ANALYSIS OF EXPEDIENT FIELD DECONTAMINATION METHODS FOR THE XMX/2L-MIL HIGH-VOLUME AEROSOL SAMPLER

THESIS

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Abstract

The XMX/2L-MIL is a high volume air sampler used by the Air Force Bioenvironmental Engineering community to collect biological aerosols. Without a verified decontamination technique, however, the XMX cannot be used effectively. The objective of this study was to evaluate several proposed methods for expedient field decontamination of the XMX. This study centered on the inactivation of *Bacillus atrophaeus* spores and vegetative *Erwinia herbicola* organisms from the XMX inner canister. The goals in this investigation were twofold: 1) to verify the antimicrobial efficacy of a 10% bleach solution and 2) to determine if wiping the components with a bleach-soaked paper towel or submerging the components directly in the bleach solution represents the optimal decontamination procedure.

Data was gathered at the Dycor Technologies facility located in Edmonton, Alberta, Canada. Their Aerosol Test Chamber was used to disseminate the surrogate agents and then sample the aerosol using three XMX devices. Counts of the microbial population were calculated at each stage of the procedure to assess the efficacy of the two proposed methods.

It was observed that 10% bleach solutions resulted in approximately 10^2-fold decreases in aggregate microbial contamination on XMX components. Of the methods tested, the submersion in a 10% bleach solution plus a 15-minute air purge showed the most efficiency. Contamination levels were consistent between all three devices during the trial and were measured at or below background levels after decontamination.

Acknowledgments

I would like to express my sincere appreciation to my faculty advisor, Lt Col David Smith, for his guidance and support throughout the course of this thesis effort. The insight and experience was certainly appreciated. I would also like to thank Diana Semler, from Dycor Technologies Ltd, for both the support and latitude she provided to me in this endeavor. Captain Casey Cooper also deserves thanks for the information he provided me regarding the XMX's sampling methodologies.

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ANALYSIS OF EXPEDIENT FIELD DECONTAMINATION METHODS FOR THE XMX/2L-MIL HIGH-VOLUME AEROSOL SAMPLER

I. Introduction

Background

Biological warfare, in one form or another, has existed nearly as long as conventional warfare itself. Though earlier peoples lacked an understanding of why these weapons were so deadly, they quickly realized how to harness them to their advantage. Contaminating water supplies with animal remains and excrement represented rudimentary attempts at conducting biological warfare (Martin, Christopher, & Eitzen, 2007). One of the most salient examples of primitive biological warfare was the siege of Caffa. A port city in what is modern Ukraine; it was besieged by the Mongols in 1345. After a year of battle, an outbreak of plague struck the invading Mongols. Gabriele de Mussi, an Italian notary who chronicled the siege, described "thousands upon thousands" of Mongols killed every day due to the ravages of plague (Wheelis, 2002). Though eventually forced to retreat, the Mongols sensed an opportunity to inflict revenge upon the Genoese holed up in Caffa and "ordered corpses to be placed in catapults and lobbed into the city in the hope that the intolerable stench would kill everyone inside (Wheelis, 2002)." Though the germ theory of infection was still centuries away, mankind's understanding of disease was still sufficient to realize that it was yet another weapon to add to his arsenal.

As microbiology advanced, so too did biological warfare. Robert Koch's famous four postulates on the relationship between microbial life and disease causation made it possible to grow pure stocks of bacteria, including pathogens suited for biological attacks (Christopher, Cieslak, Pavlin, & Eitzen, 1997). Germany was the first state to adopt a scientifically rigorous biological warfare program geared toward offense. In the early 1930's Japan founded Unit 731 and by the end of World War II it was capable of producing thousands of pounds of anthrax per year (Alibek, Lobanova, & Popov, 2005). The United States maintained an offensive biological weapons program for almost 30 years and weaponized many lethal agents such as *Francisella tularensis* and botulinum toxin as well as incapacitating agents such as *Brucella suis* and Staphylococcal enterotoxin B (Christopher, Cieslak, Pavlin, & Eitzen, 1997). The program was terminated by President Richard Nixon in 1969 and the stockpiled agents were destroyed from 1971-1972.

All of the above programs were eclipsed by that of the U.S.S.R. which maintained one of the world's most infamous state-run biological weapons programs, devoting far more time and resources than many other countries. Tularemia was rumored to have been used on the Eastern front against the Germans during the Second World War, after an unexplained outbreak affected a disproportionate number of German soldiers despite the relative proximity of the two armies (Alibek, 1999). However, the roots of the Soviet program go back as far as 1928. Following the revolution, the Bolsheviks realized that disease, not bullets and bayonets, was responsible for the vast majority of casualties during the fighting. This revelation prompted the Revolutionary Military Council to order the development of a typhus weapon, the first arrow in the Soviet's biological

quiver. From those humble beginnings eventually evolved Biopreparat, the civilian cover for the main directorate of biological weapons research. Ironically, the biggest strides in their research occurred under the command of Gen. Yuri Kalinin, years after the U.S.S.R. had become a signatory to the Biological and Toxin Weapons Convention (Alibek, 1999). It was Russian President Boris Yeltsin, in 1992, who finally made biological weapons illegal.

It is a common assertion that biological weapons represent a growing threat to the United States. The so-called "Amerithrax" incident, documents on bioterrorism recovered from al-Qaeda camps, makeshift ricin laboratories discovered in Chechnya and the attempted theft of pathogens from culture collections in Indonesia all indicate that the intention to use biological weapons still remains (Committee on Prevention of Proliferation of Biological Weapons, 2007). It also implies the biological threat is largely no longer from massive, state-sponsored programs like those of the Former Soviet Union (FSU) but smaller, more nebulous groups. When the Soviet Union collapsed in 1992, Biopreparat and its subordinate institutions had tens of thousands of weapons specialists in their service. It was believed these scientists could provide biological weapons capability to rogue nations or terrorist groups. Iran was accused of trying to recruit a Russian scientist to make biological weapons for them in late 1998 (Loeb, 2009). This belief was the genesis of the Cooperative Threat Reduction program, which aimed to stem all Weapons of Mass Destruction (WMD) proliferation in the FSU. To counter the specific biological threat, the Defense Threat Reduction Agency implements the Biological Threat Reduction Program (BTRP) which aims to prevent the proliferation of expertise, materials, equipment and technology leading to biological weapons. The BTRP

does so by employing these former weapons scientists in mutually beneficial biological research (Committee on Prevention of Proliferation of Biological Weapons, 2007).

Some professionals, however, remain extremely skeptical of the notion that biological terrorism poses as serious a threat as some of the evidence might suggest. William R. Clark, professor emeritus of immunology at UCLA, believes the threat of biological terrorism is largely embellished and the billions spent under Project Bioshield have been unnecessary. Professor Clark takes issue with the underlying assumptions that many studies utilize when predicting the sometimes cataclysmic results of a biological attack (Palmquist, 2008). A brief examination of actual biological attacks in the last 20 years seems to support this stance. Doomsday cult Aum Shinrikyo attempted to use aerosolized anthrax spores against Kameido, Tokyo, Japan in 1993 (Takahashi, 2004). During the final week of June that year, citizens began reporting foul odors, loud noises and intermittent mists coming from the Aum Shinrikyo headquarters building. Some individuals fell ill with flu-like symptoms but recovered quickly. Eventually, the complaints forced Aum's leader, Shoko Asahara, to stop the endeavor. It was only after the 1995 sarin gas attack was this event recognized as a failed biological attack. Asahara eventually admitted the mists were crude aerosolized suspensions of *Bacillus anthracis* spores. Despite having multiple doctors and nurses in the cult's employ and commanding a surprising amount of wealth (one cult leader estimated Aum Shinrikyo's net worth at over a billion dollars), producing an anthrax weapon capable of inflicting even a single casualty proved too insurmountable an obstacle (Olson, 1999). Similarly, the anthrax mail attacks in 2001 resulted in only 22 infections and 5 fatalities. These incidents illustrate that considerable time and effort must be invested to make a viable biological

weapon, even from an ideal agent like *Bacillus anthracis*. So is the collective worry about potential biological threats justified?

Examining a page from the history of the Soviet biological weapons program might provide a better indication of the deadly potential biological weapons possess. Just outside of the city of Sverdlovsk was Compound 19, a clandestine facility tasked with producing large quantities of anthrax spores for the Soviet arsenal. Within the compound, fermented spores were dried and ground into a fine power for use as an aerosol weapon (Alibek, 1999). As a consequence of this process, the building's interior was filled with spores and only a series of exhaust vent filters prevented their escape into the nearby city. Sometime during the course of operations one of the filters became clogged and was removed from the exhaust pipe. When the next shift began, the officer-in-charge was unaware of the missing filter and ordered anthrax production to be resumed. As a result, aerosolized anthrax spores were ejected into the night air and toward the unsuspecting town. Within days workers at a nearby ceramics factory began to fall ill and within one week most had died (Alibek, 1999). The communist party quickly shifted the blame to contaminated meat, a somewhat plausible explanation as the anthrax bacillus can indeed infect people through the gastrointestinal tract.

Fifteen years later and after careful epidemiological review by Matthew Meselson it was discovered that Compound 19 was indeed the cause of the anthrax outbreak. The case of Sverdlovsk illustrates how potentially devastating a concerted attack with aerosolized anthrax could be. The release killed at least 66 Soviet citizens and dozens more were infected. While 60 deaths are not by any standard "mass destruction," it can be considered a sort of proof of concept for an actual biological attack. The release

demonstrated that a bacterial aerosol represents a significant hazard, long before the anthrax attacks of 2001 in the United States. Had the soviets planned an actual attack with their anthrax weapon from Sverdlovsk, a number of factors would likely have been different. Planners would have accounted for meteorological conditions, times when the most people would be in the area or used more complicated dispersal methods, all of which would have assuredly raised the death toll several-fold. The vaccination campaign conducted soon after may have mitigated the effects, but with a surreptitious attack on an unsuspecting and immunologically naïve population much higher casualties would be expected (Meselson, et al., 1994).

