ABSTRACT

PREVOST, ROSSANA. Aerosolization and Quantification of Surrogate Biological Warfare Agents under Simulated Landfill Conditions. (Under the direction of Dr. Morton A. Barlaz and Dr. Francis L. de los Reyes III.)

The presence of harmful airborne microorganisms in indoor and outdoor environments has largely been associated with the spread of disease. However, in the past decade, harmful microorganisms have also been used as a potential weapon, as in the anthrax scare of 2001. Building debris from an attack may be disposed in landfills, yet remain contaminated with biological warfare (BW) agents. It is important to understand the transport behavior of BW agents in the landfill to evaluate the safety of landfill disposal of building debris. The objectives of this study were: 1) to detect and quantify surrogate BW agents using real time quantitative polymerase chain reaction (Q-PCR) in gas samples from a simulated landfill system that was filled with synthetic building debris (SBD) and 2) to detect and quantify the transport of surrogate BW agents, *Bacillus atrophaeus* spores and vegetative cells (used as surrogate for *Bacillus anthracis*) and *Serratia marcescens* (used as surrogate for *Yersinia pestis*), in landfill gas using an aerosol chamber. Surrogate BW agents were aerosolized individually and in mixtures under controlled conditions using a collision nebulizer in an aerosol chamber. Samples were collected on gelatin filters for detection and quantification using Q-PCR. In the second part of this study, suspended solutions of surrogate BW agents (*B. atrophaeus* and *S. marcenses* cells, or *B. atrophaeus* spores and *S. marcescens* cells) were spiked onto the surface of synthetic building debris (SBD) and subjected to four different $N_2$ flows (to simulate landfill gas migration) for aerosolization inside of the chamber. Gas flow was

kept constant to avoid back pressure. Aerosolized and non–aerosolized surrogate BW agents from spiked SBD were measured.

The results show significant variability in recovery of surrogate BW agents using gelatin filters when they were aerosolized from a suspended solution into an empty aerosol chamber. Detection and quantification of the attachment of the surrogates to chamber walls affected the calculation of recovery using the gelatin filters. The recovery of *B. atrophaeus* vegetative cells ranged from 9% to 24%, the recovery of *B. atrophaeus* spores ranged from 7% to 60%, and the recovery of *S. marcescens* ranged from 13% to 54%. In the case where surrogates where aerosolized from the surface of the SBD, *B. atrophaeus* vegetative cells and spores were barely detected at the two highest flows (13.2 L/min and 25.3 L/min) applied through the SBD, and there was no detection of *S. marcescens* at any flow. There was no significant detection of cells and spores in SBD. These results suggest that cells and spores strongly adhered to the SBD, limiting the possibility of aerosolization through landfill gas.

# Aerosolization and Quantification of Surrogate Biological Warfare Agents under Simulated Landfill Gas Conditions

by
Rossana Prevost

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APPROVED BY:

| | |
|---|---|
| Dr. Detlef Knappe. | Dr. Francis de los Reyes<br>Co-chair of Advisory Committe |

Dr. Morton A. Barlaz
Chair of Advisory Committee

# DEDICATION

To my husband for your support through this adventure and to my parents who are always pushing me to be better.

# BIOGRAPHY

Rossana Carpio Prevost was born in Lima, Peru on June 20th, 1978 to Saul Carpio and Aurea Mansen de Carpio. She graduated from the National Engineering University as a bachelor in Hygiene and Safety engineer. Upon graduation she started to work in Vega-Upaca Relima S.A., where she was in charge of the Occupational Safety and Health of the company. In 2004, she moved to the United States of America and has been working for Raleigh Housing Authority.

At the present moment, she is pursuing her master degree in North Carolina State University in Environmental Engineering; after graduation, she is looking forward to work in a company that addresses environmental issues.

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# INTRODUCTION

Biological warfare agents have been used throughout history, from the first recorded event of a Tartar army that catapulted the bodies of plague victims into the Crimean Peninsula city of Kaffa to infect the townspeople, to the attempt in 2001 where contaminated letters with anthrax were sent in the United States mail, infecting twenty-two people and killing five. The Geneva Protocol in 1925 and the Biological and Toxin Weapons Convention Treaty in 1972 restrict signatory countries from developing, producing, or acquiring biological agents to be used as biological weapons. However, not all countries are committed to these treaties.

Recent advances in biotechnology have made it easier to develop dangerous viruses, bacteria, and toxins with fewer resources, increasing concerns that renegade groups could resort to bioterrorism to attack a population. The 2001 anthrax attack has prompted researchers to develop and assess methods for the decontamination and destruction of *Bacillus* spores bound on surfaces and building material (Abu-Ashour et al., 1994; Lemieux et al., 2006; Raber et al., 2002; Rogers et al., 2005; Sangripanti et al., 2005). The decontamination activities following a bioterrorist attack on a building generate a significant amount of porous material termed "building decontamination residue" (BDR) that is likely to remain contaminated with biological warfare (BW) agents sorbed to debris constituents ( Lemieux et al., 2006; Saikaly et al., 2007).

One alternative for disposal of BDR is landfill burial. Exposure to environmental stresses such as temperature, ultraviolet radiation, and drying can reduce a BW agent's activity. However, some pathogens, like the anthrax bacteria, can exist as spores not easily susceptible to those conditions. Although significant efforts have been applied to studies of BW agents in air, surfaces, water, and leachate in the last few decades, only a few studies address the fate and transport of BW agents in landfills and porous media, and these mainly evaluate the detection, quantification and transport behavior of surrogate BW agents in leachate (see reference Saikaly et al., 2007; Saikaly et al., 2010). No literature has been found on the transport of BW agents in landfill gas.

The objectives of this study were: 1) to validate the detection and quantification of the surrogate BW agents using SYBR Green1 real time quantitative polymerase chain reaction (Q-PCR) in gas samples generated from a simulated landfill system that was filled with synthetic building debris (SBD), and 2) to detect and quantify the transport of surrogate BW agents, *Bacillus atrophaeus* spores and vegetative cells (used as surrogate for *Bacillus anthracis*) and *Serratia marcescens* (used as surrogate for *Yersinia pestis*) in landfill gas using an aerosol chamber.

Previously developed and optimized Q-PCR assays for the detection and quantification of surrogate BW agents in filters and SBD were used in this study to quantify their transport in simulated landfill gas.

# BACKGROUND ON DETECTION OF MICROORGANISM IN LANDFILL GAS

## Diversity of microbial community in the landfills

A sanitary landfill is an engineered facility where municipal solid waste is disposed in a manner that minimizes public health and environmental impacts. A complex series of chemical and microbiological reactions is initiated with the burial of refuse in a sanitary landfill. These transformations do not occur immediately. A period ranging from months to years is necessary for the proper growth conditions and the required microbiological system to become established (Palmisano & Barlaz, 1996).

Barlaz et al., (1988) characterized the microbial and chemical changes that occur in refuse decomposition. "The three major groups of bacteria identified in the four phases of the refuse decomposition were: (i) the hydrolytic and fermentative bacteria, that convert biological polymers such as cellulose and hemicellulose to sugars which are then fermented to carboxylic acids, alcohols, carbon dioxide, and hydrogen; (ii) the obligate proton-reducing acetogenic bacteria, that convert longer-chain carboxylic acids and alcohols to acetate, hydrogen, and carbon dioxide; and (iii) the methanogenic bacteria, that convert primarily acetate and hydrogen plus carbon dioxide to methane".

Other studies have focused on landfill end products, such as leachate, to compare and analyze the microbial communities prevailing in the landfill at its different stabilization phases (Calli et al., 2006); to evaluate the impact of leachate recirculation on the community structure and activity of the microorganisms (Li-Nan et al.,2002 and 2004); or to evaluate the effectiveness of new molecular techniques in the detection of microbial communities (Daly, et al., 2000; Röling, et al., 2001; Van Dyke and McCarthy, 2002).

More recent studies target not only microbial communities that contribute to the stabilization of the landfills but also those that could cause a significant impact on them. For example, Saikaly et al., (2010) measured the effect of leachate recirculation and water infiltration on the transport behavior of biological warfare agents in landfills to evaluate the suitability of the landfill for the disposal of building decontamination residue following a terrorist attack.

However, the lack of literature on detection and quantification of the microbial community in the landfill gas made the comparison of the study more difficult. A study by Kim et al., (2003) reported the predominance of *Bacteria* in landfill gas with 85.9% ± 2.4% and landfill gas condensate with 86.8% ± 7.9% of the total 16S rRNA. Archaea were detected in the landfill gas condensate with 7.2% ±1.4% of the total 16S rRNA, while there were no Archaea detected in landfill gas. Eukarya were only detected in waste material.

**Sampling of microorganisms in landfill gas**

Several studies related to the detection and quantification of airborne microorganisms have used bioaerosol monitoring. This is a rapidly emerging technique that measures viable (culturable and nonculturable) and nonviable microorganisms in different environments. The three major sampling methods for airborne microorganism are impaction, impingement, and filtration (Buttner et al., 1997).

Impaction is used to separate a particle from a gas stream based on the inertia of the particle. An impactor consists of a series of nozzles (circular or slot shaped) and a target (Jensen and Schafer et al., 1998). The bioaerosol is separated from the air stream by using the inertia of the particles to impact onto a soft agar or solid glass surface (Kim et al., 2003). The advantage of this method is that these particles impact onto growth medium and avoid the need of further dilution or plating. The disadvantage is that multiple particles, each containing one or more organisms, passing through a single hole may be inaccurately counted as a single colony, creating bias in the assessment of the viability of the microorganisms.

The impingement method is useful for the collection of culturable aerosols. Impingers use a liquid as the collection medium. Additives to the collection medium such as proteins, antifoam, or antifreeze aid in resuscitation of bacterial cells, prevent foaming and loss of the collection fluid, and minimize injury to the cells (Jensen and Schafer et al., 1998). In contrast to the impaction method, the impingement method

allows better control of the concentration of the bioaerosol because microorganisms can be diluted in the liquid. The liquid samples may also be used for additional analysis such as biochemical, immunological, and molecular biological assays (Kim, 2003). However, this method also presents some limitations such as the loss by re-entrainment in the exhaust flow caused by hydrophobicity, agitation and misting within the impinger (Muilenberg, 1998).

Filtration, in contrast with the methods described above, occurs when particles are impacted and intercepted by the fibers or surface of filter membranes. Inertial forces and other mechanisms such as diffusion and electrostatic attraction contribute to the collection of the particles on the surface of a filter (Buttner, 1997). Sampling filter media may have pore sizes of 0.01 to 10 μm and are often held in disposable plastic filter cassettes during bioaerosol sampling. The choice of a filter medium depends on the contaminant of interest and the requirements of the analytical technique (Jensen and Schafer et al., 1998). The efficiency of removing particles from the air depends on the face velocity. The overall efficiency of membrane filters is approximately 100% for particles larger than the pore size (Lippmann, 1995). A limitation of this method is the possibility of dehydration of the cells during sampling, resulting in loss of viability, damaging microbiological activity and making detection difficult (DeCosemo et al., 1992).

The methods presented above are the most common alternatives used for sampling for bioaerosols. There is no standardized method for sampling. Numerous studies have used filtration as a sampling method for the collection of targeted airborne microorganisms (bioaerosols) to allow application of molecular techniques for detecting organisms. Ambient techniques cannot be directly applied to landfill gas sampling because of the high (condensing) moisture concentration, and the enclosed nature of landfill gas collection. Recent studies by Kim and Barry (2000 and 2003) show that microorganisms from landfill gas could be collected using filtration. For the purposes of the present study, the filtration method using gelatin filters was used to capture the target microorganisms in the lab-scale simulation of landfill gas emission.

**Molecular techniques to quantify the microbial community in landfill gas**

Predominant bioaerosol detection methods often rely on culture-based analysis that provides a number of culturable microorganisms in an air sample (Williams et al., 2001; Zeng et al., 2004). However, this method could lead to biased characterization and underestimation of the total microorganism concentrations in a sample as some bacteria may not be cultured under the conditions employed. Thus, microbiological methods based on culturing may lead to an incomplete understanding of intrinsic characteristics of microbes that are not representative of the ecosystem of concern (Amman et al., 1995).

