

Bacillus anthracis Spore Inactivation by Fumigant Decontamination

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Abstract

In 2001, envelopes containing virulent Bacillus anthracis spores were placed into the U.S. mail, resulting in contamination of mail processing and distribution facilities and office buildings. These spore-contaminated facilities were subsequently decontaminated primarily by fumigation with hydrogen peroxide, chlorine dioxide, and formaldehyde. These highly-publicized incidents resulted in increased public awareness of the threat to human health posed by Bacillus anthracis, and increased interest in sampling, detection, and decontamination of indoor surfaces, rooms, and buildings.

Fumigants offer advantages over liquid application for decontaminating rooms or buildings due to the increased coverage of large surface areas and ease of cleanup. From 2001 to the present, however, no decontamination technology has been registered by the U.S. Environmental Protection Agency (EPA) for use against B. anthracis spores; rather, decontamination has been performed through the EPA's issuance of Crisis Exemptions.

Since 2001, research and testing efforts have assessed the efficacy and maturity of sporicidal fumigants as well as the maturity, chemical toxicity, material compatibility, and ventilation requirements of these technologies. Several studies have been published in the scientific literature regarding fumigant inactivation of Bacillus spores; however, most decontamination studies with fumigants have utilized surrogates for B. anthracis surrogates, which can be more resistant to a specific type of fumigant (e.g., Geobacillus stearothermophilus and vaporous hydrogen peroxide) than virulent B. anthracis. Therefore, the purpose of this review is to summarize current knowledge available in the open scientific literature describing the inactivation of virulent B. anthracis spores by various fumigating agents with respect to key operational variables that can affect fumigant decontamination efficacy, such as fumigant concentration, contact time, operational temperature, and relative humidity.

Introduction

Fumigants (e.g., gases or vapors) for decontaminating *Bacillus anthracis* spores have been sought and utilized since the 1920s to treat animal hides and skins as a

means to mitigate the health problems experienced by some workers handling contaminated wool and hair. In these early decontamination efforts, gaseous hydrochloric acid, bromine, chlorine, iodine, sulfur dioxide, ammonia, trichloroethylene, carbon tetrachloride, and phosgene (Smyth & Pike, 1923) were utilized, and gaseous hydrochloric acid, halogenated compounds, and carbon tetrachloride were found to inactivate *B. anthracis* spores (Smyth & Pike, 1923). In 1928, hydrogen sulfide gas was shown to inactivate *B. anthracis* spores and vegetative cells (Andrjevski, 1928).

More recently, the well known resistance of bacterial spores to a wide variety of treatments (Nicholson et al., 2000) has led to the development and testing of many potential decontaminant technologies including dry and moist heat, liquids, gases/vapors, and irradiation (Whitney et al., 2003). In more recent years, gaseous decontaminants such as chlorine dioxide, hydrogen peroxide, formaldehyde, methyl bromide, ozone, nitrogen dioxide, and ethylene oxide have been used to inactivate *Bacillus* spores. Fumigants such as these are advantageous for decontaminating large rooms or buildings due to their ability to cover large surface areas; however, the toxicity of the fumigant, material compatibility, contact time, concentration required, temperature, relative humidity, and ventilation needed must be considered for each type of fumigant. Fumigants (hydrogen peroxide, chlorine dioxide, and formaldehyde) were utilized in response to the 2001 intentional *B. anthracis* spore releases to decontaminate five mail facilities and two office buildings (Canter, 2005; Canter et al., 2005).

Many studies have been published in the scientific literature regarding fumigant inactivation of *Bacillus* spores; however, most of these studies have utilized surrogates for *B. anthracis*. The use of surrogates such as *B. atrophaeus*, *B. subtilis*, *B. anthracis* Sterne, and *B. anthracis* NNR1delta1 in lieu of virulent *Bacillus anthracis* spores obviates the need for special facilities such as Biosafety Level 3 (BSL-3) containment. However, the relationship between the response of a surrogate organism and the response of virulent *B. anthracis* is most often not characterized (Whitney et al., 2003), and thus the results of these studies must be viewed with some caution. In addition to the spore species utilized in assessing fumigants, four key operational variables can impact the efficacy of a fumigant decontamination run, including the fumigant

concentration, contact time, operational temperature, and relative humidity. In addition to these key operational variables, factors such as carrier (a carrier is the matrix, object, or material to which the organism or spores have been applied), material type, fumigant neutralization procedure, spore preparation method, and inoculation density can affect decontamination efficacy.

Many studies in the scientific literature describe the inactivation of *Bacillus* spores, such as the surrogate *B. subtilis*; however, relatively few studies have been published on the inactivation of virulent *B. anthracis* spores (Whitney et al., 2003). Recently, studies have directly compared the efficacy of fumigant decontaminants against *B. anthracis* Ames and surrogates on different po-

rous and nonporous materials (Rogers et al., 2004; Rogers et al., 2005; Rogers et al., 2006; Rogers et al., 2007). Therefore, the purpose of this review is to summarize current information available in the open scientific literature regarding inactivation of virulent *B. anthracis* spores by various types of fumigating agents. A summary of various fumigant decontaminants tested against virulent *B. anthracis* spores is presented in Table 1.

