Validation Study for the Use of Chlorine Dioxide Gas as a Decontaminant for Biological Safety Cabinets

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Abstract

Processes using chlorine dioxide (CD) gas for the biological decontamination of Class II laminar flow biological safety cabinets (BSCs) have been validated following a protocol developed in conjunction with NSF International. This report reviews the protocol and presents the results of the study. Trials were performed in type A1, A2, B1, and B2 cabinets from two different manufacturers. Exhaust and down-flow HEPA filters were pre-loaded with particulates to enhance the validation challenge. Two methods of CD generation were included within the trials, with one involving the injection of a specific mass of CD gas dependent upon the BSC volume, and the other involving the maintenance of a constant CD gas concentration over the duration of the exposure. In each of the more than 40 experimental trials, 12 biological indicators with ~10^6 Bacillus atrophaeus *endospores were deployed at various locations within the BSC to monitor decontamination efficacy.*

The study validated and qualified CD as an alternative to formaldehyde gas as a decontaminant for BSCs. Acceptable durations for CD exposure of less than 90 minutes were established. Neither residuals from CD nor cabinet material degradation was observed during the trials.

Introduction

A laminar flow biological safety cabinet (BSC) is a containment device routinely found within laboratories performing biological or pharmaceutical work. Its purpose is to protect the material within the cabinet from ambient biological contamination while protecting the user and the surrounding environment from contamination by biological and sometimes chemical material being purposely manipulated within the cabinet. Basic design considerations of Class II BSCs and the differentiation among various types (specifically A1, A2, B1, and B2) can be found in the literature (Kruse et al., 1991; U.S. Department of Health and Human Services, 2007). Among common characteristics are that BSCs have plenums with internal surfaces not exposed to the working space within the cabinet, HEPA filters that provide appropriately filtered air to the workspace and appropriately filtered exhaust, and blowers that provide containment and circulation. Part of the charter of NSF International, formerly the National Sanitation Foundation, is to maintain a standard for BSC manufacturing and certification (National Sanitation Foundation, 2007a).

Routinely mandated surface decontamination of the workspace and other easily accessible internal parts of the BSC is generally performed with a liquid disinfectant (U.S. Department of Health and Human Services, 2007). However, occasionally all internal parts of a BSC require decontamination. Such occasions include when maintenance or repair work requires exposing its internal parts or surfaces, such as the replacement of a HEPA filter or blower (National Sanitation Foundation, 2007b; U.S. Department of Health and Human Services, 2007). This decontamination is performed with a decontaminant in a gas or vapor state, which will be referred to collectively as "gas decontamination." The term "gas" is applied here to a chemical that is a thermodynamically stable gas at room temperature, while "vapor" is stable as a liquid at room temperature but has been converted either to a gas or to microscopic droplets by some thermal or physical means prior to its release into the cabinet. In either case, the intent is that this chemical penetrates all internal surfaces within the BSC, including through any HEPA filters. Furthermore, the chemical is typically one capable of killing bacterial endospores, which among bacteria, viruses, fungi, algae, and protozoa, are considered the most resistant to chemical disinfection (Favero & Bond, 2001; McDonnell, 2007; Prince & Prince, 2001). A gas decontamination process requires the sealing of the BSC, both to allow maintenance of the appropriate concentration of disinfectant within the BSC and to protect personnel from the typically toxic decontaminant. At minimum, it also requires a means to get the decontaminant into the enclosed cabinet and a method to circulate the decontaminant through the cabinet and the exhaust HEPA filter. The process may also require a means to get the cabinet interior to a particular humidity and temperature range (Jeng & Woodworth, 1990; Westphal et al., 2003) and a means to safely eliminate the toxic decontaminant at the end of the process.

The most common chemicals presently used for this

purpose are formaldehyde, hydrogen peroxide, and chlorine dioxide. Processes using other sporicidal chemicals that could in principle be used for this application, including methyl bromide, ozone, ethylene oxide, propylene oxide, and peracetic acid, have not been developed for this application due to various issues specific to each of those chemicals (Joslyn, 2001; McDonnell, 2007). The standard process until the last decade used formaldehyde gas, using either the solid paraformaldehyde or formalin solution as the gas source. Typically, paraformaldehyde is used within the United States, with the standard procedure using 0.3 g paraformaldehyde per cubic foot of cabinet volume, which is sufficient to generate 8,000 ppm formaldehyde gas within the cabinet once the solid has been thermally depolymerized (Luftman, 2005; Taylor et al., 1969). The actual gas concentration within the cabinet is rarely monitored, but some data indicate that the active concentration tends to be less than 3,000 ppm (Rogers et al., 2004b). Prior to the generation of formaldehyde gas, the humidity within the BSC is raised to between 60% and 85% RH (Spiner & Hoffman, 1971). The contact time for sufficient disinfection of all internal surfaces is at minimum 6 hours (Fink et al., 1988). Although formaldehyde meets the criteria of gas decontamination—having appropriate sporicidal capability, being easily circulated throughout the device, and having fairly good penetrability—there are drawbacks. Under typical conditions, a residue is left following the decontamination, much of which consists of paraformaldehyde (Ackland et al., 1980; Cheney & Collins, 1995; Luftman, 2005). This is generally removed with an ammonia solution from visible, accessible surfaces following the decontamination. The remaining residue and absorption into certain porous materials generally leaves after sufficient aeration, but until then an unpleasant odor and the possibility that residual gas will have deleterious effects upon biological samples placed within the cabinet exist. In addition, formaldehyde is currently considered a carcinogen in much of the world (IARC, 2004), so effects from both its use during the decontamination and the presence of residuals after the decontamination are of concern.

Until recently, NSF International had cited using only formaldehyde gas as a process for BSC decontamination within its NSF/ANSI 49 standard for BSCs. As other chemical processes were developed, NSF International has still specifically presented a standard formaldehyde gas process, requiring no further validation by the user as long as the given protocol has been followed. However, the document has expanded the possibility of using other chemical processes.

> "*Prior to decontamination with an alternative method (such as vaporous hydrogen peroxide [VHP]), cycle parameters and validation of those parameters must be developed for each model and size of BSC. Material compatibility in terms of degradation and absorption of an alternate decontaminant are critical for maintaining cabinet integrity and the time required for decontamination, respectively.*" (National Sanitation Foundation, 2007b)

(In the preceding, "*alternative method*" refers to a process other than the standard formaldehyde process.) The requirement for validation of each BSC model and size likely stems in part from a concern for processes using a vapor since such a decontaminant has the possibility of condensing prior to reaching all internal cabinet surfaces without circulation appropriate for the specific cabinet. Given the problems inherent with formaldehyde gas, having another gas decontaminant, as opposed to a vapor decontaminant, accepted by the NSF International without requiring validation for every cabinet model and size would be advantageous.

