Method for Studying Survival of Airborne Viable Microorganisms in Combustion Environments: Development and Evaluation

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ABSTRACT

Inactivation of airborne microorganisms due to thermal or chemical air treatment has gained considerable attention. Destruction of aerosolized biothreat agents in environments containing combustion products is particularly relevant to military and counterterrorism situations because some pathogens may survive an explosion or fire in a bio-weapon facility and be dispersed in the atmosphere. Energetic materials with biocidal properties are being sought to effectively inactivate stress-resistant aerosolized microorganisms. Consequently, appropriate methods are needed to test these materials. We designed and built a state-of-the-art experimental facility and developed protocols for assessing the survival of aerosolized microorganisms exposed to combustion. The facility uses a continuous-flow design and includes an aerosolization unit, a test (combustion) chamber, and a measurement system for bioaerosol particles exposed to combustion environments for sub-second time intervals. The experimental method was tested with *Bacillus* endospores. We assessed the inactivation of aerosolized spores exposed to a gaseous hydrocarbon flame and to combustion of aluminum-based energetic composites (including a novel iodine-containing filled nanocomposite material). Two combustion configurations were evaluated – a vertical strand containing a consolidated material and a specially designed burner in which a fuel powder is fed into a gaseous hydrocarbon flame. It was established that the bioaerosol inactivation may be overestimated due to exposure of spores on collection filters to the combustion products throughout the test. The overestimation can be mitigated by reducing the collection time and minimizing the formation of soot. The experimental facility and method developed in this study enables evaluating effects caused by biocidal products during combustion. The present version of the set-up provides the capability of detecting inactivation levels of $\sim 2.2 \times 10^5$ (over five-log viability reduction) its further design modifications can potentially enable measuring bioaerosol inactivation as high as $\sim 10^7$. The method was utilized for establishing feasibility of the new iodine-containing material for microbial agent defeat applications.

Keyword: Bioaerosols; Combustion aerosols; Hazardous air pollutants.

INTRODUCTION

Technologies based on thermal and chemical air treatment have demonstrated success in inactivating airborne microorganisms (Lee and Lee, 2006; Ma *et al.*, 2008; Wood *et al.*, 2008; Jung *et al.*, 2009a, b). The development of methods for effective destruction of aerosolized biological agents remains an integral part of defense research programs in the US (Henderson, 2004;

Hitchcock *et al.*, 2006; Koch, 2006) and abroad (Tan *et al.*, 2006; Nadasi *et al.*, 2007). Combustion product environments are of particular relevance to military and counterterrorism situations. If bio-agents released as a result of explosion or fire in a bio-weapon facility survive the stress and remain pathogenic, they may be subjected to short- or long-range atmospheric transport and contaminate large areas, which would pose a major threat to the public and infrastructure (Nelson, 2004).

An effective agent defeat weapon to counter biological agents should prevent the undesirable effect of dispersing live agents. Energetic materials are being developed with the added capability to effectively inactivate stress-resistant microorganisms when, e.g., a stockpile of stored bio-weapon agents is targeted. For instance, filled nanocomposite

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materials (FNMs) with specific combinations of halogen compounds are being presently explored because of their potential to defeat highly pathogenic airborne bacteria and viruses (Zhang *et al.*, 2010). Most of such materials are manufactured as powders to be added to energetic formulations and aerosolized upon blast (Johnson *et al.*, 2008; Obrey *et al.*, 2008). Inactivation of *aerosolized* agents, especially those dispersed in a military situation, should occur during the short time of their exposure to biocidal materials (e.g., during explosion and post-explosion combustion). Therefore, biocidal effects of combustion products are to be intimately coupled with thermal and pressure effects produced by the blast. Understanding such coupled effects represents a major challenge for the development of new energetic materials with biocidal capabilities. Equally important and challenging is the development of an adequate experimental method for testing the effectiveness of different energetic materials with biocidal reaction products.

The key component of an adequate assessment of bioaerosol inactivation is the ability to identify and enumerate microorganisms in air. Various knowledge gaps and non-standardized protocols used by researchers in diverse disciplines have triggered the need for a standard and scientifically robust approach to measuring aerosolized bio-agents which are being inactivated. There is a lack of peer-reviewed, publically accessible reports on the investigation of destruction of biological agents in aerosol phase. Available experimental methods are often too simplistic and/or do not allow quantifying inactivation with appropriate detection limit. Additionally, to our knowledge, no method has yet been developed that would discriminate between different effects causing inactivation, such as heat and combustion products. Considering that the targeted inactivation level for bio-agents is usually very high, the study design must provide sufficiently high bioaerosol concentration in control tests and sufficiently low detection limit of microorganism enumeration from the treated/ exposed samples. This enables testing formulations producing combustion products that cause substantial viability losses (e.g., 99.99% or greater). As newly-developed materials are often expensive to manufacture and available in limited amounts, a preferred evaluation method should be capable of generating credible inactivation data from combustion of small amounts of material. Finally, conventional experimental protocols for assessing microbial inactivation often measure the viability decrease over relatively long exposure time periods, which has a limited relevance to many agent defeat applications (particularly, for aerosolized agents) because the latter are often characterized by exposure time from milliseconds to about 1 s.