Methods that are independent of culturing, such as epifluorescence and direct light microscopy, are often used to determine total microorganism concentrations in air samples, but microscopy-based methods are time and labor intensive (Kildeso and Nielsen, 1997; Williams et al., 2001; Zeng et al., 2004) and not species-specific. Cultivation-independent molecular methods such as polymerase chain reaction (PCR) assay have been used to identify and quantify total bacterial load in bioaerosol samples and landfills more precisely and reliably (An et al., 2006; Buttner et al., 2004; Kim et al., 2003).

The polymerase chain reaction (PCR), is an enzymatic reaction that allows amplification of specific DNA regions through a repetitive process. This method relies on thermal cycles of heating and cooling of the reaction for DNA melting and hybridizing. Three sequences are defined: primer denaturation, primer annealing, and the extension of the annealed primers by the DNA polymerase, resulting in the duplication of the starting target material. The process generally is repeated around 20-30 cycles resulting in an exponential increase of the amount of target DNA. The amplification product is usually visualized through agarose gel electrophoresis (Barry and Kim et al., 2003). The conventional PCR assay has been applied to analyze air samples for the presence of endemic microorganisms (Alvarez et al., 1994), airbone mycobacteria (Schafer et al., 2003), biowarfare agents (Higgins et al., 2003) and fungi commonly associated with adverse health effects (Cruz-Perez et al., 2001; Williams et al., 2001).

However, conventional PCR analysis has some limitations in its accuracy, reliability and reproducibility (Birch et al., 2001). For example, the size of the region that could be amplified is limited and it is required to know the specific sequence information for the target. Another limitation is the likelihood of false positives due to contamination, making it essential to run controls along with the samples.

PCR has been evolving and its application has become more accurate. For example, quantitative real time PCR (Q-PCR) is capable of better reproducibility and accurate measurement of total microorganism concentrations in environmental samples. It allows the detection of cells irrespective of their culturability, and rapid sample quantification. Furthermore, unlike conventional PCR, Q-PCR does not require post-PCR analysis such as gel electrophoresis because its detection system combines a thermalcycler coupled to an optical module. At the end of the extension phase of each PCR cycle, the optical module measures the fluorescence intensity of each reaction which is generated by hybridization probes (TaqMan), or fluorescence resonance energy transfer (FRET), or by double stranded DNA dyes such as Sybrgreen (SYBR). The data software included with Q-PCR system calculates a threshold based on the background fluorescence, and determines the cycle number $C_T$ at which the fluorescence in the sample crosses this threshold (An et al., 2006). Finally, the $C_T$ is inversely correlated with microorganism concentration in the sample. Standard curves can be created to quantify the total microorganism concentration in a study sample.

Several Q-PCR assays have been developed and validated for the detection and quantification of the microbial community in leachate (eg. Mori et al., 2003; McDonald et al., 2008). Several studies have been validated for the detection of biological warfare (BW) agents using Q-PCR but very few address the impact of contaminated residual with BW in the landfill.

The evaluation of the fate and transport of BW agents in the landfill requires the development of specific and sensitive detection assays. However, sometimes it is not feasible to use the actual BW agents, and surrogates are required (O'Connell et al., 2006). Saikaly et al. (2007) developed Q-PCR assays for the detection and quantification of surrogate biological warfare agents in building debris and leachate using SYBR green.

Currently, the validation of Q-PCR for the quantification of microorganisms in landfill gas has not been widely investigated and there are a lack of established protocols for bioaerosol detection and quantification. Q-PCR has been selected as the detection method to evaluate the transport of surrogate BW agents in simulated laboratory scale landfill gas for the purpose of this study.

# EVALUATION OF THE EFFECTIVENESS OF DETECTING AND QUANTIFYING SURROGATE BIOLOGICAL WARFARE AGENTS IN GAS USING A BIOAEROSOL CHAMBER

## Introduction

The scarcity of literature on sampling and detection of microbial communities in landfill gas is a major constraint in evaluating and comparing the effectiveness of methods and techniques for their detection and quantification. The presence of harmful airborne microorganisms in indoor and outdoor environments not only has been associated with a variety of illnesses but also has been the focus of attention after microorganisms were used as weapons like the case that occurred in the United States in 2001. These events have motivated the development of detection platforms for BW agents, and methods for BW agent inactivation and decontamination. Delivering a BW agent requires preparing it to remain effective outside of its optimal growing conditions. Exposure to environmental stresses, such as temperature, ultraviolet radiation, and drying can reduce the agent's activity. However, some pathogens such as anthrax bacteria can encapsulate into spores not susceptible to those conditions and easily transportable through different media.

Microbial analysis of air samples present several difficulties. Bioaerosol particles, including airborne infectious agents, are usually collected into a solid liquid or agar

media (An et al., 2006). However, they may not be actively metabolizing, growing and/or reproducing during transport and, therefore may not respond to traditional culture techniques (Levin, 1997). Several studies have been developed and validated for the detection and quantification of BW agents assays using cultivation-independent molecular methods such as real time polymerase chain reaction (Q-PCR) (Tomaso et al. 2003, 2007; Wilson et al. 2005; Klee et al. 2006; Skottman et al. 2006; Olsen et al. 2007) however, there is no specific standard sampling method proven to be most efficient.

The objective of this study was to evaluate the effectiveness of a system designed to aerosolize surrogate BW agents, *Bacillus atrophaeus* (vegetative cells and spores) and *Serratia marcescens* (cells) using SYBR Green Q-PCR.

## Materials and Methods

### Bacterial strains and growth of pure culture

The bacterial strains used in this study were *B. atrophaeus* ATCC 9372 vegetative cells (formerly *Bacillus subtilis var. niger), S. marcescens* ATCC13880 obtained from the American Type Culture Collection (Manassas, VA), and purified spore suspensions of *B. atrophaeus* ATCC9372 obtained from the North American Science Association, Inc., (NAMSA) Ohio laboratory (Northwood, OH*). B. atrophaeus* in a vegetative state and *S. marcescens* were grown on Difco nutrient agar (Becton, Dickenson and Co., Sparks MD)

at 30ºC and 26 ºC respectively. Frozen stocks were used to prepare suspensions of the bacteria to avoid possible contamination of the freeze dried cultures. Cell suspensions used in the experiment were obtained by inoculating 1µL of the thawed frozen stocks into 100 mL of sterile Difco nutrient broth (Becton, Dickenson and Co., Sparks MD). Cells were incubated at 37 ºC for 16 hours with shaking (100 rpm) and then harvested at mid-logarithmic phase by centrifugation at 4000 x g for 10 min with an Eppendorf centrifuge 5810 (Eppendorf North America, Inc.). The formed pellet was washed three times with 1x PBS (phosphate-buffer saline: 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$ per distilled water, pH 7.4) by centrifugation at 4000 x g for 15 min and then re-suspended in 10 fold dilution of 1 x PBS to reach the densities desired. *B. atrophaeus* spores were diluted with 1 x PBS to obtain around $10^4$ spores/mL for the purposes of the experiment.

**Experimental Design**

Experiments were designed to measure the effectiveness of the system (nebulizer-chamber) and detection and quantification of the target surrogate BW agents using SYBR Green and Q-PCR. The chamber was designed with the objective of measuring the presence of surrogates during aerosolization from decomposing refuse. The system includes two compartments that are connected with a gas tight seal and a plexiglass slide wall that isolates one compartment from the other (Fig.1). These two components provide

a hermetic seal to avoid any possible disturbance when different flows are applied in one of the compartments (this door will be used in subsequent experiments). Compressed air with specific pressure and flows was supplied to the nebulizer (placed in the first compartment) through one inlet located at the front wall of the chamber to produce the aerosol. The flow (11.7 L/min) and pressure (22.6 psig) were selected based on the specifications of the nebulizer to produce the maximum aerosolization of the surrogate BW agents. To contribute to the suspension of the particles; a fan working at minimum speed was placed at the top wall/ceiling of the second compartment. The capture of the target surrogates was achieved using gelatin filters placed into button samplers located at the end wall of the second compartment. The filtration method comprises capturing airborne organisms suspended in aerosol on the gelatin filters through forced impact. A directional gas flow through the gelatin filter was created with the intent to promote impaction and adhesion (retention/entrapment) of the surrogate BW agents suspended in air. Two SKC universal sampling pumps connected to the outputs of the button samplers were used at their highest flow rate (5 L/min) to produce the forced impact and contributed to the maintenance of a pressure balance and avoidance of back pressure during sampling. A quick connect fitting valve was used to release the excess flow and maintain mass balance due to the inflow being higher than the outflow generated by the pumps.

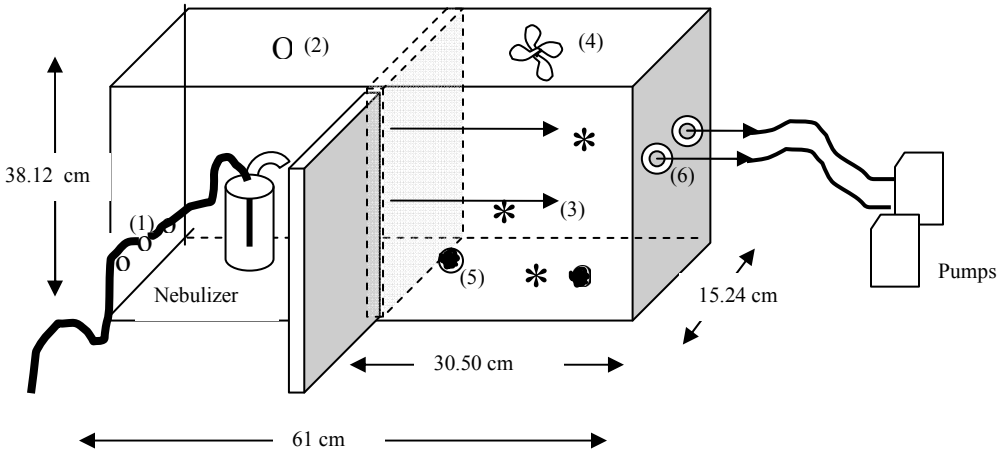


Figure 1. Designed aerosol chamber using BGI nebulizer. (1) compressor air intake; (2) open fitted valve; (3) flow of the aerosol and suspended surrogates; (4) fan; (5) Closed valves; (6) sampler buttons with gelatin filters.

Initial tests were performed in the chamber prior to aerosolization of selected surrogate BW agents at specific concentrations to (1) verify the influence of the environment and different concentrations of the saline solution and (2) determine the

most effective conditions for the aerosolization of the surrogate BW agents selected using an aerosol monitor-Dust Trak which provides readings/data of the particle concentration. *S. .marcescens* was selected for theses initial tests because of its smaller size in comparison to *B. atrophaeus* thus proving a better result in density measurements and reducing the possibility of agglomeration during the aerosolization process. Another factor considered during these tests was the concentrations of phosphate buffer. Two different concentrations were tested to evaluate the effect of the aerosolization of *S. marcescens* and the avoidance of agglomeration (see Table 1).