Hydrogen Peroxide

Fumigation with hydrogen peroxide is one of the most widely used methods for decontamination and disinfection in the scientific, medical, and pharmaceutical

Table 1

Summary of fumigant tests performed against virulent *Bacillus anthracis* spores.

Fumigant	Testing Parameters ^a	Inoculum ^b and Carrier	Efficacy ^c	Reference
Hydrochloric Acid	6 hr; 32°C	Asbestos wicks	100% inactivation	Smyth & Pike, 1923
Bromine	120 hr; 24°C	Asbestos wicks	100% inactivation	Smyth & Pike, 1923
Phosgene	120 hr; 24°C	Asbestos wicks	100% inactivation	Smyth & Pike, 1923
Carbon Tetrachloride	7 hr at 50°C-55°C; 15 hr, 30 min at 50°C	Silk threads	100% inactivation	Smyth & Pike, 1923
Iodine	18 hr	Silk threads	100% inactivation	Smyth & Pike, 1923
Iodine + Carbon Tetrachloride	18 hr	Silk threads	100% inactivation	Smyth & Pike, 1923
Hydrogen Sulfide	16-24 hr	Agar slants	100% inactivation	Andrjevski, 1928
Hydrogen Peroxide	≥1,000 ppm; 20 min; ≥60% RH ^d	1 x 10 ⁸ CFU ^e on indoor materials	3.0 to ≥7.9 LR	Rogers et al., 2005
Chlorine Dioxide	2,000 ppm; 6 hr; ≥70% RH; 23°C-27°C	1 x 10 ⁸ CFU on indoor materials	4.3 to ≥7.8 LR	Rogers et al., 2004
	3,000 ppm; 3 hr; ≥70% RH; 22°C-24°C	1 x 10 ⁸ CFU on indoor materials	≥7.1 to ≥7.9 LR	Rogers et al., 2006
Formaldehyde	1,100 ppm; 10 hr; ≥70% RH; 22°C-23°C	1 x 10 ⁸ CFU on indoor materials	5.2 to ≥7.9 LR	Rogers et al., 2007
	1.38-1.62 mL/ft ³ ; 48 hr; RH near saturation; >26°C	Textile mill	99.998% reduction	Young et al., 1970
	0.33g/ft ³ ; 2 x 6 hr; ≥50% RH	Mail equipment	100% inactivation	Canter et al., 2005
Methyl Bromide	3.4-3.9g/L; 1-72 hr; moisture present	1 x 10 ⁵ to 5 x 10 ⁷ on filter paper strips	100% inactivation	Kolb & Schneiter, 1950
	3.4-3.9g/L; 18-72 hr; moisture present	1 x 10 ⁵ to 5 x 10 ⁷ on animal hairs and bristles	100% inactivation	Kolb et al., 1952
Hydrobromic Acid	0.269 g/L; 48 hr; moisture present	1 x 10 ⁵ to 5 x 10 ⁷ on filter paper	100% inactivation	Kolb et al., 1952
Ethylene Oxide	Boil in HCl for 30 min; 10 min-18 hr	Silk suture loops	100% inactivation	Friedl et al., 1956
Nitrogen Dioxide	0.5 g/L; 15-30 min	Gauze	100% inactivation	Poliakov et al., 1962
Ozone	1%-5%; 30-150 min	2.3 x 10 ⁷ to 3.7 x 10 ⁷ per mL in raw sewage	100% inactivation	Miller et al., 1959; Rice, 2002

^aTesting at room temperature unless specified; ^bIf available; ^cLR = Log reduction; ^dRH = Relative humidity; ^eCFU = Colony-forming units

fields. Since hydrogen peroxide is considered less toxic to humans than other types of fumigants and breaks down into water and oxygen, gaseous hydrogen peroxide has been widely used as a decontaminant for treating laboratory and medical equipment, pharmaceutical facilities, hospital rooms, and animal holding rooms (Fichet et al., 2004; French et al., 2004; Hall et al., 2007; Heckert et al., 1997; Hillman, 2004; Johnston et al., 2005; Kahnert et al., 2005; Klapes & Vesley, 1990; Krause et al., 2001). Fumigation with hydrogen peroxide is typically performed at ambient temperature and relative humidity and typically involves flash evaporation of an aqueous solution (~30%) of hydrogen peroxide to generate the gaseous (or vaporous) decontaminant. Currently, commercial technologies are available to control and monitor the environmental conditions of the decontamination area as well as the concentration of hydrogen peroxide during fumigation and catalytic breakdown following the decontamination run. An analysis of the physical properties underlying the effective use of hydrogen peroxide as a decontaminating fumigant has been presented (Watling et al., 2002).

