Persistence of *Bacillus anthracis* spores and *Clostridium botulinum* and Destruction of *Francisella tularensis* and *Yersinia pestis* in Municipal Solid Waste Landfill Leachates

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INTRODUCTION

The 2001 incident with five *Bacillus anthracis* contaminated letters resulted in contaminating 56 buildings in 10 US states and Washington, D.C. Items that were contaminated were such things as: books, paper, wall hangings, staplers, telephones, furniture, computers, mail processing equipment, carpeting, ceiling panels, wallboard, paneling, nail, trash, spoiled food, contaminated decontaminate water, personal protective equipment and air scrubbing equipment. The ultimate fate of "decontaminated" building materials, that is, the permanence of the disposal technique, is of concern long after final disposal at a landfill site. As much of the decontaminated building material will end up in municipal solid waste (MSW) landfills due to capacity problems with incinerators and hazardous waste landfills, the United States Environmental Protection Agency's Office of Research and Development's National Homeland Security Research Center (NHSRC) in collaboration with the Department of Defense Edgewood Chemical Biological Center (ECBC)

are evaluating the permanence of biological and chemical warfare agents in municipal solid waste landfills. Decontaminated waste that can be verified as 100% free of residual biological contaminant should present no problem in the landfill environment short of straining national capacity. Materials that contain some residual active contaminants, however, may present a different scenario and a different concern for the landfill operator even though leachate itself is not sterile and dose and exposure risk must be considered. Exposure pathways for movement of residual contaminants out of landfills are primarily leachate and air (fugitive emission and collected gas); but the pathways may also include long-term potential for groundwater contamination or even movement through solids (e.g., soil), both from movement through leachate. If individual waste containers (sub-containment systems) become compromised in the landfill, leachate may contain components of the disposed, and presumed decontaminated, waste. Leachate is often discharged to wastewater treatment systems, and may pose a threat to human health and the environment.

Study of the permanence of the final disposal of the inactivated or active agent of terrorism must be examined by looking at the fate of various agents in the most likely pathway of escape. The agents of terrorism to be reviewed are most likely to be biological because of multiplicity and low (in most cases) infective doses, and biological agents may be the most economical to obtain in effective quantity While approximately 209 million tons of MSW are generated annually in the USA (Franklin Assoc. 1996), bacteria present in MSW are of great concern. Waste that then might contain viable biological weapons or other pathogenic microorganisms is specifically of concern especially if there is a chance that they could be transported to landfill leachate and potentially infect groundwater. Some work has been done to isolate pathogens and other bacteria in MSW and landfill leachate (Donnelly 1983). However, although various genera have been identified in MSW such as *Clostridium*, *Bacillus*, *Pseudomonas*, *Citrobacter*, *Corynebacterium*, *Aeromonas* and *Enterobacter* (Nwosu and Ladapo1999, Van Dyke and McCarthy 2002, Francis *et al.* 1980), no studies are available as to the fate of biological warfare agents in landfill leachate or MSW.

MATERIALS AND METHODS

The purpose of this research is to determine the permanence of disposal of weapons of warfare that may be introduced into municipal solid waste (MSW) landfills with insufficiently treated rubble from contaminated sites. Because of capacity problems with incinerators and hazardous waste sites, it is more likely that we will need to depend on MSW landfills to contain the uncontaminated building products. Our approach is to inoculate raw un sterilized MSW leachate with known quantities of biological agents, developing leachate microcosms in a secure laboratory, and analyzing the spiked leachates for viable agent through a 12-month time course. The samples were handled as waste would be: initially aerobically, sealed and then allowed to reach anaerobiosis. Landfill temperatures vary over their lifetime and in the waste mass. To look at two worst case scenarios, 12 and 37 ^{0} C incubations were used to simulate soil temperature and optimum temperature for pathogens. Uninoculated samples serve as negative controls and inoculated growth media (different per agent) serve as positive controls. Four bacterial agents were tested (Table 1).

Table 1. Listing of Strains and Growth Characteristics of Bacterial Cells.