Table 1. Conditions and particle concentrations of tests performed in aerosol chamber

| | **Sampling time (min)** | **Volume in nebulizer (bottle) mL** | **Average mg/m$^{3(*)}$** | **Max mg/m$^{3(*)}$** | **Min mg/m$^{3(*)}$** | **Inlet pressure and flow to nebulizer psi ( L/min)** |
|---|---|---|---|---|---|---|
| **Test #1 Air Sampling from Environmental laboratory** | 6 | N/A | 0.004 | 0.004 | 0.003 | N/A |
| **Test #2 - Tap water** | 10 | 35 | 0.38 | 0.50 | 0.02 | 20 (9.35) |
| **Test # 3 - PBS** | 10 | 35 | 32.4 | 40.4 | 1.81 | 20 (9.35) |
| **Test #4 -PBS in 10 fold dilution** | 20 | 40 | 4.59 | 5.36 | 4.26 | 22.6 (11.7) |
| **Test # 5 - PBS in 10 fold dilution with *S. marcescens* suspended** | 20 | 40 | 8.54 | 8.92 | 8.09 | 22.6 (11.7) |
| **Test # 6 - PBS in 50 fold dilution** | 20 | 40 | 1.77 | 1.86 | 1.67 | 22.6 (11.7) |
| **Test # 7- PBS in 50 fold dilution with *S. marcescens* suspended** | 20 | 40 | 4.69 | 5.16 | 4.79 | 22.6 (11.7) |

(*) Average, maximum and minimum particle concentrations were calculated after the 5$^{th}$ min where a steady state was observed

A series of experiments (trials) were conducted in the system after evaluating the environmental conditions and suspended solution (1xPBS) that could affect the aerosolization. Surrogate BW agents at specific concentrations were suspended in diluted PBS and aerosolized individually or mixed in pairs (*B. atrophaeus* vegetative cells + *S. marcescens* or *B. atrophaeus* spores + *S. marcescens*) to quantify their recovery using the gelatin filters.

Five trials were conducted using concentrations of $10^4$ cells per mL for *B. atrophaeus* and $10^3$ cells per mL for *S. marcescens* in 40 mL PBS solution (Table 2). The trials were designed to gain a better insight into the release and quantification of the surrogate BW agents selected when they are aerosolized individually and when they are mixed together in the suspended solution. Aerosolization time (20 min) during each trial was selected to assure the saturation of almost seven times the volume of the chamber (35.4 L) released by the nebulizer (11.7 L/min). Mass balance was kept during all trials using two SKC universal sampling pumps at 5 L/min each and a quick-connect fitting valve that released the remaining flow (1.7 L/min). Additional 10 minutes of pumping with a flow of 10 L/min was used to flush around three times the total volume of the chamber and capture any residual aerosolized surrogate BW agents remaining inside the chamber. Duplicate experiments for each trial at specific concentrations were conducted for *B. atrophaeus* (cells), *S. marcescens*, *B. atrophaeus* (spores), a mixture of *B. atrophaeus* cells with *S. marcescens*, and a mixture of *B. atrophaeus* spores with *S. marcescens*. The initial and

total concentration performed in each trial are presented in Table 2. After each trial the chamber was sprayed and aerosolized for 20 min with 70% ethanol. The nebulizer and SKC button sampler used in the trials were also sprayed with ethanol and subjected to UV light exposure for 3 hours before each trial.

Table 2. Initial concentration used in the aerosolization of the surrogate BW agents with nebulizer

| **BW agents solution** | **Initial Concentrations (cells/mL or spores/mL of PBS** | **Total cells or spores in solution** |
|---|---|---|
| *B. atrophaeus* vegetative cells | 3.50E+04 | 1.29E+06 |
| *B. atrophaeus* vegetative cells | 3.52E+04 | 1.37E+06 |
| *B. atrophaeus* spores | 1.26E+04 | 4.93E+05 |
| *B. atrophaeus* spores | 3.54E+04 | 1.38E+06 |
| *S. marcescens* | 4.50E+03 | 1.75E+05 |
| *S. marcescens* | 3.96E+03 | 1.55E+05 |
| *B. atrophaeus* vegetative cells + *S. marcescens* | 1.84E+04 | 6.98E+05 |
| | 1.00E+03 | 3.80E+04 |
| *B. atrophaeus* vegetative cells + *S. marcescens* | 2.25E+04 | 8.55E+05 |
| | 1.74E+03 | 6.63E+04 |
| *B. atrophaeus* spores + *S. marcescens* | 1.90E+04 | 7.23E+05 |
| | 1.21E+03 | 4.60E+04 |
| *B. atrophaeus spores* + *S. marcescens* | 1.04E+04 | 3.94E+05 |
| | 2.28E+03 | 8.67E+04 |

**Experimental equipment and operations**

The aerosol chamber used in this study is similar to the one described by Heidelberg and colleagues (1997) with several slight differences; two well defined compartments are connected with a gas tight seal and could be isolated by a plexiglass sliding wall; gelatin filters inside button samplers were placed at the end wall instead of impinger ports, no UV lamp was installed and a small fan located in the second compartment of the chamber was placed at the top to assure good mixing of the surrogates.

During spray trials, aerosolization was achieved using a 6- jet collision- type air-jet nebulizer (BGI, Inc., Waltham, MA) with air pressures ranging from 20 to 100 psig and volume of free air ranging from 12 - 42 liters per minute (lpm). Gas was supplied using compressed air at 11.7 L/min and 22.6 psig, which is suitable for the purpose of the study. Two universal sampling pumps (Model PCXR 8, SKC) connected to the outlets of the button samplers (end wall) were used with 5 L/min flow for each one to create the force impact (directional gas flow through the gelatin filter is created with the intent to promote impaction and adhesion of the surrogates suspended in a gaseous medium). The SKC pumps were run simultaneously during the aerosolization process for 20 minutes to avoid back pressure inside of the chamber and preserve mass balance. After the air to the nebulizer was turned off, the pumps were run for an additional 10 minutes to capture any residual aerosolized surrogate BW agents remaining inside of the chamber (See Figure 1).

A quick- connect valve fitting (Cole Palmer # C-06364-80) was placed on the top in the first compartment to release the remaining inflow. A fan with a very slow speed was constantly working during trials to assure suspension inside of chamber.

A DustTrak Aerosol Monitor (Model 8520) uses light scattering technology to determine mass concentration in real-time. The Aerosol Monitor was employed initially to evaluate the possible interference of the environment (environmental laboratory air) and suspension solution (1 x PBS). The monitor was operated in Survey mode, which displayed real-time readings and determined statistics such as average, minimum and maximum readings, and readings were taken every 30 seconds. Data was processed using the TrakPro Data Analysis software supplied with the equipment.

Filtration sampling with sterile 3 μm pore size gelatin filters (SKC, Inc., Eight Four, PA) placed inside the SKC button samplers were used to collect surrogate BW microorganisms for microbial analysis with SYBR Green1 and real-time PCR. Gelatin filters have a high moisture content that aids in preventing to some extent the loss of viability caused by desiccation. Gelatin filters were changed after each trial and placed in a sterile 50 mL centrifuge tube and stored at -20ºC for DNA extraction and Q-PCR.

For DNA extraction from cell solutions, 2 mL of the initial suspended solution were taken and placed in a 2 mL centrifuge tube; cells were harvested by centrifugation and stored at -80ºC. This step was repeated for each run per trial.

After each run, the nebulizer was placed in a Biosafety Level II cabinet (SterilchemGARD, Baker Co., Sandford, ME). Measured RNase-free sterile water was added to avoid possible loss of surrogate organisms inside the nozzle. One mL of the remaining suspended solution mixed with the RNase-free sterile water was taken and cells were harvested by centrifugation in a sterile microcentrifuge tube and stored at -80ºC for DNA extraction.

Four designated areas (4 $in^2$ or 25.8 $cm^2$) were wiped with a sterile buccal cotton swab (SKC, South Inc., Appomattox, VA) to recover surrogate organisms that could be attached to the chamber walls due to aerosolization. This method was applied by moving the swab back and forth across the surface with several horizontal strokes, then several vertical strokes. The swab was rotated during sampling to ensure that the entire surface of the swab was used. After sampling, the swab was returned to its original, prelabeled sampling tube for subsequent analysis. Swabs were placed in 2 mL microcentrifuge tubes at -80ºC for DNA extraction and quantification using Q-PCR.

**DNA extraction of bacterial cells and spores from suspended solution, filter and swabs**

DNA extraction from microbial cells and spores related to the suspended solution prepared for the aerosolization of the surrogate organism in each trial was performed

using a chemical lysis treatment with the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) followed by the hot detergent treatment and beat-beat homogenization (see below) as described by Saikaly et al., 2007. Chemical lysis was performed with 180 µL of enzymatic lysis buffer (20 mM Tris-Cl, pH 8.0, 2 mM sodium EDTA, 1.2% Triton X-100, and 20 mg/mL lysozyme (Sigma-Aldrich, St Louis, MO)) for 30 minutes at 37$^{o}$C, followed by digestion with 25 µL of proteinase K and 200 µL buffer AL (a buffer containing guanidine hydrochloride provided by the DNeasy kit) for 20 min at 56$^{o}$C (cells) or 70$^{o}$C for 15 min (spores). The supernatant was then transferred to a 2 mL safe lock tube containing 300 mg of zirconia/silica 0.1 mm beads (Biospec Inc., Bartleseville, OK), placed in a mini-bead beater (Biospec Inc., Bartleseville, OK) and homogenized for 3 minutes at maximum speed. After homogenization, the tube was centrifuged at 10,000 × g for 30 seconds. Supernatant was transferred into a new 2-mL microcentrifuge tube and 200 µL of ethanol (96-100%) was added, mixed by vortexing and the genomic DNA was extracted following the DNeasy kit protocol. For extraction of the DNA in sampled filters, the protocol was the same as described above for the microbial cells and spores suspension in solution.

The DNA purification from buccal swabs was done following the Spin protocol of the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA).

**Q-PCR Conditions**

Quantitative polymerase chain reaction primers for the detection and quantification of surrogate BW agents (*B. atrophaeus* and *S. marcescens*) used were the same as those used by Saikaly et al., 2007 (Table 3). Primers were obtained from Integrated DNA Technologies (Coralville, IA). The Q-PCR conditions in this study were the same as described by Saikaly et al., 2007. Real-time PCR was performed in a 25-µL reaction volume containing 12.5 µL of 2 × iQ SYBR Green Supermix (100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP, 50 U/mL iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, stabilizers) (Bio-Rad Laboratories, Hercules, CA), 0.5 µM of each primer, 5 µL of extracted DNA, and RNase-free sterile water to a final volume of 25 µL. Amplification was performed using the iQ5 real-time detection system (Bio-Rad Laboratories, Hercules, CA) using the following program: an initial denaturing step at 95$^{o}$C for 5 min, followed by 45 cycles of denaturation at 95 °C for 50 s, annealing at 63$^{o}$C for 50 s, extension at 72$^{o}$C for 45 s (Saikaly et al., 2007). Q-PCR assays with a threshold cycle (Ct) over 37 for *B. atrophaeus* spores, over 32 for *B. atrophaeus* vegetative cells and over 30 for *S. marcescens,* were considered negative. For each Q-PCR run, a negative (no template) control was used to test for false positives or contamination. The presence of nonspecific products or primer-dimers was confirmed by observation of a single melting peak in a melting curve analysis using the iCycler iQ5 optical system software v1.0.

Table 3. Q-PCR primers used in this study (Saikaly et al., 2007)

| Target gene | Primers[a] | Primer sequence(5'-3') | $T_m$[b] (°C) | Accession no. (Position on gene)[c] |
|---|---|---|---|---|
| *B. atrophaeus spacer region* | ITS_F<br>ITS_R | CATTCGATTCTTCGAGATG<br>GGTCTTACTTTTGAATGTGATGTC | 48<br>52 | AF478080<br>(259-333) |
| *S. marcescens gyrB gene* | *gyrB*_F<br>*gyrB*_R | AGTGCACGAACAAACTTACAG<br>GTCGTACTCGAAATCGGTCACA | 53<br>57 | AJ300536<br>(113-251) |

[a] Primer abbreviations: F = forward primer, R = reverse primer.
[b] Theoretical melting temperature calculated using the OligoAnalyzer 3.0 from Integrated DNA Technologies (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx; Integrated DNA Technologies, Coralville, IA).
[c] Positions of genes are given according to the accession numbers.

**Q-PCR standard curves**

Standard curves were constructed using serial dilutions of cells and spores suspensions spiked into PBS, filters, and swabs to determine the quantification and limit detection of the Q-PCR assays. Quantification by this method corrects for PCR inhibition and for losses in cell lysis and target DNA during extraction, assuming that PCR inhibition and losses of sample DNA and standard DNA during extraction are consistent (Saikaly et al., 2007). Overnight cultures of *B. atrophaeus* ATCC 9372 vegetative cells and *S. marcescens* ATCC 13880 cells were grown in nutrient broth as described previously and harvested by centrifugation at 4,000 x g for 10 min and washed three times with 1 x PBS. The final cell pellets were suspended in 1 x PBS and the cell

count was determined using the plate count method. Triplicate counts were performed for each sample. The serial dilutions for spiking experiments were based on those counts (Saikaly et al., 2007). *B. atrophaeus* spores ATCC 9372 purified suspension (10 mL, 1.7 x $10^9$ spores/ mL) obtained from NAMSA laboratories (Northwood, OH) were used in serial dilutions.