In this study, we designed and built a state-of-the-art experimental facility and developed a method for investigating the inactivation of airborne viable microorganisms exposed to combustion of various materials over a short period of time, ~0.01–l s. The test protocol is capable of quantifying viability losses of about 99.998% (with the upper detection limit as high as 99.9995%) and has a potential to target even higher levels. The newly-developed facility and method were evaluated and used for determining the inactivation of aerosolized *Bacillus* endospores exposed to combustion of energetic powders, including a novel metastable aluminum-iodine composite.

METHOD DESCRIPTION

Quantitative Characterization of Inactivation

The efficacy of a method aiming at controlling viable microorganisms can be determined by comparing the concentrations of these microorganisms with and without their exposure to a specific treatment ("control-to-test" comparison). The bioaerosol viability is usually represented by culturable count from an air sample. Thus, the loss in culturability serves as a surrogate for the viability loss, which can be quantified by the inactivation factor:

$$IF = \frac{C_{culturable}(CONTROL)}{C_{culturable}(TEST)} \tag{1}$$

where $C_{culturable}(CONTROL)$ and $C_{culturable}(TEST)$ are the culture-based concentrations of microorganisms measured without exposure and with exposure, respectively. If the challenge microorganisms are, e.g., bacterial spores, the values are expressed in colony forming units (CFU) per m^3 of air. In microbiology, microbial inactivation is often quantified in terms of log reduction, e.g., $IF = 1000$ would be referred to as a 3-log reduction. The viability loss (*VL*) — an alternative measure of inactivation used by some investigators (Green *et al.*, 1968; Lee and Lee, 2006; Jung *et al.*, 2009a, b) — can be expressed through the inactivation factor *IF* as

$$VL = \left(1 - \frac{1}{IF}\right) \times 100\% \tag{2}$$

Generally, Eq. (1) assumes that all the microorganisms treated by the method under evaluation were exposed to the same stress during the same time interval.

Experimental Facility

Fig. 1 presents the experimental setup developed for studying the inactivation of aerosolized microorganisms. The challenge bioaerosol – a specific bio-agent or its simulant – is generated from a surfactant-free, de-ionized water suspension using a six-jet Collison nebulizer (BGI Inc., Waltham, MA) at its nominal operational flow rate, $Q_{neb} = 6$ L/min. Strong shear forces inside the nebulizer created by the high-velocity air jet enhance spore de-agglomeration. The bioaerosol generation protocol has previously been validated and used in several studies (Mainelis *et al.*, 1999; Lin *et al.*, 2000; Grinshpun *et al.*, 2005, 2007). Alternatively, several other methods can be utilized for generating biological particles (Reponen *et al.*, 1997; Ulevicius *et al.*, 1997; Eninger *et al.*, 2009; Jung *et al.*, 2009c). The bioaerosol is diluted with HEPA-filtered dry air at different flow rates, which enables testing different flow regimes and different exposure times.
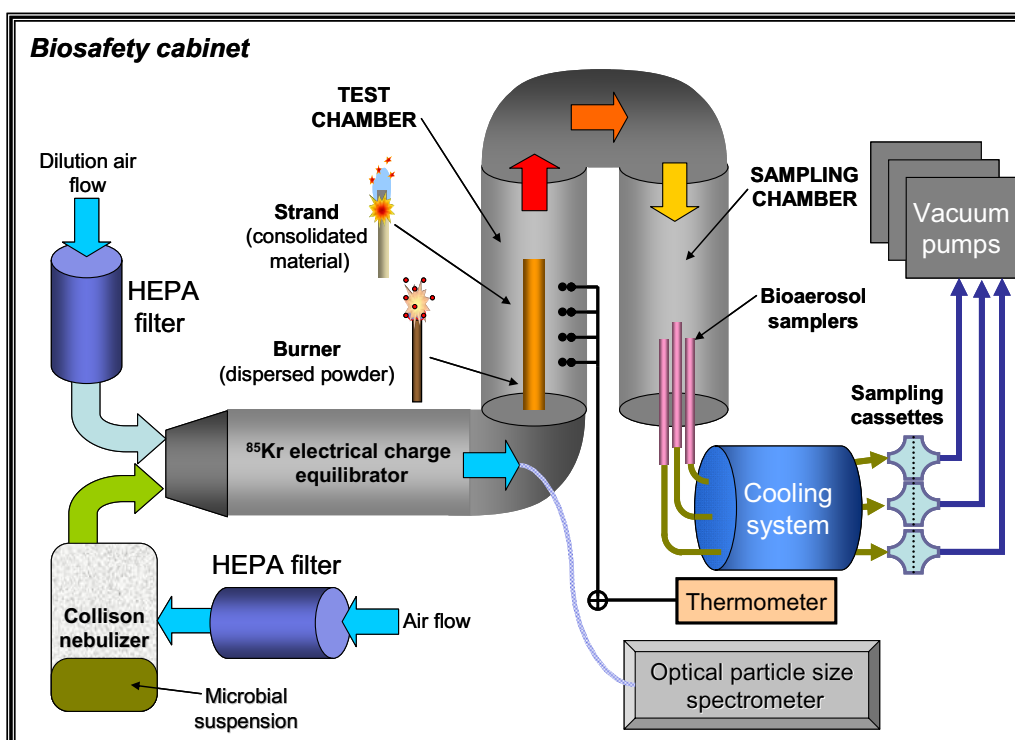
Fig. 1. Experimental facility for the evaluation of the bioaerosol inactivation due to exposure to combustion.

