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**FACULTY OF GRADUATE AND
POSTDOCTORAL STUDIES**

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M.Sc. (Microbiology and Immunology)

GRADE / DEGREE

Department of Biochemistry, Microbiology and Immunology

FACULTE, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

**A Standard Approach to Assessing Disinfection of Environmental Surfaces:
Experiments Using a Mixture of Surrogates for Bioagents**

TITRE DE LA THÈSE / TITLE OF THESIS

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**A STANDARD APPROACH TO ASSESSING DISINFECTION
OF ENVIRONMENTAL SURFACES: EXPERIMENTS USING
A MIXTURE OF SURROGATES FOR BIOAGENTS**

Safaa Sabbah

Thesis submitted to the Department of Biochemistry, Microbiology and
Immunology in partial fulfillment of the requirements for a Master of Science
degree

University of Ottawa

Ottawa, Ontario, Canada



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395, rue Wellington
Ottawa ON K1A 0N4
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Your file *Votre référence*
ISBN: 978-0-494-51655-3
Our file *Notre référence*
ISBN: 978-0-494-51655-3

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ABSTRACT

Background: Non-porous environmental surfaces accidentally/deliberately contaminated with bioagents might play a role in the spread of infections. The risk of such spread can be reduced with judicious use of liquid chemical disinfectants. Such chemicals are usually applied directly on the target surface as a liquid by immersion, flooding, spray, or by wiping with a disinfectant-wetted fabric. However, the assessments and label claims of microbicidal activities of such chemicals are often based on testing against individual classes of microbes without an appropriate soil load, while pathogens in the field are either unknown or may exist as mixtures in body fluids or other organic/inorganic debris. Further more, the influence of the mechanical action of wiping in decontaminating surfaces is hardly ever assessed. Wiping microbially-contaminated surfaces with an ineffective disinfectant may also spread localized contamination over a wider area, thus enhancing the risk of spread of infections. The potential impact of such spread remains virtually unknown. This study was, therefore, aimed at filling these three gaps in our knowledge, using a standardized and quantitative carrier test (QCT-2) known to be stringent yet applicable for use with a wide array of microbes, surface types as well as disinfectant liquids, foams and gels, with the ultimate purpose of addressing the important question of 'how clean is clean?', when it comes to deciding on the outcome of any remediation of a site contaminated with infectious bioagents.

Experimental: To simulate field conditions better, the challenge was a cocktail of bacterial spores (*Geobacillus stearothermophilus*), a vegetative bacterium (*Acinetobacter baumannii*), a mycobacterium (*Mycobacterium terrae*), and a non-enveloped virus (hepatitis A) in a soil load. Disks (1 cm in diameter) of brushed stainless steel were used as prototypical hard, non-porous environmental surface. Each disk received 10 μ L of the

microbial suspension. The inoculum was then dried prior to direct contact with the test disinfectant or assessing the effect of wiping for decontamination and transfer of contamination. In both decontamination methods, normal buffered saline (NBS) was used as the control fluid.

In direct kill tests with disinfectants, each disk with the dried inoculum was overlaid with 50 μ L of NBS or the formulation under test and held at the temperature/time specified for the experiment. The disks were then eluted with an eluent/neutralizer, the eluates assayed for viable organisms and \log_{10} reductions in viability calculated in relation to the controls.

When testing the influence of wiping, the dried inoculum on the disk was wiped while exerting a specific pressure with a piece of cotton or microfibre-based fabric pre-wetted with the control fluid or the disinfectant under test. The fabric used for wiping was then brought in contact with a clean disk to determine the extent of transfer of microbial contamination. The disks were eluted as described above to determine the level of decontamination and transfer.

Results: The microbial mixture successfully evaluated the efficacy claims and spectrum of activity of disinfectants concurrently, with no major differences in inactivation of microbes when challenged alone or as a mixture. Surprisingly, peracetic acid (PAA; 1,000 ppm) and accelerated hydrogen peroxide (AHP)-based formulations (40,000 to 70,000 ppm) reduced the viability of the spores to undetectable levels, but without complete inactivation of the virus at the same contact time and temperature. Chlorine dioxide (CD) and a 5.25% (w/v) solution of sodium hypochlorite, i.e., domestic bleach (DB), showed the broadest spectrum of microbicidal activity. However, their activities depended on air temperature. While CD (500 and 1,000 ppm) and DB (5,000 ppm) completely inactivated HAV and the spores at room temperature (\sim 22-24°C), these formulations only killed the virus at 4°C but

not the spores. *M. terrae* and *A. baumannii* were relatively readily inactivated and proved to be very similar in their sensitivities to all the tested chemicals.

In general, wiping with a disinfectant-wetted fabric was more effective than direct kill for decontamination. However, the potential transfer of contaminants to clean areas depended mostly on the microbe and the way transfer was carried out (i.e., type of fabric and wiping motion). Decontamination by wiping was more effective for the vegetative bacterium than for the virus and the spores. When disks contaminated with *A. baumannii* were wiped with a microfiber-based fabric wetted with 25 μL of NBS, a 4 \log_{10} reduction was achieved while reductions of 1.51 and 1.72 \log_{10} were recorded for the spores and the virus, respectively. Correspondingly, the transfer from the contaminated fabric to a clean disk for *A. baumannii* was at least 400 times less than that for the spores and the virus. Our data suggested that applying a formulation directly on to a contaminated surface, letting it dry, and then wiping the surface with a disinfectant helped achieve greater \log_{10} reductions while reducing substantially the risk of transfer of contaminants to clean areas.

Conclusions: This study assessed the relative strengths and the spectrum of activity of the tested formulations concurrently and under conditions more akin to those expected under field conditions. Incorporation of a cocktail of microorganisms into a single test permitted a more reliable means of classifying microbicides based on their spectrum of activity. Chlorine releasing agents proved to be more effective and have a broader spectrum of activity as compared to the peroxygens. Disinfectant activity depended on air temperature, and none of them was effective against the spores at 4°C. Decontamination by wiping with disinfectants proved to be faster and more effective than direct kill. However, a higher risk of a wider spread of contamination during wiping was also evident in this study. The foundations laid by this investigation should allow more detailed studies using actual infectious bioagents.

ACKNOWLEDGEMENTS

I thank my supervisor, Dr. Syed A. Sattar, for his kindness, understanding, valuable advice and continuous support throughout the project. I am also thankful to the members of my thesis advisory committee, Dr. Trevor Lumley, Mrs. V. Susan Springthorpe, Mr. Denis Laframboise, and Ms. Marianne Heisz for their support, kindness, and valuable advice.

I wish to thank all the staff of Dr. Sattar's laboratory for their kindness and especially Sola Adegbunrin, Justo Perez and Mohammed Ali for guidance, assistance, support and friendship.

I wish to thank my family, especially my husband, Yasser Salame, for his support and my children, Khodor, Salam, Samah and Sabrine for their understanding. I also thank my father Mohamad Ali Sabbah and my mother Afaf AlHaj Ali for their continuous encouragement, and my brother Fouad Sabbah for his support.

The study was supported in part by the CRTI program (#04-0018RD).

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LIST OF ABBREVIATIONS

ADC	Albumin Dextrose Catalase Complex
AHP	Accelerated Hydrogen Peroxide
AOAC	Association of Official Analytical Chemists
ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
BCG	Bacillus Calmette-Guérin
BSA	Bovine Serum Albumin
BW	Biological Weapon
CBW	Biological Weapons Classifications
CD	Chlorine dioxide
CDC	Centre for Disease Control and Prevention (U.S.)
CEPA	Canadian Environmental Protection Act
CFU	Colony Forming Unit
CGSB	Canadian General Standards Board
CL	Containment Level
CRA	Chlorine Releasing Agent
CREM	Centre for Research on Environmental Microbiology
DB	Domestic bleach
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic Acid
EPA	Environmental Protection Agency (U.S.)
FBS	Fetal Bovine Serum
F&DA	Food and Drugs Act (Can.)
FDA	Food and Drug Administration (U.S.)
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
GFP	Green Fluorescent Protein
HAV	Hepatitis A Virus
HIV	Human Immunodeficiency Virus
HPAC	Hazard Prediction and Assessment Capability
HVAC	Heating Ventilation and Air Conditioning
Kan	Kanamycin
LB	Lethen Broth
MEM	Minimum Essential Medium
MRSA	Methicillin-Resistant <i>Staphylococcus aureus</i>
MSSA	Methicillin-susceptible <i>Staphylococcus aureus</i>
MIC	Minimum Inhibitory Concentration
NARAC	National Atmospheric Release Advisory Centre
NBS	Normal Buffered Saline
PFU	Plaque Forming Unit
PAA	Peracetic Acid
PBS	Phosphate Buffered Saline

OADC	Oleic Acid Albumin Dextrose Catalase Complex
OECD	Organization for Economic Cooperation and Development
ppm	Part per million
QCT	Quantitative Carrier Test
QCT-2	Quantitative Carrier Test, tier 2
RH	Relative Humidity
RT	Room Temperature
SD	Standard Deviation
TPD	Therapeutic Products Directorate
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth
VHP	Vaporous Hydrogen Peroxide
WHO	World Health Organization

1. GENERAL INTRODUCTION

The battle against infectious diseases stretches as far back as recorded history, and at various stages humans spent much time and effort to counter the devastation wreaked by infections. The discovery and application of antimicrobial chemotherapy and the use of microbicides in the form of antiseptics and disinfectants, particularly in the latter half of the 20th century, allowed successful control over many infectious diseases. However, with the emergence of drug resistance, immunosuppression and other on-going societal changes (Sattar *et al.*, 1999), many previously controlled diseases (e.g., tuberculosis) have staged a comeback (Shannon, 2003) and 'new' ones have come to light (Morens *et al.*, 2004). Also, about a century ago, state-sponsored biological warfare programs turned the concept of countering infectious diseases upside down. Instead of investing in technologies to prevent and control the spread of pathogens, military researchers developed ways of making and spreading particularly dangerous microbial pathogens and their toxins into effective agents of warfare (Frischknecht, 2003).

The concept of using infectious agents to incapacitate or kill the enemy is not new in itself. Indeed, it predates the discovery of microorganisms by several centuries. However, what was recognized even then is that certain diseases were communicable and the damaging factor somehow associated with already affected humans or animals. This was the logic behind using dead or diseased animals or humans to contaminate the enemy's immediate surroundings (Broussard, 2001; Guillemin, 2005).

Relatively small quantities of such warfare agents are theoretically capable of causing levels of fatalities comparable to or even greater than that from nuclear explosive devices (Guillemin, 2005). Bioagents are also easier and cheaper to produce and deploy while being

virtually harmless to property and infrastructure. Fortunately, the use of bioagents for military purposes also has limitations. They are relatively slow-acting and unpredictable for offensive or defensive uses; they may be more suitable for use in long wars or in preparation for a war where immediate results are not required (Guillemin, 2005; Pile *et al.*, 1998).

By their very nature, bioweapons (BW) can be particularly insidious to military and civilians alike, because they are essentially invisible and can be disseminated over wide areas without any apparent smell or color while generating much psychological stress (Durban, 2002; Guillemin, 2005). Animals of economic importance can also be targets of BW; disruptions in food supplies can be caused by directly infecting animals or indirectly by contaminating animal feed (Frischknecht, 2003). Other potential targets for BW are food crops and essential vegetation (Guillemin, 2005; Stefansson, 2003).

The ability of such biological agents to cause mayhem was vividly demonstrated in 2001 (Bossi *et al.*, 2006; Cherry *et al.*, 2003). The impact of the anthrax spore-tainted letters was not limited to the U.S., but it had worldwide reverberations. The needs for preparedness and for response planning have been considered at multiple levels in many countries (Bossi *et al.*, 2006; Cherry *et al.*, 2003; Shannon, 2003). Since this release of *B. anthracis* spores, there has been growing interest in developing and assessing methods of detection, sampling and decontamination of such spores from surfaces, rooms and buildings (Rogers *et al.*, 2005; Rose *et al.*, 2004).

Chemical microbicides are often used in infection control and in remediation of sites contaminated with bioagents. Determining the effective elimination of bioagents from contaminated surfaces and objects depends on the defined objectives to be met within the decontamination approach of a specific operational scenario. How an object should be disinfected depends on its physical properties and any future intended use. Approaches can

range from no action (or decontamination by natural attenuation), a simple reduction in concentration of an agent, to sterilization by the application of various reagents or physical energy, or destruction of contaminated materials by incineration (Stuart and Wilkening, 2005; CDC, 2002).

1.1 HISTORY OF BIOLOGICAL WEAPONS

Using bioagents to infect/kill an enemy is not a new concept. Ancient humans used animal- and plant-derived toxins on arrowheads or poison darts to kill game and enemies (Dennis, 2001; Frischknecht, 2003). The use of BW took three main forms: first, deliberate poisoning of food or water with infected materials; second, use of microorganisms living or dead in a weapon system; third, use of biologically inoculated fabrics (Frischknecht, 2003; Stefansson, 2003). During the 6th century BC, the Assyrians poisoned enemy wells with a fungus (rye ergot) that would make the enemy delusional, with severe gastrointestinal problems, a burning sensation in limbs and extremities (Saint Anthony's fire), and a variant of gangrene. In 184 BC, Hannibal had soldiers throw clay pots filled with venomous snakes onto the decks of Pergamene ships (Peters and Hartley, 2002; Stefansson, 2003).

Historical records from medieval Europe detail the use of infected animal carcasses by Mongols, Turks and others to contaminate enemy water supplies (Table 1.1). The last known incident of using plague corpses for biological warfare was in 1710 when Russian forces attacked Swedes by flinging plague-infected corpses over the city walls of Reval (Tallinn) (Riedel, 2005). There are also several instances in history where the smallpox virus was used as a BW (Guillemin, 2005; Hinnebusch, 1997; Riedel, 2005).

The spread of infections remained a mystery until the discovery of microorganisms towards the end of the 19th century and firm evidence for their role as etiological agents of

several human ailments (Broussard, 2001). Soon this also provided a rational basis for the development of BW (Broussard, 2001; Guillemin, 2005). The potential for harm from use of BW was soon recognized and resulted in two international declarations: in 1874 in Brussels, and in 1899 in The Hague. However, these treaties and others (The Hague convention of 1907; the 1922 Treaty of Washington; and the 1925 Geneva Protocol) failed to prevent the development and use of BW (Durban, 2002; Fidler, 1999; Guillemin, 2005).

Table 1.1. Biological warfare recorded during the past millennium (Frischknecht, 2003)

Year	Event
1155	Emperor Barbarossa poisons water wells with human bodies
1346	Mongols catapult bodies of plague victims over the city walls of Caffa, Crimean Peninsula
1495	Spanish mix wine with blood of leprosy patients to sell to their French foes, Naples, Italy
1650	Polish fire saliva from rabid dogs towards their enemies
1675	First deal between German and French forces not to use "poison bullets"
1763	British distributed blankets from smallpox patients to native Americans
1797	Napoleon floods the plains around Nantua, Italy, to enhance the spread of malaria
1863	Confederates sell clothing from yellow fever and smallpox patients to Union troops, USA

The first use of BW occurred during WWI when German undercover agents are believed to have used *Burkholderia mallei* (glanders) and *Bacillus anthracis* to infect packhorses and mules to be used by British and French forces (Guillemin, 2005; Klietmann and Ruoff, 2001). Starting in the 1930's during the Sino-Japanese War (1937-1945) and the Second World War (WWII), Unit 731 of the Imperial Japanese Army experimented with at least 25 different disease-causing agents on more than 10,000 prisoners of war and civilians in China. Also, the army poisoned more than 1,000 water wells to study cholera and typhus outbreaks. Japanese airplanes dropped plague-infected fleas over Chinese cities or distributed them by

means of saboteurs in rice fields, along roads, and also distributed plague-contaminated foodstuffs such as dumplings and vegetables (over 580,000 victims were estimated). Some of these epidemics persisted for years and continued to kill more than 30,000 people in 1947, long after the Japanese had surrendered (Guillemin, 2005).

In WWII, the British tainted small feed cakes with anthrax as a potential means of attacks against German cattle for food, but never used that weapon (Guillemin, 2005). In 1941, the U.S., U. K. and Canada initiated a BW development program. Research carried out in the U.K. during WWII left Gruinard Island in Scotland contaminated with the spores of *B. anthracis* for the next 48 years. U.S. conscientious objectors were used during WWII as test subjects for BW in a program known as Operation Whitecoat (Guillemin, 2005). U. S. and Britain discovered plant growth regulators (i.e., herbicides) during WWII and initiated an Herbicidal Warfare program that was eventually used in Malaya and Vietnam in counter insurgency, and the Eelam war in Sri Lanka (Guillemin, 2005; King, 2005).

In 1951, China and North Korea accused the U.S. of large-scale field-testing of BWs, mainly insects, against their civilians during the Korean War (1950-1953). In the 1950s the U.S. military started open-air tests, exposing test animals and unsuspecting civilians to both pathogenic and non-pathogenic microbes. The most infamous test was the 1966 contamination of the New York's subway system with *Bacillus globigii* (now, *B. atrophaeus*) - a non-pathogenic bacterium to simulate anthrax spores - to study the spread of pathogens in a big city (Guillemin, 2005). In the summer of 1968, the U.S. forces are believed to have tested BWs, possibly anthrax spores, near Johnson Atoll in the South Pacific. In 1969, Richard Nixon terminated the BW offensive research program and signed the Biological and Toxins Weapons Convention (BTWC) in 1972 (Durban, 2002; Guillemin, 2005; King, 2005).

Although bioagents are primarily considered as weapons of international warfare, their deliberate or accidental release during peacetime can also have profound effects within a country. In the former Soviet Union in 1971, 10 people contracted smallpox in the Kazakh city of Aralsk and three of them died. It was speculated that the infection came from a BW research centre on a small island in the Aral Sea. On other occasions and in the same area, several fishermen and a researcher died from plague and glanders, respectively (Guillemin, 2005). In 1979, a biological weapons plant in Sverdlovsk (now Yekaterinburg, Russia) released airborne anthrax spores. In 1992, Russia acknowledged the release and subsequent human deaths due to an accident in a BW plant where a clogged air filter was removed but not replaced between shifts (Durbam, 2002; Guillemin, 2005). The release of anthrax spores in the U.S. in 2001 killed five, sickened 22, shut down government and other buildings for months, involved thousands of healthcare workers, environmental and law enforcement personnel, and resulted in tens of thousands of people taking and stocking antibiotics (Guillemin, 2005).

Occasionally bioagents have been used for more specific targets. In 1978 in London, England, a Bulgarian dissident, Georgi Markov, was stabbed in the leg with a ricin¹-injecting device disguised as an umbrella; he died several days after (Guillemin, 2005). His assassination was revealed later as the work of the Bulgarian secret service. In 1984, in Oregon in the U.S., the followers of the Bhagwan Shree Rajneesh (a militant religious sect) used *Salmonella* to contaminate salad bars in an attempt to influence the outcome of a local election. Although no one died, 750 became ill (Guillemin, 2005; King, 2005). In 1995 in Japan, the religious sect Aum Shinrikyo, already known to be responsible for the sarin gas

¹ Ricin toxin from *Ricinus communis* (castor beans). Please refer further to Table 1.2

attack in the Tokyo subway system, is believed to have attempted to release both anthrax spores and botulinum toxin in the centre of Tokyo (Guillemin, 2005).

The Russian bioweapons program during the 1980s applied the then novel idea of genetic engineering to BW. Their goal was to insert genes into infectious agents, which could turn the human body against itself (Alibek, 1999; Finkel, 2001). The Russian scientists inserted DNA fragments from a mouse myelin gene into *Legionella* bacteria and used the organisms to infect guinea pigs. At first, the animals exhibited a mild form of pneumonia and soon recovered. However, days after nearly all the infected animals died from brain damage (Finkel, 2001; Garrett, 2001).

The magnitude of the terrifying threats and possibilities that genetically modified BWs pose became more evident when Australian scientists, Jackson *et al.*, reported in February 2001 unexpected results from a benign pest control experiment. They used as a vector the mousepox virus and inserted into its DNA a mouse gene that controls production of interleukin-4, a powerful stimulator of the immune response. They hoped to stimulate the female's immune sensitivity so that the female mouse would reject its own eggs as a foreign object. Instead, all the infected mice died, including the controls that were vaccinated against mousepox (Dennis, 2001; King, 2005).

1.2 ENVIRONMENTAL FATE OF BIOAGENTS

The degree of health risk from an infectious agent is directly proportional to its numbers, virulence and how long it can remain viable at the contaminated site; such microbial survival is also dependent on temperature, relative humidity and exposure to light (Boone and Gerba, 2007). Where there is little understanding about viability loss mechanisms, in general, the susceptibility of vegetative bacteria to loss in viability is the greatest, followed by viruses and spores (Stuart and Wilkening, 2005; Sinclair *et al.*, 2008). When possible and depending

on the type of BW used, limiting access to the affected site may be the best means of decontamination due to a time-related decline in microbial viability. However, this approach is often not feasible because the nature of the pathogen may be unknown or could have been modified to enhance its environmental survival. Also, declaring an affected site fit for reoccupation would require some form of active decontamination and assessment of its effectiveness. There are also bioagents such as non-enveloped viruses and anthrax spores, which can readily survive in the environment for months to decades, respectively (Kim, 1994; Stuart and Wilkening, 2005; Sinclair *et al.*, 2008), and thus require elaborate site decontamination such as that undertaken following the recent anthrax episode in the U.S. (Volchek *et al.*, 2005; Simpson, 2005; McAnoy, 2006).

All infectious bioagents in Category A (Table 1.2) can be released in bodily fluids (e.g. blood, urine, saliva, nasal fluid) of infected persons or animals, but reliable data on their survival in the environment is limited (Boone and Gerba, 2007). Pathogens surviving on environmental surfaces could be released into the air and/or transferred to human hands on contact (Kim, 1994; Boone and Gerba, 2007; Stuart and Wilkening, 2005; Sinclair *et al.*, 2008; Sattar *et al.*, 1986).

Two of the primary operational responses, HPAC (Hazard Prediction and Assessment Capability) and NARAC (National Atmospheric Release Advisory Centre), represent viability loss caused by injury or death of a bioagent as exponential decay during both atmospheric transport and after surface deposition. Although it is not the only theoretical model (Stuart and Wilkening, 2005), in fact, the exponential decay model is used commonly as a general equation to represent decay when the details of a bioagent release are poorly understood and the model has been found to describe the inactivation of microbes due to exposure to active disinfectants (physicals or chemicals).

Table 1.2 Categories of ‘Select Agents’ from the U.S. Centre for Disease Control and Prevention (CBW, 2001)

Category	Characteristics	Agents
A	<ul style="list-style-type: none"> - Highest priority agents - Easily disseminated/transmitted from person to person - High mortality rate - Cause panic and disruption - Require special action for public preparedness 	<ul style="list-style-type: none"> - Anthrax (<i>Bacillus anthracis</i>) - Botulism (<i>Clostridium botulinum</i> toxin) - Plague (<i>Yersinia pestis</i>) - Smallpox (<i>Variola major</i>) - Tularaemia (<i>Francisella tularensis</i>) - Viral hemorrhagic fevers (filoviruses [e.g., Ebola, Marburg], and arenaviruses [e.g., Lassa, Machupo])
B	<ul style="list-style-type: none"> - Second highest priority agents - Moderately easy to disseminate - Moderate morbidity, low mortality - Response requires enhancement of diagnostic capacity and surveillance 	<ul style="list-style-type: none"> - Brucellosis (<i>Brucella</i> sp) - Melioidosis (<i>Burkholderia pseudomallei</i>) - Glanders (<i>Burkholderia mallei</i>) - Food safety threats (e.g., <i>Salmonella</i> spp., <i>Escherichia coli</i> O157:H7, <i>Shigella</i> spp.) - Psittacosis (<i>Chlamydia psittaci</i>) - Q fever (<i>Coxiella burnetii</i>) - Ricin from <i>Ricinus communis</i> (castor beans) - Staphylococcal enterotoxin B - Typhus fever (<i>Rickettsia prowazekii</i>) - Viral encephalitis (alphaviruses [e.g., Venezuelan equine encephalitis, eastern and western equine encephalitis]) - Water safety threats (e.g., <i>Vibrio cholerae</i>, <i>Cryptosporidium parvum</i>)
C	<ul style="list-style-type: none"> - Third highest priority agents - Emerging agents - Available - Easy to produce and disseminate - Potential high morbidity/mortality rates and major health impact 	<ul style="list-style-type: none"> - Nipah virus - Hantaviruses - Multidrug resistant tuberculosis - Tickborne encephalitis viruses - Tickborne hemorrhagic fever viruses - Yellow fever

In the exponential decay model, it is assumed that a single lethal event is responsible for death (inactivation) of any member of a population of microbes (virus, spore, or vegetative cell) and the probability of death is equal, random and constant over time.

Exponential decay can be represented by the following equation:

$$V = e^{-kt}$$

Where V is the fractional viability (the viability at any time t divided by the initial [t=0] viability) for an agent with the decay constant, k, which is determined experimentally (Stuart and Wilkening, 2005). In reality though, due to intrinsic and extrinsic factors, the decay of pathogens in the environment does not always follow the exponential decay model but often a biphasic pattern, and thus not a first-order kinetics.

1.3 POTENTIAL FORMULATIONS FOR DECONTAMINATION

In the U.S., disinfectants are regulated by the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA). All microbicidal products fall under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) of 1947 as amended (1996) and administered by the EPA (CDC, 2003). Health Canada's Therapeutic Products Directorate (TPD) is the federal regulatory authority that regulates disinfectants as drugs under Canada's Food and Drugs Act (F&DA). Ingredients in disinfectant products are regulated under the Canadian Environmental Protection Act, 1999 (CEPA) by Environment Canada.

Disinfectants are usually categorized by their degree of effectiveness. This classification is based upon the range or type of challenge microorganisms that can be inactivated by a given disinfectant. **High-level disinfectants** inactivate all classes of pathogens, with the exception of high numbers of bacterial spores, protozoan cysts/oocysts and prions. **Intermediate-level disinfectants** inactivate mycobacteria as well as vegetative bacteria and

most viruses and fungi, but do not necessarily kill bacterial spores. **Low-level disinfectants** kill only vegetative bacteria, enveloped viruses and non-filamentous fungi (Weber and Rutala, 2006; McDonnell and Russell, 1999). However, sensitivities of classes of microorganisms overlap considerably with one another and can vary depending on a wide range of factors (Springthorpe and Sattar, 2005).

Claims of microbicidal activity can be influenced by many factors such as formulation effects, concentration, presence of an soil load, synergy, contact time, temperature, surface, bioburden and test method (CDC, 2003; McDonnell and Russell, 1999). However, not following the specified dilution, contact time, method of application, or any other condition of use is considered misuse of the product (CDC, 2003).

1.3.1 Decontamination in the Healthcare System

For the prevention of healthcare-associated infection, the scheme first proposed by Spaulding forms the basis for current disinfections recommendations. Critical items (e.g. surgical instruments) that enter sterile tissue and implants should be sterilized prior to use. Semi-critical items (e.g., bronchoscopes) that come into contact with intact skin or mucous membranes should undergo at least high-level disinfection prior to use. Noncritical items including objects that may contact intact skin (e.g. blood pressure cuffs, bed rails, bed linens) and environmental surfaces (e.g., door handles, walls, floors, etc...) should receive low-level disinfection prior to use (Weber and Rutala, 2006). This classification is based upon the range of type of challenge microorganisms that can be inactivated by a given disinfectant. However, caution should be taken as sensitivities of classes of microorganisms overlap considerably with one another and can vary depending on a wide range of factors (Springthorpe and Sattar, 2005). Many microbicides have been approved for use in hospitals and other healthcare settings in the U.S. (Table 1.3).

Table 1.3 Disinfectants approved by the U.S. EPA for use in healthcare facilities listed by degree of effectiveness (McDonnell and Russell, 1999; Weber and Rutala, 2006)

High level
Glutaraldehyde (>2%) Formaldehyde (34%-38%) Glutaraldehyde (1.12%) and Phenol/phenate (1.93%) Ortho-phthalaldehyde, OPA (0.55%) Hydrogen peroxide (7.5%) Hydrogen peroxide (7.35%) and peracetic acid (0.23%) Hydrogen peroxide (1.0%) and peracetic acid (0.08%) Hypochlorite (single use chlorine generated by electrolysing saline containing >650-675 ppm of active free chlorine)
Intermediate level
Sodium hypochlorite (5.25%-6.15% household bleach diluted 1:100) Glutaraldehyde (0.1%-1%) Ethyl or isopropyl alcohols (70%-90%) Phenolic (following product label for use-dilution)
Low level
Ethyl or isopropyl alcohols (70%-90%) Sodium hypochlorite (5.25%-6.15% household bleach diluted 1:500, to give ~100 ppm available chlorine) Phenolics, quaternary ammonium germicidal detergent solutions, iodophor germicidal solution (follow product label for use-dilution)

1.3.2 Decontamination of Buildings

Buildings are attractive targets for BW attacks because of the high density of people in them and the fact that the concentration of BW tends to remain high in confined spaces for a longer period of time. In fact, the majority of people in the U.S. and other industrialized regions spend most of their time indoors (CDC, 2002, Brown *et al.*, 2007). In the event of a BW attack, environmental sampling plays an important role in the restoration of the affected area, first to determine the presence of a biological agent and risk exposure assessment, provide information on initial agent concentration and location, support the decision regarding the need for medical treatment and implementation of the most appropriate decontamination process (remediation), and provide confidence after the cleanup to permit re-entry into an area. However, some limitations do exist due to insufficient data on the efficiency of various collection and analytical methods used for the sampling of indoor

environments, especially in cases involving Category A bioagents, such as *Bacillus anthracis* (CDC, 2002; Busher *et al.*, 2008).

A study by Utrup and Frey (2004) showed that bioterrorism-relevant aerosolized viruses and bacteria, including spores, respond like small particulates to the primary (electrical) forces that control the distribution of small particulates in indoor air in a room and can be further eliminated and controlled by the same means designed to control airborne particulates. Biological agents may exist as droplets, aerosol or dry powder. The HVAC systems used in buildings can be the means through which BWs may be disseminated and transported, though these systems can be also the means by which contamination may be diluted, filtered and exhausted from buildings (Casadevall and Pirofski, 2004; Rose *et al.*, 2004; Stuart and Wilkening, 2005).

Most remediation experience is based on civilian and military involvement with anthrax research facilities. Drawing on these common facility decontamination procedures, on-site processes to remediate public facilities involved the following steps: 1) assessing the site, including environmental sampling to characterize the contamination; 2) isolating contaminated areas to prevent the sources of contamination from dissemination, including shutting the HVAC system; 3) developing and designating risk zones and levels of required personal protection equipment; 4) removing items (especially valuable ones) for off-site treatment, if necessary; 5) cleaning and remediating contaminated areas; 6) sampling the environment following remediation (post-remediation sampling); 7) additional remediation and sampling if the initial postremediation sampling showed areas that were still contaminated; and 8) disposal of decontaminated wastes (Sanderson *et al.*, 2002; Rose *et al.*, 2004; Sagripanti *et al.*, 2007; Simpson, 2005; Utrup and Frey, 2004).