In the report from the Commission on the Prevention of Weapons of Mass Destruction Proliferation and Terrorism, the authors assert that biological terrorism represents a greater threat than nuclear terrorism, due in part to the widespread proliferation of advanced biotechnologies like genetic engineering and genome synthesis (Graham, et al., 2008). Despite the successes of the BTRP in the states of the FSU there is still significant risk from many other locations including South Asia, Africa, Latin American and the Middle East (Committee on Prevention of Proliferation of Biological Weapons, 2007). Presumably, such dual-use technologies could be used to increase a microorganism's virulence or alter its genome to render current vaccines ineffective. Furthermore, increasingly smaller and more efficient equipment, like bioreactors, may reduce signatures of a developing biological weapons capability (U.S. Congress, Office of Technology Assessment, 1993). Others assert that such weapons are easier to conceal from the international community, cheaper to produce and have the potential to inflict far greater casualties (Zubay, 2005). This is not merely speculation as evidence exists that

enemies of the United States actively intend to use such weapons, though their capability to do so remains limited. In 2005 a primer on biological weapons was posted on the Iraqi al-Qaeda website, in which the author states:

> Biological weapons are considered the least complicated and the easiest to manufacture [of] all weapons of mass destruction. All the information concerning the production of these weapons is readily available in academic books, scholarly publications and even on the internet….In addition to the ease of production, these weapons are also considered to be the most affordable. With $50,000 a group of amateurs can possess a biological weapon sufficient to threaten a superpower. It is for this reason that biological weapons are called the poor man's atomic weapon (Slama & Bursac, 2009).

This growing concern is especially evident in national guidance documents. The National Security Strategy of 2002 lists among its aims the prevention of the United States' enemies from acquiring WMD. The National Strategy to Combat Weapons of Mass Destruction identifies three pillars in America's attempt to protect itself: counterproliferation, nonproliferation and consequence management. The National Military Strategy to Combat WMD (NMS-CWMD) takes these three pillars and "creates a strategic framework for combating WMD."

In support of the NMS-CWMD, the Air Force authored Doctrine Document 2-1.8 Counter-Chemical, Biological, Radiological, and Nuclear Operations (C-CBRN). AFDD 2-1.8 charges Air Force commanders and personnel with the responsibility to prevent an attack with CBRN weapons and to mitigate their effects if one is used (Air Force Doctrine Center, 2007). The Air Force has five operational pillars of C-CBRN operations which are: proliferation prevention, counterforce, active defense, passive

defense, and consequence management. This paper will focus on certain aspects of the passive defense pillar.

Should an adversary of the United States succeed in evading active defense measures and successfully launch a CBRN attack, passive defense allows commanders to neutralize, contain and manage its effects on an Air Force installation and its surroundings. There are many passive defense measures; the two of particular interest to this project are 1) detection and identification and 2) decontamination.

There is no unified career field within the Air Force which handles the planning, preparation and response to CBRN incidents. Instead, two separate AFSCs provide this capability: the civil engineering emergency managers and the bioenvironmental engineers. The bioenvironmental engineering (BEE) mission involves conducting health risk assessments which identify, evaluate and control hazards (including CBRN hazards) and then communicating the findings to commanders which contributes to informed decision-making.

Should the need arise to respond to a CBRN event, the BEE community has significant capability for each component of CBRN. To assist in the response to a biological incident, the Air Force has purchased the XMX/2L-MIL from Dycor Technologies Ltd. The XMX is a high-volume bioaerosol collector designed for operations in harsh environments. It is capable of collecting large volumes of air, removing undesirable particles (e.g. dust and debris) and concentrating aerosolized particles between 1 and 10 micrometers (i.e. respirable particles) into a 50 ml centrifuge tube, which may be used in conjunction with additional techniques such as polymerase

chain reaction (PCR) or enzyme-linked immunosorbent assay (ELISA) to identify the pathogen and enact appropriate response measures (Dycor, 2007).

The XMX was part of a two-phase test of the Chemical Biological Aerosol Warning System (CBAWS) conducted at Camp As-Sayliyah, Qatar in July 2003 and Eglin AFB, FL from October to November 2003. CBAWS is a network of biological detection, collection and identification devices designed to provide commanders with detect-to-warn and detect-to-treat capabilities (AFIOH, 2006). Testing focused on assessing system performance in environments resembling expeditionary conditions (hot, dry and windy) and in mild, humid environments with little prevailing wind.

Only during the Eglin trials were simulated biological agents released. There were 21 BW "attacks" dispersed in both point and line fashions. When the Biological Aerosol Warning Sensor (BAWS) Mk. III detected biological particles in the air, the XMX would be remotely triggered to begin sampling. The collected material would then be analyzed by the Ruggedized Advanced Pathogen Identification Device (RAPID) system. Fifteen of the samples gathered from the XMX were tested of which 7 proved positive. Of the 8 negatives, only one was from a sample registered by the referee tower, while the others were not hit and one contained a toxin simulant undetectable using RAPID.

Subsequently, ten aerosol samples were selected for identification using handheld assays (HHA). Of the ten, five were confirmed positive and the remaining five were from samples where the referee towers registered no agent cloud. Overall, it was ascertained that samples collected by the XMX work well in conjunction with the Air

Force's methods of BW identification, as demonstrated by the 100% success rate when using the HHA.

Problem Statement

Use of the XMX air sampler has been hindered by lack of a verified decontamination procedure. Without the knowledge of what decontamination techniques are effective, the Air Force runs the risk of cross-contaminating collected samples, leading to false positive results and potentially wasting time and resources on nonexistent threats.

Research Objectives/Questions/Hypotheses

The objective of this study is to evaluate proposed methods for expedient field decontamination of the XMX.

Research Focus

This study centers on the decontamination of *Bacillus atrophaeus* spores and vegetative *Erwinia herbicola* organisms from five specific XMX components.

Investigative Questions

This investigation has two principal goals: 1) to verify that a 10% bleach solution is an effective decontaminant for use with the XMX and 2) to determine if wiping the components with a bleach-soaked towel or submerging the components directly in the bleach solution represents the optimal decontamination procedure.

Assumptions/Limitations

This study was limited to only spores of gram-positive organisms and vegetative gram-negative organisms. This represents only a portion of the spectrum of potential biological threat agents which also include viruses, rickettsiae or toxins.

II. Literature Review

Chapter Overview

The purpose of this chapter is to review the efficacy of potential decontamination agents for use on the XMX/2L-MIL. Specific focus is given to sodium hypochlorite inactivation of bacteria, in both vegetative and spore form.

Background

Terms such as disinfectant or antiseptic are often used in common parlance without recognizing that strict, legal definitions exist. A disinfectant is most often, but not necessarily, a chemical agent which destroys germs and inactivates viruses (Block, 2001). Usually it is understood to affect only vegetative organisms and not bacterial endospores. Antisepsis is the inhibition or destruction of microbial growth on living tissue (Block, 2001). Sterilization refers to any chemical or physical process which results in the eradication of microbial life-both vegetative bacteria and endospores (Block, 2001). More generally, the term biocide may be applied to any chemical agent possessing disinfecting or sterilizing properties. The definition of decontamination is more operational in nature and refers to removing microbial populations to make objects, or even people, suitable for further use. A more military-oriented definition is the "reduction or removal of [biological] agents so they are no longer hazards (Hurst, 1997)."

Biological warfare agents encompass a wide variety of organisms with different morphological and physiological characteristics. Of the six agents on the Centers for Disease Control and Prevention (CDC) Category A agent list, two are gram-negative organisms, one is a spore-forming organism, one is a protein toxin and the remaining two

are viruses (Centers for Disease Control and Prevention, 2008). Each of these agents possesses various mechanisms of resistance to chemical agents. Two significant means of natural resistance include enzymatic inactivation of the antimicrobial agents and permeability barriers, which may be present in the form of a spore coat or the gram-negative cell wall. The chart in Figure 1 shows the relative resistance of various microorganisms to decontaminating agents with the most resistant at the top.

Biocides themselves differ in various properties, including on which cellular constituents they exert their lethal activity, and whether or not they can inactivate spores. Phenolic and quaternary ammonium compounds exhibit broad antimicrobial activity, but fail to eliminate spores (Russell A. , 1991). The ability to sterilize a surface is especially important for applications in biological terrorism/warfare, as the most-discussed threat, anthrax, would most likely be disseminated in spore form. Glutaraldehyde, formaldehyde, peroxygen compounds and hypochlorites have all demonstrated sporocidal effects (Russell A. , 1991).

Multiple factors govern the interaction of microorganisms with biocides. Two have already been listed (the microorganisms and the biocides themselves) but environmental factors such as pH, temperature and concentration as well as the contact time contribute to the interaction as well (Russell A. , 1991).

If one were to list the attributes of an ideal decontaminant they might select criteria such as broad spectrum antimicrobial activity, ready availability, ease-of-use, nonharmful to humans/equipment at operational concentrations, chemical stability, and rapid action. Hypochlorite compounds satisfy many of the above criteria and are the disinfectants of interest in this investigation.

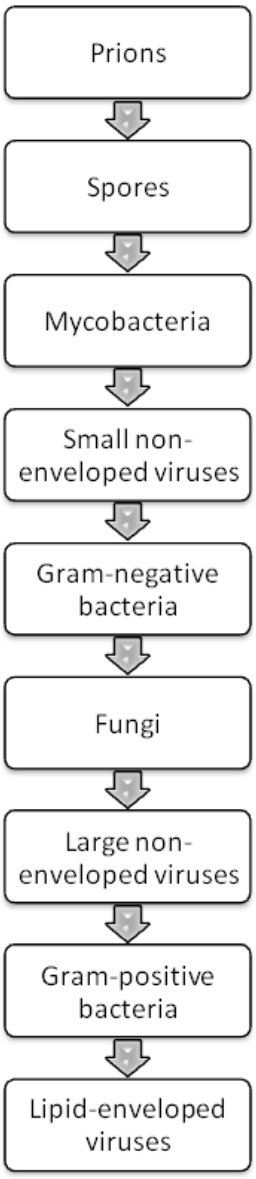


Figure 1. Relative resistance of various microorganisms to disinfectants from most resistant to least resistant.

History of Chlorine Disinfection

Chlorination has a long history as an antiseptic, a disinfectant and a sterilant. After the discovery of chlorine in 1774 by Karl Wilhelm Scheele, it soon found many applications in the fields of sanitation and medicine. By 1825, calcium hypochlorite was

being used in the general sanitation of everything from morgues to sewers to prisons. Oliver Wendell Holmes discovered the relationship between puerperal fever when he observed that a physician who regularly washed his hands with calcium hypochlorite had far fewer fatalities among his patients. To this day, chlorination is the most common method of disinfecting drinking water (Rose, et al., 2005). Inorganic hypochlorite finds extensive use in hospitals as well (Rutala & Weber, 1997). Of the many types of chlorine-releasing agents (CRA), hypochlorites are the most prominent in disinfection (Dychdala, 1991).