After passing through a 10-mCi ^{85}Kr charge equilibrator (model 3012, TSI Inc., St. Paul, MN), the bioaerosol enters the test (combustion) chamber – a vertical cylindrical channel of 50 mm inner diameter and 320 mm height. Combustion products are generated by two alternate configurations: (1) by a burning solid fuel strand or (2) from an aerosol burner located in the center of the combustion chamber.

The solid fuel strand, or "candle," was first used to test consolidated energetic materials. Materials studied in this configuration included nanocomposite powders of metallic fuel (Al) and an oxidizer (MoO$_3$) prepared by arrested reactive milling (ARM) (Dreizin and Schoenitz, 2009). Iodine was added to selected mixed composites to produce biocidal combustion products. The strands were ignited at the top and flames propagated downward. Due to very rapid reaction rates of nanocomposite materials, a strand manufactured exclusively from the nanocomposite would be consumed over a very short period, which was found impractical. Therefore, the nanocomposite powders were mixed with a slower burning organic binder, paraffin wax. The strand itself consisted of –8 + 20 mesh (approximately 0.8–2.4 mm) granules of the nanocomposite material with binder, which was contained in a wire mesh cage. To prepare the granules, the nanocomposite powder was blended with paraffin wax, consolidated, crushed, and subsequently size classified. Characteristic dimensions of the cylindrical strand were 12.5 mm (1/2") in diameter and 250 mm in height and characteristic burn times varied from 1.5 to 2.5 min. Due to variability in the packing density of the tested materials, the total mass subjected to combustion in an individual test (an energetic compound

plus wax) ranged from 20 to 28 g; however, in most cases the strand mass was rather consistent: 25 g with variability of ~2%. This strand-based design allows for testing at relatively high combustion rates. The latter is important for exploratory studies when the potential of a tested formulation to produce appreciable inactivation over short time is unknown. An electro-chemical igniter with a 300 mg load of boron-titanium ARM nanocomposite (2B·Ti) was used to initiate combustion. The burn time of the electro-chemical igniter was ~0.01 s. To remove combustion products of the electrochemical igniter from the setup, the bioaerosol sampling was started five seconds after initiating the combustion.

While sufficient for initial inactivation assessment, the strand burner is associated with some operational shortcomings. For instance, the use of an organic binder causes substantial formation of soot, exhibiting an undesirable effect on the bioaerosol particles passing by the flame as well as on those collected on the filter, as discussed in detail below. As an alternative to the strand burner, an aerosol burner was developed in which the energetic materials under investigation are fed as additives into a gaseous hydrocarbon flame (Fig. 2). In this design, the gaseous hydrocarbon flame largely determines the flame temperature and structure. While the powder additives generally change the flame characteristics, their influence is limited, which provides reproducible experimental conditions for different energetic materials tested. Although methane flame was utilized in the present experiments, a different hydrocarbon fuel (e.g., butane, acetylene, etc.) can be readily used to adjust the flame temperature and structure. Operation of the gaseous flame
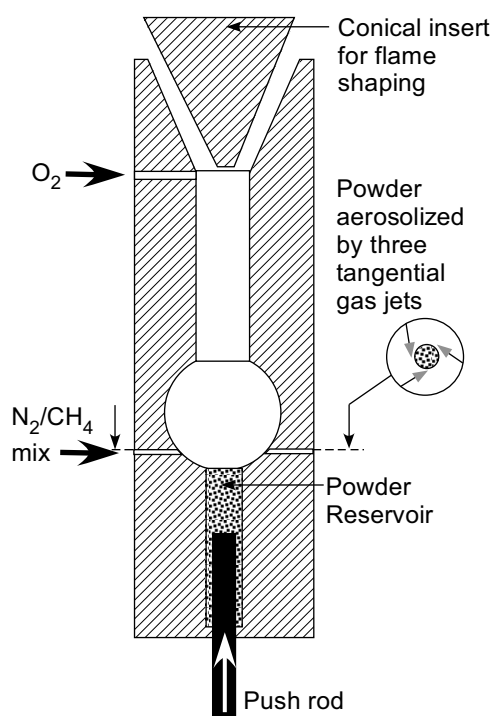
Fig. 2. Schematic diagram of the aerosol burner for generating a hydrocarbon flame seeded with energetic particles. Only the top part of the burner protrudes into the test chamber through which the bioaerosol is flown.

also enables one to readily control the soot formation with a small auxiliary oxygen flow.

The tested powder is loaded into a cylindrical channel in the center of the burner and fed into a built-in aerosolization chamber at a nominally constant rate using a cylindrical plunger, which is pushed at a controllable speed by a syringe pump. Upon entering the aerosolization chamber, the powder is lifted by three tangential jets of a methane/nitrogen mixture. Further downstream, oxygen is introduced to generate a flammable mixture. Inserted into the test chamber, the burner is capable of generating a flame with dimensions similar to that obtained with the solid fuel strand.

At a total air flow rate through the test chamber ranging from 18 to 36 L/min and the flame vertical dimensions ranging from ~30 to 100 mm (depending on operational conditions), the residence time of a bioaerosol particle in the combustion zone is ~0.1–l s. Increased air flow rates (not validated in this study) could allow testing shorter-term exposures (~0.01 s). Overall, the combustion chamber is capable of accommodating different energetic formulations and conducting tests with both consolidated (the strand-based design) and dispersed (the burner) powders at sub-second exposure time intervals.