Other on-site remediation issues include, whether to a) remediate the entire facility at one time or to remediate the facility by subsections depending on available information about air-flow in the facility, b) determine which chemical should be used to remediate the contaminated facilities (on-site specific conditions continue to be the basis for such decision, including the types of important items, e.g., books, photographs, computer equipment, especially for valuables those have to be removed off site for decontamination; c) determine which bacterium should be used to indicate the effectiveness of remediation actions, for example, by placing strips with biological indicators throughout the facility prior to remediation (Canter, 2008).

1.3.2.1 Standards for Remediation of Anthrax-Contaminated Facilities

In 2001, as the US faced an anthrax threat, there was no established acceptable limit for exposure to anthrax. This is still the case today. Trying to set such a limit of exposure is difficult. The minimum infectious dose of inhaled spores for humans is unknown, and in studies on monkeys the LD₅₀ has ranged from about 4,100 to 8,000 viable spores (Casadevall and Pirofski, 2004). It is still not known how few anthrax spores, natural or altered, if inhaled, would lead to sickness or death (Canter, 2008; Barakat *et al.*, 2002). Thus, in remediation from an anthrax incident, the general criterion continues to be the absence of any detectable anthrax spores from all post-remediation environmental samples (Canter, 2008, Busher *et al.*, 2008). Reliance cannot be placed on dry swabs as samples to detect *B. anthracis* environmental contamination. The use of aerosolized decontaminants able to reach the exterior and interior spaces of walls and difficult-to-access areas has the potential to reduce the risk of exposure and contact with infectious bioagents in heavily contaminated areas (Simpson, 2005; Sanderson *et al.*, 2002; Busher *et al.*, 2008). Nevertheless, if

contamination levels are high, thorough decontamination might require multiple repeated treatments.

In spite of lack of knowledge of MIDs for bioagents, we need to have an understanding of how well decontamination technologies function, and further, we need to understand if contamination is untreated or remains even after decontamination is attempted, then how much might be acquired from the surface upon contact, i.e., how clean is clean enough to reduce/eliminate risks from building reoccupation or reuse of objects.

Since the deliberate release of anthrax spores in 2001, the U.S. EPA has approved seven chemicals for use against anthrax only by authorized personnel following the specific requirements under 'crisis exemption', and only with approved decontamination plans. The seven chemicals are: vaporized hydrogen peroxide, chlorine dioxide, sodium hypochlorite (bleach), paraformaldehyde, methyl bromide, peroxyacetic acid with hydrogen peroxide, and ethylene oxide (Simpson, 2005; EPA, 2008). Of these, paraformaldehyde, methyl bromide and ethylene oxide are more toxic, less amenable to widespread use and will not be discussed further here.

Vaporous decontamination involves applying a chemical as a vapor (or gas) to treat enclosed spaces or sensitive pieces of equipment. The effectiveness of such a process is dependent on a number of parameters including the concentration of the decontaminant, duration of exposure, rate of reaction, air temperature, relative humidity, and the nature of the contaminated material (McAnoy, 2006; Volchek *et al.*, 2005).

After evaluation of remediation approaches, EPA reported that chlorine dioxide (ClO_2) is the most promising chemical for use as a fumigant for biologically contaminated buildings (Simpson, 2005). In fact, in response to the 2001 anthrax attacks, the EPA granted a crisis exemption for the use of gaseous ClO_2 in the remediation of buildings on a case-by-case

basis. The target conditions for the most recent crisis exemption were 750 ppm of ClO₂ for 12 h, while maintaining the temperature above 75°F (23.9°C) and the relative humidity above 75% (McAnoy, 2006; Volchek *et al.*, 2005). The 12-hour decontamination period was followed by neutralization, by passing the gas through a sodium sulfite/bisulfite solution.

ClO₂ gas acts as an oxidizing agent by a single-electron transfer process. Gaseous ClO₂ is not stable under pressure and cannot be stored in high-pressure cylinders, but it is readily soluble in water and stable for about 7 days (www.clordisys.com). In each remediation, the building was sealed to prevent further spread of the spores and windows were covered to prevent UV light from entering and decomposing the ClO₂ gas. The ClO₂ was generated outside the building with the resulting aqueous ClO₂ solution pumped into the building and passed through air strippers to release the ClO₂ gas. The humidity was also lowered during this final phase to prevent mould growth and aid aeration of the space. Environmental sampling after the first building remediation in the U.S. showed a reduction in the number of spores; however, a significant number still remained. The area was then wiped down with aqueous ClO₂ to kill the remaining spores (McAnoy, 2006).

While it is clear that ClO₂ is an effective sporicide, problems with its use in building decontamination include bleaching of synthetic fibers and photographic materials during the process and the possibility that surfaces in electrical systems may be affected by condensation (McAnoy, 2006; McDonnell and Russell, 1999). Additionally, a large volume of liquid waste is generated during the process. There is also a risk of explosion of the ClO₂ gas at concentrations above 10% by volume in air; however, such a concentration is far greater than that required for decontamination (McAnoy, 2006).

Chlorine gas is the most widely used disinfectant to treat water for drinking. As a solution of sodium hypochlorite (or bleach), it is also common as a disinfectant for

environmental surfaces and laundry. There are many types of chlorine-releasing compounds as well that are available in powder or tablet form for environmental decontamination (Warren *et al.*, 1981); certain of these chemicals release chlorine on demand and are thus more stable and safer for use than solutions of sodium hypochlorite. Even though the pungent and toxic nature of chlorine gas and its high corrosivity are recognized drawbacks in the use of most chlorine-based disinfectants for building decontamination, they can be microbicidal even at sub-zero temperatures when mixed with ethylene glycol as an anti-freeze. Jones *et al.* (1968) showed such a mixture to inactivate the spores of *Bacillus subtilis* at a temperature as low as -40°C and so it could be valuable as a BW decontaminant under certain specialized conditions.

After the anthrax attacks in the U.S., vaporous hydrogen peroxide (VHP) was also successfully used to fumigate anthrax-contaminated buildings. VHP is an oxidizing agent effective against many microorganisms including bacterial spores, while being less toxic since it catalytically decomposes to water and oxygen and so generates no persistent byproducts. Pure H_2O_2 is a colorless, thick, syrupy liquid; it is naturally generated during lightning storms and at low levels in many natural systems. VHP is generated by heating a solution of 30-35% hydrogen peroxide in water (McDonnell and Russell, 1999, McAnoy, 2006). Decontamination cycles take up between 12-24 h depending on the target organism. In addition, VHP has been used for over a decade as a sterilant technology in the medical, biological and pharmaceutical industries and continues to gain mainstream use as the technology advances (McAnoy, 2006).

Accelerated hydrogen peroxide products (AHPs) are new stabilized solutions produced to address the weaknesses of H_2O_2 , being relatively unstable and somewhat slow-acting when used on its own. The stabilizers, surfactants, anticorrosive and buffering agents in the

AHP-based compositions have high safety, compatibility and biodegradability profiles. In addition, the AHPs proved to have broad spectrum activity and faster action (Omidbakhsh and Sattar, 2006; Sattar *et al.*, 2002) than equivalent concentrations of hydrogen peroxide.

Peracetic acid (PAA) is an effective high-level disinfectant at lower concentrations. PAA also decomposes to safe by-products (acetic acid and oxygen) and it is mainly used as a low-temperature liquid sterilant for medical devices (McDonnell and Russell, 1999). After adding ethylglycol, PAA is also effective at subzero temperature against spores (Jones *et al.*, 1967).

1.4 OBJECTIVES

It is evident that after the recognized release of any bioagent, some level of decontamination is required before normal activities on the site affected can resume. Decontamination from bioagents is challenging, and there is need for reliable and simple methods for remediation. Although gaseous decontaminants are effective, it is not always possible/practical and/or safe to use them. It takes much preparation time, and needs specialized equipment. Thus, it is obvious that liquids will also need to be employed in most cases, and information on their efficacy is needed.

In real-life situation, microorganisms rarely exist as single species and most often the target organisms are not known. In addition, there is always a certain level of soil on uncleaned surfaces and their routine cleaning does not rid them of all the soil and such residual amounts of soil might be high enough to compromise proper decontamination. Therefore, this study was undertaken to address decontamination in a realistic manner: We included a soil load in the microbial suspensions to simulate naturally dirty or deliberately protected targets; and we used standardized, simple and reliable methods for testing the effectiveness of liquid microbicides against a cocktail of surrogates for infectious bioagents. We also used specific means for determining the potential transfer of bioagent surrogates

from contaminated surfaces to clean ones. The use of surrogate organisms for standardized testing is advantageous because it is neither practical to test large numbers of strains nor those that are highly pathogenic. In addition, incorporating a mixed challenge of multiple classes of microorganisms facilitates the evaluation of efficacy claims concurrently and more realistically (Best *et al.*, 1994).

The first objective of this study was to assess the efficiency of selected liquid disinfectants (peroxygens and chlorine-based agents) applied at a relatively small disinfectant to surface ratio against a mixture of microbial surrogates for infectious bioagents, at shorter contact times (minutes) and as a function of air temperature. For this, a standardized and quantitative carrier test (QCT-2) was used; it is known to be stringent yet applicable for use with a wide array of microbes, surface types as well as disinfectant liquids, foams and gels. This objective was addressed in Section 2 of the study.

The important question of "how clean is safe?" must be informed and reliable. It is therefore critical that any decontamination process be as effective and complete as possible for each unique clean-up operation. Therefore, the **second objective** was to assess the influence/efficacy of wiping with a disinfectant-wetted fabric on surface decontamination. This was addressed operationally in conjunction with the **third objective**, which was to determine the potential for spread/transfer of viable organisms over a wider and clean area during wiping. The second and third objectives are addressed in Section 3 of the study.

1.5 SURROGATE MICROORGANISMS AND THE COCKTAIL

The use of carefully selected surrogate organisms for standardized testing of disinfectants has the following advantages:

(a) Surrogates that can be handled at Containment Level-1 (CL-1) or CL-2 allow for more laboratories to generate test data than requiring the use of less commonly available CL-

3 or CL-4 facilities.

(b) Work with certain infectious bioagents requires the use of experimental animals and this further restricts work with them.

(c) In lab settings, infectious bioagents may not grow to high enough titers required for testing of microbicides.

It has been suggested that test microorganisms purchased from a single source help with eliminating one major source of inter-study variations (Springthorpe and Sattar, 2005). Therefore, all the microorganisms used in this study were ATCC strains.

In real world conditions, a bioagent will not be found in isolation from other microorganisms and a cocktail approach would simulate these conditions to assess decontamination in a realistic situation. Further, this approach would provide efficiencies in evaluating decontamination effectiveness. This mixture consisted of i) a spore-former (*Geobacillus stearothermophilus*) to represent the spores of *Bacillus anthracis*, ii) a Mycobacterium (*Mycobacterium terrae*) as a genus known for its relatively higher resistance to many disinfectants and also to represent the emerging threats from multi-drug resistant *M. tuberculosis* and other environmental mycobacteria, iii) a hardy vegetative bacterium (*Acinetobacter baumannii*) as a surrogate for *Francisella tularensis* and *Yersinia pestis*, and iv) a non-enveloped virus (hepatitis A) to represent all viruses in the list of select agents. Summarized below are relevant details on each of the surrogates selected for testing in this study.

1.5.1 *Acinetobacter baumannii*

Acinetobacter (Greek *akinetos* for non-motile) *baumannii* is responsible for increasing numbers of opportunistic nosocomial infections (Peleg *et al.*, 2008). It is a Gram-negative bacilliform bacterium, strictly aerobic, non-fermenting, non-fastidious, catalase-positive,

oxidase-negative, encapsulated and nutritionally versatile. It forms smooth, sometimes mucoid, grayish white colonies (1.5 to 3 mm after overnight culture).

A. baumannii is a common colonizer of patients in intensive care settings and the immunocompromised. Such colonization is particularly common in patients who are intubated and in those who have multiple intravenous lines or monitoring devices, surgical drains, or indwelling urinary catheters (Jawad *et al.*, 1998; Peleg *et al.*, 2008). Fatal cases of community-acquired pneumonia were reported among compromised patients. These infections are difficult to treat due to the expanding antibiotic resistance of the clinical strains. Enhanced transmissibility is mainly attributed to resistance to environmental conditions especially desiccation, multidrug resistance and the ability to use a wide range of carbon sources for nutrition (Jawad *et al.*, 1998; Peleg *et al.*, 2008). *A. baumannii* ATCC #19606 is a prototype strain that is susceptible to kanamycin. Its mean survival time (6 days) is significantly less than that of the clinical isolates (mean survival time is 27 days with significant strain-to-strain differences in survival on glass coverslips) (Jawad *et al.*, 1998). As an increasingly opportunistic pathogen, *A. baumannii* may be the best surrogate for non-spore forming vegetative bacteria for testing the effectiveness of microbicides. This organism also affects burn patients (Trottier *et al.*, 2007; Peleg *et al.*, 2008) and is also a frequent cause of serious infections in those wounded in wars (Johnson *et al.*, 2007; Peleg *et al.*, 2008).

1.5.2 *Mycobacterium terrae*

Mycobacteria are strict aerobes with curved or straight rods ($0.2-0.6 \times 1.0-10 \mu\text{m}$). They are non-spore forming, non-encapsulated and non-motile. *Mycobacterium terrae* belongs to the *M. terrae* complex (nontuberculous mycobacteria). The unique waxy coating on their cells confers on them a higher level of resistance to many types of microbicides (Falkinham,

2003). *M. terrae* has been reported to be a good choice surrogate for use in the tuberculocidal efficacy testing of disinfectants for having similar or slightly higher resistance to microbicides than *M. tuberculosis* (Best *et al.*, 1988; Gregory *et al.*, 1999; Springthorpe and Sattar, 2005). Although *M. tuberculosis* is transmitted through aerosols, little information exists on aerosolization of nontuberculous mycobacteria. Some cases of mycobacterial infections in lungs caused by *M. terrae* have been diagnosed among both immunocompromised patients and patients with normal immunologic status (Carbonara *et al.*, 2000; Gregory *et al.*, 1999).

***M. terrae* (pBEN)** is the *M. terrae* ATCC #15755 with the plasmid pBEN introduced into it by electroporation. The plasmid pBEN was constructed by Ben Chang and Lalita Ramakrishnan and was obtained as a gift from Stanford University, California. pBEN contains a PCR fragment of the gene encoding a red-shifted high-intensity mutant GFP (GFPmut3) that was inserted at the *Bam*HI-*Hind*III site of pMV262. In addition to *gfp*, pBEN contains a kanamycin (Kan) resistance gene, the origin of replication for mycobacteria (*oriM*) and *Escherichia coli* (*oriE*) and the mycobacterial promoter *hsp60*. *M. terrae* (pBEN) can grow on 7H11 agar plates containing up to 50 µg/mL of kanamycin (Zafer *et al.*, 2001; Zafer, 1999).

1.5.3 *Geobacillus stearothermophilus*

The vegetative form of this aerobic, Gram-positive, spore-former is rod-shaped (0.6-1 µm wide and 2-3.5 µm in length), occurring either singly or in short chains and motile by means of peritrichous flagella (Madigan *et al.*, 2000). Its spores are ellipsoidal in shape and located centrally in the cell. This bacterium is an obligate thermophile with a temperature optimum of 55°C for growth in the lab. It can be found in soil, hot springs and ocean sediments. ATCC #12980 is the type strain of *G. stearothermophilus* (strain 26 from the

collection of the National Canning Association). Its spores are among the most heat-resistant microbial forms known and are, therefore, commonly used as biological indicators in heat sterilization processes. The thermophilic nature of this organism makes it safe for humans, and its requirement for a higher growth temperature can be used as a selective factor to suppress the growth of other organisms in microbial mixtures.

Choice of this *Bacillus* as a surrogate for *B. anthracis* in this study was predicated by ease of separating it in the assay from other bacteria due to its growth temperature. In addition, this organism grows easily in the lab using simple culture media and also yields spores readily. It forms countable colonies on common semi-solid media in less than 48 h.

1.5.4 Hepatitis A

Hepatitis A virus (HAV) is a non-enveloped, positive-stranded RNA virus; it belongs to the genus *Hepatovirus* in the Family *Picornaviridae* (Madigan *et al.*, 2000). HAV virus mediated inflammation of the liver and cause hepatitis, an enteric infection (Madigan *et al.*, 2000; WHO, 2007). It is transmitted primarily through fecal contamination of water, ice, shellfish, fruits, vegetables, milk, or other contaminated-undercooked foods. Mollusc-like oysters and clams can concentrate HAV present in sewage-contaminated fresh or salt water, which can be an important source of infection if eaten raw or inadequately cooked. However, cooked food may become recontaminated after cooking during inappropriate handling. HAV spreads from the intestine via the bloodstream to the liver and usually results in jaundice, a yellowing of the skin and eyes and a browning of the urine as a result of stimulation of a bile pigment production by infected liver cells (Madigan *et al.*, 2000; CDC, 2008). Because it has no lipid envelope, HAV is stable when excreted from the infected liver to the bile to enter the gastrointestinal tract. High concentrations of the virus are shed in the stools of patients from 3 to 10 days prior to the onset of illness until one to two weeks after the onset of jaundice.

Faecal excretion of HAV persists longer in children and in immunocompromised persons (up to 4 - 5 months after infection) than in otherwise healthy adults, and communicability is highest during this interval (WHO, 2007).

Transfer of the virus between hands and inanimate surfaces has been shown, and inanimate surfaces are thought to play a role in the transmission of HAV (Mbithi *et al.*, 1990). HAV is relatively resistant to degradation by environmental conditions, a property that allows its maintenance and spread within populations. HAV can remain infective on nonporous inanimate surfaces for up to 2 months or longer (Boone and Gerba, 2007). Extrinsic environmental factors have no noticeable effect on the survival of HAV; however, Abad *et al.* (2001), reported that humidity variations caused a significant decline in HAV survival (T_{90} , the time required for the initial titer to decrease by 90%, changes from 35 days at 85% humidity to 11 days at 45% humidity). In a comparative study of human enteric survival on surfaces, HAV could be recovered from all types of surfaces at both 4 and 20°C. Resistance to desiccation on porous and nonporous materials showed a maximum decay rate of 1.5 log units; a rate which is significantly lower than that for other enteric viruses such as adenovirus and poliovirus, which had a 2.4 to 3.7 and 1.5 to 3.3 log units, respectively (Abad *et al.*, 2001).

In addition to its intrinsic properties listed above, which imply that it is an easy virus for manipulation and a good representative for enteric viruses, HAV was chosen in this study because of its relative safety, ability to grow to high enough titers for testing, ability to produce cytopathic effects (plaques), and its relatively high resistance to a variety of microbicides (Sattar *et al.*, 2003). Most importantly, HAV is a food safety threat and it also represents small non-enveloped viruses, which in general have a higher resistance to microbicides (Boone and Gerba, 2007; Sattar, 2007).

2. MICROBICIDAL TESTING OF CHEMICAL DISINFECTANTS USING A QUANTITATIVE CARRIER TEST METHOD

This part of the study was done to address **the first objective**, which was to assess the microbicidal activities of the selected chemicals against the test organisms using the second tier of the quantitative carrier test (QCT-2) developed earlier at CREM (Springthorpe and Sattar, 2003). QCT-2 has been used previously to assess the activity of liquid microbicides against viruses (Sattar *et al.*, 2003), mycobacteria (Springthorpe and Sattar, 2005) and the spores of several types of bacteria including *B. anthracis* (Majcher *et al.*, 2008).

2.1 INTRODUCTION

2.1.1 Microbicide Test Protocols

Prior to registration, the manufacturer must present scientific evidence of the product's safety, efficacy and quality as required by the relevant target regulatory agency. In the U.S., the official methods of AOAC (formerly, Association of Official Analytical Chemists) International and ASTM (formerly, American Society for Testing and Materials) International for testing microbicides efficacy, have evolved over many years with a completely separate and highly prescriptive test method for each class of microorganisms (Springthorpe and Sattar, 2005).

The efficacy of a microbicide is commonly examined by one of two methods. The first and the simplest is called a 'suspension test', which uses microorganisms in suspension mixed with a known concentration of test product and held for a defined contact time before terminating the action of the product either by dilution and/or neutralization, and finally sub-culturing it in liquid or semi-solid media to obtain a qualitative or quantitative assessment of effectiveness. The suspension tests estimate the *in vitro* microbicidal activity of the chemical under precise experimental conditions including the microbial strain, preparation of

inoculum, volume of inoculum vs. disinfectant, temperature, disinfectant concentration and contact period, and interfering substances (i.e., inorganic, organic matter).

The second type of tests adds more challenges to the test chemical and uses inanimate surfaces (called carriers) contaminated with dried inocula of microorganisms. The carriers are challenged with a known concentration of the test substance for a defined contact time before ending the product's action by dilution and/or neutralization.

The carrier tests estimate the *in vitro* microbicidal efficacy while reproducing surface disinfection conditions including the substrate, application technique (spray, immersion or wipe), drying time, surface area vs. inoculum and interfering substances. The main issues with the AOAC carrier tests include recovery from the surface and the surface conditions (Busher *et al.*, 2008; Springthorpe and Sattar, 2005; Sutton *et al.*, 2002).

The AOAC methods have been criticized for having many deficiencies and by using a separate approach for different classes of disinfectants, making it very difficult to compare efficacy claims across classes of microorganisms. For example, a product labeled as an EPA hospital disinfectant must pass the AOAC effectiveness tests against three target organisms: *Salmonella choleraesuis* for effectiveness against Gram-negative bacteria, *Staphylococcus aureus* for effectiveness against Gram-positive bacteria, and *Pseudomonas aeruginosa* for effectiveness against nosocomial pathogens (Springthorpe and Sattar, 2005; CDC, 2003). AOAC has no test to assess virucidal activity of chemicals.

To address many of the deficiencies in the AOAC test methodology, a two-tiered quantitative carrier test (QCT) was developed as potential replacement for the AOAC methods to evaluate the effectiveness of chemicals to inactivate bacterial spores, mycobacteria, vegetative bacteria, viruses and fungi in the presence of a soil load on disk carriers that represent environmental surfaces and medical devices (Springthorpe and Sattar,

2003). The first tier, QCT-1 (ASTM, 2000), can be used with most species of microorganisms except viruses. The second tier, QCT-2 (ASTM, 2002), is more stringent and can be used with all species of microorganisms, including viruses. Both tiers of QCT are fully quantitative with precisely measured volume of the test inoculum and test microbicide; they also avoid any loss of viable organisms through wash off and capture by membrane filtration (except viruses). Thus, highly reproducible data can be obtained (Springthorpe and Sattar, 2005).

2.1.2 The Importance of the Neutralization Step in Testing Disinfectants

An important requirement of microbicidal testing is the validation of the process applied for neutralizing the activity of the test substance immediately at the end of the contact time (ASTM E1054, 1991). The purpose of the neutralization step is to eliminate any inhibitory residual microbicidal activity. This is very important for the accuracy of a biocidal assay as microbicidal activity is commonly measured as survival with time. Incomplete neutralization could lead to inhibition of microbial growth by low levels of residual microbicide and even a low level of the chemical residue would lead to exaggerated measures of microbicidal activity (Busher *et al.*, 2008; Sutton *et al.*, 2002). Neutralization is usually accomplished by one of three methods: 1) chemical neutralization, 2) dilution, or 3) membrane filtration (Busher *et al.*, 2008).

For proper neutralization, the neutralizer must first effectively inhibit the action of the microbicide; second, the neutralizer must not itself be harmful to the challenge organisms; finally, the neutralizer and active agent must not combine to form any byproducts deleterious to the viability of the test organism(s) or any host system used to test viability.

Confirming the neutralization of the test formulation is carried out by challenge with low numbers/infective units of the test organism in all tests. Also, as the virus assay system is

indirect, an additional step is also required to ensure that prior exposure of the appropriate cell line to any neutralizer and disinfectant/neutralizer mixture does not interfere with the detection of a low level of viral challenge (Springthorpe and Sattar, 2005).

2.2 MATERIALS AND METHODS

2.2.1 Materials

Appendix I contains a list of the chemicals and other supplies used and their sources.

2.2.2 Preparation of *Acinetobacter baumannii* (ATCC #19606) Stock Cultures

A seed culture of *A. baumannii* was purchased from Gibson Labs, Inc. (1040 Manchester Street, Lexington, Kentucky, U.S.A.) as lyophilized pellets, and included culture medium and a sterile swab for rehydration and direct inoculation to a recovery medium.

2.2.2.1 Procedure

The red “snap” valve of the swab (Figure 2.1.A) was broken by bending to a 45° angle. The cap was gently squeezed until the fluid moistened the lyophilized pellet in the bottom of the tube. Gentle shaking of the tube allowed the swab to absorb the rehydrated pellets. Each swab was removed from the tube and used to inoculate one plate of trypticase soy agar (TSA). The plates were incubated at 37°C for 24 h. An isolated colony was picked with a sterile plastic loop and inoculated to another TSA plate. Colonies from this second passage were used to inoculate the cryopreservative liquid of Microbank™ vials (PRO-LAB Diagnostics, 20 Mural Street, Unit #4, Richmond Hill, Ont., Canada) containing porous beads (Figure 2.1.B). The vials were tightly capped and gently shaken to suspend the organism uniformly. The excess liquid was removed and the vials stored in a -80°C freezer.

2.2.2.2 Recovery of the Bacteria from Frozen Stock

For each experiment, a fresh bacterial culture was prepared by aseptically removing a

frozen bead and placing it into a 15 mL plastic conical tube (Sarstedt) containing 10 mL of sterile trypticase soy broth (TSB). The tube with a slightly loosened cap was incubated for 18-24 h at 37°C. The bacterial culture was then centrifuged (Beckman-Avanti™ J-25) at 6,000 rpm for 10 min (~ 4,300 x g) at 4°C; the supernatant was discarded, and the pellet resuspended in 1 mL of sterile deionized water (dd H₂O) in a 2 mL cryovial and kept at 4°C.

2.2.3 Preparation of *Mycobacterium terrae* Stock Cultures

M. terrae (ATCC #15755) and *M. terrae* (pBEN) with a kanamycin-resistance plasmid were both a part of the frozen culture collection at CREM. The former was originally obtained from the ATCC and the latter was a modified version of the same strain that was used for an earlier investigation (Zafer *et al.*, 2001).

2.2.3.1 *M. terrae* ATCC #15755

One mL of thawed *M. terrae* suspension was deposited in each of two 75 cm² vented cap sterile cell culture flasks (Sarstedt) with 20 mL of sterile growth medium containing Bacto Middlebrook 7H9 broth with Bacto Middlebrook ADC enrichment and Bacto glycerol (please refer to Appendix I). The volume of the medium in each flask was increased to a total of 100 mL and the flasks incubated at 37°C for 21 days with one control sterility flask containing only the growth medium. The resulting bacterial culture was then distributed into sterile 50 mL conical tubes and centrifuged at 2,500 rpm for 15 min (~ 2,000 x g) at 4°C (Damon/IEC). The supernatant was decanted and the pellets were resuspended in 25 mL of sterile dd H₂O and centrifuged again to wash the cells; the washing step was repeated once more. After the last wash, the supernatant was decanted and the pellets resuspended in 8 mL of dd H₂O and the final pool was put in a sterile bijoux bottle (Wheaton, Millville, N.J.) containing 7-8 glass beads (Sigma, 3 mm in diameter) (Figure 2.1.C) to store at 4°C.

Figure 2.1 Stock culture preparations

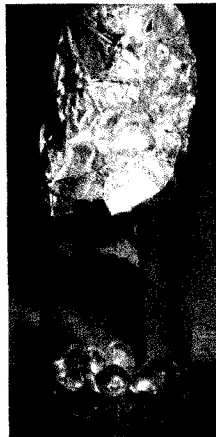
- A. Seed Culture for *A. baumannii* (ATCC #19606) in a swab from Gibson Labs Inc.
- B. Microbank vial (PRO-LAB Diagnostics) used to store *A. baumannii* stock culture.
- C. Sterile bijoux bottle (Wheaton, Millville, N.J.) containing 8 glass beads used to store *M. terrae* pools.



A



B



C

Figure 2.1

2.2.3.2 *M. terrae* (pBEN; ATCC #15755) with Kanamycin-Resistance Plasmid

A stock culture of this organism was thawed at room temperature and 100 μ L inoculated on each of two 100 mm diameter plastic Petri plates containing 7H11 agar supplemented with OADC, glycerol and 10 μ g/mL of kanamycin. The plates were incubated at 37°C for 21 days. Using a sterile plastic loop, from each plate, 3 colonies were picked to inoculate 1 mL of dd H₂O in a cryovial. After vigorous vortexing (Vortex-Genie), 100 μ L of the suspended cells were added to a 75 cm² (Sarstedt) cell culture flask and the bacteria were grown as described in section 2.2.3.1 with 30 μ g/mL of kanamycin added to the growth medium.

2.2.4 Preparation of *Geobacillus stearothermophilus* (ATCC #12980) Stock Culture

A seed culture of *G. stearothermophilus* was obtained from the stock at CREM as a one mL spore suspension. The suspension was vortexed for 20 seconds and 100 μ L of it added to a 2-mL cryovial containing 1 mL of dd H₂O; the suspension was heat-shocked at 100°C for 45 min in a water bath. Fifty μ L of it was then streaked on a TSA plate using a plastic spreader and the plate incubated for 48 h at 56°C. Three cycles of subculture and incubation for 48 h at 56°C were repeated, and in every cycle the spores were checked and counted under the microscope (CARL ZEISS, 64803) where a loopful of the suspension was taken and spread on a glass slide with a drop of dd H₂O; a glass coverslip was used to cover the suspended spores. A loop-full of discreet colonies was taken from the TSA plates with the last subculture and put in 1 mL of dd H₂O in a 2 mL cryovial and heat-shocked at 100°C for 45 min. One hundred μ L of activated spore suspension was put into two wide-necked 250 mL sterile Erlenmeyer flasks containing a sterile stir-bar, 75 mL of TSB (Oxoid CM0129) with salts (CaCl₂, FeSO₄.7H₂O, MnSO₄. H₂O, and MgCl₂.6H₂O) (please refer to Appendix I for salt solutions preparation) and pH adjusted to 7.8; each flask's neck was plugged with a

sterile cotton ball, placed over a stir plate (Bell-Stir Multi-Stir, Bellco Glass Inc. cat #6005) and incubated at 56°C for 24 h. The resulting culture was distributed into four 50 mL sterile conical tubes (Sarstedt) and centrifuged at 1,000 rpm for 20 min (~5,000 x g value) (Damon/IEC) at 4°C. The spores were then resuspended in 10 mL of dd H₂O to wash as described above by centrifugation. This was repeated twice. After the last wash, the pellets were resuspended in 8 mL of dd H₂O, a 500 µL aliquot was placed in each of 2 mL cryovials and stored at 4°C. When needed, one cryovial at a time was retrieved and the spores were activated at 100°C for 45 min.

2.2.5 Determining the Titters of Bacterial Pools Using Membrane Filtration

To determine the titers of the bacterial pools, the following membrane filtration method was done in triplicate for each bacterium (Figure 2.2): The bacterial pool was first homogenized by vortexing (*G. stearothermophilus* and *A. baumannii*) or shaking [*M. terrae* (pBEN)]; second, using a positive displacement pipette, 10 µL of the bacterial pool was suspended into 9.99 mL of NBS and vortexed; 1 mL of that suspension was added to 9 mL of NBS, vortexed and 10-fold serially diluted. Using the same filtration unit but separate filters, dilution numbers 4, 5 and 6 were filtered starting with the highest dilution. The filters were placed onto plates of appropriate recovery agar medium and incubated at the temperature and for the length of time suitable for each bacterium. Colonies were counted to calculate the titer as follows: **Titer (CFU/mL) = Average Number of Colonies x Dilution Factor x 10².**

2.2.6 Preparation of FRhk-4 cell (ATCC #CRL-1688) Monolayer Stocks

FRhk-4 cells, derived from fetal rhesus monkey kidneys, were obtained from CREM as two 75 cm² cell culture flasks. The cells were split in a 1:12 ratio to make 5 flasks with 3 of them being used for the stock cells' preparation; the rest were kept in the 37°C incubator.