Mechanisms of Chlorine Disinfection on Vegetative Bacteria

Upon the introduction of elemental chlorine into aqueous solution, the following reaction occurs: $Cl_2 + H_2O \rightarrow HOCl + H^+ + Cl^-$ (Dychdala, 1991). Free chlorine forms hypochlorous acid (HOCl) along with hydrogen and chloride ions. Salts of hypochlorous acid are referred to as hypochlorites, which will dissociate to form a hydroxide and hypochlorous acid in aqueous solutions (Dychdala, 1991).

$$Ca(OCl)_2 \rightarrow Ca^{2+} + H_2O + 2OCl^-$$
$$Ca(OCl)_2 + 2H_2O \rightarrow Ca(OH)_2 + 2HOCl$$

Equilibrium exists between hypochlorous acid and hypochlorite ion (OCl^-), $HOCl \leftrightarrows H^+ + OCl^-$ with hypochlorous acid as the moiety responsible for bacterial inactivation (Brazis, Leslie, Kabler, & Woodward, 1958). It is a highly-destructive, broad-spectrum oxidizing agent, capable of reacting with numerous biological macromolecules and other cellular components (McKenna & Davies, 1988). These include nucleotides, enzymes, cell membranes, electron transport chains and

active/structural proteins. McKenna and Davies report a study by Albrecht *et al*. which describes a specific attack by hypochlorous acid on the bacterial cell envelope (1988). It is also suggested that DNA damage or the inhibition of DNA replication may represent another inactivation mechanism. At pH levels below neutral, hypochlorous acid remains in its un-ionized form and at alkaline pH exists as hypochlorite ion. This fact is especially important to decontamination efforts as the relative sporocidal efficacy of hypochlorous acid compared to hypochlorite ion has been estimated at nearly 100:1 (Brazis, Leslie, Kabler, & Woodward, 1958).

The plasma membranes of gram-positive and gram-negative organisms are likely targets for CRAs. As mentioned previously, there are several subcellular targets that an antimicrobial agent may act on; most of which require transport through the plasma membrane. Studying the light-scattering properties of *E. coli* colonies demonstrated that after exposure to 50 µM HOCl, absorbance did not decrease, indicating the bacteria were inactivated without suffering significant insult to the phospholipids and proteins in their cell membranes (McKenna & Davies, 1988). Only after exposure to 10mM HOCl did significant membrane disruption occur, this being several times higher than the normal lethal concentration.

When exposed to chlorine in distilled water both gram-positive and gram-negative bacteria showed increased permeability in their plasma membranes (Virto, Manas, Alvarez, Condon, & Raso, 2005). The concentration of chlorine, however, which causes this permability was several times higher than what is required to kill the cells, again suggesting that membrane damage is not a significant factor in the inactivation of vegetative bacteria (Virto, Manas, Alvarez, Condon, & Raso, 2005).

When grown in the presence of ^{3}H-tagged thymidine, the uptake into a replicating DNA molecule was significantly inhibited by exposure to 50µM HOCl. This inhibition indicates a defect within the bacterium's DNA replication machinery. Within one minute, uptake of the radioactive nucleoside had decreased by 48% and by 5 minutes it had decreased by another 48% (McKenna & Davies, 1988). Furthermore, gram-negative bacteria who lack the gene recA/recB, proteins crucial for post-germination DNA repair, are much more sensitive to challenges with hypochlorous acid (Dukan & Touati, 1996). Without successful DNA replication, cells cannot complete their division, making the inhibition of DNA synthesis a good indicator of cytotoxic effects.

Similar effects are observed in the incorporation of ^{3}H-tagged leucine into bacterial proteins, which suggests than in addition to DNA proteins are another target of hypochlorite (McKenna & Davies, 1988). Low molar concentrations of HOCl act on proteins by causing oxidative unfolding in vitro. In vivo, however, it exerts its effect on proteins by encouraging irreversible aggregation (Winter, Ilbert, Graf, Özcelik, & Jakob, 2008). Heat-shock protein (Hsp) 33 is a bacterial protein activated by heat stress, which in turn prevents other proteins from aggregating and losing function. Strains of *E.coli* and *V. cholerae* defecient for Hsp33 showed increased sensitivity to hypochlorous acid exposure.

In light of the 2001 anthrax attacks, the CDC studied how well the chlorine levels maintained in public drinking water systems would protect the population if potential biological weapon agents were introduced. Strains of *Yersinia pestis*, *Brucella* and *Burkholderia* are all very sensitive to the free avialable chlorine (FAC) in potable water, with the majority of the inoculum being inactivated within 10 minutes. *Francisella*

tularensis is a somewhat hardier organism against low chlorine concentrations. When tested against *Bacillus* spores (one virulent and one attenuated), hours of exposure were required before any significant reduction took place (Rose, et al., 2005). Median FAC concentrations were calculated as well as median contact times (1.1mg/liter and 45 min respectively) and based on study findings this would be sufficient to inactivate *Burkholderia mallei, B. pseudomallei, Brucella melitensis, B. suis, Francisella tularensis* and *Yersinia pestis* by more than 3-logs under similar conditions (Rose, et al., 2005).

Spore Structure/Resistance

Before discussing how a bacterial spore may be inactivated, it would be appropriate to discuss what features in particular make spores resistant to so many forms of disinfection, especially chemical agents. The general anatomy of a spore consists of several proteinacious structures arranged in concentric layers around a central core containing the DNA and essential enzymes. The layers are (from exterior to interior): exosporium, spore coats, outer membrane, cortex, germ cell wall and inner membrane (Setlow, 2006). The exosporium is where the spore intereacts with its environment. Its surface is studded with antigens and acts as a semipermeable membrane to exclude harmful chemicals. Not all spore-forming bacteria possess an exosporium, for example *Bacillus subtilis* either lacks an exosporium entirely or it is very difficult to detect. It is made of two proteins layers: a paracrystalline basal layer and an external layer resembling a “hair-like fringe” formed by filaments of BclA, a glycoprotein (Setlow, 2006). Three enzymes are present within the basal layer, alanine racemerase, isosine-

uridine-preferring nuceloside hydrolase and superoxide dismutase. The latter may serve a protective role by ridding the spore of reactive oxygen compounds.

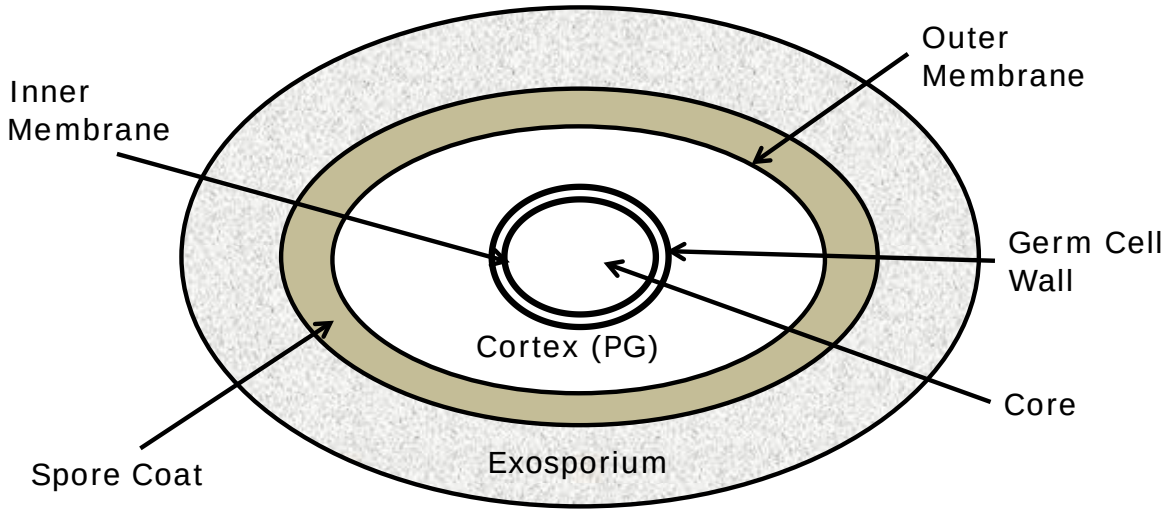


Figure 2. Generalized depiction of a *Bacillus* spore.

Beneath the exosporium sits the spore coat, an intricate structure constructed from over 50 proteins. It is a major component of spore resistance especially against oxidizing agents such as chlorine dioxide, hypochlorite and ozone (Setlow, 2006). This resistance is one of the factors which makes *Bacillus anthracis* an ideal biological weapon agent (Ghosh, et al., 2008). The morphogenesis of the spore coat is a complex process, but there are several proteins that play a significant role. SpoIVA designates the surface of the newly-forming spore as the site for all future protein deposition (Giorno, et al., 2007) (Driks, 2002). The protein shell which forms around SpoIVA is referred to as the precoat. The outermost layer of the precoat contains another imporant protein, CotE. Inner coat proteins are deposited between the layers of CotE and SpoIVA. CotE is

crucial for the formation of the outer coat and responsible for directing at least 8 structural proteins to their proper locations (Driks, 2002). SpoIVA has several fucntions in addition to protein deposition; it is required for the formation of the cortex and germ cell wall as well as for attaching the coat to the developing spore (Driks, 2002) (Giorno, et al., 2007). In the same way, CotE is responsible for the assembly of the exosporium which is fragmented or entirely absent in CotE-deficient strains of *Bacillus anthracis* (Giorno, et al., 2007). This fact may have limited consequences for biological weapons development as removal of the exosporium from the Ames strain did not result in the loss of virulence (Giorno, et al., 2007). Lastly, Cotα is another important component of the spore's outer coat. It can be visualized through thin section electron microscopy as a dark-staining region in the outer coat. With six cysteine residues, extensive intra- and intermolecular disulfide bridges form, potentially contributing to the resistance properties of the coat (Kim, Sherman, Johnson, & Aronson, 2004)

The outer membrane is an essential factor in spore growth, but may not be a significant permeability barrier to harmful molecules. The cortex is composed of a specialized form of peptidoglycan (PG), the chemical responsible for maintaining bacterial shape and counteracting osmotic pressure in bacterial cell walls (Driks, 2002). It has a low water content and is degraded during spore germination. Similarly, the germ cell wall is also composed of peptidoglycan, yet it more closely resembles the cell walls of vegetative organisms (Setlow, 2006).