Chlorine dioxide (ClO_2) gas is an appropriate alternative to formaldehyde. Properties of the gas and some of its prior applications have been discussed by Luftman and Regits (2008) and references cited therein. Chlorine dioxide (CD) is a water-soluble, yellow-green gas with a boiling point of 11°C. Dissolved in water, it has been used as a germicide and a food treatment and as a bleaching agent by the paper industry. CD is a selective oxidant, reacting primarily with those organics that are highly reduced (e.g., alcohols, aldehydes, ketones, tertiary amines, and sulfur-containing amino acids) and thus is generally not as adversely affected by typical organic loads compared to other oxidants (Knapp & Battisti, 2001). Its activity with bacterial endospores is believed to be primarily with cell membranes and not with DNA (Young & Setlow, 2003). As a selective oxidant it is also compatible with most standard materials including stainless steel, anodized aluminum, Teflon, viton, polyethylene, polypropylene, and nylon. Some discoloration of uncoated copper and cold-roll steel has been observed, comparable to what is seen when those materials are exposed to high-humidity environments. CD is stable for a limited time, so the gas is typically generated chemically at its point of use. Two typical reactions have been used for BSC decontaminations. The first involves the reaction of chlorine gas with the salt sodium chlorite:

(1) $Cl_2\ (g) + 2NaClO_2\ (s) \rightarrow 2ClO_2\ (g) + 2NaCl\ (s)$

The second reaction, requiring the presence of water, involves the reaction of chlorite ions from sodium chlorite reacting with acid:

(2) $5ClO_2^-\ (aq) + 4H^+\ (aq) \rightarrow 4ClO_2\ (g) + 2H_2O + Cl^-\ (aq)$

Several advantages result from using CD instead of formaldehyde with BSCs. Being stable as a gas at room temperature, like formaldehyde it can be easily distributed throughout a BSC and through both down-flow and exhaust HEPA filters without concern about condensation. Decontamination with CD results with no detectable residue. Therefore cleaning following its use, as is generally required for formaldehyde, is not required. CD is not a carcinogen, nor is it considered an environmental hazard.

There are a variety of ways to remove CD gas from a

cabinet following its use, including the use of a liquid chemical scrubbing system or a charcoal filter to capture and decompose the gas to a non-toxic state. Preliminary experiments with BSCs have shown that a CD exposure of 2.5 mg-hr/L is sufficient to yield a 6-log reduction of *Bacillus atrophaeus* spores on paper substrates located throughout a cabinet (Luftman & Regits, 2008). As a result, a decontamination cycle can be significantly less than 2 hours with an average CD concentration of at least 2 mg/L. This compares favorably with the minimum 6 hour, preferably 12 hour, gas exposure recommended for formaldehyde use (National Sanitation Foundation, 2007b).

In 2005 NSF International established a task group to design and implement a protocol to validate the use of CD gas as an alternative to formaldehyde gas, and to subsequently revise the NSF/ANSI 49 standard for BSCs (National Sanitation Foundation, 2007b). Active participants in this group included M. First (Harvard University), R. Gilpin (R. Gilpin, Limited), J. Hunter (Labconco Corporation), H. Luftman (previously with Micro-Clean, Inc.), D. Lupo and P. Harris (B & V Testing), W. Peters (NuAire, Inc.), D. Phillips (previously with ENV Services), J. Wagner (Controlled Environment Consulting), G. Schulling and S. Williams (NSF International), P. Lorcheim and M. Czarneski (ClorDiSys Solutions, Inc.), and M. Anand and J. P. Hobbs (Halide Group, Inc.).

Several guiding principles were used to create an appropriate protocol for this study. Given that CD is a non-condensing gas at room temperature, as is formaldehyde, the committee decided that it was not necessary to validate the use of CD for each model and size of BSC, as specified in the current standard (National Sanitation Foundation, 2007b); instead, it would be sufficient to validate its use on at least two different makes each of Class II type A2 bench and console models (where the A2 console could be replaced by a type A1), B1, and B2 biological safety cabinets, for a minimum total of 8 BSCs. The validation process needed to be general enough so purchasing equipment from a particular vendor would not be required.

Two general schemes of decontamination with CD were tested. In the first (Method 1), a specific quantity of CD gas would be generated, proportional to the volume of the enclosure. The duration of the decontamination would be a fixed time, independent of the cabinet size. The concentration of gas may vary with time, initially increasing with its generation and then potentially decreasing through a combination of leakage, absorption, and/or decomposition. This is similar to the typical method of formaldehyde gas usage, where a measured quantity of paraformaldehyde is placed within a BSC prior to decontamination. As with current formaldehyde practices, monitoring the concentration of CD within the cabinet during routine use would not be required, although it would be monitored during the validation study. In the second scheme (Method 2), the decontamination cycle would be conducted with a fixed concentration of CD gas for a specified duration. The concentration and duration would be independent of the cabinet volume. This process would require the measurement of gas concentration throughout the decontamination and a means to add more CD gas if the concentration value decayed during the exposure. The NSF task group determined that it would be sufficient to validate each method on only one set of 4 BSCs of a given make, as long as different makes were used for the two methods.

Another major guiding principle in the creation of the validation protocol was that the CD processes were to be at least as effective as the standard formaldehyde gas process. Several studies have monitored the efficacy of formaldehyde (Abraham et al., 1997; Fink et al., 1988; Munro et al., 1999; Taylor et al., 1969). These generally involved the use of bacterial endospore biological indicators (BIs). As noted above, endospores of gram-positive bacteria are among those microscopic viables that are most resistant to chemical and physical methods of decontamination. Therefore, demonstrating that a decontamination process is effective against such spores indicates that it is also effective against other viables such as viruses, fungi, yeasts, protozoa, and bacteria in their vegetative state (McDonnell, 2007; USP 31, 2008). A BI generally consists of a substrate material, such as paper, steel, or glass, which has been impregnated with a quantity of spores of a particular, typically nonpathogenic, species of bacterial spore. The number of spores on a commercially obtained BI is often ~10^6, and typical spore species include *Bacillus atrophaeus* (BA) and *Geobacillus stearothermophilus*. In decontamination validation studies, BIs are placed at several locations within a BSC prior to its decontamination. Subsequent to the decontamination, the BIs are analyzed for remaining viable spores by a microbiological laboratory, with results compared to non-decontaminated control BIs.

Two general methods are used for such analysis. Fractionation or go/no-go analysis involves placing a BI into an appropriate tube of growth media and subsequently placing the tube into an incubator set at a temperature that promotes growth of the specific BI bacteria. After 3-7 days the tube is checked for growth, with turbidity indicating that not all spores from the BI strip were deactivated. If the BI originally had 10^6 spores, a clear tube indicates that at least a 6-log reduction of spore population has been shown, while turbidity indicates a less than 6-log reduction but does not specify the results any more specifically. In the second method, enumeration analysis, the number of viable spores remaining on a BI is, in principle, counted. The viable and non-viable spores are removed from the substrate, either by macerating the substrate if it is paper, or by appropriately rinsing them off a hard substrate. A measured fraction of the effluent is added to molten agar media in a sample plate and then incubated for an appropriate time. If the remaining spores are viable, they ap-

pear as bacterial colonies on the agar plate; these can be counted and the count adjusted for the fraction of spore effluent that was used. The number of surviving spores can be compared to those from a BI control, from which a log reduction value can be calculated.

A study was performed with CD and BIs that involved two species of bacteria, paper and steel substrates, and both methods of analysis (Luftman & Regits, 2008). On the basis of that work and given that most of the prior validation work with formaldehyde involved the use of BIs having BA spores and paper substrates, it was decided to use the same type of BIs for the validation study of CD gas. Fractionation analysis was used for reasons of practicality, with replicate BIs placed at each location to compensate for the lack of more quantitative data available from enumeration analysis. The NSF task group chose a 6-log reduction as a target value for the decontamination, to be demonstrated at multiple locations within each cabinet run. The validation of a particular CD method in a particular cabinet would require at least three successful decontamination cycles.

Methods and Materials

The validation of Method 1 (fixed mass of CD) was performed at the manufacturing and laboratory facilities of NuAire, Inc. in Plymouth, MN. The five BSCs used in this study were NuAire (Plymouth, MN) models S602-600 (type A2 console, 6-foot width, 2.11m^3 volume), NU437-400 (type A2 bench top, 4-foot, 1.28 m^3), NU430-600 (type B2, 6-foot, 1.90 m^3), NU427-400 (type B1, 4-foot, 1.55 m^3), and NU427-600 (type B1, 6-foot, 1.94 m^3). Prior to the experiments, the supply and downflow HEPA filters of each cabinet were loaded with soil such that there was at least a 50% increase (~0.3 inches water gauge) of the pressure drop across the filter. These were loaded by operating the units at manufacturer-recommended airflows and introducing soil into the airstreams prior to the unit blowers. Pressure drops were measured by the pressure gauges on the BSCs.