Figure 2.2 Method used to determine the titers of bacterial pools

10 μ L of bacterial pool was dispensed into a 20 mL glass vial containing 9.99 mL of normal buffered saline, NBS (done in triplicate). 10-fold serial dilutions were prepared in NBS and the last 3 dilutions were filtered to capture countable numbers of colonies (using the appropriate filter for each organism) and filters were placed on the plates of appropriate recovery agar medium and incubated at the temperature and for the length of time suitable for each bacterium (please check Table 2.2 in the results section). Colonies were counted and the titers were calculated (CFU/mL).

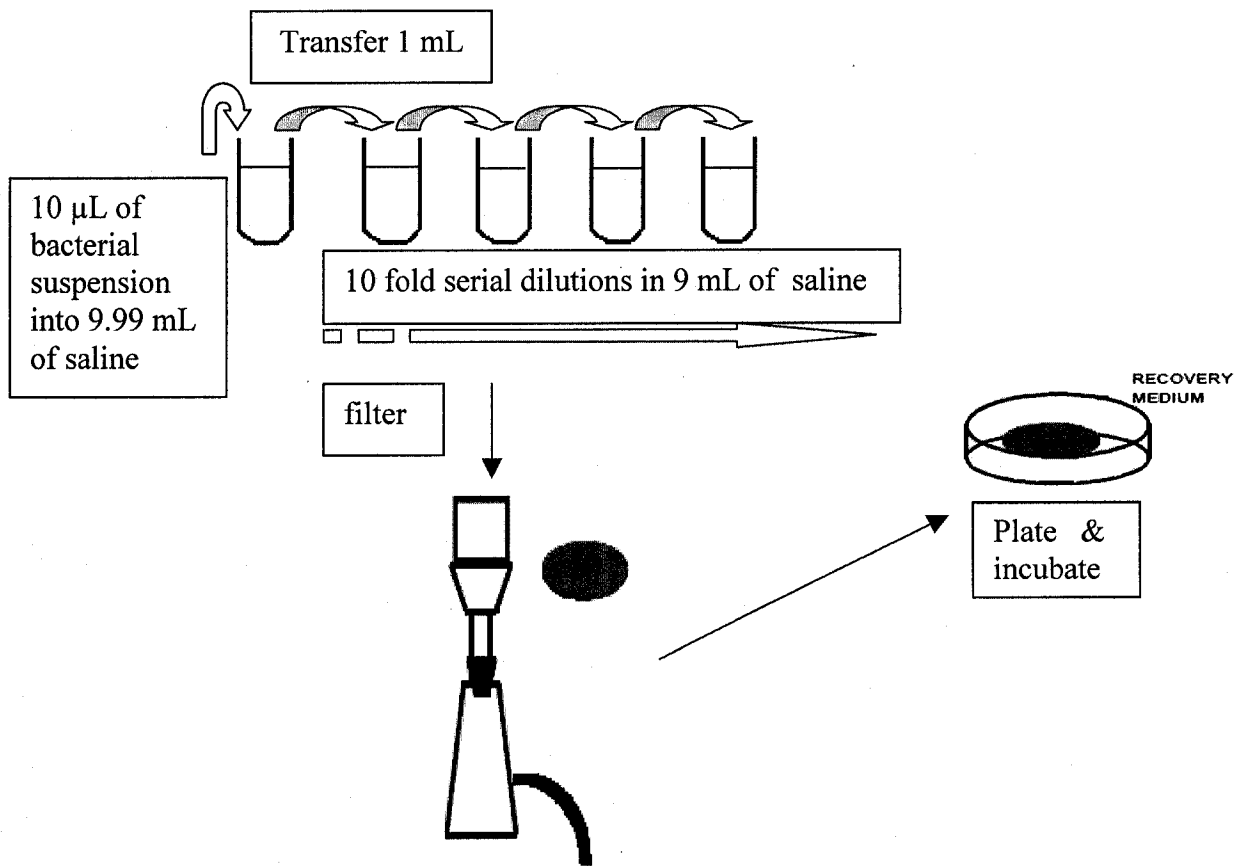


Figure 2.2

2.2.6.1 Splitting the Cells

To split the cell cultures, flasks with confluent monolayers were removed from the incubator and put inside a laminar flow cabinet; the growth medium was poured out and the monolayers washed twice with 10 mL of PBS (GIBCO; cat #21600-051) for each wash. Then, 1 mL of trypsin-EDTA (GIBCO: cat #25300) was added to each flask followed by incubation at 37°C for about 15 min. When the cells had detached, 11 mL of supplemented 1X MEM was added to each flask. Cell clumps were broken up by repeatedly sucking in and out the suspension with a sterile 10 mL disposable pipette (Sarstedt) for about 15 times. One mL was left inside each flask and 1 mL was added to each additional flask; the rest of the cell suspension was discarded into a beaker containing a 1:10 dilution of domestic bleach. A volume of 24 mL of supplemented 1X MEM was added to each flask before incubating them at 37°C.

2.2.6.2 Freezing of FRhK-4 Cells

Inside a laminar flow cabinet, flasks with three-day-old monolayers were washed first with PBS twice and 1 mL of trypsin-EDTA was added to each flask and incubated at 37°C for about 15 min. After adding 2 mL of supplemented 1X MEM to each flask, the cells were broken up by repeated pipetting of the cell suspension. Two mL of the cell suspension was removed from each flask and transferred to a 50 mL sterile conical plastic tube; then, the following was added to the cells: 1.7 mL of fetal bovine serum (FBS; GIBCO cat #12483-020) and 0.86 mL of DMSO. The cells were then dispensed as 1 mL aliquots into 2-mL cryovials, and put into a 1°C freezing container (Nalgene; Cryo 1°C) and kept at -80°C for 24-72 h. After, the vials were transferred to a cryobox and stored in liquid nitrogen.

When needed, two cryovials were removed from liquid nitrogen and let at RT to thaw their contents. After vortexing, the content of each cryovial was added to a sterile 50 mL

plastic tube (Sarstedt) containing 20 mL of 1X MEM, centrifuged (Damon/IEC) at 2,000 rpm (~1,500 x g) to wash the cells and remove the DMSO. Then, the cells were resuspended in 8 mL of 1X MEM, poured the suspension into 25 cm² tissue culture flasks to incubate at 37°C in 5% CO₂.

2.2.7 Preparation of Pools of Hepatitis A Virus Strain HM-175 (ATCC #VR-1402)

Hepatitis A virus (HAV) was obtained from the frozen stocks available at CREM. The material was thawed at room temperature and 100 µL of it added to each of two cryovials containing 900 µL of NBS. The contents of each vial were added to each flask of two-day-old FRhK-4 cell monolayer. A third flask was used as a control by receiving 1 mL of NBS. All three flasks were incubated at 37°C for 90 min. After, 9 mL of maintenance medium² (without kanamycin) was added to each flask before continuing with incubation at 37°C for 8-9 days. The monolayers were checked every day under an inverted microscope (Carson Olympus CK2). When about 80% of the infected monolayers showed virus-induced cytopathology, the flasks were subjected to three cycles of rapid freeze-thaw (-80°C to RT) to release the virus. The cell lysate from the two infected flasks was removed aseptically with a 10-mL disposable pipette and placed into a sterile 50 mL conical plastic tube and centrifuged (Damon/IEC) at 1,500 rpm for 10 min (~1,000 x g). The supernatant was dispensed into cryovials (150 µL/vial) and the vials stored at -86°C freezer (Thermo Forma).

2.2.8 Plaque Assays of HAV

2.2.8.1 Preparation of Plates with FRhK-4 Monolayers

One 75 cm² flask with a confluent monolayer of FRhK-4 cells was split at a ratio of 1:12 and 11 mL of the suspension was mixed by gently shaking with 90 mL of 1X MEM to

² Same as the growth medium (Appendix I), but with only 20 mL/L of FBS.

disperse the cells evenly. Using a disposable pipette, 24 mL of the cell suspension was removed to dispense 2 mL in each well of a 12-well cell culture plate, and 4 plates were made in total.

Alternatively, monolayers in 25 cm² flasks were used to prepare four 12-well plates from each. In this case, the trypsinized cells were first suspended in 5.5 mL of 1X MEM and the suspension then added to 95 mL of the growth medium. The plates were incubated at 37°C in a 5% CO₂ atmosphere for 24 h and this resulted in monolayers with a confluence of about 75%. The use of such incomplete monolayers yielded countable plaques in about six days rather than the usual 8-9 days reported previously (Mbithi *et al.*, 1990)

2.2.8.2 Infecting 12-Well Plates with FRhK-4 Monolayers

Ten-fold dilutions of the sample to be plaque assayed were prepared using cryovials with 0.9 mL of NBS in each. For every plate the growth medium was removed from the FRhK-4 cells monolayers by dumping into a tray lined with paper towels, and each of three wells in a row received 0.1 mL of a given dilution to be tested. Thus, three 10-fold dilutions could be tested per plate. In each plate, one row of three wells was used as cell culture control and each one of these wells received 0.1 mL of NBS. After inoculation, the plates were held for 90 min at 37°C in a 5% CO₂ atmosphere for virus adsorption to the cells.

2.2.8.3 Overlay, Incubation, Fixing and Staining of the Plates

During the period of virus adsorption, the overlay medium was prepared by mixing one part of 1.2% Noble Agar (Difco; cat #214230) with one part 2X MEM. Each well received two mL of the overlay using a disposable 25 mL pipette and held at room temperature for about 20 min to allow the overlay to solidify. The plates were then placed at 37°C in a 5% CO₂ atmosphere.

The plates were retrieved about 6 days later and the monolayers in them fixed by dispensing into each well two mL of 4% formaldehyde in saline and then holding them at room temperature for at least three hours. The overlay was dislodged and removed from each well using a stream of tap water and the cells stained with an aqueous solution of 0.1% crystal violet for about 10 min. The stain was removed by suction and the plates were dried, the plaques counted and their numbers recorded using a standard data sheet.

2.2.8.4 Calculation of the Virus Pool Titer

Plaques were counted in each well, and the average number of plaque was used to calculate the titer of the virus pool. Plaque counts from two dilutions were used in the calculation with the number of PFU from the highest dilution was divided by 10, and the average of both numbers was used to calculate the titer final value as follows:

$$\text{Titer (PFU/mL)} = \text{Average Number of Plaques} \times \text{Dilution Factor} \times 10$$

2.2.9 Validation of Neutralization

Neutralizers require validation before any microbicidal testing. This is normally accomplished by adding to the control and the neutralized active countable numbers of viable microbial units, holding the suspensions for a few min under ambient conditions and then assaying them for viability. Recovery of comparable numbers of viable units from the neutralized and control suspensions is an indication of proper neutralization and the harmless nature of the neutralizer itself to the organism(s) under test.

In validating the neutralization of bactericidal, mycobactericidal and sporicidal activities in this study, each one of three vials first received 50 μ L of the test substance and then 9.95 mL of an appropriate neutralizer to yield a 1:200 dilution; this ratio was reflective of the procedure for testing using the carrier test (Springthorpe and Sattar, 2005) employed in this

study. In parallel, three vials containing 10 mL of the neutralizer alone, and three vials containing 10 mL NBS alone, were also prepared. The mixtures were vortexed for 30 seconds. In tests for bactericidal, mycobactericidal and sporicidal activities, the corresponding microbial suspension was diluted to yield 500-1000 CFU/mL. A 100 μ L of the suspension was added to each vial, vortexed and allowed to stand at room temperature for about 15 min. The contents of the vials were separately passed through membrane filters. The filters were placed on the appropriate recovery medium and the plates incubated at the required temperature/length of time. The CFU on each plate were counted and the resulting colony counts were compared.

When testing for virucidal activity, in 2 mL cryovials, a 1:200 dilution of the test substance was prepared in duplicate by adding 5 μ L of it to 995 μ L of the neutralizer; the mixture was vortexed. In parallel, cryovials containing 1 mL of the neutralizer alone and NBS alone were prepared in duplicate. The test virus was diluted in NBS to yield 100-300 plaque-forming units (PFU) and 100 μ L of the viral dilution was added to each of the prepared cryovials (controls, neutralizer and test/neutralizer) that should yield countable plaques (approximately 10-45 plaques/well). The vials were held at RT for 15 min before starting the testing for cytotoxicity (described below).

2.2.9.1 Testing for Cytotoxicity and Interference with Plaque Formation

The culture medium was removed from the monolayers of FRhK-4 plates. From top to bottom, volumes of 100 μ L/well were dispensed starting with the first row to the right where 2 control wells received a mixture of NBS/virus with the third well received only NBS without virus. In the second row, the 3 wells received a mixture of test product/neutralizer/virus. In the third row, another mixture of product/neutralizer/virus was

dispensed into the 3 wells; the last row received the neutralizer/virus mixture. The plates were incubated for 90 min at 37°C in 5% CO₂. After this, the plates were washed with 1 mL of NBS before adding the overlay mixture (refer to 2.2.8.2) and the plates were incubated for 6 days at 37°C in 5% CO₂. If the number of plaques in the control NBS wells were significantly higher than those in the neutralizer and test/neutralizer wells that means there was interference issues which might indicate the product was not neutralized completely and/or furthermore, if the monolayer was gone, then there may have been a cytotoxicity issue which called for gel filtration as a possible solution.

2.2.9.2 Gel Filtration for Testing Cytotoxicity and Interference with Plaque Formation

The set up of the procedure include 10 mL sterile syringes (BD), 30 mL sterile plastic tubes (Sarstedt), 60 mL sterile glass tubes, sterile wool glass (Fisher Scientific cat# 11.390) and sterile Sephadex mixture (see Appendix I). Inside a safety cabinet, the syringes were mounted on the glass tubes and using a sterile forceps, just a little piece of the wool glass was put into each syringe (enough to hold the Sephadex inside and not to clog the dripping of any excess liquid inside the syringe). Using a 10 mL sterile disposable pipette (Sarstedt), about 15-17 mL of the Sephadex mixture was dispended into each syringe. 5 mL of PBS was used for washing before the tubes were sealed with Parafilm and centrifuged (Damon/IEC), at 3,000 rpm (~2500 x g) for 8 min at 4°C. The final volume of the Sephadex gel column inside the tube was between 7-8 mL. The syringes were removed from the glass tubes and mounted on 30 mL plastic tubes. The cryovials for the controls, neutralizer and test product/neutralizer mixture were prepared as described above (section 2.2.8.2). The content of the cryovials were dispensed aseptically into each of the Sephadex gel column syringes inserted into the accordingly labelled 30 mL plastic tubes and centrifuged as described above

and for the same length of time. Using 1 mL micropipette, the eluate in each plastic tube was removed and dispensed into already labelled 2 mL cryovials. The FRhK-4 plates were prepared and treated as described in the previous section 2.2.9.1.

2.2.10 Quantitative Carrier Test

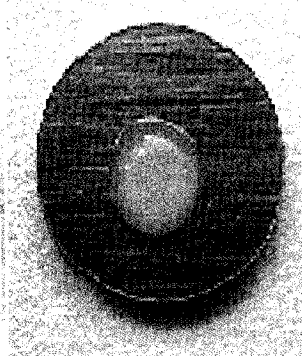
Summary of the Test Method (Figure 2.3): In a laminar flow hood and at RT, using a positive displacement pipette, 10 μ L of the test inoculum with soil load is placed at the centre of each carrier disk (refer to Appendix II for equipment used in the Quantitative Carrier Test). The inoculum was then dried for 1 h. The inoculated carrier was then placed aseptically using sterile forceps onto the inside bottom surface of a sterile Teflon vial. A 50 μ L volume of the test product was placed carefully onto the surface of the disk to cover the entire inoculum and most of the disk surface. The contact time and temperature varied as required. The contact times used for our experiments were 5, 10 and 20 min and two temperatures were tested: room temperature (22-24°C) and 4°C for products that were effective at room temperature. At the end of the contact time, 9.950 mL of the appropriate neutralizer was added to the carrier holder vial; this immediately achieved a 1/200 dilution of the test product. The vial was vortexed for 25 s and 10-fold serial dilutions were made using NBS as diluent and eluent. The appropriate dilutions (normally the last 3 dilutions that would give countable colonies) and subsequent rinses were membrane filtered. Filters were placed aseptically on the appropriate agar (100 mm plastic petri plate) and incubated at the temperature and for the length of time specific for each organism. Each test comprised of 3 control carriers (inoculum challenged with 50 μ L of NBS) and 3 test carriers (inoculum challenged with 50 μ L of the test product). Where indicated, dilutions of the test products were done with sterile water with standard hardness of 300 ppm as CaCO₃.

Figure 2.3 Main steps in the quantitative carrier test QCT-2

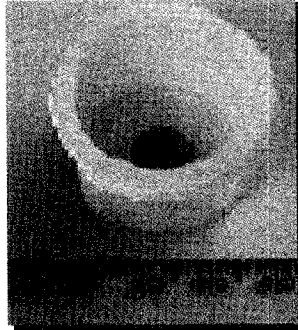
From left to right:

- 10 μ L of microbial mixture on carrier (1)
- Inoculum dried and carrier put inside a Teflon vial (2)
- Contact with decontaminant (3)
- Decontaminant neutralized (4)
- Carrier eluted by vortexing (5)
- Eluent and washings of samples captured on filter (6 and 7)
- Filter placed on the appropriate agar plate to be incubated (8)
- Count survivors and assess kill as \log_{10} reductions

MAIN STEPS IN QCT-2



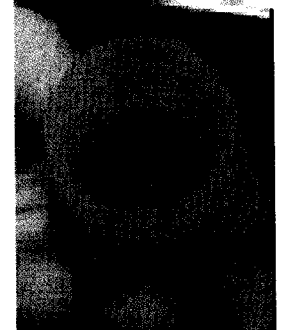
**Disk with 10 µL
inoculum**



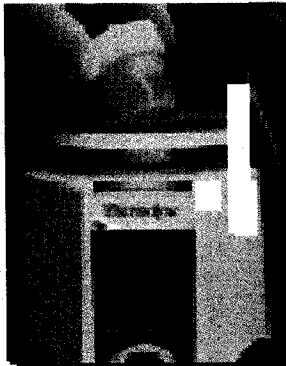
**Disk with inoculum
(dried) in Teflon
vial**



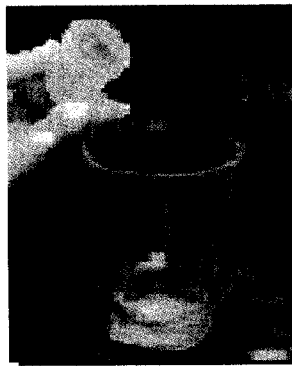
**Disk with 50 µL of
microbicide**



**Disk with 9.95 mL of
eluent/neutralizer**



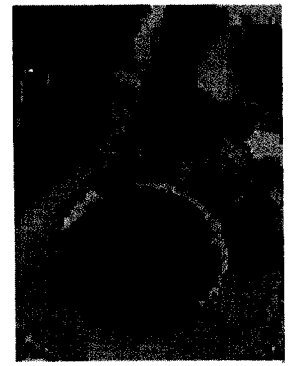
**Vortexing for
elution of
organism**



**Eluate being
poured into filter
holder**



**Filter and holder
being rinsed**



**Filter being placed
on recovery agar**

Figure 2.3

2.2.10.1 Preparation of Inoculum for Individual Testing

For each microorganism to prepare 100 μL inoculum, 68 μL of the microbial pool was combined with 20 μL of mucin, 7 μL of yeast extract and 5 μL of BSA in a 2 mL cryovial, vortexed for 20 s before inoculating each carrier with 10 μL .

2.2.10.2 Preparation of Microbial Mixtures for Testing of Microbicidal Activity

In a 2 mL cryovial, 200 μL of the mixture was prepared for each test by combining the following: 34 μL of the activated *G. stearothermophilus* spores, 34 μL of a freshly prepared *A. baumannii* pool, 34 μL of *M. terrae* (pBEN) pool, and 34 μL of the virus pool thawed at room temperature, 40 μL of mucin, 14 μL of yeast extract, and 10 μL of BSA. The mixture was vortexed for 20 second before inoculating each carrier.

2.2.10.3 Separation of the Mixture

Before starting the actual testing, it was essential to test for any problems in separating the mixture and to check for any effects of the mixture on the titer of each individual organism. The mixture was passed through a membrane filter (0.22 μm pore diameter) to retain all microbes except the virus. The filtrate was subjected to 10-fold serial dilutions and plated as described before (section 2.2.8.2). For the bacteria, 10 μL of the mixture was added to 9.99 mL of NBS, 10-fold serially diluted, membrane filtered, and then placed filters on the suitable recovery agar medium and incubated at the appropriate temperature for each organism (refer to Figure 2.4).

Two problems emerged when testing the mixture. In the initial trials, the titer of *A. baumannii* dropped by $> 5 \log_{10}$ in the mixture. The cause of this turned out to be the kanamycin present in the cell culture medium when making the pools of HAV. Therefore, the pools of the virus were prepared without including kanamycin in the cell culture medium.

The second problem in the mixture was the ability of *A. baumannii* to grow on 7H11 agar. At first, we supplemented the 7H11 agar with antibiotic(s) to inhibit the growth of *A. baumannii*. However, after several trials, using one antibiotic at a time (kanamycin, colistin) and a mixture of antibiotics (kanamycin, colistin, vancomycin, clindamycin and anisomycin), it was clear that *M. terrae* and *A. baumannii* had very similar sensitivities to antibiotics. Therefore, the only ultimate solution was to use *M. terrae* (pBEN) with the kanamycin resistance gene. From experimenting initially with antibiotics, we were able to determine the MIC level of kanamycin that inhibits *A. baumannii* growth on the 7H11 agar, and this helped in preparing 7H11 plates with kanamycin (Kan) (see the following section).

2.2.10.4 Preparation of 7H11 Plates with Kanamycin: Determining the MIC Level of Kanamycin that Inhibited *A. baumannii* Growth on 7H11 Agar

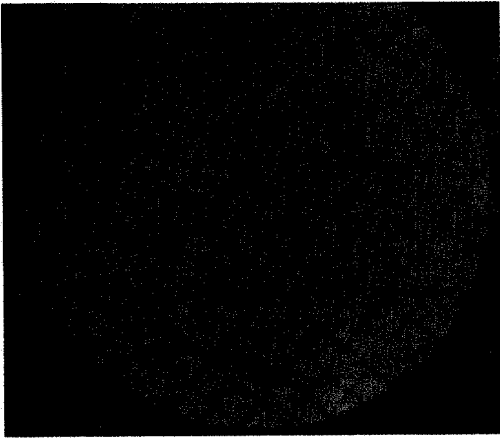
To determine the MIC level of Kan that inhibits the growth of *A. baumannii*, the following was done: first a Kan solution was prepared by dissolving 0.5g of Kan (Fisher Scientific, BP 906-5) in 50 mL of dd H₂O (10 mg/mL), filtered sterilized using a 0.22 μ m filter and stored at 4°C. One 10-fold dilution of this solution was prepared in dd H₂O. From these Kan solutions, 50 μ L, 100 μ L and 250 μ L were spread on 7H11 agar plates (containing ~ 25 mL of agar) supplemented with glycerol and OADC. The plates were left to absorb the antibiotic for 30 min before spreading 10 μ L of a fresh *A. baumannii* pool on each. Two plates without Kan were used as controls. The plates were incubated at 37°C for 24 h and checked for growth 36 h after. From the plates with the lowest Kan concentration and no observed growth, we determined the MIC level of Kan as 9.6 μ g/mL. To standardize our plates, we added 1 mL of the Kan solution (10 mg/mL) to each 1 L of sterile 7H11 agar with glycerol and OADC at 56°C before pouring it into plates to solidify. This gave 7H11 plates with ~10 μ g/mL of Kan. These plates were tested again against *A. baumannii* growth.

Figure 2.4 Method used to separate the organisms after mixing

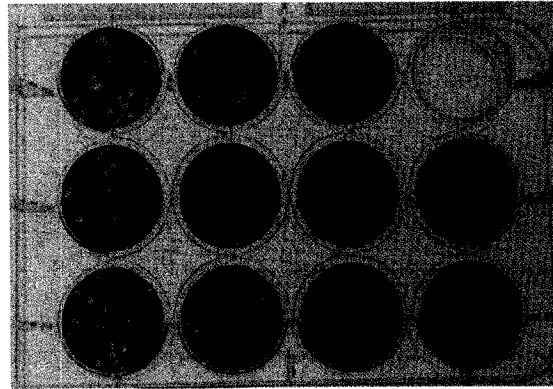
34 μL of each of the four organisms were combined to the soil load (40 μL of mucin, 14 μL of yeast and 10 μL of BSA) in a 2 mL cryovial to make a total of 200 μL .

For bacteria 10 μL of the mixture suspension was suspended in 9.990 mL of NBS, appropriate dilutions were prepared and filtered; Filters were placed on agar plates and incubates at the temperature and for the period of time suited for each organism.

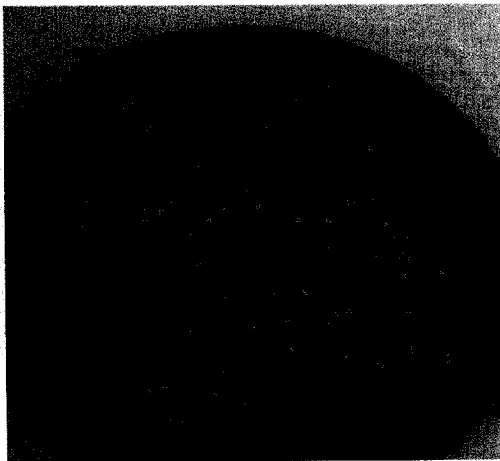
HAV was eluted through a 0.22 μm filter using 100 μL of the mixture, and then 10-fold serially diluted and plaque assayed as described before (please refer to section 2.2.8)



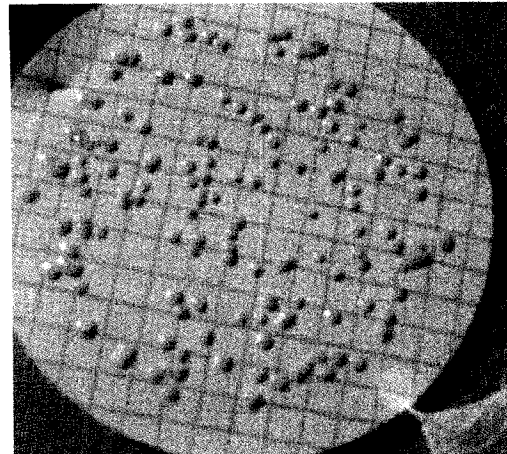
G. stearothermophilus spores
grown on TSA at $56\pm 2^{\circ}\text{C}$



For HAV, eluate passed through a
 $0.22\ \mu\text{m}$ filter and filtrate plaque
assayed in monolayers of FRhK-4 cells.



M. terrae grown on 7H11 agar
supplemented with OADC,
glycerol and Kan at $36\pm 1^{\circ}\text{C}$



A. baumannii grown
on TSA at $36\pm 1^{\circ}\text{C}$

Figure 2.4

2.2.10.5 Testing with the Mixture

A summary of the QCT-2 procedure was previously described (Section 2.2.10), including the method for separating the individual components of the mixture (Section 2.2.10). Further details in this regard are given in Appendix III.

2.2.11 Microbicides Tested

The relevant details on the liquid microbicide formulations tested in this study are shown in Table 2.1. The products were either non-chlorine-based (peracetic acid, PAA and accelerated hydrogen peroxide, AHP) or chlorine-based (chlorine dioxide, CD and domestic bleach, DB), referred to as CRAs (chlorine-releasing agents) in the study.

2.2.11.1 Dilution of the Microbicides

Unless stated otherwise on the product label, tap water is normally used to dilute germicides in the field. However, quality and disinfectant residuals in tap water can vary geographically and temporally, and therefore it is not suitable for use as a diluent in a standard test protocol. Distilled water, on the other hand, may not be reflective of field situations. In view of this, water with a standard level of hardness in it (e.g., 200-400 ppm CaCO_3) is recommended to avoid variation in results (Sattar *et al.*, 2003).

In this study, PAA, CD and DB were diluted using water with a standard hardness of 300 ppm (please refer to Appendix I for method of preparation). Hard water and dilutions were prepared fresh on the day of the test.

Note that, the AHPs were not diluted because they were labelled as ready-to-use products, with their actives being reported as percentages. However, in the results section, these values are reported as ppm when suitable to make an easy comparison with the CRAs. For example 7% AHP is equivalent to 70,000 ppm AHP, and 0.5% AHP is equivalent to 5,000 ppm.

Table 2.1. Relevant details on the disinfectants tested

Formulation	Active(s)	pH	Application(s)
^a Accel TB- Can	0.5% H ₂ O ₂	2.10	Disinfectant Cleaner (tuberculocidal 5 min)
^a Accel TB- US	0.5% H ₂ O ₂	2.72	Disinfectant Cleaner Sanitizer (tuberculocidal 5 min)
^a Virox 5 RTU, AHP	0.5% H ₂ O ₂	1.93	Disinfectant Cleaner (5 min)
^a Prevention High Level, HLP	2% H ₂ O ₂	2.70	Chemisterilant (6 h) and high- level disinfectant (5 min)
^a ALPHA-HP	4% H ₂ O ₂	1.51	Multi-Surface Cleaner
^a Accel CS-20	7% H ₂ O ₂	2.83	Chemisterilant and high level disinfectant (20 min)
*PAA	PAA	5.39 for 1,000 ppm 5.60 for 500 ppm	High level disinfectant and sterilant
**Sodium hypochlorite	^b CRA	10.16 for 5,000 ppm 9.74 for 3,500 ppm 9.47 for 2,500 ppm	Disinfectant
***Chlorine dioxide	^b CRA	3.19 for 1,000 ppm 3.85 for 500 ppm	Disinfectant/Virucidal/Tubercu- locidal

^a Ready-to-use

^b Chlorine-Releasing Agent

* PAA was prepared fresh for every test by mixing 0.68 mL of the disinfectant mixture (H₂O₂ and PAA, Ecolab Inc UN3149), 0.63 mL of the activator (NaOH, Ecolab Inc UN1824) and 13.69 mL of dd H₂O. This mixture gave ~ 2,000 ppm PAA concentration (see Appendix IV for method of testing).

** Sodium hypochlorite solution was regular domestic bleach with 5.25% free chlorine concentration.

*** Chlorine dioxide was prepared by dissolving one Exterm-1.5 tablet (ClorDiSys Solutions, Inc.) in 125 mL of dd H₂O. This would yield, according to the manufacture, a working solution containing ~ 1,000 ppm free chlorine that would be stable for about 7 days.