Bacterial Type	General Media (G) Selective Media (S)	Culture Temp. & Time
Clostridium botulinum	(G) ATCC medium 1053 Clostridial (Oxoid CM149)	35-37˚C for 24-48 hrs
	(S) PEA (phenylethyl alcohol agar) and egg yolk agar (EYA)	
Francisella tularensis	(G) ATCC medium 192 Cysteine heart agar	35-37˚C for 4-7 days
	(S) Chocolate agar (CA), Thayer Martin (TM) and Buffered Charcoal yeast extract (BCYE)	
Bacillus anthracis	(G) ATCC medium 254 Heart infusion agar	35-37˚C for 24-48 hrs
	(S) Sheep blood agar (SBA) and PLET will be used (Polymyxin-lysozyme EDTA-thallous acetate)	
Yersinia pestis	(G) ATCC medium 3 Nutrient agar	28˚C for 2-4 days
	(S) Yersinia selective agar (YSA).	

Media are listed as the general medium for culture maintenance and the selective medium used for recovery from among leachate microflora.

Inoculated leachates (triplicate samples) were tested for quantities of viable biological agent weekly for the first 2 months, then twice a month for 5 months, then monthly for 5 more months (unless data indicated otherwise) or until no detects are observed in all replicates for 2 consecutive sampling periods. This would identify the termination of the experiment for that agent. Tests of viability were performed using approved culture methods.

Numerous assumptions were made with this research, some of which are:1. Results obtained from a MSW landfill's leachate will be representative of the results we may expect to see with leachate from other landfills. We are analyzing leachates from only one site and may or may not be able to extrapolate to what would occur at a different landfill. This landfill is closed, synthetically covered, and has waste that is between 5-15 years old. However, using the one site is a start and future studies may include multiple landfill sites. 2. Triplicate microcosms will allow us to better understand the real-world situation. 3. Three ml microcosms will mimic anaerobic conditions of landfills. 4. Temperatures vary greatly in landfills. Looking at two temperatures mimics the world (average soil temperature (12^0 C) and higher incubation temperature optimized for pathogens (37^0 C)). 5. The agents will always encounter undiluted leachate in the landfill before any release is possible.

Bacterial cells were first streaked for isolation on appropriate media (General medium). A single colony was then grown in broth culture (General medium). Glycerol stocks were prepared at –80˚C for long-term storage. An overnight broth culture was grown for *F. tularensis*, *Y. pestis* and *C. botulinum* in appropriate media and incubated under appropriate aeration and temperature conditions. The cells were harvested by centrifugation, washed with phosphate buffered saline

(PBS) and resuspended in a minimal volume of PBS. The cells were used to inoculate landfill leachate samples. *B. anthracis* spores were produced utilizing the MAP protocol for spore preparation and used to inoculate leachate samples.

Viruses are propagated in either the mammalian cell line Vero or BSC-40 cells, and grown in standard cell culture media. Viruses will be collected in cellular supernatant and used to inoculate leachate samples (or can be stored indefinitely at –80^{o}C).

Inoculum Enumeration

Bacterial cells and spores were serially diluted in PBS and enumerated by plating 100-µL aliquots in triplicate on plates. Virus stocks will be enumerated by standard plaque assay (in quadruplicate) to determine concentration in plaque forming units per milliliter (pfu/ml).

Spiking of Inoculum

Bacterial cells and spores were added to appropriate aliquots of leachate such that the final concentration of the target organism was approximately 10^7 cfu/ml. Viruses will be added to appropriate aliquots of leachate such that the final concentration of the target organism is approximately 10^6 pfu/ml.

Test Matrix

Landfill leachate samples were received from the Sandy Hill Landfill (Bowie, MD) and processed within 24 hours of collection of samples. After spiking appropriate aliquots of leachate with each bacterial target organism, 3 ml aliquots were dispensed to 5 ml anaerobic culture vials until a total of 150 tubes for each target bacterium were reached. 72 tubes were then incubated at 37^{o}C and 72 tubes incubated at 12^{o}C for each bacteria. The remaining six inoculated tubes were incubated (three at 37^{o}C and three at 12^{o}C) and monitored for aerobic status (aerobic controls). An anaerobic (oxidation-reduction) indicator dye, resazurin (which turns from blue to pink to colorless as conditions go from aerobic to anaerobic), was added to the aerobic controls and their color was monitored periodically to provide a measure of the anaerobic status of the corresponding samples. Tubes were labeled (using pre-printed labels) with a control number comprised of a 6 digit start date (mmddyy), 2-3 letter organism code (from Table 2), 2 digit + C temperature (37C or 12C), and a dash (-) followed by a 2 digit tube number. Aerobic controls had AC for the organism code. Un-inoculated leachate was included as a negative control (NC for organism code), while inoculated growth media (agent specific) served as a positive control (organism code + PC). The same test matrix will be used for virus testing once testing begins.