Method 2 (fixed CD gas concentration) validation was performed within the laboratories of Micro-Clean, Inc. in Bethlehem, PA. Four BSCs manufactured by Baker, Inc. (Sanford, ME) were used. These were models B60-112 (type A1, 6-foot width, volume 2.0 m^3), SG-403 (type A2, 4-foot width, 1.4m^3), NCB-B6 (type B1, 6-foot width, 2.7 m^3), and 4-TX (type B2, 4-foot width, 1.6 m^3). The HEPA filters within these cabinets were also loaded with soil prior to the decontamination events. At least three decontamination events were run for the validation of a given BSC under a particular method of CD gas usage.

SGM Biotech, Inc. (Bozeman, MT) supplied the *Bacillus atrophaeus* (BA, ATCC #9372) biological indicators used for this study. According to the manufacturer, each strip was impregnated with a median value of 2 x 10^6 BA endospores and was contained in an envelope with Mylar on one side and Tyvek on the other. The protocol established by the NSF International task group for BI placement was followed. For each decontamination trial, a total of 12 BI strips, still within their sealed envelopes, were placed at six locations within a BSC as pairs of replicates to validate adequate penetration of CD gas throughout the cabinet. One pair of BIs was placed between the pleats on the downstream (clean) side of the exhaust HEPA filter near the center. Two more pairs of BIs were at opposite corners of the filter, placed between the pleats no more than 3 inches from the nearest outside corner of the exhaust HEPA filter. One pair of BIs was placed within a potentially contaminated positive pressure plenum. One pair of BIs was situated beneath the work surface in the plenum below the cabinet work area. The final pair of BIs was placed between the pleats near the center of the upstream (dirty) side of the down-flow HEPA filter.

Following the placement of BIs within a cabinet, a hot plate with water was placed onto the cabinet work surface to increase the relative humidity to an appropriate level (at least 60% RH) prior to the decontamination trial. A hygrometer, also placed within the cabinet, monitored the relative humidity level. The BSCs were then sealed in advance of the decontamination. Panels that had been removed for placement of the BIs were replaced. Where access panels were not replaced, plastic sheeting, duct tape, and similar sealants were used to form gas-tight containment. The BSC front face and sash were similarly sealed. The exhaust ports on A-type cabinets were sealed. For hard-ducted B-type cabinets, gas-tight dampers on the exhaust lines above the BSC were closed. As described in more detail below, ports for CD gas supply and/or circulation were incorporated within the sealing materials at designated locations.

A CD generator system (MCS), manufactured by DRS Laboratories (Allentown, PA), was used to supply and circulate the CD gas to validate Method 1. The system is comprised of a generator module, a charcoal-filled scrubber module for subsequent removal of CD from the BSC, and a regenerative blower (Gast Regenair R2103, Benton Harbor, MI) with a pumping speed of 56-71 m^3/h (33-42 cfm) to supply circulation through the BSC. CD was synthesized using sodium chlorite and an inorganic acid in water following the reaction

$$5ClO_2^- (aq) + 4H^+ (aq) \rightarrow 4ClO_2 (g) + 2H_2O + Cl^- (aq)$$

(ClorDiSys Solutions, Inc., Lebanon, NJ, supplied the proprietary pre-mixed chemical formulation.)

Method 1 involved introducing a fixed amount of CD into the BSC, proportional to the BSC size. For this validation study, 3.6 mg CD was to be generated per liter (0.10 g/ft^3) of cabinet volume. The MCS system was attached to the BSC such that gas was circulated from the generator, into the BSC through a port below its sash, exiting the BSC through a port above its exhaust HEPA filter, and back to the MCS. Prior to decontamination, the relative humidity within the BSC was increased to at least 60% RH. The MCS blower and BSC internal blower for the A2

and B1 cabinets (and only the MCS blower for the B2 cabinet) were energized during humidification to ensure adequate circulation of water vapor throughout the cabinet. During the decontamination phase, the concentration of CD was monitored by an ultraviolet spectrometer system (ClorDiSys Solutions, Inc. [Lebanon, NJ] Cloridox-EMS system), tuned to measure light absorbance at a frequency characteristic for CD. Concentration measurements, in units of mg/L, were recorded at 5, 10, 20, 30, 45, 60, and 80 minutes following the onset of CD generation. It had been previously noted that under testing conditions, CD gas concentration attained a maximum value within 20 minutes and then gradually decreased at a rate dependent upon system leakage and gas decomposition. For this study, the MCS blower and BSC internal blower (with exception for the B2 BSC, which has no internal recirculation blower) were operated for 30 minutes from the initial generation of CD, after which time gas generation ceases. The decontamination phase was continued for an additional 50 minutes, for a full exposure time of 80 minutes. The MCS blower and cabinet internal blower, where present, were activated for 2 minutes every 15 minutes during the last 50 minutes of gas exposure. The scrubber module of the MCS was then used to remove CD gas from the BSC to less than 0.3 ppm, the short-term exposure limit for CD gas as established by NIOSH. This transpired generally within 30 minutes from scrubbing initiation.

CD generation for Method 2, involving the use of CD gas at a near-constant concentration during the decontamination phase, was performed by using a ClorDiSys Solutions, Inc. (Lebanon, NJ) Minidox-M Decontamination System (Minidox). CD gas was generated through the reaction

$$Cl_2\ (g) + 2NaClO_2\ (s) \rightarrow 2ClO_2\ (g) + 2NaCl\ (s)$$

in which the chlorine gas was delivered as part of a 2% mixture in nitrogen, and the sodium chlorite was contained within cartridges with other proprietary stabilizing compounds, prepared by ClorDiSys Solutions. An internal micro-processor controlled the Minidox, allowing the presetting of the targeted humidity level (70% RH, held for 5 minutes prior to the introduction of CD gas), the concentration of CD, and the decontamination duration. The system included an integrated UV-VIS photometric measurement system and relative humidity probe to measure and control the CD concentration and relative humidity, as well as a BSC Interface Plate to facilitate the necessary connections.

Two sets of experiments were performed to allow validation at different CD concentrations, with correspondingly different durations: 4.8 mg/L for 40 minutes and 2.8 mg/L for 55 minutes. CD gas was introduced from the Minidox into the BSC through the front face of the cabinet. An external gas circulation loop was established for the B1 and B2 cabinets using a diaphragm pump and a regenerative blower, respectively. For the B1 cabinet, suction was from above the exhaust HEPA filter and supplied back though a port on the bottom of the cabinet below the down-flow filter. For the B2 cabinet, flow was from above the exhaust HEPA filter and returned above the down-flow HEPA filter. Recirculation in the A1 and A2 cabinets was provided by using only the cabinet's internal blowers. In all cases, recirculation was continued throughout the duration of the decontamination. At the conclusion of the decontamination cycle, the CD gas was vented through a ClorDiSys Solutions, Inc. charcoal-filled scrubbing unit. Scrubbing was generally complete within 45 minutes of its initiation.

The retrieval and subsequent analysis of the biological indicators were performed in the same manner for both of the decontamination methods. The BIs were removed from the CD-exposed BSC within 2 hours of completing the gas removal. Sample preparation ensued within the next 1 to 12 hours. All preparation work was performed aseptically within an operating BSC to minimize the potential for inadvertent bacterial contamination of the BIs or media. Luftman and Regits (2008) had demonstrated that CD absorbed within a BI paper substrate might reduce the population of viable spores found during subsequent analysis. Following a protocol established in that work, a 1.0 weight percent solution of sodium thiosulfate was prepared using distilled water, and then subsequently autoclaved for sterilization. Five or 10 mL aliquots were then distributed to pre-sterilized tubes, with an individual tube used for each BI to be analyzed (5 mL aliquots were used for most of the BIs within trials N14 - N20, with the remainder using 10 mL). For each BI, the BI envelope was opened and the strip directly dropped from the envelope into the thiosulfate solution without contact with any object aside from the interior surfaces of its pouch and the inside of the thiosulfate tube. The BI was left in the solution for 1.0 minute. The BI was then transferred by nominally sterilized forceps to a media tube.