After passing the test chamber and a U-shaped transportation line (50 mm diameter and 500 mm length), the aerosol is sampled into three identical cylindrical probes and transported through a 300-mm long cooling system, which reduces the air flow temperature to the initial (room temperature) level. The probes are oriented isoaxially to the incoming air flow but may not necessarily sample isokinetically. Considering the ranges of operational

parameters of the newly-developed experimental facility, non-isokinetic aspiration does not introduce measurable bias. The particles of interest (e.g., bacterial endospores) are small enough to follow the air streamlines, which provides an aspiration efficiency of 100%. The wall losses inside the sampling system are below 5% based on the estimate involving impaction, gravitation, and diffusion. The particles from each probe are collected for a subsequent culture-based analysis (AIHA, 2005). The present version of the experimental facility utilizes sterile 25-mm filter cassettes (SKC Inc., Eighty Four, PA) equipped with a 25-mm diameter autoclaved polycarbonate filters (Millipore Corp., Billerica, MA) (pore size = 0.4 μm), which provide effective collection of bacterial spores, which are usually close to 1–3 μm in diameter. The sampling flow rate of 5 L/min (for each of the three sampling lines) was found to be high enough to achieve sufficient microorganism counts on the collection filters and, at the same time, sufficiently low to minimize the desiccation-induced viability loss on filters during collection.

The temperatures on the wall of the test chamber are measured using surface thermocouple probes (Model 5TC-GG-20, Omega Engineering, Inc., Stamford, CT) with a digital thermometer (Model HH12A, Omega Engineering, Inc.). The air temperature profiles inside the test chamber are determined with non-sheathed thermocouple probes (Type J, Model 5J36-ICIN-116, Omega Engineering). The air temperature monitoring assembly allows for a cross-sectional spatial resolution as low as 1 mm. The relative humidity is measured with a thermohygrometer pen (Fisher Scientific, Pittsburgh, PA).

To monitor the ignition and combustion, a thermo-stable glass window is installed at the top of the test chamber.

The test chamber as well as the other sections of the experimental setup was made of stainless steel. The setup was assembled and operated inside a class II biosafety cabinet (Model 6TX, Baker Co., Inc., Sanford, ME).

The above experimental design does not aim at exposing bioaerosol particles exclusively to the flame zone. Given that the adiabatic flame temperature for hydrocarbon fuels is about 1900–2200°C and may be somewhat higher when metal-based energetic composites are deployed in a practical configuration, it is likely that even stress-resistant bacterial spores and viruses are completely inactivated (or disintegrated) by heat if passing directly through the flame zone (Grinshpun *et al.*, 2010a, b). The effect on bioaerosol passing in the proximity of the flame is less clear-cut. In addition, when testing a composite containing biocidal additives that are released during combustion, it is important to be able to compare the combustion-induced inactivation level of this composite to the one offered by its non-biocidal analog. From this perspective, an appreciable fraction of bioaerosol particles in the combustion test should be allowed to pass through an area between the flame zone and the wall of the combustion chamber so that the bio-particles are exposed to the tested products but not combusted themselves. These considerations were taken into account when choosing the dimensions of the experimental setup and the operational conditions.

MICROBIOLOGICAL PROCEDURES USED IN THE METHOD EVALUATION

The experimental method was evaluated with *B. atrophaeus* endospores. This microorganism is also known as *B. subtilis* var. *niger* and *B. globigii* (BG) and has been widely used as simulants of biological warfare agents (Johnson *et al.*, 1994; Franz *et al.*, 1997; Hill *et al.*, 1999; Laflamme *et al.*, 2004). The stock utilized in this study was obtained from the US Army Edgewood Laboratories (Aberdeen Proving Ground, MD). The suspension was prepared for aerosolization with freeze dried *B. atrophaeus* spores in sterile deionized water. The suspension was washed two times by vortexing and centrifuged at 7,000 rpm for 7 min at room temperature. The total bacterial concentration in suspension was adjusted to 10^7–10^9 per mL using a hemacytometer (Bright-Line Hemacytometer, Hausser Scientific, Horsham, PA). The culturable concentration [enumerated after incubation on tryptic-soy agar (TSA) at 30°C for 18 hours] was 10^7–10^9 CFU/mL. Immediately after aerosolized spores were collected, they were extracted from the collection filters with 10 mL of sterile filtered de-ionized water. Aliquots of filter extract ranging from 200 μL to 5 mL were used for cultivation. As control samples were very concentrated, the smaller aliquot of 200 μL was used with appropriate dilutions (up to 10^4). In the test samples (collected from combustion air environments), the spore recovery was low, which necessitated cultivating larger liquid volumes with no dilution. In some cases (corresponding to high inactivation), a 1-, 2-, and 5-mL aliquots were filtered through sterile filters (mixed cellulose esterase with diameter of 37 mm and pore size of 0.4 μm), which were placed face up on the agar plate. After incubation, the CFUs were counted to obtain the airborne concentration of culturable spores (CFU/m³).

For each experiment, three collection filters operating in parallel in the test trial and three filters from the control trial were analyzed. Arithmetic average values were calculated for $C_{culturable}$ (*CONTROL*) and $C_{culturable}$(*TEST*), and *IF*-value was determined as their ratio using Eq. (1). Each experiment was repeated at least three times, and the geometric mean of *IF*-value as well as the geometric standard deviation was calculated.