The inner spore membrane functions as a low-permeability barrier, further blocking small hydrophilic and hydrophobic chemicals which could damage critical cell components (Setlow, 2006). Even small, uncharged molecules like methlyamine cross

the inner membrane extremely slowly (Setlow, 2006). The reasons for the inner membrane's lack of permeablity is unknown, but it may be due to the relative immobility of its lipid consituents (Setlow, 2006).

The core contains DNA, tRNA and necessary enzymes for germination, all identical to those found in a vegetative organism (Setlow, 2006). Three molecules (in the spore's core) play an important role in the resistance of the spore: H_2O is present in much lower concentrations than in vegetative bacterial cells which restricts the movement of macromolecules. The second is dipicolonic acid. Synthesized by the mother cell it is subsequently absorbed into the spore. The last type of molecule is small acid-soluble proteins (SASP) (alpha and beta) which are assembled within the forespore before being absorbed by the mother cell. The SASP saturate the DNA, altering its structure and physical properties which contributes to its resistance to heat/chemicals.

Mechanisms of Spore Killing by Hypochlorite

One of the most useful properties of hypochlorite compounds in the context of biological warfare and bioterrorism is that they are sporocidal at many concentrations. One suggested mechanism for this activity is that hypochlorite forces the separation of the spore coat from the cortex which is soon followed by cell lysis (McDonnell & Russell, 1999).

Spores with defective or missing spore coats exhibit markedly increased sensitivity to hypochlorite disinfection. Spores with a mutation in the cotE gene (causing them to have a defective outer coat) exhibit a greater than 2-log reduction in less than 60 seconds exposure to 50 ml l^{-1} sodium hypochlorite (Young & Setlow, 2003). Outcomes

are similar when the spores have been decoated via sonication or some other physical technique. Decoated spores α-/β- spores or α-/β- *cotE* spores exhibited similar killing curves, indicating that a defective spore coat does not appear to make the core vulnerable to attack by hypochlorite.

DNA was suggested earlier as one of the potential targets for hypochlorite based on studies on *E. coli* growth (McDonnell & Russell, 1999). More recent data suggests that DNA damage does not appear to be a significant factor in the sporocidal activity of hypochlorite (Young & Setlow, 2003). Investigations comparing wild-type *B. subtilis* spores exposed to hypochlorite at pH 11 were similar to mutants which lacked α/β SASP (each demonstrated about a 3-log reduction in 45 min) (Young & Setlow, 2003). If the spore was also deficient for recA, the killing curve remained largely unchanged when compared to wild-type strains. When spores were exposed to hypochlorite at neutral pH, the lethal effects occurred more rapidly, with a 3-log reduction within 8 minutes, nearly six times faster than at alkaline pH. This is consistent with data from the inactivation of vegetative cells (Young & Setlow, 2003).

It has also been noted that hypochlorite-exposed spores tend to germinate very poorly (Wyatt & Waites, 1975). The precise mechanism remains unclear at the moment, as no hydrolysis of the cortex or release of chemicals from the core itself (such as dipicolinic acid) has been observed. It is possible that hypochlorites may damage the inner membrane in some manner which interferes with the germination pathway.

Effects of pH and Concentration

When compared to other chemical agents, such as peracetic acid, copper-ascorbate, glutaraldehyde, hydrogen peroxide, phenol and formaldehyde, hypochlorite demonstrates superior sporocidal effects (Sagripanti & Bonifacino, 1996). A 0.05% solution of hypochlorite showed maximal killing efficacy at a neutral pH, resulting in fewer than 0.01% of *Bacillus subtilis subs. globigii* surviving. Formaldehde, phenol and hydrogen peroxide (10%) each inactivated fewer than 10% of the exposed spores. It was reported that an alkaline pH would practically eliminate any sporocidal activity exhibited by hypochlorite (Sagripanti & Bonifacino, 1996). As previously mentioned, alkaline pH levels shift the equilibrium from hypochlorous acid to hypochlorite ion, the latter of which is a far less efficient antimicrobial compound. Even at alkaline pH levels, some evidence has been found to suggest some sporocidal activity remains. A 5% sodium hypochlorite solution is able to disinfect contaminated materials including tile, fabric, plastic, metal and cloth within 30 minutes at both pH 7 and 12 (Kenar, 2009). The sporocidal activity at an alkaline pH is likely due to the increased concentration of hypochlorite ion present in the solution. At 0.5% sodium hypochlorite, all samples were free of bacterial growth at neutral pH, but growth was observed in samples such as paper and soil at a pH of 12. At 0.05%, both neutral and alkaline pH levels showed some growth on various environmental samples (Kenar, 2009).

Effects of Different Solid Surfaces

Comparisons of the relative binding strength of spores to various materials have suggested that little differences existed between spores bound to rubber or metal carriers

(Sagripanti, Carrera, Insalaco, Ziemski, Rogers, & Zandomeni, 2006). Sporocidal assays comparing hypochlorite disinfection on rubber and metal coupons inoculated with *B. anthracis*, including the virulent Ames strain and several related *Bacillus* organisms, showed minimal variation in $\log_{10}$ reductions between the two materials (Sagripanti, Carrera, Insalaco, Ziemski, Rogers, & Zandomeni, 2006).

Decontamination of Other Military Biological Detection Devices

Joint Portal Shield (JPS) was the Defense Department's first automated, networked biological detection system. It was designed to provide security for high-value, fixed-location assets and can presumptively identify up to 8 separate BWA through an internal Handheld Assay (HHA). It utilizes the BAWS to determine the presence of airborne biological hazards. Though there are no documented studies regarding the decontamination strategies for the JPS, the Operator's Manual recommends using a 5% hypochlorite solution to sterilize the particle pre-separator, but not other components such as the cyclone and the sampler stack (Hamel, 2009).

The Joint Biological Point Detection System (JBPDS) is designed to replace "current force" detection systems such as the Joint Portal Shield, the Biological Integrated Detection System and the Interim Biological Agent Detector (Department of Defense, 2006). Like the JPS, it is modular in nature and affords all four services automatic collection, detection, identification and warning capabilities on the battlefield (Kauchak, 2006). Two studies were undertaken to investigate decontamination strategies for the JBPDS.

The first investigation was conducted at the West Desert Test Center, Dugway Proving Grounds, Utah. The goals of this initial study were twofold: 1) to determine if the JBPDS leaked and 2) to develop decontamination procedures for its line-replaceable units. The JBPDS was placed in their Aerosol Simulant Exposure Chamber (ASEC) which was exposed to an aerosol of *Bacillus globigii* spores at 1000 agent containing particles per liter of air (ACPLA). The JBPDS was allowed to operate for an entire cycle and then external power was cut to simulate operations in degraded conditions. The line-replaceable units were then subject to decontamination with a 5% sodium hypochlorite solution. The efforts to decontaminate the fluidics transfer system, the automated handheld assay and the inlet stack compressor created aerosol hazards that were deemed unacceptable to personnel. Due to these setbacks, the investigators shifted focus to the task of decontaminating the BAWS Mk. IV alone. Six decontamination protocols were then set forward:

1. Use of Hype-Wipe (0.94% - 5.25% sodium hypochlorite) towelettes on exterior surfaces
2. Use of Hype-Wipe (0.94% - 5.25% sodium hypochlorite) towelettes on exterior and reachable surfaces
3. Use of soaked paper towel (1.05% sodium hypochlorite)
4. Sodium hypochlorite (1.05%) mist for 20 minutes
5. Placing the BAWS in a biohazard bag with 5.25% sodium hypochlorite and expose to sunlight for 1 hour.
6. Placing the BAWS in a biohazard bag with 2.125% sodium hypochlorite and phosphoric acid to generate chlorine gas.

The tests with the Hype Wipe towelette show that it was an effective decontaminant for the majority of surfaces to which it was applied, with no *B. globigii*

spores recovered. Still several areas, such as the glass tube assembly, exhaust hose, left panel and cooling grate all had residual spores, sometimes as many as 1×10^5 CFU present on a surface following decontamination (Simmons, Hanson, & Seerup, 2008). The bleach soaked paper towels demonstrated minimal efficacy with multiple order of magnitude decreases in bacterial counts apparent on only a few components. Considering the conceptual similarity between the Hype Wipe and a paper towel soaked in a bleach solution this result appears counterintuitive. Several surfaces demonstrated no reductions at all. Similar effects on the biological loads were observed using the hypochlorite mist and incubation in a biohazard bag followed by an hour of sunlight. In fact, the latter induced such severe corrosion as to render the device inoperable (Simmons, Hanson, & Seerup, 2008). Ultimately the chlorine gas test was the most effective method of sterilizing the device; however, this method also caused severe corrosion (Simmons, Hanson, & Seerup, 2008).

A subsequent study on BAWS Mk. IV decontamination was conducted at the Massachusetts Institute of Technology's Lincoln Laboratories. The MIT-LL study examined the decontamination of just the BAWS Mk. IV as well. The criteria for selection were as follows:

1. Demonstrates sporocidal activity
2. Must not be corrosive to sensor components
3. Must not interfere with sensor operations (Tremblay, 2008)

Multiple chemical agents with various antimicrobial mechanisms as well as physical methods were compared and ranked based on the aforementioned criteria. Sodium hypochlorite scored highly for its oxidizing properties and efficacy against

spores, but received the lowest overall rank for its extremely corrosive properties, as underscored in the previous study. Still it was retested as a comparison for potentially less corrosive biocides.

In the coupon-testing portion of the investigation, individual sensor components were tested against formaldehyde (37%), hydrogen peroxide (35%) and sodium hypochlorite (10%). Sodium hypochlorite met acceptability standards for sporocidal activity but failed the standards for non-corrosivity. Only conformally-coated electronics were resistant to corrosion caused by hypochlorite (Tremblay, 2008).

Summary

CRAs, most notably hypochlorite compounds, have long been recognized as potent bactericidal and sporocidal agents. Hypochlorites, however, have several limitations to their use the most notable here being its corrosive properties when applied to delicate optics, sensors and electronics. Another factor in microbicidal efficacy is the pH of the hypochlorite solution. The XMX lacks such a complicated apparatus and runs a reduced risk of chlorine induced corrosion on the components of interest.