As noted below, a concern arose with the number of positive-testing strips from the earliest trials. The forceps had been stored within a beaker containing 70% isopropyl alcohol (IPA) between transferring strips from the thiosulfate to the broth vials. However, alcohols are not considered good sterilants for bacterial endospores. Furthermore, prior to sample N1, two positive control BIs had been transferred into broth using forceps which were subsequently placed within the IPA. Thus, a significant possibility existed that the IPA solution had been contaminated and remained so through sample N9. At this time, the IPA solution was discarded and replaced with a dilute solution of chlorine dioxide, a known sporicide, to decontaminate the forceps between BI transfers. In each subsequent trial involving 12 BIs, each strip was manipulated with a different pair of pre-sterilized forceps. All forceps were decontaminated in a chlorine dioxide aqueous solution between trials.

In the initial trials of Method 1 (N1 - N13), the growth media consisted of BBL Trypticase Soy Broth (BD,

Franklin Lakes, NJ), supplied as 10 mL aliquots within sterilized tubes. For subsequent Method 1 trials (N14 - N20) and all of Method 2 (B1 - B24, B27, and B29), SGM Biotech, Inc. (Bozeman, MT) Releasat culturing sets were used, consisting of aliquots of modified soybean casein digest broth. Sealed media tubes were then transferred to an incubator set for 30°-35°C, appropriate for cultivation of *B. atrophaeus* endospores. The media tubes were periodically examined, with the initial observation at 2 days and final recorded observation at 7 days following the initiation of the incubation. The examination consisted of looking for turbidity and, in the case of the Releasat sets, color change, both indicative of *B. atrophaeus* growth. Negative control samples, consisting of unopened media tubes, were occasionally included with experimental samples to verify the initial sterility of the media. Positive control samples to test the initial viability of spores on the BIs were prepared by taking BI strips not exposed to CD, submerging them into the neutralizing thiosulfate solution for 1 minute, and then transferring the strips to individual media tubes.

The NSF task group agreed upon the criteria for successful validation studies prior to performing these experiments. The criteria were based in part upon the statistical analysis presented within the Appendix of this paper. The result of a single trial at a specific site in a BSC was deemed successful if either one or two BIs from that site tested negative (no growth in the incubated media tube). If both strips tested positive, that site test was deemed a failure. For a single cabinet trial, the trial was considered successful (a pass) if all six site tests were successful by the criteria given above. It was considered unsuccessful (a failure) if the site tests failed at more than one location. The trial was considered a conditional pass if there was a failure at only one site. A cabinet study under a particular CD method was considered to have passed if all three trials passed. A cabinet study was also considered to pass if one or more trials had conditional passes, as long as there had not been more than one failure at any one specific site. It had also been decided that a cabinet trial could be repeated if there were a clear understanding of the reason for a trial failure that was not based upon the intended target decontamination conditions. Such reasons may have included unexpected cabinet leakage, incorrect humidity levels, or errors in BI handling.

Results

Data from the experiments using Method 1 (predetermined mass of CD, the MCS generation system, and NuAire cabinets) are presented in Tables 1, 2, and 3, with the first two tables coming from the first series of such experiments (trials N1 - N13). Rather than showing the individual CD concentration measurements as a function of time, these tables show the full "dose" of CD gas to which a BSC was exposed in a given experiment. Doses were calculated by numerically integrating the

Table 1

Method 1, BI results for A2 cabinets. Insertion of 0.1g chlorine dioxide per ft^3 of BSC with 80-minute exposure.

Cabinet	**A2 – 6 foot (console)**				**A2 – 4 foot (bench top)**			
Trial #	N1	N3	N5		N2	N4	N6	N13
CD Dose (mg-hr/L)	3.1	3.9	3.1		3.5	3.1	3.5	3.9
Position # [A]	**Number of Positive BIs (out of 2)**							
1	2	1	1		1	1	0	0
2	2	1	1		2	1	0	0
3	1	0	2		2	0	0	0
4	1	1	1		1	0	0	0
5	1	2	0		0	0	0	0
6	1	1	0		0	0	1	0
Trial Result [B]	F	CP	CP		F	P	P	P

A – Biological indicator positions:
1, 2, 3 – Within downstream side of exhaust HEPA filter, back left, center, and front right regions, respectively
4 – Beneath work surface
5 – Within positive pressure plenum
6 – Within upstream side of down-flow HEPA filter
B – Trial results: P = pass; CP = conditional pass; F = fail

Table 2

Method 1, BI results for B1 and B2 cabinets. Insertion of 0.1g chlorine dioxide per ft³ of BSC with 80-minute exposure.

Cabinet	**B1 – 4 foot (console)**				**B2 – 6 foot (console)**		
Trial #	N7	N9	N11		N8	N10	N12
CD Dose (mg-hr/L)	4.4	4.7	5.0		2.0	2.5	2.6
Position # [A]	**Number of Positive BIs (out of 2)**						
1	2	0	0		0	0	0
2	0	1	0		0	0	0
3	2	2	0		0	0	0
4	0	1	0		0	0	0
5	0	0	0		0	0	0
6	0	1	0		0	0	0
Trial Result [B]	F	CP	P		P	P	P

A and B – See Table 1 notes.

Table 3

Method 1, repeat experiments, BI results for A2 and B1 console cabinets.
Insertion of 0.1g chlorine dioxide per ft³ of BSC with 80-minute exposure.

Cabinet	**A2 – 6 foot (console)**				**B1 – 6 foot (console)**			
Trial #	N15	N17	N19		N14	N16	N18	N20
CD Dose (mg-hr/L)	3.0	3.7	3.7		2.6	3.7	3.3	3.3
Position # [A]	**Number of Positive BIs (out of 2)**							
1	0	0	0		0	0	0	0
2	0	0	0		0	0	0	0
3	0	0	0		0	0	0	0
4	0	0	0		0	0	0	0
5	0	0	0		0	0	0	0
6	0	0	0		0	0	0	0
Trial Result [B]	P	P	P		P	P	P	P

A and B – See Table 1 notes.

measured concentrations over time by the equation

$$D = \sum_{i=0}^{6} \frac{1}{2}(C_{i+1} + C_i) \times \frac{1}{60}(t_{i+1} - t_i)$$

where D is the cumulative CD dose (mg-hr/L), t_i the measurement times (0, 5, 10, 20, 30, 45, 60, and 80 minutes), and C_i the measured concentrations of CD (mg/L). Since two BIs were placed at each of six selected positions within a BSC, BI analyses following a sufficient period of incubation yielded 0, 1, or 2 positive BI strips with residual *B. atrophaeus* spores, the number of which is shown within the tables. According to the aforementioned analysis criteria, a failure occurs at a site within a single trial if there are two positive BIs. A trial passes with no site failures, has a conditional pass with one failure, and fails with more than one site failure. These

result classifications are given within the tables for each experimental trial.