2.2.12 Calculation of Log₁₀ Reduction for Assessing Microbicidal Activities

The microbicidal activity of a disinfectant was determined by comparing the number of CFU or PFU recovered from the test carriers to those from the control carriers. For both controls and tests, the average count from three carriers of the appropriate dilution was calculated. Average counts from triplicate tests were then used to calculate the CFU or PFU for controls and tests. Log₁₀ reduction for the test product was determined as follows:

$$\text{Log}_{10} \text{ Reduction} = \text{Log}_{10} (\text{average counts of controls} - \text{average counts of tests})$$

2.3 RESULTS

The relative humidity (RH) and room temperature (RT) in the lab were measured on each day of the experiment using a hygrometer and recorded. These parameters remained essentially unchanged on a given day. But over the course of this study, the RH varied between 38% and 60% and the air temperature between 22 and 24°C.

2.3.1 The Titters of the Organisms

The titers of the organisms were calculated as indicated above and are listed in Table 2.2.

Table 2.2 List of the organisms in the mixture, their recovery media and titers

Organism (ATCC #)	Recovery medium or cell line	Titer (mL)
<i>Mycobacterium terrae</i> (15755) pBEN	Middlebrook 7H11 agar with OADC; observed weekly with final reading after 28 days of incubation at 37°C	7.3 x 10 ⁸ CFU 3.7 x 10 ⁹ CFU
<i>Geobacillus</i> <i>stearothermophilus</i> (12980)	TSA agar plates incubated at 56°C for 48 h	1.5 x 10 ⁸ CFU
<i>Acinetobacter baumannii</i> (19606)	TSA agar plates incubated at 37°C for 24 h	1.2 x 10 ⁹ CFU
Hepatitis A virus (VR-1402)	FRhK-4 cells (CRL-1688) infected and incubated for 6 days	8 x 10 ⁸ PFU

2.3.2 Effect of Including Soil Load on the Titters of the Organisms

A soil load was included in the microbial suspension for inoculating the carriers when testing the organisms individually and in the mixture to represent 'dirty' conditions. The titers of the organisms, individually and in the mixture, were determined with and without the soil load. Titrations for the bacteria and the virus were performed as described in Section 2.2.5 and 2.2.8.4, respectively. For the three bacteria, the apparent drop in their titers seen in Figure 2.5 can be accounted for by the dilution caused when soil load was added to the

microbial suspension and not due to any inhibitory effects of the components of the soil load. The titers corrected for dilution factors showed this conclusion clearly. For HAV, no decrease in the titer was observed. On the contrary, the addition of the soil load appeared to aid in dispersing the virus and thus the actual counts of PFU were higher with the soil load (Figure 2.6). No major differences were noted in the results between organisms tested alone and in the mixture. Note that the dilution factor from soil load inclusion was 100/68 (please refer back to section 2.2.10.1 for more details); and this factor was considered the same for the microorganisms tested individually and in the mixture.

2.3.3 Effect of Drying on the Titers of Organisms

The viability titers of the test organisms were checked after 1 h of drying on the carrier disks at RT only for inocula with the soil load. This was done for each organism alone and in the mixture (Figure 2.7 for the bacteria and Figure 2.6 for the virus). Although the viability of *A. baumannii* was the most affected relatively to the other surrogates, the titers of the surrogates were still high enough for testing after the chosen drying time.

2.3.4 Effect of Neutralizers on Assessing Microbicidal Activities

In QCT, Lethen Broth (LB) with 1% sodium thiosulfate is usually used to neutralize oxidizing agents, and 1% sodium thiosulfate plus 0.1% Tween-80 to neutralize CRAs. But, when tested for validation in this study, LB was ineffective against 4% and 7% AHP but it adequately quenched the activity of 0.5-2% AHPs. In addition, the neutralization of chlorine dioxide by sodium thiosulfate was incomplete. In addition, LB proved to be cytotoxic to FRhK-4 cells when used as a neutralizer. LB also could not effectively neutralize the mycobactericidal activity of those formulations containing the higher levels of AHP.

The above observations made it necessary to look for a neutralizer that could effectively deal with all the actives and organisms used in this study. After experimenting with several

possible candidate neutralizers (Sutton *et al.*, 2002; Espigares *et al.*, 2003), we developed our own modification (DEBm) of the Dey-Engley broth (Sutton *et al.*, 2002) and tested it first against *A. baumannii*, which was the most sensitive of all the organisms selected for this study. Our modified neutralizer (Appendix I) indeed proved to be effective against all the actives as well as the organism tested. Further, it was non-toxic to FRhK-4 cells and exhibited tolerable levels of interference with plaque formation. Table 2.3 is a sample of the results comparing the neutralization of *A. baumannii* using LB and our modified Dey-Engley broth, DEBm.

Table 2.3 Results as CFU recovered from neutralizer validation tests on *A. baumannii* at room temperature comparing efficacy of DEBm to LB.

Microbicide	Neutralizer	CFU recovered		
		Control (Saline)	Neutralizer + Active	Neutralizer alone
7% AHP	LB	173.3	7	189.3
	DEBm	173.3	204.5	209.3
4% AHP	LB	173.3	78.5	189.3
	DEBm	173.3	181.5	209.3
2% AHP	LB	173.3	176.5	189.3
	DEBm	173.3	210.0	209.3
Bleach 5000 ppm	LB	173.3	161.5	189.3
	DEBm	173.3	211.0	209.3

Even when tested at the lower temperature, our data showed that there was a difference between DEBm and sodium thiosulfate in quenching the activity of CD when tested on *G. stearothermophilus* spores. At 4°C, using DEBm to quench the activity of CD (1,000 ppm) at

20 min contact time, there was a 3.04 log₁₀ reduction as compared to 4.01 with 1% sodium thiosulfate. However, for quenching the activity of 5,000 ppm DB the difference was insignificant, the log₁₀ reductions being 3.25 with DEBm and 3.06 with 0.1% sodium thiosulfate.

2.3.5 Organisms Individually Tested

Each organism was challenged with the disinfectant under test as described in Section 2.2.10, for QCT-2 procedure. Testing of the disinfectants started at the manufacturer's recommendation contact time whenever such information was available, and then adjusted according to the challenged organism. Testing was generally carried out with contact times of 5, 10 and 20 min. Contact times longer are considered irrelevant when considering the microbial decontamination of environmental surfaces.

All the tested products achieved a > 6 log₁₀ reduction in the viability of *A. baumannii* at a contact time of 5 min, and, even at 1 min, Accel TB U.S. achieved total kill. Therefore, *A. baumannii* proved to be the most susceptible to all the disinfectants tested. For *M. terrae* (pBEN), the same results were obtained at a contact time of 5 min except for two AHP 0.5% products. *M. terrae* ranked as second in susceptibility to the disinfectants. The spores, as expected, needed a longer contact time to achieve total kill with CRA, and AHP 4% and AHP 7% (Figure 2.8 A) while with PAA this was achieved in only 5 min.

Quite surprisingly, HAV proved to be more resistance than expected, only CRAs achieved a total kill at 5 min contact time (compared to 20 min for the spores); and PAA and AHP were poorly effective against the virus and achieved a < 1 log₁₀ reduction at 5 min contact time. Even when tested at full strength PAA (2,000 ppm) at 5 min was not effective. Only AHP 4% was able to achieve > 3 log₁₀ reduction at 20 min contact time against HAV (Figure 2.9.A). Results from individual testing at RT are listed below in Table 2.4.

Figure 2.5 The effect of including soil load on the titers of the organisms, *M. terrae* (pBEN), *A. baumannii*, and *G. stearothermophilus*, in individual suspensions and in the mixture

Suspensions of the organisms in soil load (+ SL) and without soil load (- SL) were tittered as described before in the Materials and Methods (sections 2.2.5 and 2.2.2.4) in individual testing (I) and in the mixture (M).

- A. Suspension of *M. terrae* (pBEN)
- B. Suspension of *A. baumannii*
- C. Suspension of *G. stearothermophilus*

Note that the dark columns were the titers (I + SL) and (M + SL) corrected for the dilution factor.

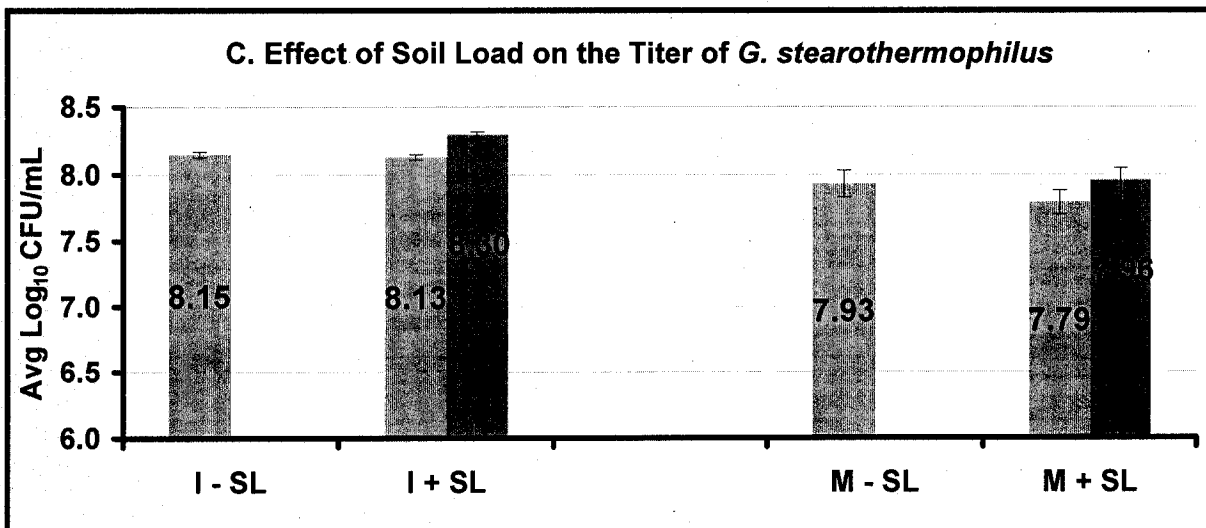
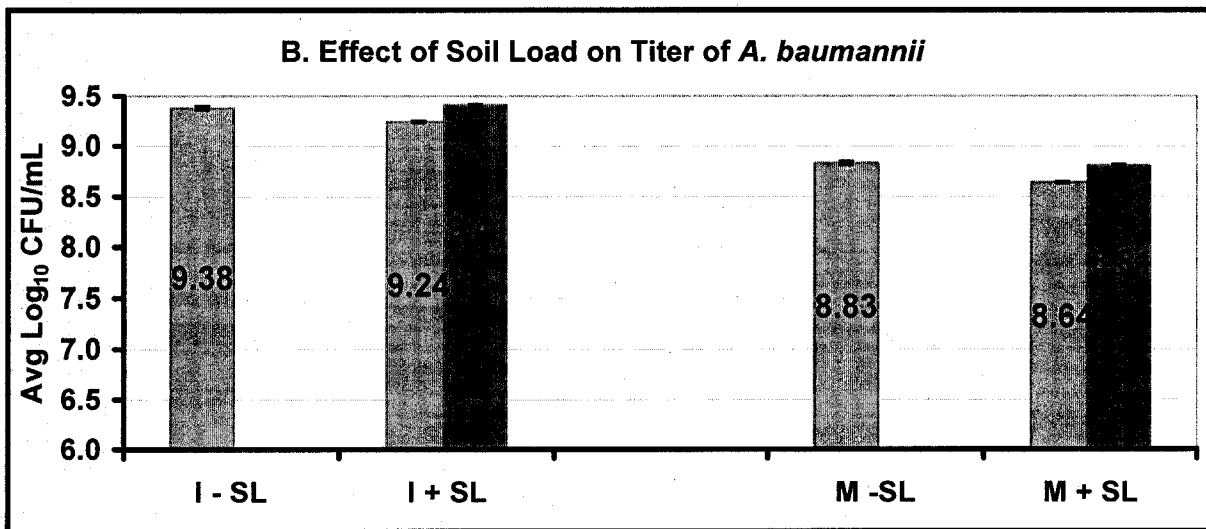
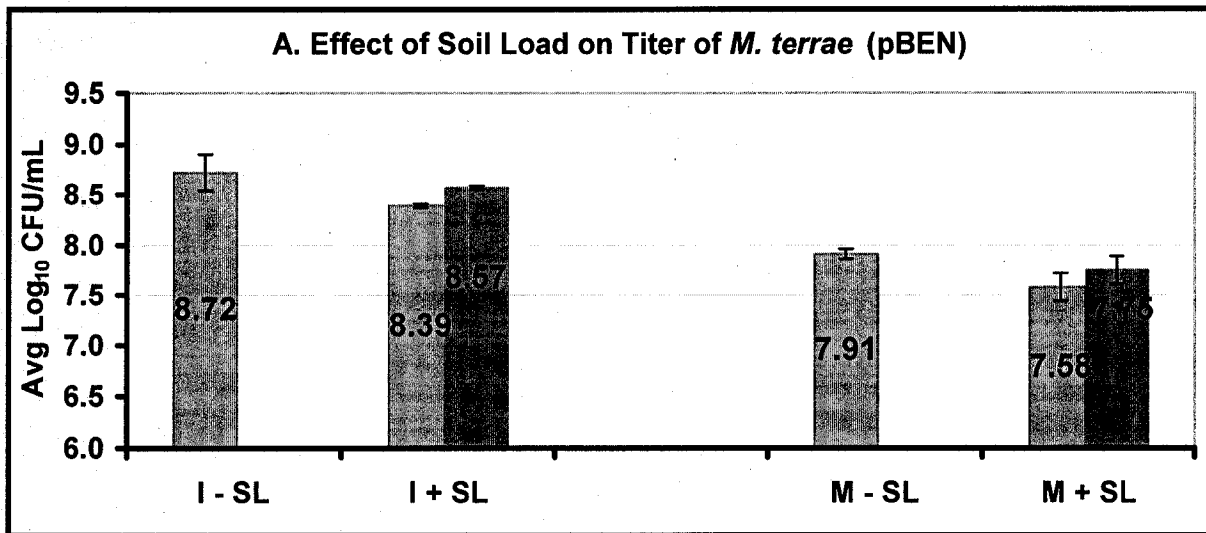


Figure 2.5

Figure 2.6 Effect of including soil load and drying on HAV in the mixture (M) and in individual (I) testing.

- A. Effect of including soil load (+ SL)
- B. Effect of drying for 1 hr on the carrier disks was only tested for suspensions in soil load (+ D). Ten μL of the viral suspension in soil load was dried first for 1 h on the carrier disks, put aseptically into a Teflon vial containing 1,000 μL of NBS, and then 10-fold serially diluted in 2 mL cryovials containing 900 μL of NBS. Dilutions were plated as described before (section 2.2.8). As controls, the disks with the virus alone (I - D) or the virus in the mixture (M - D) were immediately eluted, before allowing any drying, in 990 μL of NBS.

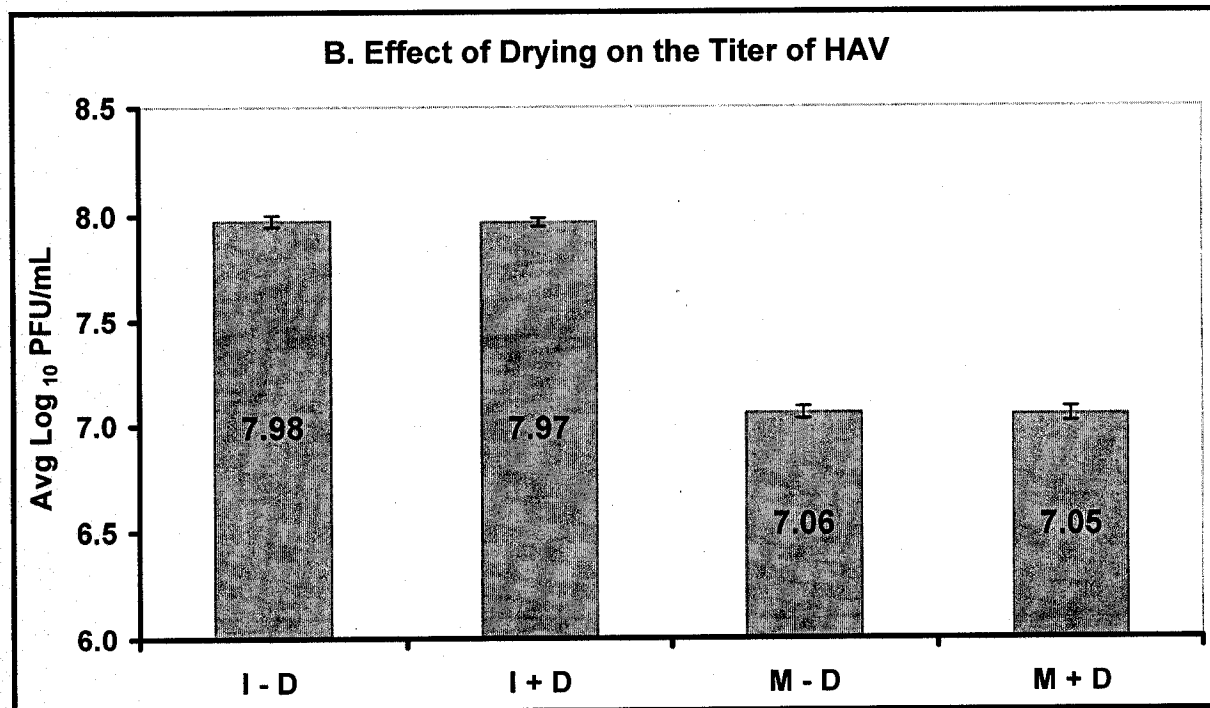
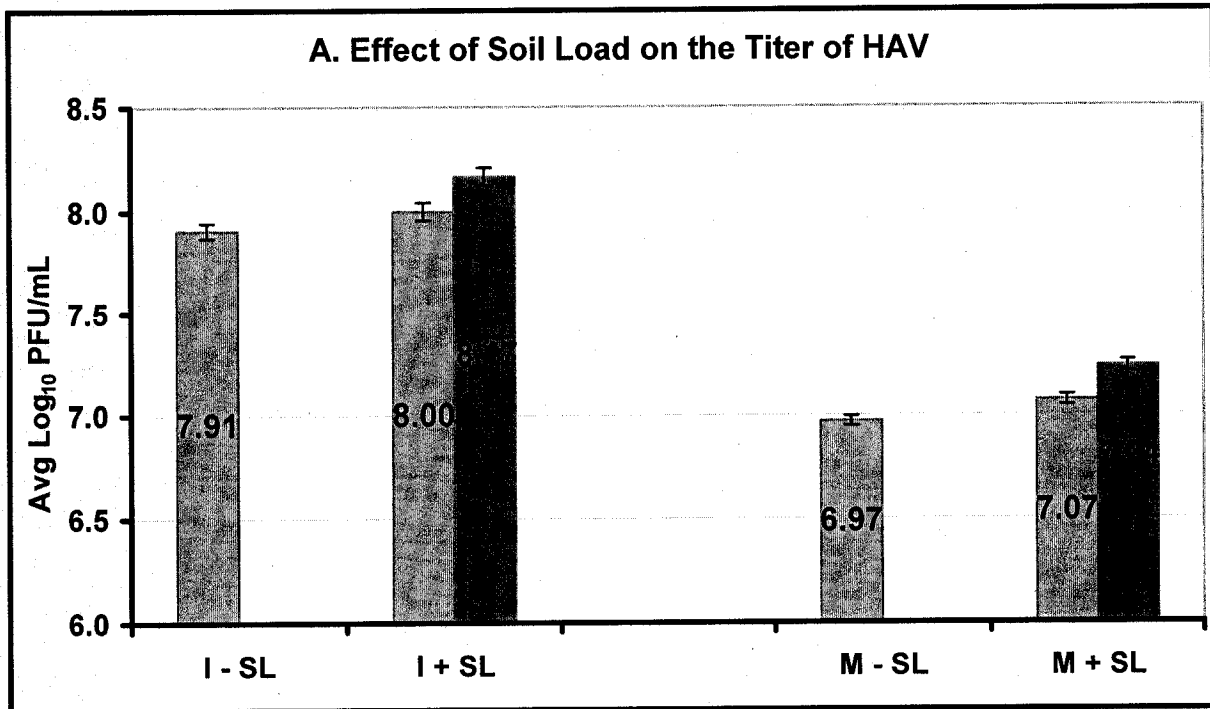


Figure 2.6

Figure 2.7 Effect of Drying for 1 h on the carrier disks on the organisms suspended in soil load.

10 μ L of the suspension was dried on the carriers as described before in Section 2.2.10; the disks were then put aseptically into a Teflon vial containing 10 mL of NBS, vials, vortexed and 10-fold serially diluted to be plated on the appropriate agar. Titters of the organisms without drying were tested by eluting immediately the suspension from the disks in 9.99 mL of NBS before allowed to dry and then treated the same as described above.

Titer of the organisms tested individually without drying (I - D)

Titer of the organisms in the mixture without drying (M - D)

Drying effect on the titters of the organisms tested individually (I + D)

Drying effect on the titters of the organisms tested in the mixture (M + D)

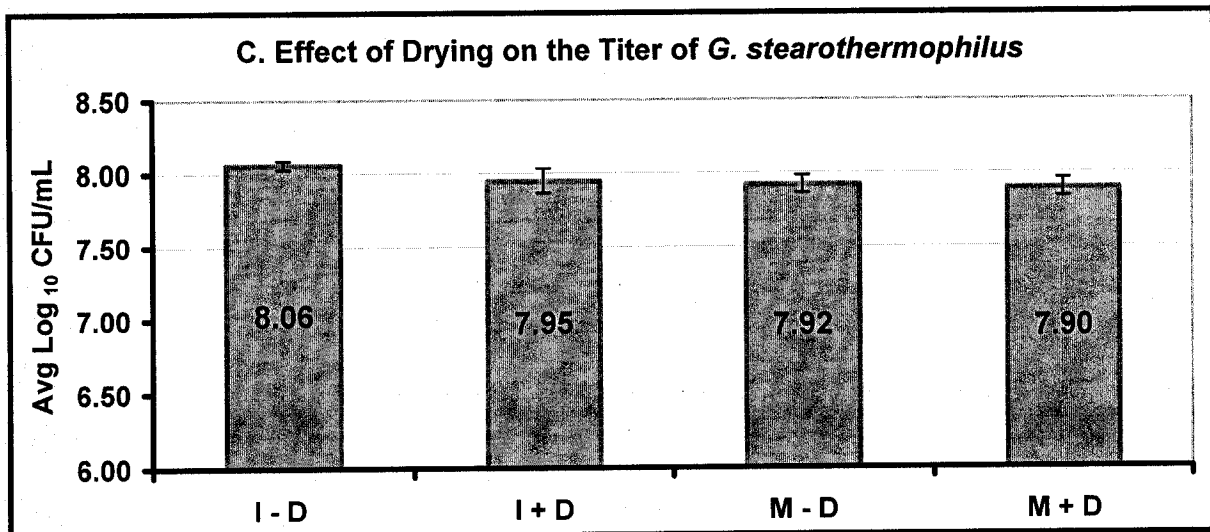
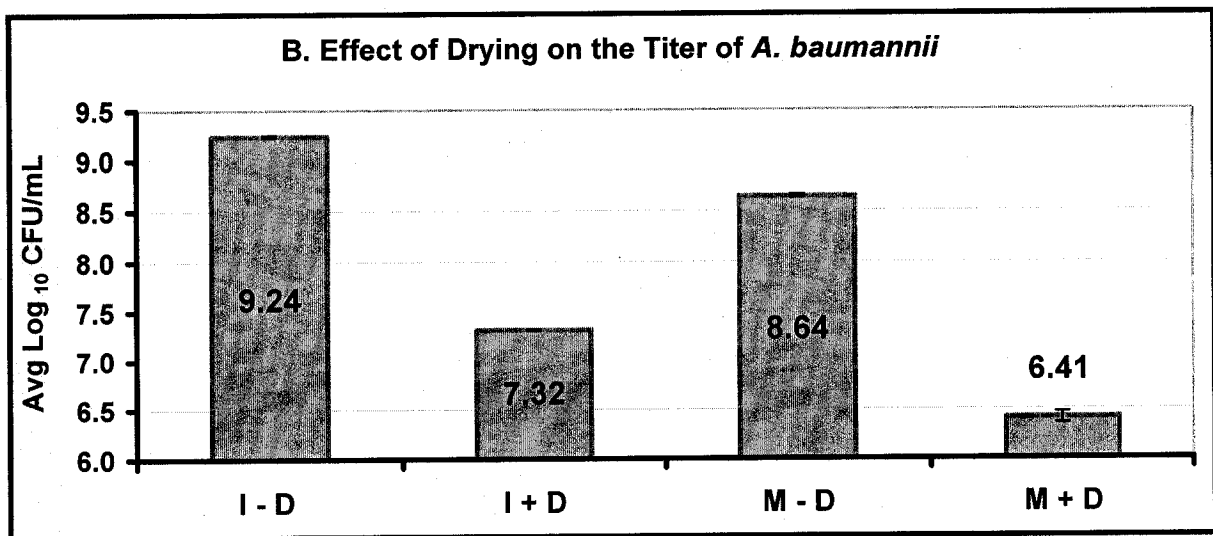
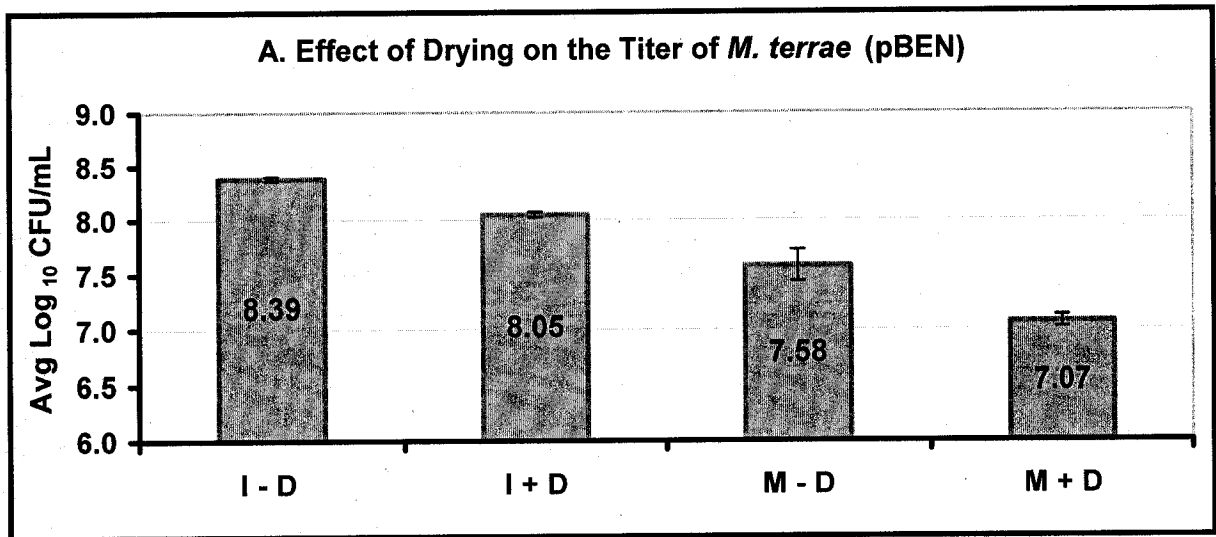


Figure 2.7.

Table 2.4 Log₁₀ reductions achieved by the disinfectants when tested on each organism individually using the standard method QCT-2

Microbicide (Contact time in min)	Mean log ₁₀ reduction ± SD			
	<i>M. terrae p</i> (BEN)	<i>A. baumannii</i>	<i>G. stearotherophilus</i>	Hepatitis A
Virox 5				
5	1.09 ± 0.22	7.22 ± 0.03	0.17 ± 0.01	0.42 ± 0.24
10	1.89 ± 0.45		0.17 ± 0.01	Not tested
20	2.45 ± 0.71		0.17 ± 0.01	
Accel TB-U.S.				
5	8.31 ± 0.02	7.19 ± 0.03	Not tested	0.72 ± 0.16
Accel TB-Can				
5	3.74 ± 0.36	7.22 ± 0.03	Not tested	Not tested
10	4.75 ± 0.57			
HLP				
5	8.31 ± 0.02	7.22 ± 0.03	0.18 ± 0.05	0.05±0.07
ALPHA-HP				
5	8.18 ± 0.19	7.22 ± 0.03	1.61 ± 0.09	1.54 ± 0.08
Accel CS20				
5	8.18 ± 0.19	7.22 ± 0.03	0.87 ± 0.04	Not tested
PAA (1000 ppm)				
5	8.18 ± 0.19	7.19 ± 0.03	8.03 ± 0.28	0.58 ± 0.22
PAA (500 ppm)				
5	8.18 ± 0.19	7.19 ± 0.03	4.03 ± 0.08	Not tested
ClO₂ (1000 ppm)				
5	8.18 ± 0.19	7.22 ± 0.03	3.07 ± 0.09	4.30 ± 0.18
ClO₂ (500 ppm)				
5	8.18 ± 0.19	7.22 ± 0.03	1.47 ± 0.45	4.30 ± 0.18
Bleach (5000 ppm)				
5	8.18 ± 0.19	7.22 ± 0.03	0.28 ± 0.01	4.41 ± 0.23
Bleach (3500 ppm)				
5	8.18 ± 0.19	7.22 ± 0.03	0.27±0.03	4.41 ± 0.23

2.3.5.1 Comparison of Susceptibility of *M. terrae* normal strain and *M. terrae* (pBEN) to Microbicides by Quantitative Carrier Test

M. terrae (pBEN) and *M. terrae* were both exposed to the same products using QCT-2 in parallel experiments to compare \log_{10} reductions achieved, and hence to compare the disinfectant sensitivity of both. There were only two products that did not achieve a total kill on the parent and transformed *M. terrae*. At 5 min contact, the first product Virox 5 achieved a 0.89 ± 0.04 as compared to a 1.09 ± 0.22 mean \log_{10} reduction on *M. terrae* and *M. terrae* (pBEN), respectively. The second product Accel TB-Can achieved a 3.74 ± 0.36 and 3.98 ± 0.25 mean \log_{10} reductions on *M. terrae* and *M. terrae* (pBEN), respectively. However, the detected differences in \log_{10} reductions were too small to be considered momentous in terms of susceptibility.

2.3.6 Organisms Tested In the Mixture

For each test, 400 μL of the mixture was prepared by combining the following in a 2 mL cryovial: 68 μL of the activated *G. stearothermophilus* spores, 68 μL of a freshly prepared *A. baumannii* pool, 68 μL of *M. terrae* (pBEN) pool, and 68 μL of the virus pool thawed at RT, 80 μL of mucin, 28 μL of yeast extract, and 20 μL of BSA. The mixture was vortexed for 20 s before inoculating each carrier with 10 μL of the mixture. After drying for 1 h, QCT-2 testing was performed as described before (Section 2.2.10 and Appendix III), the organisms were separated and \log_{10} reductions were calculated (Table 2.5).

When necessary and because of the way the separation of the organisms in the mixture was done, in cases when no colonies/plaques were seen at the first dilution and the test was repeated, the undiluted eluate was processed to confirm the complete absence of any viable organisms.

2.3.5.1 Comparison of Microbicidal Activities when Tested on Microorganisms Individually and in the Mixture

In general, the microbicidal activities when tested on the organisms alone or in the mixture were similar except for the spores. Notwithstanding the slight reduction in the titers due to dilution in the mixtures, the tested products tended to give a lower \log_{10} reduction for the spores when tested in the mixture, especially at contact times of 5 and 10 min.