III. Methodology

XMX Sample Surfaces

Three XMX/2L-MILs were selected for evaluation, designated Unit 1, 2 and 3 respectively. Each inner canister was dismantled into components labeled A-E respectively (Figure 3).

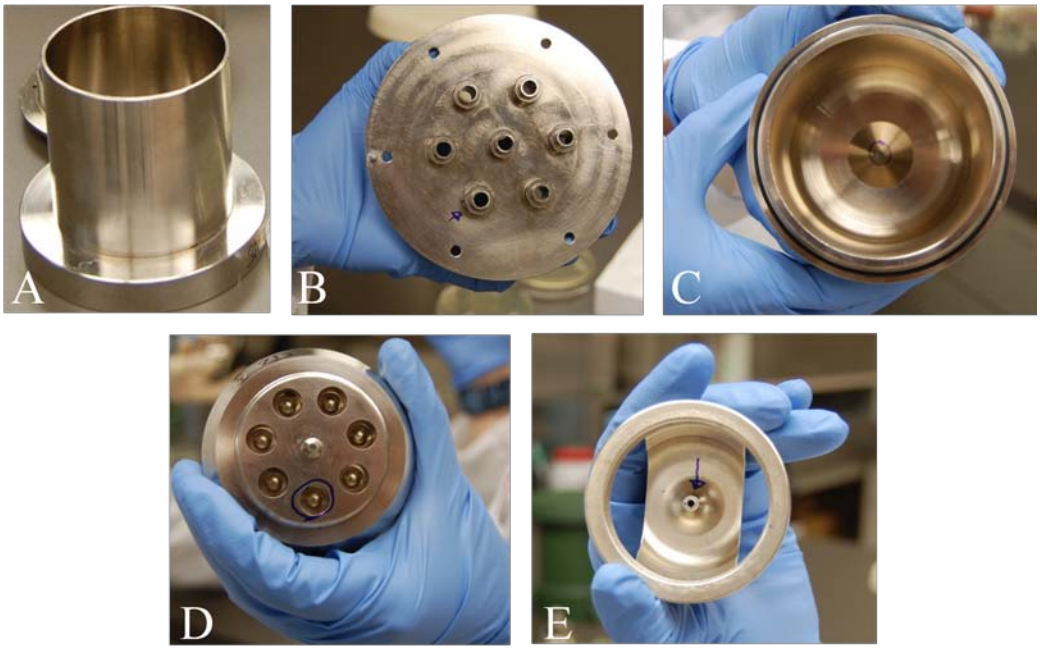


Figure 3. Components of the XMX/2L-MIL inlet stack. A - Primary inlet, B - Primary nozzle plate, C- upper canister, D - lower canister and E - final nozzle.

Biological Agent Surrogates

Bacillus atrophaeus (formerly *Bacillus globigii* and *Bacillus subtilis* var. niger) spores were provided by the Life Sciences Division, West Desert Test Center, Dugway Proving Grounds, Utah. *B. atrophaeus* is commonly used instead of *B. anthracis* in

microbicidal testing (Baird, 2004). *Erwinia herbicola*, also referred to as *Panotea agglomerans*, is a gram negative organism in the family Enterobacteracieae, used to simulate *Yersinia pestis*, the agent responsible for bubonic/pneumonic plague.

Aerosol Test Chamber

The aerosol test chamber (ATC) utilized was provided by Dycor Technologies Ltd (Figure 4). It has a volume of twelve cubic meters and has three ports to allow testing of up to three XMX devices simultaneously. Additionally the chamber has the capacity to connect two slit-to-agar devices.

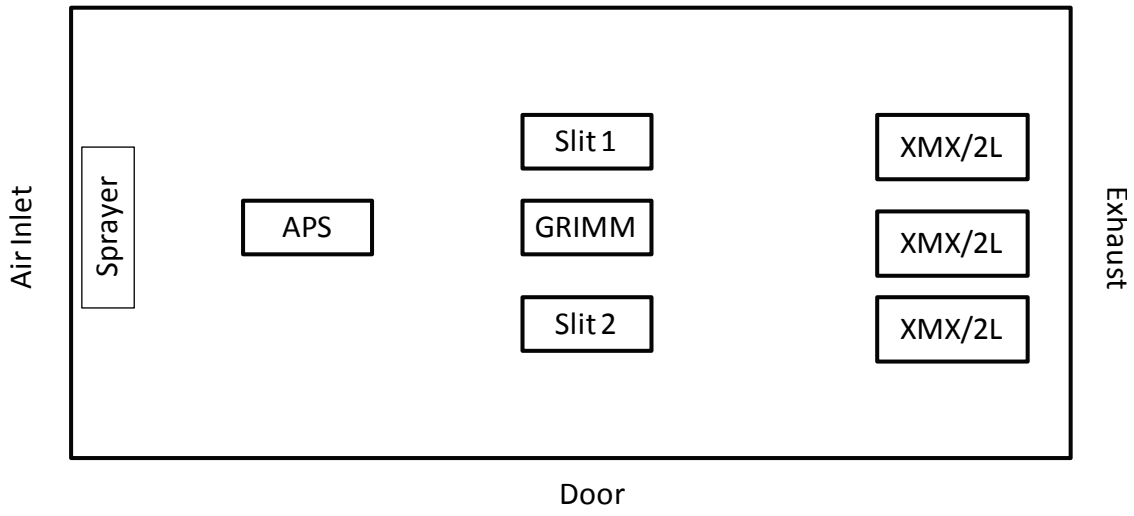


Figure 4. Aerosol Test Chamber Setup - 2009-04 XMX Decontamination Test

XMX Preparation/Testing Regimen

XMX components were sterilized between decontamination tests in a 10% bleach solution. Components were allowed to soak for 5 minutes, were placed in a water bath

and then dried in a drying oven. To ensure consistency throughout each test each component was labeled with permanent marker. To establish a baseline level of microbial contamination prior to exposure, predetermined areas of interest were sampled with sterile swabs moistened with ultrapure water. The swabs were subsequently plated on tryptic soy agar (TSA) provided by Dalynn Biological supply and incubated overnight at 37°C. After reassembly the test XMX devices were connected to the ATC and a 5-min air purge was run to allow for the measurement of the background level of microbial contamination within the ATC. After the purge, a second background measurement was taken and the XMXs were again disassembled and swabbed according to standard procedure. The swab tips were collected in Fisherbrand 50 mL centrifuge vials containing sterile water for culture / serial dilutions. During the *E. herbicola* exposures, Phosphate Buffered Saline (PBS) was used as an alternative because the organism does not survive well in water. The test matrix in Table 1 provides a brief description of each of the samples collected throughout the investigation.

Table 1. Test Matrix

Sample	Subsample	Description
1		Background: Initial sterilization for baseline
2		Background: Baseline contamination in ATC
3	a	Agent exposure, *B. atrophaeus*
	b	Post-decon (10% bleach wipedown)
	c	5-min air purge
	d	10-min air purge
	e	15-min air purge
4		Background: sterilization for baseline
5		Background: Baseline contamination in ATC
6	a	Agent exposure, *B. atrophaeus*
	b	Post-decon (10% bleach wipedown)
	c	15-min air purge
7		Background: sterilization for baseline
8		Background: Baseline contamination in ATC
9	a	Agent exposure, *B. atrophaeus*
	b	Post-decon (10% bleach submersion)
	c	15-min air purge
10		Background: sterilization for baseline
11		Background: Baseline contamination in ATC
12	a	Agent exposure, *P. agglomerans*
	b	Post-decon (10% bleach submersion)
	c	15-min air purge

Exposure

XMX inlet stacks were first inserted into the ATC, followed by the XMX base containing the five inner canister components. Two New Brunswick Scientific Biological Air Samplers (STA-203) were connected to the chamber as a means of determining the total concentration of agent in the chamber during the exposure period. Each STA sampled at a rate of 30 L/min collecting the organisms onto TSA plates. Each plate was able to collect samples for 2 minutes, requiring replacement with another plate

at the 2-minute mark. *B. atrophaeus* was maintained on a rocker platform (ZD-9550) prior to its introduction into the ATC to prevent the spores from forming agglomerates. *B. atrophaeus* spores were introduced into the chamber via a Sono-Tek Sprayer and an attached Aerodynamic Particle Sizer (TSI-3320) was used to monitor particle dimensions. The three XMX devices sampled continuously for 5 minutes to stimulate use in a biologically contaminated environment. Subsequent to each exposure run the impingement nozzle was replaced to prevent any contamination during the air purges.

Decontamination

Two methods of decontaminating critical XMX components were attempted; the first was the previously-studied wipe-down method to validate its effectiveness. The second method investigated involved submerging the components in a bleach solution for a short duration. A solution of commercially available bleach (Javex 5 bleach, 5.25% (w/v) sodium hypochlorite) was prepared at a concentration of 10% by combining one liter of bleach with nine liters of water.

Wipe-down regimen

Following the exposure sampling the XMX was removed from the ATC and the inner canister removed and dismantled into components A – E. Each component was manually wiped on all surfaces with paper towels (Scott© Shop Towels) soaked in a solution of 10% bleach. The previously determined areas of interest on each component were then swabbed with a 10% bleach solution. Following the decontamination procedure the components were wiped down with a damp paper towel to remove any

residual bleach and inhibit corrosion. Each component was swabbed once after drying with a paper towel.

Submersion regimen

As with the manual wipe-down method, the XMXs were removed from the ATC, disassembled and the appropriate components swabbed. Each component was then placed in a 5 gallon bucket containing a 10% bleach solution prepared from 1 L Javex, 9L water. After each of the five components was completely submerged in the solution a 5-minute timer was started. Upon completion, each component was then removed and placed in a bucket containing tap water for an additional 5 minutes. The components were then manually dried using paper towels and swabbed as previously described.

Quantification of Microbial Contamination

To determine the precise amount of bacterial contamination on each sampled surface, serial dilutions were performed on the samples taken from the swabbed components. The 50 mL collection tubes were vortexed to minimize any agglomeration of sample particles (i.e. spores or vegetative cells) and 1 mL was pipetted into 9 mL of sterile water creating a 1:10 dilution. This process was repeated to create 1:100 and 1:1000 dilutions when deemed necessary. Each dilution, including an undiluted sample, was plated twice. Aseptic technique was maintained throughout the experiment.