*B. atrophaeus* (cells and spores) and *S. marcescens* cell suspensions were first placed in increasing concentrations from $10^1$ to $10^8$ per mL into 2 mL centrifuge tube. Cells and spores were harvested by centrifugation; the liquid was decanted and DNA extraction protocol described above was followed. In the case of the filters and swabs, the increasing concentrations of cells or spores were placed first in the 2 mL centrifuge tube and subsequently applied to the filters or swabs. After centrifugation, 800 μL of the liquid was decanted and the protocol for extracting DNA was followed.

**Data Analysis**

Data collection and analysis for DustTrak Aerosol Monitor (Model 8520) were performed using TrakPro Data Analysis software (TSI Inc., Shoreview, MN). Data collection and analysis for Q-PCR was performed using the iQ5 optical system software v1.0 (Bio-Rad Laboratories, Hercules, CA). All statistical analyses were performed using Microsoft Excel 2003.

The calculation of the number of cells or spores per mL of PBS (suspended solution, filters and swabs) was performed using the equation presented below. The number of cells or spores per PCR reaction mix was calculated from the appropriate standard curve based on the Ct values. Each PCR reaction mix contained 5 µL of a DNA preparation of 150 µL extracted from 1 mL of suspended solution filters and swabs respectively.

Cells or spores per mL of PBS:

$$= \frac{(cells\ or\ spores\ per\ PCR\ rxn)(volume\ of\ extracted\ DNA,\ \mu L)}{(5\ \mu L\ DNA\ per\ PCR\ rxn)(mL\ of\ PBS\ used)}$$

**Results and Discussion**

**Limits of detection, linear range and quantification of the Q-PCR assays in filters, swabs and PBS samples spiked with surrogates**

To evaluate extraction efficiency in the experiments, standard curves were constructed for the quantification of *B. atropheaus* (vegetative cells and spores) and *S. marcescens* cells. Specific concentrations of the surrogate cell suspension were spiked into PBS, filters and swabs as described previously. The linear amplification ranges for each curve as well as equations for the curves are presented in Table 4.

Table 4. Quantitative amplification parameters for *B. atrophaeus* and *S. marcescens* in PBS, filters and swabs

| Q-PCR assay | Sample | Target | Amplification parameters[a] | | | Linear range (number of cells or spores per PCR reaction/ mL) |
|---|---|---|---|---|---|---|
| | | | Standard curve | E | $R^2$ | |
| ITS | PBS | *B. atrophaeus* vegetative cells[b] | y = -3.2398 log(x) + 35.756 | 1.03 | 0.99 | $3.60 \times 10^{1} - 3.60 \times 10^{8}$ |
| | | *B. atrophaeus* spores[b] | y = -2.9948 log(x) + 42.547 | 0.94 | 0.98 | $1.50 \times 10^{1} - 1.50 \times 10^{8}$ |
| | Filters | *B. atrophaeus* vegetative cells | y = -3.5612 log(x) + 33.744 | 0.87 | 0.99 | $3.60 \times 10^{0} - 3.60 \times 10^{6}$ |
| | | *B. atrophaeus* spores | y = -3.0805 log(x) + 38.034 | 0.87 | 0.99 | $5.00 \times 10^{0} - 5.00 \times 10^{6}$ |
| | Swab | *B. atrophaeus* vegetative cells | y = -3.3005 log(x) + 31.338 | 0.98 | 0.99 | $3.60 \times 10^{0} - 3.60 \times 10^{6}$ |
| | | *B. atrophaeus* spores | y = -3.6618 log(x) + 38.677 | 0.87 | 0.99 | $5.00 \times 10^{0} - 5.00 \times 10^{6}$ |
| *GyrB* | PBS | *S. marcescens*[b] | y = -2.543 log(x) + 31.313 | 0.98 | 0.99 | $1.32 \times 10^{1} - 1.32 \times 10^{8}$ |
| | Filters | *S. marcescens* | y = -2.5831 log(x) + 28.596 | 1.03 | 0.99 | $4.40 \times 10^{0} - 4.40 \times 10^{6}$ |
| | Swab | *S. marcescens* | y = -2.7356 log(x) + 30.483 | 1.03 | 0.98 | $4.40 \times 10^{0} - 4.40 \times 10^{6}$ |

[a] Each parameter is an average of triplicate standard curves. [b] Standard curve constructed using the number of cells or spores per mL.

Amplification parameters for *B. atropheaus* vegetative cells using ITS Q-PCR assay and spiked into PBS, were optimal with linearity ($R^2$ >0.99) over an 8-log dynamic range, with an amplification efficiency of 1.03 and detection limit of 3.6 x $10^1$. The limit, if detected, corresponds to the smallest mumber of cells per PCR reaction resulting in a positive amplification in all replicate reactions (Saikaly et al., 2007). For the cases where *B. atropheaus* was spiked into the filters and swabs, the amplification was also optimal ($R^2$ >0.99), linearity was over a 7- log dynamic range and efficiency ranging between 0.87 and 0.98). *S. marcescens* and *B. atropheaus* spores reported optimal amplification parameters (See Table 4).

To assess the variability of Q-PCR assays for the replicate standard curves, paired sample t- tests were used and the results showed that the difference in the slopes and y – intercepts were not statistically significant ( P>0.05). All Q-PCR assays tested negative for the non- spiked samples, indicating that *B. atropheaus* and *S. marcescens* were not naturally present during the construction of the standard curves.

The amplification parameters for *B. atropheaus* vegetative cells and spores in PBS samples were different. This suggests that the hot detergent treatment followed by bead beat homogenization was not optimal. The higher sensitivity (lower y-intercept) for *B. atropheaus* vegetative cells in comparison with spores for PBS, filters and swabs could suggest also the inefficiency of the method. Saikaly et al., 2007 evaluated several

methods for lysing and reported that all the methods were equally effective in lysing *B. atropheaus* spores. In the present study, the method mentioned above was used to extract DNA from *B. atropheaus* cells and spores.

A major limitation of Q-PCR is the occurrence of false–negative results due to the presence of PCR inhibitors in environmental samples (Saikaly et at., 2004). The development of standard curves by spiking known amounts of surrogate BW agents into the PBS, filters and swabs contributed to correct possible PCR inhibition and quantified losses due to cell lysis and DNA extraction.

**Measurement of particles and microorganisms aerosolized in the chamber**

Initial work was performed to identify the most effective conditions for aerosolization of the surrogate BW agents selected for study. Particle concentrations were measured in the chamber using an aerosol monitor DustTrak (Model 8250) to evaluate the possible impact of the environment and liquid composition in the aerosolization process. The first two tests measured the particle concentration from ambient air concentration and tap water and results ranged from 0.003 to 0.004 mg/ $m^3$and from 0.017 to 0.50 mg/ $m^3$, respectively. From these results we could infer that the particle concentrations from ambient air and water supply would not have a significant impact during the aerosolization of the surrogates. PBS was aerosolized (Test 3) as a standard to gain a

better insight of the amount of particles that would be released. The particle concentrations ranged from 1.8 to 40.4 mg/ $m^3$ during aerosolization. Two diluted concentrations of 1 x PBS (Tests 4 and 6) were also aerosolized to allow comparison of the release of the surrogate BW agents when they were suspended in these solutions. The dilution of PBS was considered because higher particle concentrations aerosolized could affect the release of the surrogates and avoid agglomeration. Particle concentrations from aerosolizations of *S. marcescens* suspended in two different concentrations of PBS (Test 5 and 7) are presented in Figure 2 and in Table 1.

The average difference in the readings between *S. marcescens* that was suspended in 10 fold dilution of PBS and 10 fold dilution of PBS without mixing (Tests 4 and 5) was 3.93 $mg/m^3$. Also, in the case where *S. marcescens* was suspended in 50 fold dilution of PBS and 50 fold dilution of PBS only (Tests 6 and 7), the average difference in the readings was 3.07 $mg/m^3$. Student's t-test showed that the difference between the means (Test 5 – Test 4 and Test 7 – Test 6) were significantly different ($P < 0.01$). Based on these experiments, surrogate BW agents were suspended in 10 fold dilution PBS for the succeeding trials. *S. marcescens* was selected for these initial tests because of its smaller size in comparison to *B. atrophaeus* thus providing better results in density measurements.

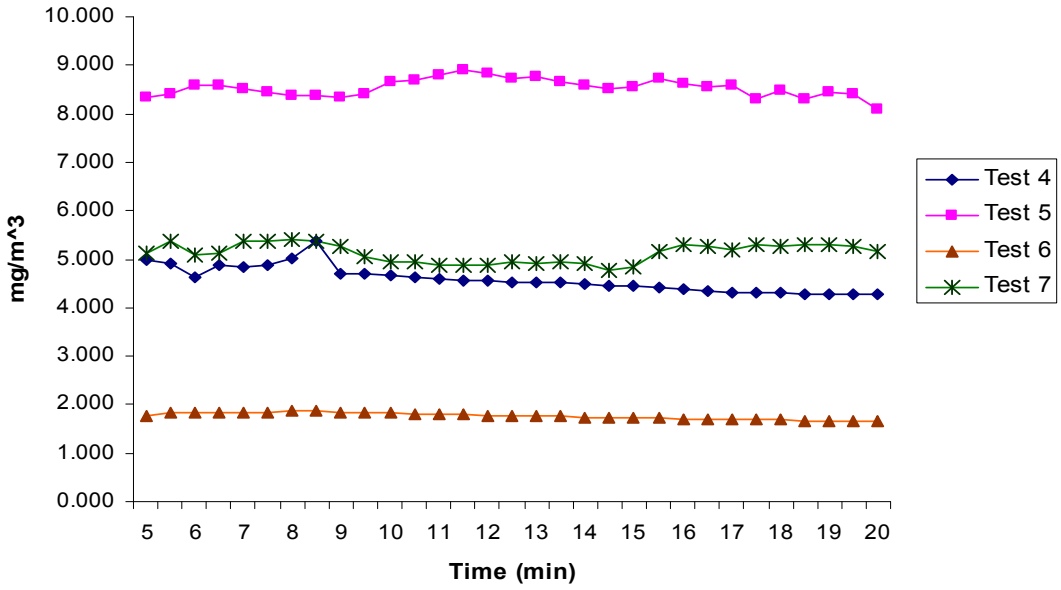


Figure 2. Comparison of total particle concentration of *S. marcescens* suspended in 10 fold dilution PBS and 50 fold dilution PBS at steady state of 5 minutes (30 second measurements) using Dust Trak aerosol monitor. Test conditions given in Table 1.

**Release and recovery of aerosolized surrogate biological warfare agents suspended in PBS**

Three different concentrations of *B. atrophaeus* cells and *S. marcescens* were aerosolized inside the chamber to evaluate the sensitivity of the detection method while maintaining the flow and pressure as described previously. The results from these trials showed that as the concentration of *B. atrophaeus* cells increased, the percentage recovery decreased, therefore a concentration of $10^4$ cells per mL was selected for this part of the experiment (see Table 5). In the case of *S. marcescens*, recovery percentages

were inconsistent with concentrations (0.5 to 30% recovery). A concentration of $10^3$ cells per mL was chosen for *S. marcescens* to maintain similarity with *B. atrophaeus* concentrations.

