# ASSESSMENT OF THE SURVIVAL OF MICROBIAL PATHOGENS IN THE ENVIRONMENT

by

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entitled: Assessment of the survival of microbial pathogens in the environment

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College.

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## ABSTRACT

The studies presented here evaluate the survival of different types of pathogens in a variety of environments. The study Appendix A focuses on the presence of enteric bacteria in reusable shopping bags. We demonstrated that ninety-seven percent of individuals surveyed never washed their reusable shopping bags and that this lack of washing can lead to the buildup of potentially harmful bacteria such as *Salmonella* spp. The hand-washing of the bags was shown to reduce seeded organisms to below detectable limits.

Appendix B examines the survival of *Bacillus altrophaeus* endospores during household laundering. It was demonstrated that detergent alone didn't significantly reduce the number of viable endospores or their spread to other garments and that bleach is necessary to significantly reduce the number of viable endospores and their spread. Risks for infection were significantly lower when bleach was used during laundering.

Appendix C details the survival of *Ascaris* ova in biosolid-amended Brazito sandy loam and clay loam. Survival of *Ascaris* ova was significantly higher in clay soil and ova inactivation increased with increasing temperature. The risk for *Ascaris* infection from consuming raw lettuce grown on such soils was calculated and it was found that annual risks for infection decreased significantly with time after harvest.

# INTRODUCTION

## Problem Definition

The spread of many pathogens depends largely upon their environmental survival before transfer to another host. (Maier et al., 2000). Pathogens transmitted through the environment may survive from hours to years outside the host, depending on the organism and the environment (Pepper et al., 2001). There are many potential routes for the transmission of enteric pathogens through the environment. Some routes can be considered "natural" routes for the transmission of waterborne diseases, but others – such as wastewater reuse or land disposal of disposable diapers – are actually new routes created by modern human activities (Maier et al., 2000).

## Literature Review

This literature review is divided into three parts. The first part focuses on bacterial cross-contamination of food products, the second part focuses on bacterial endospore survival in relation to homes and hospitals and bacterial survival during laundering, and the third part focuses on the survival of *Ascaris* ova in biosolids and biosolid-amended soils.

### Part I Cross-contamination of food products

### Pathogen transmission in a domestic setting

As many as 80% of all common infections are acquired by transmission of pathogens from fomites to the environment (U.S. EPA, 2001). Since humans from developed countries

spend 35 to 90% of their time indoors, many of these infections are acquired within the home from sources brought in from outside (Kreiss, 1990). Once an infection is established within a home, it may be spread via person-person contact or via person-fomite-person transmission. Environments in the home such as the kitchen provide a nutrient rich environment which allows populations of bacteria to grow to high numbers (U.S. EPA, 2001).

Fomites are believed to play a significant role in the transmission of some pathogens (Barker, 2001; Boone and Gerba, 2005). Contaminated fomites can also lead to the cross contamination of foods (Bidawid et al., 2000). Studies using polymerase chain reaction (PCR) have shown that pathogens can rapidly contaminate the indoor environment (Boone and Gerba, 2005).

Hygiene plays an important part in the spread of pathogens in a domestic setting. A study by van Curtis and Cairncross (2003) showed that a 30 to 50% reduction of illness can occur by providing adequate hand washing facilities and encouraging good hand washing practices. Results such as these have also been seen when alcohol gel sanitizers were used to complement handwashing (White et al., 2003; Vessey et al., 2007).

**Cross-contamination of surfaces in a domestic setting**

Many fomites within a domestic setting can be a potential source for cross-contamination of food products, especially the kitchen (De Wit et al., 1979; Scott et al., 1981; Scott et al., 1982; Scott and Bloomfield, 1990; Bloomfield, 2003). One major source of pathogens such as *Salmonella* spp., *Escherichia coli*, and *Campylobacter* spp. has been shown to be dishcloths and

sponges (Scott et al., 1981; Scott et al., 1982). Scott et al. (1982) showed that approximately half of dishcloths and cleaning cloths collected in domestic kitchens have counts greater than 100 organisms/20cm$^2$, and 30% contained enteric bacteria. It has also been shown that during preparation of chickens contaminated with *E. coli* K12 in a domestic kitchen, 74% of kitchen dishcloths used for hygiene became contaminated (De Wit et al., 1979). Surfaces in delis and meat processing facilities have been found to be contaminated with both opportunistic and frank pathogens (Tebutt, 1986; Warriner et al., 2001; Kudirkienė et al., 2010). Tebutt (1986) found that 74% of cloths used for wiping food shop surfaces contained one or more of *E. coli*, *Staphylococcus aureus, Streptococcus faecalis,* and *Clostridium perfringens* with over 50% of cloths containing more than $10^5$ colonies of *E. coli*. The use of contaminated cloths to wipe hard surfaces may result in the contamination of hands, equipment, and other surfaces (Davis et al., 1968; Gilbert, 1969; Mackintosh and Hoffman, 1984).

The contamination of kitchen surfaces (example cutting boards, worktops, chopping boards, and refrigerators) has been examined (Scott et al., 1982). Ten to 24% of such surfaces are contaminated with >200 organisms/20cm$^2$, with 10 to 20% of these surfaces having contamination with enteric bacteria (Scott et al., 1982). Cutting and chopping boards are especially notorious for contamination (van Asselt et al., 2008; Tang et al., 2010). A study of 73 homes in which a case of salmonellosis had occurred (van Schothurst et al., 1978) demonstrated that in over half, the same serotype was isolated from environmental sites including worktops, sinks and towels. Humphrey et al. (1994) showed that *Salmonella enteritidis* PT4 could be

recovered from fingers and utensils, sometimes after washing, following the preparation of egg dishes using artificially contaminated eggs. The organism was recovered from work surfaces up to 24 hours after contamination. Tang et al. (2010) demonstrated that *Campylobacter jejuni* is readily transferred from raw chicken to cutting boards and from cutting boards to cooked chicken with transfer rates as high as 44.9%. Previous studies found contamination by improper cleaning of cutting boards, kitchen cutlery and hands to be 91%, 61% and 100%, respectively (Jay et al., 1999; Clayton et al., 2003; Redmond and Griffith, 2003).

Transfer of contaminants from one surface to another can lead to many contaminated areas within the home in short periods of time and increases the chances for infection (van Asselt et al*.,* 2008; van Asselt et al*.,* 2009). Avoiding using the same kitchen utensils when raw and cooked foods are prepared is the most important step to reduce the risk of contamination by food borne pathogens (Scott, 1996); however, consumers may also use the same surfaces to prepare raw and cooked foods. Scott and Bloomfield (1991) showed that *E. coli, Salmonella* spp., and *Staphylococcus aureus* were easily transferred from a soiled laminate surface to other surfaces via fingertips. All three organisms were able to survive for up to 4 hours on a laminate surface and continued to transfer to other surfaces for 1 to 2 hours after the initial contamination. The authors also showed that significant numbers of *E. coli, Salmonella* spp., and *S. aureus* were transferred from contaminated cloths to fingertips and to laminate surfaces for up to 48 hours after inoculation, with a potential for re-growth in contaminated cloths (Scott and Bloomfield, 1991).

**Importance of hygiene in the prevention of cross-contamination of food products in the home**

A study of sporadic *E. coli* O157:H7 infection due to hamburgers prepared at home implicated poor hand and surface hygiene (Mead et al., 1997). Case households were more likely to report not washing their hands or work surfaces and being in contact with raw beef. They were also more likely to report placing cooked hamburgers back onto an unwashed plate previously in contact with the raw beef (Mead et al., 1997). Another study examined 50 homes in which children under 4 years were known to be infected with *Salmonella* spp. (Schutze et al., 1999). In 34% of homes there was also illness in other family members at the same time. Environmental sources and infected family members and pets appeared to be much more significant risk factors than contaminated foods for the development of salmonellosis in these children (Schutze et al., 1999).

In many situations (e.g., for the cleaning of cooking and eating utensils and most particularly the hands, hygienic cleaning is achievable by the use of a detergent and hot water. However, since hygiene is achieved by mechanical removal of the microbes, soap and water is only effective if applied in conjunction with a rinsing process (Bloomfield, 2003). People readily assume that wiping using a cloth rinsed in soapy water to produce a visibly clean chopping board achieves a surface which is also hygienically clean. In reality, this may remove a large

proportion of the bacteria but also spreads residual bacteria in sufficient numbers around the surface and onto the cloth to be transferred to other surfaces (Scott and Bloomfield, 1990).

A study by Barker et al. (2003) further illustrates the importance of hygiene in the home to reduce cross-contamination and the risk of infection. The authors examined work surfaces in a simulated kitchen after preparing chickens artificially contaminated with *Salmonella enteritidis* PT4. It was found that washing contaminated work surfaces and hands with water and detergent alone did not reduce levels of *S. enteritidis* to an acceptably hygienic state. Only the inclusion of sodium hypochlorite resulted in a significant reduction of the organism. Use of sodium hypochlorite in a 1 minute wash with detergent resulted in a 87.1% reduction of *S. enteritidis* in comparison to the no wash control. Detergent and water alone resulted in only a 44.3% reduction (Barker et al., 2003).

The previous results demonstrate the importance of hygiene and that the use of stronger cleaning agents such as dilute sodium hypochlorite is necessary to reduce contaminants to acceptable levels and thereby prevent cross contamination in a domestic setting.

**Part II Endospore survival and transfer in homes, hospitals, and laundry**

**Endospores and bacterial survival**

Endospores (hereafter referred to simply as spores) are highly resistant, non-reproductive, bacterial morphotypes produced by organisms in the genera *Bacillus* and *Clostridium* – both of which include several pathogenic species. The formation of these morphotypes is initiated under stressful conditions (high pH, starvation, high cell density, etc.) (Liu et al., 2004). Dormant

spores exhibit incredible longevity and can be found in virtually every type of environment on Earth (Nicholson et al., 2000). Reliable reports exist of the recovery and revival of spores from environmental samples as old as 105 years (Gest and Mandelstam, 1987; Kennedy et al., 1994), and there recently appeared a somewhat more controversial report that viable *Bacillus sphaericus* spores were recovered from the gut of a bee fossilized in Dominican amber for an estimated 25 to 40 million years (Cano and Borucki, 1995). Such high resistance to environmental stresses has prompted the use of spores for studies on environmental control and infection prevention (Gest and Mandelstam, 1987; Crawford et al., 1996; Rogers et al., 2005). Spores make bacterial control difficult and increase the probability of infection by the producing organism due to their ability to survive unfavorable environments (Liu et al., 2004). Special cleaning methods (e.g., autoclaving, bleaching, and intense steaming) are needed for their control (Perez et al., 2005; Fawley et al., 2007). For these reasons, spore formers play an important role in both food spoilage and disease (Xiao et al., 2010).

Spores germinate under environmental conditions favorable to the producing organisms (i.e., anaerobic environments for *Clostridium* species and certain amino acids and growth factors as well as physical factors for *Bacillus* and *Clostridium*). Physical factors that induce germination are heat, high hydrostatic pressure and abrasion (Setlow et al., 1991; Moir et al., 2002; Setlow et al., 2001). The cascade of events in spore germination is irreversible and leads to the formation of a viable vegetative cell; *Clostridium* species germinate at a slower rate than *Bacillus* species and often require more nutrients/germinants (Peck et al., 2009).

**Endospores in healthcare and domestic environments**

Understanding spore transmission through the environment is important to control infection patterns often seen in healthcare facilities and in the home (Crawford et al., 1996). A study by Guerrero et al. (2011) examined the potential for the spread of *C. difficile* spores in a healthcare setting. The authors found that the acquisition of spores on gloved hands was just as likely after contact with commonly touched environmental surfaces (i.e. hospital room surfaces) as after contact with commonly examined patient skin sites (Guerrero et al., 2011). In addition, there was no significant difference in the number of *C difficile* colonies acquired on hands after contact with environmental and skin sites. These findings suggest a mechanism by which contaminated surfaces could play a major role in the transmission of *C difficile* in a healthcare settings. Although previous studies have not provided direct evidence of hand contamination with *C. difficile* after contact with surfaces, both Simor et al. (1993) and Wilcox et al. (2003) demonstrated a positive correlation between the percentage of positive environmental cultures and isolation of *C. difficile* from the hands of hospital personnel.

*C. difficile* occurs quite commonly in the home where the major sources are people who are colonized or have *C. difficile* associated diarrhea (CDAD) and domestic animals (Simor et al., 2002). Up to two thirds of infants carry *C. difficile* asymptomatically during first few months of life (thought to reflect acquisition from the hospital). The organisms are also harbored by up

to 3% of healthy adults and colonization rates are higher in the over 65 age group. Carriage of *C. difficile* in household pets is also quite common (Simor et al*.,* 2002). Although carriage appears to be transient, up to 23% of household pets are affected. In most cases, however, the strains carried by pets appear to be non-cytotoxigenic strains (Otten et al*.,* 2009). *C. difficile* also occurs quite frequently in the environment outside the home and can be isolated from farm animals and vegetables, though there is no data to indicate what proportion of these strains are toxigenic (Otten et al*.,* 2009). Another potential source of *C. difficile* in the home is on the surface of the uniforms of healthcare workers that are either kept at home or are brought home for laundering. A number of studies have shown that uniforms can act as a vector for transmission of pathogens such as *C. difficile* (Mulligan et al*.,* 1980; Kim et al*.,* 1981; Malamou-Ladas et al*.,* 1983).