Hydrogen peroxide used as a fumigant has shown decontamination efficacy against a wide range of microorganisms, including bacterial spores, vegetative bacteria, and viruses (French et al., 2004; Hall et al., 2007; Heckert et al., 1997; Johnston et al., 2005; Kahnert et al., 2005; Klapes & Vesley, 1990; Melley et al., 2002; Rogers et al., 2005). Although hydrogen peroxide fumigation was used as part of the building cleanup following the intentional release of *B. anthracis* spores in the mail in 2001 (Canter, 2005), most of the available data indicative of the effects of hydrogen peroxide are based on avirulent strains of *B. anthracis* or surrogates. However, a recent study evaluated the decontamination of *B. anthracis* Ames and surrogate spores on indoor surface materials using a hydrogen peroxide gas generator (Rogers et al., 2005). In this study, approximately 1×10^8 virulent *B. anthracis* Ames spores dried on seven types of indoor surface carrier materials were exposed to ≥ 1000 ppm hydrogen peroxide gas for 20 minutes; the relative humidity ranged from 60% to 80% during this contact time. These fumigations resulted in observed log reduction values for *B. anthracis* ranging from 3.0 to ≥ 7.9 that appeared to be influenced by material porosity (Rogers et al., 2005). Significant differences in decontamination efficacy were also observed between *B. anthracis* and the surrogate spores on the porous and nonporous surfaces. No damage to the test materials was observed. As a parallel qualification of the decontamination runs, these results were accompanied by complete inactivation of biological indicators containing approximately 1×10^6 *B. subtilis* and *G. stearothermophilus* spores per carrier as noted by the lack of bacterial growth in nutrient broth.

As noted by Rogers et al. (2005), this study provided decontamination efficacy data for hydrogen peroxide fu-

migation against virulent *B. anthracis* spores on complex carrier materials. However, only a single hydrogen peroxide concentration and contact time were evaluated, suggesting the need for more testing as longer contact times may lead to complete inactivation of virulent *B. anthracis* spores on complex materials. This latter result seems possible as time-dependent spore inactivation has been observed when using aqueous hydrogen peroxide and peroxide-based formulations (Klapes & Vesley, 1990; Melly et al., 2002; Young & Setlow, 2004).

Chlorine Dioxide

Chlorine dioxide (ClO_2) is a yellow-green gas possessing strong oxidizing activity, which can be produced on-site by various methods (Gordon & Rosenblatt, 2005). It is important that ClO_2 be prepared shortly before use and protected from light during fumigation. Relative humidity, concentration, material porosity, and temperature are crucial factors that impact the sporicidal activity of ClO_2 gas (Han et al., 2001; Han et al., 2003; Jeng & Woodworth, 1990). At test conditions where the test chamber was pre-humidified to 70% to 75% RH and the temperature was held at 23°C, the sporicidal activity of ClO_2 gas was enhanced compared to ambient relative humidity of 20% to 40% (Jeng & Woodworth, 1990). Moreover, ClO_2 gas fumigation against spores exhibits decontamination efficacy when applied at temperatures ranging from 5°C to 30°C (Han et al., 1999; Han et al., 2001; Jeng & Woodworth, 1990). Porosity of the carrier material also affects ClO_2 sporicidal activity in which porous materials are less likely to be decontaminated than nonporous materials when subjected to the same application conditions (Han et al., 2003).

As with hydrogen peroxide, ClO_2 fumigation was one of the approaches used in the remediation of buildings contaminated with *B. anthracis* spores in 2001; however, most of the laboratory-generated data available for ClO_2 decontamination have also been derived using *B. anthracis* surrogates. The need for remediation of *B. anthracis*-contaminated facilities in Washington, DC in 2001 prompted the U.S. EPA to issue a crisis exemption allowing ClO_2 to be utilized. Parameters used for the fumigation were a minimum temperature of 70°F (approximately 21°C), a minimum relative humidity of 65%, and ClO_2 exposure concentration x time (CT) of 9,000 ppm-hour (U.S. EPA, 2005). As with most fumigants, maintaining a relatively constant ClO_2 concentration during the defined contact time seems to be a critical factor during the decontamination run. Simply calculating the theoretical concentration of ClO_2 based on chemical equations and dilutions factors may not be the best approach for expressing ClO_2 concentration during fumigation. Actual chemical analyses to obtain continuous or semi-continuous measurements are preferable, and these data can potentially enable manual adjustments to the

ClO₂ to maintain the target concentration and ultimate CT. A previous study demonstrated that ClO₂ gas concentrations decreased by more than 80% during the first 6 hours of a 12-hour decontamination run, which was attributed to rapid ClO₂ decomposition, absorption, or reaction with the experimental materials (Han et al., 2003). This decrease in ClO₂ affected the decontamination efficacy in which a 6-log inactivation of *B. thuringiensis* spores required 20 to 25 mg/L concentrations of ClO₂ for nonporous surfaces and 30 mg/L for porous materials over a 12-hour exposure period (Han et al., 2003). On a larger scale, a study by Luftman et al. (2006) measured the ClO₂ gas concentration at multiple time intervals and locations during exposure of a 4,800 m³ space. These investigators were unable to maintain the target CT of 1050 ppm-hours, which was potentially due to ClO₂ leakage from the decontaminated space, ClO₂ absorption into materials, or ClO₂ decomposition (Luftman et al., 2006).