Interestingly, of all the products that gave a total kill for the spores at 20 min when tested individually or in the mixture, the only one that showed lower \log_{10} reductions was ClO_2 1,000 ppm (see Section 2.3.7 and Figure 2.10). Note that in our results and only for this purpose, $\geq 3.68 \log_{10}$ and $\geq 6.62 \log_{10}$ reductions meant the product was virucidal and sporicidal, respectively, with total kill, while ≥ 6.24 and $\geq 7.17 \log_{10}$ reductions meant bactericidal and mycobactericidal, respectively, with total kill.

2.3.6 Effect of Contact Time on Microbicidal Activities

At 5 min, all tested products achieved a total kill for *A. baumannii*; the same was the case for *M. terrae* (pBEN), except for the two 0.5% AHP products -Virox 5 and Accel TB-Can. The effect of contact time was more pronounced on the spores and clearly shown by the \log_{10} reduction achieved with selected products at 5, 10, and 20 min (Figure 2.8.A tested alone) and (Figure 2.8.B tested in the mixture). As indicated before, while PAA and AHP (7% and 4%) achieved total kill of the spores, they were less effective against HAV. Figure 2.9 compares \log_{10} reductions achieved against the spores and HAV. ClO_2 (1,000 ppm) at 20 min contact time achieved a $6.07 \log_{10}$ reduction against the spores in individual testing, compared to only a $4.29 \log_{10}$ reduction in the mixture (Figure 2.10).

Table 2.5 Log₁₀ reductions achieved by the disinfectants when tested against the organisms in the mixture using the standard method QCT-2

Microbicide (Contact time in minutes)	Mean log ₁₀ reduction ± SD			
	<i>M. terrae</i> p(BEN)	<i>A. baumannii</i>	<i>G. stearotherophilus</i>	Hepatitis A
Accel TB-US 20	7.33 ± 0.16	6.65 ± 0.29	0.44 ± 0.22	0.61 ± 0.05
HLP 20	7.33 ± 0.16	6.65 ± 0.29	0.41 ± 0.04	1.01 ± 0.07
ALPHA-HP 5	7.72 ± 0.21	6.65 ± 0.29	0.28 ± 0.03	Not tested
20			7.75 ± 0.03	3.19 ± 0.02
Accel CS20 5	7.72 ± 0.21±	6.65 ± 0.29	0.11 ± 0.02	Not tested
20			7.84 ± 0.10	1.45 ± 0.04
PAA (1000 ppm) 5	7.33 ± 0.16	6.33 ± 0.03	7.21 ± 0.59	0.69 ± 0.09
PAA (500 ppm) 5 min	7.33 ± 0.16	6.33 ± 0.03	4.45 ± 0.98	0.30 ± 0.01
20			6.67 ± 0.05	Not tested
ClO₂ (1000 ppm) 5	7.72 ± 0.21	6.37 ± 0.13	1.27 ± 0.05	4.03 ± 0.19
ClO₂ (500 ppm) 5	7.72 ± 0.21	6.37 ± 0.13	0.69 ± 0.05	4.03 ± 0.19
Bleach (5000 ppm) 5	7.72 ± 0.21	6.37 ± 0.13	0.25 ± 0.02	3.97 ± 0.29
Bleach (3500 ppm) 5	7.72 ± 0.21	6.37 ± 0.13	0.25 ± 0.02	3.97 ± 0.29

Figure 2.8 Log₁₀ reductions achieved by the test formulations at different contact times on *G. stearothermophilus* spores when tested individually and in the mixture at room temperature

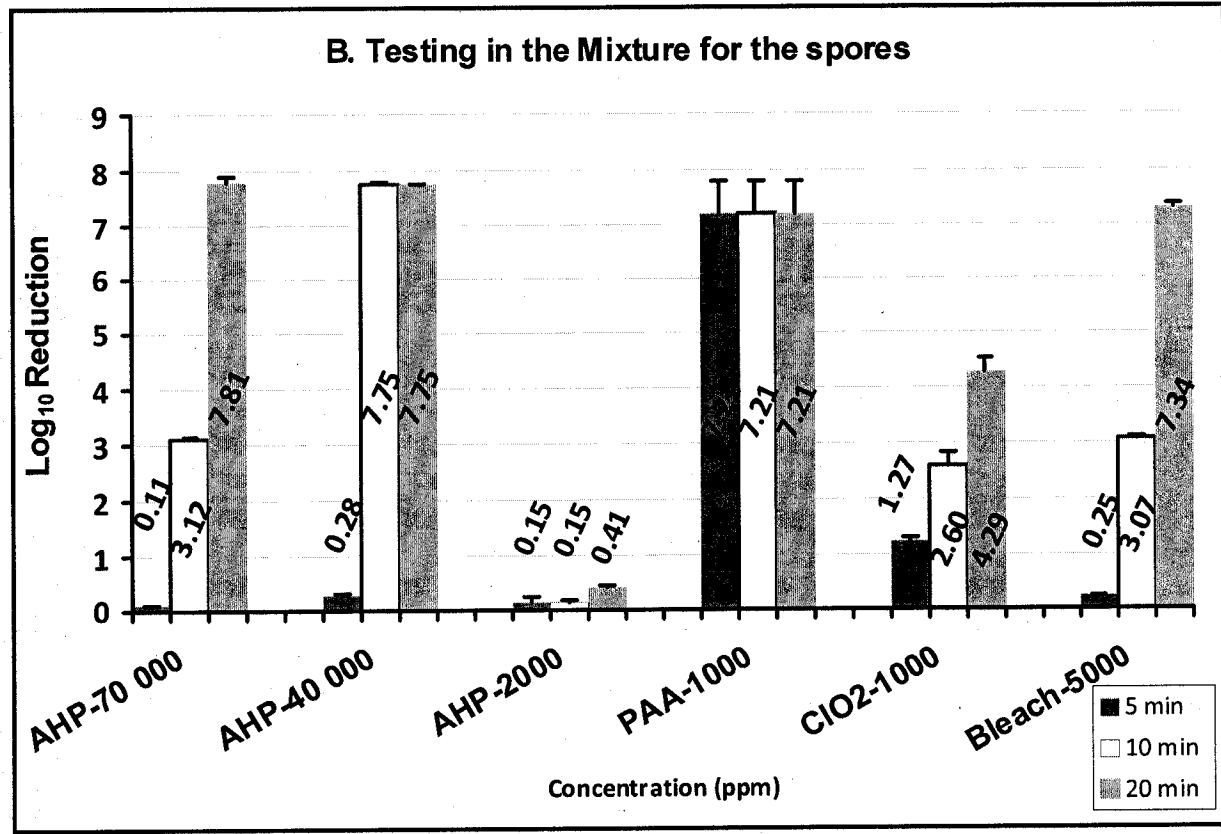
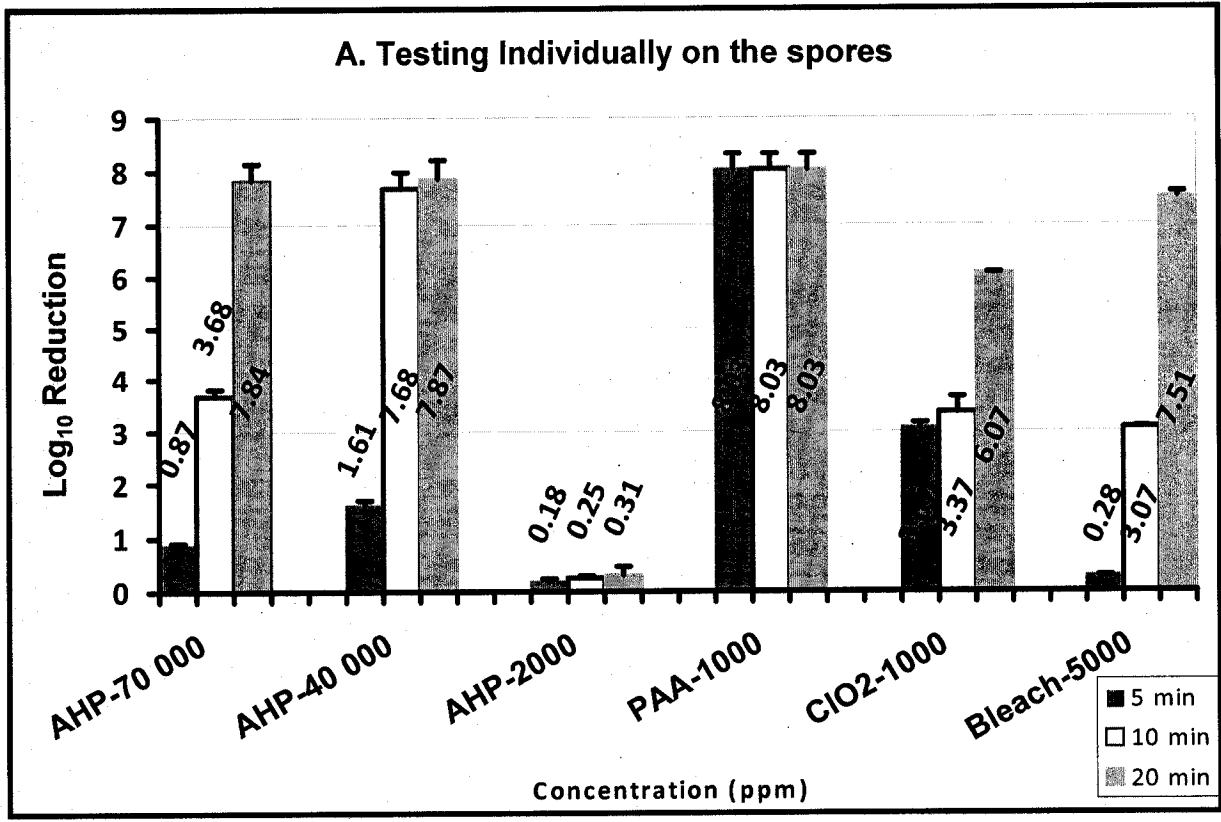


Figure 2.8

Figure 2.9 Comparison of \log_{10} reductions achieved by AHP products, domestic bleach and PAA on *G. stearothermophilus* spores and on HAV tested individually and in the mixture at room temperature

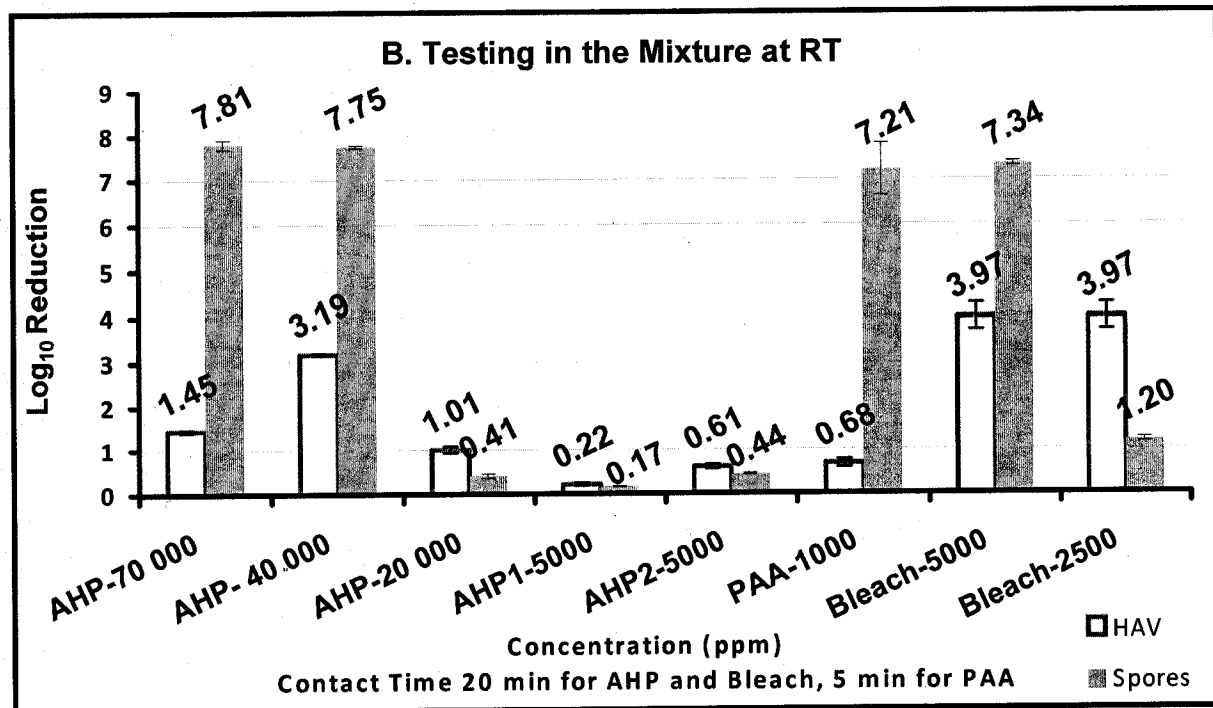
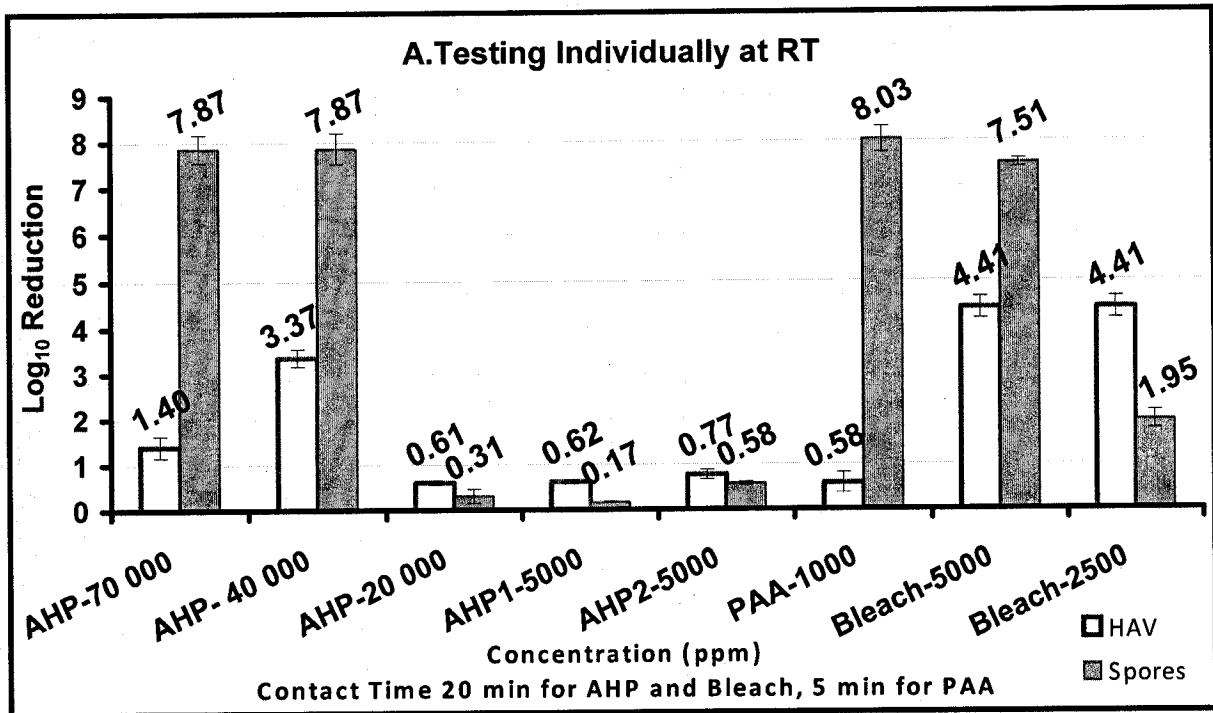


Figure 2.9

2.3.7 Effect of Dilution on Microbicidal Activities

Studying the effect of dilution on microbicidal activity was essential in this study for two important reasons: i) because variability among tests was attributed mainly to marginal products or those diluted below their recommended use concentration. And ii) to confirm the concept that the response of examining microbial kill as a function of disinfectant concentration at a fixed contact time would be a sigmoidal curve. In addition, from the dose-response curves, we could determine not only the effective concentrations against a target microbe, but also those ineffective ones that were used later on for the wipe/transfer test experiments. Dose-response curves were generated with various dilutions of DB against *A. baumannii* (Figure 2.11.A), and the spores (Figure 2.11.B). In addition, Figure 2.10 is the \log_{10} reductions achieved by CD (500-1000 ppm) on the spores at different contact times. CD (100) ppm at a 10 min contact time achieved only 0.26 ± 0.01 and 0.32 ± 0.14 \log_{10} reductions when tested individually on the spores and on HAV, respectively.

2.3.8 Effect of Lower Temperatures on Microbicidal Activities: Testing at 4°C

Carrier disks were inoculated and dried as described before (section 2.2.10). The disks were placed individually into Teflon vials and transported to a walk-in cold room (4°C) and kept there for 30 min for temperature equilibration. The test products and neutralizer were also kept in the cold room for 1.5 h where the testing was done. After neutralizing the products, the vials were transferred to RT and the rest of the experiment was finished there. Testing was done on the spores and on HAV individually for their greater resistance. The products chosen for the experiments had been shown earlier in this study to achieve a total-kill at RT at the same contact time. The low temperature had a pronounced negative effect on the microbicidal activities of AHPs and to a lesser extent on CRAs (Figure 2.12).

Figure 2.10 Effect of dilution and contact time on the activity of chlorine dioxide at room temperature against *G. stearothersophilus* spores tested individually (I) and in the mixture (M)

Figure 2.11 Dose-response curves for \log_{10} reduction as a function of the concentration of domestic bleach at fixed contact time for *A. baumannii* (2.11.A) and *G. stearothersophilus* spores (2.11.B)

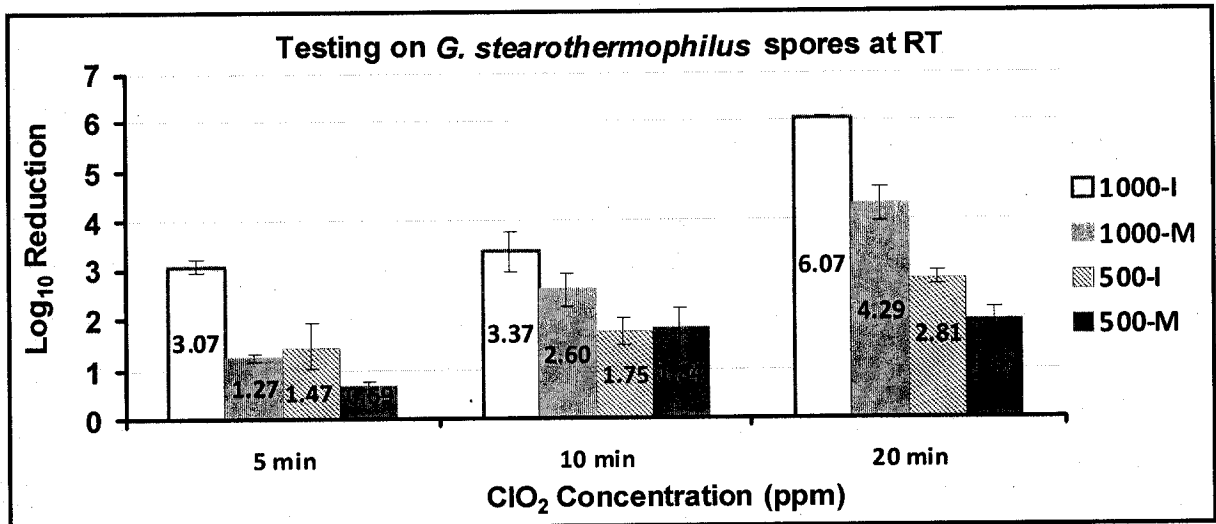


Figure 2.10

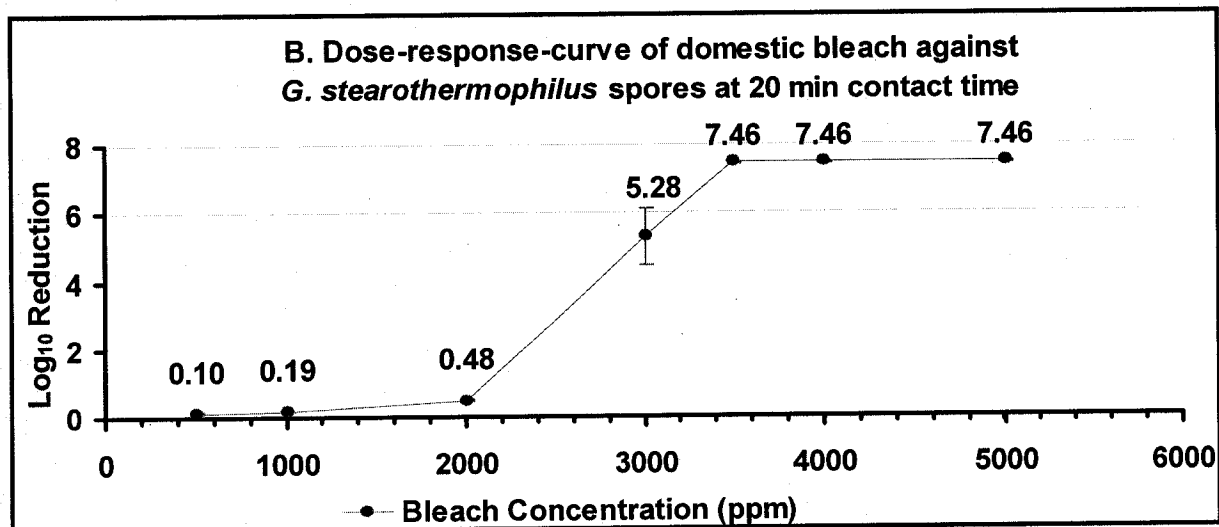
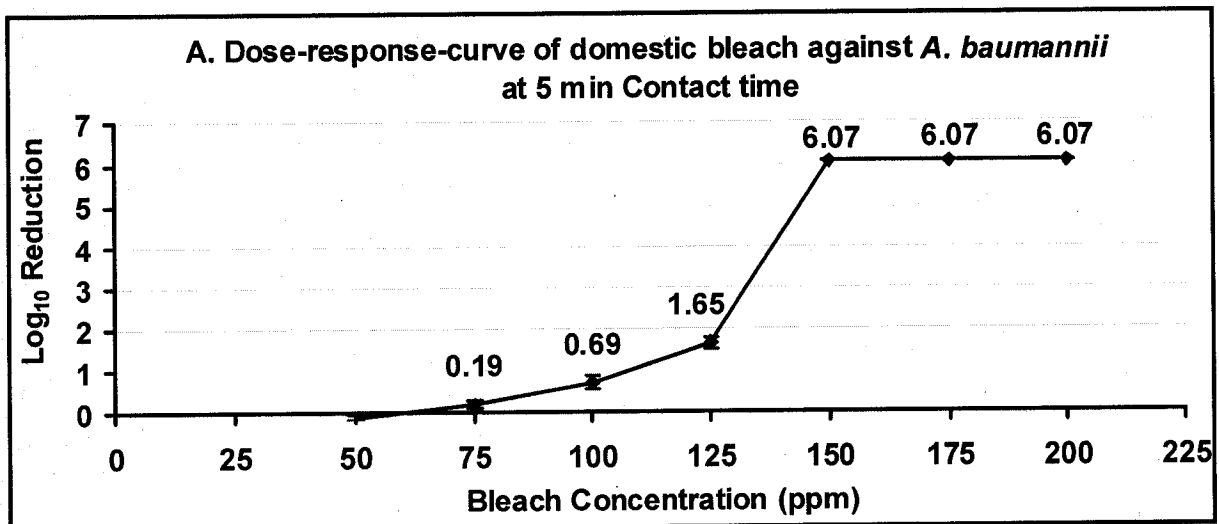


Figure 2.11

Figure 2.12 Effect of testing at a lower temperature (4°C) on the microbicidal activities of products, which achieved a total kill on *G. stearothermophilus* spores (2.12.A) and on HAV (2.12.B) at RT. Figure 2.12.C compares the log₁₀ reductions achieved by ClO₂ on HAV and on spores

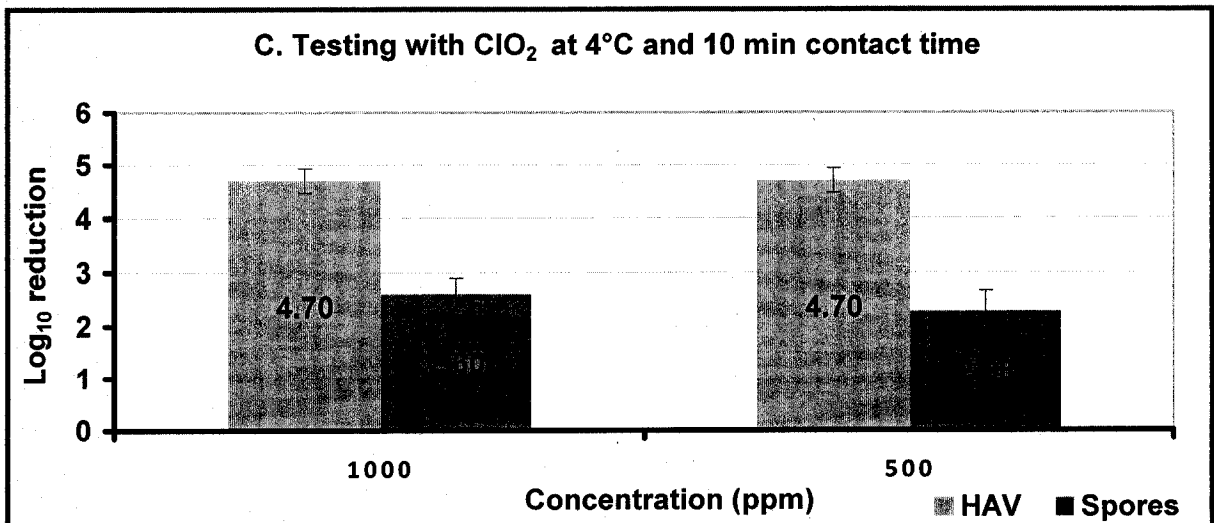
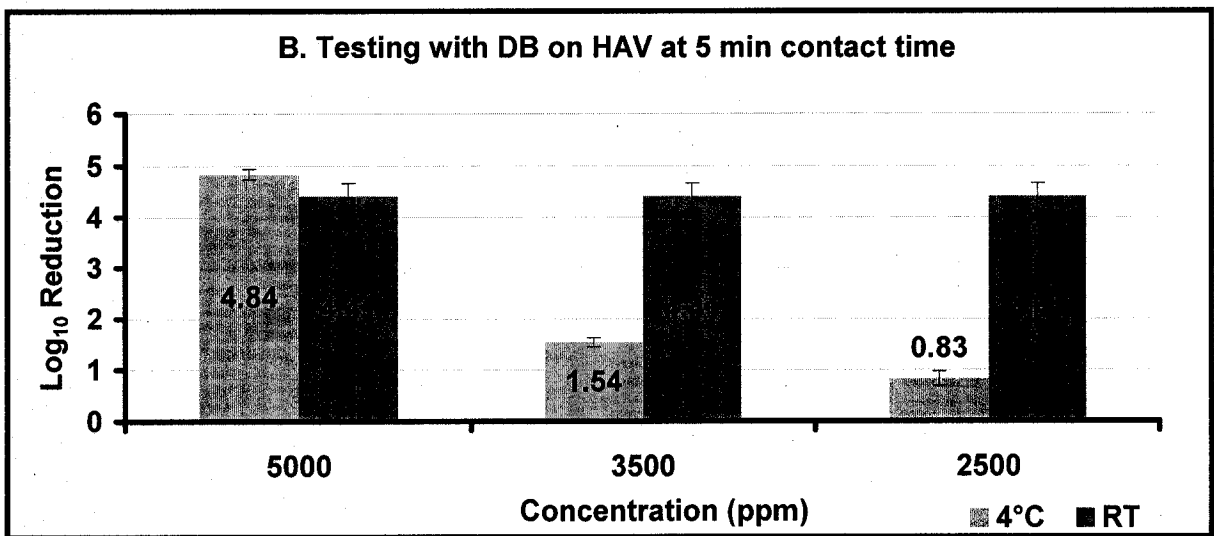
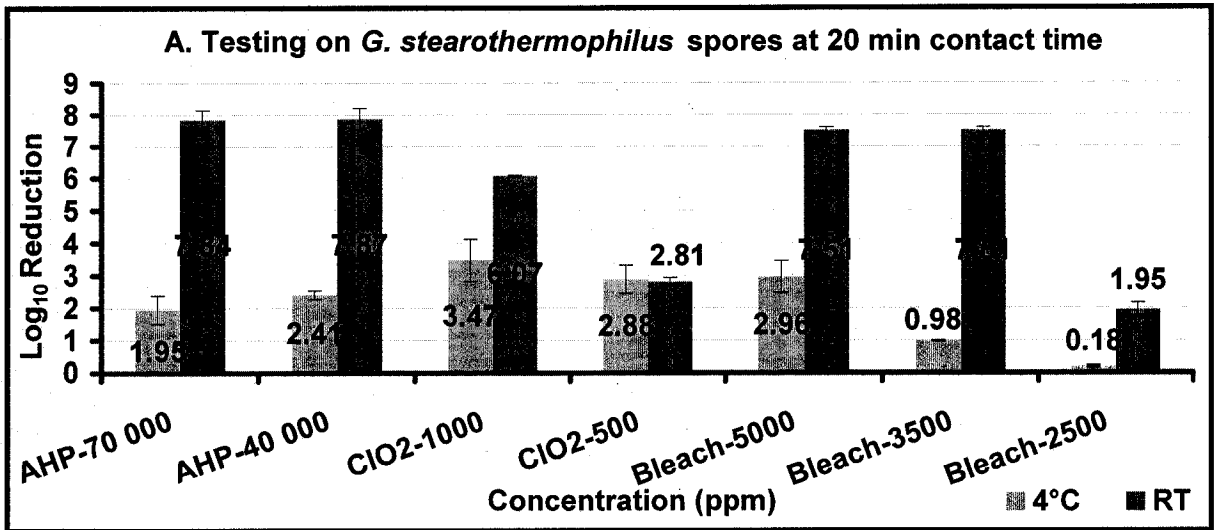


Figure 2.12

2.4 DISCUSSION

The QCT-2 test method used here was developed at CREM for testing the activities of chemicals against bacterial spores, mycobacteria, vegetative bacteria, viruses and fungi in the presence of a standardized soil load on metal disks representing environmental surfaces and medical devices (Majcher *et al.*, 2008; Springthorpe and Sattar, 2003, 2005; Sattar *et al.*, 2003). The protocol itself is simple, easy to follow, fully quantitative (Sattar *et al.*, 2003), and it has proven to be suitable for working with several organisms when tested individually or in a mixture (Best *et al.*, 1994), under different conditions and with a variety of chemical disinfectants (Springthorpe and Sattar, 2005).

Two major classes of liquid disinfectants were evaluated in this study:

(1) The peroxygens - PAA and selected commercially-available formulations based on AHP; the AHP-based products were selected because they contain not only stabilized H_2O_2 but also organic acids, detergents and anti-corrosives to speed up the activity of the H_2O_2 while making them more compatible with a wider variety of materials (Omidbakhsh and Sattar, 2006); and

(2) The two chlorine-based formulations were a 5.25% solution of sodium hypochlorite (domestic bleach) and chlorine dioxide tablets (Exterm-1.5).