**Pathogen inactivation during laundering**

Dissemination of microorganisms from fabrics to man can be brought about by bed-making, dressing, sorting laundry, and exercising (Church and Loosli, 1953; McNeil, 1963; McNeil, 1964). Several microorganisms (including Gram positive and heartier Gram negative species of bacteria) can survive the effects of laundering (Arnold, 1938). Most Gram negative bacteria and enteric viruses studied are inactivated during the drying process after laundering (Arnold, 1938; Wiksell et al., 1973). Survival of pathogenic microorganisms during laundering depends largely upon the wash-water temperature, wash-cycle design, and drying temperatures (Arnold, 1938; Wiksell et al., 1973). Arnold (1938) found that the use of several rinses and soap during laundering wash cycles significantly reduced the numbers of bacteria found in both wash

water and fabrics tested. The author also noted a significant decrease in the bacterial content of both fabrics and machinery when wash water temperatures higher than 40$^{o}$C were used in washing procedures (Arnold, 1938). A study by Wiksell et al. (1973) found that increasing wash water temperatures caused increasing inactivation on Gram positive and Gram negative bacteria as well as bacterial spores and viruses. It has also been noted that Gram positive organisms are more resistant to increasing temperatures than Gram negative organisms, with *Bacillus stearothermophilus* spores being the most resilient to the laundering process (Wiksell et al., 1972). These studies indicate that higher wash-water temperatures as well as the use of more disinfectants such as bleach are needed to prevent cross-contamination during laundering and to prevent the build-up of potential pathogens in laundering equipment.

**Part III Survival of *Ascaris* ova in biosolids and biosolid-amended soil**

***Ascaris* prevalence, lifecycle, and transmission**

*Ascaris lumbricoides* and *Ascaris suum* are very similar nematode (roundworm) parasites infecting humans and pigs, respectively (Dold and Holland, 2010). Morphologically indistinct, both parasites display a strong affinity for their respective hosts, but cross transmission studies have shown that *A. lumbricoides* can infect pigs and vice versa (Takata, 1951; Galvin, 1968). Studies have also shown that in areas non-endemic for these parasites (e.g., North America, Denmark), pigs may serve as a reservoir for *A. suum* which can infect humans, but the same is not true for endemic areas (Anderson, 1995; Nejsum et al., 2005).

*A. lumbricoides* is one of the most common parasites in the world, with an estimated 1.2 billion infected globally (Silva, 2003). This organism is most prevalent in sub-Saharan Africa, the Americas, China, and East Asia. Disease caused by *A. lubricoides* is referred to as ascariasis, and morbidity and serious health consequences resulting from infection affect 122 million people each year (Dold and Holland, 2010).

*A. lumbricoides* undergoes most of its life cycle in the human body. The organism is spread via the fecal-oral route through the ingestion of viable ova contaminating hands, food, or particularized dust (Silva, 2003). Following the ingestion of viable ova, larvae hatch in the small intestine and migrate to the cecum and proximal colon where they enter the mucosa (Murrell et al., 1997). The larvae then migrate to the liver via the portal blood supply, after which they traverse to the lungs. Advancement to the lungs occurs 6-8 days post infection (Roepstorff et al., 1997). Within the lungs, larvae penetrate the alveolar spaces and migrate to the pharynx, where they are swallowed, returning them to the small intestine (Dold and Holland , 2010). Larvae reach sexual maturity in the small intestine over a two month period and can remain active for up to one year before dying and/or being passed through the body via stool. During most of its life cycle, the adult females lay eggs that are excreted in the feces of the infected human. Upon exiting the body, ova may remain viable for several years in soil (up to 15 years in tropical soils), where they may eventually infect another human and repeat the life cycle (O'Lorcain et al., 2000). Feces from an infected human can contain 10,000 viable ova per gram of feces, while the female worm can produce over 200,000 ova daily (Dold and Holland, 2010).

*Ascaris* ova are resistant to environmental stresses because they are surrounded by several protective layers, which act as barriers to environmental conditions. Each layer is different and has defined structural and chemical characteristics (Wharton, 1980). The ova contain (from inside to outside) four layers: 1) a lipoprotein layer that is resistant to desiccation and that is impermeable to polar substances, 2) an intermediate chitinous layer that is mechanically rigid, 3) a vitelline layer that is composed of two high molecular weight glycoproteins, lending the eggshell further impermeability, and 4) an external layer composed of acid mucopolysaccharide/protein uterine that is almost impermeable (Wharton, 1980; Maya, 2010).

Infections caused by *A. lumbricoides* are usually asymptomatic, but an estimated 8-15% (120 – 220 million cases annually worldwide) cases result in morbidity (Dold and Holland, 2010). Symptoms of ascariasis can be classified as being acute or chronic. Infected persons tend to experience acute lung inflammation, dyspnea, and fever as a consequence of larval migration through pulmonary tissue. Abdominal distension and pain, nausea, and diarrhea are characteristic of chronic ascariasis (Crompton, 2001). Chronic infection with heavy worm burdens can have a negative impact on childhood development via appetite loss, lactose malabsorption, and impaired weight gain (Dold and Holland, 2010).

As a soil-transmitted helminth (STH), there are three major strategies for controlling the spread of *A. lumbricoides*: improvements in sanitation, health education, and anti-helminthic treatment. Long-term control and eradication of this parasite rely heavily on the safe disposal of

human feces. Sanitation works to interrupt transmission, prevent re-infection, and gradually reduce worm loads (Dold and Holland, 2010). Proper sanitation of human feces is becoming more important as more countries use domestic wastewater for irrigation as well as biosolids and excreta for soil fertilization and amending (Maya et al., 2010).

**Biosolids**

Domestic wastewater is primarily a combination of human feces, urine, and gray water. Gray water results from washing, bathing, and the preparation of meals. Fecal matter potentially containing pathogenic microorganisms enters community wastewater collection systems from sources like hospitals, embalming facilities, animal slaughtering operations, and dwellings (Maier et al., 2000). While these wastewaters are cleansed with treatment, pathogenic microorganisms largely become concentrated in the sludge. If that sludge is to be beneficially used on land and possibly come in contact with humans, it must be disinfected to protect the public health (Gerba et al., 2005).

Several steps are utilized at wastewater treatment facilities to ensure pathogen levels are reduced. The primary goal of wastewater treatment is the removal and degradation of organic matter under controlled conditions (Maier et al., 2000). Primary treatment is the first step in municipal sewage treatment and aims to separate large solids from the liquid component of wastewater. This is followed by secondary treatment which involves biological degradation in which the solids from the first step are decomposed by microorganisms and the level of pathogens is reduced. Secondary treatment results in the formation of a secondary sludge which

is added to sludge from primary treatment to yield biosolids (Maier et al., 2000). Biosolids are a major by-product of wastewater treatment processes. They can be further processed through screening, thickening, dewatering, and conditioning, which enhance the separation of solids from the liquid phase, and/or stabilization, which reduces the content of solids and inactivates pathogenic microbes (Maier et al., 2000). The sewage sludge must then be stabilized by additional treatment including aerobic digestion, which can result in the formation of Class A or Class B biosolids based on treatment parameters, and anaerobic digestion which results in the formation of Class B biosolids. Class A biosolids are treated to reduce the presence of pathogens to below detectable limits. Class A biosolids can be land applied without any pathogen-related restrictions at the application site. Class B biosolids are also treated to reduce pathogens but still contain detectable levels. Class B biosolids have site restrictions to minimize potential human exposure until environmental factors such as heat, sunlight, or desiccation have further reduced pathogen numbers (U.S. EPA, 1993; U.S. EPA, 1994; Maier et al., 2000).

Helminth ova are considered the main biological health risk when applying biosolids on agricultural soils (Jimenez, 2009). EPA guidelines require that the final biosolid product contains < 1000 fecal coliforms or < 3 *Salmonella* spp./4 g, < 1 pfu/4 g of enteric viruses, and < 1 helminth ova/ 4 g in order to be considered Class A. These requirements can be met through a variety of treatment processes or by the action of environmental factors and a set amount of time. Examples of the latter include: waiting 14 months before harvesting food crops whose eaten parts are above the ground and touch the sludge; waiting 20 months before harvesting food crops

whose eaten parts are below the ground, provided that the sludge was not incorporated into the soil; waiting 38 months before harvesting food crops whose eaten parts are below ground, provided that the sludge was incorporated into the soil; waiting 30 days before harvesting food crops and feed crops for animals or fiber crops; waiting 30 days before allowing animals to graze on the land; and waiting 1 year before harvesting turf, unless otherwise specified (U.S.EPA, 1994). In the past, focus has been placed on the survival of helminth ova in biosolids because they are the most difficult pathogens to inactivate. For this, the helminth *Ascaris* has been used as a standard by the EPA for determining if biosolids meet Class A requirements (U.S.EPA, 1994).

### *Ascaris* survival in biosolids and biosolid -amended soil

*Ascaris* inactivation in biosolids has been examined based on several parameters including humidity, pH, temperature, and storage time (Jimenez, 2007).

**Humidity.** Humidity and moisture are important for the survival and development of *Ascaris* ova (Feachem et al., 1983). Gaasenbeek and Borgsteede (1998) have shown that the survival of *Ascaris* ova increases significantly with increasing humidity. The authors observed a survival of 96% of ova after 12 weeks of incubation at a relative humidity of 100%. The survival of ova dropped to 62% after 12 weeks of incubation at a relative humidity of 75%. Furthermore, the survival dropped to 0% after 10 weeks of incubation at 47.5% relative humidity.

**pH.** *Ascaris* ova are highly resistant to changes in pH (Feachem et al., 1983). Studies have shown that *Ascaris* ova can survive for up to 3 months in biosolids at a high pH (from 9 to 12.6) with a constant temperature of 45$^{o}$C (Storm et al., 1981; Eriksen et al., 1995).

**Temperature.** The effects of temperature on developing *Ascaris* ova within biosolids are well documented (Schmidt and Roberts, 1981; Johnson et al*.,* 1998; Kato et al*.,* 2003; Capipizi-Banas et al*.,* 2004). Most of this data is qualitative rather than quantitative, but some trends may be seen (Maya et al., 2010). At a temperature of 35$^{o}$C, ova inactivation has been shown to be 50% in one week and up to 95% in 5 weeks (Johnson et al*.,* 1998). As temperatures are increased, ova inactivation increases (Maya et al*.,* 2010). When the temperature approaches 50$^{o}$C, complete inactivation of *Ascaris* ova may occur within as few as 3 days (Kato et al*.,* 2003; Capipizi-Banas et al*.,* 2004).

**Storage.** *Ascaris* ova in stored sludge have been shown to be highly resistant to inactivation (Jimenez, 2007). A study by Strauss et al. (1990) determined shown that long-term storage of *Ascaris* ova in biosolids at temperatures between 17 and 20$^{o}$C can cause complete inactivation of all ova after one year.

*Ascaris* survival in the soil when directly deposited has been observed (Feachem et al., 1980). Such survival in soil depends largely upon the soil type, temperature and moisture content (Feachem et al., 1980). As with other organisms, *Ascaris* ova fair better in clay soil which holds moisture better than a sandy soil (Feachem et al., 1980). When incubated at temperatures between 20$^{o}$C and 38$^{o}$C, survival times for *Ascaris* ova have been shown to be less than 90 days

in sandy soil and greater than 90 days in a clay soil (Feachem et al., 1980). Moisture is of great importance due to the differences in survival times for ova incubated in moist soil conditions (survival for 2 years) versus ova incubated in dry soil conditions (survival for less than ninety days) (Feachem et al., 1980). Again, most of these experimental values are qualitative (viable or non-viable) rather than quantitative (total ova counts) and only provide a small picture of how the soil environment affects the population of ova.

**Occurrence of *Ascaris* in biosolids within the U.S.**

Approximately 5.5 billion kg (6 million dry tons) of biosolids are produced annually in the United States, of which 60% are used for land application, with the vast majority being Class B biosolids (NRC, 2002). In a 2010 study of 18 wastewater treatment facilities in 10 different states, Pepper et al. (2010) found no *Ascaris* ova in any of the sample biosolids from facilities typical of others in the United States (Pepper et al., 2010). An older study by Pederson et al. (1981) found the *Ascaris* content in U.S. sludge to be between 1.4 and 9.7 viable helminth ova per gram of total solids. These lower numbers of ova within sewage sludge is mirrored by a low prevalence (0.8%) of infection in the United States (Jimenez, 2007).

**Methods for the isolation of *Ascaris* from environmental samples**

Four methods are available for the detection and enumeration of *Ascaris* ova in biosolids: the method recommended by the United States Environmental Protection Agency (EPA), the Tulane method, the Kato-Katz method, and simple gravity-sedimentation (Bowman et al., 2002; U.S. EPA, 2003; Goodman et al., 2007). Some of these methods have been modified for more

accuracy such as the Wisconsin flotation method (Cox and Todd, 1962), which uses a sugar solution gradient referred to as Sheather's sucrose solution, as a substitute for the EPA's testing protocol. The modification of these methods has yielded recoveries of ova (as compared to a known control) as high as ~90% compared to the 60% to 70% recovery for the EPA and Tulane methods (Goodman et al., 2007).

The EPA and Tulane test methods have been used to concentrate pathogenic *Ascaris* ova from wastewater, sludge, and compost. Samples are processed by blending with buffered water containing a surfactant. The blend is screened to remove large particulates. The solids in the screened portion are allowed to settle out and the supernatant is decanted. The sediment is subjected to density gradient centrifugation using magnesium sulfate (specific gravity 1.20). This flotation procedure yields a layer likely to contain *Ascaris* and some other parasitic ova, if present, in the sample. Small particulates are removed by a second screening on a small mesh size screen. The resulting concentrate is incubated at 26$^{o}$C until control *Ascaris* eggs are fully embryonated. The concentrate is then microscopically examined for *Ascaris* ova on a Sedgwick-Rafter counting chamber (U.S. EPA, 2003).

The Kato-Katz Method is the oldest protocol for detecting the presence of helminth eggs in feces. In this method, a small amount of fecal material is placed on newspaper or scrap paper and a piece of nylon screen is pressed on top so that some of the feces sieve through the screen and accumulate on top. A flat-sided spatula is scraped across the upper surface of the screen to collect the sieved feces. A template is placed on the slide and the sieved feces are added with the

spatula so that the hole in the template is completely filled. The spatula is passed over the filled template to remove excess feces from the edge of the hole. The template is removed carefully so that a cylinder of feces is left on the slide. The fecal material is covered with a pre-soaked cellophane strip. The slide is then inverted and the fecal sample is pressed firmly against the hydrophilic cellophane strip to spread evenly. The slide is placed on the bench with cellophane upwards to enable the evaporation of water while glycerol clears the feces. While the Kato-Katz method has shown consistent success in detecting *Ascaris* eggs, it has not been utilized on soils and composts (Goodman et al., 2007).