Performance Period and Experimental Design

Bacterial sampling was to occur every week for 2 months, then bi-weekly for 5 months and finally monthly for 5 months, or until there was no detectable target in two consecutive sampling periods. In order to assure that the absence of detectable growth was not attributable to expiration of the specific recovery media, a fresh inoculum of the target BW agent into the

specific recovery media was included in subsequent sampling periods once a "no detect" event was observed. Continued absence of detectable target in two consecutive sampling periods with positive growth observed in this second positive control was construed as evidence that the target BW agent no longer persisted under the incubation conditions, and sampling for that target BW agent was discontinued. A negative control was sampled at each time point. A positive control was also sampled at each time point or until no growth was observed in two consecutive sampling periods. The same sampling strategy will be employed for viruses.

Sampling and Analysis

At each time point for both incubation temperatures, microcosms (5 ml anaerobic culture vials) of leachate spiked with each bacterial target were removed from incubation and analyzed for viable bacteria. Samples were centrifuged, washed (pellets) and re-suspended in sterile phosphate buffered saline (PBS using aseptic technique). Samples were serially diluted in PBS and 100-µL aliquots plated in triplicate on appropriate media using aseptic technique (Selective media). Plates were labeled with the sample control number to which a dash and the 6 digit (mmddyy) date of removal from timepoint incubation was appended followed by an A, B, or C to indicate triplicate replicates. The inoculated and labeled plates were allowed to incubate at the appropriate temperature, aeration conditions, and time, and then observed for growth of target organisms . Growth of target organism indicated survival in leachate and the concentration was determined. After confirmation, all plates were autoclaved and disposed.

For samples of leachate spiked with viral targets, samples will be removed over time from both incubation temperatures and analyzed for the presence of virus, using aseptic technique at each step. The spiked leachate sample will be filtered and the virus will be eluted from the filter and added to cell culture-safe media. The virus-containing media will be serially diluted and each dilution will be used to infect monolayers of mammalian Vero cells or BSC-40 cells. Infected cell cultures will be incubated at the appropriate temperature for the appropriate amount of time after which they will be stained and observed visually for viral plaque formation.

Microbial Identification System (MIDI) Analysis

Prior to beginning any experiments, an assessment of the native microflora present in raw leachate was conducted using the MIDI. This microbial identification system compares the fatty acid profile of the unknown sample with libraries containing fatty acid profiles of known microbes in order to identify the sample. This analysis was performed to rule out the presence of any harmful bacteria and to aid in the safety of the personnel on the project. It was determined that leachate should be handled under BSL-2 conditions and posed no threat to personnel when handled at this level correctly.

CONCLUSIONS

The characterization of the aerobic normal flora of MSW landfill leachate by methods of MIDI are shown in Table 2 below. A Similarity Index (SI) of 0.5 or higher indicates a good library comparison. An SI of 0.3 to 0.5 indicates it may be a good match, but just an atypical strain, while an SI lower than 0.3 indicates that the species is not in the database. However in cases of

SI lower than 0.3, the closest relative is given. Further information about the species are also discussed below.

Table 2. MIDI Characterization of Aerobic Normal Flora of MSW Landfill Leachate

IDENTIFICATION	SI	CONCENTRATION (CFU/ML)
Bacillus thuringiensis	0.547	>1000
Aeromonas hydrophila	0.573	1000
Pseudomonas putida	0.161	1000
Chryseobacterium indologenes	0.540	10-100
Aeromonas sobria	0.307	10
Nocardia otitidiscaviarum	0.511	10
QC (*P. aeruginosa*)	0.590	

Bacillus thuringiensis: BSL 1 organism. It is a spore forming, aerobic gram-positive rod. *B. thuringiensis* is an insect pathogen (and is not associated with any human diseases) and is used in producing commercially available insecticides. *B. thuringiensis* is typically found in soils and water.

Aeromonas hydrophila: BSL 2 organism. It is also non-spore forming and is a facultatively anaerobic gram-negative rod.

Aeromonas species are ubiquitous inhabitants of natural waters and sewage. Human infections can occur primarily by contact or ingestion of water or soil. Four types of disease can occur: wound infection, septicemia, extraintestinal infections and diarrheal diseases with wound infections occurring near or in water or diarrheal disease (due to drinking untreated water) being the most common.