Two studies have employed the use of two different types of ClO₂ gas generation in evaluating the decontamination of *B. anthracis* Ames and surrogate spores inoculated on indoor surface materials using ClO₂ fumigation (Rogers et al., 2004; Rogers et al., 2006). The two ClO₂ gas-generating systems included: 1) passing 4% chlorine in nitrogen through a canister containing sodium chlorite pellets (Rogers et al., 2004); and 2) stripping ClO₂ from an aqueous solution using a liquid/air counter-current (Rogers et al., 2006). In both of these studies the operating parameters for decontamination were the same as those environmental and exposure parameters for ClO₂ outlined above. Specifically, Rogers et al. (2004) subjected *B. anthracis* Ames spores (approximately 1 x 10⁸) dried on different types of indoor surface materials to approximately 2,000 ppm ClO₂ for six hours during which the relative humidity and temperature ranged from 70% to 80% and 23°C to 27°C, respectively. The relative humidity and ClO₂ concentrations were monitored and adjusted manually during the contact time of the fumigation run. The testing promoted observed log reductions in viable *B. anthracis* spores ranging from 4.3 to ≥7.8 that varied according to the porosity of the carrier materials. Significant differences in decontamination efficacy were also observed between *B. anthracis* and the surrogate spores on the different materials. Biological indicators containing approximately 1 x 10⁶ *B. subtilis* and *G. stearothermophilus* spores per carrier were completely inactivated by the ClO₂ fumigation. The only observed damage to the test materials was a bleaching effect on the industrial carpet fibers.

Fumigation testing maintaining a target ClO₂ gas concentration of approximately 3,000 ppm for 3 hours promoted the inactivation of recoverable *B. anthracis* Ames spores from porous and nonporous indoor surface materials inoculated with approximately 1 x 10⁸ spores, corresponding to calculated log reductions ranging from ≥7.1 to ≥7.9 (Rogers et al., 2006). The inactivation of

recoverable *B. subtilis* and *G. stearothermophilus* spores from the different material types, as well as complete inactivation of biological indicators containing *B. subtilis* and *G. stearothermophilus* spores, was also observed. In all tests, the ClO₂ concentration (as measured by titration) was maintained at the target concentration for approximately three hours yielding an average CT of approximately 9,200 ppm-hour with a relative humidity range of 70% to 90% and a temperature range of 22°C to 24°C. The relative humidity and ClO₂ concentrations were monitored and adjusted manually during fumigation. Following all experimental decontamination runs, the test coupons were evaluated qualitatively for visible surface damage and no changes to any of the test materials were observed with the exception of a bleaching effect on the industrial carpet fibers. Both ClO₂ fumigation studies (Rogers et al., 2004; Rogers et al., 2006) demonstrate that semi-continuous monitoring of the ClO₂ concentration helps provide a more accurate representation of the ClO₂ gas CT exposure value during decontamination runs.

Formaldehyde

Although aqueous and gaseous formaldehyde have been used for many years in laboratory, medical, and industrial settings, formaldehyde is a known carcinogen, which may limit its use as a wide-area decontaminant. As a fumigant, formaldehyde is typically generated from the heating and depolymerization of paraformaldehyde prills (approximately 0.3 g paraformaldehyde per cubic foot of decontaminated area). The resulting gas can be neutralized with ammonium carbonate, which leads to the production and subsequent deposition of a white powder-like substance (hexamethylenetetramine) on all surfaces within the decontaminated area. The sporicidal activity of formaldehyde gas is impacted by factors such as environmental conditions, formaldehyde concentration, and test material porosity (Munro et al., 1999; Rogers et al., 2007; Spiner & Hoffman, 1971; Young et al., 1970).

With respect to sporicidal activity, many studies have been published demonstrating the efficacy of formaldehyde fumigation (Ackland et al., 1980; Braswell et al., 1970; Canter et al., 2005; Cross & Lach, 1990; Fink et al., 1988; Hoffman & Spiner, 1970; Lach, 1990; Munro et al., 1999; Rogers et al., 2007; Spiner & Hoffman, 1971; Sweet, 1971; Taylor et al., 1969; Young et al., 1970). Among these studies, only a few have evaluated the decontamination efficacy against *B. anthracis* spores. Young et al. (1970) treated a textile mill contaminated with *B. anthracis* spores by vaporizing a 37% solution of formaldehyde in a steam cleaning machine and introducing into the sealed buildings at final concentrations ranging from 1.38 to 1.62 mL per cubic foot in which the temperature was above 26°C and relative humidity near saturation. Following the two-day treatment, surface sampling results showed a significant reduction in *B. anthracis*

viable contamination. The authors note that the surface sampling used may not exclude the possibility that spores embedded in the porous matrix of concrete and wood may still be viable as formaldehyde is known to have a poor penetration capacity through porous materials (Young et al., 1970).