The simple gravity sedimentation method uses two grams of stool placed in a 100 ml polypropylene cup. The stool is then macerated in approximately 50 ml of tapwater to create a fecal slurry. The slurry is passed through a tea strainer into a polycarbonate champagne flute (a stem glass with a tall, narrow bowl similar to a champagne glass). A wooden tongue depressor is used to compress the slurry remaining in the strainer to ensure all excess water passes through the strainer into the flute. Approximately 50 ml of water is added to the cup that holds the slurry, which is then passed through the strainer into the flute. Water is added to the flute until it is full, and it is then left to sit for 30 minutes. The supernatant is removed from the sedimented material. The flute is then topped with water and left undisturbed for 30 minutes. The supernatant is removed from the sediment, and the sediment is transferred to a petri dish. Water is then added to the flute, swirled, and transferred to the petri dish. This process is repeated until all sediment is transferred to the petri dish. The dish is then placed on the stage of an inverted microscope for

examination. To assist with reading, a frame to fit within the mechanical stage and around the petri dish is cut from cardboard. The sediment in the dish should be allowed to settle for 2–3 minutes before reading. To obtain an estimated number of eggs per gram, total egg counts are divided in half (Goodman et al., 2007).

An important difference between the simple gravity sedimentation method and the EPA/Tulane Methods is the focus of test application. While the EPA, Tulane, and their modified methods focus on the number of ova contained in biosolids, the simple gravity sedimentation method focuses on pinpointing infected individuals for medical treatment (Bowman et al., 2002; U.S. EPA, 2003; Goodman et al., 2007).

## DISSERTATION FORMAT

The appendices of this dissertation report the findings of three separate studies undertaken by the candidate: A) Assessment of the potential for cross-contamination of food products by reusable shopping bags, B) Survival and transfer of bacterial endospores during household laundering: A risk assessment, and C) Survival of *Ascaris* ova in desert soils: A risk assessment. Appendices are in manuscript format and appendix A has been published in Food Protection Trends volume 31.

The dissertation author was responsible for all the research presented in the manuscripts in appendices A, B, and C.

## PRESENT STUDY

The studies presented here evaluate the survival of different types of pathogens in a variety of environments. The study in Appendix A focuses on the presence of enteric bacteria in reusable shopping bags. We demonstrated that ninety-seven percent of the individuals surveyed never washed their reusable shopping bags. This study also illustrates that this lack of washing can lead to the buildup of potentially harmful bacteria such as *Salmonella* spp. and *E. coli* when reusable shopping bags were stored under warm conditions. The hygiene intervention of hand-washing the bags was demonstrated to reduce seeded organisms to below detectable limits.

Appendix B examines the survival of *Bacillus atrophaeus* endospores during household laundering. In this study it was demonstrated that detergent alone did not significantly reduce the number of viable endospores or their transfer to other garments. Bleach was necessary to significantly reduce the number of viable endospores and their transfer to other garments. The risk for cutaneous infection from *Bacillus anthracis* endospores due to handling contaminated laundry were calculated based on the endospore inactivation observed in this study. The risks for infection were significantly lower when bleach was used during laundering.

Appendix C details the survival of *Ascaris* ova in biosolid-amended Brazito sandy loam and clay loam soils. The survival of *Ascaris* ova was significantly higher in clay soil than in sandy soil. Ova inactivation increased with increasing temperature. These data was used to

estimate the risk for *Ascaris* infection from consuming raw lettuce grown on such soils. It was found that the annual risks for infection decreased significantly with time following harvest. The results found are in agreement with previous studies estimating the risk for *Ascaris* infection under such conditions.

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# APPENDIX A:

# ASSESSMENT OF THE POTENTIAL FOR CROSS CONTAMINATION OF FOOD PRODUCTS BY REUSABLE SHOPPING BAGS

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**Abstract**

The purpose of this study was to assess the potential for cross contamination of food products by reusable bags used to carry groceries. Reusable bags were collected at random from consumers as they entered grocery stores in California and Arizona. In interviews it was found that reusable bags are seldom if ever washed and often used for multiple purposes. Large numbers of bacteria were found in almost all bags and coliform bacteria in half. *Escherichia coli* were identified in 8% of the bags as well as a wide range of enteric bacteria, including several opportunistic pathogens. When meat juices were added to bags and stored in the trunks of cars for two hours the number of bacteria increased 10-fold indicating the potential for bacterial growth in the bags. Hand or machine washing was found to reduce the bacteria in bags by >99.9%. These results indicate that reusable bags can play a role in the cross contamination of foods if not properly washed on a regular basis. It is recommended that the public needs to be educated about the proper care of reusable bags by printed instructions on the bags or through public service announcements.

## Introduction

Most foodborne illnesses are believed to originate in food prepared or consumed in the home (Buzby and Roberts, 2009; van Asselt et al., 2008). Cross contamination of foods during handling is one of the factors leading to this assumption. Cross contamination occurs when disease causing microorganisms are transferred from one food to another. For example, raw meat products are often contaminated with food borne bacteria such as *Salmonella* and *Campylobacter* (Buzby and Roberts, 2009). While cooking these foods usually destroy these bacteria, they may be transferred to other foods which may be consumed uncooked, or contaminate the hands of consumers and be directly transferred to the mouth resulting in infection. Transfer may occur by surfaces such as cutting boards, kitchen counter tops and by the hands (Buzby and Roberts, 2009). Reusable bags for transport of groceries from the store to the consumer's home have become popular in recent years. Since these bags are often reused, and used potentially for multiple purposes, the possibility for contamination of food products as well as the consumer's hands exists. The goal of this project was to assess the potential for reusable bags to cross contaminate foods carried in reusable bags.

## Materials and Methods

Collection and sampling of bags. Shopping bags were collected from consumers entering grocery stores from the San Francisco Bay area, greater Los Angeles and Tucson, Arizona.

Approximately 28 bags were collected from each location. Individuals were interviewed on bag usage, storage and cleaning procedures. In addition, five new unused bags purchased at local markets and four new plastic disposal bags were also tested. Bags were sampled using sponge-sticks (3M Corporation, St. Paul, MN) by swabbing the entire inside of the bag. Three ml of fluid was extracted from the sponge-stick by squeezing it from the sponge in a plastic bag.

Bacterial assays and identifications. Total heterotrophic plate count bacteria were determined by dilution of samples in buffered peptone water and spread plating on R2A media (Difco, Sparks, MD). This media is designed to enhance the recovery of stressed bacteria. The plates were incubated for five days at room temperature and colonies counted. Coliform and *Escherichia coli* bacteria were identified by placing one ml of the sponge sickle extract into 99 ml of Colilert media (IDDEX, Westbrook, ME) and placement in a quantitray system and inoculation overnight at 37$^{o}$C. Coliform and *E. coli* numbers were then determined using a most probable number (MPN) table provided by the manufacture. Identification was conducted by diluting positive quanti-tray samples on MacConkley's agar (Difco) to confirm the presence of coliform bacteria, since the Colilert media is not specially designed for isolation of coliform bacteria from fomites. Colonies of different morphology were selected and subcultured on Trypticase Soy Agar (Difco). The bacteria were then identified using APIE20 strips (Biomerieux, Durham, NC). *Salmonella* isolation was attempted by inoculation of one ml of sponge-stick extract into 9 ml of buffered peptone water and incubation for 24 hours at 35$^{o}$C and then subcultured into Rappaport-Vassiliadis media (Difco) and incubated at 35$^{o}$C for 24-48

hours. Positive samples, samples containing growth, were then subcultured on both Hektoen and XLD agar (Difco) at 35$^{o}$C for 24 to 48 hrs. *Listeria* isolation was attempted by inoculation of one ml of sponge sickle extract into 9 ml of UVM media (Difco) and incubated at 30$^{o}$C for 24-48 hrs. This was then passed in Frasier's broth (Difco) and incubated at 35$^{o}$C for 24-48 hrs. The broth was then streaked onto RAPID'L.mono Agar (Bio-Rad, Chicago, IL) for isolation of *Listeria*.

Assessment of bacterial growth in stored bags. To assess the potential for bacterial growth in stored reusable bags, raw chicken and beef were hand wiped with sterile gloves and the resulting juices collected in a beaker. The solution was then spiked with approximately $10^{6}$ *Salmonella typhimurium* from an overnight culture. This was then added to 8 by 7 cm swatches cut from reusable grocery bags and placed in a Ziploc bag. Half of the swatches were processed immediately by cutting into one cm sq sections, placement in 10 ml of buffered peptone water, transferred to a stomacher bag, and processed for 15 minutes in a stomacher. The sample was then diluted and assayed on XLD media and R2A media. The other set of samples was placed in the trunk of a car for two hours during the mid afternoon. To determine the potential for growth of bacteria in the meat juices, another set of swatches was processed but *Salmonella* was not added. This experiment was repeated twice on two different days.

Effect of washing on reduction of bacteria in reusable bags. This phase of the study was designed to assess proper washing conditions to eliminate bacteria from reusable shopping bags. Reusable washable cloth bags were purchased at a local grocery store and spiked with *S. typhimurium* suspended in meat juices as described in the previous section. The bottom of the

bag and sides were spiked by adding 5 ml in 0.1 ml drops. The bags were then allowed to air dry for 30 minutes. One bag was processed immediately after drying by swabbing with a sponge-stick and processed as described previously. The sponge extract was assayed directly on XLD media at 37$^{o}$C for 24 hours and black colonies counted. An additional three bags were washed with a 30 minute wash cycle with a standard household detergent (61.1g) without bleach (Tide, Procter and Gamble, Cincinnati, OH). The bags were then placed in a dryer at 55$^{o}$C for 20 minutes. The bags were then sampled using a sponge sickle and assayed as previously mentioned. Another set of bags were treated in the same manner with a detergent containing bleach (Tide, Procter and Gamble, Cincinnati, OH)

To assess the effect of hand washing another set of bags was treated in the same manner and were hand washed and rinsed in a five gallon bucket containing water using rubber gloves and allowed to dry overnight before sampling. The bags were placed in the wash water containing detergent (Tide, Procter and Gamble, Cincinnati, OH) (11.3 g in 10 L) and allowed to soak for 30 minutes before hand washing. The experiment was repeated in duplicate. The effect of adding bleach was examined as mentioned above.

**Results**

Profile of bag use**.** Interviews indicated that half the bags were used more than one day per week (Figure 1) and that 75% of consumers neglected to separate meats and vegetables (Figure 2) and only 3% regularly clean their bags (Figure 3).

Bacteria detected in bags. No bacteria were detected in new cloth reusable bags and in new plastic bags obtained from grocery stores (data not shown). However, large numbers of bacteria were detected in reusable bags collected from consumers. HPC bacteria ranged from 45 to greater than 800,000 per bag. Only one bag was negative for HPC bacteria (< 30 colony forming units). Coliform bacteria were detected in 51% of the bags tested (Figure 3). In bags containing coliform bacteria, the numbers detected ranged from 3 to 3,330 per bag. HPC bacteria averaged 22,600 and coliform bacteria 576 (Figure 4). Greater numbers of bacteria and coliform bacteria were found in reusable bags collected in California than Arizona (Figure 5). This may be due to the drier climate in Arizona, which could affect bacterial survival. The greatest numbers of HPC and coliform bacteria were found in the Los Angeles area.

A wide variety of coliform bacteria were detected in the bags including *Escherichia coli*. *E. coli* was identified in seven bags (8% of bags tested). One bag was from Tucson, AZ and the other positives were from the Los Angeles area (Table 1). Many of the bacteria isolated are capable of causing opportunistic infections in humans. No *Salmonella* or *Listeria montocytogenes* were detected in any of the bags.

Assessment of bacterial growth in stored bags. Bacteria in bags to which meat juices were added did grow within two hours of storage. Within this time the number of bacteria increased 10-fold when the temperature was 47$^{o}$C inside the trunk (Table 2). When the temperature was 53$^{o}$C there was a decrease in the number of viable bacteria. The warm temperatures and presence of food in the bags can encourage rapid growth of bacteria.

Effect of washing on reduction of bacteria in reusable bags. Machine or hand washing even without the presence of bleach was effective in reducing coliform and other bacteria in the bags to levels below detection (Tables 3 and 4).

**Discussion**

It is estimated that there are about 76,000,000 cases of foodborne illness in the United States every year (Buzby and Roberts, 2009). Most of these illnesses are believed to originate in the home from improper cooking or handling of foods (Buzby and Roberts, 2009; van Asselt et al., 2008). Reusable bags if not properly washed during uses, create the potential for cross contamination of foods, especially when raw meat products and foods traditionally eaten uncooked (fruits and vegetables) are carried in the same bags, either together or between uses. This risk can be increased by the growth of bacteria in the bags. The results of this study indicate that large numbers of bacteria occur in reusable bags and are capable of increasing 10-fold in a trunk within a two hour period of time. Slightly more than half of the bags contained coliform bacteria, indicating contamination by raw meats or other uncooked food products. *E. coli*, used to indicate fecal contamination, was detected in 8% of the bags. The presence of these bacteria demonstrates reusable bags do get contaminated by enteric organisms and a risk from food borne pathogens does exist. Attempts to isolate *Salmonella* and *Listeria* bacteria from the bags were not successful in this study, but this may only represent the limited number of samples that were collected.

Greater numbers of bacteria were present in bags in California than in Arizona. A similar study in Canada also found fewer numbers of total bacteria and coliform bacteria in reusable bags (Health Canada, 2010) than were found in this study. The lower numbers of bacteria found in bags in the Canadian study may represent some differences in methods or that the warmer temperatures in California and Arizona encourage growth of the bacteria in the bags. The greater numbers of bacteria in the bags in California vs. Arizona may reflect the higher relative humidity in California.