An average CFU value was determined from at least three replicate counts. The lowest countable number was 1 CFU per plate while some plates showed zero count. When averaging from a set of plates containing zero and non-zero counts, ½ CFU was designated for the former. The limit of detection (LOD) was calculated using 1 CFU per 5 mL aliquot sample, which is the largest aliquot transferred in the study on a single agar plate. This results in the LOD of 2 CFU per filter sample (1 CFU/5 mL = 2 CFU/10 mL of total extraction volume). Similar to our recent study (Grinshpun *et al.*, 2010a), we adopted the Practical Quantification Limit (PQL) = 5 × LOD = 10 CFU/sample as the lowest level that can be reliably achieved within specific precision and accuracy limitations provided by routine laboratory operating conditions (McBean *et al.*, 1998; Wayman *et al.*, 1999; Benjamin and Belluck, 2001).

The upper limit of the detectable IF was determined as the ratio of Cculturable (CONTROL) to the counting LOD and referred to as the Highest Detection Limit (HDL). Similarly, the upper limit of the reportable IF was derived from PQL = 5 × LOD and referred to as the highest practical quantification limit (HPQL).

The maximum viable spore count on the control filter can be reached by aerosolizing from a single Collison nebulizer charged with suspension of the highest achievable concentration of 10^8 per mL (which is still below the aggregate-producing level) and mixing it with dry air at the lowest dilution ratio of 1:2 (which is sufficient to provide adequate water evaporation). Based on this maximum and the above-listed LOD and PQL, the following upper limits of the detectable *IF* were calculated: HDL of ~2.2 × 10^5 (~99.9995% viability loss) and HPQL of ~4.4 × 10^4 (~99.998% viability loss). It is feasible to achieve ~10^1- to 10^2-fold higher bioaerosol concentrations by using alternative bioaerosol generation and air mixing systems. It is also possible to increase HDL and consequently HPQL, at least within an order of magnitude, by adjusting the sampling conditions. Therefore, the HPQL can be potentially further increased allowing for quantifying *IF*-values as high as ~10^7.

EVALUATION RESULTS

In the course of evaluating the newly-developed method and experimental facilities, the following topics were covered:

(i) Characterization of the challenge aerosol and particle losses in the system;

(ii) Effectiveness of the facility decontamination conducted between the tests;

(iii) Inactivation of spores exposed to combustion of strands filled with conventional and potentially biocidal energetic materials (pilot data on comparison);

(iv) Inactivation of spores collected on filters exposed to combustion products during the test;

(v) Role of metal powder dispersed into hydrocarbon flame in inactivating spores (pilot data).

Characterization of the Challenge Aerosol and Particle Losses in the System

First, it was verified whether the particles aerosolized by the nebulizer and dried by the HEPA-filtered air flow represented single *B. atrophaeus* spores. The aerosol particle size distribution was measured with an optical particle size spectrometer (Dust Monitor 1.108, Grimm Technologies, Inc., Douglasville, GA) approximately 640 mm downstream of the nebulizer, at the entrance to the test chamber. The real-time aerosol measurements were conducted at four dilution flow rates, $Q_{dilution}$, of 12, 18, 24, and 30 L/min. Thus, the total flow rate through the system, $Q = Q_{neb} + Q_{dilution}$, was 18, 24, 30, and 36 L/min, respectively. Fig. 3A shows the particle size distributions measured over a 1-min time period initiated 5 min after the nebulization began. The peaks of these distributions occurred at approximately the same particle size, $D_p = 0.71$ μm, representative of intact endospores [existing literature refers to the aerodynamic diameters of the tested *Bacillus* spores ranging from 0.7 to 0.9 μm (Li *et al.*, 1999; Aizenberg *et al.*, 2000) and microscopic optical diameter of ~1 μm for non-germinated endospores (Daniels *et al.*, 2006)]. These data suggest that the tested dry dilution flows were sufficient to shrink the nebulizer-generated particles to the spore size as water content evaporated between the aerosolization point (nebulizer) and the chamber's entry point. The particle number concentration decreased with increasing dilution ratio. Thus, it was concluded that the aerosol entering the test chamber was representative of the intended bioaerosol with respect to its particle size and concentration characteristics.

Second, by tracking the peak aerosol concentration in 6-second increments, 50% variability was observed during the first 30 s of nebulization. While decreasing with time, the variability remained relatively high (approximately 15%) for the next 2–3 min. It was determined that the concentration stabilized approximately by the 4th minute and remained essentially constant (variability < 4%) during at least 30–45 min of continuous aerosol generation. The data suggest that the nebulizer should be turned on at least 5 min prior to the test, and the single test time should generally not exceed 30 min.