In 2002, a mail sorting machine and stamping device contaminated with *B. anthracis* spores in the U.S. Department of Justice mail facility in Landover, Maryland, were decontaminated using formaldehyde (from heated paraformaldehyde) fumigation (Canter et al., 2005). For fumigation the machines were enclosed in a tent and treated for two 6-hour intervals with 0.33 g heated paraformaldehyde per cubic foot of decontaminated space at a minimum of 50% relative humidity. All post-fumigation samples were negative for *B. anthracis* growth and no damage to the machines was observed.

Recently, Rogers et al. (2007) exposed *B. anthracis* Ames spores (approximately 1×10^8) dried onto both porous and nonporous indoor surface materials to formaldehyde fumigation. During the 10-hour contact time, the average formaldehyde vapor phase concentration was measured at approximately 1,100 ppm with a relative humidity range of 70% to 75% and a temperature range of 22°C to 23°C. The mean log reduction of extractable viable *B. anthracis* spores ranged from 5.2 to ≥ 7.9 for different porous and nonporous test materials in which significant differences in log reduction values were observed between *B. anthracis* and the surrogates, *B. subtilis* and *G. stearothermophilus*. Parallel evaluation of biological indicators showed a partial inactivation where only a little over 50% exhibited no growth, which may be due to lack of penetration through the biological indicator packaging (e.g., Tyvek). There was no observable damage to any of the test materials following exposure to formaldehyde gas with the exception of the white powder deposit formed following neutralization.

Methyl Bromide

Methyl bromide (MeBr) is a colorless, odorless gas that is extremely hazardous if inhaled or exposed to the eyes and skin. While used in the United States as an agricultural fumigant, MeBr is also capable of inactivating *B. anthracis* spores (Juergensmeyer et al., 2007; Kolb & Schneiter, 1950; Kolb et al., 1952). Kolb and Schneiter (1950) demonstrated complete inactivation of 1×10^5 to 5×10^7 *B. anthracis* spores dried on filter paper strips when exposed to 3.4 to 3.9 g/L MeBr at room temperature in the presence of moisture for 24 hours. Animal hair and bristles inoculated with 1×10^5 to 5×10^7 *B. anthracis* spores were completely inactivated following exposure to 3.4 to 3.9 g/L MeBr at room temperature in the presence of moisture for 18 hours (Kolb et al., 1952). Furthermore, *B. anthracis* spores (5×10^5 to 5×10^7) dried on filter paper strips were completely inactivated by expo-

sure to 0.269 g/L of another bromine-containing compound, gaseous hydrobromic acid, at room temperature in the presence of moisture for 48 hours (Kolb et al., 1952).

Ethylene Oxide, Nitrogen Dioxide, and Ozone

With respect to ethylene oxide, nitrogen dioxide (NO_2), and ozone, little information is available in the scientific literature regarding the decontamination efficacy against *B. anthracis* spores. Most studies evaluating these fumigants have utilized various *Bacillus* species other than *B. anthracis* (Rice, 2002; Whitney et al., 2003). A study conducted by Friedl et al. (1956) evaluated the sporicidal activity of ethylene oxide against *B. anthracis* spores following the AOAC sporicidal test. For this testing, silk suture loops were contaminated with spores, subjected to boiling in hydrochloric acid for 30 minutes, and then exposed to the ethylene oxide gas for up to 18 hours. The testing demonstrated that *B. anthracis* spores were completely inactivated at 60 minutes of exposure to ethylene oxide in which ethylene oxide appeared more effective in dry air compared to wet air (Friedl et al., 1956). However, some investigators provided varying results on the effect of relative humidity on the decontamination efficacy of ethylene oxide. For example, Ernst and Shull (1962) demonstrated that the inactivation of *B. subtilis* var. *niger* spores was increased by pre-humidification, followed by high relative humidity at 54°C for sterilization. Kereluk et al. (1970) observed decontamination efficacy against *B. subtilis* var. *niger* spores exposed to ethylene oxide (concentration range of 200 to 1,200 mg/L) in which no optimum relative humidity ranging from 15% to 90% was observed. However, Doyle et al. (1970) demonstrated that relative humidity does play a role in ethylene oxide decontamination depending on whether or not *B. subtilis* var. *niger* spores were maintained in hermetically sealed polymeric films. Furthermore, Oxborrow et al. (1983) claimed that at temperatures above 50°C, relative humidity ranging between 30% to 70% had little effect on ethylene oxide sterilization of *B. subtilis* var. *niger* spores.