Contamination of raw meat products with *Salmonella*, *Campylobacter* and *E. coli* is common. Studies have shown that children are at increased risk of both *Salmonella* and *Camplyobacter* infections if they ride in a shopping cart carrying meat products and eating fruits and vegetables prepared in the home (Fulterton, 2007; Jones et al., 2006). This suggests that improper handling of raw food products during shopping and transport to the home is a route of exposure for the transmission of these pathogens. Package meats can leak during transport and contaminating the bag. In addition, pathogenic bacteria can also occur on the outside of the packaged meats (Harrison et al., 2001). The common use of bags for other purposes than carrying groceries is also a potential concern. Transporting gym clothes or other clothing may result in cross contamination of bacteria such as MRSA (methicilin resistant *Staphylococcu aureus*).

Cross contamination problems associated with reusable bags for carrying groceries has been recognized by health departments (Health Canada, 2010; Minnesota Department of Health,

2007), and they have made recommendations about proper handling and cleaning. In this study it was demonstrated that hand and machine washing were able to reduce the bacteria in the bags below detection. Unfortunately, almost no one interviewed ever washed their reusable bags. Public unawareness of the potential risks seems almost universal. Approaches such as printed instructions on reusable bags for cleaning between uses or the need to separate raw foods from other food products, public service announcements, and health advisories are recommended. With increased use of reusable grocery bags, more research is needed to elucidate the bacterial profiles of bags in certain areas and to further illustrate the risk of transmission of some of these potential pathogens.

**Conclusions and Recommendations**

- Consumers almost never wash reusable bags
- Large numbers of bacteria were found in 99% of reusable bags tested, but none in new bags or plastic bags
- Coliform bacteria including *E. coli* were found in half of the bags tested
- Bacteria were capable of growth when stored in the trunks of cars
- A potential risk of bacterial cross contamination exists from using reusable bags to carry groceries
- Hand or machine washing reduced the numbers of bacteria in reusable bags by >99.9%
- Requiring printed instructions on reusable bags that they be washed between uses or the need to separate raw foods from other food products

**Acknowledgments**

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**Table 1. Identity of coliform bacteria detected in reusable bags**

| Type of Coliform | Number of bags detected |
|---|---|
| *Leclercia Adecarboxylata* | 1 |
| *Enterobacter aerogenes* | 1 |
| *Enterobacter cloacae* | 2 |
| *Enterobacter sakazakii* | 1 |
| *Escherichia vulneris* | 4 |
| *Escherichia coli* | 7 |
| *Klebsiella pneumoniae ssp pneumonia* | 6 |
| *Pantoea spp 3* | 4 |
| *Serratia ficaria* | 8 |
| *Serratia (ruidae or plymuthica)* | 1 |

**Table 2. Effect of Car Trunk Storage on the Growth of Bacteria in Reusable Bags (HPC)**

| Trial | Trunk Temperature ($^{o}$C) | Colony forming units (CFU)* | |
|---|---|---|---|
| | | Before | After |
| 1 | 47 | 7.11 +/- 0.026 | 8.19** +/- 0.105 |
| 2 | 53 | 7.17 +/- 0.025 | 6.25** +/- 0.088 |

*Colony forming units (Log Transformed)
**P≤0.001

**Table 3. Effect of Washing on Bacterial Reduction in Reusable Cloth Bags: Without Bleach**

| Wash Method | Before* | | After* | |
|---|---|---|---|---|
| | HPC | *Salmonella* | HPC | *Salmonella* |
| Machine | 5.33 | 5.89 | <1.48** | <1.48** |
| Hand | 5.48 | 5.47 | <1.48** | <1.48** |

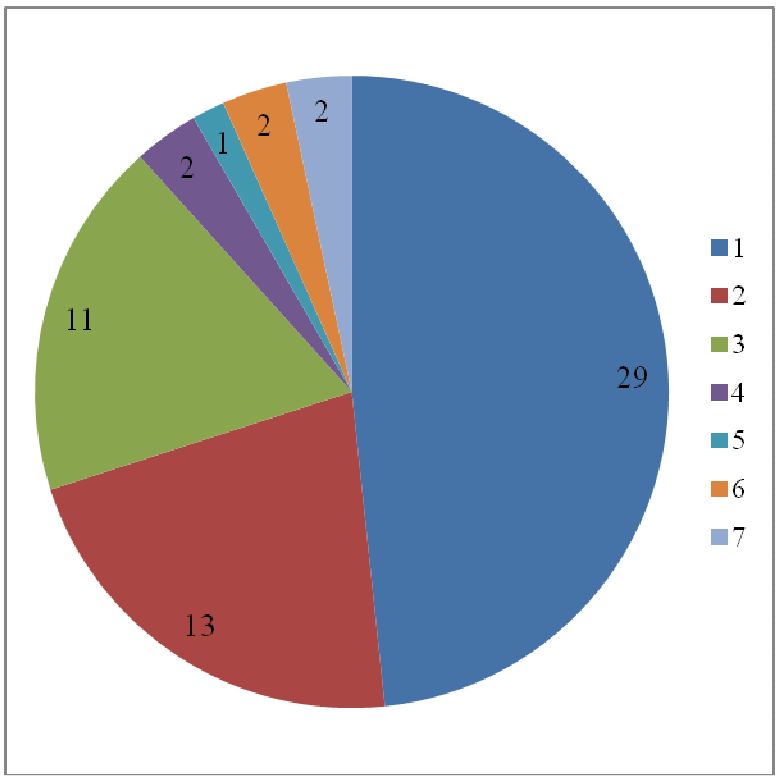
*Colony forming units (Log Transformed)
**P≤0.001

**Table 4. Effect of Washing on Bacterial Reduction in Reusable Cloth Bags: With Bleach**

| Wash Method | Before* | | After* | |
|---|---|---|---|---|
| | HPC | *Salmonella* | HPC | *Salmonella* |
| Machine | 4.95 | 4.66 | <1.48** | <1.48** |
| Hand | 4.58 | 4.79 | <1.48** | <1.48** |

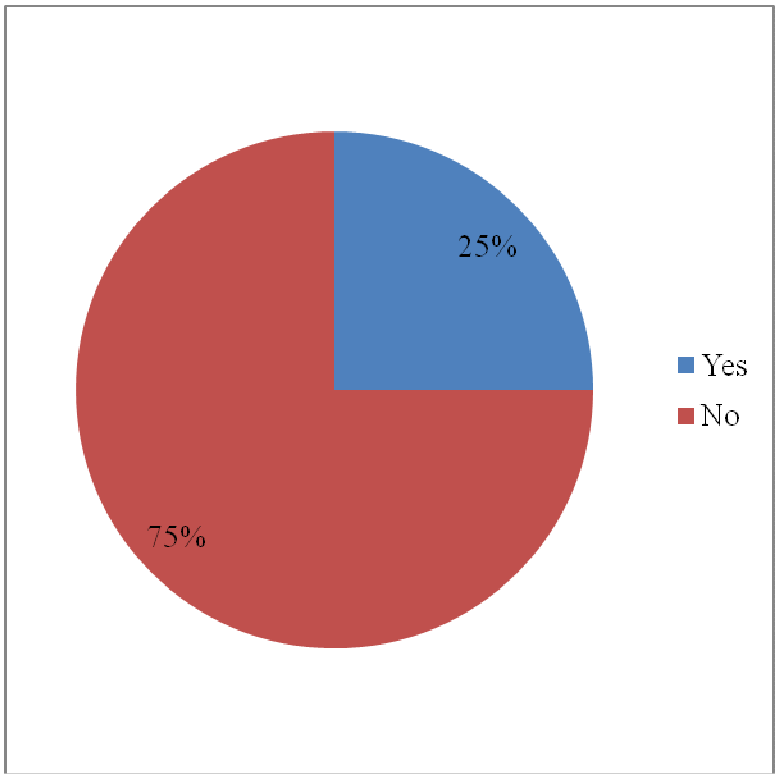
*Colony forming units (Log Transformed)
**P≤0.001

**Figure 1. Number of days per week reusable shopping bags were used**



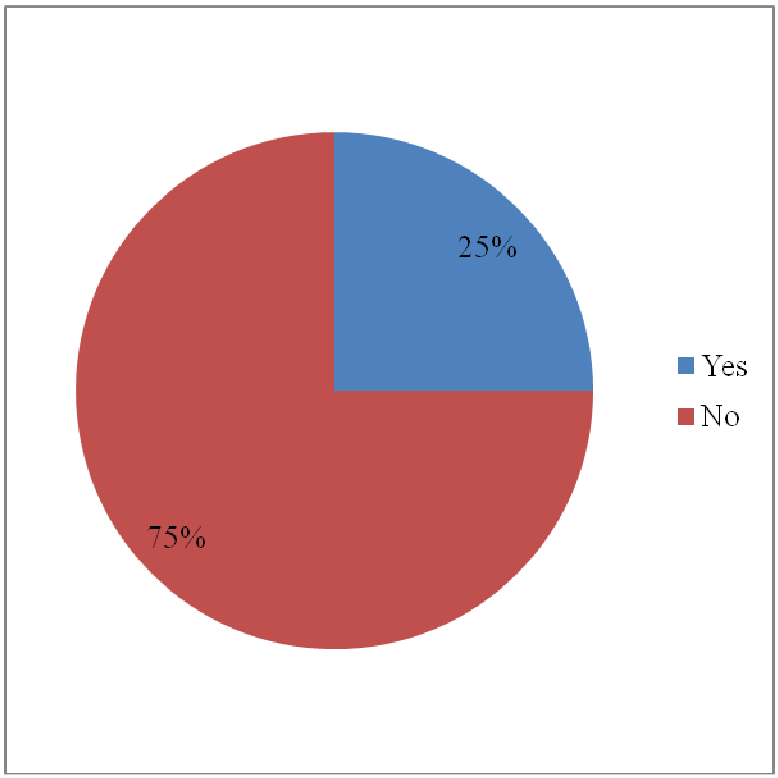

Interviews indicated that more than half of individuals used their reusable shopping bags more than one day per week.

**Figure 2. Percentage of consumers that used separate resuable shoppings bags for produce and meat**



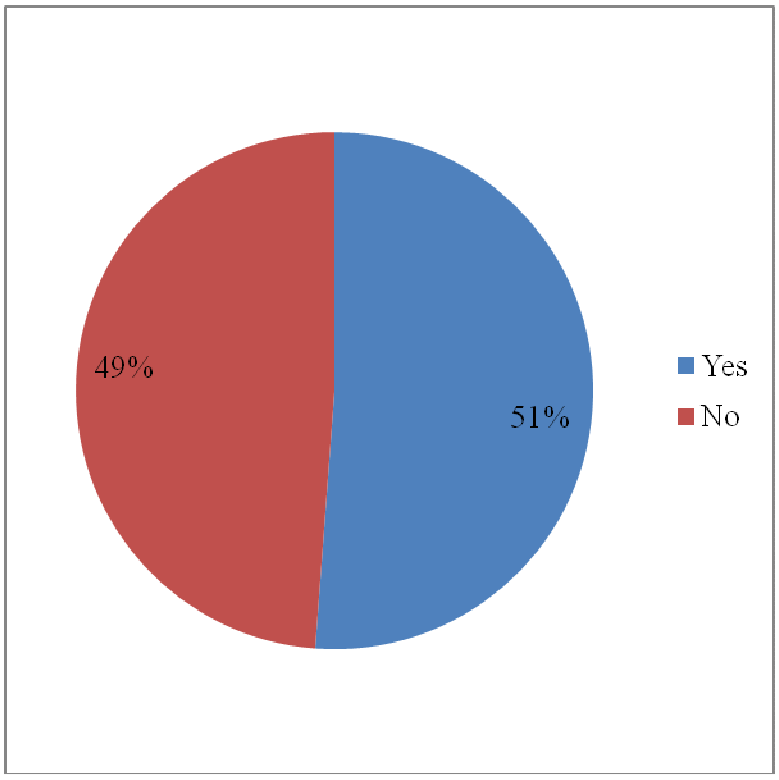

Seventy-five percent of individuals questioned do not use separate bags for meats and vegetables.

**Figure 3. Percentage of consumers who cleaned their resuable shopping bags regularly**



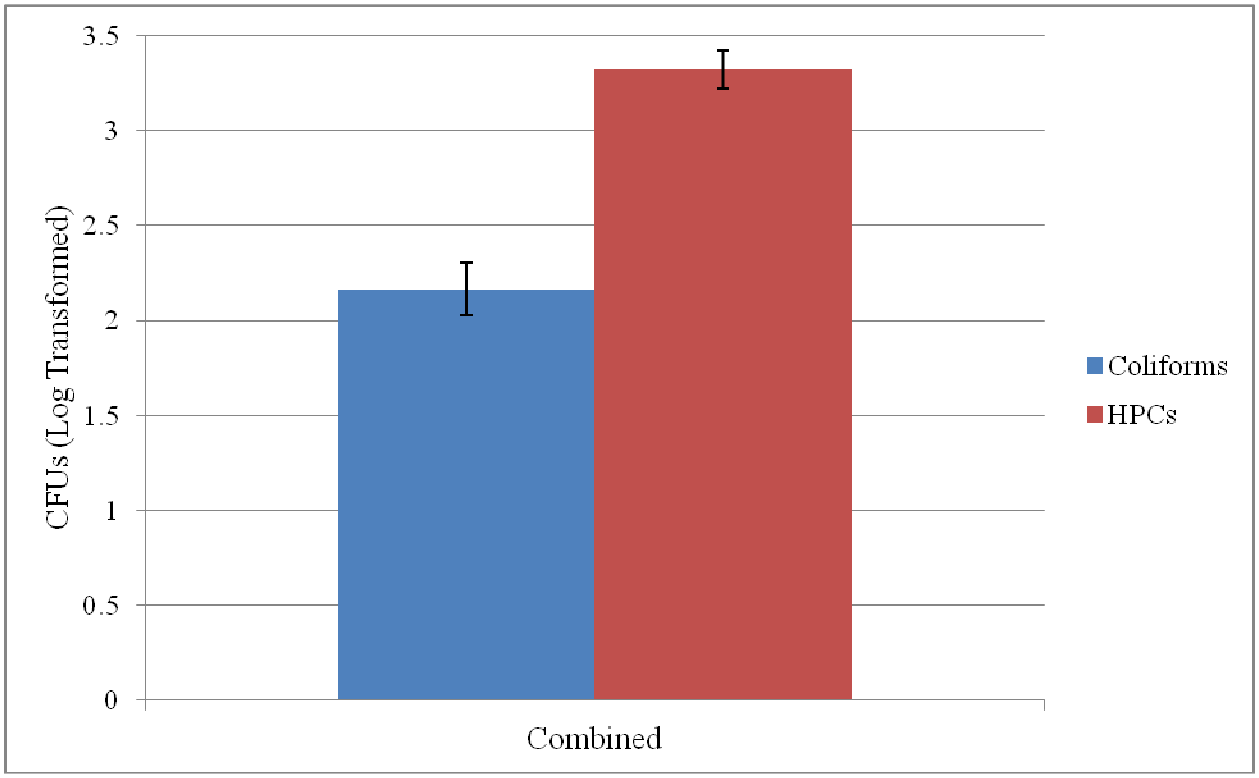

Ninety-seven percent of individuals interviewed admitted that they never wash their reusable bags.