Table 5. Recovery of *B. atrophaeus* cells and *S. marcescens* cells at different concentrations using gelatin filter during nebulization

| BW agents solution | Initial Concentrations (cells/mL of PBS | Total initial cells in suspended solution[a] | Total cells remaining in suspended solution after nebulization[b] | Total cells recovery from filters | Total Recovery[c] |
|---|---|---|---|---|---|
| *B. atrophaeus* cells | 1.20E+08 | 4.55E+09 | 3.08E+09 | 4.14E+07 | 2.81% |
| *S. marcescens* | 2.57E+07 | 9.76E+08 | 8.23E+08 | 1.81E+07 | 11.85% |
| *B. atrophaeus* cells | 7.05E+08 | 2.68E+10 | 1.84E+10 | 6.02E+07 | 0.71% |
| *S. marcescens* | 1.90E+07 | 7.22E+08 | 6.01E+08 | 1.00E+07 | 8.31% |
| *B. atrophaeus* cells | 7.58E+06 | 2.88E+08 | 1.80E+08 | 6.02E+06 | 5.59% |
| *S. marcescens* | 9.28E+05 | 3.53E+07 | 2.85E+07 | 1.84E+06 | 27.05% |
| *B. atrophaeus* cells | 4.95E+06 | 1.88E+08 | 1.31E+08 | 8.29E+06 | 14.48% |
| *S. marcescens* | 6.87E+05 | 2.61E+07 | 8.46E+06 | 9.45E+04 | 0.54% |
| *B. atrophaeus* cells | 9.34E+04 | 3.55E+06 | 2.97E+06 | 4.18E+04 | 7.24% |
| *S. marcescens* | 1.00E+04 | 3.81E+05 | 3.30E+05 | 1.59E+04 | 30.81% |
| *B. atrophaeus* cells | 7.90E+04 | 3.00E+06 | 2.56E+06 | 9.67E+04 | 21.74% |
| *S. marcescens* | 7.42E+03 | 2.82E+05 | 2.15E+05 | 6.51E+03 | 9.68% |

[a] the total initial cells for *B. atrophaeus* and *S. marcescens* cells was calculated considering a initial volume of 38 mL. [b] The final volume used to calculate total cells remaining in suspended solution include the addition of 8 mL of sterile water to wash out any possible cells attached to the nebulizer and a correction of 0.37 mL of possible volume lost due to pippeting. [c] Total recovery percentage was calculated from the ratio between the total cells recovery from filter during nebulization over the difference between the total initial cells suspended in solution and the total cells remaining in solution after nebulization.

A set of trials was performed and the quantification of the initial suspended solution was calculated using Q-PCR to validate the effectiveness of the system in the aerosolization of the surrogate BW agents. The percentage of cells released in each trial was determined with the following equation:

$$= \frac{\left(\begin{array}{c}\textit{Total initial cells or spores in} \\ \textit{suspended solution}\end{array}\right) - \left(\begin{array}{c}\text{Res}\textit{idual volume} * \quad \textit{Cell concentration remaining} \\ \textit{in nebulizer}\end{array}\right)}{\left(\textit{Total initial cells or spores in suspended solution}\right)}$$

The average release of *B. atrophaeus* vegetative cells was 23.9% and 40.1% when it was suspended with *S. marcescens* cells. For *B. atrophaeus* spores, the average release was 46.0%, and 28.9 % when suspended with *S. marcescens*. Finally, the average release for *S. marcescens* was 40.6%, and 89.8% when mixed with *B. atrophaeus* cells, and 36.1% when mixed with *B. atrophaeus* spores (see Table 6).

The volume of the residual suspended solution left in the nebulizer bottle varied with each run although the set ups for each run during the trials were constantly monitored. A 10 mL pipette was used to quantify the volume of the residual suspended solution after conclusion of each run. This technique however could cause a variation in the quantification residual suspended solution due to the loss of volume that is left inside of the pipette. The loss of this volume due to pipetting was measured and a correction of 0.37 mL was added to the residual suspended solutions volume in each run for calculation purposes. The calculations of the total cells/spores remaining in the suspended

solution are very sensitive with the respect to the final volume (residual suspended solution added with RNase-free sterile water and the loss of volume for pipetting) which could be a possible reason for the high variation in the percentages of release of the surrogate BW agents.

As discussed previously, a quick- connect valve fitting was used to preserve mass balance and avoid back pressure. As a result, some cells were lost through opening # 2 (Fig. 1) and this loss was not quantified. Rather, this loss was estimated by assuming a homogeneous suspension of the released surrogate BW agents during nebulization.

To calculate the amount of cells lost through the valve the following equation was used;

$$= \frac{\begin{pmatrix} Total\ \ cells\ or\ spores \\ re\,\mathrm{cov}\,ered\ \ from\ \ filters \end{pmatrix} * \begin{pmatrix} Flow\ rate\ \ through \\ connected\ \ valve \end{pmatrix} * \begin{pmatrix} Time\ used\ during \\ nebulizati\ on \end{pmatrix}}{\begin{pmatrix} Total\ \ flow\ rate\ \ used \\ by\ the\ two\ sampler\ \ pumps \end{pmatrix} * \begin{pmatrix} Total\ operation\ \ \ time\ of \\ sampler\ \ pumps\ \ during\ \exp eriment \end{pmatrix}}$$

Total amount of cells lost through the valve are presented in Table 6. Percentage recovery and concentrations profile (duplicate runs per trial) of *B. atrophaeus* vegetative cells, *S. marcescens*, *B. atrophaeus* spores and the combination of *B. atrophaeus* cells with *S. marcescens* and *B. atrophaeus* spores with *S. marcescens* are presented in Table 5. The equation below was used to calculate the percentage recovery without accounting for the losses through connected valve

$$= \frac{\left(\begin{array}{c}Total\ \ cells\ \ or\ \ spores \\ re\,\mathrm{cov}\,ered\ \ from\ \ filters\end{array}\right) + \left(\begin{array}{c}Total\ \ cells\ \ or\ \ spores\ \ \mathrm{det}\,ected\ \ in \\ chamber\ \ walls\end{array}\right)}{\left(\begin{array}{c}Total\ \ cells\ \ or\ \ spores\ \ in \\ suspended\ \ solution\end{array}\right) - \left(\begin{array}{c}Total\ \ remain\ \ cells\ \ or\ \ spores\ \ in \\ suspended\ \ solution\end{array}\right)} * \ \ 100$$

In the first trial, the recoveries of the aerosolized *B. atrophaeus* vegetative cells (single suspension) were 23.8% and 23.7%. However, it was noticeable that when *B. atrophaeus* and *S. marcescens* mixed suspended solution was aerosolized, the percentage recoveries for *B. atrophaeus* decreased to 8.5% and 9.3%. In the case of *B. atrophaeus* spores (single suspension), the percentage recoveries for Trial 2 were 7.2% and 21.1% and when they were mixed with *S. marcescens* the recoveries were 11.9% and 59.6% (Trial 5). It could be suggested from these results that during the mixture of *B. atropheaus* with *S. marcescens,* the recovery of *B. atrophaeus* cells diminished in comparison with *B. atrophaeus* spores. A possible explanation could be the difference in the size of the cells and spores. *B. atrophaeus* spores are smaller than the vegetative cells therefore they could continue to be suspended easily and able to be captured by the filter during the impact force process.

The percentage recoveries for Trial 3, *S. marcescens* in single suspension, were 13.4% and 17.2%. In Trial 4 (*S. marcescens* mixed with *B. atrophaeus* vegetative cells) and Trial 5 (*S. marcescens* mixed with *B. atrophaeus* spores), the recoveries for *S. marcescens* were 32.0%, 21.9%, 45.0% and 53.8%.

It is not clear why there is a high variability in the percentage recoveries; however a possible explanation could be the electrostatic charge of the cells during aerosolization in the chamber. Aerosol particles dispersed by nebulization are usually electrostatically charged and if these are in high levels it contributes to particle loss through the sampling system (chamber walls) which could affect the filter efficiency measurements. Several studies have pointed out this issue (Griffiths et al., 1993; Willeke and Baron et al., 1993; Weis et al., 2002; Krauter and Biermann et al 2007;) and Mainelis (2001) has stated that the electrical charge of airborne particles is one parameter that is often overlooked. Electron microscopy of *Escherichia coli* and other Gram-negative bacterial cells has revealed the presence of three distinct layers in the cell walls. The innermost layer is a cell membrane which is surrounded by a rigid peptidoglycan layer.

The outermost layer (outer membrane) contains lipopolysaccharides, phospholipids and lipoproteins and is about 80 Å thick (Sherbet & Lakshmi et al., 1973; Mainelis et al., 2001). The coat of a Gram-positive endospore such as *Bacillus subtilis var niger*, is organized in three layers: an amorphous undercoat; a lamellar inner structure; and a striated electron-dense outer coat (Henriques & Moran et al., 2000; Mainelis et al., 2001) and some evidence suggests that this outer layer has distinct properties from the rest of the structure. According to Shebert and Lakshmi et al. (1973), ionizable groups of the polysaccharide-phospholipid- protein complex occur in the 60 Å thick isoelectrical zone of the outermost layer. Therefore, it has been suggested that the electric charge on the

surface of a bacterial cell is attributable to a large extent to the kind of ionizable groups present on the cell surface and to their spatial distribution (Mainelis et al., 2001). Weis et al. (2002) observed that *B. anthracis* spores deposited on the charged monitors, indicating the influence of electrostatic effects on spore behavior.

As it was previously referred, the estimated cells or spores lost through the fitting valve for *B. atrophaeus* vegetative cells, *S. marcescens* cells and *B. atrophaeus* spores were calculated for each trial. These amount of cells or spores were used in the equation presented bellow to calculate a new percentage recovery (Table 5) and to evaluate the possible increment of the total recovery in the experiment.

$$= \frac{\left(\begin{matrix}\text{Total cells or spores} \\ \text{recovered from filters}\end{matrix}\right) + \left(\begin{matrix}\text{Total cells or spores detected in} \\ \text{chamber walls}\end{matrix}\right) + \left(\begin{matrix}\text{Estimate of cells / spores} \\ \text{lost through fitting valve}\end{matrix}\right)}{\left(\begin{matrix}\text{Total cells or spores in} \\ \text{suspended solution}\end{matrix}\right) - \left(\begin{matrix}\text{Total remain cells or spores in} \\ \text{suspended solution}\end{matrix}\right)} * 100$$

The increment of the percentage recovery for Trial 1, *B. atrophaeus* vegetative cells, was around 2% and for Trial 2 (*B. atrophaeus* spores) was 0.4% and 2%. In the case of *S. marcescens* single suspension (Trial 3), the increment in recovery fluctuated between 0.02% and 0.05%. In Trial 4 (*S. marcescens* mixed with *B. atrophaeus* vegetative cells) and Trial 5 (*S. marcescens* mixed with *B. atrophaeus* spores), the increment of recovery for *B. atrophaeus* cells was 0.6% and 0.8%, for *B. atrophaeus* spores was 1% and 1.8% and for *S. marcescens* ranged between 0.02% and 6.1%.