A fairly high frequency of positive BIs was observed in trials N1 through N9. For the A2 console BSC, two sites had failures for trial N1, and one each for trials N3 and N5, with no consistency concerning the location of the site failures. Under the criteria set by the NSF task group, the validation study for the CD decontamination of this cabinet by Method 1 had failed on the basis of these data as there had been a trial failure (N1). The A2 bench top cabinet study had also failed on the basis of trials N2, N4, and N6 as there had been two site failures for N2. The B1 cabinet study (using model NU427-400, a 4-foot wide cabinet) failed on the basis of trials N7 and N9 as there were two site failures for N7 and the one site failure for N9 coincided with a failed site in the N7 trial. As noted within the Methods section above, it was decided that the cause for much of these failures was a procedural error in the decontamination of forceps between BI preparations for analysis. This procedural problem was corrected, as noted, by using an aqueous CD solution for further decontamination of forceps. As seen in Tables 1 and 2, the following trials N10, N11, and N12 showed no positive BIs. The B2 cabinet study with Method 1 passed the validation criteria. As a cause for prior trial failure had been identified, the NSF task group criteria permitted the repeat of trials in an attempt to achieve three trial passes to achieve a pass in a cabinet study. Due to limited available material and time in this phase of studies, only one more trial for Method 1 could be attempted in this sequence. Trial N13 (Table 1), performed with the A2 bench-top cabinet, produced no positive BIs. On the basis of trials N4, N6, and N13, the A2 bench-top cabinet passed the validation criteria for Method 1.

A second sequence of trials was subsequently performed at the NuAire facility, following the successful validation of Method 2 reported below, to validate the use of Method 1 with the NuAire A2 console and B1 cabinets. For this part of the study, a model NU 427-600 BSC was used for the B1 BSC, a 6-foot wide cabinet rather than the 4-foot cabinet used previously. Results from these trials are presented in Table 3. Aside from correcting the methodology in the handling of the BIs, conditions similar to the previous trials were used. The A2 console cabinet was studied in trials N15, N17, and N19. Analysis of the BIs after 7 days of incubation showed no viable spores on any of the strips. The B1 cabinet was tested four times, as the CD dose on the first of these trials, N14, was slightly low. Nevertheless, all 48 BIs associated with the B1 cabinet trials N14, N16, N18, and N20 tested negative after 7 days of incubation. Two positive control indicators, prepared by immersing BIs not exposed to CD in the sodium thiosulfate solution for 1 minute prior to transferring them to growth media and incubation, tested positive. Three negative control samples, consisting of sodium thiosulfate solution in media with incubation, tested negative. On the basis of these results, the A2 console-top cabinet and B1 cabinet passed the validation criteria for Method 1.

Data from the experiments using Method 2 (fixed concentration of CD gas, the ClorDiSys Minidox generation system, Baker cabinets, performed at Micro-Clean) are presented in Tables 4, 5, and 6, with the first two tables coming from the series of experiments having a targeted CD concentration of 4.8 mg/L with an exposure duration of 40 minutes and Table 6 corresponding to a targeted CD concentration of 2.8 mg/L for a duration of 55 minutes. As nearly constant CD concentrations were maintained for these trials, the CD dose is calculated as the product of the CD concentration with the duration of CD exposure. The three trials for each of the Baker A1 and A2 cabinets using Method 2 at 4.8 mg/L passed, as shown in Table 4, thus validating that method for those cabinets. Four trials were performed for the B1 BSC at 4.8 mg/L, as the pair of BIs from site 6 in trail B4 were dropped within the cabinet during retrieval (Table 5). All four of these trials passed on the basis of task group criteria. The first trial on the B2 cabinet, trial B12, was performed using a diaphragm pump to drive the gas circulation that had a lower pumping speed than that of the Gast blower that had been used in previous trials. Trial B12 failed as there were failures at three sites, all within HEPA filters for which gas penetration might be expected to be most challenging. Three more trials for this cabinet were performed with the Gast blower, and all passed. Thus, Method 2 at 4.8 mg/L for 40 minutes had been validated for all four Baker BSCs. Table 6 shows the data demonstrating the successful validation of all four cabinets of Method 2 at 2.8 mg/L for 55 minutes.

For all trials, little or no material degradation was found within the subject BSCs as a result of the decontamination sequences. Furthermore, no chemical residuals attributable to the CD gas were evident.

Discussion

Intrinsic within the protocol for this validation study was the definition of a successful decontamination event at a particular site within a given cabinet. Noted previously, two paper-strip biological indicators, each impregnated with ~10^6 endospores of *B. atrophaeus*, were placed at each site. A concern had been that even with an ideal decontamination event, the possibility of getting a positive BI result from a go/no-go analysis due to sample mishandling or other more random natural events remains. The likelihood of more than one such random event is greatly reduced, prompting the use of BI replicates at each site. If one or two strips return positive because in fact the decontamination event was not ideal, what can be surmised as an appropriate lower limit to the spore log reduction of that event? A detailed description of the relevant statistics involving the use of

Table 4

Method 2, BI results for A1 and A2 console cabinets. Constant CD gas concentration of 4.8 mg/L for 40 minutes.

Cabinet	**A1 – 6 foot (console)**				**A2 – 4 foot (console)**		
Trial #	B1	B6	B7		B2	B5	B15
CD Dose (mg-hr/L)	3.2	3.2	3.2		3.2	3.2	3.2
Position # [A]	**Number of Positive BIs (out of 2)**						
1	0	0	0		0	0	0
2	1	0	0		0	0	0
3	0	0	0		0	0	0
4	0	0	0		0	0	0
5	0	0	0		0	0	0
6	0	0	0		0	0	0
Trial Result [B]	P	P	P		P	P	P

A and B – See Table 1 notes.

Table 5

Method 2, BI results for B1 and B2 console cabinets. Constant CD gas concentration of 4.8 mg/L for 40 minutes.

Cabinet	**B1 – 6 foot (console)**					**B2 – 4 foot (console)**			
Trial #	B3	B4	B10	B11		B12	B13	B14	B16
CD Dose (mg-hr/L)	3.2	3.2	3.2	3.2		3.2	3.2	3.2	3.2
Position # [A]	**Number of Positive BIs (out of 2)**								
1	0	0	0	0		2	0	1	0
2	1	0	1	0		1	0	0	0
3	0	0	0	0		2	0	0	0
4	0	0	0	0		1	0	0	1
5	0	0	0	0		0	0	0	0
6	0	ND	0	0		2	0	0	0
Trial Result [B]	P	P	P	P		F	P	P	P

A and B – See Table 1 notes; ND – no data

such strips is presented in the Appendix to this paper. Summarizing its conclusion, we assume that the BIs in an experiment have an initial spore population of $2x10^6$, which is typical for most commercially available indicators labeled as 10^6. The analysis indicates that if one strip returns negative and the other positive after go/no-go analyses, then there is a 95% probability that the decontamination event produced at least a 5.7 log reduction of the test spores and a 50% probability that the log reduction was at least 6.2. With both strips testing negative, there is a 95% probability of at least a 6.1 log reduction and a 50% probability of at least a 6.8 log reduction. If both strips return positive, no lower limit for log reduction can be made. As a result of these statistics, it was deemed sufficient to have one of two BIs at a site test negative to declare a successful decontamination at that site. If only one of six sites in an experimental trial failed and that site did not fail in other trials, it was surmised that the one failure was most likely from random events or an analysis error, or would not in itself give

Table 6

Method 2, BI results for A1, A2, B1, and B2 console cabinets.
Constant CD gas concentration of 2.8 mg/L for 55 minutes.

Cabinet	A1				A2				B1				B2		
Trial #	B8	B9	B27		B23	B24	B29		B17	B19	B22		B18	B20	B21
CD Dose (mg-hr/L)	2.8	2.8	2.8		2.8	2.8	2.8		2.8	2.8	2.8		2.8	2.8	2.8
Position # [A]	Number of Positive BIs (out of 2)														
1	0	0	0		0	0	0		0	0	0		0	0	0
2	0	0	0		0	0	0		0	0	0		0	0	0
3	0	0	0		0	1	0		0	0	0		0	0	0
4	0	0	0		0	0	0		0	0	0		0	0	0
5	0	0	0		0	0	1		1	0	0		0	0	0
6	0	0	0		0	0	0		0	0	0		0	0	0
Trial Result [B]	P	P	P		P	P	P		P	P	P		P	P	P

A and B – See Table 1 notes.

cause to consider the trial itself a failure.