**Figure 4. Percentage of reusable shopping bags found to have coliforms**



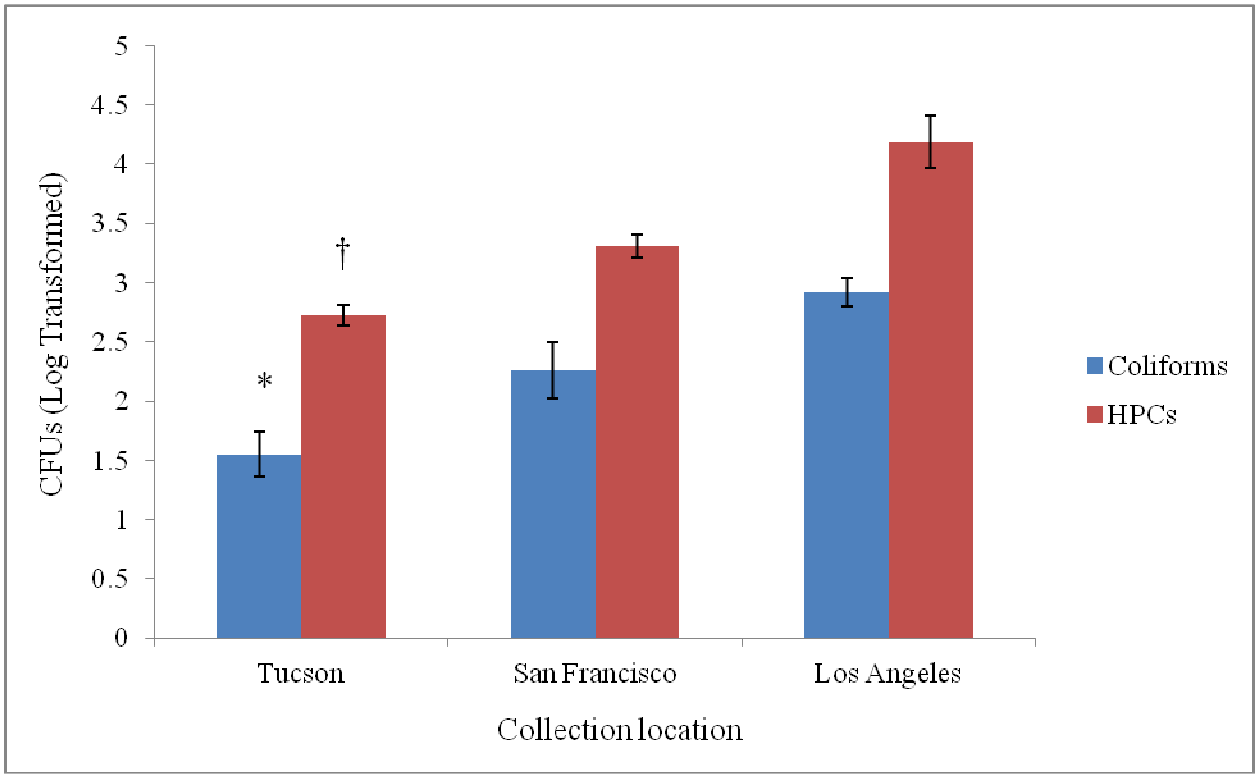

More than half of the bags processed were positive for the presence of coliforms with *Escherichia coli* making up 8% of the coliform pool.

**Figure 5. Coliform and HPC bacteria found on reuseable shopping bags for all collection sites.**




HPC bacteria ranged from 45 to greater than 800,000 per bag with an average of 22,600. ). In bags containing coliform bacteria the numbers detected ranged from 3 to 3,330 per bag with an average of 576.

**Figure 6. Average coliforms and HPC bacteria detected in reusable shopping bags by collection location**




Greater numbers of bacteria and coliform bacteria where found in reusable bags collected in California than Arizona – most likely attributable to the milder climate in California. One way ANOVA with Tukey Posthoc: $P \leq 0.05$

*: $P \leq 0.05$ Tucson Coliforms vs. San Francisco and Los Angeles

†: $P \leq 0.05$ Tucson HPCs vs. San Francisco vs. Los Angeles

# APPENDIX B

## SURVIVAL AND TRANSFER OF BACTERIAL ENDOSPORES DURING HOUSEHOLD LAUNDERING: A RISK ASSESSMENT

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**Abstract**

The purpose of this study was to assess the transfer potential and survival of bacterial endospores during household laundering practices. The data obtained were used to estimate the risk for infection from *Bacillus anthracis* endospores while handling contaminated laundry and the impact of using chlorine bleach. *Bacillus atrophaeus* spores were used as a surrogate for *B. anthracis*. We also examined the use of silver in conjunction with bleach to determine if any further reduction in viable endospores could be obtained using this combination. Less than a one $\log_{10}$ reduction of endospores occurred during laundering. When bleach was used this resulted in a 1.92 $\log_{10}$ reduction. Silver had no significant effect on endospore inactivation with or without the addition of bleach. The risk of infection from laundering was found to be 10 to 100 fold less when bleach was used.

**Introduction**

The survival of pathogenic bacteria and viruses during laundering has always been of interest (Nordstrom et al., 2011). It has been shown that some bacteria and viruses are capable of survival and transfer to uncontaminated clothing during household laundering (Nordstrom et al., 2011). Many pathogens and opportunistic pathogens may survive both washing and drying of fabrics (Wiksell et al., 1973). Bacteria can persist on fabrics for six months or longer and contaminated fabrics have been linked to outbreaks of Q-fever, salmonellosis, and other gastrointestinal infections (Buford et al., 1977; Standaert et al., 1994). Wash water temperature plays an important role in killing microorganisms during laundering (Wiksell et al., 1973; Jaska et al., 1980; Christian et al., 1983). Hospital laundering practices once held that water temperature must be ~75$^{o}$C, but more recent studies have shown that wash water temperatures ranging from 47$^{o}$C to 60$^{o}$C may be used as effectively. The one caveat to lower temperatures is that higher concentrations of chlorine bleach and/or detergents must be used (Christian et al., 1983). Substantial survival of *B. stearothermophilus* and *Staphylococcus aureus* at a wash water temperature of 68$^{o}$C and *Serratia marcescens* at a temperature of 46$^{o}$C has been demonstrated by Wiksell et al. (1973). The potential for transfer of organisms surviving the wash cycle and contaminating washers is high when cooler water temperatures are used (Buford et al., 1977; Christian et al., 1983). Many cleaning agents have higher efficacy at elevated temperatures, and

the use of lower temperature washes can lead to accumulations of pathogens in the washer (O'Toole et al., 2009). Reducing the number of bacteria in the washer is not only a concern for individual consumers but also for those individuals using self-service laundering facilities. The potential for transfer of pathogenic organisms from one household to another exists if improper sanitation measures are practiced in such facilities.

Bacterial endospores are environmentally resistant and have the potential of surviving the laundering process. Endospores (or simply, spores) are highly resistant, non-reproductive, bacterial morphotypes produced by organisms in the genera *Bacillus* and *Clostridium*, both of which include several pathogenic species. Spores are of concern because of their prevalence in hospitals and nursing homes (Guerrero et al*.,* 2011). A widespread outbreak of *Bacillus* infection at the National University Hospital of Singapore was linked to linens contaminated with spores of *Bacillus cereus*. Towels in the hospital were shown to be heavily contaminated with *B. cereus* spores even after laundering (Balm et al., 2011). Interventions focusing on laundry protocols (increased wash-water temperature, use of bleach, etc.) resulted in concentrations of *B. cereus* spores returning to baseline levels (Balm et al., 2011). *C. difficile* has also been shown to occur in the home, and has been linked to infants and domestic animals. These occurrences may have been a result of contaminated animal bedding and/or infant garments (Simor et al*.,* 2002).

Of added concern today is the presence of pathogens that could be used as bioterrorist agents. Clothing can be contaminated during exposure and the potential exists for cross contamination to other clothing during laundering. *B. anthracis*, the causative agent of anthrax,

is a Gram-positive spore forming bacterium is considered a Category A select agent by the Centers for Disease Control (Inglesby et al., 2002). Three modes of transmission exist for *B. anthracis* spores: inhalation, gastrointestinal, and cutaneous (Twenhafel, 2010). With an incubation period directly related to the exposure load and an infectious dose as low as one inhaled or contacted spore (Wilkening, 2008), cross contamination and possible spread to and within the home and other buildings may be a major concern for this lethal pathogen during a bioterrorism event or following an accidental release.

*Bacillus subtilis* has been used in the past to study the biochemical/molecular pathways which are common across the genus and as a model organism for *B. anthracis* sporulation due to its similarity and ease of culture ( Madigan et al., 2009; Greenberg et al*.,* 2010). The thermophile *Bacillus stearothermophilus* has been used in previous studies regarding the effectiveness of laundering in reducing spores (Wiksell et al., 1973), but other *Bacillus* species may be a better surrogates that are mesophiles (Madigan et al., 2009). *B. atrophaeus* has been used as a model for *B. anthracis* in more published studies than any other *Bacillus* species (Greenberg et al., 2010). *Bacillus atrophaeus* (formerly *Bacillus subtilis* var. *globigii* or *B. globigii*) (Greenberg et al., 2010) is an excellent model for such studies due to its fast growth rate and high spore production (Hayward et al., 1946). This organism produces a very distinctive, deep orange pigment which also allows for easier tracking during such studies (Hayward et al., 1946).

The purpose of this study was to assess the effects of wash water temperature and laundering with or without detergent, bleach and silver against *B. atrophaeus* spores. The data

obtained was then incorporated into a quantitative microbial risk assessment to determine the risks for someone handling laundry contaminated with spores under each washing scenario.

**Materials and Methods**

Lyophilized *B. atrophaeus* spores were suspended in tryptic soy broth (TSB, Difco, Sparks, MD) and subsequently streaked for isolation on tryptic soy agar (TSA, Difco). The plates were incubated at 37$^{o}$C for 48 hours to distinguish between strains producing rough ($F_2$) and smooth (S) colonies (Hayward et al., 1946). Rough and smooth colonies were selected and subcultured again for isolation. The strains producing these two colony types were shown by Hayward et al. (1946) to have differences in endospore production, with the rough strains producing approximately twice as many endospores. For this reason, the $F_2$ strain of *B. atrophaeus* was chosen for spore production in the current study.

Spores of this organism were produced using methods identical to those outlined by Nicholson and Setlow (Nicholson and Setlow, 1990). Sporulation Medium (DSM, previously referred to as Schaeffer's Sporulation Medium) (100 µl) was inoculated with an isolated colony after 18 to 24 hours of growth and inoculated on a DSM plate using the spread plate method and incubated overnight at room temperature (~25$^{o}$C). The resulting lawn was collected by addition of 5 ml of DSM broth and gentle scraping with a glass spreader. The $OD_{600}$ of the collected lawn was measured via spectrophotometry (Spectronic GENESYS 5, Milton Roy, Ontario, Canada) and the suspended lawn diluted in DSM broth to an $OD_{60}$ of 0.1. A DSM broth was then inoculated with the proper dilution of lawn (for an $OD_{600}$ of 0.1) and incubated until an $OD_{600}$ of

1.8 was reached (Time = 0; $t_0$). The spores were harvested after 24 hours of further incubation (Time = 24; $t_{24}$) after an examination of the culture via Gram stain and light microscopy to ensure that ≥ 90% cells contained endospores. The cells and spores were then heat shocked at 80°C for 10 minutes in 1.5 ml sterile Eppendorf tubes (Eppendorf, Hauppauge, NY) in a water bath, centrifuged (10,000 rpm for 10 minutes) in an Eppendorf 5415C microcentrifuge (Eppendorf, Hauppauge, NY), and resuspended in cold deionized water. The temperature difference allows for the release of spores from the parent cells. After centrifugation, the supernatant containing the spores was collected while the vegetative cell debris was discarded. The spores were washed by suspending in cold, deionized water and centrifuging as mentioned above 4-6 times to ensure release from the parent cells. The spores were stored in sterile deionized water in 15 ml conical tubes at 4°C.

Susceptibility to silver and bleach

Spores of *B. atrophaeus* were inoculated into 10 ml of deionized water containing bleach (31 µl scaled down from the manufacturer's instruction of 236 ml in a 70 L capacity washer) and incubated at room temperature and shaken in sterile 50 ml polypropylene conical tubes (VWR International, Radnor, PA). After 7.5 minutes, silver was added as 1 to 3000 mg/L $AgNO_3$ and the solution shaken for another 7.5 minutes. After the incubation period, spores were serially diluted in DE neutralizing broth (Difco, Sparks, MD) with 1% $Na_2S_2O_3$, heat shocked (80°C for 10 minutes), and assayed on TSA plates. All experiments were conducted in triplicate.

