

2.4.4.1 Photometric Monitoring

The ClorDiSys ClO_2 monitor is a photometric system operating in absorbance mode with a fixed path cell. A pump provides flow of the test gas from the test point to the analytical cell. The maxima and minima of an unspecified, proprietary ClO_2-specific absorbance band are continuously monitored and used to calculate the absorbance. Calibration was performed by the manufacturer with National Institute of Standards and Technology (NIST)-traceable transmission band-pass optical filters and was performed in-house every six months with manufacturer reference filters. The monitor includes a photometer zero function to correct for detector aging and accumulated dirt on the lenses. Daily operation of the photometers included moments when clean, ClO_2-free air was being cycled through it. If the photometer read ≥ 0.1 mg/L during these zero air purges, then the photometer was re-zeroed. The photometer was cleaned if the concentration measurements were not within 10% of the mSM-4500 values.

2.4.4.2 mSM-4500-ClO_2

Two variations of the 1992 18th edition Standard Method (SM)-4500-ClO_2 titration were used during this testing: (1) amperometric titration (SM-4500-ClO_2-**E**) and (2) iodometric titration (SM-4500-ClO_2-**B**). The SM-4500-ClO_2 collection method has been modified (mSM) to include gas-phase sampling based upon a buffered potassium iodide bubbler sample collection, and restricting the official method to a single titration based upon analyzing the combined chlorine, ClO_2, and chlorite as a single value. This method can only be applied only where chlorine and chlorite are not present. Since the modified method described below is applied to gas-phase samples, the presumption of the absence of chlorite and chlorate is valid. The presence of chlorine would be indicated by a difference in ClO_2 concentration as measured by the photometer and titration.

The modified method was performed as follows:

a. Add 20 mL of phosphate buffer solution, pH 7.2, containing potassium iodide (KI) (KIPB solution, 25 g KI/ 500 mL of phosphate buffer) to two impingers. (P/N PRG-5795, Prism Research Glass, Inc., Raleigh, NC, USA)

b. Set ClO_2 gas flow from the chamber into the impingers containing KIPB solution in series at a flow rate of 1 L/min for two minutes.

c. Combine the 20 mL of KIPB solution from each impinger into a 200 mL volumetric flask and rinse the impingers thoroughly with deionized water.

d. Add 5 mL of 6 N hydrochloric acid (HCl) to the solution.

e. Place solution in dark for five minutes.

f. Titrate the solution with 0.1 N sodium thiosulfate. The end point is determined visually (yellow to clear for mSM-4500-ClO_2-B) or amperometrically (mSM-4500-ClO_2-E).

g. Record the volume of sodium thiosulfate titrated. Conversion calculations from titrant volume to ClO_2 concentration are based on SM 4500-ClO_2:

$$ClO_2\ (mg/L) = \frac{\text{Volume of Sodium Thiosulfate (mL)} \times N \times 13.490}{\text{Volume of Gas impinged (L)}} \qquad \text{(Eqn 2)}$$

2.5 Sampling Strategy

2.5.1 Sampling/Monitoring Points

Photometer and mSM-4500-ClO_2 samples were taken from ports in the isolation chamber. Each port from the well-mixed chamber was expected to be representative of the bulk concentration.

The RH and temperature sensors were co-located on the meter. The meter was placed far enough from the walls of the chamber to be unaffected by any difference between wall temperature and the bulk atmosphere within the chamber. A HOBO sensor was placed in the center beside the BIs. Table 2-10 details the parameters for the monitoring methods.

Table 2-10. Monitoring Methods

Monitoring Method	Sampling Flow Rate	Measurement Range	Measurement Frequency and Duration
Photometer	5 Lpm nominal	50 -10,000 ppmv ClO_2	Real-time; six per minute
mSM-4500-ClO_2	0.5 Lpm	36 -10,000 ppmv ClO_2	Every 30 – 60 minutes; four minutes each
Vaisala RH/ Temperature Meter	NA	0 -100% RH, -40 °C to 60 °C	Real-time; six per minute
K-type thermocouple (Omega Engineering, Stamford, CT, USA) for chamber and coolant temperature	NA	-200 °C to 1350 °C	Real-time; six per minute
HOBO RH/ Temperature Sensor	NA	5 - 95% RH, 0-100 °C	Real-time, three per minute
BIs	NA	0 to >1 x 10^6 spores	Growth/No Growth determinations Viable population evaluated at end of experimentation as compared with time 0

Table 2-11 lists the critical and non-critical measurements for each sample.

Table 2-11. Critical and Non-Critical Measurements

Sample Type	Critical Measurements	Non-critical Measurement
mSM-4500-ClO_2	Collected gas volume, titrant volume	Temperature, collection time
Fumigation Conditions	RH, temperature, photometric ClO_2 reading	
BIs	Exposure time, proximity*, lumen length*, vortexed or not vortexed*	

* Measurements critical for specific tests only.

2.6 Sampling Handling and Custody

2.6.1 Preventing Cross-Contamination

Cross-contamination of BIs during fumigation was prevented by the Tyvek® or glassine (manufacturer dependent) envelopes that enclosed each BI. Samples were also separated and organized by attachment to 12.7 mm diameter stainless steel springs.

Each extractive ClO_2 sample was placed in its own sample jar. Glassware was triple-rinsed with deionized water before reuse.

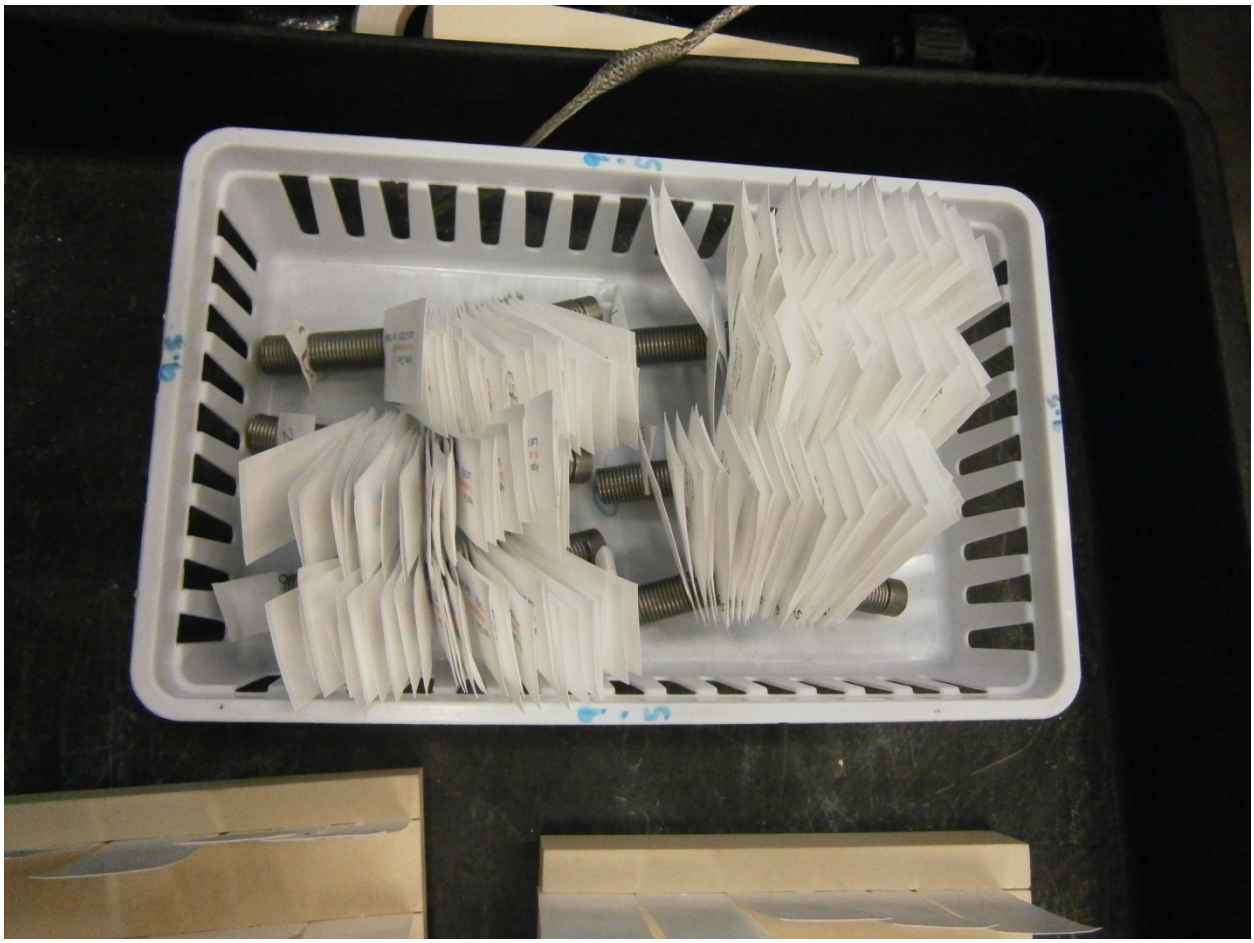

Figure 2-6. Springs used to Organize BIs

All work involving the growth/culturing and analysis of the samples was performed while using the proper personal protective equipment (PPE). Processing and analysis was completed within the confines of a BSC. During transfer of the samples from the Tyvek® envelopes to sterile tubes containing the culturing broth, aseptic technique was used to prevent contamination or cross-contamination of samples. To prevent any form of cross-contamination, all *B. atrophaeus* samples were manipulated separately from *G. stearothermophilus* samples. Prior to any analysis or processing, the workspaces were cleaned and made free of debris. The BSC was thoroughly cleaned by wiping surfaces in the following order: pH-adjusted bleach (pAB), deionized water, and a 70-90 % solution of denatured ethanol. The BSC was cleaned in this manner before work began, after each use, between sample sets involving different species of bacteria, and any other time in which a contamination event was suspected to have occurred. An ultraviolet (UV) light was used in the BSC for decontamination only after all work was completed and no personnel were working in the immediate area to prevent exposure to UV light. All biological waste material that was accumulated from the processing and analysis was properly disposed of in order to prevent possible contamination.

Samples were manipulated in the following order to prevent any form of cross-contamination: negative controls (lowest concentration of bacteria), sample sets (all unknown and variable concentrations of bacteria), positive controls (highest concentration of bacteria). Samples that were visually identified as No

Growth were streak-plated to confirm the absence of growth prior to the streak-plating of any samples positive for growth. Every BI that was incubated for qualitative analysis was placed in a new sterile, disposable tube. In addition, all glass borosilicate tubes used for quantitative analysis and extraction were used only once and then disposed of to ensure no cross-contamination occurred between samples.

2.6.2 Sample Containers

Tyvek®-wrapped BIs provided by the vendors were delivered by the manufacturer in plastic bags containing silica desiccants. These bags served as the sample containers until analysis. BIs were segregated by organism and stored in a stable indoor humidity-, light-, and temperature-controlled secondary containment. ClO_2 extractive samples were typically processed immediately and hence were not stored in a container. Coupons were aseptically placed in a 50 mL conical tube and transported to the NHSRC RTP Microbiology Laboratory for analysis.

2.6.3 Sample Identification

Each BI, coupon or sample was identified by a unique sample ID that was documented in an explicit laboratory log that included records of its associated test number, inoculum level, sampling method, and the date sampled. Each BI was marked with the material descriptor and unique code number. Sample IDs included descriptors, for project number (WA 51), test ID, inoculum type, burden type, burden concentration, material type, sample purpose (test, control, field blank, etc.) and replicate number as applicable. Once samples were transferred to the NHSRC RTP Microbiology Laboratory for microbiological analysis, each plate was additionally identified by replicate number and dilution. The NHSRC RTP Microbiology Laboratory also included on each plate the date it was placed in the incubator.

2.6.4 Information Recorded by DTRL Personnel

DTRL personnel were responsible for recording data collected during the fumigation such as sample volumes, titration volumes, and other data used to characterize the fumigation conditions. Field personnel also recorded the times that BIs were exposed and removed from the fumigation chamber.

2.6.5 Sample Preservation

BIs were placed inside a permeable envelope that allows penetration of the fumigant but prevents movement of microorganisms from the outside to the inside, and vice versa, thereby preserving the BI from contamination. Before use, BIs were stored in packaging containing desiccant, preventing hydration of the spores. After exposure or use, BIs were stored under ambient laboratory conditions before analysis.

2.6.6 Sample Holding Times

After sample collection for a single test was complete, all biological samples were transported to the NHSRC RTP Microbiology Laboratory immediately, with appropriate chain of custody (COC) form(s). Samples of other matrices were stored no longer than five days before the primary analysis. Typical hold times, prior to analysis, for most biological samples was ≤ two days. ClO_2 extractive samples were typically processed immediately and hence were not stored.

2.6.7 Sample Custody

Careful coordination with the NHSRC RTP Microbiology Laboratory was required to achieve successful transfer of uncompromised samples in a timely manner for analysis. Test schedules were confirmed with the NHSRC RTP Microbiology Laboratory prior to the start of each test. Accurate records were maintained whenever samples were created, transferred, stored, analyzed, or destroyed. The primary objective of these procedures was to create a written record used to trace the possession of the sample from the moment of its creation through the reporting of the results. Details of the chain of custody procedures were documented in the approved QAPP.

2.6.8 Sample Archiving

All coupons were archived for a minimum of two weeks following completion of analysis. This time allowed for review of the data to determine if any re-plating of selected samples was required. Samples were archived by maintaining the primary extract at 4 ± 2 °C in a sealed extraction vessel. Incubated BIs were not typically archived after the seven-day plating.

3 Results and Discussion

Tests results are arranged below for the sake of clarity rather than in chronological order. Test A and Test B were scoping tests designed to identify burdens or carrier materials that might modify the behavior (resistance to ClO_2) of a BI towards the target BI. Subsequent investigations consisting of a test or a series of tests provided more in-depth knowledge of the behaviors of BIs and their constituent parts.

3.1 Fumigations

All fumigations with the exception of Tests N, Z, and AB were intended to have fumigation conditions at 2.77 mg/L (1000 ppm) ClO_2 and 75% RH at 23.8 °C. The range of exposure times varied depending on the purpose of the test. Fumigations are complex operations, and are difficult to replicate. Fumigation conditions for all tests are summarized in Table 3-1.

3.2 Test A – Burden Scoping Test

The effect of both low and high concentrations (see Table 2.1) of ten different burdens on the survivability of *B. atrophaeus* BIs is shown in Table 3-2 and Figures 3-1 and 3-2.

These tests were conducted to identify burden materials that 1) increased the survival of the test organism, and 2) demonstrated a dose-dependent increase in BI survival. Burdens demonstrating dose-dependent effects on BI survival were more desirable as protection from the fumigant could be increased or decreased by altering the burden concentration. The data demonstrate that many burdens provided too much protection, resulting in 100% survival rates even at 9000 ppm*hours. Increasing concentration increased protection for three burdens: carrageenan (CAR), glutathione (GLU), and humic acid (HMA). The custom BIs that had a low concentration of CAR looked promising, and were evaluated further (Tests C and E). HMA also showed great promise, with increased protection with the increase in burden concentration. However, HMA also showed a tendency towards false positives as indicated by turbidity control BIs. The turbidity control BIs included burden but no inoculum, and were incubated along with test BIs. HMA, as well as FCL, produced an effect that was interpreted as growth, even though the BIs did not include inoculum. These materials thus demonstrated a tendency towards false positives and were rejected. The HMA BIs also seemed to interfere with the growth of low inoculum spikes of fumigated BIs, with only 19 of 24 of the spiked control samples showing growth. For these reasons, HMA was not studied further as a burden.

Similarly, the results from *G. stearothermophilus* BIs are shown in Table 3-3 and Figures 3-3 and 3-4. Overall, *G. stearothermophilus* BIs showed similar survival rates to *B. atrophaeus* BIs, with CLB, DTT, FCL, GEL, and GLU significantly increasing both organisms resistance to ClO_2. The low concentration amino acid coctail (AAC) provided protection to the *G. stearothermophilus* BI, but the reliability at lower exposure times was too low to be considered for further investigation. Similar to the *B. atrophaeus* BIs, carrageenan (CAR) provided partial protection for the *G. stearothermophilus* BIs, as did HMA.

Table 3-1. Average Conditions during Fumigations

		ClO_2 (mg/L) Titration Data		ClO_2 (mg/L) Photometer Data		RH (%)		Temperature (°C)	
Test ID	Test Date	Average	SD*	Average	SD	Average	SD	Average	SD
A	1/20/2010	2.6	0.04	2.5	0.1	75.1	0.1	23.7	0.1
B	2/3/2010	2.6	0.3	2.5	0.3	74.6	0.0	23.2	0.2
C	4/14/2010	2.8	0.4	2.8	0.2	75.1	0.8	25.8	0.3
D	5/5/2010	2.7	0.1	2.7	0.0	75.3	0.2	25.6	0.1
E	7/21/2010	2.9	0.1	NA	NA	75.4	0.3	23.9	0.1
F	9/15/2010	3.0	0.1	2.8	0.1	75.2	0.2	24.0	0.1
G	11/10/2010	2.8	0.1	2.7	0.2	75.1	0.1	22.6	0.1
H	2/22/2011	2.7	0.1	2.6	0.1	58.0	0.3	23.8	0.1
I	3/8/2011	2.7	0.3	2.5	0.3	75.5	1.0	23.5	0.5
J	4/26/2011	2.7	0.0	2.8	0.1	75.6	1.8	23.8	0.1
K	5/24/2011	2.8	0.1	2.8	0.1	75.1	0.1	23.9	0.1
L	8/1/2011	2.9	0.1	2.9	0.1	75.3	0.3	24.8	0.2
M	9/21/2011	2.8	0.1	2.9	0.1	75.1	0.1	24.1	0.04
N	11/1/2011	0.7	0.1	0.6	0.0	73.8	0.4	24.1	0.1
O	2/27/2012	3.2	0.5	3.0	0.3	74.2	0.2	23.8	0.2
P	3/12/2012	2.8	0.1	2.5	0.1	75.5	0.7	23.8	0.1
Q	3/20/2012	2.9	0.1	2.8	0.5	75.2	0.4	23.8	0.1
R	7/24/2012	3.0	0.1	3.1	0.1	75.3	0.0	24.0	0.0
S	10/9/2012	2.9	2.9	2.9	0.1	75.1	0.0	23.8	0.1
T	10/30/2012	2.9	2.9	2.9	0.1	75.1	0.1	23.2	0.3
U	1/22/2013	2.9	0.1	2.7	0.1	75.0	0.0	23.7	0.2
V	3/13/2013	2.7	0.1	2.8	0.1	75.0	0.1	23.7	0.3
W	8/20/2013	2.5	0.1	2.4	0.1	75.1	0.0	22.9	0.2
X	8/27/2013	2.8	0.2	2.8	0.2	75.4	0.5	23.5	0.4
Y	9/9/2013	2.9	0.1	2.8	0.1	75.0	0.2	23.7	0.1
Z	1/28/2014	5.7	0.5	5.5	0.2	73.9	2.4	23.6	0.3
AA	3/5/2014	2.8	0.1	2.8	0.1	75.4	0.5	20.5	0.7
AB	3/19/2014	1.5	0.3	2.1	0.4	74.1	2.8	23.8	0.0

*Standard deviation.

Table 3-2. Survivability of *B. atrophaeus* BIs with Burdens

	Low Concentration Burden			High Concentration Burden		
ClO_2 ppm*hours (nominal)	**5000**	**7000**	**9000**	**5000**	**7000**	**9000**
Amino Acid cocktail (AAC)	20%	0%	20%	0%	0%	0%
Alginate (sodium salt) (ALG)	0%	0%	0%	0%	40%	0%
Carrageenan (CAR)	100%	80%	20%	100%	100%	80%
Cellobiose (CLB)	100%	100%	100%	100%	100%	100%
Dimethyl Sulfoxide (DMS)	0%	0%	0%	0%	0%	0%
Dithiothreitol (DTT)	100%	100%	100%	100%	100%	100%
Ferrous Chloride (FCL)	100%	100%	100%	100%	100%	100%
Gelatin (GEL)	100%	100%	100%	100%	100%	100%
Glutathione (GLU)	100%	100%	80%	100%	100%	100%
Humic Acid (sodium salt) (HMA)	20%	0%	0%	100%	100%	100%

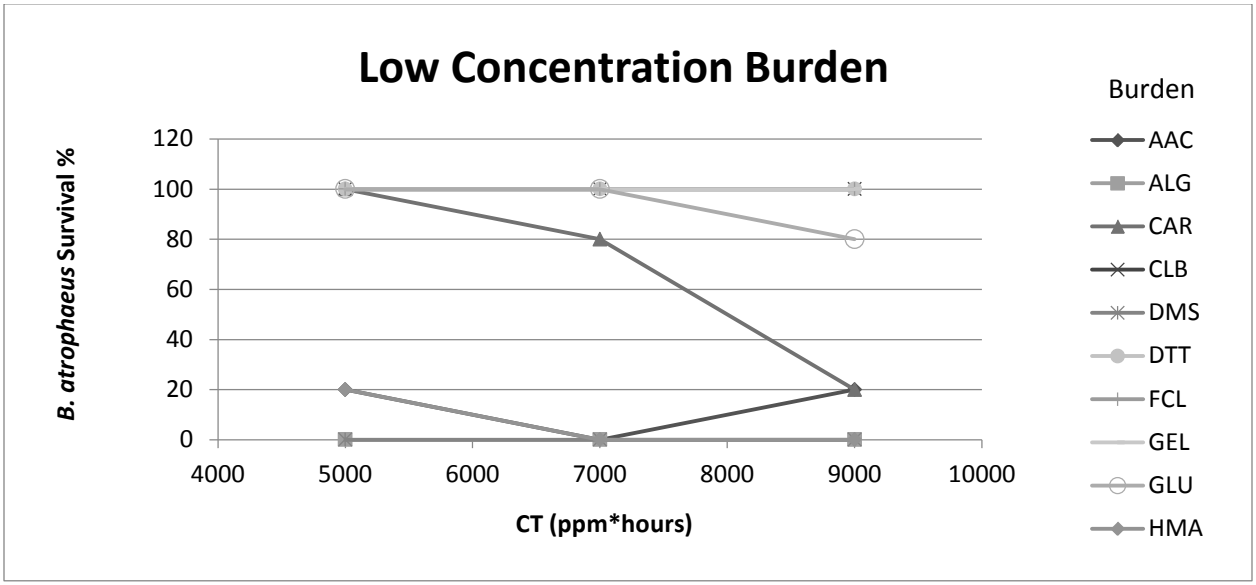

Figure 3-1. Survival (1000 ppm ClO_2) of *B. atrophaeus* BIs (n=5) with Low Concentration Burdens

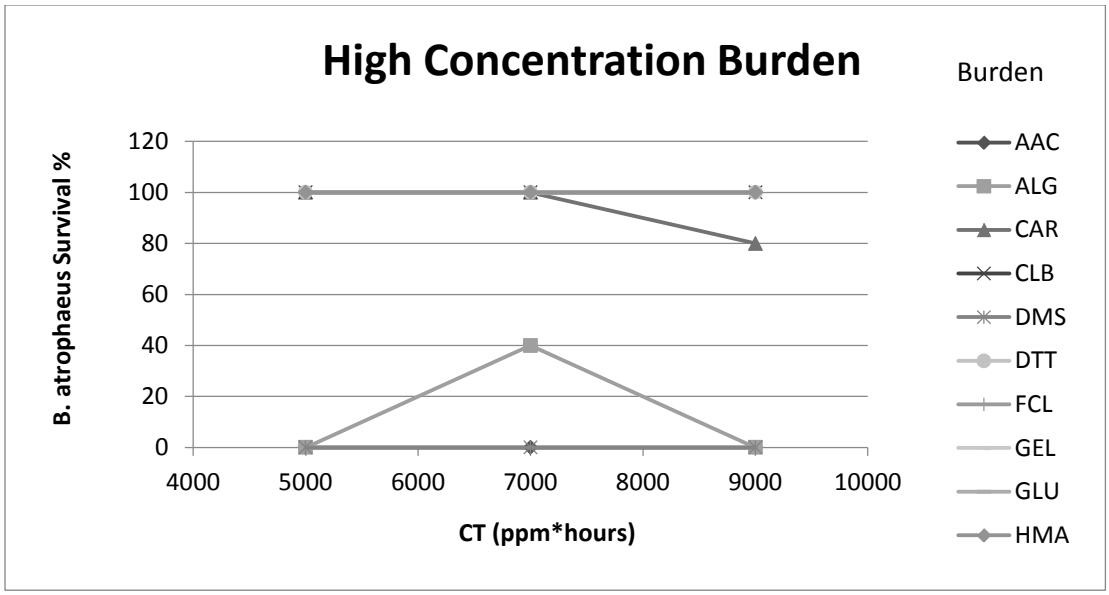

Figure 3-2. Survival (1000 ppm ClO_2) of *B. atrophaeus* BIs (n=5) with High Concentration Burdens

Table 3-3. Survivability of *G. stearothermophilus* BIs with Burdens

	Low Concentration Burden Survival Rate (%)			High Concentration Burden Survival Rate (%)		
ClO_2 ppm*hours (nominal)	**5000**	**7000**	**9000**	**5000**	**7000**	**9000**
Amino Acid cocktail (AAC)	20	40	0	100	100	100
Alginate (sodium salt) (ALG)	0	0	0	0	0	20
Carrageenan (CAR)	60	0	40	100	60	80
Cellobiose (CLB)	100	100	100	100	100	100
Dimethyl Sulfoxide (DMSO)	0	0	0	0	0	0
Dithiothreitol (DTT)	100	100	100	100	100	100
Ferrous Chloride (FCL)	100	100	100	100	100	100
Gelatin (GEL)	100	100	100	100	100	100
Glutathione (GLU)	100	100	100	100	100	100
Humic Acid (sodium salt) (HMA)	100	80	20	100	100	100

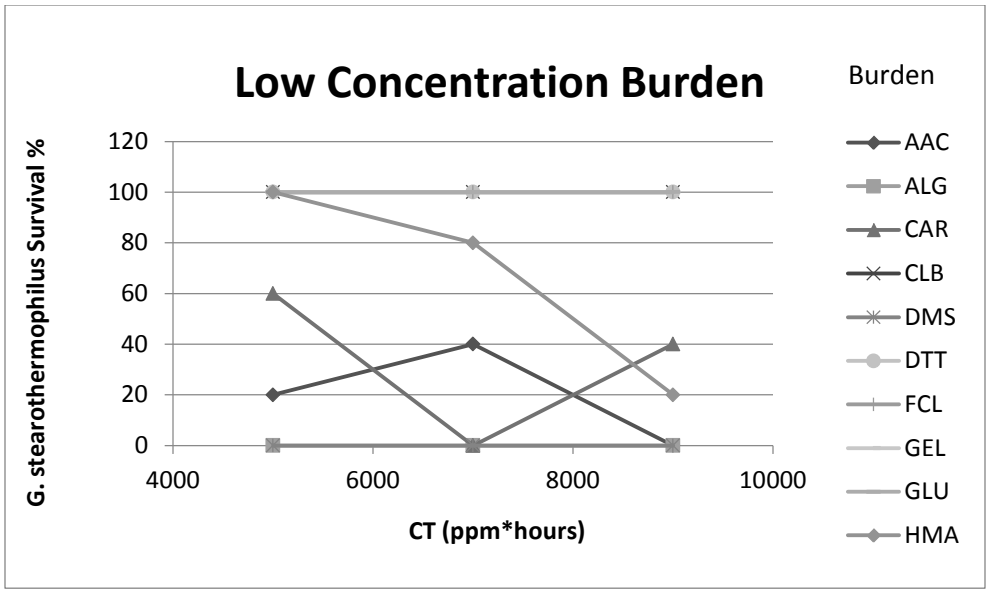

Figure 3-3. Survival (1000 ppm ClO_2) of *G. stearothermophilus* BIs (n=5) with Low Concentration Burdens

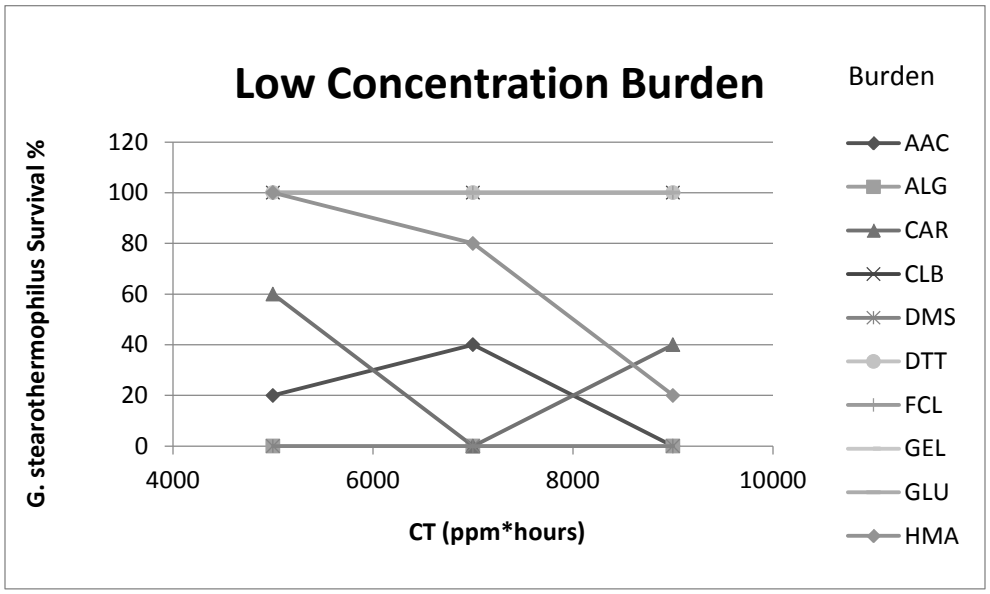

Figure 3-4. Survival (1000 ppm ClO_2) of *G. stearothermophilus* BIs (n=5) with High Concentration Burdens

3.3 Test B – Material Scoping Test

Table 3-4 shows the survival rates of *B. atrophaeus* and *G. stearothermophilus* on carrier materials.