Pseudomonas putida: BSL 1 organism. It is a non-spore forming, aerobic, gram-negative rod. *P. putida* is usually associated with soil, water and plants and animal sources. Although some pseudomonads are important opportunistic pathogens, such as *P. aeruginosa*, *P. putida* is non-pathogenic.

Chryseobacterium indologenes (Flavobacterium): BSL 2 organism. It is a gram-negative rod. It is aerobic and non-spore forming. Flavobacteria are ubiquitous in soil and water. *F. indologenes* has rarely been associated with human infection.

Aeromonas sobria: BSL 1 organism. It is a non-spore forming, gram-negative rod. It is facultatively anaerobic.

Nocardia otitidiscaviarum: BSL 2 organism. It is an aerobic actinomycete. It is non-spore forming and stains gram-positive. *N. otitidiscaviarum* is considered a human pathogen; however, it is not the most common species associated with human infection (pulmonary and systemic).

Several actinomycetes can cause infection, but *Nocardia* species usually account for the majority of reported cases with *N. asteroides* causing over 90% of all infections. *Nocardia* are inhabitants of soil and water and infection in humans can occur after inhalation or inoculation through breaks in the skin. It should be noted however, that human infections typically only occur in immunocompromised individuals.

The characterization of the anaerobic normal flora of MSW landfill leachate by methods of MIDI are shown in Table 3 below. Again a similarity Index (SI) of 0.5 or higher indicates a good library comparison. Further information about the species are discussed below.

Table 3.MIDI Characterization of Anaerobic Normal Flora of MSW Landfill Leachate

IDENTIFICATION	SI	CONCENTRATION (CFU/ML)
Aeromonas hydrophila	0.786	1000
Aeromonas sobria	0.346	10
Corynebacterium glucuronolyticum	0.117	10
Brevundimonas diminuta	0.510	10
QC (P. aeruginosa)	0.755	

NOTE: Both ***Aeromonas*** species are discussed above. Since they are facultative organisms, they were present under both anaerobic and aerobic conditions.

Corynebacterium glucuronolyticum: BSL2 organism. It is a gram-positive, non-sporeforming, facultative anaerobic rod. The organism is usually associated with the human urinary tract. Other members of the genus *Corynebacterium* are more notable such as *C. diphtheriae,* which causes diphtheria. The genus includes other human pathogens and opportunistic species, but information on *C. glucuronolyticum* is limited.

Brevundimonas (Pseudomonas) diminuta**:** BSL 1 organism. It is a gram-negative, non-spore forming bacillus. Although most Pseudomonads are aerobic, some strains of *B. diminuta* may be anaerobes. *B. diminuta* has been isolated from water, hospital equipment and human clinical specimens. *B. diminuta* has been the cause of at least one reported case of septicemia, but clinical significance of *B. diminuta* has not been determined.

Fate of BWA in MSW Landfill Leachate

Bacillus anthracis

At both 12 and 37 ^{0} C, the MSW leachate allowed for consistent survival of *B. anthracis* for at least 24 weeks.

Clostridium botulism

Clostridium botulism survived at both 12 and 37 ^{0} C for at least 22 weeks. The survival was not

as stable as it was for *B. anthracis*. This may suggest that it sometimes exists in an anaerobic vegative state instead of a spore formation.

Yersinia pestis

At 37 ^{0} C *Y. pestis* was non viable at 2 weeks. At 12 ^{0} C, it was non viable at 6 weeks. The cooler soil-like temperature seems to protect it more.

Francisella tularensis

F. tularensis was non viable at 7 weeks at both 12 and 37 ^{0} C in the MSW landfill leachate.

Thus the spore-forming agents *B. anthracis* and *C. botulinum* are surviving in the MSW leachate for longer periods than the non- spore forming bacteria. Longer term studies will be performed on these organisms. Incubation temperatures are not much of a variable for any of the agents as the degradation rates were similar with different temperatures. Using raw leachate allowed for simulation of competition by normal flora.

In conclusion, although it would be hoped that survival of biological weapons and pathogens would not be insured in landfill leachates, the persistence of some of these agents must be considered before deciding to place them into municipal solid waste landfills. However, landfills are never sterile. Thus concentration of agent, infective and lethal doses, and likelihood of exposure to a sensitive population must be taken into account.

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