Washing machine experimental design

Cotton swatches, (five per experimental run) measuring 56 $cm^2$ (7 cm x 8 cm) were inoculated with approximately 2.0 X $10^7$ colony forming units (CFU) and allowed to air dry for 30 minutes. One of the swatches served as a control for the total inocula for four experimental swatches and was processed immediately (process described below). Four inoculated swatches and four sterile swatches were added to a Whirlpool front-loading washing machine (Duet steam model, Whirlpool Corporation, MI). Included in the load were 2.23 kg of sterile (autoclaved) pillowcases for the simulation of a normal load. One of the pillowcases contained a simulated organic load (336.6 g urea, 2.4 g triethoanolamine, 31.2 g sebum base, 3.78 g gelatin, 157.5 g black charm clay, and 336.6 g sodium chloride, 118.8 g calcium chloride, and 72.6 g magnesium chloride) to simulate a normal dirt load (Gerba and Kennedy, 2007). The wash cycle was run on a normal, quick wash of 30 minutes. This cycle consists of a 12 minute wash, a 10 minute rinse, and 8 minute spin. The water temperature was set at cold, which was measured as 77$^oC$ to represent how most individuals launder clothes in the home (Gerba and Kennedy, 2007). Detergent was added as per the manufacturers' instructions (61.1 g). In experiments including bleach, 236 ml (1 cup) of 6% sodium hypochlorite (chlorine bleach) was injected during the wash cycle. Materials were left in the washer for 30 minutes after the wash cycle was completed to duplicate the conditions commonly occurring in households. All materials were then placed in a dryer on high and allowed to dry for 30 minutes. The swatches were then cut into one $cm^2$ sections and stomached (Stomacher 3500, Seward, NE) for 10 minutes in Buffered Peptone Water (BPW) containing 1% $Na_2S_2O_3$ on medium speed to neutralize any residual free chlorine

which may have been present. The BPW was then serially diluted, heat shocked (80$^{o}$C for 10 minutes), and inoculated on tryptic soy agar using the spread plate method. Samples of wash drainage water were also collected and examined for the presence of spores. The total inoculums were calculated by multiplying the CFU calculated on the swatch processed immediately by the number of inoculated swatches added to each load. Percent recovery was calculated by dividing the total CFU recovered (CFU from the inoculated swatches + CFU transferred to the sterile swatches + CFU recovered from the drainage water) by the total inoculum. All experiments were performed in triplicate.

Quantitative Microbial Risk Assessment (QMRA)

A beta-Poisson model has been developed for *B. anthracis* to estimate the response to exposure on the calculations of Haas using animal models (Bartrand et al., 2008;Weir and Haas, 2011). The probability of infection can be expressed as $P(\text{infection}) = 1 - [\ 1 + \mathbf{d}(2^{1/\alpha} - 1)/\mathbf{N_{50}}]^{-\alpha}$. Where $\alpha = 0.55$ and $\mathbf{N_{50}}$ (median infective dose) = 28,472. The intranasal Guinea pig experiments (Altboum et al., 2002) were used as the dose response model as other models are more representative of an airborne outbreak event rather than spread via contaminated linens (contact). Dose is expressed as **d** in this model. Risk from multiple exposures is given by $P(\text{multiple}) = 1 - [1 - P(\text{infection})]^{n}$, where n = the number of exposure events. The variable, n, was given the value of one or ten to simulate one or ten exposures.

The median human incubation period and median monkey time-to-death versus *B. anthracis* dose has been determined by Wilkening (2008). Initiation of infection can occur with

as few as 360 spores inhaled or contacted spores, with an incubation period of nine days. The incubation period dramatically decreases with increased dose with a minimum of three days with doses of one million and 10 million spores (Wilkening, 2008).

The following assumptions were used to estimate the risk of infection resulting from spore contamination of clothing and/or laundry equipment:

- The concentration of spores which may be found on contaminated surgical scrubs or other clothing was based on previous studies with bacteria (Nordstrom et al., 2011)
- The log inactivation of spores in laundry with or without the use of bleach found in this study.
- The transfer efficiencies of spores from fabrics to hands (Gerardo Lopez, unpublished data)
- A one time or ten time exposure to spores during laundering

Details key assumptions may be found in Table 1.

Data and Statistical Analysis

A one-way ANOVA with was used to compare significance between laundering without bleach versus laundering with bleach. In the experiments with silver, two variables were examined – the use of bleach and silver. For this, a two-way ANOVA was used for analysis. For both statistical tests, a difference was considered to be statistically significant if $P \leq 0.05$.

**Results**

Washing with and without bleach. No significant reduction of spore viability occurred when using a standard wash cycle of 30 minutes, with detergent only (Table 2). Significant differences ($P \leq 0.05$) were seen between the use of detergent and bleach versus detergent alone (Table 2). The use of detergent alone resulted in only a 0.73-$\log_{10}$ reduction in viable spores after washing. The addition of bleach resulted in a 1.93-$\log_{10}$ reduction in viable spores after washing. The use of bleach also reduced the amount of viable spores transferred to sterile swatches with a 1.29-$\log_{10}$ greater transfer of CFU occurring when bleach was excluded (Table 2).

Silver had no significant effect on spore inactivation with or without the addition of bleach (Table 3).

Risk of *B. anthracis* infection from handling contaminated laundry. The one-time and ten-time risks of *B. anthracis* infection from handling contaminated laundry are shown in Tables 4 and 5. Table 4 shows the risks involved when laundering without the use of bleach while Table 5 shows the risks involved when laundering with the use of bleach. Significantly higher risks are seen in scenarios where bleach is excluded from washing. Risks calculated for scenarios where bleach is excluded are ten to 100 times higher (Tables 4 and 5) than those where bleach is used.

When bleach was excluded from washing (Table 4), the one-time exposure to *B. anthracis* spores through handling contaminated laundry leads to a risk of infection greater than one in 10,000 when spore concentration was at least 500 CFU/$cm^2$ and transfer efficiency was at

least 5%. At higher concentrations of spores (1,000 CFU/cm$^2$), risks were greater than one in 10,000 with transfer efficiencies as low as 1%. For ten exposure events, the risk of infection was greater than one in 10,000 with as little as 100 CFU/cm$^2$ and transfer efficiencies of 1%.

The inclusion of bleach in the laundering process resulted in a decreased risk of *B. anthracis* infection from handling contaminated laundry (Table 5). All calculated risks for infection for one-time exposure events were less than one in 10,000. For ten exposure events, the risk for infection was less than one in 10,000 except at high spore concentrations combined with high transfer efficiency rates. This demonstrates the need for bleach when laundering clothing suspected of being contaminated with spores.

**Discussion**

Environmental contamination is a common route of transmission for pathogens producing endospores. Isolation of contaminants may prove to be impractical or difficult if there are many people involved in handling or if the source of infection is unknown (Simor et al., 2002). Consequently, interrupting transmission via indoor fomites is one of the more practical methods for limiting or preventing infections (Bloomfield, 2003; van Curtis and Cairncross, 2003; Guerrero et al., 2011). Results from this study show that the use of bleach is necessary for a significant reduction of spores during household laundering with cold water. A maximum of 2-$\log_{10}$ reduction in viable spores was seen when bleach was used. Similar results have been observed in other laundry studies by (Barrie et al., 1994; Ohsaki et al., 2007). In an investigation of hospital linens by Barrie et al (2007), it was found that viable *Bacillus cereus* spores persisting

in laundered linens were responsible for outbreaks of meningitis. The spores were traced to prewash water entering the facility, and the study showed that hypochlorite intervention reduced the numbers of spores in wash-water, linens, and machines. Ohsaki et al. (2007) examined the use of bleach during laundering and its impact on the cross-contamination of *Bacillus cereus* spores. A concentration of 0.13% bleach (170 ml/135 L) was not enough to inactivate *B. cereus* spores or to prevent cross-contamination through laundering. As bleach levels increased, so did the inactivation of *B. cereus* spores. Concentrations closer to the manufacturer's suggested one cup (236 ml) of bleach resulted in a ten-fold decrease in spores; two cups of bleach reduced viable spores to below detectable limits (Ohsaki et al., 2007). Wiksell et al. (1973) found that detergent had no significant effect on the numbers of Gram positive and Gram negative organisms in polyester-cotton sheeting. It was also found that neither high wash-water temperature nor detergent had any significant effects on the viability of *B. stearothermophilus* spores in laundered polyester-cotton sheeting (Wiksell et al., 1973). This further demonstrates the need for chlorine bleach when spore contamination is suspected.

Use of silver with bleach did not have a synergistic antimicrobial effect on spores at low concentrations but had an antagonistic effect on bleach at higher concentrations leading to more spore survival. This antagonistic effect is likely due to silver nitrate reacting with bleach to form silver oxide and neutralizing sodium hypochlorite's mode of action.

The current study also demonstrates the potential for transfer of bacterial spores to other clothing items during laundering. There was a transfer of 3.7-$\log_{10}$ CFU of spores to sterile

swatches during laundering without bleach. The use of bleach reduced the transfer of spores to 2.41-$\log_{10}$ CFU. Barrie et al. (1994) found that *B. cereus* contamination in hospital linens. The authors illustrated that the use of high temperatures (80$^{o}$C) and detergent alone was not sufficient to inactivate and prevent the spread of spores to other garments during laundering. Similar results were seen in our study as detergent alone had no effect on spore viability. Our study further demonstrates the importance of the use of chlorine bleach in reducing spores and spore transfer during laundering.

Risks of infection increased with increasing spore concentration and were significantly higher when bleach was excluded due to increased spore survival under those conditions. Even at low concentrations of spores, a risk existed for infection. This is due to the fact that the infectious dose for *B. anthracis* is so low (as little as 1 to 360 spores) (Wilkening, 2008). These results indicate that more rigorous restrictions need to be made for hospitals which allow healthcare workers to launder scrubs at home to prevent the possible spread of spores in the home. This may also be of concern to homes with elderly or individuals who may be taking antibiotics since *Clostridium difficile* associated diarrhea (CDAD) is becoming more common (Simor et al., 2002). *C. difficile* causes diarrhea in individuals whose resident gastrointestinal flora have been disrupted by certain antibiotics (Simor et al., 2002).

The current study shows that unlaundered hospital scrubs harboring spores of *B. anthracis* pose a much greater risk of infection during laundering if bleach is excluded from standard washing practices. Likewise, *C. difficile* spores which may be present on unlaundered

scrubs might offer a higher risk of infection if bleach is excluded from the laundering process of these garments. Other studies should be done to further extend our knowledge of infection risk from spores on unlaundered scrubs and include scenarios for CDAD in the home. Furthermore, healthcare workers should be given specific instructions on how to properly launder their scrubs in the home or the facility needs to ensure that all the scrubs/contaminated clothing are washed on site.

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**Table 1. Assumptions used for QMRA**

| **Assumption** | Spore Concentration* | Spore Inactivation** | Transfer Efficiency† |
|---|---|---|---|
| **Details** | 1 - 1000 CFU/$cm^2$ | 0.73 $log_{10}$ or 1.93 $log_{10}$ reduction | 0.1% - 5% |

* Values used fall within the range of heterotrophic plate counts found for the bacterial content of home-laundered hospital scrubs (Nordstrom et al., 2011).
** Inactivation values for spore laundered in the presence or absence of chlorine bleach (current study).
† Transfer efficiency range of spores from fomites to fingers using cotton and polyester (Gerardo Lopez, unpublished data)

**Table 2. Survival and transfer of spores after washing**

| Treatment | Before washing and drying (CFU Log transformed) | After washing and drying (CFU Log transformed ± SEM) |
|---|---|---|
| Inoculated Swatch (No Bleach) | 7.38 ± 0.01 | 6.59 ± 0.06 |
| Sterile Swatch (No Bleach) | 0.00 ± 0.00 | 3.70 ± 0.11 |
| Inoculated Swatch (With Bleach) | 7.32 ± 0.05 | 5.39 ± 0.06 * |
| Sterile Swatch (With Bleach) | 0.00 ± 0.00 | 2.41 ± 0.27 † |

* = $P \leq 0.05$ for inoculated swatch (with bleach) vs. inoculated swatch (no bleach) after washing and drying.

† = $P \leq 0.05$ for sterile swatch (with bleach) vs. sterile swatch (no bleach) after washing and drying.

**Table 3. Effects of silver and bleach on spore survival**

| Treatment | After washing (CFU Log Transformed ±SEM) |
|---|---|
| Control 1 (Saline) | 10.38 ± 0.02 |
| Control 2 (Bleach) | 8.42 ± 0.03 |
| 500 ppb Ag(NO3) + Bleach | 8.31 ± 0.04 |
| 1 ppm Ag(NO3) + Bleach | 8.26 ± 0.04 |
| 5 ppm Ag(NO3) + Bleach | 9.36 ± 0.02 * |
| 10 ppm Ag(NO3) + Bleach | 9.46 ± 0.01 * |
| 3 ppt Ag(NO3) + Bleach | 10.31 ± 0.02 * |

*There was a statistically significant difference ($P \leq 0.05$)between the number of viable spores recovered in comparison to both control and the treatments with lower levels of $AgNO_3$ (i.e. 0.5 mg/l and 1mg/l).