Table 6. Initial concentrations and mass balance of surrogate BW agents aerosolized in chamber

| | BW agents solution | Initial concentrations (cells/mL or spores/ml of PBS | Total cells or spores in suspended solution | Total remaining cells or spores in suspended solution | Average release of cells or spores[a] | Total cells or spores recovered from filters | Total cells or spores detected on chamber walls | Estimate of total cells or spores lost through the valve[c] | Total cells or spores not detected[d] | Total recovery[e] | Total recovery considering estimate cells or spores lost through the valve[h] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Trial # 1 | *B. atrophaeus* vegetative cells | 3.15E+04 | 1.23E+06 | 1.04E+06 | 23.89% | 3.56E+04 | 8.37E+03 | 4.03E+03 | 1.41E+05 | 23.82% | 26.01% |
| | *B. atrophaeus* vegetative cells | 1.01E+04 | 3.94E+05 | 2.65E+05 | | 2.17E+04 | 8.81E+03 | 2.46E+03 | 9.84E+04 | 23.65% | 25.56% |
| Trial # 2 | *B. atrophaeus* spores | 1.26E+04 | 4.93E+05 | 1.72E+05 | 46.03% | 9.82E+03 | 1.34E+04 | 1.11E+03 | 2.98E+05 | 7.23% | 7.57% |
| | *B. atrophaeus* spores | 3.54E+04 | 1.38E+06 | 1.01E+06 | | 6.94E+04 | 8.95E+03 | 7.87E+03 | 2.93E+05 | 21.09% | 23.21% |
| Trial # 3 | *S. marcescens* | 4.50E+03 | 1.75E+05 | 8.62E+04 | 40.61% | 1.60E+02 | 1.18E+04 | 1.81E+01 | 7.72E+04 | 13.41% | 13.43% |
| | *S. marcescens* | 3.96E+03 | 1.55E+05 | 1.08E+05 | | 1.98E+02 | 7.88E+03 | 2.25E+01 | 3.88E+04 | 17.22% | 17.27% |
| Trial # 4 | *B. atrophaeus* vegetative cells + S. marcescens | 1.84E+04 | 6.98E+05 | 4.33E+05 | 40.08% | 1.37E+04 | 8.95E+03 | 1.55E+03 | 2.43E+05 | 8.53% | 9.11% |
| | | 1.00E+03 | 3.80E+04 | 4.35E+03 | | 1.76E+02 | 1.06E+04 | 2.00E+01 | 2.29E+04 | 32.01%[f] | 32.07%[i] |
| | *B. atrophaeus* vegetative cells + S. marcescens | 2.25E+04 | 8.55E+05 | 4.95E+05 | 89.81%[b] | 2.49E+04 | 8.49E+03 | 2.82E+03 | 3.27E+05 | 9.26% | 10.05% |
| | | 1.74E+03 | 6.63E+04 | 5.91E+03 | | 1.05E+02 | 1.31E+04 | 1.19E+01 | 4.71E+04 | 21.88%[f] | 21.90%[i] |

Table 6 (Continued)

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Trial # 5 | *B. atrophaeus* spores + | 1.90E+04 | 7.23E+05 | 3.32E+05 | 28.88% | 3.56E+04 | 1.09E+04 | 4.03E+03 | 3.45E+05 | 11.88% | 12.91% |
| | S. marcescens | 1.21E+03 | 4.60E+04 | 1.58E+04 | | 2.53E+03 | 1.11E+04 | 2.87E+02 | 1.66E+04 | 45.09%[g] | 46.04%[j] |
| | *B. atrophaeus spores +* | 1.04E+04 | 3.94E+05 | 3.80E+05 | 36.12%[b] | 2.31E+03 | 6.23E+03 | 2.62E+02 | 5.79E+03 | 59.59% | 61.42% |
| | *S. marcescens* | 2.35E+03 | 8.93E+04 | 8.35E+04 | | 3.13E+03 | N/D | 3.54E+02 | 2.68E+03 | 53.80%[g] | 59.90%[j] |

[a] Release percentages were calculated from the ratio between the difference of total cells/spore in suspended solution and total remain cells/spores in suspended solution over total cells/spore in suspended solution . [b] Average release of *S. marcescens* cells when it is mixed with *B. atrophaeus*. [c] Calculated from the ratio of total cells or spores recovered from filter multiplied by flow rate released through connected valve (1.7 L/min) and the time used during nebulization (20 min) over the product of the total flow rate used by the two sampler pumps (10L/min) and total operation time of sampler pumps during experiment (30 min) and assuming a homogeneous aerosol suspension leaving the valve. [d] Calculation does not include the estimate cells or spores lost through the valve. [e] Calculated from the ratio between the addition of the total cells or spores recovered by the filter and total cells or spores detected on chamber walls over the total amount of cells or spores released during experiment. [f] Total recovery of *S. marcescens* cells in suspended solution mixed with *B. atrophaeus* vegetative cells. [g] Total recovery of *S. marcescens* cells in suspended solution mixed with *B. atrophaeus* spores. [h] Calculated from the ratio between the addition of the total cells or spores recovered by the filter, total cells or spores detected on chamber walls and the estimate cells or spores lost through the valve over the total amount of cells or spores released during experiment. [i] Total recovery considering the estimate cells or spores lost through fitting valve of *S. marcescens* cells in suspended solution mixed with *B. atrophaeus* vegetative cells. [j] Total recovery considering the estimate cells or spores lost through fitting valve of *S. marcescens* cells in suspended solution mixed with *B. atrophaeus* spores. N/D: No detection

As described previously, four specific areas inside the second compartment of the chamber were scraped with a sterile buccal cotton swab to determine the loss of cells/ spores on the chamber walls at the end of each run. The total cells/ spores in the chamber was calculated assuming the amount of cells/spores per square inch obtained from these four specific areas multiplied by the total surface area of the chamber. A mass balance was conducted for each trial to determine the percentage of cells/ spores that adhered to the walls (the difference between the total initial cells or spores in solution and the total remaining cells or spores in the solution after each run was considered as 100%). The average percentage recoveries for *B. atrophaeus* vegetative cells (single suspension) and spores (by itself) from the walls were 5.7 % and 3.3 %. When *B. atrophaeus* (vegetative cells) were mixed with *S. marcescens,* the average percentage was 2.9% and in the mix of *B. atrophaeus* spores with *S. marcescens* the average percentage was 23.1%. In the case of *S. marcescens* (single suspension), the average percentage recovery from the walls was 15.0% and when it was mixed with *B. atrophaeus* vegetative cells and spores the recoveries were 26.6% and 18.6%, respectively. These results indicate that a significant percentage of *B. atrophaeus* (cells and spores) and *S. marcescens* were not recovered.

A number of experiments and studies have previously indicated the capacity of bacteria to adhere to different surfaces. Adhesion of bacteria spores (Hüsmark and Rönner et al., 1992, 1993) and vegetative cells (Texeira and Oliviera et al., 1999; Sinde and Carballo et al., 2000) was shown to increase with hydrophobic surfaces. Bacterial adhesion to inert surfaces is generally believed to consist of successive steps involving:

(a) physicochemical interactions, including van der Waals', Lewis acid-base hydrophobic and electrostatic interactions; and (b) adhesion reinforcement through polymer excretion (Flint et al., 1997; Faille, Jullien and Fontaine et al., 2002). Buchard et al., (1990) also showed that adhesion strength was affected by the material's hydrophobicity and Faille et al., (2002) after analyzing the properties of adhesion of *Bacillus* spores and *E. coli* through six materials with very different surface properties, concluded that hydrophobic interaction seemed to be a major influence in the bacterial adhesion. Saikaly et al., (2010) evaluated the hydrophobicity of *B. atrophaeus* (vegetative cells and spores) and *S. marcescens* and from the data it was suggested that *B. atrophaeus* spores are relatively hydrophobic, *S. marcescens* cells are relatively hydrophilic, and *B. atrophaeus* vegetative cells showed an intermediated hydrophobicity. Since the same bacterial strains and growing conditions were used in the present experiment, it could be suggested that hydrophobicity interactions influenced the recovery of the target surrogates using gelatin filters. Also, the lower percentage recovery of the cells/ spores found on the walls for *B. atrophaeus* (spores and cells) in comparison with *S. marcescens* affected by this factor.

The method used to obtain cells and spores from the chamber walls could be another possible explanation of the lower percentage recovery and the significant percentage of the undetected target surrogates during this experiment. Sanderson et al. (2002) reported that the sampling method using a dry swab performed poorly in the detection of *B. anthracis* spores (failing to detect spores >66% of the time) in comparison with other methods like a vacuum sock sample used on non porous surfaces. Based on these results,

they stated dry swabs are not the most recommended method to sample for *B. anthracis* environmental contamination. Aerosolization deposits spores more uniformly across smooth surfaces like glass or polycarbonates and their recovery depends on the interaction of individual spores sticking to the swab fibers, leading to lower recovery rates. Therefore, decreased recovery at lower spore densities could be explained by the poor adhesion of the biological material to the swabbing materials (Edmonds et al., 2009) and strong attachment to the wall.

**Conclusions**

- Aerosolization of the surrogate BW agents in the system was achieved but release percentages were variable depending of the surrogate. The average release of *B. atropheaus* vegetative cells was 23.9% when tested alone and 40.6% when it was mixed with *S. marcescens*. For *B. atrophaeus* spores the average release was 46.0% and when it was mixed with *S. marcescens* was 28.9%. In the case of *S. marcescens* average releases when they were in single suspension, mixed with *B. atrophaeus* vegetative cells and mixed with *B. atrophaeus* spores were 40.6%, 89.8% and 36.1%, respectively.

- The total recovery of the surrogate BW agents when they are aerosolized from a suspended solution was variable, ranging for *B. atrophaeus* vegetative cells from 8.5% to 23.8%; *B. atrophaeus* spores from 7.2% to 59.6% and for *S. marcescens* from 13.4% to 53.8%.

- Surrogate BW agents were detected on chamber walls, and adhesion of cells/ spores to the walls possibly due to ionic charges during nebulization limited the efficiency of collection.

- The fraction of cells or spores estimated to have been lost through the chamber opening was between 0.02% and 2% of the cells recovered on the filters plus chamber walls.

# DETECTION AND QUANTIFICATION OF AEROSOLIZED SURROGATE BIOLOGICAL WARFARE AGENTS IN SIMULATED LANDFILL GAS MIGRATION

## Objective

The objective of this stage of the study was to detect and quantify the aerosolized surrogate biological warfare agents (*B. atrophaeus* and *S. marcescens*) spiked onto the surface of SBD as present in the gas phase in response to simulated landfill gas migration and remaining on the SBD.

## Material and Methods

### Bacterial strains and growth of pure culture

Bacterial strains and growth conditions were the same as described in the previous chapter. The initial densities of cells in the mixed suspended solution used in this part of the experiment are presented in Tables 7 and 8.

Table 7. Initial amount of cells in solution poured into the SBD for bioaerosol experiment (Trial 1)

| | Initial amount of cells in solution ( 598 mL) |
|---|---|
| ***Bacillus atrophaeus* vegetative cells** | 1.06E+10 |
| ***Serratia marcescens*** | 1.10E+10 |

Table 8. Initial amount of cells/spores in solution poured into the SBD for bioaerosol experiment (Trial 2)

| | Initial amount of cells in solution ( 598 mL) |
|---|---|
| ***Bacillus atrophaeus (spores)*** | 3.77E+11 |
| ***Serratia marcescens*** | 1.10E+10 |

**Experimental Setup**

The experiment was conducted in the previously described aerosol chamber with some modifications. A removable stainless steel sheet was installed between the two compartments to support the SBD, and three ¼" tubes with eleven holes, each with a diameter of 0.3 mm, were placed in the nitrogen inlets. Half of the first compartment (around 8.8 L) in the aerosol chamber was filled with synthetic building debris (SBD). At the end wall, located in the second compartment of the aerosol chamber, gelatin filters were placed inside the SKC button sampler for filtration of the gas.

For aerosolization, a 598 mL mixed suspended solution (*B. atrophaeus* and *S. marcescens* vegetative cells in 10 fold dilution of 1 x PBS) was spiked onto the surface of

the SBD. To apply the suspension, a 50 mL syringe was used to spray the cells over the surface of SBD using back and forth movements and from the right to left. Three perforated tubes inserted into the SBD (almost 2 cm from the bottom base of the aerosol chamber) were fed with $N_2$ at 14.7 psig and four specific flow velocities simulating the migration of landfill gas were applied flow rates through a porous media.

Dr. Paul Imhoff at the University of Delaware developed a simulation of gas flow in a landfill to estimate the range of velocities that could be expected in response to a gas collection system. The modeled pumping scenario assumed a gas flow rate at the well of 100 $ft^3$/min (CFM) or 2.83 $m^3$/min. The average and maximum velocities in the flow field at this pumping rate were estimated at 3.4 x $10^{-5}$ and 6.75 x $10^{-3}$ m/sec, respectively. To calculate the flows utilized for this experiment (Table 8), the present study focused on the maximum velocity which represented a worst case for microbe aerosolization. This velocity was considered as the velocity at the surface of the SBD surface placed in the chamber (A = 464.5$cm^2$). The gas flow rates selected in this study were calculated assuming that SBD had a porosity of 0.35 as follows:

$$= \begin{pmatrix} Surface\ \ area\ \ of\ \ SBD \\ placed\ \ in\ \ chamber\ ,cm^2 \end{pmatrix} * (Selected\ \ velocity\ ) * \begin{pmatrix} Selected\ \ porosity \\ of\ \ the\ \ SBD \end{pmatrix}$$

This results in a flow of 6.6 L/min. The flows tested were 50%, 100%, 200% and close to 400% of this value. The highest gas flow rate was set to the maximum capacity of the rotameter which was slightly less than four times the base flow rate of 6.6 L/min.