Third, the dimensions of the elements of the experimental setup and the air flow conditions were selected to assure that the particle losses inside the system are minimal. To test whether this was actually achieved, the particle concentrations and size distributions measured at the entrance of the test chamber were compared to those measured at its exit and in the sampling chamber. The latter are shown in Figs. 3B and 3C, respectively. The measurements were conducted with the combustion strand installed in the test chamber but not ignited (having approximately the same dimensions as the strand, the burner represents the same obstacle in the air flow causing about the same particle deposition). We found that the particle losses inside the test chamber (including the deposition on the strand surfaces) did not exceed 3.9% by number; the particle transport to the sampling point caused no more than 5.2% losses. This low internal deposition is considered acceptable for the purpose of the present study.
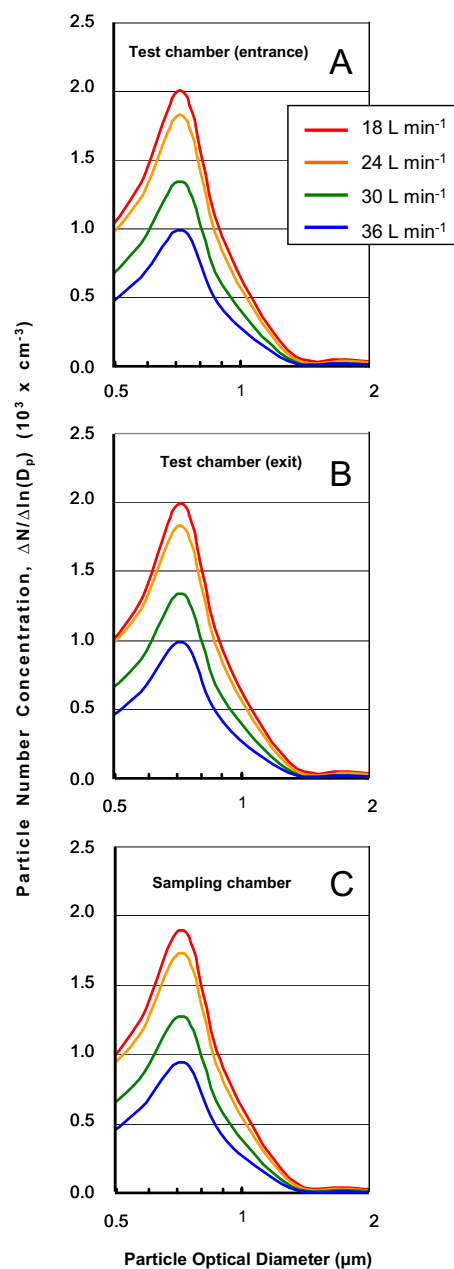

Fig. 3. The particle size distributions of aerosolized *B. atrophaeus* endospores measured at three different points of the experimental system and at four different air flow rates.

Effectiveness of the Facility Decontamination Conducted between the Tests

To ensure that the results of a subsequent experiment were not affected by the residual contamination of the system from a previous experiment, the system was cleaned between consecutive trials by blowing HEPA-filtered air at 85 L/min for 20 min. In addition, the internal surfaces were sprayed and wiped with 70% ethanol. The effectiveness of these procedures was assessed through a post-cleaning air sampling conducted during a 10-min period at 5 L/min. From a total of nine collection filters analyzed, only two developed any colonies (with 2 and 1 CFUs in 5-mL samples). The average count from these nine filters translates into the culturable concentrations lower than the one derived from the LOD = 2 CFU per filter established in the experimental protocol of this study. Thus, it was concluded that the implemented decontamination procedures were appropriate.

Inactivation of Spores Exposed to Combustion of Solid Fuel Strands Filled with Conventional and Potentially Biocidal Energetic Materials (Pilot Data on Comparison)

The newly-developed experimental facility and method were deployed to generate pilot data on the inactivation of *B. atrophaeus* endospores passing the test chamber, in which a strand filled with a consolidated energetic material ($12Al·MoO_3$ nanocomposite powder with about 20 wt-% paraffin wax) was burned. The $12Al·MoO_3$ nanocomposite powder was prepared in a shaker mill according to a procedure described elsewhere (Umbrajkar *et al.*, 2008). The iodine was incorporated by adding 5 wt-% at the preparation phase. The tests were conducted at a total flow rate (nebulizer flow plus dilution flow) of 36 L/min. Following the first 5 s, during which the flame was stabilizing, the sampling was initiated. The combustion continued for approximately 2 min with a mass burn rate ranging approximately from 200 to 300 mg/s. The sampling was stopped when the entire strand had burned out or when 2 min sampling time limit was reached (whatever occurred first).

Noticeable differences were observed in the structure and consistency of the flames generated by burning different solid fuels; furthermore, using organic binders resulted in substantial generation of soot. The collection filters were covered with soot, which caused additional concern regarding the continuing soot effect on the collected bioaerosol particles.

The spore exposure to the burning strand containing $12Al·MoO_3$ nanocomposite with no iodine produced relatively low *IF*-values: geometric mean (GM) = 52.3, geometric standard deviation (GSD) = 1.51, number of replicates n = 5. The solid-strand combustion of the same nanocomposite with embedded iodine produced much higher *IF*-values: GM = 9,849, GSD = 4.9, n = 6. It is acknowledged that a part of the above difference occurred due to the temperature effect because combustion of the iodinated formulation created a higher temperature in the test chamber. As a reference, the average wall temperature measured at the end of the 2-min experiment was ~150ºC