Nitrogen dioxide (NO_2) is a red-brown gas with oxidizing properties that is formed by combining atmospheric nitrogen and oxygen or by burning synthetic ammonia in oxygen. A study by Poliakov et al. (1962) evaluated NO_2 in which virulent *B. anthracis* spores inoculated onto sterile gauze were subjected to 0.5 g per liter of NO_2 for 15 or 30 minutes contact time at room temperature and normal atmospheric pressure. Following NO_2 exposure, the inoculated gauze samples were rinsed with phosphate-buffered saline and assessed for viability by qualitative growth in culture medium and virulence assessment in mice (Poliakov et al., 1962). Exposure to NO_2 promoted complete inactivation of *B. anthracis* as indicated by lack of bacterial growth after 10 days as well as

100% survival of all mice injected with NO₂-treated spores (Poliakov et al., 1962). Based on these results by Poliakov et al. (1962), the authors suggest that it may be worth testing NO₂ as a disinfectant of buildings infected with *B. anthracis* spores; however, to our knowledge, it does not appear that such testing has been performed.

Ozone is a strong oxidizing agent that has been used to disinfect drinking water, wastewater, food products, and bio-clean rooms (Kim et al., 2003; Rice, 2002). The sporicidal activity of ozone has been studied extensively using *B. subtilis* as a surrogate for *B. anthracis* (Currier et al., 2001; Ishizaki et al., 1986; Masaoka et al., 1982; Pelleu et al., 1974; Rickloff, 1987). These studies consistently report the requirement for high relative humidity where the optimum level needed during gaseous ozone decontamination was determined to be 90% to 95%, with decreasing efficacy at less than 50% relative humidity (Ishizaki et al., 1986; Rice, 2002). Currently, little information is available for *B. anthracis* spore inactivation by ozone, especially in the gaseous form; however, *B. anthracis* inactivation was demonstrated by passing 1% to 5% ozone through an aqueous medium with a contact time of 90 minutes (Miller et al., 1959; Rice, 2002).

Material Compatibility

An important concern with respect to fumigation of a contaminated site (e.g., office building) is the potential for short- and long-term damage or corrosion to various materials. Although there are many types of fumigants that have been or are currently in use, there is the potential that some of these chemicals may not be suitable for remediating an area that may contain sensitive equipment or delicate furnishings. The fumigant having the most data reported with respect to material compatibility is hydrogen peroxide. French and coworkers (2004) fumigated portions of a hospital ward with a hydrogen peroxide vapor contaminated with methicillin-resistant *Staphylococcus aureus* (MRSA) in which no damage was observed within the hospital rooms and showers. Similarly, Rogers and coworkers (2005) reported no observable damage to indoor surface materials treated with hydrogen peroxide vapor fumigation. Hall and coworkers (2007) decontaminated a room and biological safety cabinet (BSC) in which no damage to the BSC was observed and a computer remained fully functional after a total of three decontamination cycles. Moreover, in the case of animal rooms treated with vaporous hydrogen peroxide corrosion or functional damage to heat-sensitive equipment was not observed after 10 decontamination runs (Krause et al., 2001). However, Kahnert et al. (2005) note that it is important that when using vaporous hydrogen peroxide fumigation that the chemical concentration be kept below the condensation point as hydrogen peroxide condensation may be damaging to surfaces and present residual safety risks.

When compared to hydrogen peroxide fumigation studies, the available data and information on material damage from exposure to other gaseous decontaminants are limiting. When used as a fumigant decontaminant, ClO₂ promoted bleaching of industrial carpet fibers during decontamination of *B. anthracis* spores for 9,000 to 12,000 ppm-hour (Rogers et al., 2004; Rogers et al., 2006) that was noted at the end of the three-hour decontamination run, which may not necessarily be indicative of material damage from longer-term fumigations. Moreover, Han et al. (2003) reported no damage to paper, wood, epoxy, or plastic surfaces contaminated with *B. thuringiensis* spores treated with 5 to 30 mg/L ClO₂ gas for 12 hours at 21°C and 85% to 92% relative humidity. These authors note that ClO₂ gas treatment would be suitable for decontaminating furniture or wooden structures, but rugs, cloths, and computers may be challenging (Han et al., 2003).

As noted above, the formaldehyde fumigant is typically generated from heating paraformaldehyde prills in which the resulting gas following decontamination is typically neutralized with ammonium carbonate. This neutralization produces a white powder-like substance (hexamethylenetetramine) that forms on all exposed surfaces and is irritating to the eyes and skin. Besides the potential for hexamethylenetetramine deposition, no damage to exposed laboratory rooms, mobile trailers, mail equipment, or indoor surface materials has been observed (Canter et al., 2005; Rogers et al., 2007; Taylor et al., 1969). For MeBr, Kolb et al. (1952) exposed *B. anthracis*-contaminated animal hair and bristles to 3.4 to 3.9 g/L for 18 hours in the presence of moisture, which resulted in complete kill and no observable material damage. Moreover, vaporous forms of iodine or iodine with carbon tetrachloride have demonstrated disinfectant properties against *B. anthracis* without causing any damage to animal hides (Smyth & Pike, 1923).