Table 9. Set of flow rates to aerosolize surrogate BW agents spiked on the surface of the SBD

| | **L/min** | **Duration of $N_2$ flow (min)** |
|---|---|---|
| Flow 1 | 3.4 | 23 |
| Flow 2 | 6.6 | 12 |
| Flow 3 | 13.2 | 6 |
| Flow 4 | 25.3 | 3 |

The time set for feeding the $N_2$ gas into the SBD contained in the chamber for each flow was calculated considering the time that would be required to saturate three times the free volume remaining in the chamber with the selected flow. These conditions were established to assure total saturation and transport of the aerosolized surrogates inside the chamber. To measure equally distributed feeding flows of the $N_2$ through experiments, rotameters were installed at each line connected to the three ¼" perforated tubes. The flow per tube was one third of the flows listed in Table 9.

Two SKC universal sampling pumps (Model PCXR 8), with average flows of 4 L/min each, were connected to the outlets of the button samplers (end wall) and run simultaneously to avoid back pressure inside the chamber and preserve mass balance. An additional opening was set in the second compartment of the aerosol chamber to balance the remaining inflow; however, to avoid any possible loss of aerosolized surrogates a gelatin filter was placed across this opening.

The $N_2$ tank was closed and SKC universal sampling pumps were stopped after the conclusion of the time set for the first flow velocity. A plexiglass door was slid to

separate the two compartments, and the gas tight seal was activated to assure the isolation between compartments. The gelatin filter located on the side wall of the chamber was removed, placed into a 50 mL centrifuge tube and stored at -20ºC for subsequent DNA extraction and Q-PCR.

A vacuum, using the SKC universal sampling pumps at 4 L/ minute each, was induced in the second compartment to remove any remaining aerosolized surrogate for 7 minutes (time required to assure the pumping of two times the volume in the second compartment) while keeping the same gelatin filter placed inside of the button samplers. The fan, located in the second compartment, was maintained at slow speed the entire time to suspend aerosolized surrogates. After 7 min, the gelatin filters inside the button samplers were removed, placed in a labeled 50 mL centrifuge tube and stored for subsequent work. The same sequence was performed for the other three flow velocities.

After all four experiments, the SBD was sampled to quantify BW surrogates remaining on the solids. The SBD placed in the first compartment was divided into three approximately similar layers (6.35 cm thick), weighed, and stored in -20ºC for DNA extraction. An experiment using the mix of *B. atrophaeus* spores and *S. marcescens* was performed in the same manner as it described above (See Figure 3). After each experiment, the chamber was sprayed and subsequently aerosolized for 20 minutes with 70% ethanol. All removable parts inside the chamber and SKC button samplers were

also sprayed with ethanol and sterilized in UV light for 3hours. The chamber was not used for 24h between experiments.

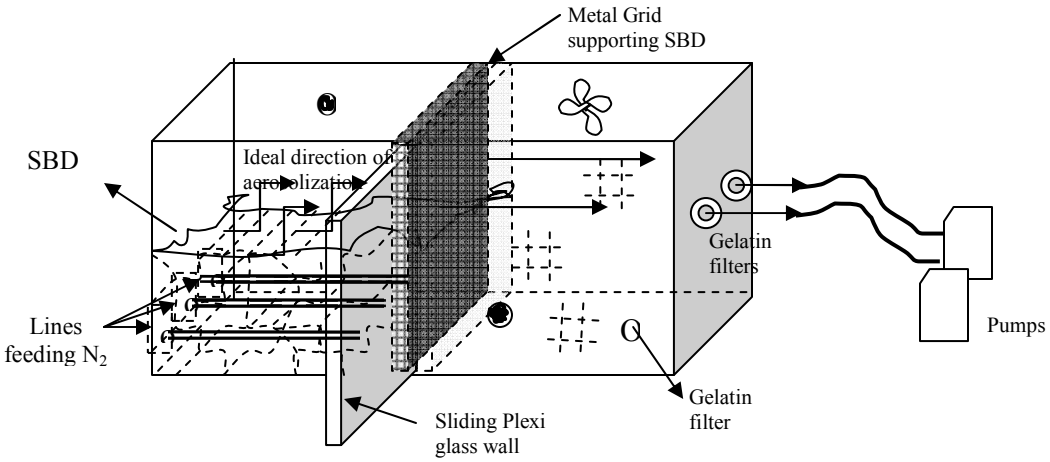


Figure 3. Aerosolization of surrogate BW agents simulating landfill gas mitigation through the SBD

**SBD Composition**

The synthetic building debris was developed based on the documentation for the Building Decontamination Residue (BDR) Disposal Decision Support Tool provided by the EPA. The SBD composition was as follows (number in parenthesis represents the percentage in dry weight): ceiling tile (12.79), carpet (4.47), vinyl (0.78), electronics (5.56), furniture (33.68), white office paper (34.29), folders/cardboard (4.22), and mixed office paper (4.21). The SBD materials were shredded using a slow speed, high torque shredder and mixed in the proportions given above. The product particle size was typically on the order of 2 cm wide by 3 to 7 cm long, allowing the material to be well mixed prior to be used in the experiments (Saikaly et al., 2010).

The SBD was incubated under anaerobic conditions for about a year prior to use. Unfortunately, the volume of $CH_4$ was not measured due to leakage.

**Processing Method**

SBD samples were subjected to an indirect sample processing technique prior to DNA extraction as described by Staley et al., (2009), with a small modification as presented below.

Indirect cell separation method using Sucrose-TritonX-100

SBD used in the experiment was sliced in three layers each approximately 6.35 cm in depth. Each layer was homogenized in a Waring blender for 2 minutes. From each layer, a 250g wet weight subsample was combined with 600 mL of Sucrose-Triton X-100

(1.22 g mL-1 sucrose plus 0.5% Triton X-100) and homogenized in a Waring blender for 2 minutes. Sucrose – Triton X -100 was used as an alternative detergent/high specific gravity solution to improve the extraction of spores from the SBD and evaluate the recovery efficiency of vegetative cells (Dragon and Rennie et al., 2001; Ryu et al., 2003).

The blended mixture was poured into a 3.8 L 75-µm nylon paint strain bag (Trima Co.) and hand squeezed. The supernatant was collected in a 1.9-L sterile plastic container, transferred to sterile centrifuge tubes and centrifuged at 15,000 x g for 15 minutes using the Sorvall Ultra 80 $^{TM}$ centrifuge. The supernatant was decanted and the pellets were combined into a centrifuge tube. The residual, in emptied tubes, was suspended with DI water and combined with the pellet and centrifuged for 15 minutes at 15,000 x g.

The resulting combined pellet was mixed well by hand using a sterile spatula and stored at -20 ºC for DNA extraction. The squeezed solid of the SBD remaining from the previous step was weighed in a Ziploc bag and stored at -20 ºC for DNA extraction. The plastic container, spatula and the Waring blender were wiped with ethanol and exposed to UV light for 3 hours before the start of a new process to avoid cross contamination. This process for the combined pellet was conducted in triplicate for each layer.

**DNA extraction from processed SBD samples**

From the combined pellet and the squeezed SBD remaining from the subsample; 2.5 g (wet weight) aliquots from each one were used for DNA extraction. The protocol used for DNA extraction was the same as described in Saikaly et al. (2010). The protocol combined the QIAamp DNA Mini kit and the QIAamp DNA Blood Maxi kit (QIAGEN, Valencia, CA).

The protocol consists of a hot detergent treatment followed by bead-beat homogenization. The hot detergent treatment was performed with 17.5 mL of Aamp Stool Lysis buffer (QIAamp DNA Stool kit) for 20 min at 70 ºC (cells) or 85 ºC for 15 min (spores). This was followed by bead- beat homogenization using 2.5 g of zirconia/silica 0.1 mm beads (Biospec Inc., Bartlesville, OK). After homogenization, the tube was centrifuged at 3,220 x g for 8 min and the genomic DNA was extracted using the Stool Mini kit and the QIAamp DNA Blood Maxi kit.

**Q-PCR Conditions**

The extracted genomic DNA was amplified using the Q-PCR conditions described in the previous chapter. The number of cells or spores per g of processed SBD was determined using the formula shown below. The number of cells or spores per PCR reaction mix was calculated from the appropriate standard curve based on the Ct values. Each PCR reaction mix contained 2.5 g of pellet (wet weight), respectively (Saikaly et al., 2010).

Cells or spores per dry g of processed SBD

$$= \frac{(\text{cells or spores per PCR rxn})(\text{volume of extracted DNA, } \mu\text{L})(\text{total dry mass of combined pellet, g})}{(5\ \mu\text{L DNA per PCR rxn})(\text{dry mass of pellet used for DNA extraction, g})(\text{dry g of processed SBD})}$$

**Results and Discussion**

**Detection and quantification of aerosolized surrogate BW agents**

Four specific flows that simulate landfill gas flow through the SBD were selected to evaluate detection and quantification of aerosolized surrogate BW agents. Q-PCR was used to detect and quantify target surrogate BW agents in gelatin filters. This method allows the detection of targeted DNA whether or not cells are dead, non-culturable or viable. The detection and quantification of the *B. atrophaeus* vegetative cells and *S. marcescens* are given in Table 8. The two filters placed in the SKC button samplers and the filter located at the opening in the second compartment of the chamber (to preserve mass balance and avoid back pressure) did not capture any cells during the first two flow rates (3.4 L/min and 6.6 L/min), therefore there was no detection or quantification of the surrogates. A very low number of the *B. atrophaeus* vegetative cells ($8.12 \times 10^1$) were detected with a flow of 13.2 L/min only in the filters placed in the SKC button sampler. Finally, a very small concentration of *B. atrophaeus* cells ($2.54 \times 10^2$ cells from filters in button samplers and $8.55 \times 10^1$ cells from filter located on the wall) were detected and quantified with the highest flow (25.31 L/min). In the case of *S. marcescens* there was no

detection during these flows. From these results, it appears that there was no significant aerosolization of *B. atropheaus* or *S. marcescens* vegetative cells (see Table 10).

Detection and quantification results for the experiment where *B. atrophaeus* spores and *S. marcescens* were combined in a suspended solution and spiked into the surface of the SBD at the four flow rates, are given in Table 11. For flow rates of 3.4 L/min and 6.6 L/min, there was no detection of the surrogate BW agents. At a flow of 13.2 L/min, the total *B. atrophaeus* spores recovery using gelatin filters was $6.24 \times 10^2$ spores from the filters in the button samplers and $1.06 \times 10^3$ from the filter located on the wall. A slightly higher level was observed with the highest flow (25.31 L/min) with $9.35 \times 10^4$ spores from the filter located in the button samplers and $6.77 \times 10^1$ spores from the filter located on the wall. For *S. marcescens*, there were no cells detected using the gelatin filters in either of the combined experiments at any flow rate. It could be observed that the recovery of cells/ spores aerosolized is very low in comparison to the initial concentration spiked into the SBD.