**Table 4. Risk of infection from *B. anthracis* spores transferred to hands during laundering without bleach**

| Concentration of spores (CFU/cm$^2$) | Dose after wash | Transfer efficiency | Dose transferred to hands | One time exposure risk | Ten-time exposure risk |
|---|---|---|---|---|---|
| 1 | 0.27 | 0.001 | 2.70E-04 | 1.32E-08 | 1.32E-07 |
| 1 | 0.27 | 0.005 | 1.35E-03 | 6.59E-08 | 6.59E-07 |
| 1 | 0.27 | 0.01 | 2.70E-03 | 1.32E-07 | 1.32E-06 |
| 1 | 0.27 | 0.05 | 1.35E-02 | 6.59E-07 | 6.59E-06 |
| 100 | 27 | 0.001 | 2.70E-02 | 1.32E-06 | 1.32E-05 |
| 100 | 27 | 0.005 | 1.35E-01 | 6.59E-06 | 6.59E-05 |
| 100 | 27 | 0.01 | 2.70E-01 | 1.32E-05 | 1.32E-04 |
| 100 | 27 | 0.05 | 1.35E+00 | 6.59E-05 | 6.59E-04 |
| 500 | 135 | 0.001 | 1.35E-01 | 6.59E-06 | 6.59E-05 |
| 500 | 135 | 0.005 | 6.75E-01 | 3.29E-05 | 3.29E-04 |
| 500 | 135 | 0.01 | 1.35E+00 | 6.59E-05 | 6.59E-04 |
| 500 | 135 | 0.05 | 6.75E+00 | 3.29E-04 | 3.29E-03 |
| 1000 | 270 | 0.001 | 2.70E-01 | 1.32E-05 | 1.32E-04 |
| 1000 | 270 | 0.005 | 1.35E+00 | 6.59E-05 | 6.59E-04 |
| 1000 | 270 | 0.01 | 2.70E+00 | 1.32E-04 | 1.32E-03 |
| 1000 | 270 | 0.05 | 1.35E+01 | 6.58E-04 | 6.56E-03 |

**Table 5. Risk of infection from *B. anthracis* spores transferred to hands during laundering with bleach**

| Concentration of Spores ($CFU/cm^2$) | Dose after bleach wash | Transfer efficiency | Dose transferred to hands | One time exposure risk | Ten-time exposure risk |
|---|---|---|---|---|---|
| 1 | 0.01 | 0.001 | 1.00E-05 | 4.88E-10 | 4.88E-09 |
| 1 | 0.01 | 0.005 | 5.00E-05 | 2.44E-09 | 2.44E-08 |
| 1 | 0.01 | 0.01 | 1.00E-04 | 4.88E-09 | 4.88E-08 |
| 1 | 0.01 | 0.05 | 5.00E-04 | 2.44E-08 | 2.44E-07 |
| 100 | 1 | 0.001 | 1.00E-03 | 4.88E-08 | 4.88E-07 |
| 100 | 1 | 0.005 | 5.00E-03 | 2.44E-07 | 2.44E-06 |
| 100 | 1 | 0.01 | 1.00E-02 | 4.88E-07 | 4.88E-06 |
| 100 | 1 | 0.05 | 5.00E-02 | 2.44E-06 | 2.44E-05 |
| 500 | 5 | 0.001 | 5.00E-03 | 2.44E-07 | 2.44E-06 |
| 500 | 5 | 0.005 | 2.50E-02 | 1.22E-06 | 1.22E-05 |
| 500 | 5 | 0.01 | 5.00E-02 | 2.44E-06 | 2.44E-05 |
| 500 | 5 | 0.05 | 2.50E-01 | 1.22E-05 | 1.22E-04 |
| 1000 | 10 | 0.001 | 1.00E-02 | 4.88E-07 | 4.88E-06 |
| 1000 | 10 | 0.005 | 5.00E-02 | 2.44E-06 | 2.44E-05 |
| 1000 | 10 | 0.01 | 1.00E-01 | 4.88E-06 | 4.88E-05 |
| 1000 | 10 | 0.05 | 5.00E-01 | 2.44E-05 | 2.44E-04 |

# APPENDIX C

## SURVIVAL OF ASCARIS OVA IN DESERT SOILS: A RISK ASSESSMENT

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**Abstract**

The goal of this study was to determine the effects of temperature and soil type on the survival of *Ascaris* ova in two biosolid-amended, desert soils. Two soils with different soil textures were utilized: a sandy loam and a clay loam. Survival data was subsequently used to estimate the risks for *Ascaris* infection that would result from the consumption of vegetables grown on such soils under different conditions. In laboratory studies, ova viability decreased faster in the sandy loam soil. In 60 days, ova viability decreased by 2.2 $\log_{10}$ at 37 $^{o}$C and 1.25 $\log_{10}$ at 24 $^{o}$C in the sandy loam soil. A similar inactivation of ova was observed in an outdoor microcosm study. Utilizing this inactivation data, the annual risk of infection by *Ascaris* from consuming raw lettuce grown on biosolid-amended soil increased with increasing ova content in biosolids, increased consumption of lettuce, and reduced time intervals between biosolid amendment of soil and crop harvesting. The results of this study suggest that a waiting period of 120 days at average soil temperatures of 25$^{o}$ C or 90 days at 37$^{o}$ C after land application of biosolids on fields to which lettuce is planted would result in yearly risks of less than 1:10,000 for *Ascaris* from consumption of the lettuce with a ova concentration of 4 ova g dry solids.

## Introduction

*Ascaris* ova are believed to be the most resistant enteric pathogens to inactivation during sludge and wastewater treatment. Not only are *Ascaris* ova more resistant to treatment processes than other pathogens, but they are also more resistant than other helminthes (Maya et al., 2010). As a result, *Ascaris* ova are one of the microbial parameters utilized for classification of Class A biosolids under the Part 503 regulations for land application of biosolids in the United States (U.S. EPA, 1993). These guidelines suggest that helminth ova concentrations should be limited to less than one helminth ova/4 g (0.25/g) total solids (TS) in Class A biosolids. However, there are no limits on helminth concentration in Class B biosolids. In 2006, the World Health Organization (WHO) published guidelines on safe use of fecal material and sludge in agriculture and aquaculture utilizing wastewater. These guidelines require that the helminth ova content should be less than one viable ova per liter in wastewater used for irrigation (WHO, 2006). Previous studies have shown that the inactivation of *Ascaris* ova during various wastewater treatment processes is dependent upon temperature, pH and humidity (Maya et al., 2010), but most of the published data is qualitative rather than quantitative, and focuses on helminth survival in biosolids, ignoring the effects of the soil environment on the survival of ova. More quantitative data are necessary to fully understand the relationship between environmental conditions and ova survival so that a more accurate estimate of risk can be made for exposure to ova via land application of Class B biosolids.

Quantitative microbial risk assessment (QMRA) is emerging as a useful tool for developing guidelines for human exposure to pathogens (Haas, 2002; Navarro et al., 2009). QMRA has shown its effectiveness in assessing the risk of transmission of water and food-borne infections. The best probabilistic estimates have been obtained using epidemiologic data from actual outbreak studies (Navarro et al., 2009). When applying QMRA, the first step is to define the best distribution model that fits observed infection rates as a function of pathogen exposure dose. The most commonly used models to estimate the probability of infection are the exponential and the Beta-Poisson. Major sources of uncertainty in QMRA involve hygiene-related behavior and consumption patterns (water or food) for a targeted population, and the actual pathogen doses under consideration (Navarro et al., 2009).

The goal of this study was to assess the inactivation of ova in two Arizona soils to which biosolids had been applied, and use this information to assess the risks of *Ascaris* infection from ingesting contaminated raw vegetables grown on the amended soils.

**Materials and Methods**

Sample Preparation

Dewatered, mesophilic, anaerobically digested sludge was obtained from Ina Road Wastewater Reclamation Facility (Tucson, AZ). The biosolids contained 7% total solids as determined via moisture content analysis. The sludge was brought to a pH of 7 before by addition of 1 N NaOH. Two different soil types were used: a sandy loam and a clay loam collected from plots within the University of Arizona Campbell Agricultural Center in Tucson,

AZ. One gram of soil (either sandy loam or clay loam) was added to 9.0 g of biosolids and one ml of *Ascaris suum* ova (Excelsior Sentinel, Inc., Trumansburg NY) added at a concentration of 6,500 ova/ml within a sterile pre-weighed petri dish or 50 ml glass beaker. The average moisture content measured for biosolid-amended soil samples was 93%.

Experimental Conditions

Sandy loam samples were held either at room temperature (25$^{o}$C), 37$^{o}$C or left exposed to the outdoor environment for thirty days (microcosm). Clay loam samples were incubated at 37$^{o}$C for thirty days. Samples incubated under controlled conditions were prepared as mentioned above. All samples demonstrated a greater than 90% loss of moisture within 24 hours of incubation regardless of conditions. The microcosm consisted of placement of the soil/ biosolid mixtures (1 g/9 g) in 50 ml glass beakers placed 2.4 cm below the surface of either soil in a 30 x 20 x 12.5 cm. plastic container. Triplicate samples were processed at time intervals of 0 (control), 1, 5, 10, 20, and 30 days. Weather data (temperature and precipitation) were collected from Tucson International Airport (located next to the test site) for the duration of the experiment.

Enumeration of *A. suum* Ova within Biosolid-Amended Soil

Samples of soil/biosolid mixtures were weighed and subsequently processed according to a modified Wisconsin flotation method described previously (Cox and Todd, 1962). Samples were suspended in deionized water in 50 ml conical tubes for 30 min and then centrifuged (Beckman CS-6, Beckman Coulter, Indianapolis, IN) for 5 min at 3,200 rpm. The resulting

supernatant was poured off, and this process repeated 2 to 3 times until the supernatant was clear. Sheather's sucrose solution (specific gravity = 1.27) (Cox and Todd, 1962) was added to the biosolid/soil pellet and mixed by vortexing. Additional Sheather's sucrose solution (≃ 35 ml) was added to the sugar biosolid mixture, and tubes were then spun at 3200 rpm in a swinging bucket rotor for 15 minutes (Beckman CS-6, Beckman Coulter, Indianapolis, IN). The upper portion of the mixture (containing Sheather's sucrose solution, microscopic debris and *Ascaris* ova) was poured into two nested sieves (Erie Scientific, Portsmouth, NH) (63 µm and 38 µm, respectively). The sieves were rinsed with deionized water, and the contents on the 38 µm sieve were rinsed into a 15 ml conical tube. The 15 ml conical tubes were filled with water and spun at 3200 rpm (Beckman CS-6, Beckman Coulter, Indianapolis, IN) for 5 minutes. The resulting supernatant was aspirated off down to 1, 2, or 3 ml, and 0.1 to 0.3 ml of ovum solution was mixed with an equal volume of 0.2 N $H_2SO_4$, serially diluted, and placed in a 24-well culture plate. Ova were incubated in the 24-well culture plates at room temperature and examined for viability (development) after 12, 15, and 30 days. Ova were enumerated and examined for viability by scanning each well under light microscopy. Each well was examined for viable ova, non-viable ova, and total ova. For each count, ova were multiplied by the appropriate serial dilution factor to determine the amount of viable and non-viable ova contained in the original sample.

Risk Assessment

A dose-response function has been developed for *Ascaris lumbricoides* based on outbreak data and epidemiological studies (Navarro and Jimenez, 2011). The dose–response relationship developed for *A. lumbricoides* infection consists of a Beta-Poisson model with $\alpha = 0.104$ in the equation $P(d) = 1 - [\ 1 + (d/\boldsymbol{N_{50}})(2^{1/0.\alpha} - 1)]^{-\alpha}$ (Equation 1), where $d$ = the estimated dose of *A. lumbricoides* ova and the alpha parameters derive from the use of the beta distribution to model non-constant pathogen survival probabilities. This relationship was the end result of fitting the observed infectivity and the exposure dose estimated for the Mezquital Valley in Mexico (Navarro et al., 2009; Navarro and Jimenez, 2011). The estimated annual risk is given by $P_{annual} = 1 - [1 - P\ (d)]^n$ (Equation 2), where $P_{annual}$ = the annual risk of infection in an individual from n exposures per year to pathogen dose; $d$ is the total number of organisms in a known consumed amount of either vegetables or soil, based on *A. lumbricoides* concentration for each exposure scenario; $\boldsymbol{N_{50}}$ is the median infective dose (Navarro et al., 2009).

The following assumptions were also used in estimating the probability of an *A. lumbricoides* infection from eating raw lettuce grown on biosolid amended soil:

- *A. suum* inactivation in desert soils calculated from the present study
- Use of three different helminth concentrations in biosolids (0.25. 1, and 4 ) based on content found in the United States (Pederson et al., 1981; Navarro et al., 2009; Pepper et al., 2010).

- An *Ascaris* concentration of 0.0065, 0.0257, and 0.0959 ova/g lettuce based on 0.25, 2 and 4 ova g total solids and a 10% transfer of ova to lettuce leaves at these rates (Jimenez et al. 2006).
- Data for lettuce was used with a mean intake range estimate of 30–54 g per event (U.S. EPA, 2002). 30 g, 65 g and 100 g.
- Consumption of uncooked lettuce is once a week (U.S. EPA, 2002).
- A one $\log_{10}$ (WHO, 2006) *A. lumbricoides* reduction due to crop washing in addition to ova inactivation within biosolid amended soil seen in this study: Times chosen for QMRA were 30 and 60 days after biosolid application in addition to the 60 day period of lettuce cultivation (Kerns et al., 1999).

Statistical Analysis

Statistical significance was assessed via a two-way ANOVA with Tukey's posthoc test ($\alpha = 0.05$) using SigmaPlot software (San Jose, CA). Linear regression values were also obtained using SigmaPlot software.

**Results**

Temperature and rainfall data for samples exposed to the outdoor environment can be found in Table 1. The lowest temperature occurred on day 9 of the experiment (14 $^{o}$C). The highest temperature occurred on days 1 and 2 (34 $^{o}$C). The average temperature for the duration of the experiment was 21$^{o}$C, and this value was used for statistical comparisons of temperature. The median minimum and maximum temperatures were calculated to be 14 $^{o}$C and 29 $^{o}$C,

respectively. Only one rainfall event occurred during the experiment. This event resulted in 7 mm of rain and occurred on day 9 of the experiment.

Soil effects on *Ascaris* viability. Significant differences in $\log_{10}$ reduction were observed between sandy loam and clay loam soils at 37 $^{o}$C on days 10 and 30 (Figure 1). Inactivation rates, as determined by the slope of the best fit line as calculated by linear regression, were similar in each type of soil with inactivation being higher in the sandy loam. The $r^2$ values (obtained from best fit line, SigmaPlot) were 0.972 and 0.998 for the sandy loam and clay loam, respectively.

Temperature effects on *Ascaris* viability. Significant differences in $\log_{10}$ reductions were observed in the sandy loam (incubated at 37 $^{o}$C, 25 $^{o}$C, and outdoor ambient temperatures) after 10 and 30 days (Figure 2). However, after 20 days, $\log_{10}$ reduction at 25 $^{o}$C and ambient outdoor temperature were similar but significantly different than the reductions at rates for 25 $^{o}$C and outdoor ambient incubation were similar, while the highest inactivation rate occurred at 37°C. The $r^2$ values were 0.972, 0.980, and 0.987 for incubations in the sandy loam at the temperatures 37 $^{o}$C, 25 $^{o}$C, and outdoor ambient, respectively.