Decontamination Effectiveness, Residual Risk, and Future Directions

Currently, official registered tests such as the AOAC 966.04 Sporicidal Activity Test and the ASTM Quantitative Carrier Test Method E 2111-00 are specifically designed for testing liquid decontamination products against spores. However, there are no such methods for the evaluation of fumigants, although there are several potential reasons why no such official testing methods, or standardized technology operational parameters, are available. First, the amount of research that has been conducted with fumigants when compared to liquids is minimal; however, an increase in research efforts with fumigant decontamination testing has occurred since the 2001 intentional release of *B. anthracis* spores in the mail. Second, the operational parameters (fumigant concentration, relative humidity, temperature, and duration) are more

difficult to control in laboratory-scale fumigations compared to liquid decontaminant testing. Some commercially available technologies utilize equipment that has the capability of controlling relative humidity, chemical concentration, and contact time while operating at room temperature. Typical examples of these are technologies that generate some form of a vapor-phase hydrogen peroxide fumigant. However, other commercially available technologies that utilize equipment to generate formaldehyde, ClO_2 , or MeBr gas are not equipped to control operational parameters, placing a higher demand on the end user. When conducting laboratory studies with gases such as these, researchers often present results based on initial theoretical (or calculated) determinations of parameters such as chemical concentration, relative humidity, and temperature and not actual measured results. Measuring these parameters throughout the experiment can be a difficult task, especially when some of these fumigants require many hours of contact time. Nevertheless, some studies do report monitoring some or all of these operational parameters, and in certain cases the study results have elaborated on the difference between the “expected” versus measured results (Han et al., 2003; Luftman et al., 2006; Rogers et al., 2004; Rogers et al., 2006; Rogers et al., 2007).

A third potential reason for lack of operational parameter standardization is the physical size of the equipment needed for testing fumigants. Decontaminant technologies and decontamination chambers are large in size, which may prevent the ability to perform larger scale fumigant decontamination testing in some laboratories. These technologies and decontamination chambers typically will not fit inside a biological safety cabinet and require more extensive engineering controls to handle many of the toxic gases as compared to that required for liquids testing. Some gases, such as MeBr, may not have a commercially available technology that would present additional safety hazards due to the lack of a standard delivery mechanism for these colorless and odorless gases, thereby requiring extensive environmental monitoring to ensure the safety of laboratory personnel.

Test system standardization for fumigant testing is an additional aspect that should also be addressed. For example, operational parameters that might be modified, optimized, and standardized to some degree, such as spore preparation and inoculation, carrier material type, environmental conditions, and fumigant concentration, have not been fully evaluated. Spore production conditions and the mechanism of spore deposition onto surfaces could potentially influence spore inactivation. Spore production methods can vary between laboratories and these differences can influence spore resistance to decontamination (Cazemier et al., 2001; Melly et al., 2002; Palop et al., 1999). Moreover, the addition of bio-burden to a spore suspension prior to applying to a test coupon is considered by some to be necessary for challenging the effectiveness of decontaminants. For example, it has been

noted that the presence or absence of debris in spore preparations can also influence spore inactivation by formaldehyde gas (Spiner & Hoffman, 1971). In contrast, the absence of biological debris in the spore preparation does not appear to significantly affect gaseous ozone inactivation of clumped spores (Currier et al., 2001). Another factor that could potentially influence fumigant decontamination efficacy is the method of spore carrier inoculation. Most studies utilize an approach where spores in liquid suspension are applied to a carrier material either by spot inoculation or submersion of the carrier in the spore suspension. These inoculated carriers are then allowed to dry for a period of time prior to decontamination. However, in the 2001 release of *B. anthracis* spores in the mail, the spores were delivered as a dry powder and the subsequent “binding” or other interactions of the dry powder with various materials differs from the case where spores are delivered to a carrier by applying an aqueous suspension. The efficacy of the decontamination technology may be markedly influenced by how the spores are applied to the material. Similarly, inoculation density per carrier may lead to spore clumping, stratifying, or layering that may affect the ability of a fumigant to interact with the spores.

The test material type and porosity have also been shown to influence the decontamination efficacy of a fumigant (Han et al., 2003; Rogers et al., 2005; Spiner & Hoffman, 1971; Young et al., 1970). For nonporous materials, inoculated spores will predominantly reside on the material surface, while porous materials will enable spores to penetrate and stratify within the test material. Such penetration and embedding of spores into the test materials could interfere with fumigant-spore contact, thereby potentially decreasing decontamination efficacy. For fumigant decontamination studies, exclusive use of nonporous test materials, such as glass or steel, may limit the ability to fully evaluate or identify potential limitations of new or existing fumigant technologies. Therefore, the use of both porous and nonporous test materials could provide both best- and worst-case scenarios for challenging fumigants in decontaminating against *B. anthracis* spores.