Table 10. Total *B. atrophaeus* and *S. marcescens* cells obtained from filters at four different flows of $N_2$ in the SBD (Trial 1)

| | **Test 1 flow (3.4 l/min)** | | **Test 2 flow (6.6 l/min)** | | **Test3 flow (13.2 l/min)** | | **Test 4 flow (25.31 l/min)** | |
|---|---|---|---|---|---|---|---|---|
| | Filter in Button sampler | Filter on the wall | Filter in Button sampler | Filter on the wall | Filter in Button sampler | Filter on the wall | Filter in Button sampler | Filter on the wall |
| ***Bacillus atrophaeus*** | ND | ND | ND | ND | 8.124E+01 | ND | 2.53E+02 | 8.54E+01 |
| ***Serratia marcescens*** | ND | ND | ND | ND | ND | ND | ND | ND |

ND: No detection. Note: The initial concentration in the suspension spiked on the surface of SBD for *B. atrophaeus* cells was $1.06 \times 10^{10}$ and for *S. marcescens* cells was $1.10 \times 10^{10}$

Table 11. Total *B. atrophaeus* spores and *S. marcescens* cells obtained from filters injecting four different flows of $N_2$ in the SBD (Trial 2)

| | **Test 1 flow (3.4 l/min)** | | **Test 2 flow (6.6 l/min)** | | **Test3 flow (13.2 l/min)** | | **Test 4 flow (25.31 l/min)** | |
|---|---|---|---|---|---|---|---|---|
| | Filter in Button sampler | Filter on the wall | Filter in Button sampler | Filter on the wall | Filter in Button sampler | Filter on the wall | Filter in Button sampler | Filter on the wall |
| ***Bacillus atrophaeus (spores)*** | ND | ND | ND | ND | 6.24E+02 | 1.05E+03 | 9.35E+04 | 6.77E+01 |
| ***Serratia marcescens*** | ND | ND | ND | ND | ND | ND | ND | ND |

ND: No detection. Note: The initial concentration in the suspension spiked on the surface of SBD for *B. atrophaeus* spores was $3.77 \times 10^{11}$ and for *S. marcescens* cells was $1.10 \times 10^{10}$

It is should be noted that no prior study of this kind has been published; therefore it is difficult to compare the results of this study with others. The literature available only investigated detection of bacteria from the landfill gas system. Huang et al. (2002) analyzed the airborne biological (bioaerosol) contamination in closed landfill sites

and reported that levels of airborne bacteria were above $10^3$ CFU/$m^3$ from samples obtained from passive venting tubes placed in closed and undergoing-closure areas, concluding that there is aerosolization of airborne culturable microbes even after post closure. Barry et al. (2008), reported detection of bacteria in landfill gas under the microscope using Acridine Orange (AO) and 4',6-diamidino-2-phenylindole (DAPI) and concluded that microorganisms could be carried to the surface by landfill gas. The studies mentioned above suggest the possibility that some bacteria could be transported by through the landfill gas system. However they do not specify the type and characteristics of these bacteria making comparisons difficult. In addition, the reported data are not sufficient to calculate the fraction of cells released from the solids.

A potential explanation for the low detection of *B. atrophaeus* vegetative cells and spores could be the ionic strength between the cells/spores and the SBD. Several studies (Sobsey et al. 1975; Fontes et al., 1991) have reported that an eluant with higher ionic strength could enhance the attachment of the cells to solid waste components. Also, the possibility of adhesion of cells and spores to the walls during aerosolization should be considered. As was mentioned before, hydrophobicity interaction with inert surfaces and electrostatic charges of the cells or surfaces could affect the recovery of the surrogate BW agents with the filters.

The aerosolization of *S. marcescens* results in a wide range of percent recovery, suggesting that although the cells were aerosolized they were attached to the chamber walls before they could reach the gelatin filters.

**Recovery of cells and spores from SBD**

Two experiments were conducted inside the chamber to simulate the aerosolization of the surrogate BW agents present in the waste (mix of *B. atrophaeus* and *S. marcescens* vegetative cells and the mix of *B. atrophaeus* spores and *S. marcescens*). The chamber was filled around one fourth of its volume with SBD and $N_2$ was injected with a specific flow using three perforated tubes placed almost at the bottom of the SBD. The same batch of SBD was used for each experiment and fresh SBD was used for each new experiment. After the gas flow was stopped, the SBD placed inside the chamber was divided into three layers and the number of cells per layer was determined using Q-PCR. In the experiment where a solution of *B. atropheaus* vegetative cells and *S. marcescens* were spiked on the surface of the SBD, the retained surrogates were found only in the first layer ($1.05 \times 10^5$ cells for *B. atrophaeus* and $2.50 \times 10^5$ cells for *S. marcescens*). Further quantification of the cells was performed in the squeezed residual SBD material during the processing of the SBD prior to DNA extraction (blended SBD used in the indirect method). Once again detection was only found in the squeezed SBD

material from the first layer and the total concentrations for *B. atrophaeus* was 1.11 x $10^5$ cells and for *S. marcescens* was 2.65 x $10^5$ cells (Table 12).

For the experiment of *B. atrophaeus* spores mixed with *S. marcescens,* cells were detected in the first and second layer (see Table 13). Concentrations for *S. marcescens* retained in the SBD were in the same order of magnitude as in the experiment where it was mixed with *B. atrophaeus* vegetative cells (1.50 x $10^5$ cells in the first layer and 1.78 x $10^5$ cells in the second layer). In the case of the squeezed residual SBD material, the total concentration of *S. marcescens* was 1.46 x $10^5$ cells for the first layer and an order of magnitude increase was determined in the second layer (8.93 x $10^6$ cells).

For *B. atrophaeus* spores, the total concentration retained by SBD was 9.18 x $10^6$ in the first layer and 7.50 x $10^6$ in the second layer and in relation to the residual squeezed SBD material the concentration of spores was 2 orders of magnitude higher in the first layer compared to the second layer (2.13 x $10^7$ and 9.10 x $10^5$).

Table 12. Total *B. atrophaeus* and *S. marcescens* cells obtained from SBD after complete experiment (Trial1)

| | **Layer 1** | | **Layer 2** | | **Layer 3** | |
|---|---|---|---|---|---|---|
| | Cells from pellet | Cells from squeezed solid | Cells from pellet | Cells from squeezed solid | Cells from pellet | Cells from squeezed solid |
| ***Bacillus atrophaeus*** | 1.05E+05 | 1.11E+05 | ND | ND | ND | ND |
| ***Serratia marcescens*** | 2.50E+05 | 2.65E+05 | ND | ND | ND | ND |

ND: No detection. Note: The initial concentration in the suspension spiked on the surface of SBD for *B. atrophaeus* cells was 1.06 x $10^{10}$ and for *S. marcescens* cells was 1.10 x $10^{10}$

Table 13. Total *B. atrophaeus* spores and *S. marcescens* cells obtained from SBD after complete experiment (Trial2)

| | Layer 1 | | Layer 2 | | Layer 3 | |
|---|---|---|---|---|---|---|
| | Cells from pellet | Cells from squeezed solid | Cells from pellet | Cells from squeezed solid | Cells from pellet | Cells from squeezed solid |
| ***Bacillus atrophaeus (spores)*** | 9.18E+06 | 2.13E+07 | 7.50+06 | 9.10E+05 | ND | ND |
| ***Serratia marcescens*** | 1.50E+05 | 1.46+05 | 1.78E+05 | 8.93E+06 | ND | ND |

ND: No detection. Note: The initial concentration in the suspension spiked on the surface of SBD for *B. atrophaeus* spores was $3.77 \times 10^{10}$ and for *S. marcescens* cells was $1.10 \times 10^{10}$

This study did not focus on the analysis of the moisture content of the SBD, however a possible explanation for the detection of *B. atrophaeus* spores and *S. marcescsens* in the second layer in comparison with the experiment with mixed *B. atrophaeus* cells could be that the moisture content of the SBD used from one experiment to another varied, allowing the cells to be transported deeper and be sorbed by drier areas.

Recoveries from the filters were negligible (< 0.00000001 %) in both experiments in comparison to the initial concentration of surrogate BW agents spiked onto the surface of SBD. Because suspended solutions were spiked into the SBD it was not possible to determine the amount of cells that aerosolized inside of the chamber during each trial. From the results, it could be suggested that a large amount of the surrogate BW agents were not recovered.

A potential explanation of such a low detection and recovery could be that the cells and spores adhered to the SBD components. As was reported in the study by Saikaly et al., (2010), *B. atrophaeus* cells and spores were shown to be hydrophobic, which facilitate their adherence to the SBD. Van Loosdrecht et al. (1987) reported that adhesion of bacteria (vegetative cells and spores) to surfaces with different levels of wettability increases the bacterial hydrophobicity. Surface electrical charge also was shown to have some influence in bacterial adhesion. The heterogeneous nature of the SBD could be a factor that limits the aerosolization of the surrogates and enhance their adherence. Nevertheless, there are a number of other factors (cell size, the presence of extracellular polyssacharides, cell mobility) that could contribute to the binding between bacteria and solid surfaces. Additional experiments are needed to evaluate the hydrophobicity in cells and spores in heterogeneous porous media.

Another possible explanation for the low recovery of the target surrogates could be the inability to efficiently separate cell and spores from the solid phase using the indirect cell separation method. However, the cells in the squeezed solids were also quantified, and adding the cells quantified in the squeezed solids did not change the negligible recoveries. Direct methods assume the material placed into extraction tubes are representative of sampled ecosystems; however, this assumption is tenuous for heterogeneous materials like the SBD (Staley et al., 2010, in press). For indirect methods, the separation of the samples from the material matrix (in this case 3 layers of

SBD) allows concentration of cells from larger samples; in this study 250 g sub samples were used to quantify the amount of cells or spores in the SBD. The results show that a direct measurement approach would not result in higher recoveries in the SBD. The results suggest that cells and spores are still attached to the SBD components.

The use of sucrose/tritonX-100, a solution that contains a non-ionic detergent (to disrupt hydrophobic interactions) and buoyant concentrations of sucrose (to lift the separated spores) as replacement of chilled 1 x PBS solution in the indirect method, led to a slight increase in the recovery of spores from SBD in comparison to *B. atrophaeus* vegetative cells (around 0.0083 % more *B. atrophaeus* spores were recovered from the pellet and squeezed SBD than vegetative cells). However, the treatment did not increase the recovery of *S. marcescens*. Previous studies have reported improvement of the recovery of spores only from field soil which has a less heterogeneous composition than SBD and municipal solid waste (Dragon and Rennie et al., 2001; Ryu; Lee; Yoo; Seong and Oh et al., 2003). Future work is necessary to identify and optimize more efficient methods in the extraction of the DNA of cells and spores from SBD.

**Conclusions**

- Detection and quantification of the surrogate BW agents (only *B. atrophaeus* vegetative cells and spores) were achieved only during the two highest flows injected in the SBD (13.2 L/min and 25.3 L/min). This likely represents a worst

case scenario because it is unlikely that we could find in the landfill concentrations of surrogate BW agents at ranges of $10^6$ to $10^7$ per g of SBD.

- The detection of the surrogate BW agents in the residual squeezed SBD indicates that the indirect method was not effective in the extraction of cells and spores from SBD. However, the results from the squeezed solids indicate that direct methods for extraction of DNA directly from SBD would not significantly increase recovery from SBD.

- High percentages of surrogate BW agents were not detected during the experiments in the enclosed system (chamber) suggesting the possibility of attachment of cells and spores to the walls or SBD components and the inability of our collection and separation methods to extract them.

- The use of both non-ionic detergent and buoyant concentration of sucrose (Triton X-100/sucrose) could contribute in 0.08% to the extraction of *B. atrophaeus* spores. However more research with extraction of DNA and cells from heterogeneous porous media needs to be done.

# RECOMMENDATIONS

- Evaluate the changes in the magnitude of electrical charges carried by *B. atropheaus* (vegetative cells and spores) and *S. marcescens* during aerosolization and how it could affect the recovery efficiencies collection.

- The influence of hydrophobicity of surrogate BW agents on adhesion to heterogeneous porous media like SBD or solid waste should be studied in depth evaluating the variation of type of proteins in the outer cell surface, hydrophobic pigments and temperature.

- Evaluate different extraction techniques that contribute to a more efficient recovery in the surrogate BW agents from heterogeneous porous media.

- Further studies on assessing and quantifying BW agents and microorganisms transported through landfill gas are needed to be able to evaluate the possibility of reaerosolization during different stages of the sanitary landfill.

- Viability of the surrogate BW agents during their transport through landfill gas should be evaluated.

- If solids contaminated with biological warfare agents are to be buried in a landfill, then the exposure of these solids to relative high gas velocities can be eliminated by the strategic placement of gas wells relative to the contaminated waste.

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