(varied from 109ºC to 160ºC) while testing $12Al·MoO_3$ without iodine, and ~240ºC (varied from 200ºC to 253ºC) while testing the iodinated nanocomposite. In the latter case, the air temperature profiles measured in the test chamber identified the space in the vicinity of the wall (~1–2 mm wide at Q = 36 L/min) with temperatures of ~200–300ºC. These temperatures are not nearly as high to produce the spore inactivation of *IF*~10^3–10^4 exclusively due to heat. Based on the previously reported findings (Grinshpun *et al.*, 2010a), close to 10% of spores should have remained viable after exposure to a heated air flow (no combustion) at the above-specified temperatures in the pre-wall layer. After accounting for the cross-sectional area represented by this layer relative to the total cross-section area of the flow, we estimate that at least ~1% of spores passing the test chamber should have survived if viability loss was caused only by the heat exposure. This translates into *IF* of ~10^2. The experimental values obtained with the strand containing the iodinated nanocomposite revealed much higher inactivation levels, thus pointing to additional (non-thermal) contribution. The latter is believed to be associated with the products released during combustion of the solid strand containing $12Al·MoO_3$ nanocomposite with iodine. In contrast, combustion of the non-iodinated composite produced the viability losses closer to the levels, which can be explained exclusively by the heat-induced stress.

To summarize, the almost 200-fold difference in *IF*-values between the iodinated and non-iodinated composites obtained in these pilot tests can be explained, at least to a significant extent, by the effect of released iodine on airborne viable spores of *B. atrophaeus* passing the test chamber. Generally, there is a need in developing appropriate approaches to separate the thermal and chemical effects causing inactivation of airborne microorganisms. Nevertheless, these results show the feasibility of iodine-containing material for agent defeat applications.

Inactivation of Spores Collected on Filters Exposed to Combustion Products during Tests

While the pilot data generated in this study provide evidence that iodine in the FNM enhances the inactivation of spores exposed to combustion, there is an important limitation in attributing this effect solely to the *aerosol* interaction. Indeed, the spores, which remain viable after passing through the system, are collected on filters and continue being exposed to combustion products including smoke and soot until the test ends (the cooling system eliminates exposure of the collected spores to hot air but does not remove the combustion products from the incoming air flow). To quantify the survival of spores, which are exposed to combustion products on the collection filter, we performed the following experiment. First, the originally aerosolized endospores passed through the system (at a total air flow rate of 36 L/min) and were collected on five polycarbonate filters with no combustion in the chamber. Two of these five pre-loaded filters served as controls, while three others were exposed to combustion products released when a strand filled with the $12Al·MoO_3$

nanocomposite and paraffin wax was burned in the test chamber at a mass rate (R), of approximately 250 mg/s with clean air supplied at 36 L/min. The exposure time (equal to the established collection time period) varied approximately from 20 to 80 s. Combustion products were aluminum oxide, molybdenum oxide, water, carbon oxides and soot [further details on this material and its products are given in Umbrajkar *et al.* (2008)]. The spores were extracted from the filters using a standard extraction method using a vortex touch mixer (Fisher Scientific, Pittsburgh, PA), serially diluted, and subjected to the culture-based analysis using TSA media (Grinshpun *et al.*, 2010a). The survived spore fraction was calculated based on the bacterial colony forming units counted from the control and treated samples.

The microbial inactivation occurring on the collection filters was found to depend on the time during which the filter was exposed to combustion products. The survived fraction [can be expressed as $(IF_{filter})^{-1}$] decreased exponentially with time and fell below 10% within a minute due to the high amount of combustion products released when a $12Al\cdot MoO_3$ strand was burned (Fig. 4). Collection times as short as 20 s caused inactivation of more than 50% of the initially viable spores on the filter. Thus, at high mass burn rates, the viability loss on filters became comparable to the primary (aerosol) inactivation. To eliminate this problem, the collection time should be minimized or the burn mass rate reduced considerably. Otherwise, alternative bioaerosol collection methods (e.g., an impinger or wet cyclone) should be chosen. Among several products released during the strand combustion, considerable amount of soot is expected to have a pronounced effect on the viability of microorganisms collected on filters.