Table 3-4. Survival rates of BIs based on Carrier Material

	B. atrophaeus Survival Rate (%)			*G. stearothermophilus* Survival Rate (%)		
ClO_2 ppm*hours (nominal)	**5000**	**7000**	**9000**	**5000**	**7000**	**9000**
CBD- Chipboard	0	0	0	0	20	0
CEM – CE Membranes	0	0	0	0	0	0
CER- Ceramic Tile	0	0	0	0	0	0
CRK – Adhesive Cork	0	0	0	40	20	20
CUP- C14500 Copper	0	0	0	0	0	0
FLT – Adhesive Felt	20	0	0	0	0	0
RUB- Adhesive Rubber	100	100	100	100	100	100
SST- Stainless Steel	0	0	0	0	0	0
WOD- Wooden Discs	0	0	0	20	0	0
XYZ - Porous Polypropyl	0	0	0	40	40	0

Rubber provided complete protection to both spore types, and was thus unsuitable for further study. Porous polypropyl (XYZ) provided partial protection to *G. stearothermophilus* BIs, but survival rates at 5000 ppm*hours were considered too low for follow-up. Subsequent testing was performed on stainless steel carriers only.

All tests included control BIs (see Section 1.3.1) to validate the test methods. The performance controls, a type of control BI, were fumigated for the entire duration of the test, placed in extraction fluid, and then the extraction fluid was spiked with spores of the target organism. Fumigated wooden BIs of this type would not support growth of spiked *B. atrophaeus* or *G. stearothermophilus* spores, thereby indicating a tendency towards false negative results. Wooden BIs would thus require different laboratory methods. Because wood is a natural material with natural variation, and is often treated prior to consumer usage, different laboratory methods might be necessary for each batch to avoid false negative results. Based on these data, wood would not be a viable carrier.

Overall, the data suggest that finding a suitable carrier material to develop the target BI successfully was not likely, and the quest for a suitable carrier material using this approach should be discontinued. Lower protection factors of the materials (as compared to the burden approach) and the inability to adjust the protection were the two leading reasons for abandoning this approach. The results do suggest that decontamination of rubber materials may be extremely difficult, as 100 % of the BIs survived all exposure points, for both organisms tested.

3.4 Barrier Investigation

During Test K, physical barriers instead of burdens were tested to determine if physical barriers could be used to provide protection to spores dried in micro-titer plate wells. Five levels of inoculum, from 100 CFU/micro-titer plate to 1 x 10^6 CFU/micro-titer well plate were tested. Three barriers were used: one layer of Breathe-Easy (Diversified Biotech, Dedham, MA, USA), two layers of the Breathe-Easy membrane, and 1 layer of NuFab (DuPont, Wilmington, DE, USA, discontinued product). The negative control samples on the Breath-Easy well plates showed growth, so results from the Breathe-Easy are not reported due to data quality concerns. Figure 3-5 shows the survival rates for micro-titer plates protected by the 1 layer of NuFab.

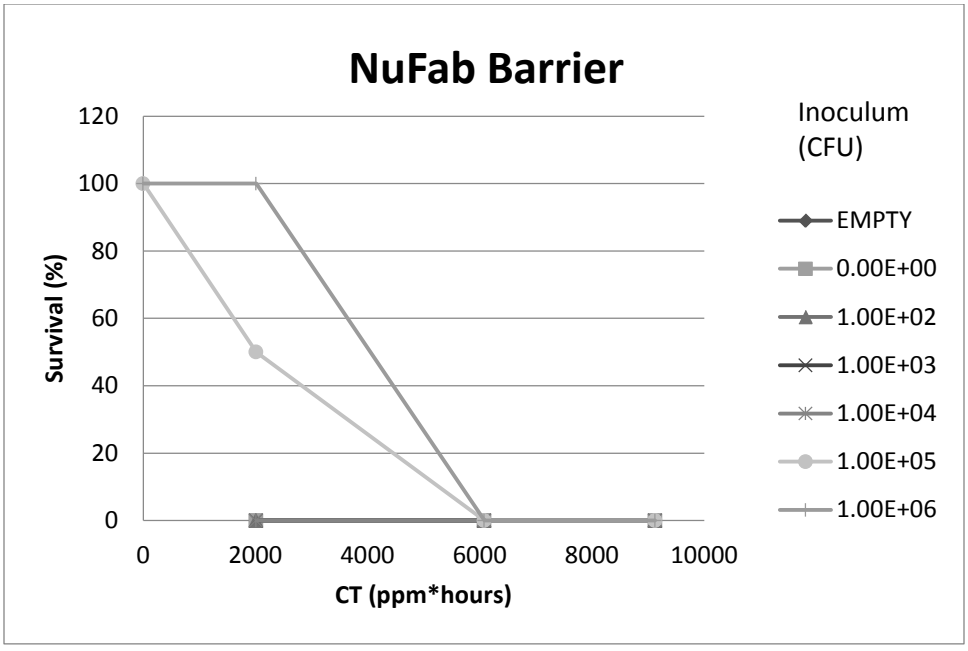

Figure 3-5. Survival (1000 ppm ClO_2) for *B. atrophaeus* Protected by NuFab Barrier (Test K) (n=8)

While lower inocula did not survive even short fumigation exposure, the higher two inocula showed some protection by the NuFab barrier. The 1 x 10^6 inoculum showed the desired response curve, but complete kill was achieved at a time point earlier than the target nine hours. Follow-up tests could not be performed because the product had been discontinued.

3.5 COTS BI Comparisons

None of the COTS BIs tested showed promise as an ideal BI candidate for the purposes of this study (i.e., inactivation following exposure to 9000 ppm*hours ClO_2). The sections below give detailed results.

3.5.1 COTS Yakibou *B. atrophaeus BIs*

The D-value evaluation of COTS Yakibou BIs (Test K) was inconclusive, with only two time points showing fractional survival rates. All BIs survived the longest fumigation exposure time (five hours), in contrast to previous tests and studies that showed kill points in the first few hours of exposure.

3.5.2 Mesa EtO BI

Test U included an investigation of the Mesa EtO BI as compared to the COTS Yakibou *B. atrophaeus* BI. Survival rates are shown in Figure 3-6.

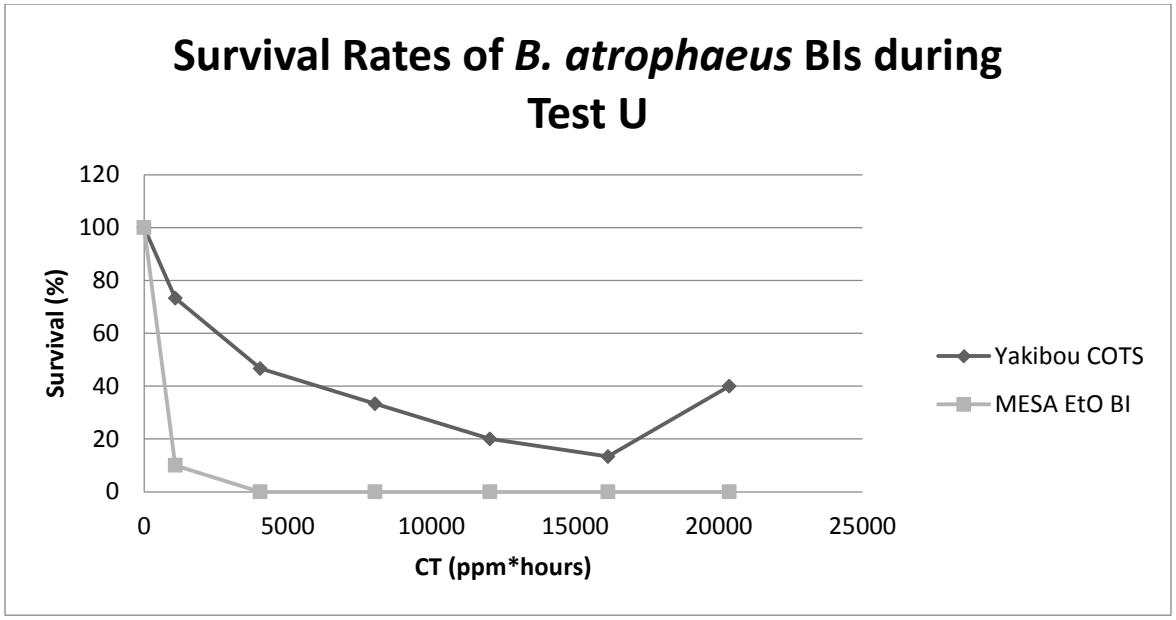

Figure 3-6. Survival (1000 ppm ClO_2) of Two COTS BIs (Test U) (n=30)

Test U suggested the Yakibou BI was hardier than the Mesa EtO BI. The Yakibou COTS BI was so hardy that it showed a 40% survival rate after 20 hours at 1000 ppmv, or 20,000 ppm*hours.

3.5.3 RCT B. atrophaeus BIs

RCT BIs performed very similarly to the stainless steel Raven BI. The results are discussed in Section 3.7 where the CT investigation results are discussed.

3.5.4 MesaStrip and Releasat® BIs

Three COTS BIs were fumigated during Test V. The survival rates are shown in Figure 3-7.

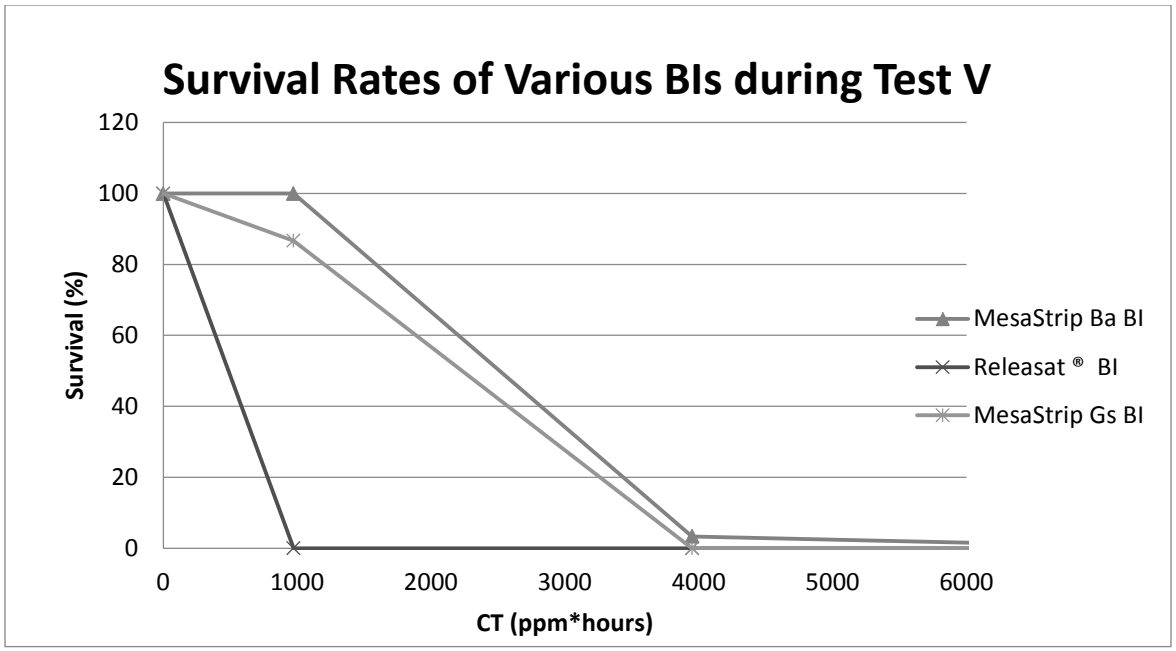

Figure 3-7. Survival (1000 ppm ClO_2) of 3 Commercially-Available BI types (Test V) (n=30)

None of the COTS BIs used in Test V are representative of the ideal BI. Survival rates are very low at four hours (4000 ppm*hours), whereas the ideal BI would have a kill point close to the 9000 ppm*hour mark. There are large differences in the ability of the BI to survive a one-hour fumigation, with the two paper strip BIs showing more hardiness.

3.5.5 ProLine PCD BIs

ProLine PCD BIs, fumigated in Test W, were very resistant to ClO_2 fumigation and may not show promise as a surrogate BI. Survival rates are shown in Figure 3-8.

Based on the limited data from the 6000 and 8000 ppm*hour marks, the lumens did provide some protection to the BI. The approach of physical barriers using lumens could be further investigated with a less hardy organism. Interestingly, this BI, though similar to MesaStrip BG BIs, was much hardier, possibly due to the spore preparation. There was no difference in the behavior of the removed nozzle BI (see Table 2-4) and the original BI as evaluated in Test X.

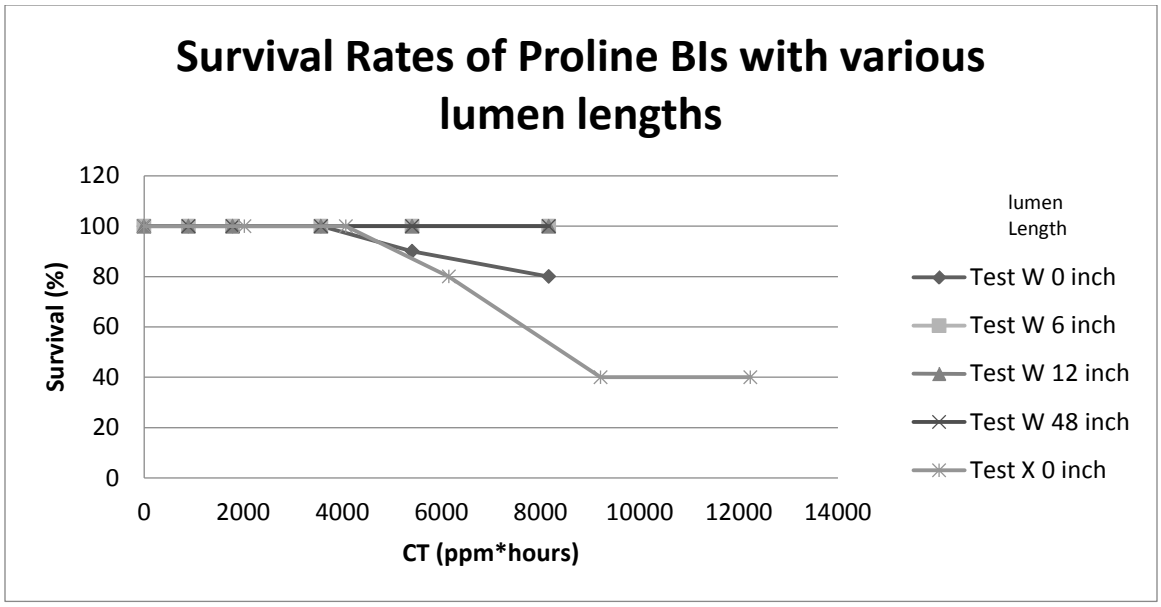

Figure 3-8. Survival (1000 ppm ClO_2) of Proline BIs (n=10) with Various Lumen Lengths

3.6 Proximity Investigation

Test L was conducted to determine if placing BIs very close together biased survivability. While the distance between the closest BIs (0.5 mm) was much larger than the mean free path of the gas molecules, there was some concern that a demand for the fumigant by the BI itself or the BI packaging could create a localized minimum in gas concentration. Thirty (30) COTS BIs of two types (Apex BG and Raven BG) were placed in two configurations: one that had all BIs packed closely together (0.5 mm apart) and one that had one cm between BIs. The results are shown in Table 3-5. Figure 3-9 shows the results of the Apex BI.

Most Raven BIs were deactivated, even after one hour of exposure, leaving no basis for determining the effect of the proximity of other BIs. The Apex BIs resulted in fractional kill data, which are more suited for D-value evaluation. Survival rates at one- and two-hour exposures were very similar for both BI configurations, suggesting that the close proximity of other BIs did not provide any protection. D-values, shown in Figure 3-10, also suggest no difference between the two configurations.

3.7 CT Investigation

The concentration-time (CT) investigation tested the survival rate of two types of BIs (Apex *B. atrophaeus* and Raven *B. atrophaeus*) after exposure to ClO_2 at common CTs but at two fumigant concentrations, 1000 ppmv and 250 ppmv ClO_2. Mesa Laboratories RCT culture test kits were also tested at 250 ppmv ClO_2. The exposure times and resulting CT for the two fumigations are shown in Tables 3-7 and 3-8.

Table 3-5. Survival (1000 ppm ClO_2) of Apex and Raven BIs (n=30) Placed Close (compact) and Widely Dispersed

BI Type	Distance Between BIs	Hours Exposed	No. Surviving	Survival (%)	Maximum Survival Rate (%)	Paired t-test p value
Apex	0.5 mm	1	17	57	57	0.14
		2	4	13	13	
		3	5	17	17	
		4	2	7	7	
		5	0	0	3	
		6.5	1	3	3	
	10 mm	1	16	53	53	
		2	4	13	13	
		3	0	0	7	
		4	0	0	7	
		5	2	7	7	
		6.5	0	0	0	
Raven	0.5 mm	1	1	3	3	0.50
		2	0	0	3	
		3	0	0	3	
		4	1	3	3	
		5	0	0	3	
		6.5	1	3	3	
	10 mm	1	1	3	3	
		2	1	3	3	
		3	0	0	3	
		4	1	3	3	
		5	0	0	0	
		6.5	0	0	0	

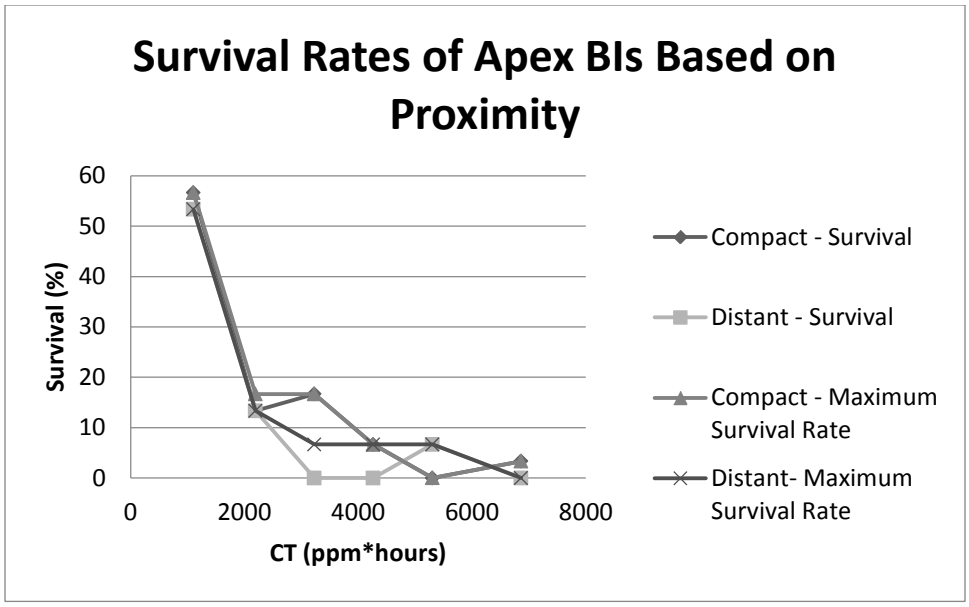

Figure 3-9. Survival (1000 ppm ClO_2) of Apex BI (n=30) Based on Proximity to Other BIs (Test L)

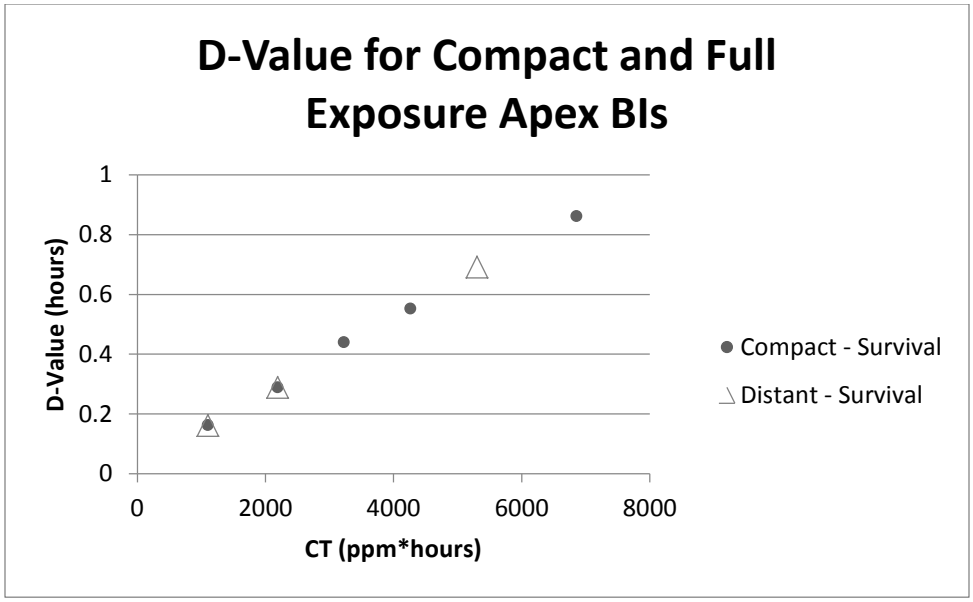

Figure 3-10. D-Value (1000 ppm ClO_2) for *B. atrophaeus* BIs from Proximity Investigation (Test L)

Table 3-6. Concentration*Time Values and Survival Rates for Test M (1000 ppmv ClO_2)

BI Type	Minutes Exposed	Nominal ppm*hours	No. Surviving	Survival (%)	Maximum Survival Rate (%)
Apex	0	0	10	100	100
	15	250	30	100	100
	30	500	29	97	97
	45	750	26	87	87
	60	1000	22	73	73
	90	1500	8	27	27
	120	2000	3	10	13
	180	3000	3	10	13
	240	4000	0	0	13
	300	5000	1	3	13
	360	6000	4	13	13
Raven	0	0	10	100	100
	10	167	16	53	53
	20	333	2	7	7
	40	667	0	0	3
	60	1000	1	3	3
	90	1500	0	0	3
	120	2000	0	0	3
	180	3000	0	0	3
	240	4000	0	0	3
	300	5000	0	0	3
	360	6000	1	3	3

Table 3-7. Concentration*Time Values and Survival Rates for Test N (250 ppmv ClO_2)

BI Type	Minutes Exposed	Nominal ppm*hours	No. Surviving	Survival (%)	Maximum Survival Rate (%)
Apex	60	250	30	100	100
	120	500	30	100	100
	180	750	22	73.3	73.3
	240	1000	23	76.7	76.7
	300	1250	12	40	40
	360	1500	9	30	30
	480	2000	5	16.7	16.7
Raven	10	42	30	100	100
	20	83	28	93.3	93.3
	30	125	20	66.7	66.7
	60	250	6	20	20
	120	500	2	6.7	13.3
	180	750	1	3.3	13.3
	360	1500	4	13.3	13.3
	480	2000	1	3.3	3.3
RCT	10	42	30	100	100
	20	83	30	100	100
	30	125	30	100	100
	40	167	25	83	83
	60	250	10	33	33
	120	500	2	6.7	6.7
	240	1000	0	0	0
	480	2000	0	0	0

The results of these two fumigations are shown in Figure 3-11.

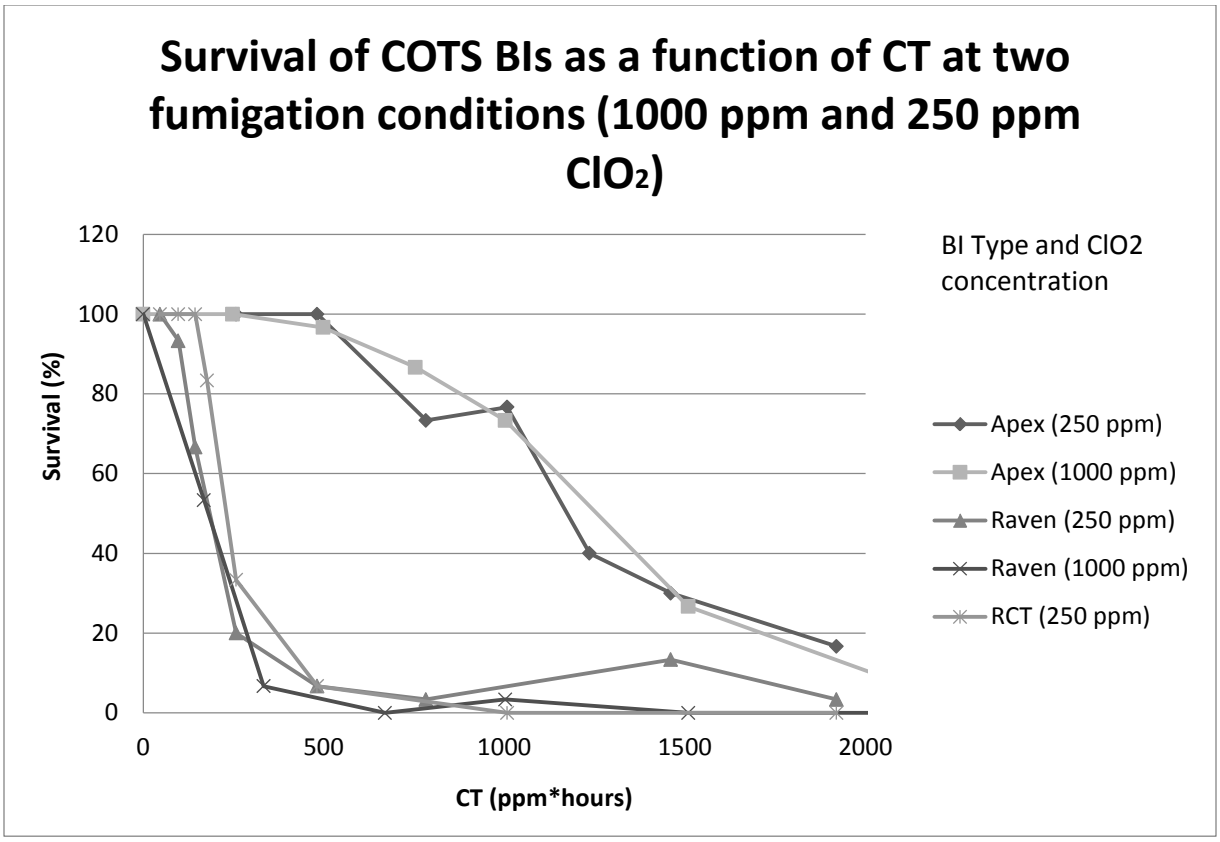

Figure 3-11. Survival of BI (n=30) Types at Various CT Values (Tests M and N)

These two tests showed similar response of BI survival rates based on CT exposure for both BI types, tested at the two concentrations rather than exposure time. The results suggest that kill kinetics are exposure-dependent, not concentration or time (alone) dependent. Figure 3-11 also demonstrates the difference between different manufacturers of BIs, with Apex *B. atrophaeus* BIs being significantly hardier than BIs from Raven or Mesa Laboratories (RCT). The cause of the hardiness of the Apex *B. atrophaeus* BIs is unknown.

3.8 Burden Investigation

Many burdens were evaluated on *B. atrophaeus* and *G. stearothermophilus* BIs. The following sections describe the fumigation process and the results of each burden.

3.8.1 Cellobiose

The effect of cellobiose (CLB) burden was tested on both *B. atrophaeus* (Tests A, D, E, F, G) and *G. stearothermophilus* (Test A, D, E) BIs. Burden concentrations ranged from 0.10 % to 11.3 %.

Survival rates of the *G. stearothermophilus* BIs with CLB concentrations lower than 0.5 % are shown in Figure 3-12. All higher concentrations exhibited 100 % survival rates. Not all time points were tested at all concentrations.

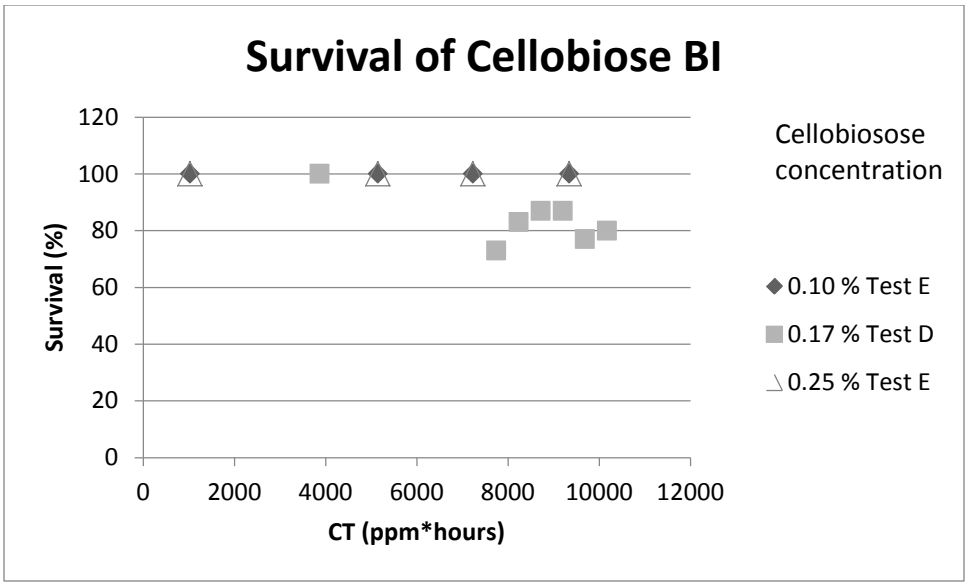

Figure 3-12. Survival (1000 ppm ClO_2) of *G. stearothermophilus* BIs with CLB Burden Test D (n=30) and Test E (n=10)

The ideal BI would show high survival rates at five- and seven-hour exposures but poor survival rates at nine hours. A BI with the ideal BI characteristics would indicate the likelihood that *B. anthracis* spores survived a decontamination attempt. While CLB protected the BI at the seven- or eight-hour mark, as shown by the high survival rates at those time points, use of CLB did not culminate in the eventual kill by the fumigant by the nine-hour time point. CLB burden at 0.17 % provided too much protection to BIs of both types, with survival rates over 80 % even after 10,500 ppm*hours. *G. stearothermophilus* BIs with a CLB burden could be further investigated at concentrations less than 0.10 % but did not look promising based on the Test D results.