The validation protocol devised for this study met the requirements set by NSF International. It included the use of BSCs spanning the typical configuration of Type A and B cabinets from more than one manufacturer. HEPA filters had been loaded with soil to represent typical conditions when cabinets in current use require decontamination. Biological indicators were placed at positions that included those considered most challenging for gas decontamination. Multiple trials for each cabinet under specific decontamination conditions were invoked. The methods of validated gas generation did not require the use of chemicals or equipment tied to particular manufacturers. NSF International is planning to use the general guidelines of this protocol design when validating the use of other chemicals for gas decontamination in the future.

A major goal of the CD validation study, if it was successful, was to then use the validation protocol in designing appropriate protocols for the routine use of CD gas in the decontamination of BSCs. All CD decontaminations should be preceded by a humidification step bringing the relative humidity to within the range of 65%-80% RH. The quantity of CD and durations of exposure used in the study should be augmented to fully ensure a successful decontamination. Specifically, for Method 1 it is recommended that 4.7 mg CD per liter of cabinet volume (0.13 g/ft^3) and decontamination duration be at least 85 minutes. For Method 2, two condition sets are recommended: either a concentration of 5.0 mg CD/L be maintained for 45 minutes or a concentration of 3.0 mg CD/L be maintained for 60 minutes. Method 1 would not require the use of a spectrometer to monitor the CD concentration during decontamination, but Method 2 would. The need for gas recirculation during decontamination appears dependent upon the cabinet type and CD method. An external recirculation loop extending from above the exhaust HEPA filter to or below the cabinet front face and the cabinet internal circulation blower, when present, is to be used for all Class II BSCs when using Method 1 throughout the duration of humidification and CD gas generation, and used for at least 2 minutes every 15 minutes after gas generation until the end of the decontamination period. A similar loop is to be used throughout Method 2 decontaminations of B1 and B2 cabinets and with A-type cabinets without operable blowers, and while not necessary, may be used for A-type cabinets with operable internal blowers, as well. When an operable internal recirculation blower is present in the BSC, it is to be left operating throughout a Method 2 decontamination process. Removal of CD following the decontamination by the use of an absorption or chemical scrubber is recommended.

As noted above, there were little or no obvious effects of material degradation within the BSCs of this study attributable to the use of CD gas during the validation trials. Supporting this observation are multiple references to the use of CD gas for device, room, and building decontamination showing no adverse effects to a significant list of materials including stainless steel, anodized aluminum, Teflon, viton, polyethylene, polypropylene, and nylon, as well as to electronic devices within the decontamination space (Buttner et al., 2004; Czarneski & Lorcheim, 2008; Leo et al., 2005; Luftman

et al., 2006; Rogers et al., 2004a). However, as CD gas is somewhat oxidative, as hydrogen peroxide or ozone, and is utilized in a relatively humid environment, some oxidation effects have been observed following a substantial number of decontamination cycles (Hawley & Kozlovac, 2005; U.S. Environmental Protection Agency, 2005). Non-coated non-stainless steel and other ferrous materials will gradually show corrosion after multiple CD decontamination cycles. This is similar to corrosive effects seen after similar exposure to a highly humid environment and much less than would be observed by exposure to other chlorine-containing chemicals such as sodium hypochlorite (bleach) or chlorine gas. Within BSCs this would be eventually manifested on surfaces including the internal blower shaft, rolled steel paneling, and occasional hardware. Surface oxidation is also found on non-anodized aluminum and copper surfaces, which after multiple exposures may have an effect on electronic devices and on the coil of cabinet blowers. The CD dose levels within the protocols of this work were chosen to give an appropriate balance between decontamination efficacy targeted to a 6-log reduction of spore population and minimizing material concerns, thus resulting in CD exposures significantly lower than had been used in some prior work. Evidence here and in extensive field work suggests these conditions are suitable for more than 10 decontamination cycles of a new BSC, which are more such cycles than typically encountered by a BSC during its lifetime.

Conclusion

Processes using chlorine dioxide gas for the decontamination of laminar flow biological safety cabinets have been successfully validated by this study. Protocols were established in conjunction with the Chlorine Dioxide Task Group of NSF International, including representatives from BSC manufacturers, BSC certification organizations, consulting groups, and academia. Two methods of control for the CD process, as well as two methods of synthesizing CD gas, were included in the study to allow the greatest flexibility in the application of CD by the technical community. The first method involved the introduction of a specific amount of CD gas (3.6 mg/L) into a BSC, dependent upon its volume, allowing exposure for 80 minutes. In the second method, two specified concentrations of CD gas within the cabinet were achieved, monitored, and maintained for durations dependent upon the concentrations (specifically, 4.8 mg/L for 40 minutes and 2.8 mg/L for 55 minutes). Particular attention was given to having a means of gas circulation throughout the cabinet for much of the decontamination cycles. Validation was achieved through a strategy using biological indicators placed within multiple locations of the BSCs during each experimental run.

Chlorine dioxide gas has been demonstrated to be suitable for the decontamination of BSCs as an alternative to formaldehyde gas, the only other chemical system that has been considered validated for this use by NSF International. The duration of gas exposure in the final processes likely to be invoked will be less than 90 minutes, compared with a 6-hour minimum for the current formaldehyde practice. In place of the chemical neutralization for the formaldehyde procedure, gas removal through scrubbing is recommended for CD, allowing the full-time that CD gas is present for a BSC decontamination being less than 2 hours. With the additional lack of chemical residuals, the CD decontamination process is a very practical method for general use.

Appendix—Probability Analysis of Biological Indicator Results

In establishing the merit of a particular decontamination scheme, it is of interest to determine the probability **p** of a single bacterial spore on a biological indicator (BI) surviving the decontamination process. More specifically, if one has several BIs, each initially with **N** viable spores on it (often on the order of 10^6), what can be inferred about this probability if some BIs analyzed by a go/no-go methodology show viable spores (positive growth) and some show no viable spores (negative growth)? A typical target for gas decontaminations is to demonstrate that a log-reduction, **L**, of a specific spore population exceeds a value of 6, where **L** is defined as **-log (p)**.

While in the laboratory there may be some physical differentiation among spores on BIs prior to a decontamination event (DE) due to such effects as variation of viability among spores, non-uniformity of spore distribution on a BI, and lack of uniformity of the BI substrate surface, it is assumed for this statistical analysis that all of the spores among the BIs have an identical probability of survival **p** following a DE. Then the probability of a single spore dying is **(1-p)**. The probability of no surviving spores, P_0, on a BI with an initial population of **N**, is then given by

(1) $P_0 = (1-p)^N$

The probability of 1 or 2 spores surviving become

(2) $P_1 = Np(1-p)^{N-1}$ and

$P_2 = \frac{1}{2} N(N-1)p^2(1-p)^{N-2}$

and for greater numbers of survivors by the binomial expansion equation

(3) $P_n = N! / [(N - n)! (n)!] p^n (1-p)^{N-n}$

(Halvorson & Ziegler, 1932). In the case of **N** being large, (3) can be well approximated by the use of the Poisson distribution function

(4) $P_n = (pN)^n e^{-(pN)}/n!$

where **e** is the base of natural logarithms.

This equation has some interesting ramifications. Consider the case for a large **N** where it is already known that for a specific DE, **p = 1/N** or **pN = 1**. For example,

assume that it is known that there are 10^6 spores on a BI and that each spore has the probability of survival of 10^{-6} following a DE. Then (4) gives the probability P_0 of no surviving spores after the DE of e^{-1}, or approximately 0.37. That is, there would be only a 37% chance that a single BI strip will be negative upon analysis or a 63% chance the BI will test positive. (This may be counter to one's intuition that it is unlikely to have a positive BI result in such a case with $p = 10^{-6}$.) In the case of $pN = 1$, (4) simplifies to $P_n = e^{-1}/n!$ The probability of having a single spore survive, P_1, is also 0.37. The probabilities of having more than one spore survive drops off quickly with n. Also note that, as long as N is large, this result is independent of N.