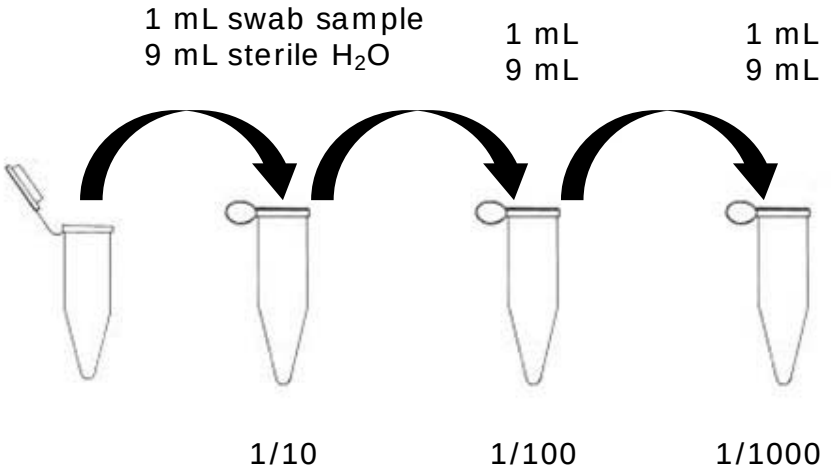


Figure 5. Serial dilution procedure

Analysis

Microbial counts in colony forming units per milliliter (CFU/mL) were recorded and then the average between each plate count was calculated. These values were then scaled according to their dilution factor, i.e. a 1/100 dilution would be multiplied by 100 to obtain the true number of colonies. These values were then normalized to 2500 liters of air per 5 minutes. From these normalized values, the contamination on each XMX component (A-E) for all three test units were summed to yield total contamination values for each subsample. These values were plotted on a log scale. Differences in mean bacterial counts in CFU/ml following application of the decontamination procedure and the air purge were examined using single-factor analysis of variance (ANOVA). Significance of differences was placed at $P < 0.05$ (5%).

IV. Results/Discussion

The application of a sodium hypochlorite solution to contaminated XMX surfaces by moistened paper towels showed considerable variation in its ability to reduce microbial contamination to background levels or below. In Figure 6, microbial contamination actually increased in one test after the decontamination procedure had been applied. Combined with the 30-minute air purge, however, contamination was reduced to near-background levels.

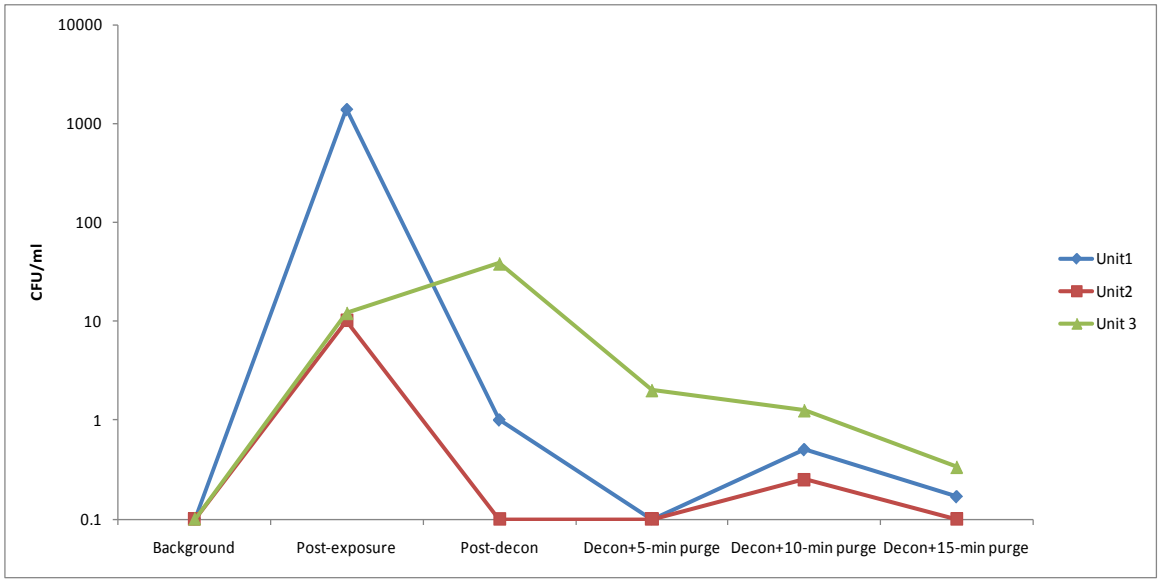


Figure 6. *B. atrophaeus* concentrations collected with the XMX/2L-MIL prior to exposure, during exposure, post decontamination by bleach wipedown and throughout an aggregate 30 minutes of air purge.

Also of note is that Unit 1 collected a bacterial load two orders of magnitude higher that the remaining devices. A possible explanation might be the air flow patterns within the test chamber itself, which may cause one device to "see" more of the bacterial

aerosol than the other devices. Dycor had previously examined how ACPLA (agent-containing particles per liter of air) differs between the ports in their aerosol test chamber. As the concentration of suspended *B. atrophaeus* spores increased, one of the ports tended to collect more spores than the other (see Appendix 1). This might explain why the contamination for XMX Unit 1 was so large when compared to the other two. It is important to note however, that the previous investigation by Dycor utilized a differing spraying device (a Hudson nebulizer versus a Sono-Tek sprayer) and a different sprayer position than the XMX decontamination setup. A comparison of the two chamber configurations can be found in Appendix 1.

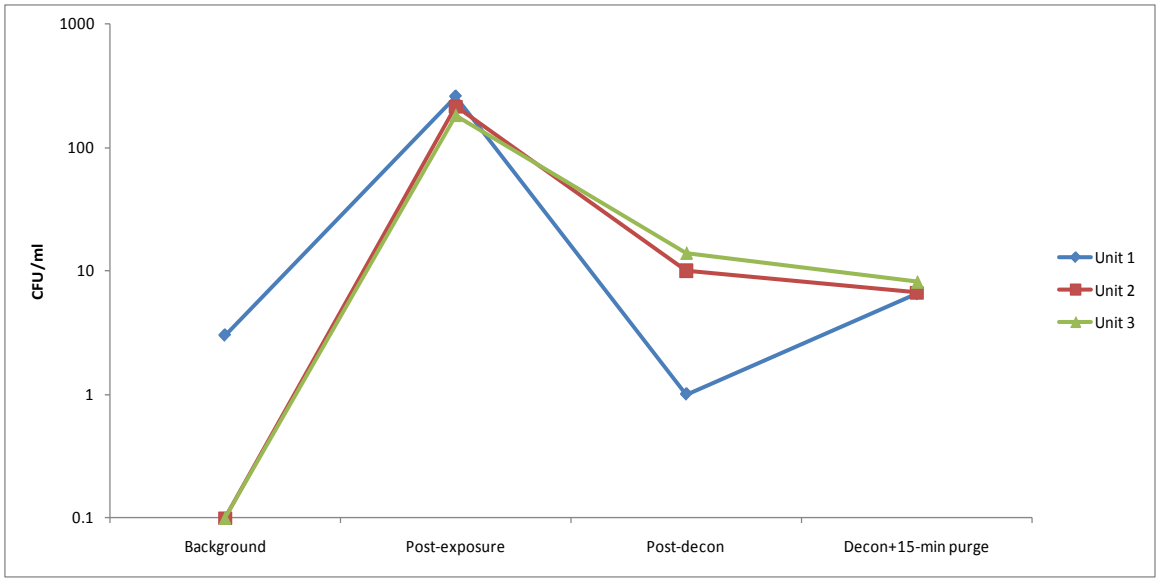


Figure 7. *B. atrophaeus* concentrations collected with the XMX/2L-MIL prior to exposure, during exposure, post decontamination by bleach wipedown and throughout 15-minutes of air purge.

Subsequent trials, however, did not demonstrate such disparate data. As shown in Figures 7 and 8, the contamination levels were generally consistent among all three

devices. While the wipedown procedure combined with only a 15-minutes air purge is capable of reducing bacterial contamination, the levels present are not comparable to background contamination. Figure 6 shows microbial growth in the post-decontamination condition, potentially indicating that bleach itself is not entirely effective or that preparation of the solution was inadequate. It may also suggest an error in technique such as an inconsistency between how thoroughly specimens were decontaminated and how thoroughly they were swabbed, or that a longer air purge is helpful.

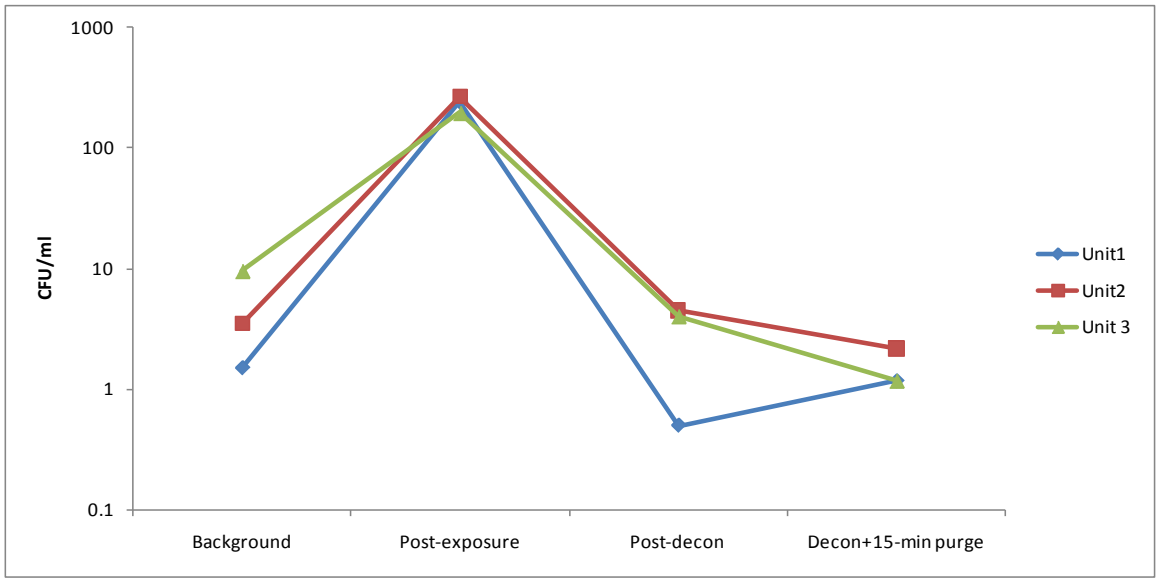


Figure 8. *B. atrophaeus* concentrations collected with the XMX/2L-MIL prior to exposure, during exposure, post decontamination by bleach submersion and throughout 15-minutes of air purge.