The survival rates of the *B. atrophaeus* BIs with concentrations of CLB lower than 0.25 % are shown in Table 3-8. All *B. atrophaeus* BIs with CLB burdens of 0.25 % and higher showed 100 % survival rates at all time-points.

Concentrations of CLB below 0.05% did not provide enough protection for the *B. atrophaeus* BI to survive even one hour of fumigation during Test G. The results from fumigation G indicate that CLB must be more than 0.05 % to survive seven hours of fumigation. Test E demonstrated that 0.1 % CLB provided too much protection, even with the inadvertent spike in ClO_2 concentration experienced during this test.

However, CLB BI data from Test G are not consistent with Test E or Test F.

CLB is not recommended as a candidate burden due to the inconsistencies seen in Test G and the lack of sensitivity to fumigation time.

Table 3-8. Survival Rates of *B. atrophaeus* BIs with Less Than 0.25% CLB burden

	0.005% CLB	0.01% CLB	0.05% CLB		0.06% CLB	0.07% CLB	0.10% CLB		0.17% CLB
Hours Exposure	**Test F**	**Test F**	**Test G**	**Test F**	**Test G**	**Test G**	**Test G**	**Test E**	**Test D**
1	0 %	0 %	0 %	50 %	20 %	40 %	20 %	100 %	NA
4	NA	NA	NA	NA	NA	NA	NA	NA	100 %
5	0 %	0 %	0 %	20 %	0 %	0 %	20 %	100 %	NA
7	0 %	0 %	0 %	30 %	10%	0 %	0 %	100 %	NA
8	NA	NA	0 %	NA	5%	0 %	0 %	NA	97 %
9	0 %	0 %	5 %	10 %	5%	5%	5 %	90 %	100 %

3.8.2 Dithiothreitol

Concentrations of dithiothreitol (DTT) used as a burden on both *B. atrophaeus* and *G. stearothermophilus* BIs ranged from 5 mM to 250 mM.

Figure 3-13 shows the survival rates for *B. atrophaeus* BIs with DTT burden. The 10 mM DTT burden exhibited nearly perfect behavior during Test E, with modest survival rates at 5000 ppm*hours and no survival at 9000 ppm*hours. However, 20 mM of DTT provided full protection, with 100 % survival at 9000 ppm*hours.

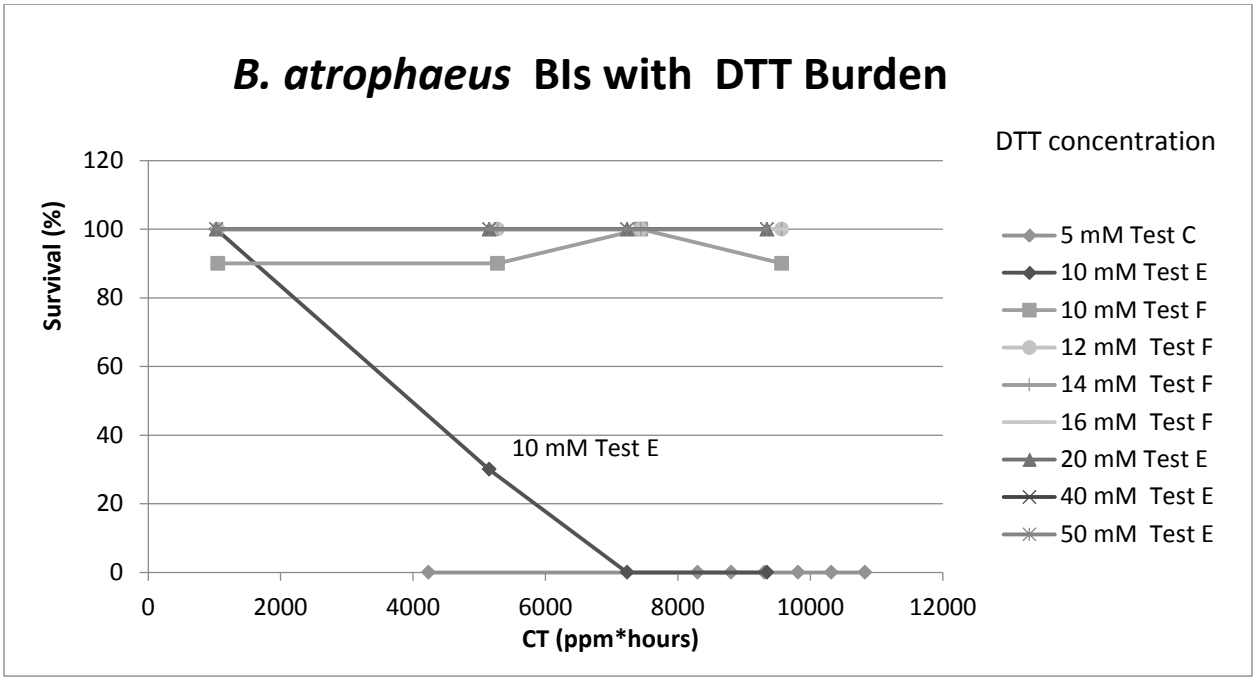

Figure 3-13. Survival (1000 ppm ClO_2) of *B. atrophaeus* BIs with DTT Burden (Test C (n=30), Test E (n=10), and Test F (n=10))

In Test F, *B. atrophaeus* BIs with DTT burden showed very high survival rates (90-100 %) at all fumigation time points, in contrast to results from Test E, which did experience a high-concentration spike of ClO_2. Survival rates from 10 mM DTT *B. atrophaeus* BIs from the two tests are shown in Table 3-9. Because the results of Test E were not repeated during Test F, the effect of the burden was masked by some other unknown stronger variable, possibly the variations in fumigation conditions. DTT was not further evaluated as a burden on *B. atrophaeus* BIs, but may be of interest, especially at higher ClO_2 concentrations.

Table 3-9. Survival Rates of 10 mM DTT on *B. atrophaeus* BIs (n=10)

Hours	Test E	Test F
1	100 %	90 %
5	30 %	90 %
7	0 %	100 %
9	0 %	90 %

Little protection of *G. stearothermophilus* BIs was offered by 5 mM DTT in Test C. All other concentrations of DTT showed 100 % growth. Based on these results, concentrations of DTT between 5 mM and 63 mM could be of interest as burdens on *G. stearothermophilus* BIs.

3.8.3 Carrageenan

Carrageenan (CAR) was used as a burden on both *B. atrophaeus* and *G. stearothermophilus* BIs at concentrations ranging from 0.01 %-0.25 % and 0.05 % and 1 % respectively.

The survival rates from *G. stearothermophilus* BIs with CAR burden are shown in Figure 3-14. The seven-hour time point seems to be an outlier. However, poor survival rates after one hour of fumigation and non-linear response to fumigation time indicate this BI should not be included for further study.

Figure 3-15 shows the survival rates for CAR *B. atrophaeus* BIs. As discussed earlier, the Test A results showed promise. However, Test C was not consistent with Test A, and the Test E results are counter-intuitive, with survival rates of nine-hour exposure higher than survival rates of one-hour exposure. Nonetheless, survival rates did generally vary as a function of burden concentration. These results could be indicative of the effects of post-exposure handling, unusual sensitivity to variations in fumigation conditions, or protection of the BI by a high-concentration burst of ClO_2 (as experienced early in the Test E fumigation).

Indeed, the Test E results are so counterintuitive, that they are presented in Figure 3-16, assuming an inadvertent switch of one-hour and nine-hour samples during laboratory evaluation. Evaluation of these results indicates that CAR may be a very promising burden, with a concentration between 0.1 % and 0.25 %. Due to the lack of confidence in the CAR data, this BI was not further evaluated. Future studies may consider further investigation of the 0.1 % and 0.25 % CAR BI.

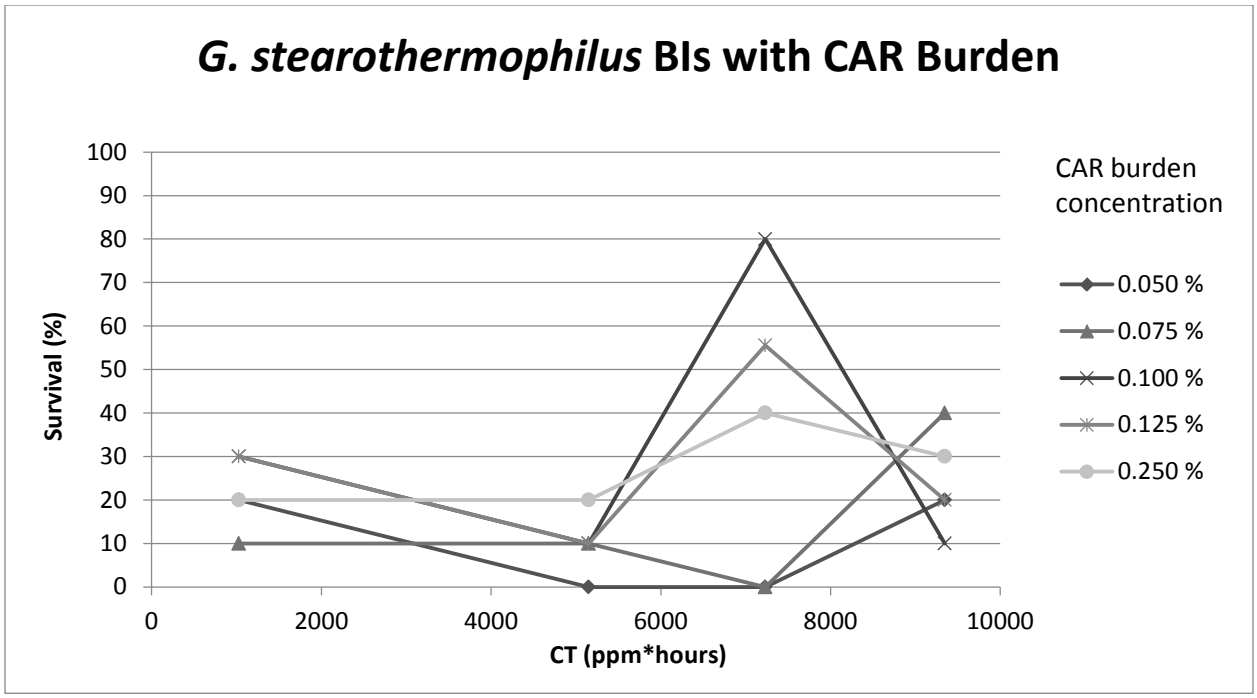

Figure 3-14. Survival (1000 ppm ClO_2) of *G. stearothermophilus* BIs (n=10) with CAR Burden (Test E)

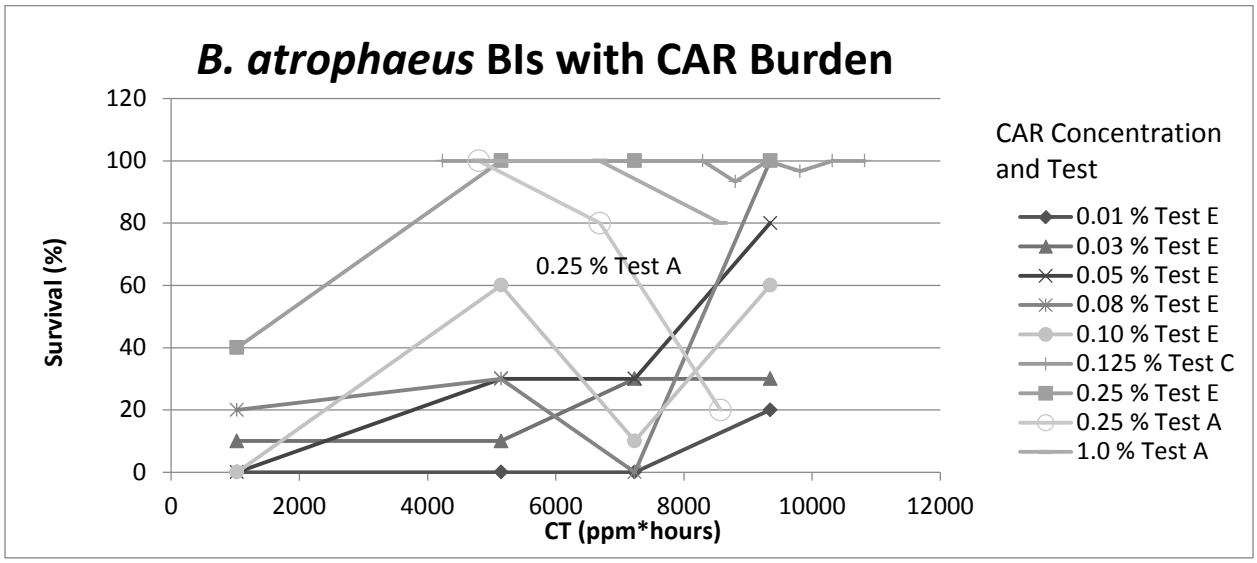

Figure 3-15. Survival (1000 ppm ClO_2) for *B. atrophaeus* BIs with CAR Burden from Test C (n=30), Test E (n=10) and Test F (n=10)

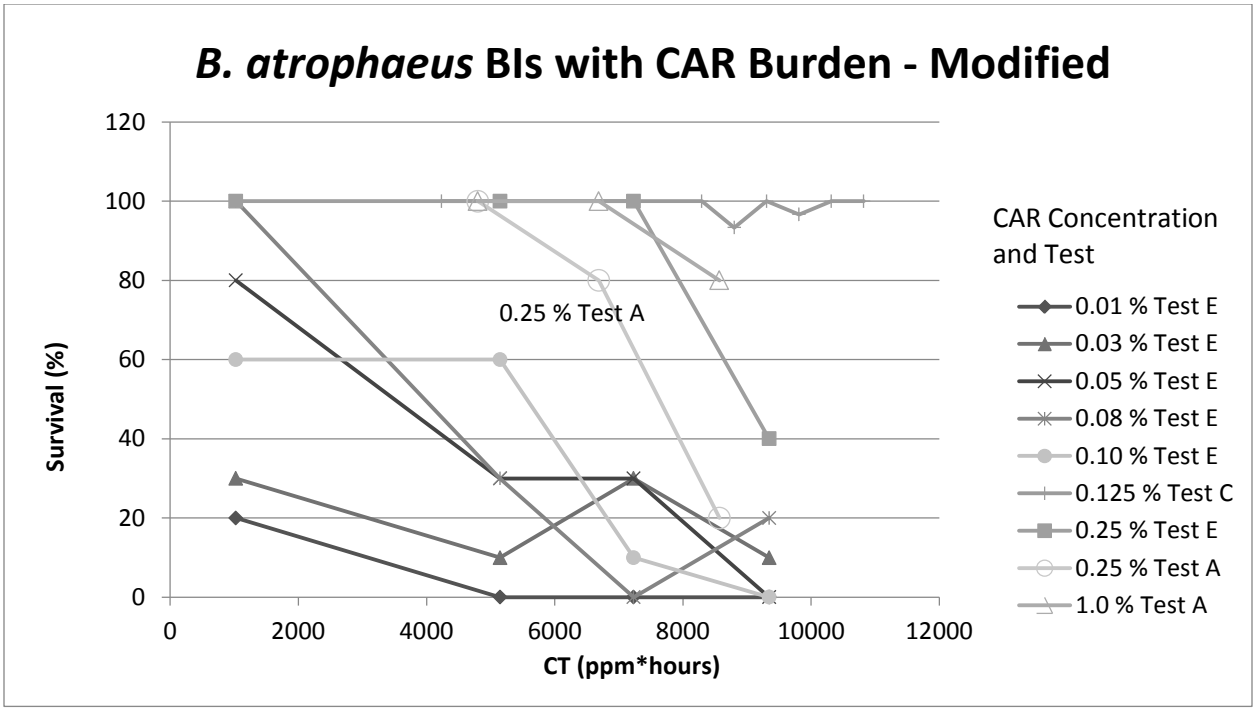

Figure 3-16. Reinterpretation of Figure 3-15

3.8.4 Glutathione

Test A demonstrated that glutathione (GLU) could provide protection to both *B. atrophaeus* and *G. stearothermophilus* BIs. The lower concentration (25 mM) provided full protection at seven hours and 80 % protection at nine hours for Test A. As a follow-up, Test C at a lower concentration of 5 mM on both BIs was tested, with the supposition that the lower concentration would provide less protection. However, GLU BIs of both species had 100 % survival rates after nine hours of exposure.

GLU BIs also showed high variability and insensitivity to fumigation time (see Table 3-10). For these reasons, GLU was not considered a candidate burden for subsequent tests.

3.8.5 Gelatin

Table 3-11 shows the concentrations of gelatin (GEL) that were tested as burdens on both *B. atrophaeus* and *G. stearothermophilus* BIs. Replicate concentrations have been shaded.

Table 3-10. Survival Rates of Test C *B. atrophaeus* and *G. stearothermophilus* BIs with 5 mM GLU Burden

Exposure Time (Hours)	*B. atrophaeus* BI	*G. stearothermophilus* BI
	% Surviving	*% Surviving*
4.0	93	100
8.0	87	83
8.5	77	93
9.0	97	93
9.5	97	100
10.0	97	100
10.5	100	100

Table 3-11. Tested Concentrations of GEL as a Burden on *B. atrophaeus* and *G. stearothermophilus* BIs

Test A	Test D	Test E	Test F	Test G	Test H	Test I
Both BIs		*B. atrophaeus* BIs only				
2.5 %	0.1 %	0.25 %	1.6 %	1.0 %	0.8 %	0.8 %
10.0 %	1.0 %	0.50 %	1.7 %	1.5 %	0.9 %	0.9 %
		0.75 %	1.8 %	1.6 %	1.0 %	1.0 %
		1.00 %	1.9 %	1.7 %		
		1.25 %	2.0 %			
		1.50 %				
		2.00 %				

As discussed in Section 3.2 gelatin provided full protection during Test A, allowing nearly full survival at 2.5 %. Successive tests were conducted with lower concentrations of GEL.

Figure 3-17 shows the results of *G. stearothermophilus* BIs with GEL burden. Increasing the GEL concentration from 0.1 % to 1 % did not increase the survival of the *G. stearothermophilus BI*, though during Test A these BIs with 2.5 % GEL showed complete protection (100 % growth). The GEL *G. stearothermophilus* BI is indicative of a sigmoidal BI and is not recommended for further study. Complete kill was never achieved for *G. stearothermophilus* GEL BIs, even after 10,000 ppm*hours.

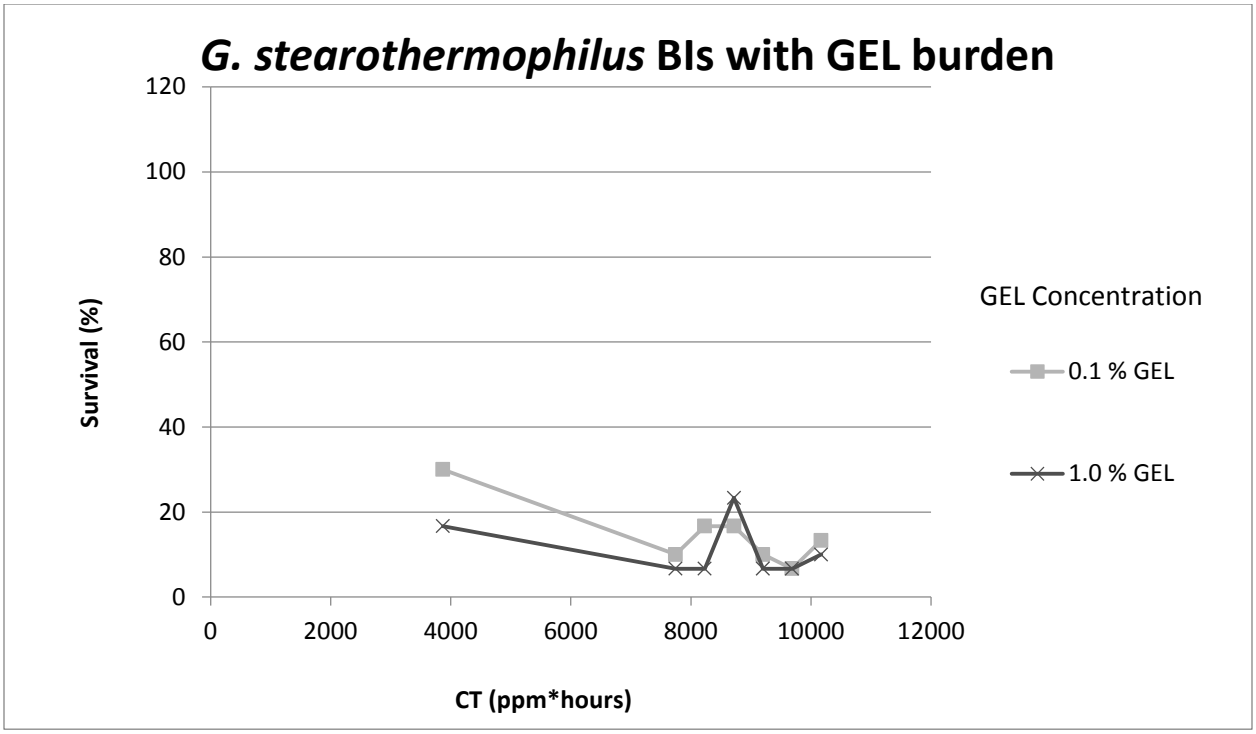

Figure 3-17. Survival (1000 ppm ClO_2) of *G. stearothermophilus* BIs (n=30) with GEL Burden (Test D)

As seen in Table 3-12, both 0.1 % and 1.0 % GEL provided too little protection to *B. atrophaeus* BIs.

Table 3-12. Survival Rates GEL *B. atrophaeus* BIs (Test D)

Hours	0.1% GEL	1.0% GEL
4.0	0 %	20 %
8.0	0 %	7 %
8.5	0 %	3 %
9.0	0 %	0 %
9.5	0 %	10 %
10.0	0 %	0 %
10.5	0 %	0 %

Figure 3-18 shows the promise of GEL as a burden on *B. atrophaeus* BIs. From Test A, 2.5 % GEL provided too much protection (100 % survivability), while Test D suggested 1.0 % was too low (20 % survival after just four hours). Taken within the context of this test alone, GEL is very promising: a nice correlation of survivability to concentration at one hour and strong protection up to seven-hour exposure. However, combining the results from Test E and Test D suggests variability between the two data sets. Two sources of variability are changes in fumigation conditions and changes in manufacturer. The survival rates of some GEL *B. atrophaeus* BIs are shown in Table 3-13. The GEL BI results from Test G were consistent with Test F, but not consistent with Test E.

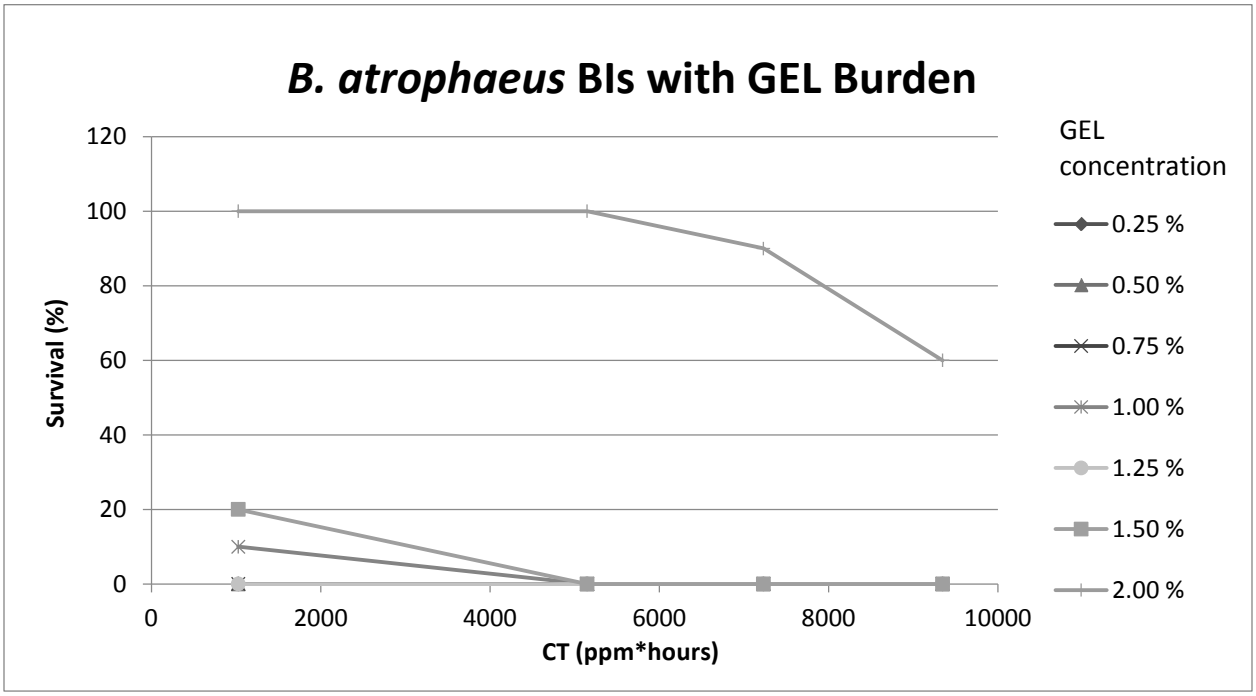

Figure 3-18. Survival (1000 ppm ClO_2) of *B. atrophaeus* BIs (n=10) with GEL Burdens (Test E)

Table 3-13. Survival Rates of Gelatin *B. atrophaeus* BIs for Some Concentrations

	1.0 % GEL					1.5 % GEL		1.6 % GEL		1.7 % GEL	
	Test										
Hours Exposure	D	E	G	H	I	G	E	G	F	G	F
1	NA	10 %	100 %	100 %	100 %	100 %	20 %	100 %	100 %	100 %	100 %
5	20 % (four hour exposure)	0 %	80 %	100 %	100 %	100 %	0 %	100 %	100 %	100 %	100 %
7	NA	0 %	100 %	100 %	100 %	100 %	0 %	100 %	100 %	100 %	100 %
8	7 %	NA	85 %	100 %	97 %	100 %	NA	100 %	NA	100 %	NA
9	0 %	0 %	35 %	100 %	100 %	100 %	0 %	100 %	100 %	100 %	100 %

Figure 3-19 shows a graphical representation of the survival rates of a single BI (1.0 % gelatin on *B. atrophaeus* BIs) over a range of fumigations. As indicated in Table 3-13, there was a wide range of responses for this BI. As discussed earlier, there was a spike in ClO_2 concentration during Test E, which could explain the lower survival rates, but the remaining fumigations had no known significant anomalies. Each manufactured batch of 1.0 % GEL BIs behaved differently (Test H and Test I were manufactured on the same date, from the same spore lot).

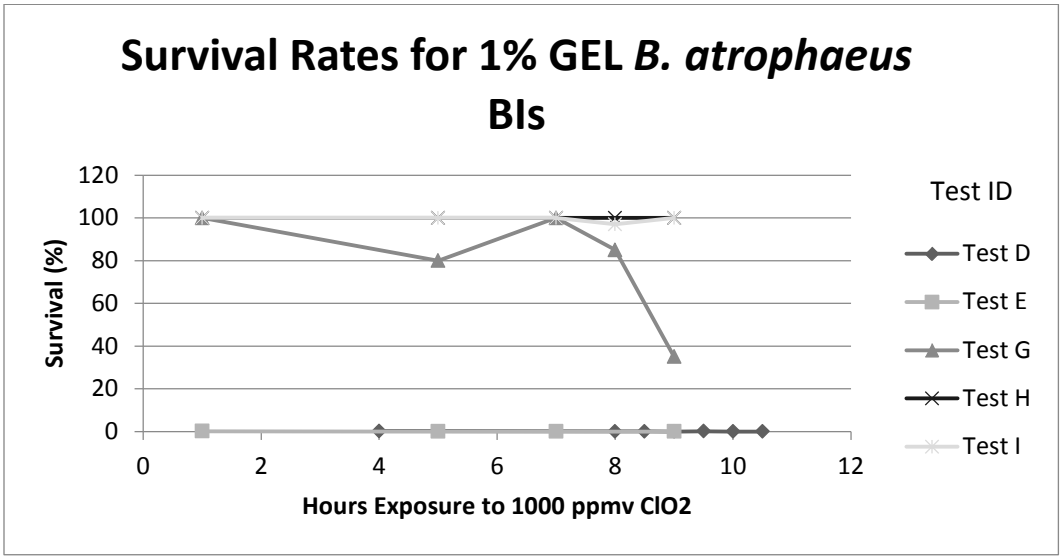

Figure 3-19. Survival (1000 ppm ClO_2) of 1.0% GEL *B. atrophaeus* BIs from Test D (n=30), Test E (n=10), Test G (n=5), Test H (n varies between 5 and 30), and Test I (n varies between 5 and 30)

Test H and Test I *B. atrophaeus* BIs with GEL burden showed high survival rates (90% or higher) at all fumigation conditions. GEL BIs did not produce repeatable results, though the cause of variability is unclear.