AHPs act as oxidants by producing hydroxyl free radicals (HO^\bullet), which attack essential cell components including proteins, lipids and DNA with exposed sulfhydryl groups and double bonds being particular targets (McDonnell and Russell, 1999). PAA is considered a more potent biocide than AHP and unlike H_2O_2 , it is free from decomposition by peroxidases. PAA decomposes to safe byproducts (acetic acid and oxygen) and remains active in presence of organic loads. Its main application is as a low-temperature liquid sterilant for medical devices, flexible endoscopes and hemodialyzers. PAA, like H_2O_2 ,

denatures proteins and enzymes and increases cell wall permeability by disrupting sulfhydryl and sulfur bonds (McDonnell and Russell, 1999).

Sodium hypochlorite is the most common type of chlorine-based disinfectant for environmental decontamination. In water, it ionizes to produce Na^+ and ClO^- (hypochlorite ion), which establishes equilibrium with HOCl (hypochlorous acid). Between pH 4 and 7 chlorine exists predominantly as HOCl , and above pH 9, ClO^- predominates. HOCl , long considered the active moiety responsible for bacterial inactivation, disrupts oxidative phosphorylation. It also causes oxidation of sulfhydryl groups and amino acids, ring chlorination of amino acids, loss of intracellular contents, inhibition of protein synthesis, and depresses DNA synthesis. Chlorine dioxide is another important CRA; it inhibits protein synthesis (McDonnell and Russell, 1999; Weber and Rutala, 2006). Exterm is a relatively new formulation of sodium chlorite (NaClO_2) in its dried state. When dissolved in water, it produces an acidic solution, which breaks down NaClO_2 rapidly to produce chlorine dioxide. ClO_2 gas is readily soluble in water and lasts at most a few days, and then breaks down into chlorites and chlorates (www.clordisys.com).

On any unit area of an environmental surface it is quite normal, although not apparent, to have surface soiling far greater than the mass of microbial contamination. Such soil, which is usually a mixture of organic and inorganic substances, may not only enhance the survival of microbes, but also shield them from the deleterious effects of physical and chemical agents. Further, and of particular relevance to chemical disinfectants, various components of the soil can create a demand on, and consume the active before it could accomplish the task of achieving the desired degree of microbial decontamination (Springthorpe and Sattar, 2005; Selkon *et al.*, 1999; Rutala and Weber, 2001). The level of soil on uncleaned surfaces may vary widely. However, routine cleaning of surfaces does not rid them of all the soil and such

residual amounts of soil may still be high enough to compromise proper decontamination (Springthorpe and Sattar, 2005), especially when dealing with infectious bioagents. Therefore, it was essential to incorporate a certain level of soil in the testing to better simulate field conditions.

Animal serum at a final concentration of about 5% is normally used in testing the microbicidal activities of chemicals (ASTM, 1989; CGSB, 1991), but sera used in such testing are relatively expensive and may also contain specific or non-specific inhibitors for test organisms (Best, 1994). Additionally, the quality of sera may vary from lot to lot. In view of these constraints, this study relied on the following three types of proteins available readily in a relatively pure form (Springthorpe and Sattar, 2003): i) *mucin* (to represent mucilaginous substances common to many body fluids); ii) *albumin* (to represent proteins of a relatively large size also common in body fluids), and iii) *yeast extract* to simulate peptides which can readily react with and negatively affect the microbicidal activity of many types of disinfectants.

As described in Appendix II, stock solutions of the proteins were prepared in NBS (pH 7.2) to incorporate an inorganic load, and the final protein in the soil load was about 5%, which is roughly equal to that in 5% serum (Best, 1994). More importantly, the soil load was found to be compatible with all the organisms tested in this study. Another change to the QCT-2 method in this study included the use of NBS as diluent, eluent and for washings instead of unbuffered normal saline, and the use of 300 ppm as the desired level of water hardness as a diluent for test products instead of the original 400 ppm, a concentration recommended by the EPA in 1993 (Springthorpe and Sattar, 2003). The first change was made after noticing that the pH of normal saline varied considerably and was also hard to adjust to the desired level of about pH 7.2. Using the NBS allowed us to have a standard pH

of 7.2 ± 0.2 throughout the study, which eliminated any effect of pH variations on the survival and recovery of the organisms. The second change was introduced as a result of recent attempts at the harmonization of the disinfectant test methodology around the globe (OECD: http://www.oecd.org/department/0,3355,en_2649_32159259_1_1_1_1_1,00.html).

A very crucial requirement in microbicidal testing is the validation of the neutralization step to quench the antimicrobial activity of the test formulation immediately at the end of the contact time (ASTM E1054; 1991). In this study, nearly all commonly available neutralizers were tried but with limited success in that they either did not work effectively, proved toxic to the cell cultures and/or interfered with plaque formation by HAV. This presented us with a considerable challenge and thus required much initial work to come up with a suitable neutralizer and validate its activity against all actives and microbes used here. DEBm, the formulation we developed for use in this study, is a modified form of the DE broth (Sutton *et al.*, 2002), we removed ingredients that were no longer useful and altered the concentration of other compounds for better activity and wider compatibility. It should be noted that this is a significant achievement towards the development of a universal neutralizer, a goal that has eluded many thus far (Espigares, *et al.*, 2003; Traoré *et al.*, 2002, Dey and Engley, 1983).

Here we would like to report two findings we experienced while working with DEBm in this study. Firstly, the DEBm was able to quench the activity of the 4% and 7% formulations of AHPs and showed no cytotoxicity to the FRhk-4 monolayers when used in the usual 1:200 ratio (as disinfectant to neutralizer) set for QCT-2. However, when used in a 1:20 ratio to determine effectiveness of the tested formulations at a lower dilution, the cell monolayers incubated with the lowest dilution of the virus became unstable, especially with the 4% AHP. Secondly, the original recipe of DEB from Sutton *et al.* (2002) made the cell monolayer unstable even at a 1:200 dilution and with a strong interference with plaque formation. In

fact, we could not see any plaques at all; this strong interference was traced back to the use of a higher percentage of Tween-80 in the original recipe for DEB.

Decontamination of dried inocula on surfaces is much more challenging than when the microbes are in suspension. In this study, the microbes suspended in the soil load were dried for 1 h on the carrier disks. The effect of such drying was evaluated for each of the four organisms used here alone and in the mixture (Figures 2.6 and 2.7). All the surrogates retained sufficiently high titers after the drying time, with the highest loss noted with *A. baumannii*.

The development and application of a mixture of four suitable and representative surrogates for infectious bioagents makes this study rather unique, because it allowed for a standardized and simultaneous assessment of the microbicidal activities of the test formulations under identical conditions. The actual test conditions also incorporated several levels of stringency. For example, the carriers had a relatively uneven surface to better 'hide' the targets. As well, the volume of the disinfectant applied on each disk was considerably smaller per unit surface area than what is normally used in many standard tests for microbicidal activity, and the soil load incorporated exerted a greater demand on the active to better simulate field conditions. Moreover, target organisms in real-life situations may be unknown and may also be found as mixtures rather than as mono-specific contaminants.

A suitable low-risk surrogate for biothreat agents that could be used under less stringent conditions can greatly advance biological restoration science. Typically spores of surrogate indicator species are used as biological indicators. However, a species that is more resistant than spores to remediation could be used to add a margin of safety to the remediation process (e.g., if a species or a pathogen that is more resistant to chemicals than the anthrax spores is

killed by the treatment, then there is a greater insurance that the anthrax spores will be killed by that treatment) (Rose *et al.*, 2004; Utrup and Frey, 2004; Simpson, 2005, Nola, 2003).

The surrogates chosen for inclusion in the microbial mixture covered the spectrum of microbicide resistance of all infectious bioagents known currently. The findings reported here would also permit the selection of a suitable disinfectant in case the target is already known or is strongly suspected to belong to a given microbial class (Sattar, 2007). However, even closely related organisms may exhibit differences in their susceptibility to disinfectants, and would require a more careful review of the situation before selecting a particular type and in-use concentration of the decontaminating agent. In addition, microorganisms grown on different media are perceived to have differences in their susceptibility to microbicides (Springthorpe and Sattar, 2005). Therefore, we used ATCC strains and standardized growth media and methods in order to eliminate any variations in our testing and to make it easy for any possible comparison with future studies.

Caution is also recommended against a blanket acceptance of the hierarchy of microbial resistance to disinfectants (Weber and Rutala, 2006; McDonnell and Russell, 1999). The traditional ranking of microbial classes classifies microorganisms starting from the least to the most susceptible as follow: prions, coccidia, spores, mycobacteria, cysts, small non-enveloped viruses, Gram-negative bacteria (non-sporulating), fungi, large non-enveloped viruses, Gram-positive bacteria, and enveloped viruses. This ranking is based on resistance to microbicides from results of studies with individual types of organisms using limited types of chemicals/formulations and different test protocols (Weber and Rutala, 2006; McDonnell and Russell, 1999). Therefore, such ranking of susceptibility/resistance of various classes of pathogens must be regarded only as rough guides with the prospect of successful decontamination being dependent on specific field conditions and the actual decontamination

process applied. In the case of infectious bioagents, one must err on the side of caution for a greater degree of confidence for success. Further confirmation of the findings of this study with representative CL-3 agents is highly recommended for an even greater confidence in any remedial process required.

The recovery of infectious HAV in the absence of any viable spores is somewhat surprising. While further work would be necessary to properly explain this anomaly, we do not believe that HAV *per se* has a higher level of disinfectant resistance than bacterial spores. It is quite conceivable that in our testing, the relatively small particle size of HAV afforded it greater physical protection in the dried inoculum. Based on the traditional rankings, one would assume that products such PAA and AHP (4-7%) that were sporicidal at 5 and 20 min, respectively, would work on HAV. McDonnell and Russell (1999) expected hydrogen peroxide, which reacts strongly with amino acids, to possess virucidal activity by acting on capsid proteins. But this was obviously not the case here. Another finding of this study was that the *Mycobacterium* proved to be more susceptible to all the different classes of microbicides tested as compared to HAV. This proves to another weakness in the traditional rankings of disinfectant susceptibility where mycobacteria are considered more resistant than non-enveloped viruses.

Interestingly, the mycobacterium *M. terrae* and the Gram-negative bacterium *A. baumannii* proved to be very close in their susceptibility to the disinfectants tested. In our initial testing on the mixture *A. baumannii*, a metabolically versatile pathogen, could grow on TSA plates as well as on 7H11 agar. Therefore, to successfully separate the components of the mixture and not to allow the growth of *A. baumannii* on the 7H11 plates, we used *M. terrae* (pBEN). The latter is *M. terrae* (ATCC 15755) with the plasmid pBEN containing a kanamycin-resistance gene that allowed the transformed bacterium to grow on 7H11 agar

with up to 50 µg/mL of the antibiotic. It should be noted here that the disinfectant resistance/susceptibility profile of *M. terrae* and the transformed *M. terrae* (pBEN) proved to be similar. The transformed mycobacterium also grew faster than its parent when inoculated onto plates of 7H11 agar with kanamycin than on those without it; readily visible colonies were produced in only 5-6 days (Appendix V).

Three formulations each with 0.5% AHP were evaluated in this study and two of them had slightly different effectiveness towards vegetative bacteria and mycobacteria. Even though they had the same sensitivities to antibiotics, *M. terrae* proved to be more resistant to two AHPs (0.5%) than *A. baumannii*. While Virox 5 and Accel TB-Can were both bactericidal at 5 min of contact time against *A. baumannii*, they were barely effective against *M. terrae* (pBEN) even at 20 min contact time (Table 2.4). This difference in sensitivity was indicative of the presence of different mechanisms of resistance against antibiotics and microbicides, although these mechanisms might be similar and have an effect on each other.

Similarly for the two 4-7% AHP products, one product (Alpha HP) was more effective than the other (Accel CS20) even at a 5 min of contact against the spores. The former was sporicidal at 10 min of contact, while the latter took an additional 10 min to achieve the same level of kill. Note that the pH of Alpha HP was more acidic than that of Accel CS20. In a recent study that used QCT-2 to assess the sporicidal activities of chemicals, a 7% AHP product showed similar sporicidal activity at 5, 10 and 20 min contact times to our data, although spores of different *Bacillus* spp. were used as a challenge (Majcher *et al.*, 2008). The 2% AHP proved to be bactericidal and mycobactericidal; however, it worked very poorly on the spores and the virus. In fact, our data showed that it was as ineffective as the 0.5% products towards the spores and HAV. From that point, one cannot assume that products with the same formulation and the same (or higher) percentage of actives would

have the same effectiveness, and thus it is very important to evaluate each product individually. These differences in the activity for products with the same types and levels of the listed actives on the labels could well be due the differences in the unlisted 'inerts' or excipients in them (Springthorpe and Sattar, 2003). Inerts are not required to be listed by regulatory agencies, nor are their details readily available due to trade secrets.

Although the concentrations of PAA used in this study were lower than those used earlier (Jones *et al.*, 1967; Majcher *et al.* 2008), at 1,000 ppm, it achieved a total kill at 5 min against all the surrogates in the mixture, including the spores; even 500 ppm proved to be sporicidal at a contact time of 20 min.

The dose-response curves of domestic bleach against *A. baumannii*, as well as the spores, followed an expected sigmoidal pattern. At the lower concentrations of the disinfectant there was no detectable kill. As the disinfectant concentration was increased, while holding the contact time and other experimental conditions constant, the level of kill gradually increased until the extent of inactivation plateaued.

For *A. baumannii*, the difference between the exposure concentration that failed totally in the specified contact time and the one that always succeeded was very narrow and the slope of the curve quite steep (see Figure 2.11). Therefore, any dilution errors, slight change in the formulation of the product, surface or soil interaction effects could be risky and cause performance to fall to the edge, particularly under field situations. In contrast, for the spores, even though the same formulations were used with much higher concentrations, the difference between the concentrations in the plateau to those at the other end of the curve was large, and the slope was not as steep as the one for the *A. baumannii* curve. Therefore, choosing a use concentration well into the plateau (>4,000 ppm DB) would not be compromised by any of the effects described above.

A very good example for a compromised concentration effectiveness was the 3,000 ppm DB concentration which was very well onto the linear portion of the curve, the SD at this concentration was very wide compared to the other concentrations; the kill at this point sometimes reached $\geq 6 \log_{10}$, and at other times dropped dramatically to $< 4.5 \log_{10}$. The use-concentration should be well onto the plateau of the concentration-kill curve (> 175 ppm). Then, the shape of the concentration-kill curve did not depend only on the disinfectant formulation but also on the challenged organism. Failure to sterilize critical items and adhere to guidelines for high-level disinfections of semi-critical items have led to serious infections like outbreaks of nosocomial infections due to contaminated prosthetic heart valves, intraocular lenses, bronchoscopes and endoscopes (Weber and Rutala, 2006; McDonnell and Russell, 1999).

These findings reinforce the importance of not only selecting the right concentration of the active, but also applying it properly to achieve the desired level of microbial kill. This is especially important when dealing with infectious bioagents. It should also be noted here that most commercial microbicides for environmental surface disinfection may contain the lowest levels of actives required to pass the registration process and to compete economically in the market. This, together with the recognized deficiencies in microbicide test protocols, requires greater caution in the selection and use of microbicides when considering remediation of contaminated sites.

Results from the study of Rogers *et al.* (2005) indicate that *G. stearothermophilus* spores are more significantly resistant to being killed by hydrogen peroxide gas as compared with *B. subtilis* and *B. anthracis* spores. Another study also reported that *G. stearothermophilus* spores were more resistant to being killed by H_2O_2 gas as compared to *B. subtilis* spores

(Klapes and Vesley, 1990). Spore-production methods, including growth medium and heat treatment have been shown to influence spore resistance (Rogers *et al.*, 2005; Palop *et al.*, 1999; Cazemier *et al.*, 2001; Melly *et al.*, 2002). Our findings on the sporicidal activities of the formulations tested were in general agreement with those of Majcher *et al.* (2008) who also used the QCT-2 test method. However, some differences were also noted. For example, in our tests, 5,000 ppm DB achieved only a $\sim 3 \log_{10}$ reduction in the spore titre at 10 min of contact while the other study reported a $>6 \log_{10}$ reduction at the same contact time. In addition, we used Exterm-1.5 tablets dissolved in 125 mL of dd H₂O to yield 1,000 ppm of ClO₂ (1,000 ppm); in the other study the volume of water to dissolve the tablets was not clearly stated. However, in both studies, ClO₂ showed limited sporicidal activity as it achieved $\geq 6 \log_{10}$ only at 20 min of contact. Our data showed lower \log_{10} reductions after 5 and 10 min of contact as compared to those obtained by Majcher *et al.*, (2008). While the exact reasons for these differences remain unknown at this stage, a possible explanation may be the differences in the neutralizers used.

As a general rule, the microbicidal potency of chemicals is directly proportional to ambient temperature (Springthorpe and Sattar, 2003). Therefore, the findings of test data from experiments at RT ($22 \pm 2^\circ\text{C}$) may not be valid for decontamination of sites at low temperatures, with the additional practical difficulty of keeping the formulations from freezing under sub-zero condition. Thus far, this aspect of environmental decontamination has not received adequate attention. The very limited work that has been carried out has focused on oxidative chemicals where ethylene glycol was added as an anti-freeze (Jones *et al.*, 1967; Jones *et al.*, 1968).

As anticipated, our testing also showed lower sporicidal activities of both CRAs and

AHPs at lower temperatures, with CRAs being less affected than the AHPs (Figure 2.12). While at RT both categories achieved $>6 \log_{10}$ reduction at 20 min of contact, 7 and 4% AHP products achieved \log_{10} reductions of only 1.95 ± 0.44 and 2.41 ± 0.13 , respectively, at 4°C ; for ClO_2 (1,000 ppm), ClO_2 (500 ppm) and DB (5,000 ppm) the reductions were 3.47 ± 0.65 , 2.88 ± 0.43 and 2.96 ± 0.49 , respectively.

Our findings are in agreement with previous studies regarding the effect of lower temperatures on the sporicidal activity of free available chlorine (Rice *et al.*, 2005; Rose *et al.*; 2005); in both of these studies *B. anthracis* (Ames) showed slightly higher resistance than the Sterne strain.

Even though the primary focus of this investigation was remediation of surfaces deliberately contaminated with infectious bioagents especially spores, its findings are highly relevant to dealing with decontamination in health-care facilities against nosocomial infections and food-handling establishments.

Using a mixture of surrogate organisms for assessing the microbicidal activity of two classes of disinfectants by the quantitative carrier proved to be very practical and useful. It helped identify many issues with regards to microbicidal testing in addition to clarifying many misconceptions around relative strength and sensitivity of microbes and microbicides.

Summing up, the findings summarized here fulfill the requirements of the first objective of this investigation. The protocols as applied and refined should be suitable to extend the work to CL-3 and possibly CL-4 agents to confirm that the results obtained with the carefully chosen surrogates are indeed applicable to various classes of infectious bioagents.

3. DECONTAMINATION WITH WIPING: WIPE/TRANSFER TESTS

This part of the study was performed to meet **the second and third objectives of this thesis**, and which were to assess the influence of wiping with a disinfectant-wetted fabric and to determine the **potential of the used fabric to transfer** viable organisms to clean areas during wiping.

3.1 INTRODUCTION

Although gaseous decontaminants are effective, it is not always possible/practical to use them. As mentioned before, after the anthrax incident in the U.S. in 2001, in one of the buildings, to kill the remaining spores thorough wiping of the areas with liquid chlorine dioxide solution was used after decontamination using gaseous chlorine dioxide. Thus, it is evident that wiping with liquid disinfectants will also still be needed in many cases, and information on the efficacy of wiping with a disinfectant-wetted fabric is a gap that needs to be filled.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of stock culture

In this part of the study our main target was the *G. stearothermophilus* spores. However, within the time frame of the project, we also assessed decontamination and transfer by wiping of *A. baumannii* and HAV, trying to obtain a better perception of what wiping and transferring could achieve against the most and the least resistant microbes.

The same stock cultures prepared for QCT-2 (Section 2.2.2 for *A. baumannii*, 2.2.4 for the spores and 2.2.7 for HAV) were used for the wipe/transfer test. Also, preparing inocula with the soil load, drying time and ambient conditions - RT (22-24°C) and RH (37-78%) - were the same as those described in Section 2.

3.2.2 Fabrics

Two types of fabrics were used. One was microfiber-based (blend of 80% polyester and 20% polyamide) and the other was 100% cotton. From each type of fabric, several circular pieces of 2.3 cm diameter were cut with a pair of scissors. Each fabric disk was used only once and discarded after decontamination by autoclaving.

3.2.3 Fabric Carriers

Custom-made tubular stainless steel holders and Teflon inserts, together with commercially-available rubber 'O' rings (Figure 3.1.A), were washed with 7X liquid detergent (MP Biomedicals, Inc., cat# 76-670-94), rinsed with deionized water and air dried (see Appendix II for more information on the custom-made parts). One Teflon insert and one stainless steel holder were put together and a circular piece of the test fabric was mounted over the mouth of the assembled holder and secured in place with an 'O' ring. The fabric carriers were placed in a glass Petri dish, wrapped with aluminium foil and sterilized by autoclaving at 120°C for 45 min and then dried for at least 4 h in a dryer. After each experiment, the fabric carriers were decontaminated by autoclaving, the fabric pieces discarded and their carriers cleaned in 7X detergent, rinsed with deionized water, and then air-dried before being used for the next experiment.

3.2.4 Wipe/transfer Test Method

In a laminar flow cabinet, each disk carrier was inoculated with 10 μ L of the test microbial suspension and the inoculum dried for 1 h. At the end of the drying time, using sterile forceps, two inoculated disks and two sterile clean disks were mounted on a sterile plastic Petri-dish that was placed over two magnets on a digital read-out top-loading balance (Mettler PC2000) (Figure 3.1.B). Each piece of fabric was wetted with 25-50 μ L of NBS or

the test formulation (Figure 3.1.C). The fabric-mounted carrier was picked up with a gloved hand and used to wipe an inoculated disk (donor disks) as shown in Figure 3.1.

The test formulations or their dilutions used in the wiping tests were chosen because they had already been determined to be ineffective against the target microorganism(s) in prior assessments in this study using QCT-2 (Section 2).

Figure 3.2 shows the main steps of the wipe/transfer method. Two types of motion were assessed for wiping and transfer: First, a diagonal wiping motion, where the donor disk (the carrier disk with the dry inoculum) was wiped diagonally for 10 s from top right to left 5 times, and from top left to right 5 times, while exerting a pressure of 50-100 g.cm⁻². The fabric carrier used for the wiping was immediately brought in contact for 10 s with a clean, sterile disk (recipient disk) while exerting the same pressure as that in the wiping test. In the circular wiping motion, the wiping consisted of four circular moves in 5 s and then immediate transfer to a clean disk using the same type of motion and contact time. The donor and recipient disks were eluted separately in NBS with vortexing for 20 s. Ten-fold serial dilutions were prepared in NBS; the dilutions were membrane-filtered when working with bacteria and plaque-assayed when working with the virus as described for QCT-2, using the same recovery methods and growth conditions.

With each wipe test, a QCT-2 experiment was run in parallel to better assess and compare the influence of wiping on decontamination. The QCT-2 experiments in this part of the study were done as described before in Section 2.2.10. NBS was the control solution. Counts obtained from the controls in the QCT-2 experiments represented the initial inoculum recovered from the stainless steel disk surface. For QCT-2 and the wipe test experiments, we calculated, using a standard spreadsheet, the average CFU/PFU recovered from the disks. Results obtained from the control disks and donor disks after wiping were used to calculate

the average \log_{10} reduction achieved by wiping as follows:

Average \log_{10} reduction by wiping =

Average \log_{10} (CFU/PFU recovered by QCT-2 \div CFU/PFU recovered from donor disks)

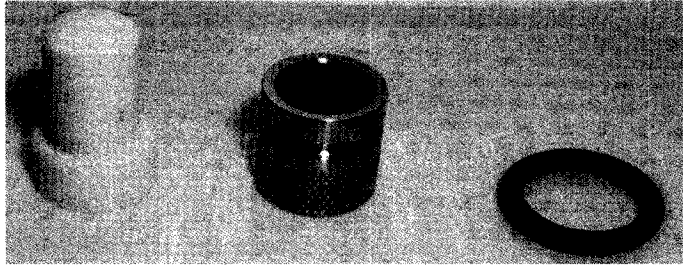
The percentage of transfer was calculated by relating the CFU/PFU recovered on the recipient disk to that recovered from the control disks in the QCT (referred to as the initial inoculum) as follows:

Average % transfer =

[CFU/PFU recovered from recipient disk \div CFU/PFU recovered by QCT-2] x 100

Figure 3.1 Wipe/transfer method equipments

- A Custom-made tubular stainless steel holder and Teflon insert and commercially-available rubber 'O' ring.
- B Two inoculated disks and two sterile, clean disks were mounted on a sterile plastic Petri-dish that was placed over two magnets on a digital read-out top loading balance (Mettler PC2000). A fabric carrier held by fingers was used for wiping and transferring.
- C Microfiber fabric (1) and cotton fabric (2) were wetted with 25 μ L of a crystal violet solution to show how the disinfectant would soak onto the two types of fabric, i.e., microfiber and cotton.



A



B



C

(1)

(2)

Figure 3.1

Figure 3.2 Main steps in the wipe/transfer test

From left to right

- Inoculum dried onto donor disk (1)
- Donor disk and sterile recipient disk placed on digital balance inside a laminar flow hood (shown more clearly in Figure 3.1.B)
- Fabric wetted with test product (2) (Note that in the figure the fabric was wetted with dye to show how a liquid product would soak onto the fabric).

With standard friction and pressure

- Donor disk wiped with sterile mounted fabric carrier wetted with test product (shown better in Figure 3.1)
- Immediately transferred fabric carrier to contact sterile recipient disk (3)
- Transferred carrier disks to separate Teflon vials for elution (4)

Eluent and washings of samples captured on filters (5, 6 and 7). Filters placed on the appropriate agar (8), and incubated at the right temperature and for the length of time suitable for each microorganism as described above in Table 2.2 (the results section of QCT-2 method). Note that steps 1 and 4 to 8 were performed exactly as described in the QCT-2 method (please refer to Figure 2.3 in Section 2.2.10.)

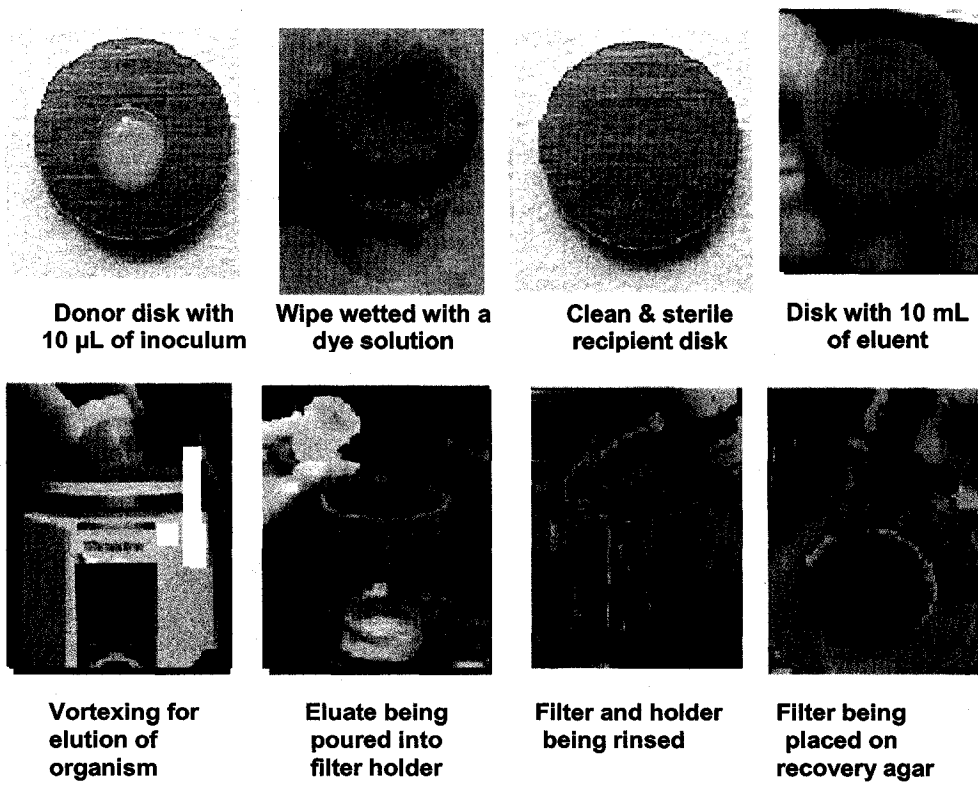


Figure 3.2

3.3 RESULTS

Each experiment was repeated at least three times. The relative humidity (RH) and room temperature (RT) in the lab were measured on each day of the experiment using a hygrometer and recorded. These parameters remained essentially unchanged on a given day. However, over the course of this study, the RH varied between 38 and 60% and the air temperature between 22 and 24°C. All the results from the wipe/transfer tests are summarized in Table 3.1.

3.3.1 Testing with *A. baumannii*

A freshly prepared pool ($2-4 \times 10^9$ CFU/mL) was used for each experiment. When preparing the inoculum in the soil load, the suspension was diluted with dd H₂O to achieve a titre of $\sim 10^7$ CFU/mL on the carrier disk after the inoculum had dried. Microfiber fabric was used to assess decontamination (as log₁₀ reduction - Figure 3.3.A) by diagonal wiping method. Fabrics were wetted with 50 µL of either 50 ppm or 100 ppm of DB, with NBS used as the control solution. These concentrations of the bleach were chosen on the basis of the data from the dose-response curve obtained in the QCT-2 experiments (Figure 2.11.A) where 50 ppm and 100 ppm achieved average reductions of 0 and 0.69 log₁₀, respectively. However, wiping achieved average log₁₀ reductions of 3.52, 4.60 and 4.26 for wipes wetted with NBS, 50 and 100 ppm of DB, respectively, and with respective average percentages of transfer of 1.26×10^{-1} , 5.7×10^{-3} and 4.6×10^{-3} of the initial inoculum.

In another set of experiments, 25 µL of the test solutions were used to wet the fabric. In these trials, we applied a circular motion both for wiping the donor disk and also for transfer of the contaminant(s) to a clean surface (the sterile recipient disk), simulating use in practice when applying decontamination by wiping. In this instance, wiping achieved average log₁₀ reduction of 4.18, 5.61 and 4.57 for the microfiber fabric wetted with NBS, 50 ppm DB and

100 ppm DB, respectively. The percentages of transfer from the same fabric samples were 1.47×10^{-3} , 2.5×10^{-4} and 4.86×10^{-6} , respectively of the initial inoculum.

3.3.2 Testing with *G. stearothermophilus* spores

For the spores, 500 ppm of DB and 5,000 ppm of Virox-5 were used to wet the fabric pieces, based on the dose-response curve (Figure 2.11.B) and QCT-2 results (Table 2.4): both products achieved almost zero \log_{10} reductions on the spores. NBS was used as the control fluid. One set of experiments was performed with diagonal wiping with microfiber fabric, and three sets of experiments were conducted with a circular motion. Data obtained from the diagonal wiping are discussed in section 3.3.2.1 below.