Unlike in a strand of consolidated FNM, the aerosol burner design provides a slow feeding of the tested material in powder form directly to a hydrocarbon flame, thus producing much lower mass burn rates (in our tests conducted at a total air flow rate of 36 L/min, R varied from 12 to 22 mg/s). The filter samples obtained in 1-min experiments involving the aerosol burner did not have a distinctive black/grey deposit that was observed when burning solid strands, apparently due to an extensive generation of soot. Fig. 4 shows that when testing with the aerosol burner, the detrimental effect associated with the viability loss on the collection filter was smaller as compared to the tests conducted with the solid strand. The difference between the respective slopes, $k = 0.0034$ s^{-1} (burner, $R \approx 20$ mg/s) versus $k = 0.0538$ s^{-1} (strand, $R \approx 250$ mg/s), is clearly appreciable. By using the aerosol burner, the time of bioaerosol collection from combustion air environments could be at least 1 min. This sampling time is considered reasonably practical while still short enough to avoid a sizable loss of viability on the filter. At the same time, the experiment with pre-loaded filters produces a conservative estimate of the collection time limit because in a routine test the spores collected at the end of the sampling period have much shorter exposure to combustion products on the filter than those collected at the beginning of the sampling period. The aerosol burner demonstrated several advantages over the strand design as the former provides a more stable/uniform combustion at a sufficiently low mass burn rates. The measured viability loss is more representative of the spore *aerosol* exposure.
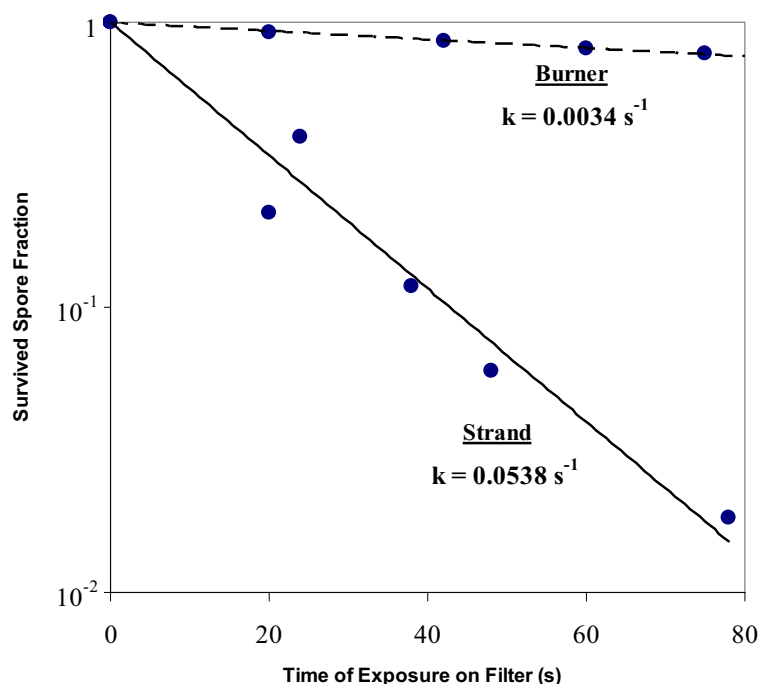

Fig. 4. Survival of *B. atrophaeus* endospores on a 25-mm polycarbonate collection filter (pore size: 0.4 µm) exposed to combustion products of $12Al\cdot MoO_3$ while burning a dispersed powder (aerosol burner) and a pellet of consolidated material (solid strand).

The test conditions established by this validation study are applicable to stress-resistant bacterial spores. Microorganisms that are more susceptible to environmental stress may require establishing different test conditions, in particular shorter collection time periods.

Role of Metal Powder Dispersed into Hydrocarbon Flame in Inactivating Spores (Pilot Data)

The aerosol burner is capable of providing two combustion environments:
(i)   a gaseous (e.g., hydrocarbon) flame and
(ii)   the flame seeded with particles of an energetic material.

In both cases, exposure to the heat and combustion products may cause significant inactivation of the aerosolized *B. atrophaeus* endospores. However, the presence of a dispersed energetic material (e.g., seeded metal particles) in the aerosolization chamber of the burner has a potential to enhance inactivation due to higher energy input. The new method developed in this study was evaluated with respect to its ability to obtain a measurable difference in *IF*-values associated with metal particles in the flame. Identical tests were performed using the burner producing a hydrocarbon flame with and without spherical aluminum particles (Alfa Aesar, 97.5%, nominal diameter 3–4.5 μm) burned at R ≈ 20 mg/s. At a total air flow rate of 36 L/min, the pure flame expanded 10–20 mm downstream from the top edge of the burner and about 6 mm radially. The flameless area along the wall was also about 6 mm wide. When feeding aluminum powder particles, the flame configuration did not change appreciably. Luminous streaks of heated and burning particles were apparent in the flame zone and above the gaseous flame. In these tests, a 1-min sampling period was established for collecting spores on filters.

The spore exposure to the pure flame produced moderate inactivation: GM of *IF* was 50.2 (GSD = 1.49; n = 3), which corresponds to approximately 2% survival. Dispersing of aluminum powder enhanced the inactivation considerably: GM = 857 (GSD = 1.58; n = 3), which translates to approximately 0.12% survival. This substantial difference between the *IF*s determined with and without the aluminum powder reflects an enhancement in inactivation taking place in the aerosol phase (as shown above, a low rate provided by the aerosol burner and a short sampling time of 1 min minimize the influence of the powder combustion on the representativeness of the filter samples). The difference brought in by dispersing aluminum powder in the flame can be attributed to a combination of two factors: (i) increase of air temperature due to the energy output from combustion of aluminum particles in the gas mixture and (ii) aerosolized combustion products (alumina smoke). Both may directly affect the viability of the exposed bioaerosol particles.

CONCLUSIONS

In summary, we designed and built a state-of-the-art experimental facility and developed a method for assessing the survival of aerosolized microorganisms exposed to combustion for a short time (<1 s). In spite of its ability to measure high inactivation levels, the newly-developed method does not aim at exposing bioaerosol particles exclusively to the flame zone, in which even stress-resistant microorganisms are expected to be completely inactivated (or disintegrated) by heat. The design allows assessing the bioaerosol inactivation in the periphery or in close proximity to the flame zone. It is particularly advantageous when testing fuels modified with biocidal additives, which are released during combustion, against their unmodified analogs. The method and experimental facility were evaluated with aerosolized *B. atrophaeus* (BG) endospores exposed to a pure hydrocarbon flame and to combustion of different aluminum-based energetic composites (including novel compositions with added iodine). The method was used in establishing feasibility of the new iodine-containing material for microbial agent defeat applications. Although in this study we tested the new method with bacterial spores, its use can be expanded to other challenge bioaerosols (including viruses).

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