3.8.6 Casein

Casein (CSN) was used as a burden on *B. atrophaeus* BIs for 19 tests starting with Test E. Concentrations tested ranged from 0.1 % to 10 %.

Figure 3-20 shows survival rates for *B. atrophaeus* BIs with CSN burden from Test E. This new burden showed promise, providing full protection at 10 % and partial protection at lower concentrations. Interestingly, the presence of the CSN on the BI resulted in encapsulated bacterial growth during the first days of growth, preventing the broth from becoming cloudy but creating a visible bubble on the surface of the BI. Nonetheless, CSN was chosen for follow-up testing in Test F, which was designed to pinpoint a concentration of CSN that might better approximate the 9,000 ppm*hours kill point.

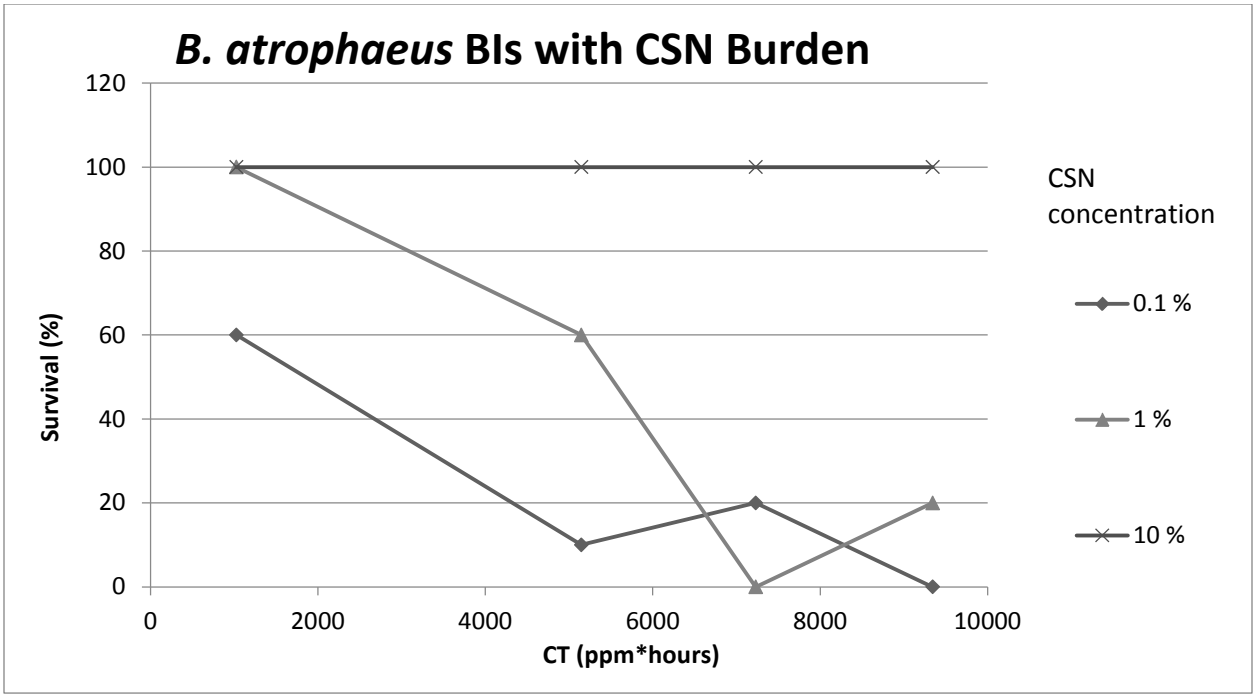

Figure 3-20. Survival (1000 ppm ClO_2) of *B. atrophaeus* BIs (n=10) with CSN Burden (Test E)

Figure 3-21 displays the survival rates of the *B. atrophaeus* BIs burdened with CSN from Test F. As in Test E, CSN clearly demonstrates an ability to protect the spores, allowing growth at conditions that inactivate the unburdened BI. In general, there was a dose-dependent response to increased concentration of CSN. While the 1.0 % shows a nearly perfect response, the 100 % growth at seven hours seems an outlier taken in the context of the 40 % growth rate of the BI with 1.2 % CSN. Also visible in Figure 3-21 is the fact that increased protection of the BI reduces the ability to reach complete kill conditions after 9000 ppm*hours.

Table 3-14 shows the survival rates of *B. atrophaeus* with selected concentrations of CSN burden across several fumigations, demonstrating the variability encountered between different batches of BIs and between different fumigations. Figure 3-22 shows the survival rates of 1.0 % casein BIs for five different fumigations. Tests H and I were of the same batch of BIs.

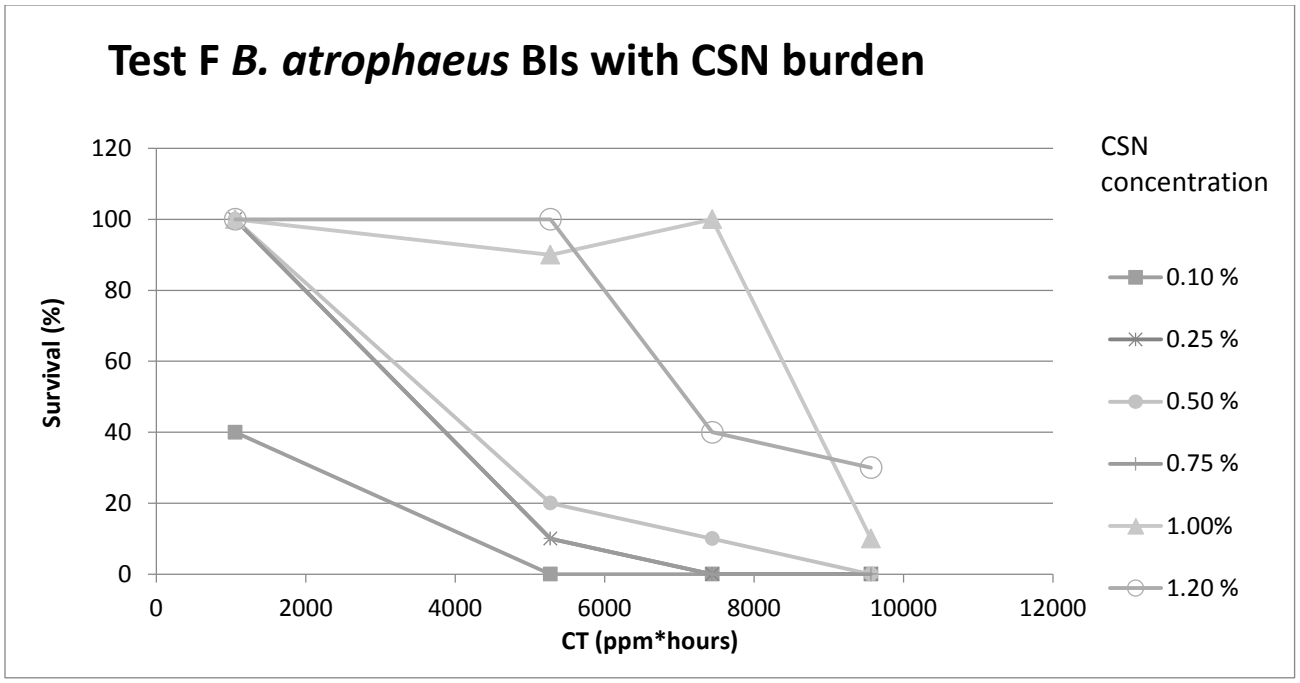

Figure 3-21. Survival (1000 ppm ClO_2) of *B. atrophaeus* BIs (n=10) with CSN Burden (Test F)

Table 3-14. Survival Rates of *B. atrophaeus* BIs with CSN Burdens

	0.8 % CSN	0.9 % CSN			1.0 % CSN					1.1% CSN		
Hours Exposure	% Surviving Test G	% Surviving Test G	% Surviving Test H	% Surviving Test I	% Surviving Test E	% Surviving Test F	% Surviving Test G	% Surviving Test H	% Surviving Test I	% Surviving Test G	% Surviving Test H	% Surviving Test I
1	100	100	100	100	100	100	100	100	100%	100%	100%	100%
5	40	0	100	60	60	90	80	100	100%	80%	100%	100%
7	10	50	100	50	0	100	40	100	100%	70%	100%	100%
8	0	0	100	17	NA	NA	0	100	100%	0%	100%	100%
9	0	0	100	17	20	10	0	100	100%	0%	100%	97%

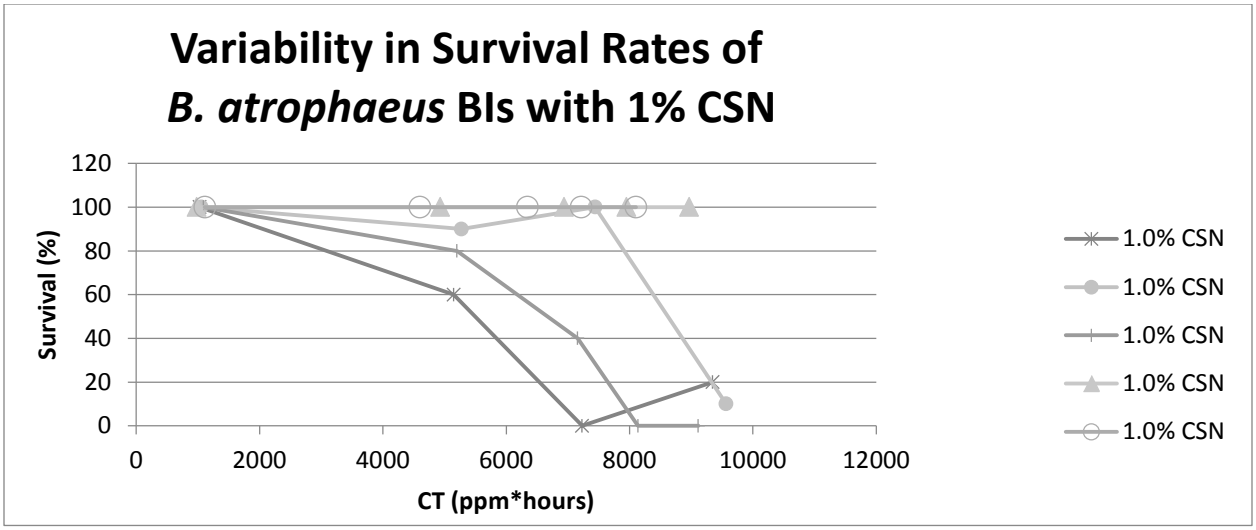

Figure 3-22. Variability in Survival Rates of *B. atrophaeus* BIs with 1.0% Casein

Table 3-15 shows the fumigation conditions associated with the BI survival curves, shown in Figure 3-22, as well as some additional fumigations to be discussed in Section 3.9. Table 3-15 also shows the correlation between the survival rate of the 1% Casein *B. atrophaeus* BI and some fumigation conditions. The strongest correlation is with ClO_2 concentration, even though the difference between the highest and lowest average concentration is less than 10%. Furthermore, the highest survival rates occur at the lowest concentrations, which suggest coincidence rather than correlation. The correlation in the ppm*hours belies the true source of variation. For instance, it would seem that the lower CT at the 9 hour mark of Test H and I could have contributed to the high survival rate, but the 8-hour exposure during Test G, for instance, had a CT of 8136 ppm*hours, and still had a 0% survival rate. Variations in fumigation conditions do not seem to be the source of variations in survival rates.

Table 3-15. Fumigation Conditions for the 9-hour Exposure of Multiple Tests

9 Hour Exposure	Test E	Test F	Test G	Test H	Test I	Test P	Test Q	Test R	Test S	Test T	Survival Rate Correlation (Pearson correlation coefficient – r)
Average ClO_2 (mg/L)	2.9	3.0	2.8	2.7	2.7	2.8	2.9	3.0	2.9	2.9	-0.78
ppm*hours	9345	9566	9113	8968	8665	94823	9805	9746	9285	93234	-0.70
Max ClO_2 (mg/L)	10.8	3.1	3.1	2.9	3.5	2.9	8.0	3.1	3.1	3.2	-0.14
Average RH (%)	75.4	75.2	75.1	58.0	75.5	75.5	75.2	75.0	75.1	75.1	-0.64
Max RH (%)	77.8	76.2	76.1	59.0	81.1	77.7	76.9	75.3	75.6	75.7	-0.44
Average Temperature (°C)	23.9	24.0	22.6	23.8	23.5	23.8	23.8	23.7	23.8	23.2	0.18
Max Temp (°C)	24.0	24.4	23.0	23.9	24.2	24.0	24.0	24.0	24.0	24.2	0.19
Survival Rate of 1.0% CSN *B. atrophaeus* BI	20 %	10 %	0 %	100 %	100 %	15 %	15 %	20 %	5 %	10 %	

While the 1.0 % casein *B. atrophaeus* BI shows promise, variability was a problem as evidenced by the high survival rates in Test H and Test I. Additional tests discussed in Section 3.9 were conducted to identify (and remove) preventable sources of variability.

3.9 Variability Investigation

A number of additional fumigations and tests were performed to better understand the variability resulting from the BI tests. The majority of these tests were performed using *B. atrophaeus* BIs with and without CSN burden.

3.9.1 Spore Preparations

Tests O, P and Q were designed to investigate whether the source of BI variability might arise from unavoidable variations in fumigation conditions or from unavoidable variations in spore preparations. A single batch of BIs for all three tests was prepared from two different sources of spores, Raven and Yakibou. Other than the source of the liquid inoculum, BIs were prepared identically by Yakibou.

Test O was aborted early due to ClO_2 generator error, so only the five hour time point was valid. The results are shown in Figure 3-23.

Survival was aided with increasing CSN concentration. Surprisingly, the Raven spore preparation was more resistant to fumigation than the Yakibou spore preparation, in contrast to the Raven *B. atrophaeus* BIs, which had typically been less resistant to fumigation than Yakibou BIs (Sections 3.5 and 3.6).

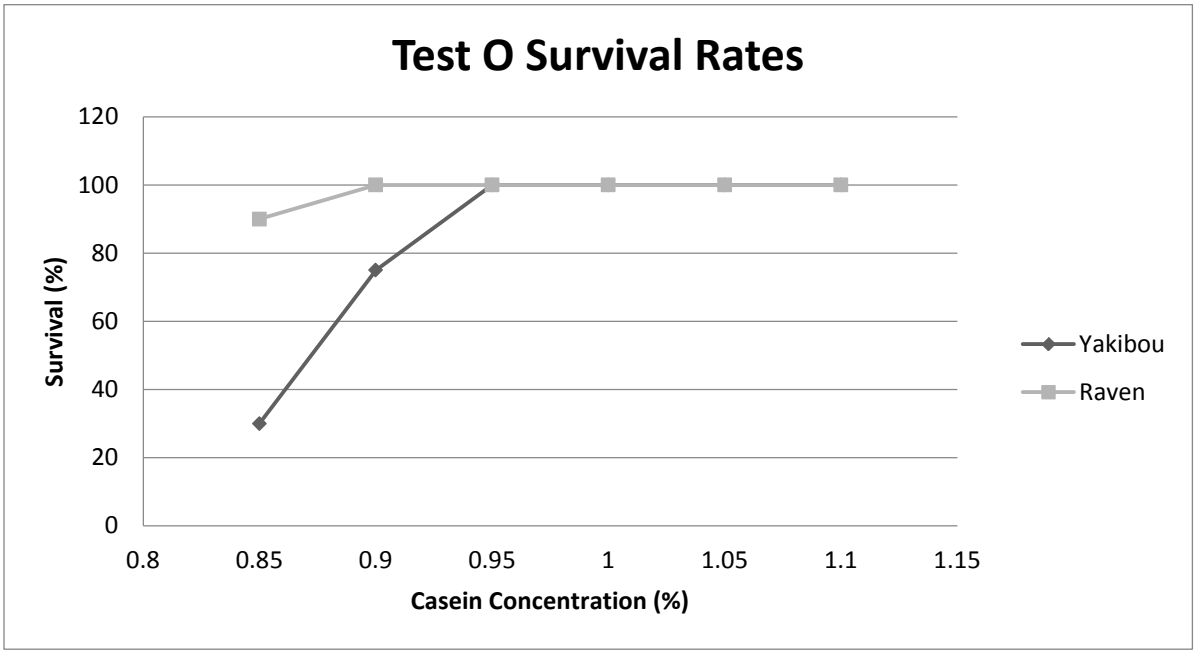

Figure 3-23. Survival (5000 ppm*hours, 1000 ppm ClO_2) of Two Spore Preparations (n=10) as a Function of CSN Concentration (Test O)

Figures 3-24 and 3-25 shows the survival rates for Test P BIs with casein burden with Yakibou and Raven spore preparations, respectively.

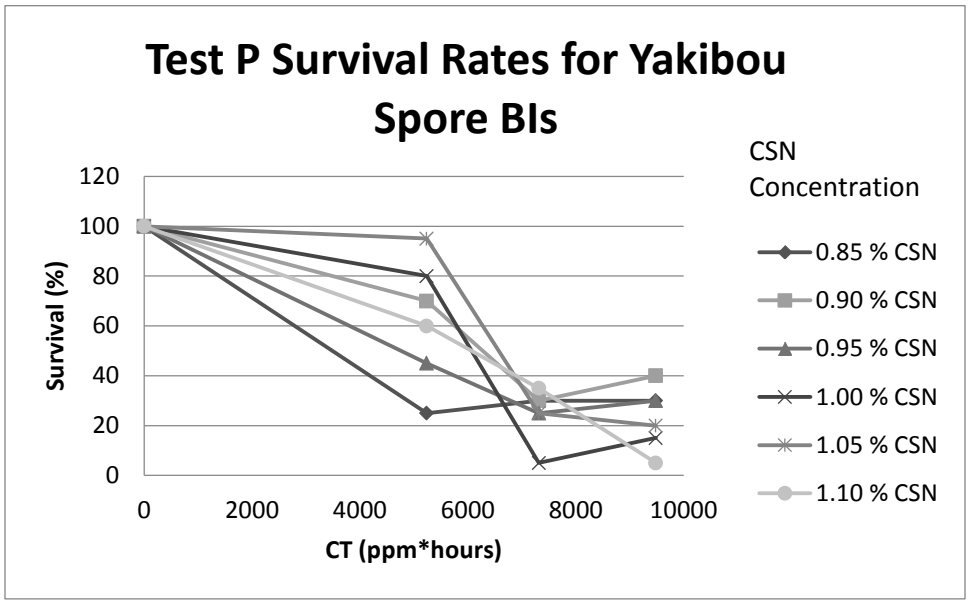

Figure 3-24. Survival (1000 ppm ClO_2) of Yakibou Spore BIs (n=20) with CSN Burden (Test P)

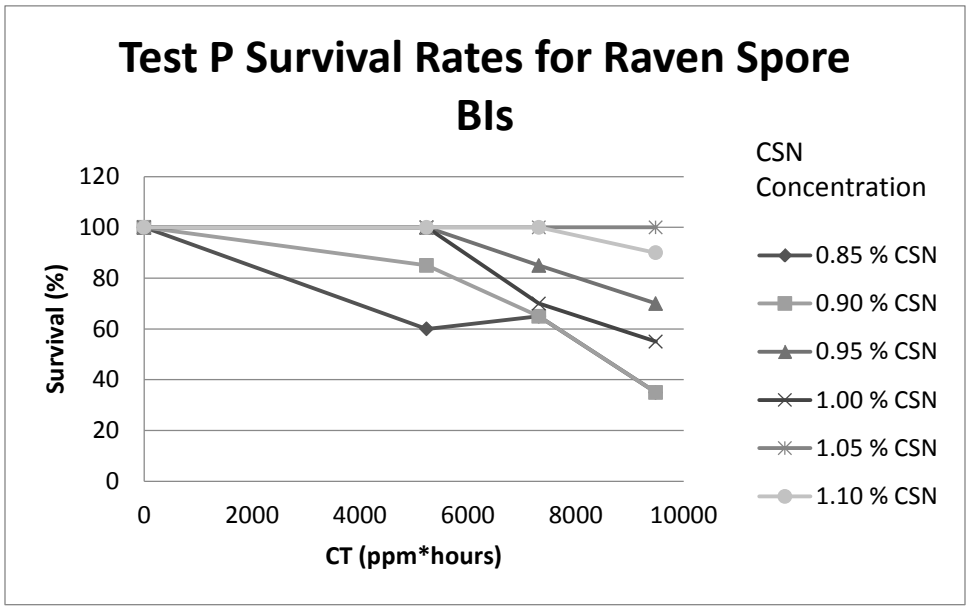

Figure 3-25. Survival (1000 ppm ClO_2) of Raven Spore BIs (n=20) with CSN Burden (Test P)

Again, the Raven spore preparation was hardier than the Yakibou spore preparation. The nine-hour time point for the Yakibou spores showed unexpected behavior, with higher concentrations of CSN burden demonstrating lower survival rates than lower concentrations. Test Q survival rates were similar to Test P. These results suggest differences in spore preparation procedures may have a significant impact on BI survival. Such results draw into scrutiny the utility of BIs for evaluating fumigation efficacy, as subtle differences in between-batch or between-vendor spore preparation conditions could significantly alter the outcome of these indicators.

D-values were calculated using the Most Probable Number method proposed by Stumbo [4]. The D-values for Test P and Test Q are shown in Figure 3-26.

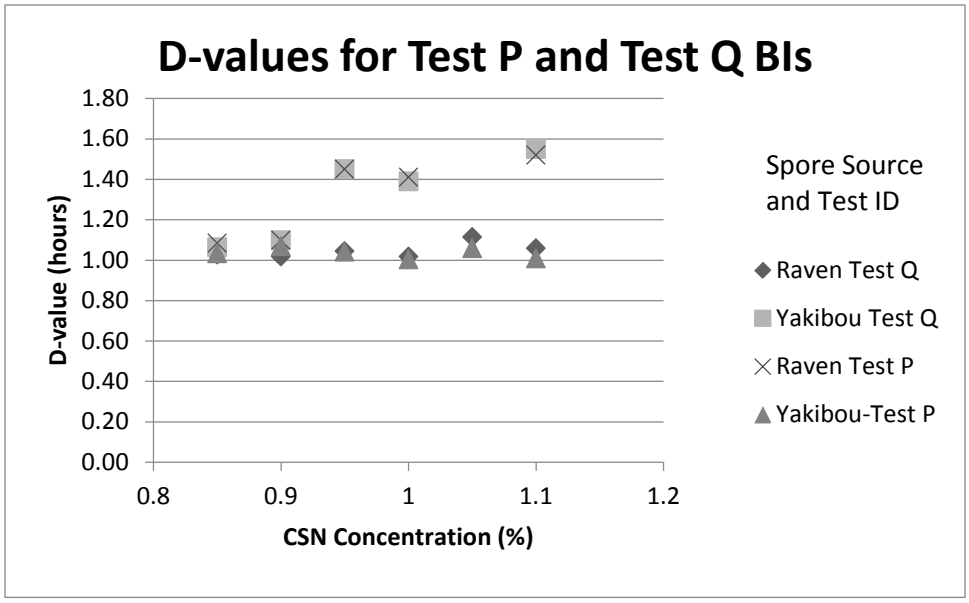

Figure 3-26. Calculated Average D-Values for Test P and Test Q BIs

Average D-values of Yakibou BIs are inelastic to CSN concentration, whereas increasing burden concentration increases D-values for the Raven BIs. The inelasticity of the Yakibou BIs is contrary to previous Tests E, F, and G.

Figure 3-27 shows the calculated D-values of Test P as a function of exposure time. The D-values of a BI with linear response should be the same at all time points. A negative slope of D-value over time (sloping down) is indicative of a time lag or a shoulder. Such a BI would have no response to fumigation until after a certain minimum exposure has been reached. The BIs in Figure 3-27 have a D-value over time with a positive slope. Similar to the findings of Rastogi et al (2010) [1], these data suggest that kill curves are not first order reactions and that the survivors are the result of tailings.

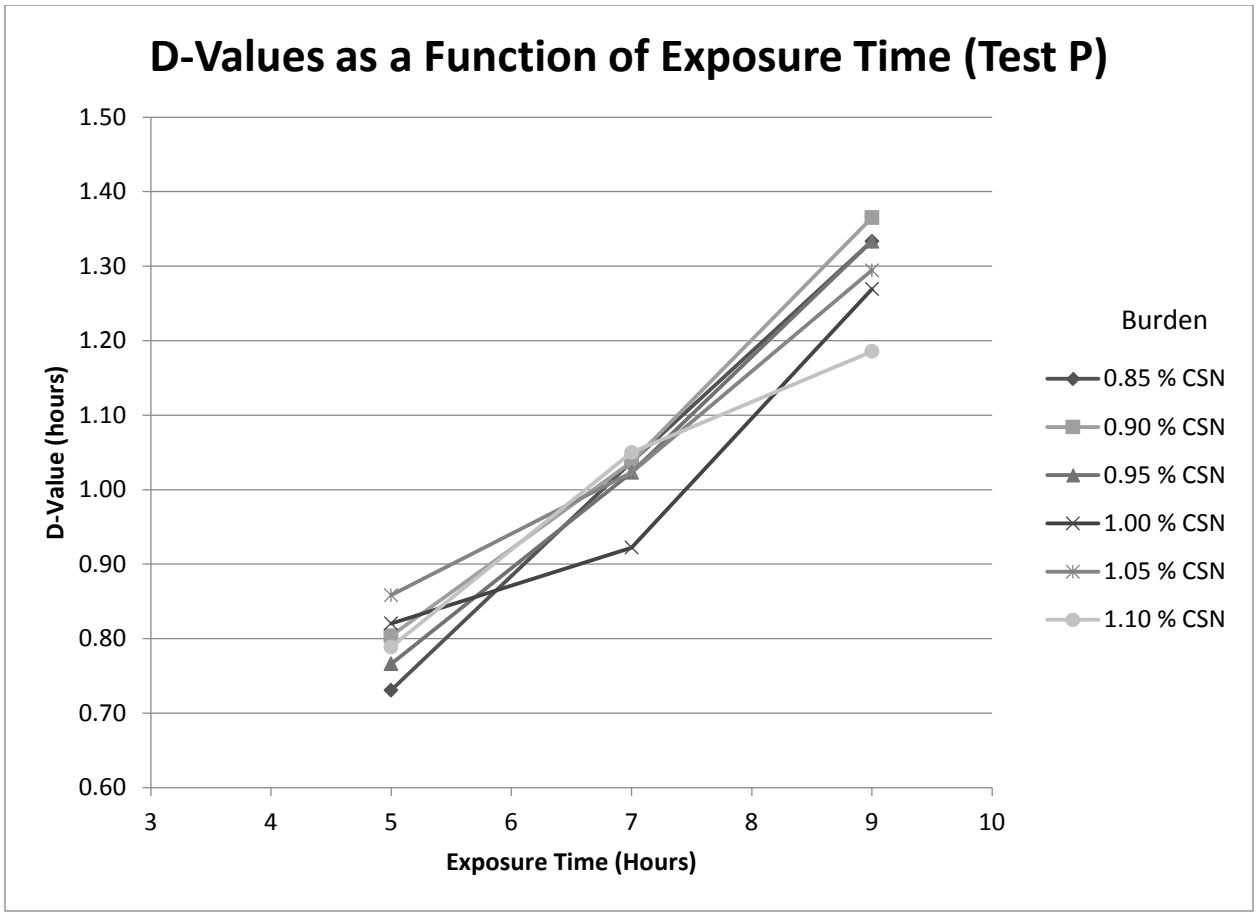

Figure 3-27. Calculated D-Values of Yakibou BIs per Exposure Time

Tests O, P, and Q demonstrate that spore preparations can react quite differently to similar fumigation conditions and can contribute to the survival rates of an organism or BI. There are no publicly available differences in the spore preparation procedures used by different laboratories to explain different survival rates. However, spore preparations have been shown by other researchers to affect resistance. Young and Setlow [4] determined that “spores prepared at higher temperatures were more resistant”. Bloomfield and Arthur [5] showed that both spore coat and the cortex affects the resistance of *B. subtilis* spores to chlorine-releasing agents. Future investigations could help develop protocols which would remove this variability.

However, spore preparation does not account for all of the variability, perhaps best shown in a comparison of Test U and Test V. Survival rates are shown in Figure 3-28.