With the circular motion, in the first set of experiments (Figure 3.4.A), wetted microfiber fabrics were used to wipe donor disks carriers for 5 s and immediately transferred to wipe the sterile recipient disks for 5 s. In the second set of experiments (Figure 3.4.B), a modification was used inspired by the decontamination case of anthrax where gaseous chlorine dioxide was used followed by wiping with liquid CD (see Sections 3.1 and 1.3.2.1). Here, aliquots (25 μL) of the test solution/control were deposited over the dried inoculum on the donor disks, and then allowed to dry completely (~ 45-55 min) under ambient conditions. These disks were then wiped with a microfiber fabric wetted with 25 μL of the same test or control solution for 5 s as described before for the first set, and immediately after, the fabric carriers were transferred to wipe the sterile recipient disks for 5 s. In a third set of experiments (Figure 3.4.C), to investigate the effect of fabric type on decontamination and transfer of microbes, a cotton fabric was used instead of the microfiber.

Figure 3.3 Wipe/Transfer test results as average CFU recovered/carrier for *A. baumannii* using the microfiber fabric and domestic bleach (50 ppm and 100 ppm). The exerted pressure was 50-100 g cm⁻². Results from parallel QCT-2 experiments are also included to determine the initial CFU recovered from the disks and which we considered as the initial inoculum recovered after drying

- A. Wiping the donor disk with the dried inoculum on with the microfiber fabric wetted with 50 μL of DB for 10 s using a diagonal motion method and immediately transferring the fabric to contact the sterile recipient disk for 10 s.
- B. Wiping the donor disk with a circular motion for 5 s with the microfiber fabric wetted with 25 μL of DB and immediately transferring the fabric to wipe by a circular motion to the sterile recipient disk for 5 s.

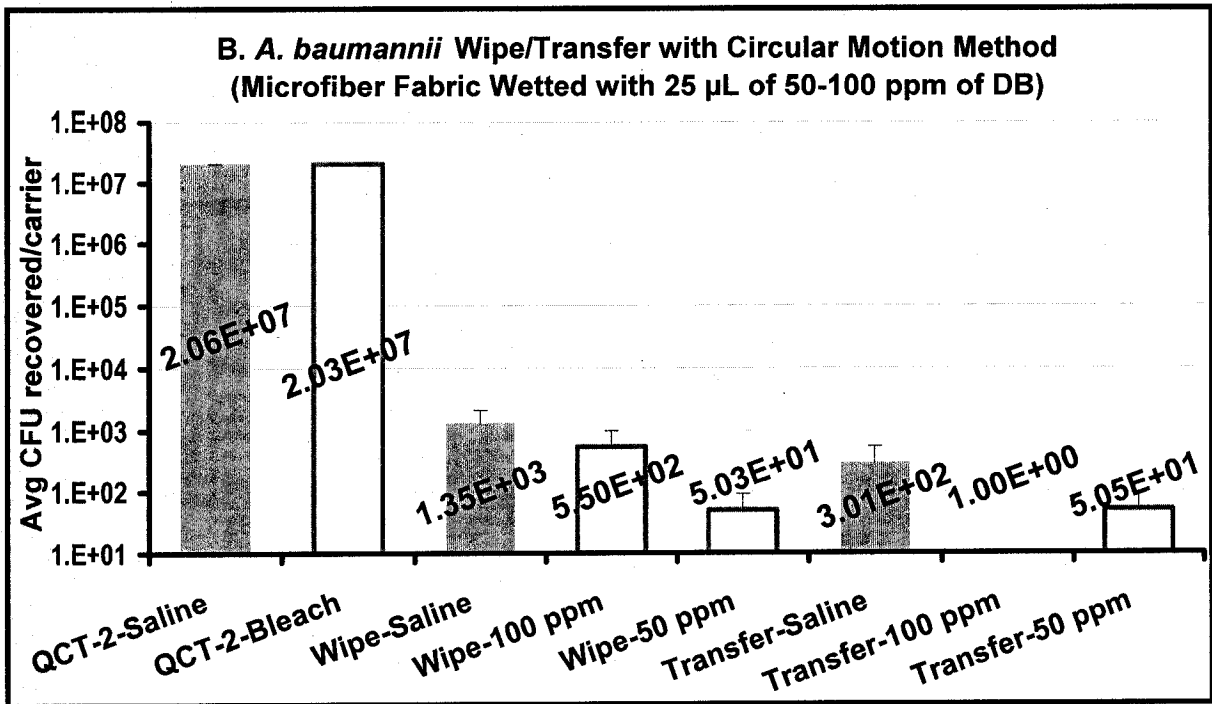
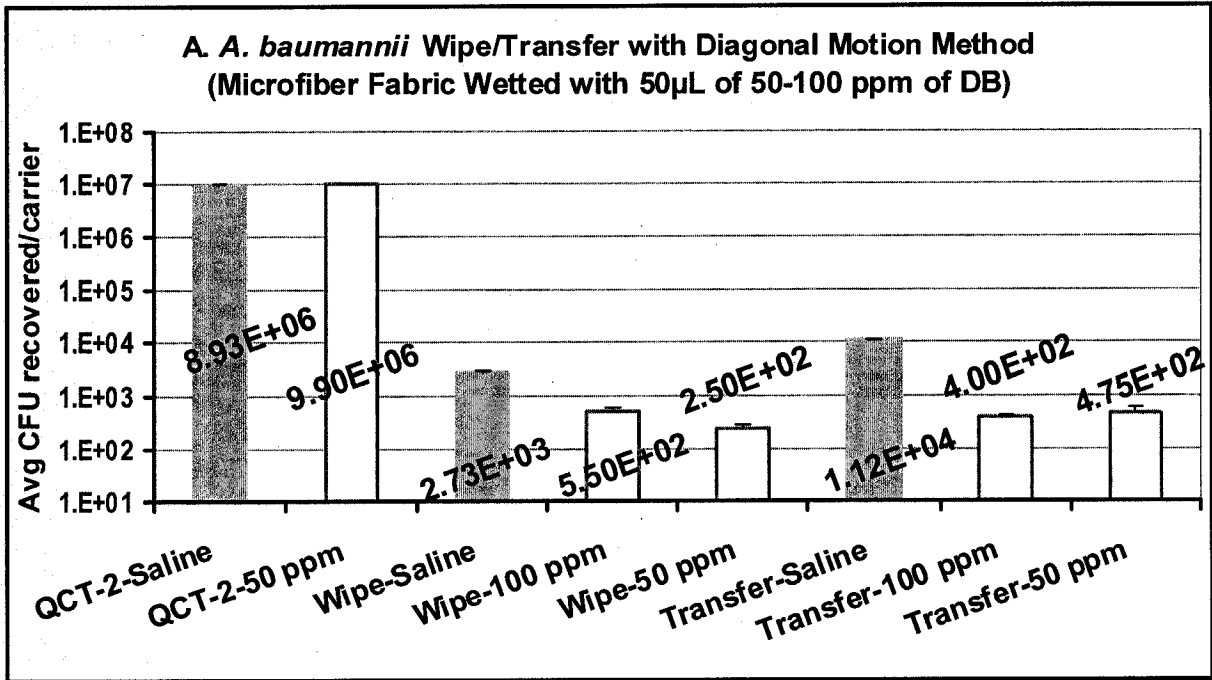


Figure 3.3

3.3.2.1 Effect of Type of Motion Used for Wiping on Transfer of the Spores

For the diagonal wiping method as compared to circular wiping method using the microfiber fabric wetted with 25 μ L of 500 ppm DB, the average \log_{10} reductions achieved by wiping the donor disks were 1.62 and 2.46 for control and test, respectively, for diagonal wiping as compared to 1.51 and 1.50 for the circular wiping. The average percentage of transfer by diagonal wiping method (transfer by contact) was 0.58 and 0.46 as compared to 0.58 and 0.37 by circular motion for controls and 500 ppm DB, respectively.

3.3.2.2 Effect of Drying the Formulation on the Carriers before Wiping

The percentage of transfer with a circular motion was 0.58, 0.37, and 0.51 for NBS, 500 ppm DB, and Virox-5, respectively, with average \log_{10} reductions of 1.51, 1.50, and 1.53, respectively. However, when 25 μ L of the test fluid was dried on the carriers before wiping the average percentage of transfer was reduced to 0.25, 0.07, and 0.001 for NBS, 500 ppm DB and Virox 5, respectively, and with the respective average \log_{10} reduction of 2.18, 2.48, and 3.87.

These data clearly show that drying the formulation directly on the inoculum and then wiping helped to reduce transfer of the contamination by 5.29 times for the bleach and 510 times for the AHP product, while achieving a higher log reduction when wiping the donor disks.

3.3.2.3 Effect of the Type of Fabrics Used for Wiping

When a cotton fabric was used for wiping, the average percentage of transfer was 0.34, 0.36 and 0.38 for NBS, DB and Virox 5, with average \log_{10} reductions of 1.54, 1.7 and 1.24 respectively. There was no large difference in transfer results or \log_{10} reduction results when compared to those with microfiber fabrics.

3.3.3 Testing with Hepatitis A virus

Knowing from the QCT-2 results that HAV and the spores were similar in sensitivity to microbicides, the same concentrations of test formulations used for testing spores were used for HAV wipe/transfer tests with the microfiber fabric applying a circular motion (Figure 3.5). The \log_{10} reductions achieved were 1.72, 2.05 and 2.17 for wiping with the microfiber fabric wetted with NBS (control), 500 ppm DB, and Virox-5, respectively, and with the percentage of transfer being 0.5, 0.42 and 0.28, respectively. These results were similar to those of the spores under the same experimental conditions. However, the percentage of HAV transferred when using Virox-5 was clearly less than that of the spores.

Figure 3.4 Wipe/transfer test results for *G. stearothersophilus* spores with the circular motion method and fabrics wetted with 25 μ L of tests (domestic bleach 500 ppm and the 5,000 ppm AHP Virox-5) or NBS for controls

- A. Testing with the microfiber fabric
- B. Testing the effect of applying and drying first 25 μ L of the test or control on the dried inocula combined with wiping using wetted microfiber fabric with the same solutions.
- C. Testing with the cotton fabric

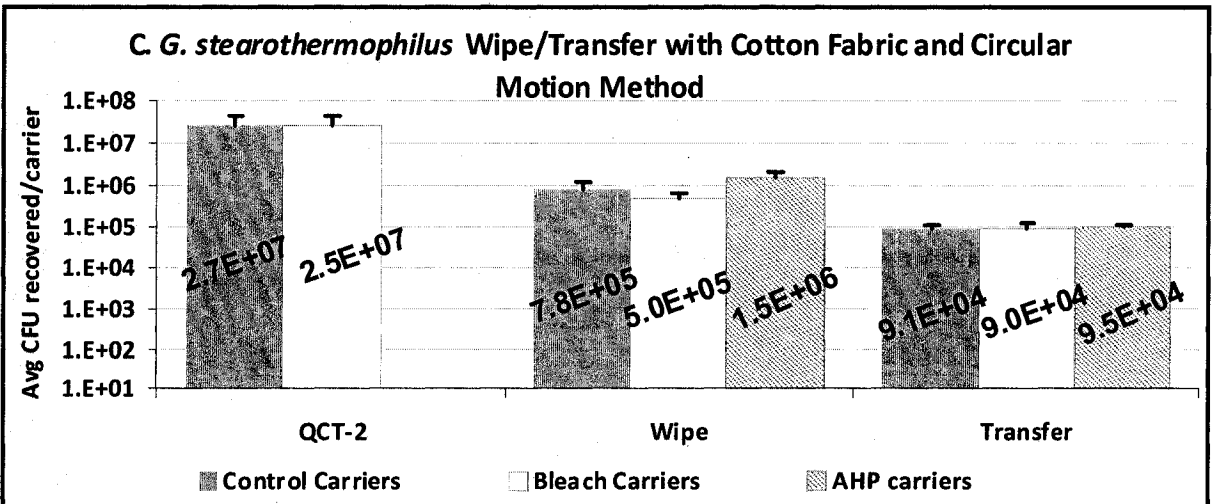
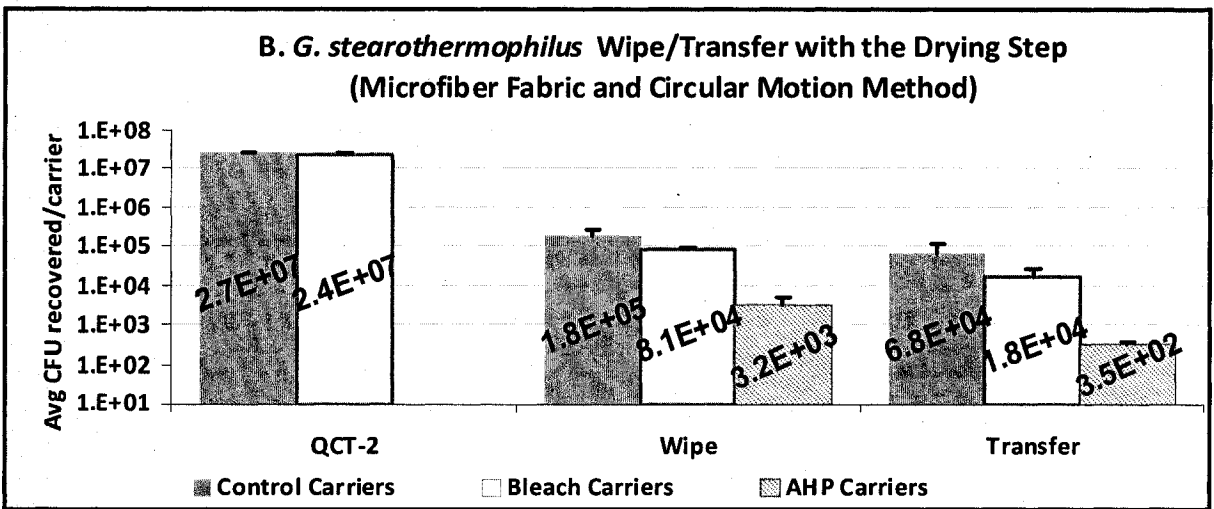
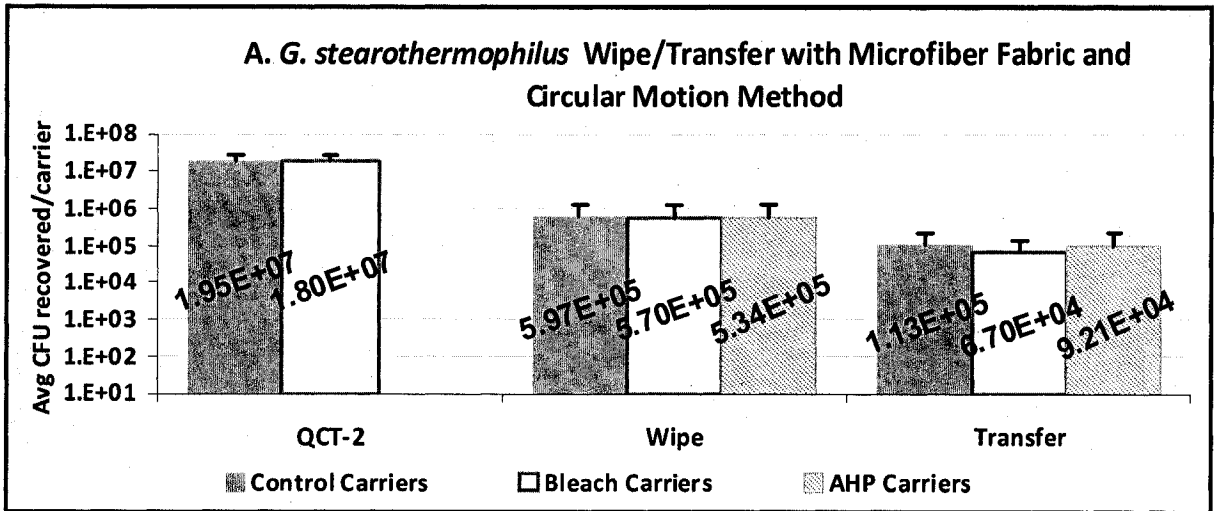


Figure 3.4

Figure 3.5 Wipe/transfer test results for HAV with the circular motion method using the microfiber fabric wetted with 25 μ L of domestic bleach (500 ppm) or the 5,000 ppm AHP Virox 5, and NBS for controls

After wiping and transfer, the disks (donors and recipients) were transferred to separate Teflon vials containing 1 mL of NBS. 10-fold serial dilutions with NBS in 2 mL cryovials were prepared and plaque assayed

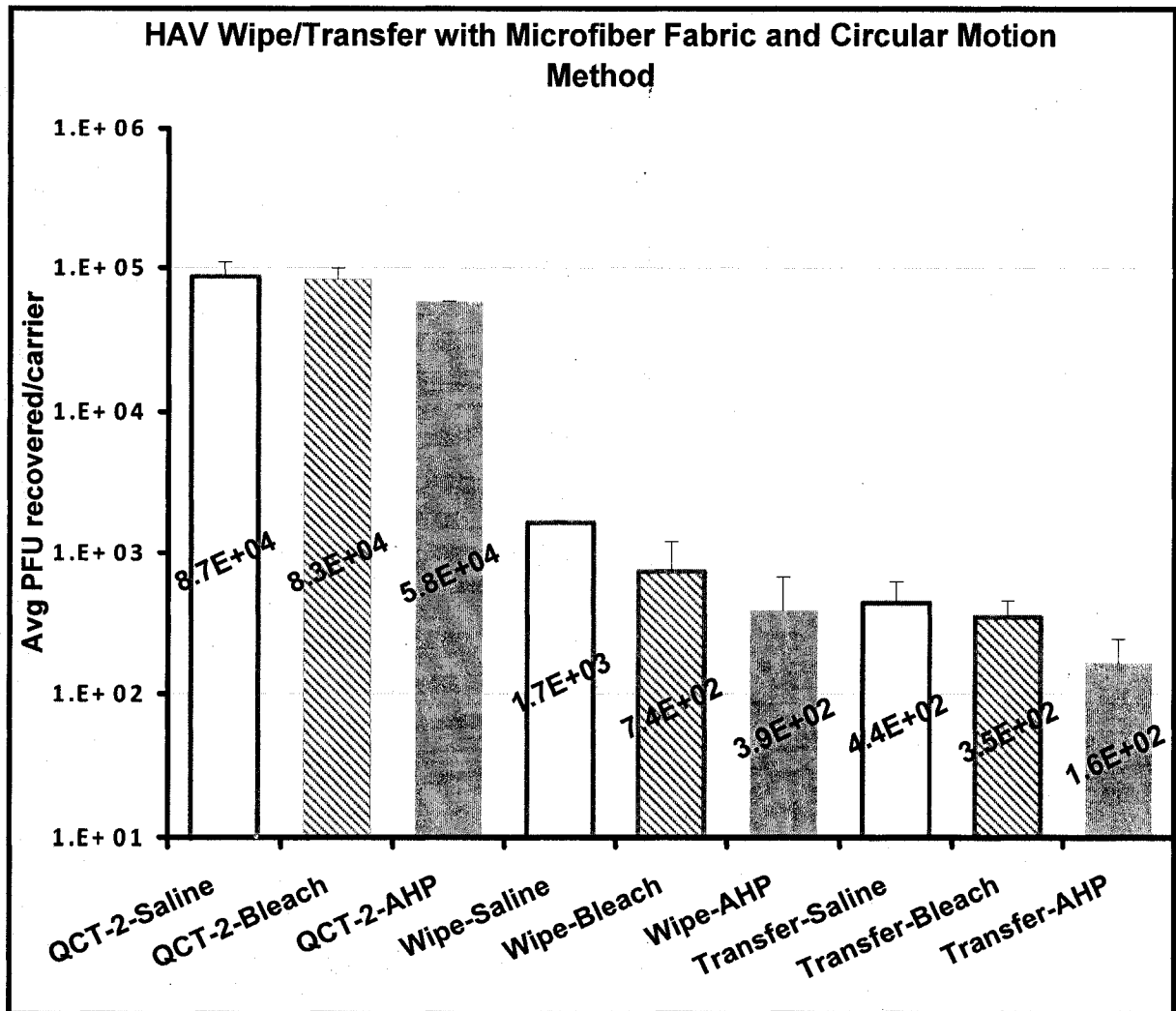


Figure 3.5

Table 3.1. Summary results of all wipe/transfer tests done in this study against *A. baumannii*, *G. stearothermophilus* spores and HAV

Unless stated otherwise, the volume used to wet the fabrics was 25 μ L.

- All the data presented in this Table were calculated as the average of at least three replicate experiments.
- The initial inoculum was the CFU/PFU recovered from the control disks after drying the using QCT-2 method.
- Mass balance is the sum of the CFU/PFU recovered from the donor disks and recipient disks after wiping and transfer, respectively.
- Log_{10} reductions represented the reductions achieved by wiping the donor disks with the respective test solution (please refer to Section 3.2.4 for calculation method).
- % of transfer to the sterile recipient disk achieved by the same fabric that was used to wipe the donor disk in relation to the initial inoculum (please refer to Section 3.2.4 for calculation method).

Table 3.1

Type of fabric and method of wipe/transfer	Microbe	Test solution (ppm)	Initial Inoculum	Mass Balance	Log ₁₀ reduction	% of transfer
Microfiber and circular method	<i>A. baumannii</i>	Saline	2.06 x 10 ⁷	1.65 x 10 ³	4.18	1.47 x 10 ⁻³
		DB-100		5.51 x 10 ²	4.57	4.86 x 10 ⁻⁶
		DB-50		1.01 x 10 ²	5.61	2.50 x 10 ⁻⁴
Microfiber (wetted with 50 µL) and diagonal Method	<i>A. baumannii</i>	Saline	8.93 x 10 ⁶	1.39 x 10 ⁴	3.52	1.26 x 10 ⁻¹
		DB-100		9.50 x 10 ²	4.26	4.60 x 10 ⁻³
		DB-50		7.25 x 10 ²	4.60	5.70 x 10 ⁻³
Microfiber and diagonal method	<i>G. stearothersophilus</i>	Saline	2.7 x 10 ⁷	7.99 x 10 ⁵	1.62	0.56
		DB-500		1.43 x 10 ⁵	2.46	0.46
Microfiber and circular method	<i>G. stearothersophilus</i>	Saline	1.95 x 10 ⁷	7.10 x 10 ⁵	1.51	0.58
		DB-500		6.37 x 10 ⁵	1.50	0.37
		AHP-5000		6.26 x 10 ⁵	1.53	0.51
Microfiber and circular method plus the drying step	<i>G. stearothersophilus</i>	Saline	2.7 x 10 ⁷	2.45 x 10 ⁵	2.18	0.25
		DB-500		9.90 x 10 ⁴	2.48	0.07
		AHP-5000		3.55 x 10 ³	3.87	0.001
Cotton and circular method	<i>G. stearothersophilus</i>	Saline	2.7 x 10 ⁷	8.66 x 10 ⁵	1.54	0.34
		DB-500		5.92 x 10 ⁵	1.70	0.36
		AHP-5000		1.55 x 10 ⁶	1.24	0.38
Microfiber and circular Method	HAV	Saline	8.7 x 10 ⁴	2.09 x 10 ³	1.72	0.5
		DB-500		1.08 x 10 ³	2.05	0.42
		AHP-5000		5.49 x 10 ²	2.17	0.28

3.4 DISCUSSION

We used a fully quantitative method with stringent testing conditions, where the fabric carriers and disk carriers were designed in such a way to provide a standard and relatively small surface area for microbial wiping and transfer from disks to fabrics to disks. This also allowed the use of relatively small volumes of disinfectants to wet the fabrics. The level of pressure used in these experiments reflected that applied in practice while wiping environmental surfaces. A comparable study by Williams *et al.*, (2007) reported the use of exerting weight between stainless steel disk and wipe of 100 ± 5 g for 10 s.

We used titers of the surrogate organisms higher (or in line) than the levels of bacterial contamination reported for organisms from each group. Williams *et al.* (2007) used 6-7 log₁₀ of bacterial suspension, and Sattar *et al.* (2001) used 10⁵ CFU of *S. aureus* on each donor surface. A recent study showed that tables, wheelchairs, shower chairs, bathtubs and foot washbowls in a hospital ward were commonly contaminated by 100-10⁵ CFU of MRSA or MSSA (Oie *et al.*, 2005). Hodges *et al.* (2006) used ~10⁶ CFU/mL to serially dilute and attained inocula ranging from 4.0 x 10⁰ to 6.0 x 10⁴ spores/mL when tested removal of spores from steel surface using microfoam swabs. Abad *et al.* (2001) used virus inocula ranging from 1-5 x 10⁵ PFU/mL.

We used inocula suspended in a soil load (5% FBS) in all tests to mimic dirty conditions. It had been suggested that, in general, the presence of large amounts of organic material on surfaces could reduce the ability of wipes to remove bacteria (Williams *et al.*, 2007).

It has been well documented that premoistened wipes are more efficient in removing/sampling for microbes than dry ones (Sattar *et al.*, 2001; Busher *et al.*, 2008). The physical action of wiping is meant to augment the decontaminating potential of the microbicide by loosening up any dried contamination and providing better access to

pathogens therein. Wiping in conjunction with an appropriate microbicide can result in a more efficient process of decontamination. However, wiping surfaces with a weak or ineffective formulation runs the risk of spreading localized contamination over a wider area during wiping. By using NBS to wet control wipes and choosing test formulation concentrations that proved of being poorly effective when tested in this study on the same organisms by the QCT-2 method with contact times much longer than that used in the wipe/test method, we were able to determine that the higher \log_{10} reductions achieved by wiping was due mainly to mechanical removal of the microbes from the contaminated donor disks. This conclusion is in agreement with that of Williams *et al.* (2007).

Decontamination by wiping proved to be more efficient for vegetative bacteria than for the virus and the spores. With microfiber wipes wet with 25 μL of NBS, *A. baumannii* achieved an average \log_{10} reduction of 4.18; however, the reductions for the spores and HAV were 1.51 and 1.72 \log_{10} , respectively. Additional reductions were achieved with wipes wet with disinfectants for *A. baumannii* (an average of 0.39-1.43 for 100 ppm and 50 ppm DB) and to a lesser extent for HAV (an average of 0.33-0.45 for the 500 ppm DB and Virox-5), but this was not the case with the spores, where no further reductions were observed. Correspondingly, for *A. baumannii*, the percentage of transfer from microfiber wetted with NBS to a clean disk was at least 400 times less than that for the spores and HAV. Our results were in agreement with the study of Mackintosh and Hoffman (1984), where differences between transfers of species were documented. For example, *Staphylococcus saprophyticus* transferred well to hands but not as well from hands to fabrics as the other species, and it survived well on the skin. *Pseudomonas aeruginosa*, *Klebsiella aerogenes* and *Serratia marcescens* transferred moderately well overall and also survived on the skin, while opposite

results were observed with a strain of *Escherichia coli* and another of *Streptococcus pyogenes* (Mackintosh and Hoffman, 1984).

We do not think that difference in the levels of microbes affected our results, but we feel that the intrinsic properties of each organism, including the outer layer hardness of the cells and their hydrophobic nature might have played a role. Wiping with friction might have caused injury to the cell outer membranes of *A. baumannii*, but not to the virus and to the hard coats of the spores. Further studies should be done to determine if the lower transfer of *A. baumannii* was caused by injuries to the cells or hydrophobic forces. This can be done by comparing the number of CFU left on the fabrics after wipe and transfer to that of the CFU recovered from the QCT-2 control and the total number of CFU recovered from both donor and recipient disks. Although this was not done in this study, we did investigate qualitatively what remained of the bacteria on the microfiber pieces of fabric after transfer, and it was substantial. This could also be seen clearly in Table 3.1 by comparing the numbers of CFU/PFU recovered by QCT-2 to those obtained from the sum of the CFU/PFU recovered from the donor disks and recipient disks after wiping and transfer (referred to as the mass balance in Table 3.1), respectively. Interestingly, fabrics retained much larger numbers of *A. baumannii* as compared to those of the spores and the virus.

These data confirmed again that HAV was as tough as the spores for surviving decontamination by wiping, surviving friction and transferring at an equal rate to clean surfaces. Even with a shorter contact time and a small surface contact area, the number of PFU transferred were as high as 160 for transfer with the microfiber wetted with Virox-5 (a commonly used concentration for the purpose of regular disinfection in hospitals, health-care and day care centres) and even higher for 500 ppm DB (350 PFU) and NBS (440 PFU). This can possibly explain why some viral infections - transmitted through the fecal-oral route and

spread by means of continual low-level transmission through the environment - have persisted in the environment and continue to be a major health problem. In fact, with the currently limited availability of antiviral drugs and vaccines for most common enteric and respiratory viruses, evidence suggests that hygiene and disinfection are crucial for preventing and interrupting the spread of viral infections (Boon and Gerba, 2007). Therefore, choosing the wrong product for decontamination or any misuse would continue to have appalling outcomes. Studies have verified naturally occurring outbreaks for 8 out of 10 viruses (HAV, RSV, norovirus, rotavirus, influenza virus, coronavirus, astroviruses and adenoviruses). Disease outbreak investigations and disinfection intervention studies have documented indoor environmental surfaces as reservoirs for pathogenic viruses with the potential for spread of infectious disease (Boone and Gerba, 2007; Ansari *et al.*, 1988). In 2002 norovirus caused consecutive outbreaks of gastroenteritis on cruise ships, which required discontinuation of service and aggressive environmental disinfection to stop further infection (Hota, 2004). In a study by Barker *et al.* (2004), cleaning surfaces with a detergent solution spread norovirus to clean surfaces; however, cleaning with a 5,000 ppm chlorine solution was effective in eliminating norovirus from environmental surfaces and preventing cross-contamination. It should be noted that studies have shown that very small amount of enteric viruses (e.g., norovirus estimated at 10 to 100 virions) can cause human infection (Barker *et al.*, 2001; Rzezutka and Cook, 2004). Interestingly, during the smallpox eradication campaign era, contact with contaminated smooth surfaces did not appear to be an effective mode of smallpox transmission (Busher *et al.*, 2008).

In addition, although it has been impossible to distinguish whether contamination of the healthcare workers' hand was due to direct patient contact or to contact with contaminated environmental surfaces, it is believed that MRSA, VRE and *C. difficile* are often transmitted

from patient-to-patient via the hands of healthcare workers (Weber and Rutala, 2006; Rutala and Weber, 2001; Rochon-Edouard *et al.*, 2004). Tested under simulated hospital environment conditions, *A. baumannii* was found to survive much better on fingertips or on dry surfaces as compared with other genera of Gram-negative bacilli. Although health-care worker hand carriage with *Acinetobacter* is typically transient, it may be more prolonged in individuals with damaged skin (Patterson *et al.*, 1991).

For the spores, drying a small volume of NBS before wiping with the wet microfiber fabric reduced transfer by almost a half and increased the average \log_{10} reduction by 0.67. This effect of drying was even more pronounced when test products were used, even though these products were poorly effective when assessed by QCT-2.

The data presented here suggests that aerosolizing disinfectants and letting them air-dry accompanied by wiping might be the key to achieving a faster and more effective means of environmental surface decontamination.

The role of the type of fabric in transferring contamination was also studied. However, for the spores, no significant difference was found between the cotton and microfiber wipes, neither for the percentage of transfer, nor for the \log_{10} reductions achieved for both controls and tests.