Following decontamination and remediation of a building, an obvious concern is the safety of all personnel re-entering the decontaminated site. However, this concern could be mitigated by demonstrating that the residual levels of biological agent are within established “how clean is safe?” guidelines. Nevertheless, the perception of those individuals who would be entering the post-remediation site is also important. The current assessment for effective remediation is no growth of *B. anthracis* spores from post-decontamination air and surface sampling (Canter, 2005). However, due to the lack of official, verified surface sampling methods, accepted limits of detection required for assessment of “how clean is safe?” and standardized fumigant application, the criterion of a “no growth” level may not be fully-discernable based on the current state of decontamination assessments.

Therefore, risk assessment-based acceptable levels of decontamination (e.g., 6-logs or higher) prior to building re-occupancy or use may ultimately need to be established. However, the testing process and organisms used in attaining such a level of acceptance needs to be considered as well. That is, are biological indicators that contain surrogate spores suitable for demonstrating decontamination efficacy, or should the organism of interest be tested on defined materials or matrices? For example, treatment of a methicillin-resistant *Staphylococcus aureus* (MRSA)-contaminated hospital environment with hydrogen peroxide fumigation promoted complete kill of *G. stearothermophilus* biological indicators, while 1.2% of the sample sites exhibited positive MRSA growth following fumigation (French et al., 2004). Similarly, hydrogen peroxide fumigation promoted 100% inactivation of biological indicators, while complete kill was not observed for *B. anthracis*, *B. subtilis*, or *G. stearothermophilus* on various porous and nonporous carrier materials (Rogers et al., 2005). In contrast, formaldehyde gas exposure promoted only about 50% inactivation of biological indicators; however, greater than a 6-log inactivation was observed for *B. anthracis*, *B. subtilis*, or *G. stearothermophilus* on multiple porous and nonporous materials (Rogers et al., 2007). Similar results have been reported where biological indicators decontaminated with formaldehyde gas did not parallel the inactivation of *Bacillus* spores dried on stainless steel carriers (Munro et al., 1999). Ultimately, decisions will need to be made as to whether acceptable levels of decontamination should be based on the use of biological indicators or be based on the log kill obtained for many types of carrier materials. As indicated previously in this review, additional laboratory and field studies are needed to obtain data that will form the basis for addressing future decontamination scenarios that involve fumigation or other decontamination technologies.

An important ideological argument that needs to be resolved in the future for decontamination testing and implementation to keep moving forward is whether or not data should be generated against *B. anthracis* spores, or will data generated using suitable surrogates be sufficient. The lack of standardized testing makes it difficult to make direct comparisons between studies using *B. anthracis* and those using only surrogates due to differences in test conditions, carrier materials, quantitative versus qualitative data, inoculum load, etc. Due to the lack of an "official" or "standard" surrogate for *B. anthracis*, individuals within regulatory and scientific communities share the view that the definitive efficacy of decontamination against *B. anthracis* using a fumigant or liquid should be determined by data using *B. anthracis*. This view may change in the future if an "official" surrogate is identified and subsequent testing data are accepted for use in making informed risk-based and biosafety decisions. In any case, there needs to be a formal decision process for determining which type(s) of fumigant decontamination

technologies are suitable for remediating *B. anthracis*-contaminated areas in the event of another public health emergency as that which occurred in 2001.

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The Microbiological Validation of a New Containment Level 4 Cabinet Line

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Abstract

The aim of this study was to quantify microbiologically the operator protection factor (OPF) and measure the extent of cross-contamination provided by a new Advisory Committee on Dangerous Pathogens (ACDP) containment level 4 cabinet line system (glove box line). To accomplish this goal, the cabinet line was filled with microbial tracer aerosol, and microbial air samplers were used to detect any release of microbial tracer, both inside and outside the cabinet line when procedures were carried out. These procedures mimicked normal working conditions and realistic accident scenarios. The operator protection factors (OPFs) and internal cross-contamination ratios were calculated.

The cabinet line gave OPFs of between $>10^6$ and $>10^7$ in all tests. No cross-contamination was detected when internal doors remained closed between the individual cabinets and the spine. However, low levels of

microbial tracer were detected moving from the contaminated cabinet to the spine but not to other individual cabinets when cabinet doors were opened.

These experiments demonstrated that the cabinet line provides a high level of protection for the operator and that multi-agents can be manipulated simultaneously within the cabinet line without cross-contamination. In addition, data have been produced, within a large primary containment system, from which informed risk assessments and procedures can be written. Such data provide a basis with which to compare primary containment with alternative ways of working with dangerous pathogens, such as positive pressure-suited systems.

Keywords

Containment, level 4, biosafety, microbiological safety cabinet, protection