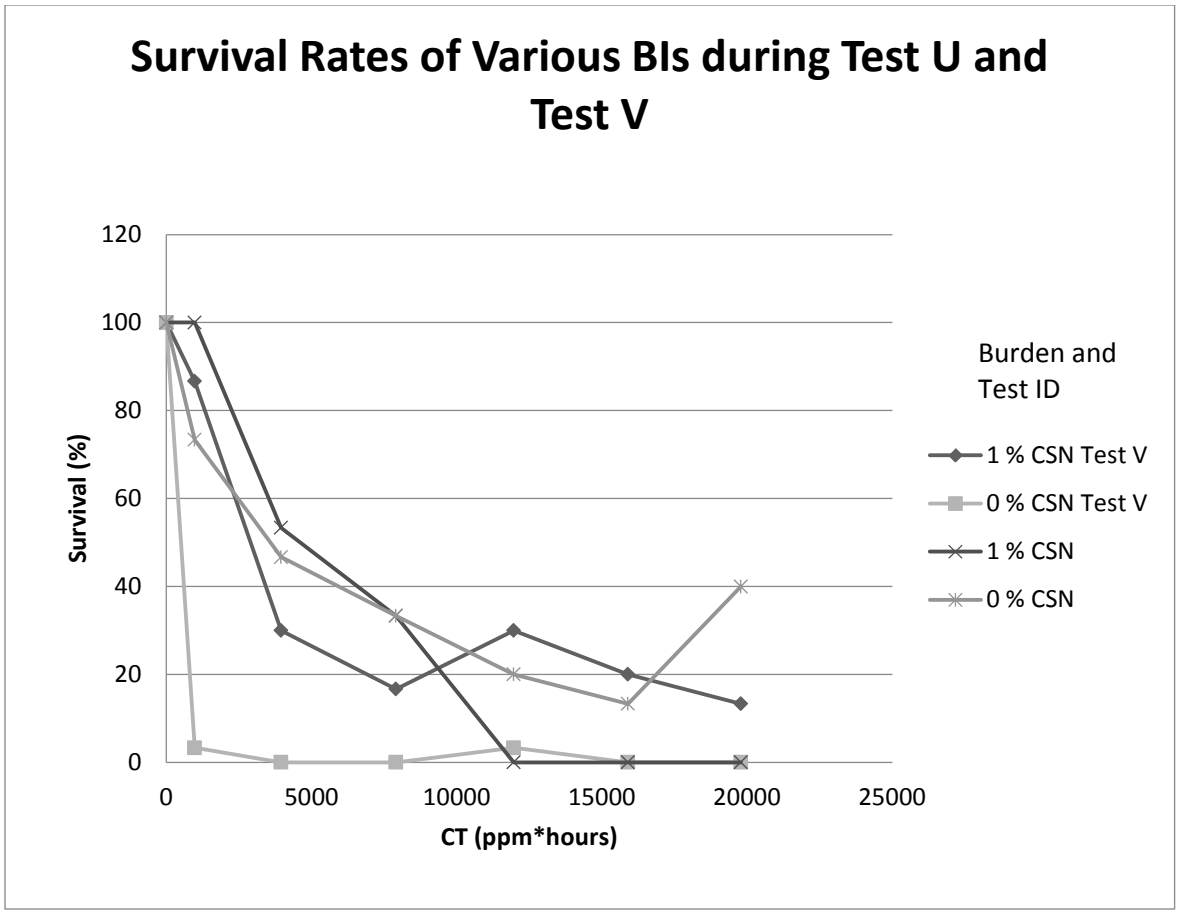

Figure 3-28. Survival (1000 ppm ClO_2) of Test U and Test V BIs (n=30)

The BIs used for Test U and V were prepared on different dates approximately five months apart, but with the same stock solution. The difference in response of the 0% casein is remarkable. Some unknown difference in the preparation of the BIs may account for the moderate survival rates even after long fumigations.

3.9.2 Effect of Vortex Mixing BIs during Analysis Procedures

During incubation, some burdens would form a layer which would encapsulate the BI growth. One cause of the variability could be the difficulty in recognizing the growth of a BI when the encapsulation was happening. Test J included BIs that were vortex-mixed, using the highest setting on the vortex mixer, before plating the seven-day incubated broth in addition to BIs that were analyzed with the previously used procedure without vortex mixing. The number of BIs per type varied between two and five replicates based on availability. The results, shown in Table 3-16, suggest that the use of vortex mixing did not systematically affect survival rates.

Table 3-16. Effect of Vortex Mixing on Survival Rate Determination

*B. atrophaeus BI*s with GEL Burden			*B. atrophaeus BI*s with CSN Burden		
BI ID	Survival Rate (%)		BI ID	Survival Rate (%)	
	Vortex Mixed	Not Vortex Mixed		Vortex Mixed	Not Vortex Mixed
GEL-025-286+V-7-01	0	0	CSN-010-239+V-7-01	0	0
GEL-050-286+V-7-01	0	0	CSN-010-286+V-7-01	33	0
GEL-075-286+V-7-01	0	0	CSN-050-239+V-7-01	0	0
GEL-100-117+V-7-01	100	33	CSN-050-239+V-9-01	0	0
GEL-100-286+V-7-01	0	0	CSN-090-117+V-5-01	0	20
GEL-200-239+V-9-01	100	100	CSN-090-117+V-7-01	0	0
			CSN-090-117+V-9-01	0	0
			CSN-100-117+V-5-01	100	100
			CSN-100-117+V-7-01	100	100
			CSN-100-117+V-9-01	100	100
			CSN-120-117+V-7-01	100	100
			CSN-120-117+V-9-01	100	100
			CSN-120-239+V-7-01	0	0
			CSN-120-239+V-9-01	0	0
			CSN-1000-286+V-9-01	100	100

3.9.3 Effect of RH

The effect of RH during fumigation was not in the original test matrix, but an operator error led to an RH of 60 % in the fumigation chamber for Test H. While all *B. atrophaeus* and *G. stearothermophilus* BIs survived the lower RH fumigation, some BIs did not survive the fumigation at 75 % RH (Table 3-17). The survival rates for the higher RH test are shown in Figure 3-29 and Figure 3-30 for the GEL burden and CSN burden BIs, respectively. These results may corroborate previous reports [6, 7, 8] of the sensitivity of BIs to RH during ClO_2 fumigation.

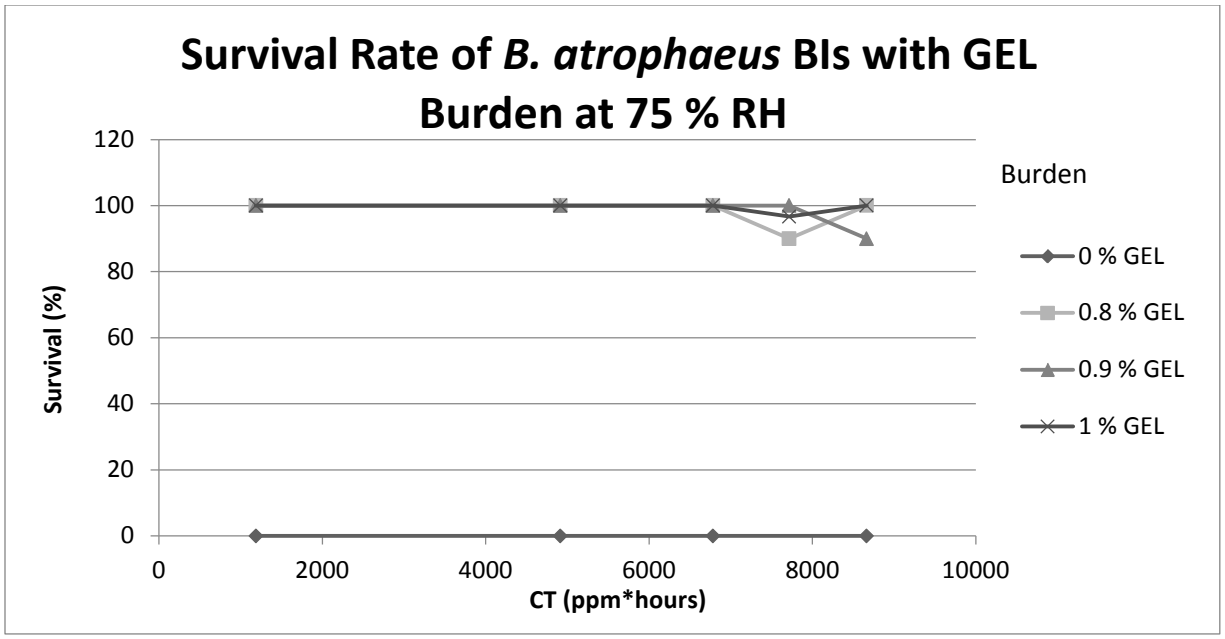

Figure 3-29. Survival (1000 ppm ClO_2) of *B. atrophaeus* BIs (n =30) with GEL Burden at 75% RH* (Test I)

*** All BIs survived fumigation at 60 % RH**

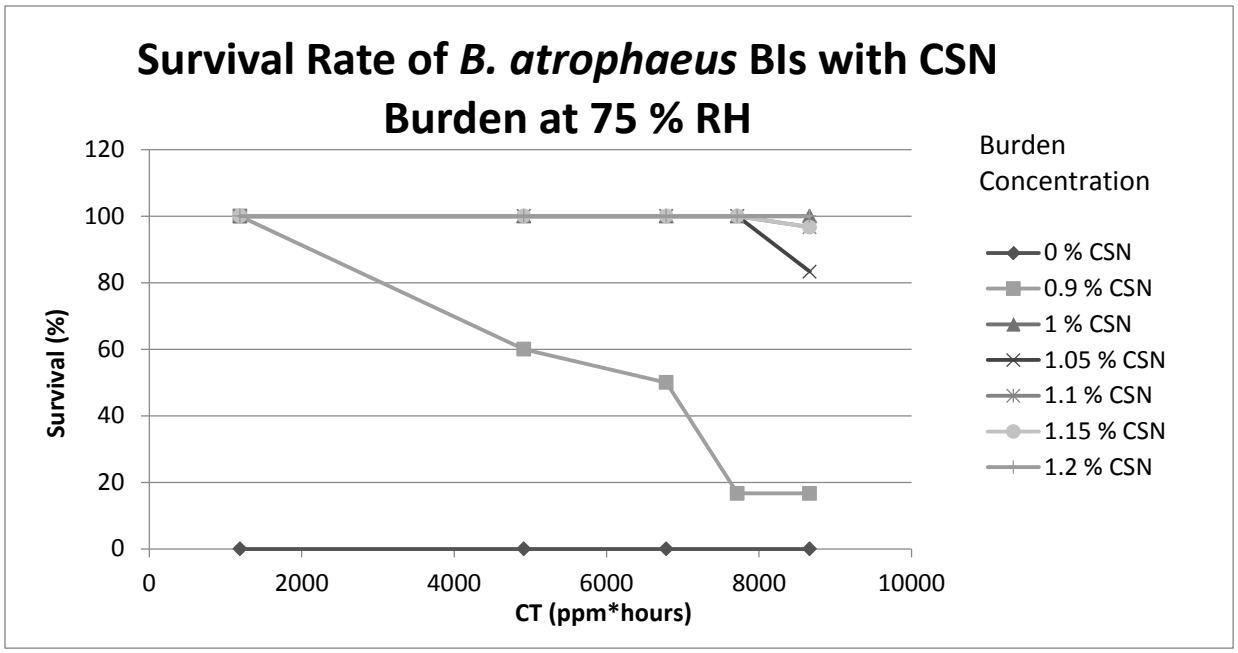

Figure 3-30. Survival (1000 ppm ClO_2) of *B. atrophaeus* BIs (n=30) with CSN Burden at 75% RH* (Test I)

* All BIs survived fumigation at 60 % RH

Table 3-17. Survival of BIs after 60% RH and 75% RH Fumigations

Burden	***% Concentration***	***Hours at 1000 ppm ClO_2***	***Survival (%) after 75% RH fumigation (Test I)***	***Survival (%) after 60% RH fumigation (Test H)***
Gelatin	0.00	1	0	0
		5	0	0
		7	0	0
		9	0	0
	0.80	1	100	100
		5	100	100
		7	100	100
		8	90	100
		9	100	100
	0.90	1	100	100
		5	100	100
		7	100	100
		8	100	100
		9	90	100
	1.00	1	100	100
		5	100	100
		7	100	100
		8	97	100
		9	100	100
CSN	0.00	1	0	0
		5	0	0
		7	0	0
		9	0	0
	0.90	1	100	100
		5	60	100
		7	50	100
		8	17	100
		9	17	100
	1.00	1	100	100
		5	100	100
		7	100	100
		8	100	100
		9	100	100
	1.05	1	100	100
		5	100	100
		7	100	100
		8	100	100
		9	83	100
	1.10	1	100	100

Burden	% Concentration	Hours at 1000 ppm ClO_2	Survival (%) after 75% RH fumigation (Test I)	Survival (%) after 60% RH fumigation (Test H)
		5	100	100
		7	100	100
		8	100	100
		9	97	100
	1.15	1	100	100
		5	100	100
		7	100	100
		8	100	100
		9	97	100
	1.20	1	100	100
		5	100	100
		7	100	100
		8	100	100
		9	100	100

3.9.4 Quantitative Analysis

Tests R, S, T, U and V were conducted to quantify the spores remaining after fumigation and to correlate D-values calculated from qualitative results to quantitative log reductions (LRs). Test R included BIs that were analyzed in two ways. For each time point and BI type, half were analyzed qualitatively (Section 2.4.1) like most BIs in this study, and the other half were analyzed quantitatively (Section 2.4.3) to determine the number of spores generating the “growth” result. Figure 3-31 shows the quantitative results from Test R. Figure 3-32 shows the same data, but the quantitative data are interpreted as Growth/No Growth and presented in the same format of “Survival (%)’ as many of the other figures in this text. For example, any replicate with one or more detected viable spores would be interpreted as “Positive Growth” for that replicate. The percentage of replicates with ≥1 CFU is therefore reported as “Survival (%)”.

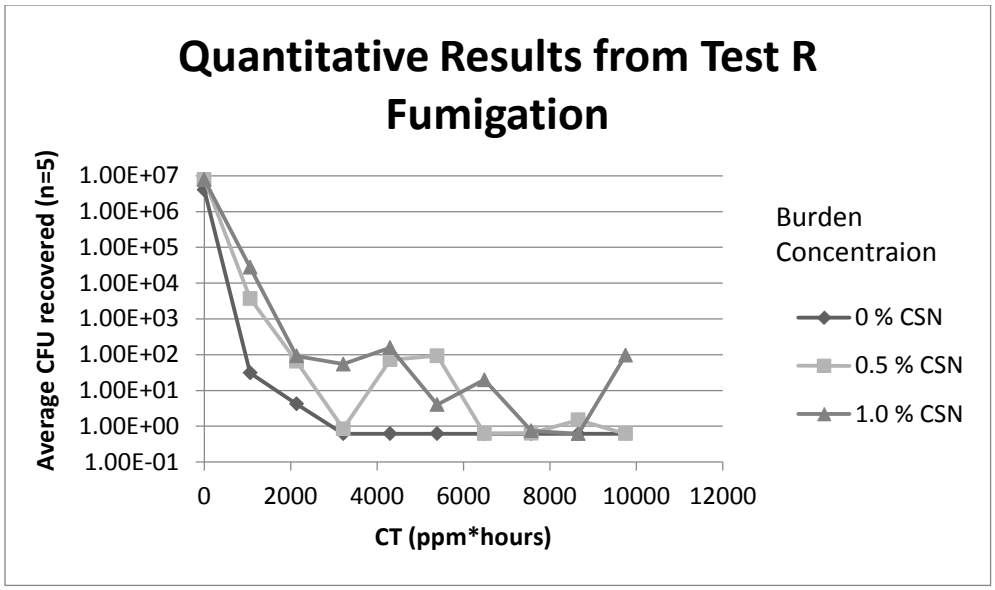

Figure 3-31. Quantitative Analysis of CFU (n-5) following 1000 ppm ClO_2 fumigation (Test R)

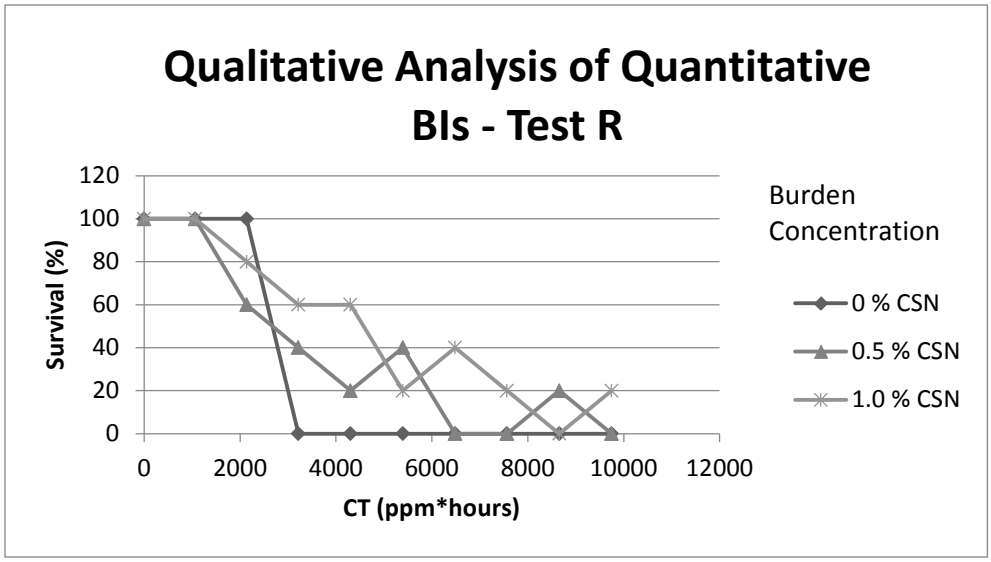

Figure 3-32. Qualitative Interpretation of Figure 3-31 Quantitative Results

As expected, unprotected *B. atrophaeus* BIs (0% CSN) did not survive fumigation conditions longer than three hours. As seen in Figure 3-31, the unprotected spores in 0% CSN *B. atrophaeus* BIs also demonstrated the expected kill curve or decay rate. While the spores are being killed on the BI, until complete kill (inactivation of all spores on the BI), the BI itself would still present a "Positive Growth" result if analyzed qualitatively. As a comparison to Figure 3-32, Figure 3-33 shows the survival rate of the BIs that were analyzed qualitatively.

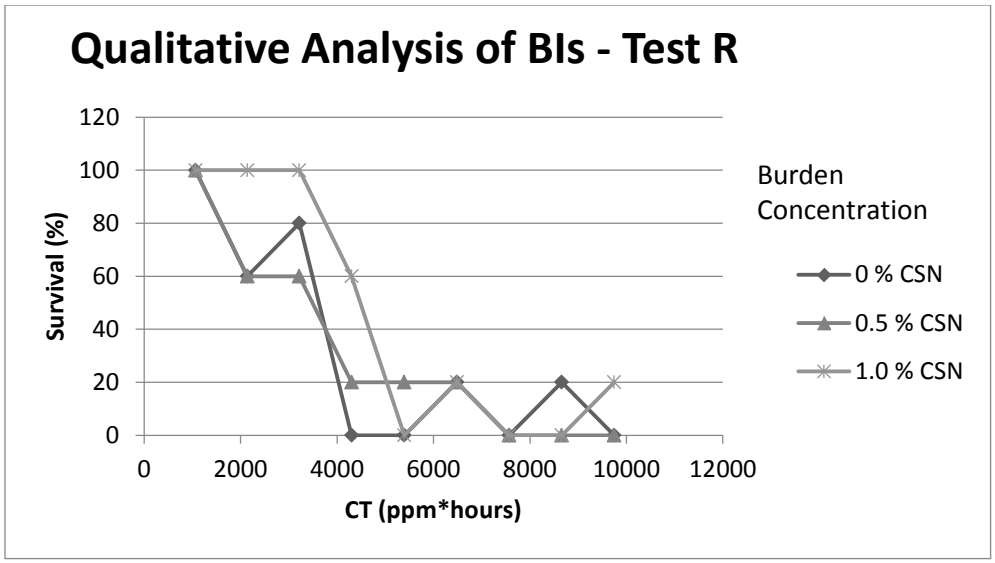

Figure 3-33. Test R Qualitative Results from Qualitative BIs (n=5)

The quantitative results allowed both D-value methods (Sections 1.4.1 and 1.4.2) to be calculated for Test R. The D-values, calculated by each of the methods, are shown in Figure 3-34.

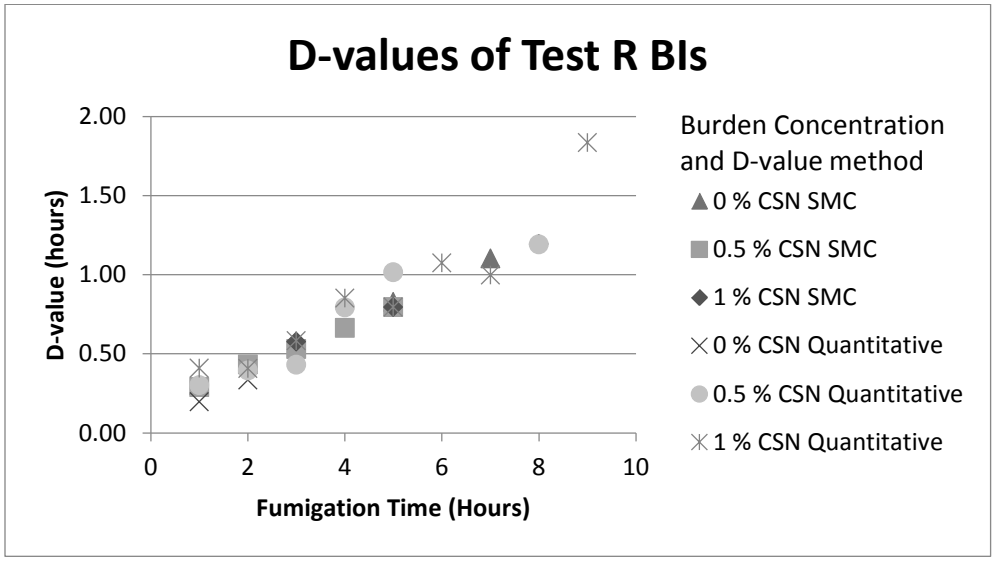

Figure 3-34. D-Values (1000 ppm ClO_2) of *B. atrophaeus* BIs versus Fumigation Time (Test R)

Figure 3-34 shows the sigmoidal response of the BIs with the strong relationship of the D-value to fumigation time. Figure 3-34 also shows no sensitivity to the concentration of the CSN burden, but does show a good correlation of the two methods of D-value estimation.

The similarities of Figure 3-32 and Figure 3-33, and the similar values for D-values as shown in Figure 3-34, suggest that both qualitative and quantitative BI methods can be used to assess the efficacy of a fumigation, suggesting in turn that the survival rate of qualitatively analyzed BIs can hinge on the presence or absence of very few spores. Designing a BI and predicting the response of 99 % or even 99.9 % of the spores on the BI is rather easy, but predicting the response of just a few protected spores with special circumstances is exceedingly difficult. This second population of resilient spores drives the Growth/No Growth response.

3.9.5 Fumigation Repeatability (Tests S, T, U and V)

Tests S and T were originally designed to be triplicate fumigations of the same set of BIs, designed to detect differences in D-value and survival rates of the BIs due to variations in fumigations. Only two of these tests were performed; the third test was performed for longer exposure times. Like Test R described in Section 3.9.4, this test included BIs to be analyzed both quantitatively and qualitatively. Unlike some of the previous differences in survival rates of burdened and unburdened BIs from previous fumigations, the results of these two replicate fumigations were very similar. Figures 3-29 and 3-30 show a comparison of BI results between the two fumigations.

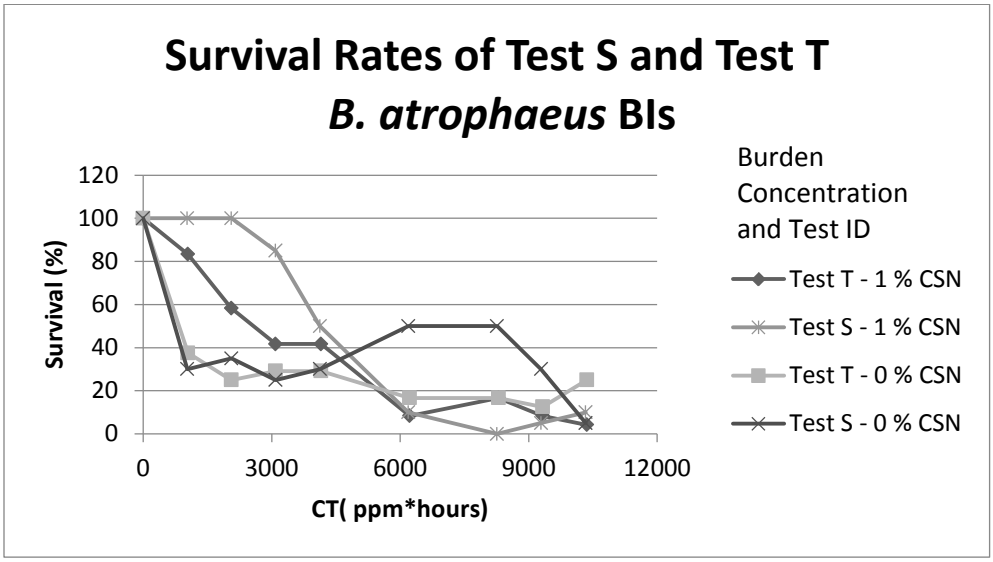

Figure 3-35. Survival (1000 ppm ClO_2) from Test S and Test T BIs (n=20)

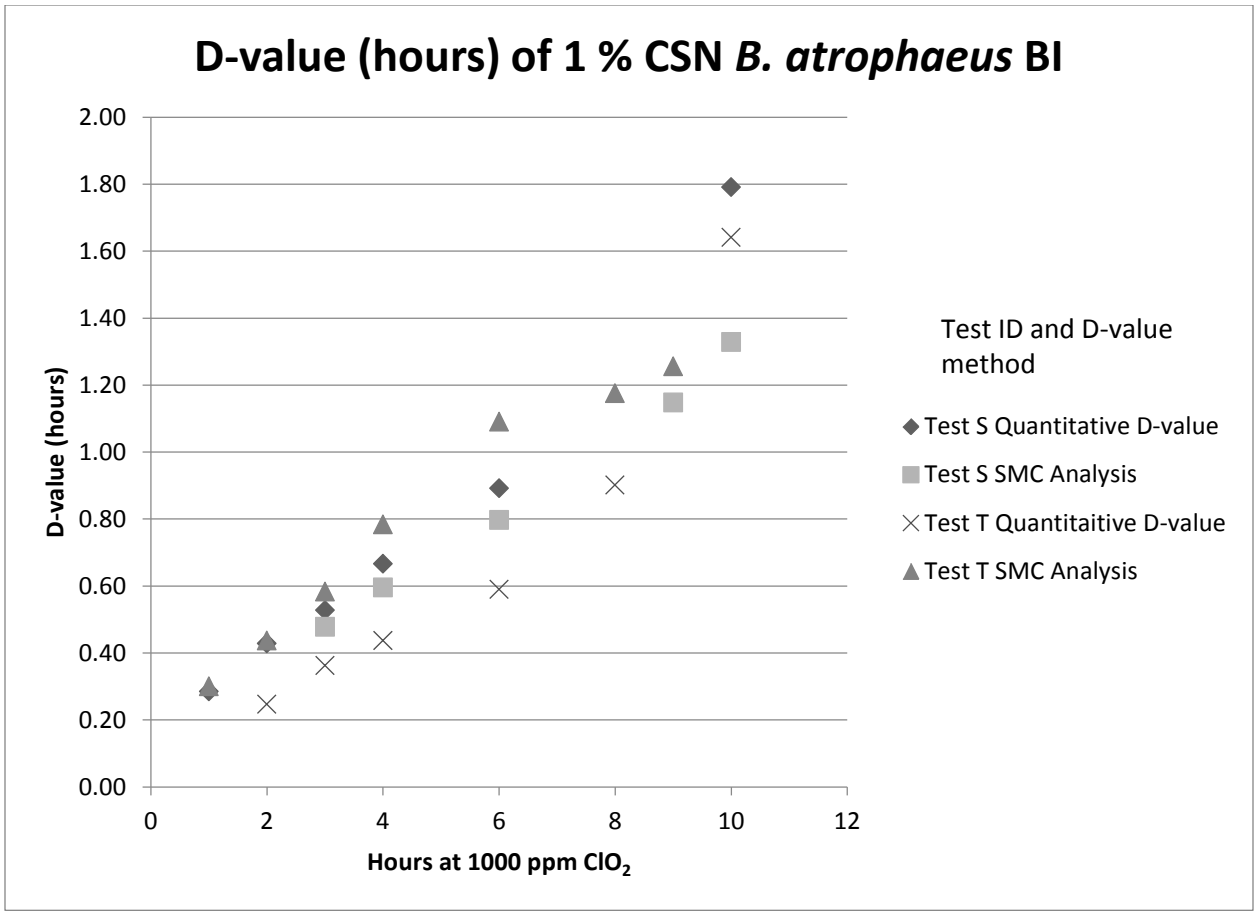

Figure 3-36. Two D-Value Methods of 1 % CSN *B. atrophaeus* BIs from Two Fumigations

The positive slope of D-values over time again suggests that the survival curve of the 1% casein BI has a tailing. Survival rates at long fumigation times are driven by a subset of very resistant spores. This subset of spores may possess an intrinsic resistance to fumigation, or may be protected due to location or proximity to other spores.

Tests U and V investigated longer fumigation times (up to 20 hours) to determine the length of the tailing. The survival rates of 1% casein BIs over the four tests are shown in Figure 3-37.

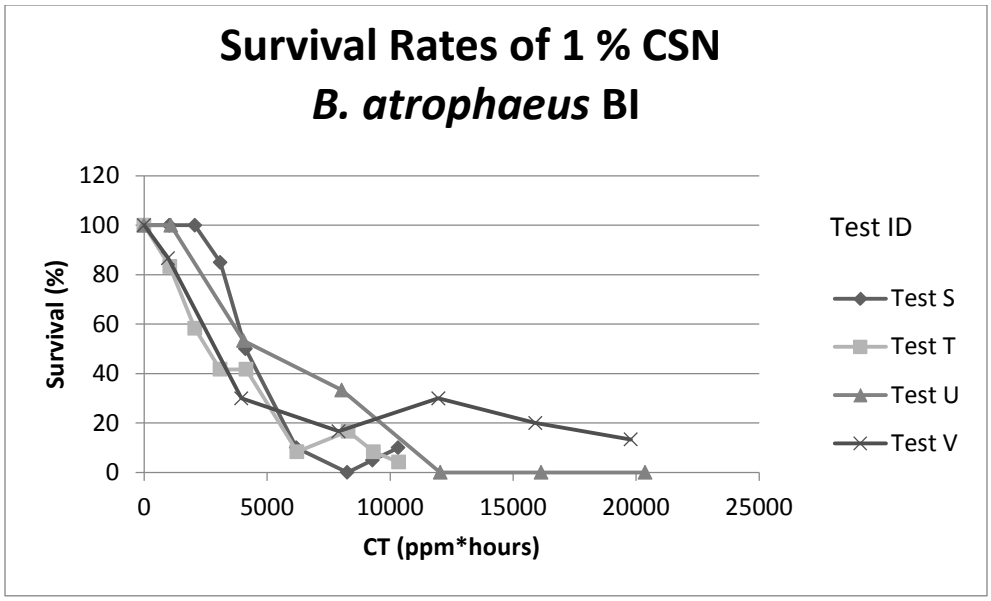

Figure 3-37. Survival (1000 ppm ClO_2) of 1% Casein BIs (Qualitative Analysis)

While there are some obvious differences in the survival rates, the Tests S and U showed a lag time of greater than one hour and Tests S, T, and U all had survival rates under 10 % after ten hours. In contrast, the Test V batch of BIs had a time lag less than one hour and had a tailing with survival rates near 20 % for fumigation times between eight and twenty hours. Differences in production may have more of an effect than differences in fumigation parameters

3.9.6 Effects of Spore Population Density

A series of tests (Tests W, X and Y) was performed to determine if the tailing effects were due to a relatively small number of surviving spores protected by clumping or some other mechanism due to the large spore population on the BI. This series of tests used two populations of B. *atrophaeus* (1.2×10^3 and 1.1×10^5 CFU) rather than the approximately 2×10^6 population inoculated onto previous tests.