The bleach wipedown regimen initially appears to be somewhat more efficient than the wipe down procedure. It reduced microbial contamination on XMX surfaces by approximately two orders of magnitude, with final bacterial counts at or below

background levels. This stands in stark contrast with the previous trial. Eliminating the human element and simply exposing all surfaces to the chlorine solution equally may be one of the factors explaining the apparent increase in effectiveness. The extended contact time (5 minutes) for the bleach submersion procedure may also be a factor. Though the wipedown method was not timed, no more than 2 minutes were spent decontaminating any individual component.

Single-factor ANOVA revealed no significant difference ($p = 0.09$) between the two proposed decontamination methods (including air purge) in reducing microbial populations (Figure 9). With this outcome it cannot be concluded that one method results in a lower mean bacterial count that the others.

ANOVA: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Decon by 10% bleach wipedown + 30 min air purge	3	0.6	0.2	0.014439
Decon by 10% bleach wipedown + 15 min air purge	3	15.3334	5.111133	15.50921
Decon by 10% bleach submersion + 15 min air purge	3	4.5001	1.500033	0.333333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	38.84936	2	19.42468	3.674976	0.090785	5.143253
Within Groups	31.71397	6	5.285662			
Total	70.56333	8				

Figure 9. Influence of decontamination procedure on microbial contamination of the XMX inlet stack.

A limitation of the current investigation is reproducibility. Relatively few samples were run which can hinder arriving at a reasonable conclusion. With more trials

it may have been easier to determine if there is indeed a bias in the ATC or if some other indeterminate error was to blame. Furthermore, limitations on the efficiency of spore recovery by swabbing have been identified. Many factors contribute to spore recovery, including the initial seed of bacteria, the surface being sampled and the swab itself. Cotton swabs sampling on steel surface with liquid-deposited *B. atrophaeus* spores had a recovery efficiency of only 47% (SD = 9.3) (Edmonds, Collett, Valdes, Skowronski, Pellar, & Emanuel, 2009). When water is eliminated from the spores deposited on a surface, they tend to aggregate and can be drawn into miniscule crevices in the material, rendering them effectively lost to most sampling techniques (Edmonds, Collett, Valdes, Skowronski, Pellar, & Emanuel, 2009). It is possible that data was lost during this investigation due to the inherent difficulties in sampling spores.

Another factor which may have affected the results is the alkalinity of the bleach solution used during the investigation. The Material Safety Data Sheet for Javex 5 bleach lists the pH as 12.5-13 (Clorox, 2009). As previously mentioned, pH affects the antimicrobial activity of sodium hypochlorite. The literature search indicated that neutral bleach is preferable due to the fact hypochlorite exists preferentially as hypochlorous acid and not as hypochlorite ion. Recall that $HOCl \leftrightarrow H^+ + OCl^-$. This fact may account for some of the shortcomings in bleach disinfection noted earlier. Addition of vinegar, a substance which would likely be present in deployed as well as domestic environments, will reduce the pH and should subsequently increase antimicrobial action (Sliwa, 2006). Acidifying the solution may also decrease its stability, necessitating that bleach solutions be prepared fresh and not stored for long periods so as to retain their killing power.

Testing for available chlorine, a task well within BE capabilities, should prove helpful in maintaining the solution's disinfecting properties.

Perhaps one of the most significant limitations was the method used to arrive at the baseline contamination values, the bleach bath. By using this procedure, the tests were essentially comparing the efficacy of bleach to itself. A superior method of determining baselines would be a non-chemical method, such as autoclaving, which has documented sporocidal effects. Studies have demonstrated that autoclaving can successfully reduce spore contamination by at least 4-logs (Lemieux, Sieber, Osborne, & Woodard, 2006). The authors reported that two standard cycles of 40 minutes at 31.5 lb/in^2 and 275° F (135° C) were effective in decontaminating building materials (Lemieux, Sieber, Osborne, & Woodard, 2006).

V. Conclusions and Recommendations

Conclusions of Research

In this investigation two field-expedient methods of decontaminating the XMX/2L-MIL were tested: soaking a paper towel in 10% bleach and wiping down available surfaces and submerging the parts entirely in the solution with a five minute contact time. Hypochlorite is confirmed to be bactericidal and sporocidal at concentrations as low as 0.05%. During the study, it was observed that 10% bleach solutions resulted in approximately 10^2-fold decreases in aggregate microbial contamination on XMX components. Of the methods tested, the submersion regimen in conjunction with a 15-minute air purge showed the most efficiency. Contamination levels were consistent between all three devices and were measured at or below background levels after decontamination.

However, in certain aspects bleach disinfection did not appear to match the results reported in the literature. Though a 10^2-fold decrease represents a 99% kill probability, upwards of five orders of magnitude reductions are commonly reported in disinfection studies (Sagripanti, Carrera, Insalaco, Ziemski, Rogers, & Zandomeni, 2006) (Sagripanti & Bonifacino, 1996). The results obtained in the trials for 10% bleach wipedowns also showed peculiar trends, such as increases in microbial contamination after applying the bleach solution as well as failure to reach baseline levels. Such outcomes are can likely explained as artifacts from errors in procedure or the inadequate pH level of the bleach solution, rather than any weakness in bleach's ability to inactivate microorganisms. Though the differences in overall decontamination efficacy between the methods did not

reach statistical significance at 95% confidence, the author feels that certain recommendations may still be made.

Recommendations for Action

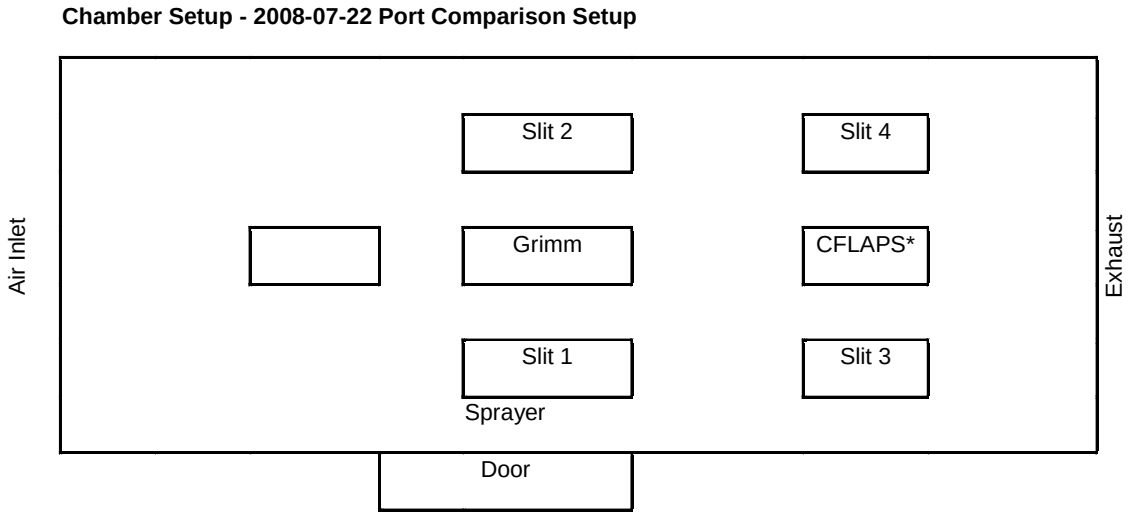
At this juncture, the bleach submersion procedure represents a simple and rapid method of decontaminating the XMX/2L-MIL after sampling a biological incident. The materials are readily available in both home-station or deployed environments, and the procedure requires minimal instruction to apprehend and properly execute. It eliminates much of the potential for human error which was present in the wipe down procedure and gives around two orders of magnitude reduction in the microbial population on the device. Furthermore, lowering the pH using vinegar is strongly recommended; it represents a simple, non-toxic method for increasing bleach's ability to sterilize surfaces with significant support from scientific literature.

Recommendations for Future Research

While a 10% bleach solution fits many of the criteria for an ideal decontaminating agent (inexpensive, easy to use, relative non-toxicity, broad antimicrobial effect etc.), what this investigation does not address is, how clean is safe? A clear metric for deciding what qualifies as "officially" decontaminated was not reached before the commencement of this project. Contamination levels were reduced to near background levels, but as previously mentioned, this baseline was determined arbitrarily using one of the methods undergoing evaluation. Further study should combine this decontamination procedure with the method of detection and identification currently used by the Air Force and its

sister services, the Joint Biological Agent Identification and Diagnostic System (JBAIDS). Doing so embraces the "Garrison equals Deployed" concept employed by the BE career field. Recall that the Eglin trials studied the XMX as part of the CBAWS alongside the BAWS Mk. III and the Rugged Advanced Pathogen Identification Device (RAPID) system, the predecessor to JBAIDS. The samples collected by the XMX/2L-MIL are suitable for analysis using the JBAIDS which is able to more reliably identify several biological weapon agents simultaneously than RAPID (Idaho Technology Inc., 2001-2009). Ideally, after decontaminating an XMX unit using the protocol recommended above, analysis using the JBAIDS would come back negative. It is entirely possible, however, that the JBAIDS process is sensitive enough to detect the level of residual contamination on the XMX that were characterized as "background." If future studies are undertaken, a set amount of microbial reduction should be predetermined with the results compared to that mark.

Appendix 1 – Supplemental Data



* The CFLAPS is a biological aerosol detector that continuously samples.