"Most Probable" Value of p

Returning to the general situation where p is unknown, assume one performed a DE using r total BIs and with q BIs being negative, or showing no growth, after analysis. One can estimate p_{mp}, a most probable value for p given a set of BI results with r total BIs and q testing negative. Assume that a reasonable approximation of P_0 is given by the ratio q/r. Then from (4) and using p_{mp} for p,

(5) $q/r = P_0 = \exp(-p_{mp}N)$ or
$p_{mp} = N^{-1}\ln(r/q)$

where $\ln(x)$ is the natural logarithm of x. This analysis is similar to that given by Pflug et al. (2001), referring to the original work by Halvorson and Ziegler (1932). There are a couple of significant limitations in this analysis for p_{mp}. There is no analysis of likely error limits for p_{mp} given here, although one would assume that such limits would decrease with r, the total number of BIs used in the DE experiment. Secondly, the analysis is of predictive value only if $0 < q < r$. If $q = 0$, that is all BIs test positive, one cannot predict whether only a few or virtually all of the N spores on each BI survived the DE. If some, but not all BIs test negative and N is large, it can be assumed that there were only a few surviving spores on the positive BIs, and that p is on the order of N^{-1}. If all BIs are negative, (5) predicts $p_{mp} = 0$, as the probability of spore survival can be arbitrarily small to give such a result. One could give a practical lower bound for p under such conditions by assuming that if one more BI had been run, it would have come back positive. Then, by (5),

(6) $p_{mp}(q=r) < (1/N)\ln((r+1)/r)$

which for large r simplifies to $p_{mp}(q=r) < 1/(N\ln(r))$.

In the experimental procedure invoked within the attached paper, $r = 2$ BIs at a particular site in a given DE. (Gas exposure and/or humidification may be different among separate sites in a given trial, so that each site should be treated as having a distinct DE.) There are typically $N = 2 \times 10^6$ spores on each of the BIs according to their manufacturer. If one BI returns negative and the other positive ($q = 1$), by (5) $p_{mp} = 3.5 \times 10^{-7}$, corresponding to a most probable log reduction, L_{mp}, of 6.5. If both strips return positive ($q = 0$), one would assume that $p > N^{-1}$, or that $L < 6.3$, but it could be as small as 0, i.e., no decontamination occurred. If both return negative ($q = 2$), then by the method of (6), $p_{mp} < 2.0 \times 10^{-7}$, or $L_{mp} > 6.7$.

Mean Value of p

There are alternate, potentially more informative ways to analyze data from BI experiments involving go/no-go BI characterization. We limit discussion here to potential experimental outcomes involving the use of one or two BIs for a DE. Having one BI return positive in a one-BI experiment or two BIs being positive from a two-BI experiment provides no upper limit below 1 for p, the probability of survival of a single spore, and thus no lower limit above 0 for L, the likely log reduction of a spore population. Such results suggest the need to change the experimental conditions or method of decontamination so that more quantitative results can be attained. Equation (4) is used to formulate probability functions for each of these cases. To simplify notation, we use the substitution $m = pN$. For case 1, a single BI is tested and returns as negative. The probability distribution function $f(m)$ for this case becomes

(7) Case 1: $f_1(m) = P_0 = e^{-m}$

For case 2, two BIs are tested and both return negative.

(8) Case 2: $f_2(m) = 2(P_0)^2 = 2e^{-2m}$

In case 3, two BIs are tested, with one returning negative and one returning positive.

(9) Case 3: $f_3(m) = 2P_0(1-P_0) = 2(e^{-m} - e^{-2m})$

For each of the above, the appropriate factor has been used so that these distribution functions are normalized, i.e.,

$$\int_0^\infty f(m)\, dm = 1$$

These functions then present probability distributions for values of m for the three experimental results.

A mean value for m, $\langle m \rangle$, corresponds to an average value of m for a given probability distribution, with each value of m weighted by its probability:

$$(10) \quad \langle m \rangle = \int_0^\infty m\, f(m)\, dm$$

Given $\langle m \rangle$ and N, one then has an average predicted value for p, the probability of survival for a single spore. The uncertainty of $\langle m \rangle$ is given by the square root of the distribution's variance,

$$(11) \quad \mathrm{Var}\langle m \rangle = (\langle m^2 \rangle - \langle m \rangle^2)/r = \{\int_0^\infty m^2 f(m)\, dm - \langle m \rangle^2\}/r$$

where r is the total number of samples, or in this case, BIs. The integral equations above can be solved using the general result

$$(12) \quad \int_0^\infty m^n e^{-am}\, dm = n! / a^{(n+1)}$$

The following results are obtained:

Case 1: $\langle m \rangle_1 = 1$ $\quad Var\langle m \rangle_1 = 1$

Case 2: $\langle m \rangle_2 = 1/2$ $\quad Var\langle m \rangle_2 = 1/8$

Case 3: $\langle m \rangle_3 = 3/2$ $\quad Var\langle m \rangle_3 = 5/8$

For the specific case encountered in the attached validation experiment, with $N = 2 \times 10^6$, then these yield $\langle p \rangle_1 = (5 \pm 5) \times 10^{-7}$, $\langle p \rangle_2 = (2.5 \pm 1.8) \times 10^{-7}$, and $\langle p \rangle_3 = (7.5 \pm 4.0) \times 10^{-7}$, for cases 1, 2, and 3, respectively.

Probability Limits for p and L

The distribution functions $f_i(m)$ from (7), (8), and (9) can be used to determine the probability P_U that the true value for m from an experiment is less than an upper limit value that one chooses for m, namely m_U. This is given by

$$(13) \quad P_U = P(m < m_U) = \int_0^{m_U} f_i(m)\, dm$$

From this, one can calculate upper limit values m_U for m such that we have U = 50%, 90%, and 95% confidence that the "true" m is less than m_U. For the three cases involving biological indicators described above,

(14) Case 1: $P_U = 1 - \exp(-m_U)$

or $m_U = -\ln(1 - P_U)$

(15) Case 2: $P_U = 1 - \exp(-2m_U)$

or $m_U = -\frac{1}{2} \ln(1 - P_U)$ and

(16) Case 3 $P_U = 1 + \exp(-2m_U) - 2\exp(-m_U)$

Values for m_U can now be calculated for a given P_U. (Note that for case 3, a table can be constructed of P_U versus m_U, from which appropriate values of m_U for a given P_U can be read.)

Case #	$\langle m \rangle$	$m_{50\%}$	$m_{90\%}$	$m_{95\%}$
1	1.0	0.69	2.30	3.0
2	0.5	0.35	1.15	1.5
3	1.5	1.25	3.0	3.7

$m_{50\%}$ gives the median value for each case, which is somewhat less than the mean value of each distribution, $\langle m \rangle$. For Case #3 one can also show that there is a 95% probability that $m > 0.25$, which makes sense given that one BI fails in that case, so that there is a lower limit to the probability of spore survival.

For the specific case encountered in the attached validation experiment, with $N = 2 \times 10^6$, one can use this table to calculate limit values p_U (from $m = pN$) such that there is a 50%, 90%, or 95% probability that the true probability of spore survival is less than the corresponding limits. Similarly, lower limit values for the log reduction of spore populations can also be determined for these cases, presented below.