Figure 3-38 shows the survival rates of the lowest inoculum BIs for all three fumigations.

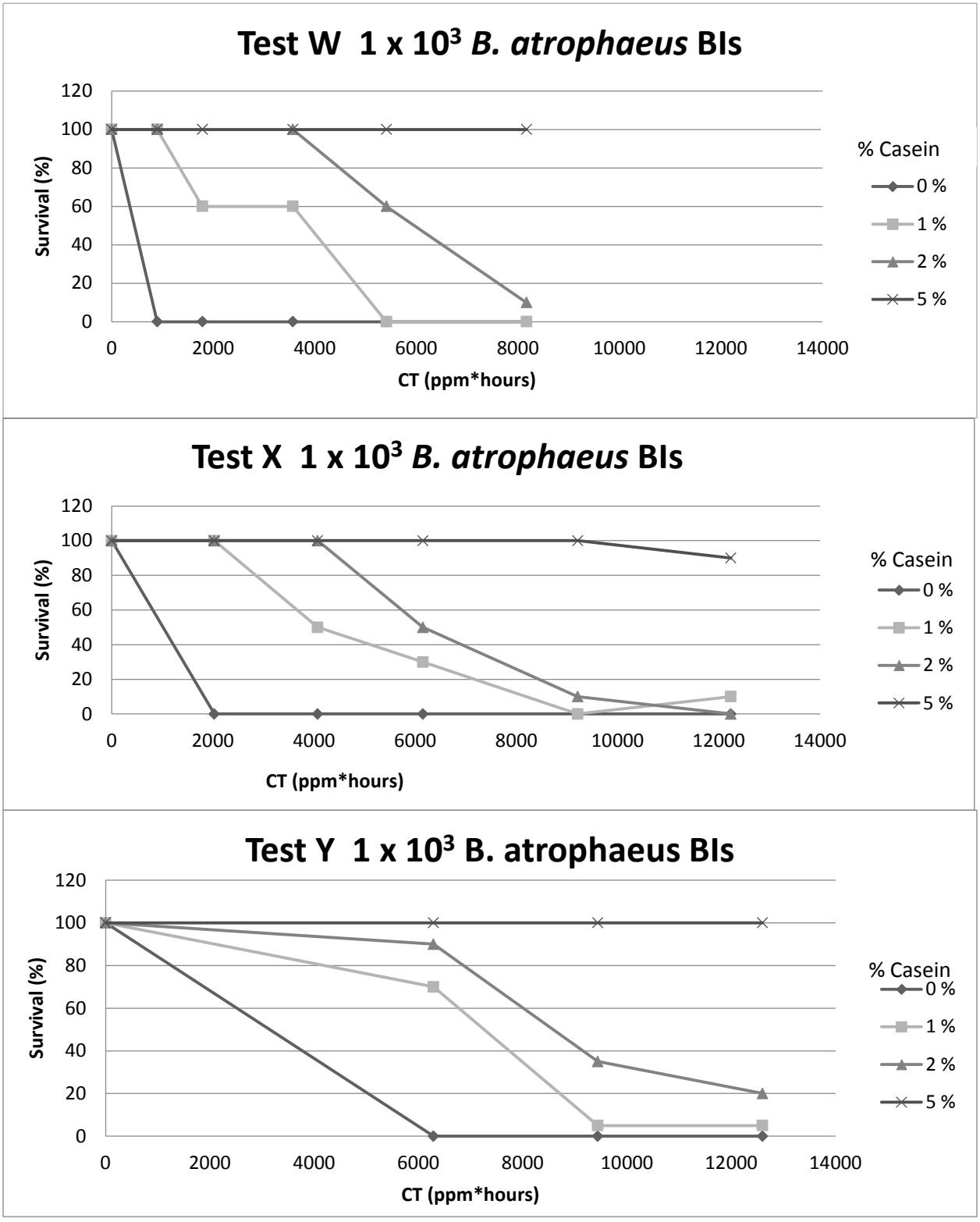

Figure 3-38. Survival (1000 ppm ClO_2) of 10^3 CFU Inoculum *B. atroph*aeus BIs (n=10)

A clear relationship between survival rates and casein concentration can be seen in Figure 3-38. Higher burden concentrations impart increased chances for survival by increasing the time lag. These data do not suggest that the burden also changes the D-value once the time lag has been met, though more data points would be necessary to fully test that hypothesis. Again, BIs with a 1% - 2% casein burden show promise, with resistance to ClO_2 fumigation for four to six hours, and with high probability of deactivation at nine hours. Such results may warrant further investigation of this BI.

A second batch of the low inoculum BIs were produced for the CT investigation described in Section 3.9.7. The survival rates for Test AA are shown in Figure 3-39. This second batch of low inoculum BIs had a response similar to the prior batch.

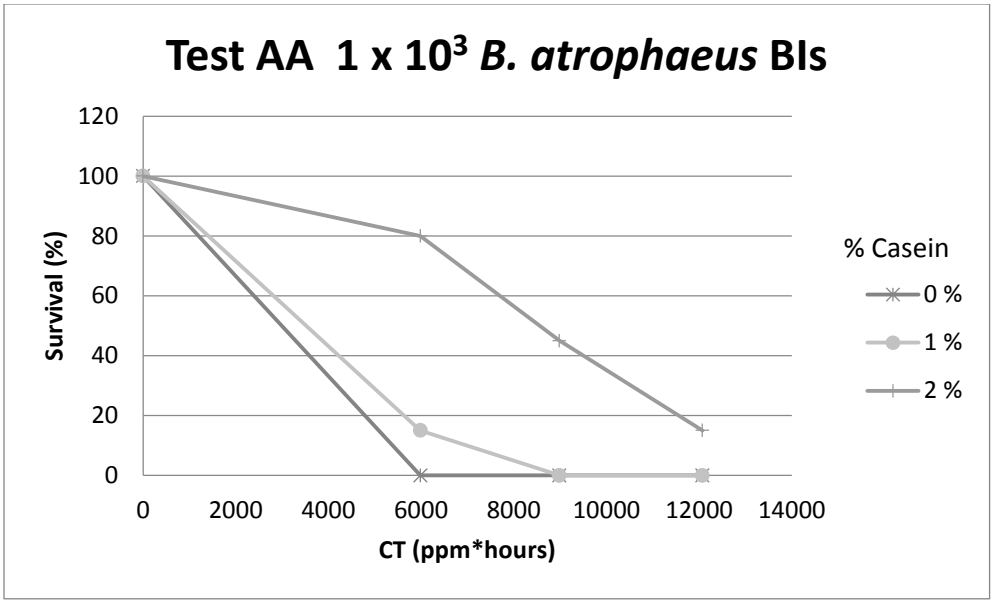

Figure 3-39. Survival (1000 ppm ClO_2) for Low inoculum BIs (n=20) in Test AA

While further testing is needed, the 1 % and 2 % casein BIs with a 1 x 10^3 inoculum could be an excellent model for *B. anthracis* spores. Side-by-side testing of the two species could provide information for a confidence model, including guidance on the number of replicates needed and whether one concentration or a combination of both burden concentrations offers a better prediction.

Figure 3-40 shows the response of BIs with 100x more spores (i.e., 1 x 10^5 *B. atrophaeus*) in the original inoculum (see Figure 3-38). Clearly, the higher inoculum levels provide a greater chance for survival. Interestingly, the unburdened BIs in some cases had higher survival rates than BIs with CSN. Behavior of these BIs was not unlike previous tests with the higher 2 x 10^6 inoculum.

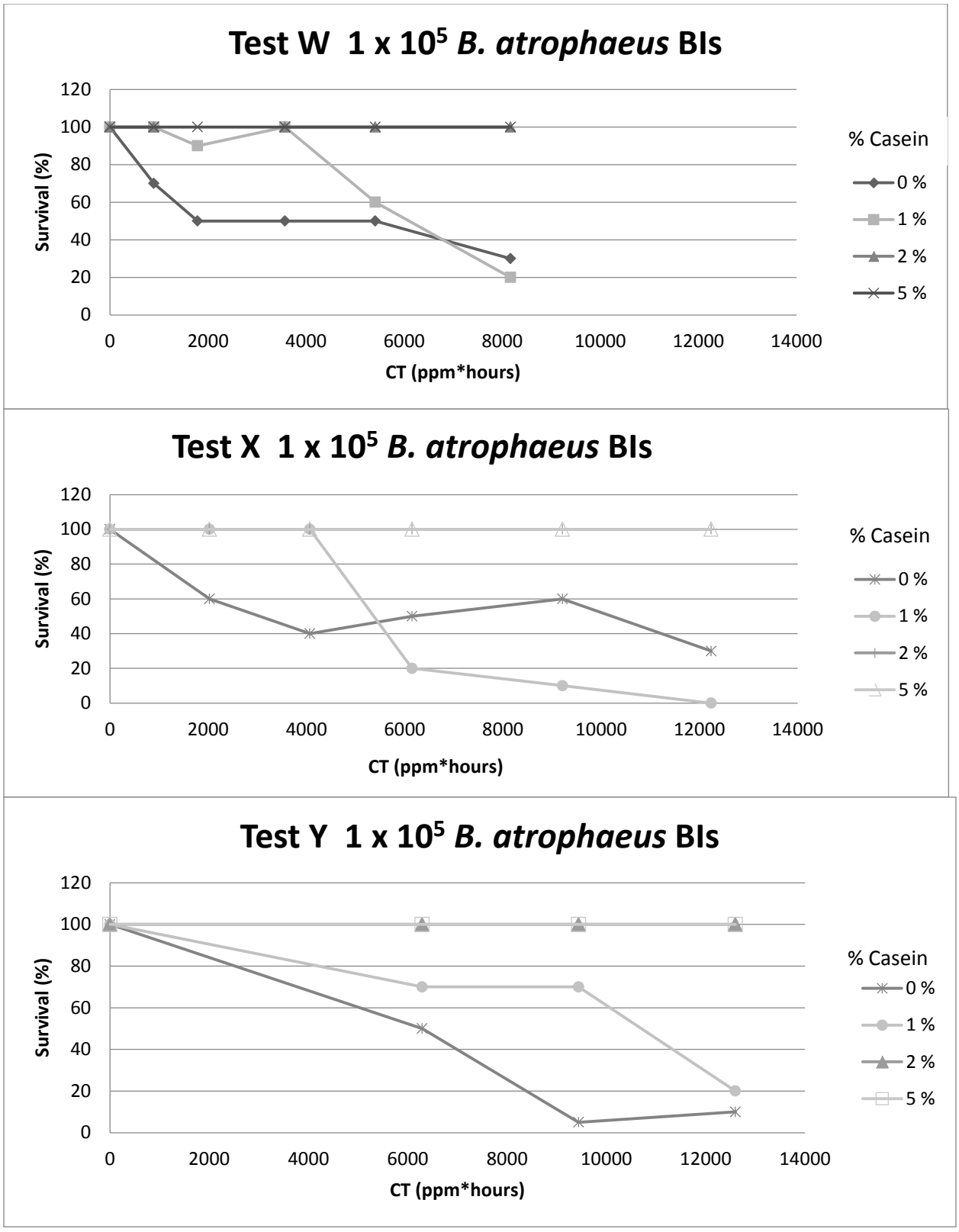

Figure 3-40. Survival (1000 ppm ClO_2) of 10^5 CFU Inoculum *B. atroph*aeus BIs (n=10)

The W. X, and Y fumigations were not of sufficient duration to determine if tailings might be more likely with the higher inoculum, though tailings with the higher inoculum are certainly suggested with the 0% casein results.

3.9.7 CT Investigation of Low Inoculum

Tests Z, AA, and AB used the low inoculum BIs discussed in Section 3.9.6 at three ClO_2 concentrations, 500 ppm, 1000 ppm, and 2000 ppm. For these tests, exposures were targeted towards the same CT for the different concentrations. The target times and the actual CTs are listed in Table 3-18.

Table 3-18. Target and Actual CT Exposure for Tests Z, AA, and AB

Target CT (ppm*hours)	2000 ppm		1000 ppm		500 ppm	
	Test Z		Test AA		Test AB	
	Exposure Time (hours)	Actual CT (ppm*hours)	Exposure Time (hours)	Actual CT (ppm*hours)	Exposure Time (hours)	Actual CT (ppm*hours)
6000	3	6470	6	5980	12	5840
9000	4.5	9720	9	8980	18	9410
12000	6	13070	12	12070	24	13350

Most of the *B. atrophaeus* BIs with no burden or 1 % casein burden were deactivated by the 6000 ppm*hour CT for all three fumigations. The survival rate of the 2 % casein BI is shown in Figure 3-41, plotted against both CT and fumigation time.

The response of this BI to CT exposure looks similar at 2000 ppm and 1000 ppm, but does not look similar for the 500 ppm fumigation. The long fumigation times required to reach target CT at 500 ppm proved more effective than the shorter fumigation times at the two higher concentrations, possibly related to increased permeability of the spore coat by extended exposure to high RH, as there seemed to be no benefit to raising the ClO_2 concentration from 500 ppm to 1000 ppm at a 12 hour fumigation time. This is not to say that the BIs were insensitive to increased concentration, and the 2000 ppm fumigation conditions provided a similar decontamination efficacy in a shorter amount of time.

The material coupons (See Section 2.1.6) that were included in this test series responded similarly to the 2% casein BIs. Table 3-19 shows the CFU recovered from coupons from Test AA and Test AB. The longer fumigation at 500 ppm was more effective than the shorter fumigation at 1000 ppm, though both were effective at providing a 6 LR for at least some coupon types.

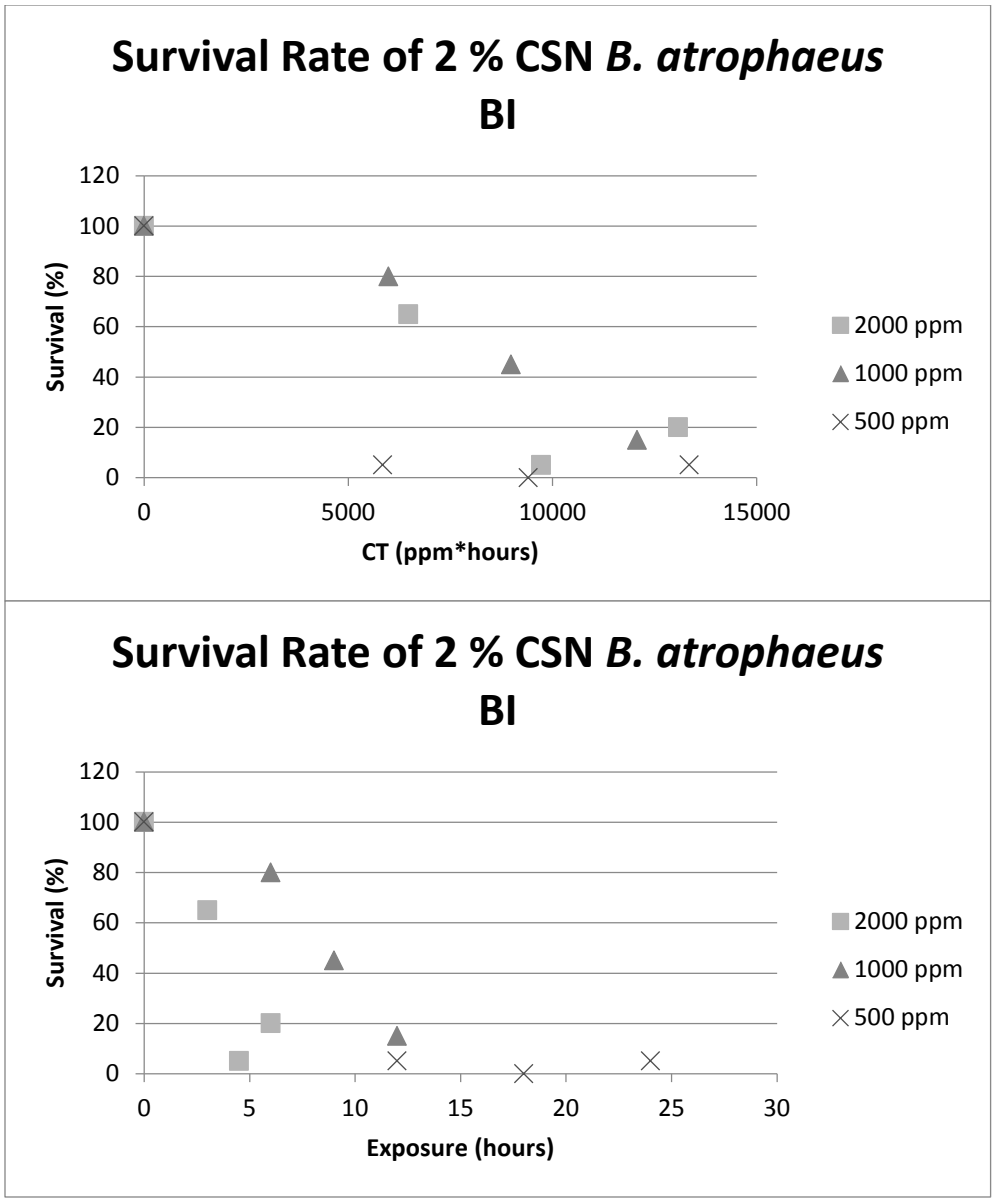

Figure 3-41. Survival of 2 % CSN *B. atrophaeus* BI (n=20) at Three ClO_2 Concentrations (Test Z, AA, and AB)

Table 3-19. Recovery (CFU) from Test AA and Test AB Coupons

	CT (ppm*hours)	0	6000	9000	12000
500 ppm	Aluminum	6.03E+06	ND	43	50
	Carpet	4.87E+06	73	ND	ND
	Wood	6.99E+06	ND	ND	ND
1000 ppm	Aluminum	2.41E+07	109	10	ND
	Carpet	6.98E+06	316	113	164
	Wood	6.96E+06	6	7	ND

Carpet coupons were the most difficult to decontaminate, with spores surviving a 12-hour fumigation at 1000 ppm ClO_2 (12,000 ppm*hours).The carpet coupons did not show growth after an 18-hour fumigation at 500 ppm (9,000 ppm*hours).

3.9.8 Age of BI

Test J was conducted with a constricted test matrix due to the limited availability of BIs remaining from prior tests. BIs of various batches and ages were subjected to the same fumigation (1000ppm, 75% RH, 25°C) during Test J to identify any effect of age on BI survival rates. For all BIs, survival rates following fumigation of aged BIs were lower than rates from the original fumigation. Figure 3-42 shows the change in the survival rates as a function of the age of BIs with various concentrations of CSN burden. A change of 100% means that 100% of the BIs survived the original fumigation (when new) and 0 % of the same batch survived the fumigation when old.

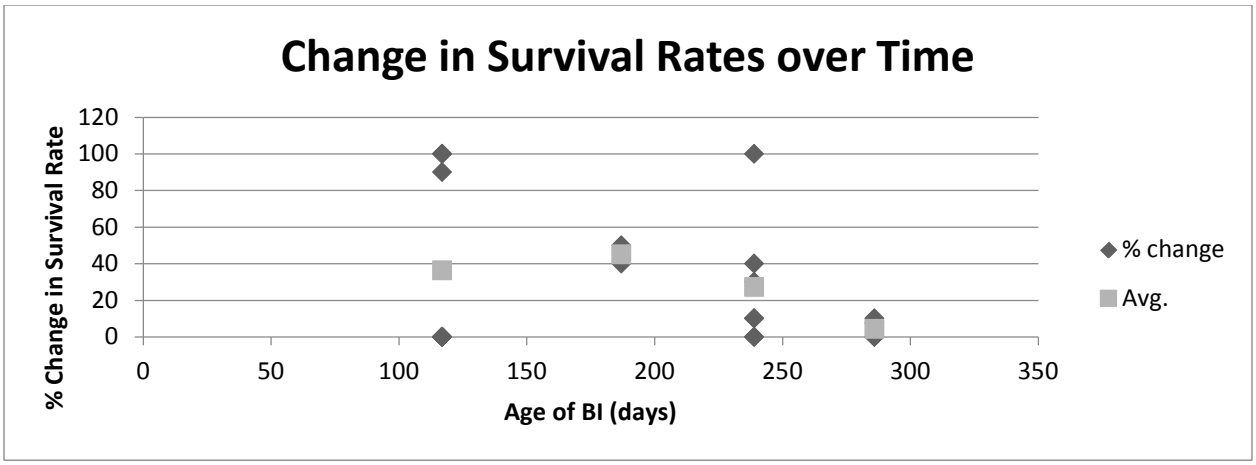

Figure 3-42. Effect of Age of CSN BI on Survival Rates (Test J)

In general, there was a change in survival rates for CSN-burdened BIs, but there does not appear to be a trend with respect to the age of the BI, suggesting that BIs with burdens may have a relatively stable shelf life, though the number of variations, including differences in burden concentration, fumigation conditions, and BI batches, lessen the confidence in this conclusion. No age-related trends were detected in either gelatin (GEL) or CSN *B. atrophaeus* BIs.

The Test WXY series also permitted triplicate fumigations of the same batch of BIs. Fumigation conditions are shown in Table 3-20.

Table 3-20. Fumigation Conditions for Low Inoculum Tests

	Test W	Test X	Test Y
Average RH (%)	75.1	75.4	75.0
SD RH	0.0	0.5	0.2
Average Temp (°C)	22.9	23.5	23.7
SD Temp	0.2	0.4	0.1
Average mSM 4500-ClO_2 (mg ClO_2/L)	2.5	2.8	2.9
Average Photometer (mg ClO_2/L)	2.4	2.8	2.8
6 Hour ppm*hours	5410	6150	6290

D-values (Table 3-21) were calculated for all BIs that demonstrated partial survival; D-values cannot be calculated for conditions where all BIs show growth or all BIs show no growth. The Pearson correlations between the D-values and fumigation conditions were calculated and are shown in Table 3-22.

Table 3-21. D-Values (hours) for BI Types during Low Inoculum Tests

BI	Test W	Test X	Test Y
1 % CSN 10^2 CFU	0.96	1.47	2.27
2 % CSN 10^2 CFU	2.09	2.06	2.71
0 % CSN 10^4CFU	1.01	1.52	1.81
1 % CSN 10^4CFU	1.06	1.27	1.71

Table 3-22. Correlation between D-Values of Selected BIs and Fumigation Conditions

BI	RH corr	T corr	4500 corr	EMS corr	ppm*hours corr	Age of BI corr
1 % CSN 10^2 CFU	-0.16	0.90	0.82	0.72	0.87	1.00
2 % CSN 10^2 CFU	-0.57	0.62	0.49	0.36	0.58	0.92
0 % CSN 10^4 CFU	0.12	0.99	0.95	0.89	0.98	0.95
1 % CSN 10^4 CFU	-0.23	0.87	0.78	0.67	0.84	1.00

Corr = Correlation.

The strongest correlation with D-value was the age of the BI, with increasing resistance to fumigation as the BI aged on the shelf. Unlike Test J, this series was conducted with much shorter shelf lives, on the order of weeks rather than months. Coincidentally, the actual fumigation concentration also increased with increasing age of the BI. Further testing should be conducted before drawing conclusions about shelf stability, though shelf stability would be essential for a custom BI.

There was no change in D-value for the series of Test S, Test T, and Test U as a result of age.

4 Quality Assurance

This project was performed under two approved Category III Quality Assurance Project Plans (QAPP) titled *Decontamination Process Indicators. Part 1 – Biological Indicators. Part 2 – Process Parameter Correlations (*December 2009) and *Part III – Determination of the Effect of Spores Storage Time on Susceptibility to Inactivation by* ClO_2 (September 2010)..

4.1 Sampling, Monitoring, and Analysis Equipment Calibration

Documented operating procedures were used for the maintenance and calibration of all laboratory and NHSRC RTP Microbiology Laboratory equipment. All equipment was certified by the manufacturer as calibrated or had the calibration verified by EPA's Air Pollution Prevention and Control Division (APPCD) on-site (RTP, NC) Metrology Laboratory prior to use. Standard laboratory equipment such as balances, pH meters, BSCs and incubators were routinely monitored for proper performance. Calibration of instruments was done at the frequency shown in Table 4-1. If deficiencies were noted, the instrument was adjusted to meet calibration tolerances and recalibrated within 24 hours. If tolerances were not met after recalibration, additional corrective action was taken, including recalibration and/or replacement of the equipment.

Table 4-1. Sampling and Monitoring Equipment Calibration Frequency

Equipment	Calibration/Certification	Expected Tolerance
Thermometer	Compare to independent NIST thermometer (this is a thermometer that is recertified annually by either NIST or an International Organization for Standardization (ISO)-17025 facility) value once per quarter	± 1°C
Stopwatch	Compare against NIST Official U.S. time at http://nist.time.gov/timezone.cgi?Eastern/d/-5/java once every 30 days.	± 1 min/30 days
Clock	Compare to office U.S. Time @ time.gov every 30 days.	± 1 min/30 days
pH meter	Compare to NIST-traceable buffer solutions daily	± 0.2 units
Micropipettes	All micropipettes will be certified as calibrated at time of use. Pipettes are recalibrated by gravimetric evaluation of pipette performance to manufacturer's specifications every year.	± 5%
BSC	The BSC will be verified to be within certification dates at the time of use. BSC are adjusted yearly to be within flow tolerances established by the manufacturer.	± 10%
Titration Equipment	Titration equipment and reagents will be calibrated weekly against a known standard solution of 1000 ppmv chlorite.	± 15%
Scale	Compare reading to Class S weights	± 1%

The metering device used for ClO_2 extractive sample collection was calibrated annually by the APPCD Metrology Laboratory.

4.2 Data Quality

The data quality objectives (DQOs) of this project are three-fold:

- Collect data to permit development of a custom BI that matches *B. anthracis* response to fumigation. (Part 1)

- Collect data to examine the sensitivity of a fumigation process to variability in environmental factors, specifically temperature and relative humidity. (Part 2)

- Collect data to determine the effect of BI storage time on susceptibility to inactivation by ClO_2. (Part 3)

The objective of this project was to develop a custom BI that would provide reliable results of No Growth after fumigation with 9000 ppm*hours ClO_2, while at the same time providing Growth results at fumigation conditions unlikely to deactivate *B. anthracis* spores. This section discusses the QA/QC checks (Section 4.3) and Acceptance Criteria for Critical Measurements (Section 4.4) considered critical to accomplishing the DQOs.

4.3 QA/QC Checks

Uniformity of the test materials was a critical attribute for assuring reliable test results. Uniformity was maintained by obtaining a large enough quantity of material that multiple material sections and carriers could be constructed with presumably uniform characteristics. Samples and test chemicals were maintained to ensure their integrity. Samples were stored away from standards or other samples which could cross-contaminate them.

Supplies and consumables were acquired from reputable sources and were NIST-traceable when possible. Supplies and consumables were examined for evidence of tampering or damage upon receipt and prior to use, as appropriate. Supplies and consumables showing evidence of tampering or damage were not used. All examinations were documented and supplies were appropriately labeled. Project personnel checked supplies and consumables prior to use to verify that they met specified task quality objectives and did not exceed expiration dates.

Quantitative standards do not exist for biological agents. Quantitative determinations of organisms in this investigation did not involve the use of analytical measurement devices. Rather, CFU were enumerated manually and recorded. QC checks for critical measurements/parameters are shown in Table 4-2. Acceptance criteria (see Section 4.4) were set at the most stringent level that could be routinely achieved. Positive controls and procedural blanks were included along with the test samples in the experiments. Other background checks were also included as part of the standard protocol. Replicate BIs were included for each set of test conditions. Operating procedures were performed by qualified, trained and experienced personnel were used to ensure data collection consistency. The confirmation procedure, controls, blanks, and method validation efforts were the basis of support for biological investigation results. If necessary, training sessions were conducted by knowledgeable parties, and in-house practice runs were used to gain expertise and proficiency prior to initiating the research.

Table 4-2. QA/QC Sample Acceptance Criteria

QC Sample	Information Provided	Acceptance Criteria	Corrective Action
Negative Control BI (coupon or BI without biological agent)	Controls for sterility of materials and methods used in the procedure.	No observed CFU	Reject results, identify and remove source of contamination.
Positive control (BI or inoculated material not fumigated)	Shows ability of incubation tubes to support and show growth	Growth.	Reject results. Identify and correct problem.
Turbidity Control (BI with material or burden, not inoculated but fumigated)	Provides information about the tendency towards false positives of candidate BI	Both outcomes were accepted.	Materials or burdens which show growth were rejected for subsequent study.
Performance Control (BI with burden spiked after fumigation)	Provides confirmation that the fumigated burden is compatible with bacterial growth	Growth	Reject burden.
Blank tryptic soy agar Sterility Control (plate incubated, but not inoculated)	Controls for sterility of plates.	No observed growth following incubation.	All plates are incubated, so any contaminated plates were discarded.