The role of the type of motion when wiping was studied on the spores and on *A. baumannii*. Two motions were assessed: a circular motion for wiping and transfer (5 s each) versus a diagonal wiping with transfer by contact (10 s each). For the spores, although the timing was not equal, there was no significant difference between the controls for both types of motion neither for the percentage of transfer nor for the \log_{10} reduction achieved by wiping. When using a diagonal motion, with microfiber fabrics wet with 500 ppm DB, transfer of the spores were higher than when applying a circular motion, eventhough the

\log_{10} reductions achieved by wiping the donor disks using both motions were in the same range. Similarly, tests with *A. baumannii* showed that when using a circular motion, the reduction was higher and the transfer was much lower as compared to those obtained with a diagonal motion even though the timing for transfer was longer and the volume to wet the fabric was higher. Combined data from tests on the two microorganisms suggested two things: firstly, transfer by pressurized contact with no friction lead to a higher transfer than transfer with friction; and secondly, timing of mechanical removal was not a factor, but the hardness of the cells of the microbe was. Thus, for an organism such as *A. baumannii*, the type of motion made a difference; the continual friction in the circular motion could have caused injuries to the cell wall, so more reduction was achieved leading to less transfer. However, for the much hardier spores, friction did not effect the survival, so a circular motion while wiping continued to circulate the spores on the donor disks (therefore less reduction), and so the fabric retained less spores for transfer to the sterile recipient disk. This was in contrast to what happened with diagonal wiping, where the fabric retained more spores for transfer to the clean disk. This conclusion was similar to the previous one about the effect of type of fabric on spores wiping and transfer, since motion and friction exerted were the same for both type of fabrics. Therefore, for decontamination purposes, we suggest the use of a circular motion; however, for documenting the effectiveness of a decontamination process against anthrax spores, for example, the diagonal motion would be more suitable because of the repeated removal movement of the swab without re-spreading the spores as compared to a circular motion.

Contrary to our results, a study by Sattar *et al.*, (2001), using *S. aureus* as a model, concluded that friction increased the level of transfer from fabrics to fingerpads, and a higher bacterial transfer from polycotton as compared to all-cotton was observed. Interestingly, the

results of a study of Williams *et al.* (2007) showed some strain-dependent variability for *A. baumannii* when it came to removal/transfer, and suggested that some phenotypic differences might influence removal and destruction of certain strains.

While other methods either qualitatively described transfer of bacteria (Williams *et al.*, 2007) or used a quantitative approach that was difficult to standardize (Marples and Towers, 1979; Mackintosh and Hoffman, 1984), the method we used in this study quantitatively described transfer using a simple, rapid and reproducible procedure. More importantly, this method can be used for bacterial and viral testing under a various of conditions.

4. CONCLUSIONS

As summarized in the preceding sections, this study successfully met its three objectives. In the process, we developed and validated several experimental approaches directly related to assessing the microbicidal activities of chemicals and to testing the influence of wiping on the inactivation/removal of infectious agents on environmental surfaces.

The selection and application of a microbial mixture was particularly unique, as it allowed the evaluation of the microbicidal activity of liquid chemicals in a simple yet standardized and fully quantitative format. The levels of stringency incorporated in the test protocol made the data generated much more relevant to the challenges a microbicide may encounter in the field. This aspect is particularly significant in any attempts at remediation of sites contaminated with infectious bioagents, because the risk of failure could be quite serious both in terms of economics and public safety.

The microbicides tested are all available commercially and they were chosen for their relatively rapid and broad-spectrum microbicidal activity. Limitations of time and resources did not permit us to include more formulations, other concentrations of the actives selected or to widen the types of substrates and temperatures. While our findings differentiate formulations based on their microbicidal activity, this should be only one factor in the ultimate selection of a given microbicide because material compatibility, human and environmental safety, as well as the potential for generating harmful disinfection byproducts cannot be ignored.

Another important contribution of this study is the development of a neutralizer suitable for use with a variety of microbicidal chemicals, as well as with a wide array of infectious agents. Testing the virucidal activity of chemicals presents a major challenge when it comes

to neutralizing the active(s) rapidly and effectively without harming the virus and its host cells. The formula developed here has eliminated the ingredients once considered necessary to deal with now phased-out chemicals, such as hexachlorophene, while adjusting other essential components for optimal activity and reduced potential for cytotoxicity. Nevertheless, further improvements in this regard are on-going for a more extensive assessment of the neutralizer.

While wiping of an environmental surface to clean and decontaminate it is certainly not new, there is recent renewed interest in the marketing of pre-soaked fabrics for wiping animate and inanimate surfaces to prevent the spread of infections. The advent of microfiber technology (Rutala *et al.*, 2007) has further enhanced the interest in wipes. Microbicide-impregnated wipes in general can be potentially quite beneficial by combining chemical and mechanical actions during decontamination. However, there is a lack of suitable test protocols to quantitatively assess the decontaminating ability of such wipes. This study adapted a previously reported protocol (Sattar *et al.*, 2001) to test wiping action for decontamination of hard environmental surfaces.

Our findings reinforced the potential benefits of combining the microbicidal activity of a chemical with the physical action of wiping. Such a combination, could not only lead to more efficient decontamination, but also to substantially reducing the concentration of microbicides needed. On a cautionary note, we also showed that the use of an ineffective microbicide could readily spread microbial contamination over a wider area during wiping.

The experiments on wiping as reported here should be regarded as preliminary and directed more towards the refinement of test protocols. The groundwork laid here should enable more elaborate studies on this topic.

As far as we are aware, there is a definite lack of studies on the microbicidal activities of chemicals at low temperature for use in cold rooms, and freezers and also in the case of bioagent decontamination outdoors during cold weather. Much of what we know on microbicides comes from tests carried out at air temperatures of no less than 20°C. As mentioned before, such information is virtually useless when considering application of microbicides under colder and subzero environments. Greater effort is needed on the part of researchers as well as manufacturers to develop innovative yet safe formulations and technologies for use under cold conditions.

This study was a part of a larger investigation with the overall objective of developing standards to determine 'how clean is clean?', so that after remediation a site could be assessed as safe enough for reoccupation. Several aspects of the information generated by us will be suitable for developing models to address the question discussed above. It is also anticipated that the protocols developed by us may soon be applied for work with CL-3 agents to validate the data generated with their surrogates.

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APPENDIX I: LIST OF SOLUTIONS (COMPOSITIONS, CHEMICALS AND SUPPLIERS)

CHEMICAL/SOLUTION	COMPONENTS	PREPARATION	SUPPLIER & CATALOGUE No
Crystal violet, 0.1%	Crystal violet dd H ₂ O	1.0 g 1 L	Fisher Scientific: C581-100
DMSO	Dimethyl sulfoxide	1.10 g/ mL	ICN Biomedicals Inc: Cat # 191418
Formaldehyde solution For fixing FRhK-4 cells	Sodium chloride (crystals) Formaldehyde Deionized water	34 g 160 mL 3.84 L Mix well together	Sigma-Aldrich: EC231-598-3 Fisher Scientific: F79-4, 37% w/w
Dulbecco's Phosphate Buffered Saline, PBS	PBS powder without Calcium chloride and magnesium chloride	9.6 g, add to 1 L of dd H ₂ O and filter sterilize	GIBCO™: 21600-051
Fetal Bovine Serum, FBS	FBS liquid (500 mL)	Heat inactivated in a water bath at 56°C for 1 h.	GIBCO™: 12483-020
<u>FRhK-4 cells Growth Media and Solution for Plaque Assay:</u>			
10X MEM Stock solution	Minimum Essential Medium Eagle (MEM) dd H ₂ O	10.6 g 1L Stir to dissolve and filter sterilize through a 0.22 µm filter	Sigma: M4642

Hepes (1.5M)	Hepes crystals dd H ₂ O	35.7 g 100 mL Stir to dissolve and filter sterilize	Fisher Scientific: BP310-500
Sodium bicarbonate, NaHCO₃ (7.5%)	Sodium bicarbonate dd H ₂ O	7.5 g 100 mL Stir to dissolve and filter sterilize	Fisher Scientific: M33-500
kanamycin sulfate	10 mg/mL in 0.85% saline, 100X	100 mL	GIBCO: 15160
1X MEM supplemented growth medium	10X MEM stock solution FBS (Fetal bovine serum) MEM NEAA (Non-essential aminoacids) Hepes (1.5 M) Sodium bicarbonate (7.5%) dd H ₂ O	100 mL from Stock 10X MEM 70 mL 10 mL 10 mL 15 mL 795 mL Mix and store at 4°C	Sigma: M4642 Fisher Scientific: M33-500 Gibco: 11140 Fisher Scientific: BP310-500 Fisher Scientific: M33-500
2X MEM for Overlay medium for plaque assays	10X MEM stock solution MgCl ₂ 6H ₂ O 1.12 g/mL FBS MEM NEAA (100X solution) L-Glutamine Kanamycin sulfate NaHCO ₃ dd H ₂ O	100 mL 4.78 mL 20 mL 10 mL 10 mL 10 mL 15 mL 330.22 mL Mix and store at 4°C; make a total of 500 mL	Sigma: M4642 Fisher Scientific: M33-500 GIBCO™: 12483-020 Gibco: 11140 Gibco: 25030 GIBCO: 15160 Fisher Scientific: M33-500

<p>1.2 % Agar Noble</p>	<p>Agar Noble dehydrated powder dd H₂O</p>	<p>4.8 g 400 mL Dissolve by stirring on a hot plate. Sterilize by autoclaving at 121°C</p>	<p>Difco™: 214230</p>
<p>4% Formaldehyde in saline</p>	<p>Formaldehyde 37% w/w Sodium chloride Deionized water</p>	<p>160 mL 34 g 3.84 L Mix well</p>	<p>Fisher Scientific: F79-4 Sigma-Aldrich: EC 231-598-3</p>
<p><u>Hard water (300 ppm):</u></p>	<p>MgCl₂ · 6H₂O CaCl₂ · 2H₂O dd H₂O</p>	<p>0.1984 g 0.4624 g 10 mL Filter sterilized through a 0.22 µm filter</p>	<p>BDH: B10149 Fisher Scientific C-70 CAS-10035048</p>
<p>Hard water stock solution I:</p>	<p>NaHCO₃ dd H₂O</p>	<p>0.3502 g 10 mL Filter sterilized through a 0.22 µm filter</p>	<p>Fisher scientific: S233-500</p>
<p>Hard water, 300 ppm</p>	<p>Solution I Solution II dd H₂O</p>	<p>0.3 mL 0.4 mL 50 mL Add solution I and II to water. Mix well</p>	

<p><u>Growth media and Agar</u></p> <p>Middlebrook 7H9 broth to grow <i>M. terrae</i></p>	<p>Solution I: Bacto® Middlebrook 7H9 dehydrated powder Glycerol dd H₂O</p> <p>BBL™ Middlebrook ADC-Enrichment</p>	<p>0.94 g 0.4 mL 180 mL Stir to dissolve, autoclave at 120-124°C to sterilize. 20 mL, added aseptically to solution I after allowing its temperature to cool down to 45°C.</p>	<p>Difco: 0713-13 Fisher scientific: G33-500 BD: Ref 211887 Quelab: QB-39-5206</p>
<p>TSB to grow <i>A. baumannii</i></p>	<p>Tryptone-soy broth powder dd H₂O</p>	<p>15 g 500 mL Stir to dissolve on a hot plate. Sterilize by autoclaving at 120-124°C. Aliquot into 15 mL conical tubes (10 mL in each). store at 4°C.</p>	<p>Quelab: QB-39-2974 Fisher scientific: G33-500 In-house made (see below)</p>
<p>Mycobacteria 7H11 agar</p>	<p>Middlebrook 7H11 powder Glycerol dd H₂O OADC</p>	<p>21 g 6 mL 900 mL 100 mL powder and glycerol added to water, stir to dissolve completely on hot plate. Autoclave at 120°C. Allow to cool down to 50-55°C, then add OADC.</p>	<p>Quelab: QB-39-2974 Fisher scientific: G33-500 In-house made (see below)</p>

<p>Tryptone-soy agar plates, TSA</p>	<p>Tryptone-soy broth powder Bacteriological agar dd H₂O</p>	<p>30 g 15 g 1000 mL Add all to water; stir to dissolve on a hot plate. Sterilize by autoclaving at 120°C.</p>	<p>Quelab: QB-39-5206 Quelab: QB-39-0221</p>
<p><u>Media Supplement for 7H11 Agar</u> Albumin Dextrose Catalase Complex (ADC)</p>	<p>Solution I: Bovine serum albumin, fraction V NaCl Glucose Catalase (Bovine) dd H₂O</p>	<p>50 g 8.1 g 20 g 0.04 g 900 mL</p>	<p>Fisher Scientific: BP1600-100 Sigma-Aldrich: EC 231-598-3 Sigma® : EC 200-075-1 Sigma: C-10 EC1.11.1.6</p>
<p>6M NaOH</p>	<p>Sodium hydroxide crystals dd H₂O</p>	<p>40 g 250 mL Stir to dissolve</p>	<p>Sigma: EC215-185-5; S 5881</p>
<p>Sodium oleate solution</p>	<p>Solution II: Oleic Acid 6 M NaOH dd H₂O</p>	<p>0.6 mL 0.6 mL 30 mL Incubate at 50°C until solution is clear</p>	<p>Fisher Scientific: CAS 112-80-1</p>

<p>Oleic Acid Albumin Dextrose Catalase Complex (OADC)</p>	<p>ADC Sodium oleate solution</p>	<p>solution II solution I Add solution II to solution I. Adjust pH to 7 with 1N NaOH, add dd H₂O up to 1 L, and filter sterilize. Store at 4°C</p>	<p>Home made solution Home made solution</p>
<p><u>Normal Buffered Saline, (Dilution Water)</u> Phosphate Buffered Stock Solution</p>	<p>Potassium dihydrogen phosphate, KH₂PO₄, crystals dd H₂O</p>	<p>34.0 g ~1 L Dissolve KH₂PO₄ in 500 mL of deionized water, adjust pH to 7.2 ± 0.2 with 1N NaOH or 1N HCl and bring to 1000 mL with deionized water</p>	<p>Fisher Scientific: P-382</p>
<p>Normal Buffered Saline (Dilution Water)</p>	<p>NaCl Phosphate Buffer Stock Solution dd H₂O</p>	<p>8.75 g 1.25 mL bring up to 1000 mL, adjust pH to 7.2 ± 0.2 with 1 N NaOH. Sterilize by autoclaving at 120°C.</p>	<p>Sigma-Aldrich S9888 Home made solution</p>
<p><u>Soil load</u> Yeast extract 5% Bovine serum albumin 5%</p>	<p>Yeast extract powder BSA powder</p>	<p>0.5 g 0.5 g</p>	<p>BD: 212750 Sigma: B-4287</p>

<p>Bovine mucin 0.4%</p>	<p>Mucin powder</p>	<p>0.04 g Each dissolved in 10 mL of NBS and filter sterilized through 0.22 µm filter, aliquot in 2 ml cryovial tubes (0.5 mL/tube) and store in a -20°C freezer.</p>	<p>Sigma type I: M4503-1G</p>
<p>Sephadex mixture for gel filtration</p>	<p>Sephadex powder dd H₂O</p>	<p>50 g 500 mL Soak sephadex powder in dd H₂O over night at 4°C. Sterilize mixture by autoclaving at 120°C.</p>	<p>Sephadex™ LH-20, Cat# 17-0090-02</p>
<p><u>Stock solutions for <i>G. stearothermophilus</i> culture medium</u> Tryptone soy 2.5%</p>	<p>Tryptone soy powder dd H₂O</p>	<p>2.5 g 1 L Stir to dissolve and sterilize by Autoclaving at 120°C.</p>	<p>Oxoid: CM0129</p>
<p>Calcium chloride 25%</p>	<p>CaCl₂ dd H₂O</p>	<p>25 g 100 mL Stir to dissolve and sterilize by Autoclaving at 120°C.</p>	<p>BDH Analar® B10070</p>
<p>Ferrous sulfate 31%</p>	<p>FeSO₄·7H₂O H₂SO₄ (concentrated) dd H₂O</p>	<p>31 g 0.5 mL 100 mL Stir to dissolve and sterilize by filtration through a 0.22 µm filter.</p>	<p>BDH Analar B10112 Fisher Scientific A300 S212</p>

<p>Magnesium sulfate 30%</p>	<p>MnSO₄.H₂O dd H₂O</p>	<p>30 g 100 mL Stir to dissolve and sterilize by autoclaving at 120°C.</p>	<p>BDH: ACS492</p>
<p>Magnesium chloride 11%</p>	<p>MgCl₂.6H₂O dd H₂O</p>	<p>11 g 100 mL Stir to dissolve and sterilize by autoclaving at 120°C.</p>	<p>Fisher Scientific: M33-500</p>
<p>Trypsin</p>	<p>0.05% Trypsin-EDTA, 1X</p>	<p>100 mL</p>	<p>GIBCO: 25300</p>

APPENDIX II: LIST OF EQUIPMENTS & THEIR MANUFACTURERS

EQUIPMENT	MANUFACTURER AND SPECIFICATIONS
<p><u>Pipettes</u> Air Displacement Pipettes Positive Displacement Pipette</p>	<p>10-100 µL: Transferpette® 100-1000 µL: Transferpette® 1-20 µL: Eppendorf</p>
<p><u>Centrifuges</u> Beckman-Avanti™ J-25 Damon/IEC-PR-J</p>	<p>Beckman Coulter, Mississauga, Ontario, Canada Damon/IEC Division, Midland, Ontario, Canada</p>
<p><u>Materials for OCT-2</u> Glass Vials (20 mL) and Caps Flat-bottomed (15 mL) Teflon Vials Stainless Steel Disks (1 cm in diam., made from 0.7 mm thick sheets of magnetized and brushed stainless steel) 10 mL Bottle Dispenser Membrane Filters to Capture: <ul style="list-style-type: none"> - spores and vegetative bacteria (47 mm in diameter with pore diameter 0.22 µm) - Mycobacteria and fungi (Black gridded, 47 mm in diameter with pore diameter 0.45 µm) </p>	<p>Galaxy Glass, vials: cat# 5260-GV caps: cat# 5299- G Cole Parmer Vernon Hills, IL: cat# PK-08936-30 Muzeen and Blythe Ltd., AISI brushed stainless steel type 430 Fisher brand®, cat# 03-692-179 PALL Life Science Corp: cat# P/N 66234 Millipore Corp: cat# MSP000814</p>
<p><u>Materials for Wipe/transfer test</u> Custom-made tubular stainless steel holder O-ring Nitrile Custom-made Teflon insert Perfect® Clean^{AM} (80% polyester, 20% polyamide) 100% cotton (purchased locally)</p>	<p>Rehabilitation Centre Workshop, Ottawa, Canada Spaenaur, Kitchener, Canada Rehab Workshop, Ottawa, Canada</p>

Digital balance, Mettler PC2000	Mettler Instruments, Zürich, Switzerland
<u>Materials for FRhK-4 cells and HAV</u> 12 well cell culture plates 75 cm ² Tissue Culture Flasks 25 cm ² Tissue Culture Flasks Cryo 1°C freezing container	Corning, ref 3513 Sarstedt, ref 83.1813 Falcon [®] , ref 353109 NALGENE [™] , cat# 5100-001
<u>Materials for gel filtration</u> 10 mL syringe 30 mL plastic conical tube 60 mL glass tube (2.3 cm in diameter)	BD: Luer-Lok [™] tip ref. 309604 Sarstedt, ref 60545
PH meter	Brinkmann B, METROHM 632
Bijoux bottle	Wheaton, Millville, N.J.
<u>Disposable pipettes</u> 25 mL 10 mL 5 mL	Sarstedt, ref 86.1685.001 Sarstedt, ref 86.1254.001 Sarstedt, ref 86.1253.001
<u>Disposable conical tubes</u> 15 mL 50 mL	Sarstedt, ref 62.554.002 Sarstedt, ref 62547.004
2 mL cryovial	Sarstedt, ref 72.694.006
Hot plate	Fisher Scientific: Isotemp [®]
Hygrothermometer	ABBEON relative humidity and temperature Indicator, model No M2A4. ABBEON Incorporated, West Germany
<u>Freezers</u> -80°C -20°C	Thermo Forma -86°C UTL Kenmore
Laminar flow cabinet	Class II type A
Vortex	Vortex-Genie: cat# 12812V1
Water Bath	Fisher Scientific, Isotherm 210

Appendix III

FLOW CHART SHOWING THE MAIN STEPS IN THE QUANTITATIVE CARRIER TEST APPLIED TO TESTING WITH THE MICROBIAL MIXTURE

Mixture of *M. terrae* (pBEN), *A. baumannii*, *G. stearothermophilus* spores, and HAV in soil load



10 μ L inoculated onto 3 control and 3 test carriers



Inoculum dried for 1 h at room temperature



50 μ L of disinfectant (NBS for control) placed on dried inoculum for desired time



Carrier placed in Teflon vials containing 9.950 mL of neutralizer to arrest disinfectant activity and elute the microorganisms



2 mL of eluate passed through 0.22 μ m filter



Filtrate 10-fold serially diluted and plaque assayed



1 mL eluate serially diluted, filtered and filters placed on appropriate agar



1) *M. terrae* (pBEN) grown on 7H11 agar supplemented with OADC, glycerol and kanamycin at $37 \pm 1^\circ\text{C}$

2) *A. baumannii* grown on TSA at $36 \pm 1^\circ\text{C}$

3) *G. stearothermophilus* spores grown on TSA at $56 \pm 2^\circ\text{C}$

CFU/PFU on test and control plates counted and \log_{10} reductions calculated.

APPENDIX IV: METHOD FOR DETERMINING THE RELATIVE CONCENTRATIONS OF PERACETIC ACID AND HYDROGEN PEROXIDE

A quality assurance test method to calculate the concentrations of peracetic acid and hydrogen peroxide by titration with potassium permanganate.

Principle: In peracetic containing products, hydrogen peroxide (H_2O_2) content is determined by an oxidation-reduction titration with potassium permanganate (KMnO_4). At the end point of this titration, an excess of potassium iodide (KI) is added to the solution. The potassium iodide reacts with peracids to liberate iodine, which is titrated then with a standard solution of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$).

Equipment:

- Analytical balance
- Erlenmeyer flask, 250 mL
- Volumetric flasks, 250 mL, 500 mL and 1000 mL
- Pipettes, 10 mL, 10 mL and 25 mL
- Burettes, 25 mL for KMnO_4 ,
 - o 10 mL for $\text{Na}_2\text{S}_2\text{O}_3$
- Magnetic stirrer and stir bars
- Hot plate
- Thermometer
- Ice
- Oven
- Graduated cylinders, 10 mL and 100 mL

Reagents:

- KMnO_4 , 0.1334 N standardized (refer to standardization method below)
- Sulfuric acid, H_2SO_4 (1 N)
- $\text{Na}_2\text{S}_2\text{O}_3$, 0.025 N
- KI , 2.5 N
- Starch indicator solution, 1%
- Potassium hydroxide, KOH concentrated
- 1% H_2O_2 standardized solution
- PAA disinfectant

Preparation of Reagents:

- In a 1 L volumetric flask diluted a stock solution of KMnO_4 (~ 0.5336 N) (Matheson Coleman and Bell, FW 15304) to give 1 L of KMnO_4 (0.134 N).
- Slowly added 28 mL of concentrated sulfuric acid (Fisher Scientific A300 S212) to a 1 L volumetric flask (kept on ice bath) containing approximately 700 mL of dd H_2O . The solution was allowed to cool, diluted to volume (1 L) and mixed thoroughly.
- In a 500 mL volumetric flask diluted 0.1 N $\text{Na}_2\text{S}_2\text{O}_3$ (Sigma-Aldrich, 319546-500 mL) 1 in 4 to give 500 mL of $\text{Na}_2\text{S}_2\text{O}_3$ (0.025 N).
- In a 250 mL Erlenmeyer prepared starch indicator by adding 1.25 g of

- soluble starch powder (Analar: 2008550) to 125 mL of dd H₂O. Completely dissolved by stirring on a hot plate until solution is clear.
- Added slowly 0.0015 g of salicylic acid (Sigma Aldrich CAS 69-72-2). Cooled to room temperature.
- Prepared KI (2.5 N) by adding 41.5 g of KI crystals (Sigma ReagentPlus™, 99%, cat# 207969-500G) to 100 mL of dd H₂O. Add 50 μL of KOH concentrated (?).
- Prepared 1% of H₂O₂ by taking 1 mL from an H₂O₂ (30 wt% stabilized) stock solution (Sigma-ALDRICH, 216763-100 mL) in 100 mL of dd H₂O.
- Prepared KOH concentrated by adding 40 g of KOH crystals (Anachemia Chemicals LTD, AC7650) to 100 mL of dd H₂O. Dissolved and mixed well.
- Prepared PAA disinfectant as described before (section 2.2.10) by mixing
- 0.68 of the disinfectant mixture (H₂O₂ and PAA, Ecolab Inc UN3149),
- 0.63 mL of the activator (NaOH, Ecolab Inc UN1824) and 13.69 mL of
- dd H₂O.

Sample Analysis Procedure:

- *To standardize the titration method, first run analysis on a solution of known concentration as follow:*
- Pipetted 10 mL of H₂O₂ 1% solution into a 250 mL Erlenmeyer containing 75 mL of 1N H₂SO₄. Cooled immediately by putting on ice to 0°C.
- *With continuous agitation:*
- Titrated with KMnO₄ (0.1345 N) to the first faint pink colour (with one drop and must persist for at least 10 s).
- Recorded the mL of KMnO₄ used for the titration.
- Added 10 mL of KI solution. Should get a yellow colour solution.
- Titrated with Na₂S₂O₃ (0.025 N) kept adding till the yellow colour was not noticed anymore.
- Added 1 mL of starch indicator.
- Continued titration with Na₂S₂O₃ (0.025 N) till the absence of starch (solution should be clear at the end point)
- Recorded the mL of Na₂S₂O₃.
- Each analysis was repeated in triplicate and their averages were used to calculate the amount of H₂O₂ and peracetic acid.

The same analysis was repeated with 1 mL of the prepared PAA solution used in the QCT-2 experiments.

Calculations:

The amount of H₂O₂ was calculated as follow:

$$\% \text{H}_2\text{O}_2 = \frac{(\text{mL KMnO}_4) (\text{N KMnO}_4) (17) (100\%)}{(\text{g sample titrated}) (1000)}$$

Where 17 = equivalent weight of H₂O₂

1000 = factor to convert equivalents to milliequivalents

The amount of Peracetic acid was calculated as follow:

$$\%PAA = \frac{(\text{mL Na}_2\text{S}_2\text{O}_3)(N \text{ Na}_2\text{S}_2\text{O}_3) (38) (100\%)}{(\text{g sample titrated}) (1000)}$$

Where 38 = equivalent weight of peracetic acid

1000 = factor to convert equivalents to milliequivalents

Note that before doing the calculation the weight of 1 mL of the prepared PAA disinfectant was taken as follow: a small Erlenmeyer flask (25 mL) was put on an analytical digital balance (Mettler AE240), cancel the balance then add using an air displacement pipette 1 mL of the prepared PAA. This was done in triplicate and the average was taken to do the calculations above.

Standardization of KMnO₄ (0.1334 N) solution used in titration:

- In an analytical balance (Mettler AE240) three 0.10g samples (to the nearest 0.1 mg) of sodium oxalate (Sigma-ALDRICH, cat # 379735-5G) previously dried at 105°C (Blue M Stabil-therm, part No-C-2630-0, Germany) for 4 h. The sample were transferred to separate 250 mL volumetric flasks and brought up to volume with dd H₂O.
- Pipetted 25 mL of the above solution into a 250 mL Erlenmeyer flask and added 100 mL of 1N H₂SO₄. Titrated drop wise at RT until the first pink color appeared throughout the solution (~ several drops). Allowed to stand until colorless (~ 10 min). Warmed the solution to 55-60°C and continued titration to a permanent pink color.
- An approximate volume of 1.05 mL of total permanganate was required. The titration was done drop wise near the endpoint to ensure that each drop became decolorized before the next drop was added.
- The normality of KMnO₄ was calculated as follow:

$$N = \frac{(\text{gram wt. Sodium oxalate titrated}) (1000)}{(67) (\text{mL KMnO}_4)}$$

Where 67 = equivalent weight of Sodium oxalate.

1000 = factor to convert equivalents to milliequivalents.

$$\text{Gram wt titrated} = \frac{(\text{Sodium oxalate wt.g}) \times 25 \text{ mL}}{250 \text{ mL}}$$

- Average of the three titrations was calculated and used this value as N for KMnO₄ used in titration of the PAA.

APPENDIX V: *M. terrae* (pBEN) Grown On 7H11 Agar with/without Kanamycin



A. *M. terrae* (pBEN) grown for 11 days on 7H11 agar without kanamycin.



B. *M. terrae* (pBEN) grown for 11 days on 7H11 agar with kanamycin.

10-fold dilutions of *M. terrae* p(BEN) were made in duplicates. Same dilutions were filtered and filters placed onto 7H11 agar without kanamycin (A), and 7H11 agar with kanamycin (B).

CURRICULUM VITAE

Safaa Sabbah

Education

Bachelor of Science Honours (June 2005),
Carleton University, Ottawa, ON, Canada.
Major: combined Biochemistry and Biotechnology.

Bachelor of Science Honours (September, 1993),
Lebanese University, Faculty of Science Section II, Fanar, Beirut, Lebanon.
Major: Chemistry.

“Licence D’Enseignement” in Chemistry (May, 1990),
Lebanese University, Faculty of Science Section I, Beirut, Lebanon.
Major: Chemistry.

Biotechnology Skills

PCR amplification, cloning and tagging, primers/probes design, TOPO[®] cloning using different vectors with sticky or blunt ends DNAs. Plasmid isolation, subcloning, restriction endonuclease digests and analysis. Transformation/co-transformation of *N. crassa* cells, preparation and transformation of *N. crassa* wild type spheroplasts, electroporation of conidia. Animal cell culture. Agarose/SDS-polyacrylamide gel electrophoresis, protein overexpression, Southern/Northern/Western blot, electrophoretic mobility shift assay, HPLC, immunofluorescence staining, immunofluorescence assays.

Have experience with reagents preparation, use of pH meters, microscopes, column chromatography, spectrometers, use of centrifuges including SORVAL SS-34- rotor and Eppendorf centrifuges.

Computer Skills

Proficient in Microsoft Word, Excel, PowerPoint, Adobe Photoshop, Blast, NCBI Search Engine.

Languages: Arabic, English and French.

Work experiences

- Taught Chemistry, Biology and Physics in public and private high schools in Lebanon (1990-1994).
- Chemistry, Biology and Mathematics Tutor: Assisted university students with courses, notes taking and lab reports (1987-1993, 1998-2005).

Written Work

- Directed Studies and Seminar Biol 4900 (Summer 2003) research paper (57 pages). Title: Role of cadherins in cell biology. Supervisor: Dr. David Miller (Carleton University)
- Biochemistry Research Project, Bioc 4908 (fall-winter 2004-2005) (56 pages). Title: Tagging *le-3(sbr)* Responsive Genes For Immunolocalization. Supervisor Dr. P. J. Vierula (Carleton University).
- Literature review "Biological Weapons" (Winter 2007) (54 pages).
- Literature review "Decontamination of Biological Agents" (Winter 2008) (33 pages).
- Presentation at the students' symposium at the University of Ottawa, February 2008.
- Presentation at the CBRN decontamination workshop, April 2008.
- Presentation at the 31st AMOP technical seminar, June 2008.