Case #	$L_{50\%}$	$L_{90\%}$	$L_{95\%}$
1	6.5	5.9	5.8
2	6.8	6.2	6.1
3	6.2	5.8	5.7

For the specific case of $N = 2 \times 10^6$ spores on a strip and, in a two-BI experiment one coming back negative and the other positive, there is a 95% probability that a log-kill enumeration of a strip from that decontamination condition would be greater than 5.7.

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References

Abraham, G., Le Blanc Smith, P. M., & Nguyen, S. (1997). The effectiveness of gaseous formaldehyde decontamination assessed by biological monitoring. *Journal of the American Biological Safety Association, 2*(1), 30-38.

Ackland, N. R., Hinton, M. R., & Denmeade, K. R. (1980). Controlled formaldehyde fumigation system. *Applied Environmental Microbiology, 39*(3), 480-487.

Buttner, M., Cruz, P., Stetzenbach, L., Klima-Comba, A., Stevens, V., & Cronin, T. (2004). Determination of the efficacy of two building decontamination strategies by surface sampling with culture and quantitative PCR analysis. *Applied Environmental Microbiology, 70,* 4740-4747.

Cheney, J. E., & Collins, C. H. (1995). Formaldehyde disinfection in laboratories: Limitations and hazards. *British Journal of Biomedical Science, 52,* 195-201.

Czarneski, M. A., & Lorcheim, P. (2008). Validation of chlorine dioxide sterilization. In J. Agalloco and F. Carleton (Eds.), *Validation of pharmaceutical processes* (3rd ed.) (pp. 281-287). New York: Inform Healthcare, Inc.

Favero, M. S., & Bond, W. W. (2001). Chemical disinfection of medical and surgical materials. In S. Block (Ed.), *Disinfection, sterilization, and preservation* (p. 888). Philadelphia: Lippincott, Williams & Wilkins.

Fink, R., Liberman, D. F., Murphy, K., Lupo, D., & Israeli, E. (1988). Biological safety cabinets: Decontamination or sterilization with paraformaldehyde. *American Industrial Hygiene Association Journal, 49,* 277-279.

Halvorson, H. O., & Ziegler, N. R. (1932). Application of statistics to problems in bacteriology. *Journal of Bacteriology 25,* 101-120.

Hawley, R. J., & Kozlovac, J. P. (2005). Decontamination. In L. Lindler, F. Lebeda, & G. Korch (Eds.), *Infectious diseases: Biological weapons defense: Infectious diseases and counterterrorism* (pp. 333-348). Tottowa, NJ: Humana Press, Inc.

International Agency for Research on Cancer. (2004). Formaldehyde, 2-butoxyethanol and 1-*tert*-butoxy-2-propanol. *IARC monographs on the evaluation of carcinogenic risks to humans. 88*. Available at: www.iarc.fr/ENG/Press_Releases/archives/pr153a.html. Accessed online 2008.

Jeng, D. K., & Woodworth, A. G. (1990). Chlorine dioxide gas sterilization under square-wave conditions. *Applied Environmental Microbiology, 56,* 514-519.

Joslyn, L. J. (2001). Gaseous chemical sterilization. In S. Block (Ed.), *Disinfection, sterilization, and preservation* (pp. 337-360). Philadelphia: Lippincott, Williams & Wilkins.

Knapp, J., & Battisti, D. (2001). Chlorine dioxide. In S. Block (Ed.), *Disinfection, sterilization, and preservation* (pp. 215-228). Philadelphia: Lippincott, Williams & Wilkins.

Kruse, R. H., Puckett, W. H., & Richardson, J. H. (1991). Biological safety cabinetry. *Clinical Microbiology Reviews, 4,* 207-241.

Leo, F., Poisson, P., Sinclair, C. S., & Tallentire, A. (2005). Design, development and qualification of a microbiological challenge facility to assess the effectiveness of BFS aseptic processing. *PDA Journal of Pharmacy Science Technology, 59,* 33-48.

Luftman, H. S. (2005). Neutralization of formaldehyde gas by ammonium bicarbonate and ammonium carbonate. *Applied Biosafety: Journal of the American Biological Safety Association, 10*(2), 101-106.

Luftman, H. S., Regits, M. A., Lorcheim, P., Czarneski, M. A., Boyle, T., Aceto, H., et al. (2006). Chlorine dioxide gas decontamination of large animal hospital intensive and neonatal care units. *Applied Biosafety: Journal of the American Biological Safety Association, 11*(3), 144-154.

Luftman, H. S., & Regits, M. A. (2008). *B. Atrophaeus* and *G. Stearothermophilus* biological indicators for chlorine dioxide gas decontamination. *Applied Biosafety: Journal of the American Biological Safety Association, 13*(3), 143-157.

McDonnell, G. E. (2007). *Antisepsis, disinfection and sterilization—Types, action and resistance*. Washington, DC: AMS Press.

Munro, K., Lanser, J., & Flower, R. (1999). A comparative study of methods to validate formaldehyde decontamination of biological safety cabinets. *Applied Environmental Microbiology, 65*(2), 873-876.

National Sanitation Foundation. (2007a). *National Sanitation Foundation: Standard No. 49 for Class II (Laminar Flow) Biosafety Cabinetry*.

National Sanitation Foundation. (2007b). Annex G—Recommended microbiological decontamination procedure, *National Sanitation Foundation: Standard No. 49 for Class II (Laminar Flow) Biosafety Cabinetry,* G1-G3.

Pflug, I. J., Holcomb, R. G., & Gómez, M. M. (2001). Principles of the thermal destruction of microorganisms. In S. Block (Ed.), *Disinfection, sterilization, and preservation* (pp. 112-123). Philadelphia: Lippincott, Williams & Wilkins.

Prince, N., & Prince, D. L. (2001). Principles of viral control and transmission. In S. Block (Ed.), *Disinfection, sterilization, and preservation* (pp. 543-571). Philadelphia: Lippincott, Williams & Wilkins.

Rogers, J., Sabourin, C. L., Taylor, M. L., Riggs, K., Choi, Y. W., Richter, W. R., et al. (2004a). Environmental technology verification report: CDG Research Corporation bench-scale chlorine dioxide gas: Solid generator. Battelle. www.epa.gov/etv/pubs/11_vr_cdg.pdf. Accessed online 2008.

Rogers, J., Sabourin, C. L., Taylor, M. L., Riggs, K., Choi, Y. W., Richter, W. R., et al. (2004b). Environmental technology verification report: CERTEK, Inc. 1414RH formaldehyde generator/neutralizer. Battelle. Available at: www.epa.gov/etv/pubs/11_vr_certek.pdf. Accessed online 2008.

Spiner, D. R., & Hoffman, R. K. (1971). Effect of relative humidity on formaldehyde decontamination. *Applied Microbiology, 22,* 1138-1140.

Taylor, L. A., Barbeito, M. S., & Gremillion, G. G. (1969). Paraformaldehyde for surface sterilization and detoxification. *Applied Microbiology, 17,* 614-618.

U.S. Department of Health and Human Services. (2007). *Biosafety in microbiological and biomedical laboratories* (5th ed.) (pp. 303-341). Washington: U.S. Government Printing Office.

U.S. Environmental Protection Agency. (2005). *Compilation of available data on building decontamination alternatives*. Available at: www.epa.gov/NHSRC/pubs/600r05036.pdf. Accessed online 2008.

USP 31/NF 26. (2008). <1035> Biological indicators for sterilization. United States Pharmaceutical Corporation, 399-402.

Westphal, A. J., Price, P. B., Leighton, T. J., & Wheeler, K. E. (2003). Kinetics of size changes of individual *Bacillus thuringiensis* spores in response to changes in relative humidity. *Proceedings of the National Academy of Sciences, 100,* 3461-3466.

Young, S. B., & Setlow, P. (2003). Mechanisms of killing *Bacillus subtilis* spores by hypochlorite and chlorine dioxide. *Journal of Applied Microbiology, 95,* 4-67.

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