In addition, Appendix C contains the *DTRL – QC Checklist for Data Reviewers*, which was used to review the data presented in this report.

4.4 Acceptance Criteria for Critical Measurements

DQOs are used to identify the critical measurements needed to address the stated objectives and specify tolerable levels of potential errors associated with simulating the prescribed decontamination environments. The following measurements were deemed to be critical to accomplish the project objectives:

- Real-time fumigant concentrations
- Temperature
- RH
- Fumigation time sequence
- Determination of spore survival for selected samples

The Data Quality Indicators (DQIs) listed in Table 4-3 are specific criteria used to quantify how well the collected data met the DQOs. The accuracy of the real-time ClO_2 monitors as assessed with respect to the mSM 4500-ClO_2 Methods. Precision of the EMS real-time ClO_2 monitor could not be accessed due to unavailability of a constant-concentration source and the feedback nature of their operation in this specific testing. The accuracy of the extractive methods was assessed using standards of known concentration. RH sensors were compared to a calibrated standard humidity sensor and a standard saturated salt

solution producing a 75 % RH atmosphere. Failure to provide a measurement method or device that met these goals resulted in the rejection of results derived from the critical measurement. For instance, if the plated volume of a sample was not known (i.e., is not 100% complete), then that sample was deemed invalid.

Table 4-3. Accuracy and Completeness DQIs for Critical Measurements

Measurement Parameter	Analysis Method	Accuracy	Detection Limit	Completeness %
Real-time ClO_2 concentration inside the test chamber (high concentration tests)	ClorDiSys EMS monitor (0.1 – 30 mg/L)	15 % of mSM-4500-E	0.1 mg/L 36 ppmv	100
Extracted ClO_2, high concentration	mSM 4500-ClO_2	5% of Standard solution	0.1 mg/L (solution)	90
RH	RH probes (0-100 %)	± 5 % of 75% standard salt solution	NA	95
Differential time	Computer clock	1 % of reading	0.5 sec	95
Temperature inside the test chamber	Thermocouple	± 2 $^{\circ}$C	NA	90
Bacterial Growth	NHSRC RTP Microbiology Laboratory MOP 6560. 6566	Categorical (presence or absence)	1 CFU	99

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Table 4-4 lists how well the critical measurements collected during testing met the completeness criteria described in Table 4-3.

Table 4-4. Completeness of DQIs

Test ID	Real-time ClO_2 Concentration Inside the Test Chamber	Extracted ClO_2	RH	Differential Time	Temperature Inside the Test Chamber
	DQI Completeness Goals				
	100%	90%	95%	95%	90%
Test A	100.0	0	100	100	100
Test B	90.0	0	100	100	100
Test C	90.2	0	100	100	100
Test D	92.8[a]	0	100	100	100
Test E	0.0	0	100	100	100
Test F	100.0	0	100	100	100
Test G	97.2	0	100	100	100
Test H	97.0	100	0	100	100
Test I	94.5	100	100	100	100
Test J	97.9	100	100	100	100
Test K	100.0	100	100	100	100
Test L	99.9	0	100	100	100
Test M	100.0	0	100	100	100
Test N	51.7	0	100	100	100
Test O	95.3	100	100	63.7	100
Test P	74.1	0	100	100	100
Test Q	87.4	0	100	100	100
Test R	100.0	0	100	100	100
Test S	100.0	0	100	100	100
Test T	99.4	0	100	100	100
Test U	94.9	0	100	100	100
Test V	100.0	0	100	100	100
Test W	100.2	100	100	100	100
Test X	98.1	0	100	100	100
Test Y	99.8	0	100	100	100
Test Z	100	0	100	100	100
Test AA	100	0	100	100	100
Test AB	10.0[b]	0	100	100	100

[a] The unavailability of primary photometer data records required the real-time ClO_2 concentration data recorded by the ClO_2 generator (secondary photometer) be used.

[b] During Test AB, approximately eight hours into the 24 hour exposure, the primary photometer began to fail, resulting in 10% completeness for the category

In most instances, when the real-time ClO_2 concentration measurement did not meet the completeness goal, fluctuations with the ClorDiSys primary photometer were the cause. However, in Test E, a ClorDiSys EMS monitor malfunction resulted in 0% completion of the real-time ClO_2 concentration DQI. A ClO_2 generator data file was also unavailable for this test. Extracted ClO_2 samples were therefore used to

monitor the ClO_2 concentration. Also, screenshots of the real-time plot generated by the ClO_2 generator provide a graphical representation of ClO_2 concentrations throughout exposure time. Records for the validation of the titration equipment could not be found for many tests

The completeness for the Test H RH was 0 % because the RH during the exposure time was lower than intended. Since the acceptance criteria for precision were met, the data set was used as a low relative humidity test for comparison with the other relatively high RH tests.

The DQIs for bacterial growth are determined by the presence of turbidity associated with the growth of target microorganisms within TSB media. Every TSB media tube is visually inspected prior to the addition of any BI and is inspected again 7-9 days after a BI has been aseptically transferred to a sterile TSB tube, and the tube has been allowed to incubate at the temperature most favorable for growth of the target microorganism. Visual inspection of the tubes, both before and after the addition of the BI samples, is 100 %. The accuracy and ability to detect and allow for growth and proliferation of the target organism in the TSB media (to determine the presence or absence of viable microorganisms) is 1 CFU.

Further, all TSB media tubes that were found to be negative for turbidity (growth) were subjected to homogenization by either inversion of the sample tube or by vortex mixer and were directly plated (either by sterile loop or pipette) to confirm the absence of growth (especially by the target microorganism). Also, 10 % of all samples that were turbid upon visual inspection and therefore indicative of growth were plated (either by sterile loop or pipette) to confirm that the growth was consistent with the colony morphology for the target microorganism. MOPs 6566 and 6566 rev 1 were followed to complete the analytical methods for processing the BI samples.

The quantitative acceptance criteria were associated with targeted setting conditions in the test chambers during the entire exposure time. These acceptance criteria are listed in Table 4-5.

Table 4-5. Precision Acceptance Criteria for Critical Measurements

Measurement Parameter	Analysis Method	Precision RSD (%)
Real-time ClO_2 concentration inside the test chamber	ClorDiSys EMS monitor (0.1 – 30 mg/L)	$\pm$ 10 %
Extracted ClO_2 inside the test chamber	mSM 4500-ClO_2	± 15 %
RH inside the test chamber	RH probes (0-100 %)	± 15 %
Temperature inside the chamber	Thermistor	± 2 °C
Incubator temperature	Type K thermocouple	± 2 °C
Refrigerator temperature	Type K thermocouple	± 2 °C
Plated media (incubated before inoculation)	Visual	NA
Microbiological material blank	Visual	NA
Positive Control BIs	Visual	NA

Table 4-6 details the precision of the critical measurements for each test.

Table 4-6. Observed Precision of Critical Measurements

Test ID	Real-time ClO2 Concentration Inside the Test Chamber	Extracted ClO2, High Concentration	RH	Temperature Inside the Test Chamber
	Precision RSD Acceptance Criteria Goals			
	± 10 %	± 15 %	± 15 %	± 2 °C
Test A	3.8	1.5	0.1	0.8
Test B	12.4	14.2	0.1	1.2
Test C	5.6	13.8	1.1	2.7
Test D	1.7	3.6	0.3	1.6
Test E	NA	2.2	0.4	0.2
Test F	2.0	3.0	0.2	1.5
Test G	6.6	5.1	0.2	0.5
Test H	5.4	4.3	0.5	2.6
Test I	13.6	11.7	1.4	2.7
Test J	3.5	6.4	2.4	2.6
Test K	3.1	3.5	0.1	2.9
Test L	3.8	3.7	0.4	0.6
Test M	14.7	2.5	1.2	1.2
Test N	35.8	13.4	1.9	1.1
Test O	16.4	15.7	0.3	1.0
Test P	4.6	2.9	0.9	0.5
Test Q	17.8	2.9	0.5	0.5
Test R	3.3	2.2	0.3	0.4
Test S	3.5	3.2	0.1	0.4
Test T	3.2	5.5	0.1	1.5
Test U	5.4	4.6	0.1	1.2
Test V	3.0	2.9	0.1	1.9
Test W	5.0	5.8	0.1	0.4
Test X	6.5	7.6	0.6	5.4
Test Y	8.9	3.5	1.3	0.6
Test Z	3.4	9.1	3.3	1.1
Test AA	3.3	4.6	0.3	1.4
Test AB	20.1	32.1	3.3	0.3

During Test E, all BIs experienced a spike in ClO_2 concentration to 6.6 mg/L at the beginning of this fumigation. They were tested at 1, 5, 7 and 9 hours. The primary photometer failed to report data, so the only photometer data available are manually recorded values.

The RH during Test H was incorrectly controlled due to operator error, leading to an average RH of 60%.

Several anomalies were encountered during the Test I fumigation due to equipment failure, leading to a spike of nearly twice the target concentration.

The photometer reading of the GMP chlorine dioxide generator during Test O was erratic, leading to the cancellation of the test and higher than anticipated exposure.

Instances when the real-time ClO_2 concentration measurement was not within QA specifications can be attributed to mechanical failures which, in some cases, were resolved by performing regular equipment maintenance. The majority of the tests that did not meet the real-time ClO_2 concentration inside the test chamber precision requirement met the precision requirement for extracted ClO_2, further indicating mechanical failure for the real-time measurements that did not meet the acceptance criteria. The two instances (i.e. Test O and Test AB) where both the real-time and extracted ClO_2 measurements are indicative of a poorly controlled fumigation which should be considered when examining the results. The temperature measurements for consecutive tests, Test H, Test I, Test J and Test K, were only slightly outside of the precision requirement.

As before mentioned, data from both photometers were unavailable for Test E. Therefore, a value for precision could not be applied

Test N had a target ClO_2 concentration of 250 ppm. However, the operating range for the ClO_2 generator is ± 35 ppm, and the generator is not optimized to control ClO_2 levels below 300 ppm. As a result, the acceptance criteria for the ClO_2 concentration tests were not met.

Plated volume critical measurement goals were 100 % completion. All pipettes are calibrated yearly by an outside contractor (Calibrate, Inc., Carrboro, NC, USA) and verified gravimetrically at the conclusion of testing.

Plates were quantitatively analyzed (CFU/plate) using a manual counting method. For each set of results (per test), a second count was performed on 25 % of the plates with significant data (data found to be between 30-300 CFU). All second counts were found to be within 10 %of the original count.

Many QA/QC checks are used to validate microbiological measurements. These checks include samples that demonstrate the ability of the NHSRC RTP Microbiology Laboratory to culture the test organism, as well as to demonstrate that materials used in this effort do not themselves contain spores. The checks include

- Field blank samples: sterile BIs or coupons sampled at the same time as inoculated BIs or coupons.

- Laboratory Media and Supplies: includes all materials, individually, used by the NHSRC RTP Microbiology Laboratory in sample analysis.

4.5 Data Quality Audits

This project was assigned QA Category III and did not require technical systems or performance evaluation audits.

4.6 QA/QC Reporting

QA/QC procedures were performed in accordance with the QAPP for this investigation.

4.7 Amendments to Original QAPP

All amendments to the original QAPP were submitted by e-mail to the EPA QA officer for formal approval.

5 Summary

Biological indicators (BIs) have often been used to indicate the efficacy of a sterilization technique, especially in cases such as fumigations where distribution may not be uniform. Many COTS BIs and modifications to BIs were tested under this multi-year investigation for their suitability to determine the efficacy of a ClO_2 fumigation for *B. anthracis*-contaminated building materials. These modifications included chemical burdens, changes in coupon material (carriers), and physical barriers. Based on previous data, a kill point of 9,000 ppm*hours exposure to ClO_2 gas was used as the target exposure to model *B. anthracis* spore kill on building material surfaces. No modification was identified capable of achieving a repeatable and precise (± 500 ppm*hours) BI deactivation at 9000 ppm*hours, but not before. However, results from tests investigating the sources of variance may be used to focus future decontamination and sterilization research.

Burdens can have the effect of increasing survival rates and D-values of BIs. Burdens that seemed to increase survival rates of seven-hour fumigations and yet did not provide protection such that all BIs survived nine-hour fumigations included cellobiose, dithiothreitol, carrageenan, gelatin, and casein, all of which could be further evaluated. Most promising was 1 % casein as a burden on low inoculum (10^3 CFU) *B. atrophaeus* BIs. Results were variable, however, with large variations between batches and fumigations due to unidentified factors seemingly most related to production. Such results draw into question the utility of BIs for evaluating fumigation efficacy, as subtle differences in between-batch or between-vendor spore preparation conditions could significantly alter the outcome of these indicators.

While coupon materials did affect the survival rates of BIs, none of the carriers showed promise, providing either too much or too little protection. Unlike burdens, carriers could not be tested at different concentrations, therefore making it more difficult to adjust kill points. Fumigated wooden carriers would not support growth of the target organism and were therefore not a suitable carrier material. Carriers made of rubber were highly resistant to inactivation by ClO_2, suggesting this material may be difficult to decontaminate.

Either semi-permeable barriers or lumens can be used as physical barriers, and types of both were demonstrated to extend the survival rates of spores. More research should be conducted on semi-permeable membranes, as incorporation of membranes into BI manufacture would be easy to implement. BIs incorporating tortuous paths such as lumens should also be further investigated.

Various COTS BIs were investigated, including *B. atrophaeus* BIs from Apex Laboratories, Raven Labs, and three variations from Mesa Laboratories. Some were more hardy and some were less hardy than the target 9000 ppm*hour full kill. Moreover, the spore preparation was found to have an impact on spore survival rates. Because spore preparations exhibit this variability, the behavior of BIs can fluctuate from batch to batch, though there is also variability between batches using the same spore preparation, which suggests some other production factor may be causing the variation in survival rates. Future research should focus on removing variation from spore preparations or focus on species that are more easily destroyed, thus removing the significance of spore variation. Regardless, the apparent variability in kill points amongst COTS BIs suggests that BI variability may be unavoidable and therefore to some degree acceptable.

Two COTS BIs (Apex and Raven stainless steel *B. atrophaeus*) behaved very similarly on a concentration*time (CT) basis for two fumigations at two different fumigant concentrations (250 ppmv and 1000 ppmv ClO_2). This similar behavior is promising because field conditions in an actual event are expected to affect the maximum concentration any one fumigation technology can meet, and others have demonstrated that *B. anthracis* spore kill is more dependent upon the product of concentration and time (CT) than either concentration or time alone. Further, the spacing (distance between) of BIs within the exposure chamber did not affect kill kinetics in the current study. This is another important finding that can inform field-use of BIs.

Consistent with previous reports, the current study found that D-values are not linear over the duration of the fumigation. Many studies [9, 10, 11] have found curvilinear responses to chemical sterilizations, rather than a generally linear response to thermal sterilizations. Many BIs resilient enough to survive a seven-hour fumigation would also tend to survive a nine-hour fumigation. Put another way, a subpopulation of spores on a BI may have higher resistance than the main population, due either to a protective location or inherent hardiness, producing a biphasic response with most spores deactivated in an early portion of the sterilization cycle and a subset deactivated in a much later portion of the cycle. While a BI with very hardy spores may predict the behavior of bacterial spores in an actual event, a BI with overly hardy spores may also falsely predict the negative outcome of what in reality was a successful fumigation. One possible explanation of hardy spores was the protective bio-burden of clumping in high-inoculum BIs. Lower inoculum BIs were tested and showed a trend of lower survival rates. Further testing was conducted on these lower inoculum BIs, suggesting a lower tendency towards the long-surviving tail and an increasing resistance with increasing casein burden.

This work identified several techniques to allow BIs to survive longer fumigation durations. These techniques may be used to tune a BI to better model the inactivation of any target organism. To produce a good model of *Bacillus anthracis* with ClO_2 fumigations, the authors would recommend side-by-side comparisons of *Bacillus anthracis* to BIs with low inoculum and 1 % and 2 % casein burden.

Testing with COTS BIs showed that perceived issues (tailing effects) during custom BI developmental tests are not uncommon, as such effects are observed for COTS BIs as well. This effect has been observed during BI exposure to gaseous fumigation technologies, and may be less prevalent in heat/steam-based sterilization technologies such as autoclaving (personal communication, Joe Dalmasso – Yakibou Labs, Inc.). Tailing effects can have significant implications for BI use during real-world decontaminations, where BIs may be used as partial evidence to suggest fumigant effectiveness against an infectious agent. Designing a BI and predicting the response of 99 % or even 99.9 % of the spores on the BI is rather easy, but predicting the response of just a few protected spores with special circumstances is exceedingly difficult. This second population of resilient spores drives the Growth/No Growth response.

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Appendix A: Miscellaneous Operating Procedures

MOP 6535a	Serial Dilution: Spread Plate Procedure to Quantify Viable Bacterial Spores
MOP 6560	Biological Indicator (BI) Tests using Nutrient Broth and Analysis of Results
MOP 6562	Preparing Pre-Measured Tubes with Aliquoted Amounts of Phosphate Buffered Saline with Tween20 (PBST)
MOP 6566	Culturing of Apex Laboratories Tyvek Packaged Biological Indicators (Rev 0) and Culturing Biological Indicator Strips (Rev 1)
MOP 6570	Use of STERIS Amsco Century SV 120 Scientific Prevacuum Sterilizer
MOP 6576	Determination of Spore Thermal Challenge (Heat Shock) Resistance

Miscellaneous Operating Procedure (MOP) 6535a:

Serial Dilution: Spread Plate Procedure to Quantify Viable Bacterial Spores

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MOP 6535a

TITLE: SERIAL DILUTION: SPREAD PLATE PROCEDURE TO QUANTIFY VIABLE BACTERIAL SPORES

SCOPE: Determine the abundance of bacterial spores in a liquid extract

PURPOSE: Determine quantitatively the number of viable bacterial spores in a liquid suspension using the spread plate procedure to count colony-forming units (CFU)

Materials:

- Liquid suspension of bacterial spores
- Sterile centrifuge tubes
- Diluent as specified in QAPP or Test Plan (e.g., sterile water, Phosphate Buffered Saline with Tween 20 (PBST))
- Media plates as specified in QAPP or Test Plan (e.g., Trypticase Soy Agar (TSA) plates)
- Microliter pipettes with sterile tips
- Sterile beads placed inside a test tube (used for spreading samples on the media surface according to MOP 6555 (*Petri Dish Media Inoculation Using Beads)* or cell spreaders
- Vortex mixer

1.0 PROCEDURE (This protocol is designed for 10-fold dilutions.)

1. For each bacterial spore suspension to be tested label microcentrifuge tubes as follows: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}... (The number of dilution tubes will vary depending on the concentration of spores in the suspension). Aseptically, add 900 uL of sterile diluent to each of the tubes.

2. Label three media plates for each dilution that will be plated. These dilutions will be plated in triplicate.

3. Mix original spore suspension by vortexing thoroughly for 30 seconds. Immediately after the cessation of vortexing, transfer 100 uL of the stock suspension to the 10^{-1} tube. Mix the 10^{-1} tube by vortexing for 10 seconds, and immediately pipette 100 uL to the 10^{-2} tube. Repeat this process until the final dilution is made. It is imperative that used pipette tips be exchanged for a sterile tip each time a new dilution is started.

4. To plate the dilutions, vortex the dilution to be plated 10 seconds, immediately pipette 100 uL of the dilution onto the surface of a media plate, taking care to dispense all of the liquid

from the pipette tip. If less than 10 seconds elapses between inoculation of all replicate plates, then the initial vortex mixing before the first replicate is sufficient for all replicates of the sample. Use a new pipette tip for each set of replicate dilutions.

5. Carefully and aseptically spread the aliquotted dilution on the surface of the media either by use of glass beads (MOP 6555) or cell spreader (the method used may be directed in the QAPP or Test Plan) until the entire sample is distributed on the surface of the agar plate. Repeat for all plates.

6. Incubate the plates for the optimum time period at the optimum growth temperature for the target organism (incubation conditions will vary depending on the organism's optimum growth temperature and generation time. This information can be found in Bergey's Manual of Determinative Bacteriology or it will be provided with the ATCC certification.

7. Manually enumerate the colony forming units (CFU) on the media plates by manually counting with the aid of a plate counting lamp and a marker (place a mark on the surface of the Petri dish over each CFU when counting, so that no CFU is counted twice). A hand held tally counter or an electronic counting pen may be used to assist the person counting, but may not be used as the primary source for the count.

   Quality control (QC) requirements for bacterial enumeration will be addressed per QAPP or test plan. However, in general, the following QC practices should always be adhered to:

   a. The arrangement of plates and tubes, and the procedure for preparing dilutions and enumerating CFU should be done the exact same way each time. This helps prevent systematic errors and often helps determine the cause of problems when a discrepancy is found.

   b. A visual check of the graduated pipette tip should be made during each use to ensure the pipette is pulling properly.

   c. Samples should acclimate to room temperature for 1 hour prior to plating.

   d. Samples should be processed (extracted and plated) from the least contaminated to the most contaminated.

   e. When a target range of CFU is known, three dilution factors are plated to bracket the expected results (0, -1, and -2, if the -1 dilution factor was the target).

   f. Enumerated colonies and results should be verified that the results are the target organism, and that second counts have been performed. Second counts must be completed on 25% of significant data, and must be within 10% of the first count. If CFUs are found to have more than a 10% difference between first and second counts, then a third count is to be completed.

g. Pictures should be taken of any plates that are contaminated or have results out of the normal

8. Record all quantitative data in the "Serial Dilution/Plating Results Sheet". Target range for statistically significant counts is 30-300 CFU. Data that fall out of the 30-300 CFU range are addressed in MOP 6584 (*Procedure for Replating Bacteria Spore Extract Samples*) and MOP 6565 (*Filtration and Plating of Bacteria from Liquid Extracts*).

2.0 CALCULATIONS

Total abundance of spores (CFU) within extract:

(Avg CFU / volume (mL) plated) × (1 / tube dilution factor) × extract volume

For example:

Tube Dilution	Volume plated	Replicate	CFU
10^{-3}	100 µL (0.1 mL)	1	150
10^{-3}	100 µL (0.1 mL)	2	250
10^{-3}	100 µL (0.1 mL)	3	200

Extract total volume = 20 mL

(200 CFU / 0.1 mL) × $(1/10^{-3})$ × 20 mL =

(2000) × (1000) × 20 = 4.0×10^7 CFU

Note: The volume plated (mL) and tube dilution can be multiplied to yield a 'decimal factor' (DF). DF can be used in the following manner to simplify the abundance calculation.

Spore Abundance per mL = (Avg CFU) × (1 / DF) × extract volume

Serial Dilution/Plating Results Sheet **Page 1 of _____**

TEST INFORMATION			
EPA Project No.		PI	
Technician Name		Test Date	
Technician Signature		Test No.	

RESULTS								
Date:	**Volume Plated:**							
		Tube Dilution						
Sample ID	**Plate Repl.**	**10^{0}**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**
	A							
	B							
	C							
	A							
	B							
	C							
	A							
	B							
	C							
	A							
	B							
	C							
	A							
	B							
	C							
	A							
	B							
	C							

NOTES: _____

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Page 2 of ______

Sample ID	Plate Repl.	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
	A							
	B							
	C							
	A							
	B							
	C							
	A							
	B							
	C							
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	A							
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	C							

NOTES: ____

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Page _____ of ______

Sample ID	Plate Repl.	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
	A							
	B							
	C							
	A							
	B							
	C							
	A							
	B							
	C							
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NOTES: ____

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Page _____ of ______

Sample ID	Plate Repl.	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
	A							
	B							
	C							
	A							
	B							
	C							
	A							
	B							
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	B							
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	A							
	B							
	C							

NOTES: ____

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Miscellaneous Operating Procedure (MOP) 6560:
Biological Indicator (BI) Tests Using Nutrient Broth and Analysis of Results

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MOP 6560

TITLE: BIOLOGICAL INDICATOR (BI) TESTS USING NUTRIENT BROTH AND ANALYSIS OF RESULTS

SCOPE: This MOP provides the procedure for testing biological indicators (BIs) in 25 mL nutrient broth tubes and then obtaining results from the BIs in the tubes.

PURPOSE: This procedure will ensure that that the BIs are properly placed in 25 mL nutrient broth tubes aseptically and that the results obtained are accurate.

1.0 PREPARING 25 ML NUTRIENT BROTH TUBES

Refer to MOP 6556 from Biolab Facility Manual.

2.0 PLACING BI's IN NUTRIENT BROTH TUBES

Always note which flat the 25mL nutrient broth tubes are coming from and make certain that if more than one batch of tubes (from one bottle) is used, several (preferably three) tubes are taken from each as negative material controls to test the sterility of the broth. These tubes will serve as negative controls.

Sample sets should have positive controls as well as negative controls. The positive controls will not be subjected to any testing variable and will be placed straight into the tubes. The positive controls should come from the same batch as the negative controls. At least three positive controls should be placed with a sample set or test.

1. Prepare the hood by wiping down with ethanol and a clean Kimwipe. Then stock the hood with the following items if they are not already there:

    - The flats of 25mL nutrient broth tubes
    - Sharpie marker
    - The BI samples
    - Tweezers
    - Ethanol
    - Burner and striker

2. Label the 25 mL tubes with a Sharpie marker before you start. Put all pertinent information, such as the sample number and the date on the tubes. Each BI will go into one tube.

3. Light the burner and adjust the flame for a width adequate to flame tweezers if needed.

4. Unscrew the caps to the nutrient broth tubes and place the lid top side down on the benchtop. Quickly open the BI (usually one side says “peel” or “open here”) and without touching the BI, let it fall into the 25mL nutrient broth tube. If the BI is paper, due to static electricity, this technique may not work, so instead, hold the open BI while with a free hand place the tweezers into ethanol and flame them. Then use the disinfected tweezers to promptly extract the BI from the lining and drop it into the tube.

5. Replace the cap to the tube immediately.

6. Repeat with all samples.

7. Place all tubes into proper incubator for 7-9 days. (BI’s of *Bacillus subtilis*, *Bacillus atrophaeus* or *Bacillus anthracis Sterne* go into the 32 °C incubator, while those of *Geobacillus stearothermophilus* go into the 60 °C incubator). Seven days is preferable, but if the sample pull falls on a weekend, 9 days is fine for recovery.

3.0 ANALYSIS OF RESULTS FROM INCUBATED BIs IN NUTRIENT BROTH TUBES

1. Carefully take BIs in 25 mL nutrient broth tubes out of the incubator in which they were placed. Try not to stir up the contents of the tubes.

2. Look at the negative controls by holding them up to light and gently swirling the tube. The tube should not have any turbidity or debris in it. Record results.

3. Look at the positive controls by holding them up to light and gently swirling the tube. The tube should be turbid and should have growth/debris indicative of the BI that was used. The positive controls will be the basis fo comparison to positive samples. Record results.

4. All test samples will then be resulted in the same visual manner. Make certain that the growth in each tube resembles the same type of growth as seen in the positive control.

5. After all tubes are viewed and the results of turbidity recorded, the tubes must be plated. Using tryptic soy agar petri dishes, label one plate for each tube.

6. Prepare a biohood by wiping down with ethanol and a clean Kimwipe. Then stock the hood with the following items if they are not already there:

    - The labeled plates
    - The BI samples in 25 mL tubes
    - Sterile swabs
    - Vortexer

7. Using the vortexer, vortex each tube prior to opening it.

8. Open the tube and lay the cap top side down on the benchtop.

9. Using the sterile swab and making certain it does not touch anything but the inside of the 25 mL tube, place the swab into the broth with the BI.

10. Take the swab and make a zigzag motion on the appropriately labeled petri dish agar.

11. Throw the swab away in the appropriate receptacle.

12. Replace the cap on the tube.

13. Put all plates and tubes in the appropriate incubator. Save the tubes until after the plate results are considered valid and finalized.

14. After 12 to 24 hours, check the plates for growth and record results. The results should be consistent with the visual turbidity results.

15. If further investigation of the plate growth is needed, complete gram stain or other physiological tests.

16. Report results.

Miscellaneous Operating Procedure (MOP) 6562:

Preparing Pre-Measured Tubes with Aliquoted Amounts of Phosphate Buffered Saline with Tween 20 (PBST)

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MOP 6562

TITLE: PREPARING PRE-MEASURED TUBES WITH ALIQUOTED AMOUNTS OF PHOSPHATE BUFFERED SALINE WITH TWEEN 20 (PBST)

SCOPE: This MOP provides the procedure for preparing PBST.

PURPOSE: This procedure will ensure that that the PBST is prepared correctly and that all measured tubes are filled aseptically.

1.0 PREPARING STERILE PHOSPHATE BUFFERED SALINE WITH TWEEN 20 (PBST)

Phosphate Buffered Saline with Tween 20 (PBST) is prepared 1 L at a time in a 1 L flask.

1. Add 1 packet of SIGMA Phosphate Buffered Saline with Tween 20 (P-3563) to 1 L of deionized (DI) water.

2. Shake vigorously to mix until dissolved.

3. Label bottle as “non-sterile PBST” and include date and initials of person who made PBST.

4. Filter sterilize into two 500 mL reagent bottles using 150 ml bottle top filter (w/ 33mm neck and .22 µm cellulose acetate filter) for sterilization. Complete this by pouring the liquid into the non-sterile PBST into the top portion of the filtration unit 150 ml at a time, while using the vacuum to suck the liquid through the filter. Continue to do this until 500 ml have been sterilized into a 500 ml bottle. Change bottle top filter units between each and every 500 ml bottle.