Figure 10. Chamber Setup - 2008-07-22 Port Comparison Setup

Table 2. Port comparison trial, static cloud with 100 ACPLA load

		ACPLA			
Setpoint	**Trial #**	**Slit 1**	**Slit 2**	**Slit 3**	**Slit 4**
100	1	80.9	69.5	69.5	71.9
	2	90.3	80.9	81.5	84.9
	3	77.8	68.1	66.9	70.9
	4	94.6	86.5	82.0	84.8
	5	77.4	62.2	67.2	63.2
	Average	84.2	73.4	73.4	75.1
	Minimum	73.4		**Maximum**	84.2
	Range	10.8		**% diff.**	12.8

Table 3. Port comparison trial, static cloud with 200 ACPLA load

		ACPLA			
Setpoint	**Trial #**	**Slit 1**	**Slit 2**	**Slit 3**	**Slit 4**
200	6	122.3	101.0	103.4	115.5
	7	114.4	106.4	104.6	125.8
	8	105.3	101.6	103.1	120.6
	9	110.0	97.0	87.0	103.8
	10	109.2	99.6	100.9	111.9
	Average	112.2	101.1	99.8	115.5
	Minimum	99.8		**Maximum**	115.5
	Range	15.7		**% diff.**	13.6

Table 4. Port comparison trial, static cloud with 300 ACPLA load

		ACPLA			
Setpoint	**Trial #**	**Slit 1**	**Slit 2**	**Slit 3**	**Slit 4**
300	11	122.0	110.9	114.5	133.8
	12	140.6	123.2	114.0	119.7
	13	138.1	124.0	120.1	141.8
	14	143.8	129.3	113.7	144.4
	15	131.6	124.0	121.3	131.1
	Average	135.2	122.3	116.7	134.2
	Minimum	116.7		**Maximum**	135.2
	Range	18.5		**% diff.**	13.7

Appendix 2 – Raw Data

Table 5. Raw counts of microbial contamination during B.g. trials

The following samples were collected using *Bacillus atrophaeus* (Bg)										
Background 01	Unit 1	Unit 2	Unit 3							
A	0	0	0							
B	1	0	0							
C	0	0	0							
D	0	0	0							
E	0	0	0							
Sample 3A CT (raw)	**Unit 1a**	**Unit 1b**	**Unit 2a**	**Unit 2b**	**Unit 3a**	**Unit 3b**				
d0	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC				
d1	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC				
d2	13	25	26	22	21	26				
d3	2	3	2	2	0	3				
Sample 3A (raw)										
Unit 1	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
d0	2	2	13	9	10	14	9	13	1	2
d1	1	0	1	1	1	4	2	0	0	0
d2	0	0	0	0	0	0	0	0	0	0
d3	0	0	1	0	0	0	0	0	0	10
Sample 3A (raw)										

Unit 2	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
d0	3	2	14	21	0	0	1	0	0	0
d1	0	0	3	1	0	0	0	0	0	0
d2	0	0	0	0	0	0	0	0	0	0
d3	0	0	0	0	0	0	0	0	0	0
Sample 3A (raw)										
Unit 3	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
d0	2	1	24	27	5	2	4	2	5	5
d1	0	0	1	1	0	0	0	0	0	0
d2	0	0	0	0	0	0	0	0	0	0
d3	0	0	0	0	0	0	0	0	0	0
Sample 3B (raw)	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
Unit 1	1	0	1	0	0	0	0	0	0	0
Unit 2	0	0	0	0	0	0	0	0	0	0
Unit 3	0	0	0	0	0	0	39	38	0	0
Sample 3C (raw)	**Unit 1a**	**Unit 1b**	**Unit 2a**	**Unit 2b**	**Unit 3a**	**Unit 3b**				
Collection tube	0	0	0	0	4	6				
Sample 3C (raw)	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
Unit 1	0	0	0	0	0	0	0	0	0	0
Unit 2	0	0	0	0	0	0	0	0	0	0
Unit 3	0	0	0	0	0	0	3	1	0	0

Sample 3D (raw)	**Unit 1a**	**Unit 1b**	**Unit 2a**	**Unit 2b**	**Unit 3a**	**Unit 3b**				
Collection tube	0	0	1	2	5	4				
Sample 3D (raw)	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
Unit 1	0	0	0	0	2	0	0	0	0	0
Unit 2	0	0	1	0	0	0	0	0	0	0
Unit 3	1	0	1	0	0	0	2	0	1	0
Sample 3E (raw)	**Unit 1a**	**Unit 1b**	**Unit 2a**	**Unit 2b**	**Unit 3a**	**Unit 3b**				
Collection tube	0	0	1	2	5	4				
Sample 3E (raw)	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
Unit 1	0	0	1	0	0	0	0	0	0	0
Unit 2	0	0	0	0	0	0	0	0	0	0
Unit 3	1	0	0	0	1	0	0	0	0	0
Background 4	**Unit 1**	**Unit 2**	**Unit 3**							
A	0	0	0							
B	1	0	0							
C	0	0	0							
D	0	0	0							
E	0	0	0							
Background 5 (raw)	**Unit 1a**	**Unit 1b**	**Unit 2a**	**Unit 2b**	**Unit 3a**	**Unit 3b**				

Collection tube	1	0	0	1	2	1				
Background 5 (raw)	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
Unit 1	1	1	0	0	0	0	3	0	1	0
Unit 2	0	0	0	0	0	0	0	0	0	0
Unit 3	0	0	0	0	0	0	0	0	0	0
Sample 6A CT (raw)	**Unit 1a**	**Unit 1b**	**Unit 2a**	**Unit 2b**	**Unit 3a**	**Unit 3b**				
d0	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC				
d1	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC				
d2	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC				
d3	21	13	18	28	22	36				
Sample 6A (raw)										
Unit 1	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
d0	13	3	194	157	21	30	73	91	19	24
d1	3	0	10	12	2	4	2	6	1	1
Sample 6A (raw)										
Unit 2	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
d0	1	0	155	162	8	3	61	67	6	5
d1	0	1	13	16	1	0	2	5	1	0
Sample 6A (raw)										

Unit 3	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
d0	10	9	171	151	29	26	14	28	31	32
d1	1	1	7	4	0	2	1	2	3	2
Sample 6B (raw)	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
Unit 1	0	0	1	1	0	0	0	0	0	0
Unit 2	4	1	0	0	4	3	1	1	4	2
Unit 3	5	5	4	2	4	3	1	0	4	0
Sample 6C CT (raw)	**Unit 1a**	**Unit 1b**	**Unit 2a**	**Unit 2b**	**Unit 3a**	**Unit 3b**				
d0	6	4	*	*	16	11				
d1	1	1	*	*	1	0				
Sample 6C (raw)	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
Unit 1	2	10	4	4	2	4	5	2	4	2
Unit 2	8	0	5	2	6	0	6	6	5	2
Unit 3	2	2	5	7	5	4	8	4	6	6
Background 7	**Unit 1**	**Unit 2**	**Unit 3**							
A	0	0	0							
B	0	0	0							
C	0	0	0							
D	0	0	1							
E	0	0	0							
Background 8 CT (raw)	**Unit 1a**	**Unit 1b**	**Unit 2a**	**Unit 2b**	**Unit 3a**	**Unit 3b**				

d0	1	0	0	0	1	1				
Background 8 CT (raw)	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
Unit 1	0	0	1	0	0	0	0	2	0	0
Unit 2	0	0	1	0	3	1	1	1	0	0
Unit 3	1	0	8	1	0	0	1	0	7	1
Sample 9A CT (raw)	**Unit 1a**	**Unit 1b**	**Unit 2a**	**Unit 2b**	**Unit 3a**	**Unit 3b**				
d0	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC				
d1	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC				
d2	81	92	114	94	105	133				
d3	6	6	11	7	8	3				
Sample 9A (raw)										
Unit 1	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
d0	4	2	130	120	70	63	43	52	4	9
d1	1	0	7	12	10	6	3	5	2	0
Sample 9A (raw)										
Unit 2	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
d0	4	2	252	226	6	9	25	28	14	9
d1	0	0	19	22	0	0	2	2	3	1
Sample 9A (raw)										

Unit 3	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
d0	2	2	190	140	22	18	8	6	10	12
d1	1	0	17	15	2	2	0	0	0	0
Sample 9B (raw)	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
Unit 1	0	0	0	0	0	0	1	0	0	0
Unit 2	2	0	1	0	0	1	3	0	2	0
Unit 3	2	0	2	0	2	0	1	0	1	0
Sample 9C CT (raw)	**Unit 1a**	**Unit 1b**	**Unit 2a**	**Unit 2b**	**Unit 3a**	**Unit 3b**				
d0	5	6	*	*	5	8				
Sample 9C (raw)	**Aa**	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Ea**	**Eb**
Unit 1	0	0	2	0	1	2	1	0	1	0
Unit 2	6	0	2	0	2	1	1	0	1	0
Unit 3	1	0	0	0	0	2	1	0	2	1

*indicates evaporation of the sample medium

TNTC = too numerous to count

14-Apr-09

Nomenclature

d0	No dilution	Sample A: post-exposure
d1	1/10 dilution	Sample B: post-decon
d2	1/100 dilution	Sample C: post-decon + 5 min
d3	1/1000 dilution	Sample D: post-decon + 10 min
		Sample E: post-decon + 15 min
a	First split sample	
b	Second split sample	Label Part
σ	Standard deviation	A Inner stack
		B Nozzle plate
CT	Collection tube	C Upper canister
		D Lower canister
		E Final nozzle

Figure 11. Nomenclature describing the raw data table

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14. ABSTRACT
The XMX/2L-MIL is a high volume air sampler used by the Air Force Bioenvironmental Engineering community to collect biological aerosols. Without a verified decontamination technique, however, the XMX cannot be used effectively. The objective of this study is to evaluate several proposed methods for expedient field decontamination of the XMX. This study centers on the inactivation of *Bacillus atrophaeus* spores and vegetative *Erwinia herbicola* organisms from the XMX inner canister. The goals this investigation sought to achieve were twofold: 1) to verify the antimicrobial efficacy of a 10% bleach solution and 2) to determine if wiping the components with a bleach-soaked paper towel or submerging the components directly in the bleach solution represents the optimal decontamination procedure.

Data was gathered at the Dycor Technologies facility located in Edmonton, Alberta, Canada. Their Aerosol Test Chamber was used to disseminate the surrogate agents and then sample the aerosol using three XMX devices. Counts of the microbial population were calculated at each stage of the procedure to assess how effective the two proposed methods were.

It was observed that 10% bleach solutions resulted in approximately 10^2-fold decreases in aggregate microbial contamination on XMX components. Of the methods tested, the submersion in a 10% bleach solution plus a 15-minute air purge showed the most efficiency. Contamination levels were consistent between all three devices during the trial and were measured at or below background levels after decontamination.

15. SUBJECT TERMS
Bacillus anthracis, *Bacillus atrophaeus*, *Yersinia pestis*, *Erwinia herbicola*, decontamination, sodium hypochlorite, XMX/2L-MIL, bioaerosols

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