5. Change label to reflect that the PBST is now sterile. Include initials and date of sterilization. The label should now include information on when the PBST was initially made and when it was sterilized and by whom.

6. Each batch of PBST should be used within 90 days.

2.0 PREPARING 20 ML/5 ML PBST TUBES FOR USE DURING EXPERIMENTATION

Twenty (20) ml or five (5) ml of the prepared PBST will be added to each sterile 50-ml conical tube as detailed below. Each flat of conical tubes contains 25 tubes, so one 500 ml sterile bottle of PBST should fill approximately one flat when 20 ml tubes are needed and four flats when 5 ml tubes are needed.

1. Prepare the hood by wiping down with ethanol, followed by bleach, followed by DI water and a clean Kimwipe or Techwipe. Then stock the hood with the following items if they are not already there:

    - The flats of sterile conical tubes you need to fill with PBST.
    - Sufficient bottles of sterile PBST to fill these tubes.
    - Ample 25 ml serological pipettes (at least 3 per flat) for 20 ml transfers and 10 ml serological pipettes for the 5 ml transfers.
    - Serological pipetter (automatic, hand-held pipette).
    - Burner and striker.

2. Light the burner and adjust the flame for a width adequate to flame the lips of the PBST bottles.

3. Take one flat of sterile conical tubes and loosen each cap on the outside edges (about ½ turn).

4. Open a serological pipette and insert into the serological pipetter, taking care to not touch the tip to any surface.

5. Hold the pipetter with the first three fingers of your right (or dominant) hand. With your left hand (or non-dominant hand), pick up a bottle of the PBST and use the bottom of your right hand to unscrew the lid. Place the lid upside down on the benchtop and quickly flame the lip of the bottle. Turn the bottle and repeat, taking care to thoroughly flame the lip without getting the glass so hot that it shatters.

6. Inset the tip of the pipette into the bottle and fill to the 20 ml line. Flame the bottle lip and place the bottle on the benchtop.

    NOTE: If the tip of the pipette touches the outside of the bottle or any other surface in the hood, consider it contaminated. Discard the pipette and reload a new one.

7. Quickly pick up one of the tubes that you have loosened the cap on, and use the bottom of your right hand to remove the cap. Completely discharge the entire pipette into the tube, taking care to not touch anything with the tip of the pipette. Recap the tube and place back into the flat (the lid does not have to be tight – you will tighten the lids after you have completed filling the 10 outside tubes).

    NOTE: If the tip touches the outside or rim of the tube (or any other surface in the hood), consider the tube and pipette contaminated. Discard both the tube and the pipette.

8. Pick up the PBST bottle and flame the lip. Repeat Steps 6 and 7 until all 10 of the tubes on the outside of the flat have been filled. Flame the lip of the PBST bottle and replace the cap. Slide the used pipette back into the plastic sleeve and put to the side of the hood for disposal. Then tighten the lid of each tube you just filled. But rather than placing it back into its original spot in the flat, switch it for the empty tube from the next row. When this has been completed, go around the outside of the flat again and loosen the lids of these 10 tubes. Repeat steps 4 through 7 to fill and cap these tubes.

9. This same procedure is used to fill the middle row of tubes from the flat, and if more than one flat of tubes is being filled, can be done at the same time as the outside rows of a second flat.

10. When all tubes have been filled, label each flat as follows, and place on the shelf in room E390B:

    "PBST Tubes (20 ml or 5 ml)"
    Date prepared
    Your initials

11. These tubes should be made at least 14 days before they need to be used so that they can be verified as sterile. Any tubes that are cloudy or that have any floating matter/turbidity should be discarded. The tubes are stable for and should be used within 90 days.

3.0 CLEANUP FOR 20 ML/5 ML PBST TUBES

1. Dispose of the used pipettes in the nonregulated waste.

2. Plug in the serological pipetter so that it can recharge.

3. Replace any unused PBST in the liquid containment on the shelf. Make sure that the bottle is labeled as having been opened (date opened and initials of whomever used it).

4. Turn off the burner.

5. Wipe down the hood benchtop with ethanol, followed by bleach, followed by DI water and a clean Kimwipe or TechWipe.

4.0 PREPARING 900µL PBST TUBES FOR USE DURING EXPERIMENTATION

1. Prepare the hood by wiping down with ethanol, followed by bleach, followed by DI water and a clean Kimwipe or Techwipe. Then stock the hood with the following items if they are not already there:

- A sterile beaker of microcentrifuge tubes.
- Sufficient tubes of sterile PBST to fill these tubes (PBST may be aseptically transferred to 50 ml conical tubes for an easier aseptic transfer to the microcentrifuge tubes- it is easier than working from a 500 ml reagent bottle. Make certain that these 50 ml conical tubes are labeled to when the PBST was made, sterilized, etc.).
- 1000 µL micropipette.
- 1000 µL sterile pipette tips
- Microcentrifuge tube racks.
- Labeled beaker or waste container used to hold non-regulated waste, such as tips, under the hood.

2. Carefully remove the microcentrifuge tubes one at a time from the beaker and close the top on each one before placing it in the tube rack. Place the tubes in the rack skipping every other row. Fill up two racks doing this.

3. Add 900 µL of PBST to the microcentrifuge tubes by aseptically transferring the PBST from the sterile 50 ml conical tube containing the PBST. Do this by using the 1000 µL micropitte and tips. Change tips whenever after two rows of tubes are completed or whenever a contamination event (such as touching the outside of the 50 ml tube or the microcentrifuge tube) occurs. Put the dirty tips in the beaker or container used to contain waste (tips, tubes) in the hood. If any 900 µL tubes are contaminated during the transfer, dispose of them in the waste container used to hold tips under the hood. If a new box of tips has to be opened, make certain the date it was opened and initials of the person who opened it are clearly labeled on the box.

4. After both racks are full, carefully move all the tubes from one rack to fill in the empty rows on the other rack. In this manner, one rack should be completely filled with tubes at this point.

5. Label the rack of tubes as “Sterile 900 µL PBST Tubes”, along with the name of the person who completed the transfer, along with the date. Also, include the date that the original stock of PBST was made and the date it was sterilized, along with the initials of the person who completed those steps.

5.0 CLEANUP FOR 900µL PBST TUBES

1. Dispose of the waste that was put in the labeled beaker or waste container (micropipette tips and tubes) in the nonregulated waste. Then, place this beaker in the “To be decontaminated via sterilization- contaminated glassware” bin or if it is a disposable container, then it can be put in the non-regulated waste container.

2. Put the unused sterile tips and the micropipetter back in its original location.

3. Replace any unused 50 ml conicals of PBST in the liquid containment on the shelf. Make sure that the tube is labeled as having been opened (date opened and initials of whomever used it). If the tube could possibly be contaminated in any way, dispose of it in non-regulated waste.

4. Wipe down the hood benchtop with ethanol, followed by bleach, followed by DI water and a clean Kimwipe or TechWipe.

MOP 6566

TITLE: CULTURING OF APEX LABORATORIES TYVEK PACKAGED BIOLOGICAL INDICATORS

SCOPE: This MOP outlines the procedure for culturing Apex Laboratory Biological Indicators (BIs)

PURPOSE: Repeatable, aseptic transfer of BIs to growth media to determine viability

Materials:

- PPE (gloves, lab coat, safety goggles)
- Vortex mixer
- Sterile forceps (optional)
- Culture tubes with presterilized bacterial growth media (Tryptic Soy Broth or Nutrient roth as determined by study)
- Incubator set to appropriate growth temperature for indicator organism (i.e., 55 - 60ºC for *Geobacillus stearothermophilus*)
- Biological Safety Cabinet (Class II)

1.0 PROCEDURE (without the use of forceps)

1. Label all culture tubes, prior to start, with date, sample ID, organism, and processors initials.

2. Begin by donning PPE (gloves, lab coat, and protective eyewear).

3. Clean the workspace (biological safety cabinet) by wiping surfaces with pH-adjusted bleach, next with diH_2O, and lastly with a 70-90 % solution of denatured ethanol. Make sure the workspace is clean and free of debris. Gather all necessary items to perform the task, place these items within arms reach of the biological safety cabinet so that, once the procedure has begun, the task may be performed without interruptions and travel about the laboratory.

4. Discard gloves and replace with fresh pair.

5. Using proper aseptic techniques, loosen the lid of the first sample's culture tube, careful not to completely remove the lid.

6. Locate the corresponding first biological indicator (still concealed in a Tyvek envelope), confirm that the label of the biological indicator matches that of the culture tube; then, grasp the offset tabs at the end of the indicator with thumb and index finger of both hands.

7. Open the pouch slowly, with equal force, by pulling the two tabs apart until the enclosed indicator coupon is just visible. Peeling the envelope too far may result in the indicator coupon falling from the packaging and becoming contaminated.

   NOTE: If an indicator coupon should come in contact with any surface **other than** the untouched inner portion of the Tyvek envelope or the inside of the culture tube, the indicator coupon must be discarded, and noted in the laboratory notebook.

8. Once the Tyvek envelope has been peeled open, and the indicator coupon slightly exposed; gently grip the coupon by squeezing the outside of the Tyvek envelope between the thumb and index finger of one hand. (take extreme care not to touch the coupon with fingers).

9. Being careful not to touch the coupon to any surface, including the exterior of the culture tube, transfer the coupon to the inside of the culture tube by removing the culture tube lid with one hand, holding the envelope and pouch inverted above the open culture tube, and then releasing the coupon from the Tyvek envelope. Immediately replace the culture tube lid, confirm again that label of the indicator matches that of the culture tube. Save all empty Tyvek envelopes, and catalog them by date of experiment.

   NOTE: Sterile forceps can be utilized in this procedure to transfer the indicator coupon from the Tyvek envelope to the culture tube. If using forceps, it is important to remember that when working with bacterial spores, forceps and other items are not readily sterilized with an ethanol soak followed by flaming. More stringent measures should be utilized to prevent cross contamination if reusing forceps or other items. (i.e., bleach soak 10 minutes, followed by dH_20 rinse, followed by ethanol soak for 1 minute, and flamed to remove ethanol). Alternately, a new disposable sterile forceps can be utilized for each sample.

10. Repeat procedure (from step 4) for remaining samples. Periodically replace gloves, especially any time gloves come into contact with items outside the biosafety cabinet.

11. Once all samples have been successfully transferred to culture tubes, place tubes in incubator at appropriate temperature.

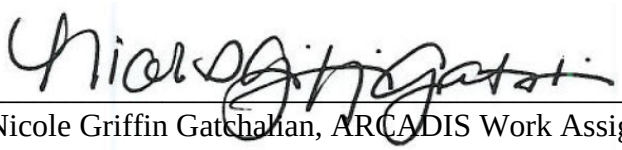
12. After one (1) and seven (7) days, check the growth status of each tube (Or other interval as determined by Quality Assurance Practice Plan (QAPP). Record results and observations in laboratory notebook. Growth is deemed ‘positive’ if the media is visibly turbid. Growth is

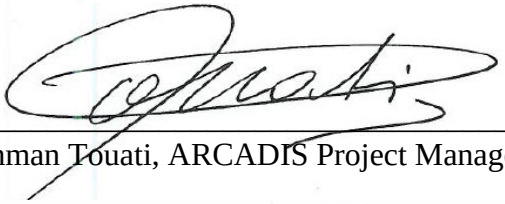
deemed 'negative' if the growth media is lucid, displays no turbidity, and is indistinguishable from that of the negative controls.

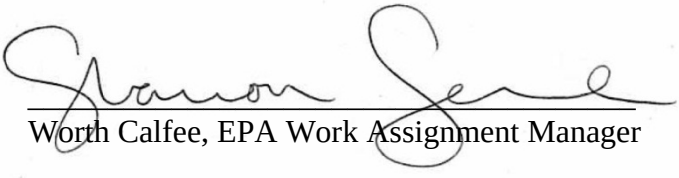
NOTE: Turbidity is best detected by holding each culture tube above eye-level, bringing the tube between a light source and the technician's eyes, and then swirling the tube. Turbidity can be characterized by clearly visible clumps of cells, or by faintly visibly rolling clouds of cells.

13. Optional: To confirm the turbidity of the culture tube is from growth of the test organism, a loop-full of the culture may be streaked onto an agar plate and incubated overnight to confirm colony morphology. To confirm the absence of growth in tubes that display no visible turbidity, the entire contents of the culture tube can be filtered using a 0.2 or 0.45 µm pore-size analytical filter, and the filter subsequently placed on the surface of an agar plate (collection side up) and incubated overnight. Step 12 is to be used at the discretion of the PI, or as outlined in the QAPP.

Miscellaneous Operating Procedure (MOP) 6566: Culturing Biological Indicator Strips

Prepared by:  Date: 5/16/2013
Nicole Griffin Gatchalian, ARCADIS Work Assignment Leader

Reviewed by:  Date: 5/16/2013
Dahman Touati, ARCADIS Project Manager

Approved by:  Date: 5/16/2013
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MOP 6566

TITLE: CULTURING OF BIOLOGICAL INDICATOR STRIPS

SCOPE: This MOP outlines the procedure for culturing biological indicator (BIs) strips.

PURPOSE: Repeatable, aseptic transfer of BIs to a growth media to determine viability.

Materials:

- PPE (gloves, lab coat, safety goggles)
- pH-amended bleach
- DI water
- 70% Ethanol
- Vortex mixer
- Sterile thumb forceps
- Culture tubes with ~10 mL of pre-sterilized bacterial growth media as determined by manufacturer or QAPP (i.e.,Tryptic Soy Broth, Nutrient Broth)
- Incubator set to appropriate growth temperature for indicator organism as determined by manufacturer or QAPP (i.e., 55 - 60ºC for *Geobacillus stearothermophilus*)
- Class II Biological Safety Cabinet (BSC)
- Quality Assurance Project Plan (QAPP) or Test Plan

1.0 PROCEDURE

1. Prior to start, label all culture tubes with sample ID as determined by QAPP or Test Plan, along with the date. Sort the culture tubes in logical manner, so that it will be easy to place them into tubes. They must be placed in tubes from least possibly contaminated to most possibly contaminated.

2. Begin by donning PPE (gloves, lab coat, and protective eyewear).

3. Clean the workspace (BSC) by spraying surfaces with pH-amended bleach and allow it to sit for 3 minutes minimum. Next, spray the surfaces with DI water, and then wipe it clean with a KimWipe. Lastly, spray the surfaces with 70% ethanol and wipe it clean with a KimWipe. Make sure the workspace is clean and free of debris. Gather all necessary items to perform

the task, place these items on a cart beside the BSC so that, once the procedure has begun, the task may be performed without interruption.

4. Discard gloves and replace with fresh pair.

5. Place the prelabeled culture tubes in the BSC. Make certain they are in a logical order so that placing them into the culture tubes progresses from least contaminated to most contaminated.

6. Locate the first BI strip and its corresponding culture tube. Confirm that the label of the BI matches that of the culture tube.

7. Place the culture tube in a rack, and aseptically remove its cap. Immediately grasp the offset tabs at the end of the corresponding BI with both the thumb and index finger of both hands. Separate the packaging containing the BI and allow the BI to aseptically free fall into the tube's culture media.

   NOTE: Open the pouch slowly, with equal force, by pulling the two tabs apart until the enclosed indicator coupon is just visible. Peeling the envelope too far may result in the indicator coupon falling from the packaging and becoming contaminated. If an indicator coupon should come in contact with any surface **other than** the untouched inner portion of the BI packaging or the inside of the culture tube make a note of it on both the tube and in the laboratory notebook.

8. Immediately replace the culture tube lid, confirm again that label of the indicator matches that of the culture tube. Discard all empty BI packaging into non-regulated waste.

   NOTE: Sterile disposable thumb forceps can be utilized in this procedure to transfer the indicator coupon from BI packaging to the culture tube, especially in the event that the transfer is difficult due to BI packaging issues. Use a new pair of sterile disposable thumb forceps for each sample and dispose of all sterile thumb forceps in non-regulated waste.

9. Repeat steps 6-8 for remaining samples.

10. Once all samples have been successfully transferred to culture tubes, place tubes in incubator at appropriate temperature as determined in the QAPP or Test Plan.

11. Check the growth status of each culture tube at an interval as determined by the QAPP or Test Plan. Record results and observations in laboratory notebook. Growth is deemed 'positive' if the media is visibly turbid. Growth is deemed 'negative' if the growth media is lucid, displays no turbidity, and is indistinguishable from that of the negative controls.

    NOTE: Turbidity is best detected by holding each culture tube above eye-level and bringing the tube between a light source and the technician's eyes, and then

swirling the tube. Use the positive and negative controls to aid in determining turbidity.

To confirm that all ‘negative’ samples are free of growth and to confirm that all ‘positive’ turbidity from the culture tubes are from growth of the target organism, the culture tubes must be plated and any and all colony morphology observed. These steps, however, are to be directed by the QAPP or Test Plan.

1. Agitate the culture tube with the vortex mixer for 3 to 5 seconds, and then immediately aseptically transfer 100 µL from the culture tube to prelabeled media plates. Incubate for appropriate time and temperature, as determined either by the manufacturer or the QAPP or Test Plan.

2. To further confirm the absence of growth in tubes that display no visible turbidity, the entire contents of the culture tube can be filtered using a 0.2 or 0.45 µm pore-size analytical filter, and the filter subsequently placed on the surface of an agar plate (collection side up) and incubated for appropriate time and temperature.

Miscellaneous Operating Procedure (MOP) 6570:
Use of Steris Amsco Century SV 120 Scientific Prevacuum Sterilizer

Prepared by: _____ Date: 3/21/2013
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Reviewed by: _____ Date: 3/21/2013
Dahman Touati, ARCADIS Project Manager

Approved by: _____ Date: 3/21/2013
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MOP 6570

TITLE: USE OF STERIS AMSCO CENTURY SV 120 SCIENTIFIC PREVACUUM STERILIZER

SCOPE: Basic instructions for use of the large Steris autoclave.

PURPOSE: To outline proper procedural use of the autoclave, using preprogrammed cycles, to effectively sterilize items, while complying with quality control standards.

Materials:

- Amsco Century SV 120 Scientific Prevacuum Sterilizer
- Items to be sterilized (liquids, solids, waste, etc)
- Pouches to contain materials during sterilization and maintain sterility until use
- Aluminum foil
- Autoclave indicator tape
- Sterilization verification ampoules (such as Raven ProSpore Ampoules)
- Thermally resistant gloves
- De-Ionized (DI) water

1.0 PROCEDURE

1.1 Start Up

1. Turn on the autoclave. The power switch is located behind the door in the top right corner. The digital touch screen on the front of the unit will power up and indicate that a memory test is in progress.
2. After the memory test is complete, the device will request that it be flushed. This should be conducted daily to minimize scaling inside the boiler. The flush valve is located behind the door on the bottom, left of the device (yellow handle). Move the valve to the open position and then press the “Start Timer” button on the touch screen. The flush will run for 5 minutes and will alert at completion with a single chime.
3. Once the flush is complete, close the flush valve and press the “Continue” button on the touch screen. The screen should then return to its default menu which has 2 choices “Cycle Menu” and “Options”

1.2 Basic Operation

1. Prepare any items that need to be sterilized. The items must be carefully wrapped or sealed in sterilization pouches in order to maintain sterility when removed from the autoclave. Examples of this include: wrapping any orifices with aluminum foil, placing whole items in autoclave pouches, loosely applying a cap on a bottle (to allow for the pressure changes inside).
2. Once prepared, each item should be outfitted with a sterility indicator such as a small piece of autoclave indicator tape; or by utilizing an autoclave pouch with a built-in sterility indicator strip. These indicators provide a visual verification that the sterilizing temperature (121°C) was reached.
3. To add items to the autoclave, open the autoclave door by pressing down on the foot pedal on the bottom right corner on the front of the device.
4. Place items that need to be sterilized into the autoclave, adding or moving racks to accommodate the load. If liquids are being autoclaved, then they must have secondary containment (usually a large plastic autoclave-safe tray) to contain any fluids in the event of a leak, spill or boil-over. Add an indicator ampoule to the first autoclave cycle of the day, regardless of the type of cycle.
5. Once the autoclave is loaded, press the foot pedal to close the autoclave door.
6. Once the door is sealed, a menu of the cycles can be seen by pressing the button on the touch screen labeled “Cycle Menu”. Then choose the appropriate cycle by touching the corresponding button. If the cycle chosen is the one desired for the sterilization process, press the “Start Cycle” button. Otherwise, press “Back” to return to the prior menu screen.
7. After the cycle has started, the type of cycle, the number of the cycle, the items placed in the autoclave during the cycle, the time, whether or not an indicator ampuole was included in the load, and the initials of the person starting the cycle must be recorded in the autoclave log book, located in the drawer across form the unit labeled “Autoclave Supplies.”
8. Quality control (QC) indicator ampoules, usually Raven ProSpore Ampoules with *Geobacillus stearothermophilus* (at a concentration 10E6), are added to one cycle each day to ensure that the autoclave is functioning properly. These ampoules are used according to manufacturer’s instructions. These ampoules must be properly labeled with the date in which they were autoclaved and the initials of the individual that completed the cycle. At the beginning of each week, a positive control ampoule must be processed, where the ampoule is placed directly into the 55°C water bath, without being autoclaved. The positive control indicator ampoule should change from purple to yellow in color, indicating growth. All test ampoules should be placed in a water bath following the end of the cycle in which they are run. These ampoules should not change color (from purple to yellow, but instead should remain a purple to

purple-brown color). Ampoules should be checked at both 24 and 48 hour intervals for growth and then finally recorded and disposed of after 48 hours. All QC information concerning ampoules should be recorded in the autoclave notebook.

9. Upon completion of any cycle, the autoclave will alarm with a repeating beep for approximately one minute. Any time after this alarm starts, it is safe to open the main door (take caution because the steam escaping the chamber will be very hot when the door is opened). The contents from the autoclave will be very hot; use protection to remove items from the autoclave (thermally resistant gloves).
10. Place the contents of the autoclave in an appropriate place to cool, and close the autoclave door using the foot pedal.

1.3 Cycles

1.3.1 Gravity Cycles

Gravity cycles are used to sterilize glassware and other utensils, which are not submerged in nor contain any volume of liquid. These cycles are typically used for “dry” materials. Currently there are two different gravity cycles programmed for daily operations: a 1-hour cycle and a 30-minute cycle. The time that the chamber is held at the sterilization temperature (121 °C) is the only difference between these two cycles. The different sterilization times allow for the compensation of the various sizes of materials and more resilient organisms. The 30-minute cycle is primarily used for a small quantity of material. The 1 hour cycle is used for large loads or items containing a large amount of contamination. The 1 hour cycle is recommended for inactivation of gram positive spore-forming bacteria.

1.3.2 Liquid Cycles

Liquid cycles are used to sterilize a variety of liquids and solutions. The solutions are typically mixed prior to sterilization. It is important to have secondary containment to contain any fluids in the event of a leak, spill or boil-over. The 30-minute liquid cycle is used to sterilize small volumes of liquid (usually less than 2L total). When attempting to sterilize any volume larger than 2L, the 1-hour liquid cycle should be used to ensure complete sterilization. The 1-hour liquid cycle is the preferential cycle used as the destruction cycle for waste. In the event of materials (liquid or otherwise) being contaminated/exposed microorganisms, the 1-hour liquid cycle will be used as the initial means of decontamination. When completing a decontamination cycle, if there is no liquid inside of a container, then deionized water must be added to the container or the item must be submerge prior to the start of the cycle. Only items that are being decontaminated can go in destruction cycles. Decontamination cycles cannot be mixed with sterilization cycles.

Miscellaneous Operating Procedure (MOP) 6576: Determination of Spore Thermal Challenge (Heat Shock) Resistance

Prepared by: _____ Date: 6/12/2013
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Reviewed by: _____ Date: 6/12/2013
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MOP 6576

TITLE: DETERMINATION OF SPORE THERMAL CHALLENGE (HEAT SHOCK) RESISTANCE

SCOPE: This MOP outlines the procedure for determining the ability of a spore suspension to withstand a thermal challenge (heat shock)

PURPOSE: A quality control measure to ensure spores used for experimentation display the robust phenotype (heat resistance) characteristic of bacterial spores.

Materials:

- PPE (gloves, lab coat, safety goggles)
- Biological Safety Cabinet (Class II)
- Liquid suspension of bacterial spores
- Sterile microcentrifuge tubes
- Diluent (sterile deionized water, BPW, PBS, or PBST)
- Vortex mixer
- Water Bath (set to 80°C)
- Micropipette
- Sterile pipette tips
- NIST Traceable timer

1.0 PROCEDURE

1. Determine the titer of the spores suspension to be tested using BioLab MOP 6535a.

2. Using fresh, sterile diluent, prepare 1.0 ml aliquots of the spore suspension in microcentrifuge tubes, each containing between 1E4 and 1E5 CFU per ml (calculations to determine volume of spore suspension vs diluents based upon results from step 1). Use the following equation:

$$\frac{\text{Target spore titer (i.e, 2E4)}}{\text{Determined Stock Titer (i.e, 1E8)}} \times 1\text{ ml} = \text{Spike volume (in ml)}$$

(Add this spike volume to enough diluent to total 1 ml)

NOTE: Vortex stock samples 20 seconds prior to pulling spike volume to ensure homogeneity.

3. Prepare six 1 ml sample tubes for each spore stock to be analyzed. Three will be exposed to the treatment (heat), three will be controls (unexposed). Label tubes accordingly.

4. Once all tubes are assembled, and the temperature ($80 \pm 2^{\circ}C$) of the water bath has been verified and recorded; insert all treatment tubes into the water bath, using floating or submersible racks as necessary. Be sure that all of the liquid in the tube is below or equal with the level of the water in the water bath. Start the NIST Traceable timer. Keep control (unexposed) samples refrigerated or on ice.

5. Exactly following 20 minutes of exposure, remove treatment tubes, and place on ice (or refrigerate) for 5 minutes.

6. Determine spore titer in each sample (exposed and unexposed) using BioLab MOP 6535a.

7. The mean log titer of exposed samples should be not be greater than 0.5 log less than the mean log titer of unexposed samples (Equation 1). Data should be reported as the mean log of exposed samples minus the mean log of unexposed samples (Equation 1). Note: it is not uncommon to observe greater than 100% titer (ratio of exposed to unexposed samples), since heat shock may increase germination efficiency of bacterial endospores.

$$\text{Log}_{10}\,(\text{Mean Unexposed Tubes}) - \text{Log}_{10}\,(\text{Mean Exposed Tubes}) \leq 0.5 \qquad (1)$$

8. In addition, data should be reported as CFU/ml for all samples and as percent survival according to equation 2:

$$\frac{\text{Mean titer of exposed samples}}{\text{Mean titer of control samples}} \times 100 \qquad (2)$$

Appendix B: DTRL – QC Checklist for Data Reviewers

DTRL –QC CHECKLIST FOR DATA REVIEWERS

Spreadsheet checks:

- ❒ Before beginning QC, make sure the WAL (or Task Leader as appropriate) has briefed you on the task. DQI goals should be noted and understood by both parties.
- ❒ Create a QC tab in the spreadsheet. Notes should be recorded in this QC tab as described in these checks. When a QC reviewer adds information directly to a data file, it should be noted in the QC tab.
- ❒ Column headers present and sufficiently detailed.
- ❒ Units noted in each column/row, as required.
- ❒ Spot check that raw data were entered correctly (10%). Make sure digitally recorded data has the correct time stamp. For recorded data such as Labview files, simply sourcing the data to its original file is sufficient.
- ❒ Proper CFU acceptance criteria used, and all required re-plates and filter plates done.

Note: It is the responsibility of the QC reviewer to make sure they are using the correct criteria. Currently, the following general rules apply:

- Acceptable plate counts are from 30 to 300 CFU (unless noted).
- There should be **no colored cells** in the spreadsheet, unless noted as to why this is acceptable. If the replicate counts on a dilution plate don't agree within 50% of each other, the ID cell turns blue. If the RSD is outside of 50%, the cell turns yellow. Once an acceptable count agreement/RSD has been obtained by a re-plate, the cell will no longer be blue/yellow.
- Filter plates or higher volume plates must be run when there are between 0 and 29 CFU at the zero dilution. For filter plate data, the highest volume should be used for 0 counts. All non-zero filter data must be listed.
- If reportable counts seem like an outlier, these results are turned to text by the enterer, and become left aligned (rather than right aligned).

- ❒ Check the accuracy of each formula in one cell. Then randomly click other cells in that row/column to verify that the formula was copied correctly. Check for cells indicated by Excel with a green corner as inconsistent formulae.
- ❒ Document the source of any constants in formulae, if not apparent.
- ❒ The location of the status worksheet is noted (or the location of the raw data files, if a status worksheet is not used).

- ❒ Project deviations are noted and detailed.
- ❒ There are no links to external spreadsheets. These should be turned to fixed numbers, and have the source listed in a comment or other method. The absolute path (including sheet and cell reference) to the value should be noted in a comment for easy updating using macros. These macros can be found in DTRL/Facility/Macros.
- ❒ Add name and date to data spreadsheet as QC Reviewer.
- ❒ Check results against DQI goals for the project. For any DQI goal not met, specify the failure in the QC section.
- ❒ Ensure all necessary calibrations are performed according to the QAPP. It is also important to check that these calibrations (pre and post as required) have been appropriately applied to all test data collected.
- ❒ Witness (sign and date) each page of the laboratory notebook associated with this data.

Project Documentation:

Depending on the project in question, "Project Documentation" can involve any or all of the following:

- Raw data files
- Chain of Custody
- Data collection forms
- Laboratory notebooks
- Living documents
- QAPP
- MOPs
- Calibration files

The QC check will usually require that you at least look through the laboratory notebook to see if there were any deviations that need to be detailed in the data spreadsheet. The other documents may be needed for project details, procedures, instrumentation, etc.

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