QMRA of ingesting raw vegetables grown in biosolid-amended soil

Scenario 1: Annual risk of *Ascaris* infection from ingesting raw lettuce grown on biosolid-amended sandy loam assuming a soil temperature of 37 $^{o}$C. Table 2 shows that the risk of infection from consuming lettuce grown on biosolid-amended soil with variable concentrations of *Ascaris*. Risks are also calculated for two scenarios, 90 and 120 days post-

biosolid application. Data show that risks increase with increased ova concentrations, increased consumption of lettuce. Conversely risks decrease when harvesting is delayed from 90 to 120 days post-biosolid application, All annual risks for this scenario are less than 1:10,000 even at the highest *Ascaris* concentration (4 ova/g TS) and at maximum rates of lettuce consumption.

Scenario 2: Annual risk of *Ascaris* infection from ingesting raw lettuce grown on the biosolid-amended sandy loam assuming a soil temperature of 25 $^{o}$C. Table 3 shows similar trends as those shown in Table 2. However, data also show that risks are greater for soil temperatures of 25 °C relative to 37 °C, due to enhanced inactivation at the higher temperature. *Ascaris* concentrations at or above 1 ova/g TS in conjunction with lettuce consumption rates at or above 65 g/d resulted in risks greater than 1:10,000 when the post-application period was 90 days. At 120 days post-application the calculated risk of infection were less than 1:10,000 for this scenario.

Scenario 3: Annual risk of *Ascaris* infection from ingesting lettuce grown on biosolid-amended Brazito sandy loam assuming an outdoor ambient soil temperature. Table 4 shows similar trends to those noted earlier. Actual risks at outdoor ambient temperatures are similar to those shown in the laboratory indoor study at 25°C. This is a reflection of the fact that ambient temperatures were closer to 25°C than 37°C. The calculated risk of infection for 90 days post-application was greater than 1:10,000 at *Ascaris* concentrations of 4 ova/g TS and consumption rates of at least 65 g/d. However, the calculated risk of infection was less than 1: 10,000 under any scenario at 120 days post-application.

**Discussion**

*Ascaris* survival in biosolid amended soils

Soil type was shown to play a role in the survival of *A. suum* ova (Figure 1). Ova incubated in biosolid-amended clay loam had a lower inactivation rate than ova incubated in biosolid-amended sandy loam. Clay and silt particles retain moisture very efficiently and, therefore pathogens introduced into a soil with moderate or high clay and/or silt content shows enhanced survival (Pepper et al., 2006). When incubated at temperatures between 20 $^{o}$C and 38 $^{o}$C, survival times for *Ascaris* ova have been shown to be less than 90 days in sandy soil and greater than 90 days in soils with higher clay content (Feachem et al., 1980).

Soil temperature also influenced the survival of *A. suum* ova (Figure 2). Ova incubated at 25 $^{o}$C in biosolid amended sandy loam showed lower inactivation rates than those incubated outdoors. Ova incubated at 37 $^{o}$C had the highest inactivation. Similar results have been reported in other studies (Maya et al., 2010), and show that higher temperatures significantly affect the survival of *Ascaris* ova. *Ascaris* ova have been shown to survive for up to one year in biosolids held at temperatures between 17 $^{o}$C and 20 $^{o}$C (Strauss et al., 2003). Johnson et al. (1998) found a 95% inactivation of *Ascaris* ova in five weeks when incubated at 35$^{o}$C in sludge. Studies have shown that temperatures above 40 $^{o}$C result in *Ascaris* inactivation in only two days (Kato et al., 2003). Temperatures above 50 $^{o}$C have been shown to result in *Ascaris* inactivation in as little as 2 hours (Schmidt and Roberts, 1981; Kato et al., 2003).

Risk Assessment

Infectious risks from helminth ova are dependent on ova concentrations in biosolids, lettuce consumption, and *Ascaris* ovum inactivation. It takes 30 to 60 days for lettuce to be harvest ready after planting. With a one $\log_{10}$ reduction due to crop washing, risk of infection ranged from a low of 8.1E-10 at a helminth ovum concentration of 0.25 g TS in biosolids to a high of 6.1E-04 at a helminth ovum concentration of 4 g TS in biosolids. The risk of infection may be underestimated by using lower than actual transfer rates of *Ascaris* to lettuce and also by underestimating lettuce consumption. Conversely, the risk of infection can be overestimated by overestimating transfer rates of *Ascaris* to lettuce and lettuce consumption.

The lowest risk resulted from the scenario of eating raw lettuce grown on biosolid-amended sandy loam soil which was incubated at 37 $^{o}$C (Table 2). This is due to *Ascaris* ovum inactivation being greatest under these conditions. The greatest risk resulted from eating raw lettuce grown on biosolid-amended sandy loam soil incubated at 25 $^{o}$C (Table 3). *Ascaris* ovum inactivation under these conditions was the lowest yielding a greater risk. The United States Environmental Protection Agency has used a 1:10,000 yearly risk of infection as guidance for treatment of surface water intended for drinking (Regli et al., 1991) Currently the U. S. EPA requires up to two years before food crops can be grow on land to which Class B biosolids have been applied (U.S. EPA, 1993). The results of this study suggest that a waiting period of 120 days at 25 $^{o}$C or 90 days at 37 $^{o}$C after land application of biosolids on fields to which lettuce is planted would result in yearly risks of less than 1:10,000 for *Ascaris* from consumption of the

lettuce if ova concentration in the biosolids was 4 ova g dry solids. With reported ova concentration generally less than 0.25 ova g TS in Class B biosolids in the United States (Pepper et al., 2010), a considerable margin of safety exists from land application of Class B biosolids and risk from *Ascaris* infection from production of food crops eaten raw in desert regions.

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**Table 1. Weather data during biosolid amended Brazito sandy loam incubation under outdoor conditions**

| Day | Air Temperature ($^{o}$C) | | Precipitation (mm) |
|---|---|---|---|
| | Maximum | Minimum | |
| 1 | 34.44 | 15.00 | 0.00 |
| 2 | 34.44 | 16.67 | 0.00 |
| 3 | 29.44 | 18.33 | 0.00 |
| 4 | 27.78 | 12.22 | 0.00 |
| 5 | 32.78 | 10.56 | 0.00 |
| 6 | 28.89 | 16.67 | 0.00 |
| 7 | 26.11 | 15.00 | 0.00 |
| 8 | 25.00 | 13.33 | 0.00 |
| 9 | 13.89 | 5.00 | 7.00 |
| 10 | 15.00 | 2.78 | 0.00 |
| 11 | 28.89 | 3.89 | 0.00 |
| 12 | 27.78 | 10.56 | 0.00 |
| 13 | 27.22 | 10.56 | 0.00 |
| 14 | 23.33 | 11.67 | 0.00 |
| 15 | 30.00 | 8.89 | 0.00 |
| 16 | 32.78 | 10.56 | 0.00 |
| 17 | 33.89 | 15.00 | 0.00 |
| 18 | 32.22 | 15.56 | 0.00 |
| 19 | 31.11 | 17.22 | 0.00 |

| | | | |
|---|---|---|---|
| 20 | 31.67 | 13.33 | 0.00 |
| 21 | 30.56 | 14.44 | 0.00 |
| 22 | 31.11 | 15.00 | 0.00 |
| 23 | 31.11 | 12.78 | 0.00 |
| 24 | 28.89 | 16.11 | 0.00 |
| 25 | 27.22 | 14.44 | 0.00 |
| 26 | 28.33 | 15.00 | 0.00 |
| 27 | 28.33 | 12.78 | 0.00 |
| 28 | 33.33 | 12.22 | 0.00 |
| 29 | 33.33 | 15.00 | 0.00 |
| 30 | 26.11 | 15.00 | 0.00 |

Temperature varied throughout the month of April during the experiment with a maximum of 34.4$^{o}$C and a minimum of 2.8$^{o}$C. Only one rainfall event occurred (day 9) producing 0.28 inches of rain.

**Table 2. Annual risk of infection from *Ascaris* ova after various harvesting times in biosolid amended sandy loam soil at 37$^{o}$C.**

| Assumptions | | | Annual risk | |
|---|---|---|---|---|
| Biosolids (ova/g TS) | Lettuce (*ova*/g) | Lettuce Consumption (g/d) | 90 Days Post-application | 120 Days Post-application |
| 0.25 | 0.0065 | 30 | 8.3E-08 | 8.1E-10 |
| 0.25 | 0.0065 | 65 | 1.8E-07 | 1.8E-09 |
| 0.25 | 0.0065 | 100 | 2.8E-07 | 2.7E-09 |
| 1 | 0.0257 | 30 | 3.3E-07 | 3.2E-09 |
| 1 | 0.0257 | 65 | 7.1E-07 | 6.9E-09 |
| 1 | 0.0257 | 100 | 1.1E-06 | 1.1E-08 |
| 4 | 0.0959 | 30 | 1.2E-06 | 1.2E-08 |
| 4 | 0.0959 | 65 | 2.6E-06 | 2.6E-08 |
| 4 | 0.0959 | 100 | 4.1E-06 | 4.0E-08 |

The log inactivation for these calculations represent the log inactivation obtained from experimentation plus 1 $\log_{10}$ for loss during washing (Total log removal due to inactivation and loss after crop washing).

**Table 3. Annual risk of infection from *Ascaris* ova after various harvesting times in biosolid amended sandy loam soil at 25$^{o}$C**

| Assumptions | | | Annual risk | |
|---|---|---|---|---|
| Biosolids (ova/g TS) | Lettuce (ova/g) | Lettuce Consumption (g/d) | 90 Days Post-application | 120 Days Post-application |
| 0.25 | 0.0065 | 30 | 1.3E-05 | 8.2E-07 |
| 0.25 | 0.0065 | 65 | 2.7E-05 | 1.8E-06 |
| 0.25 | 0.0065 | 100 | 4.2E-05 | 2.7E-06 |
| 1 | 0.0257 | 30 | 4.9E-05 | 3.2E-06 |
| 1 | 0.0257 | 65 | 1.1E-04 | 7.0E-06 |
| 1 | 0.0257 | 100 | 1.6E-04 | 1.1E-05 |
| 4 | 0.0959 | 30 | 1.8E-04 | 1.2E-05 |
| 4 | 0.0959 | 65 | 4.0E-04 | 2.6E-05 |
| 4 | 0.0959 | 100 | 6.1E-04 | 4.0E-05 |

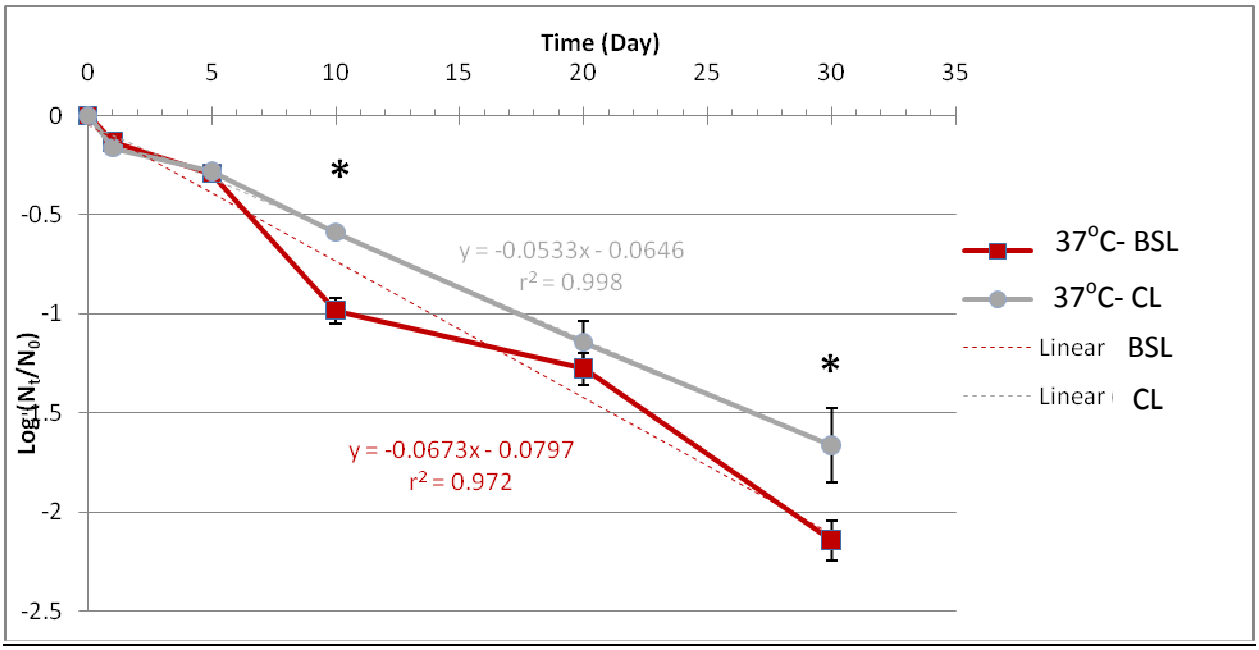
The log reduction represent the log inactivation obtained from experimentation plus 1 $\log_{10}$ for loss during washing (Total log removal due to inactivation and loss after crop washing).

**Table 4: Annual risk of infection from *Ascaris* ova after various harvesting times in biosolid amended sandy loam soil under outdoor conditions**

| Assumptions | | | Annual risk | |
|---|---|---|---|---|
| Biosolids (ova/g TS) | Lettuce (ova/g) | Lettuce Consumption (g/d) | 90 Days Post-application | 120 Days Post-application |
| 0.25 | 0.0065 | 30 | 6.2E-06 | 2.0E-07 |
| 0.25 | 0.0065 | 65 | 1.3E-05 | 4.4E-07 |
| 0.25 | 0.0065 | 100 | 2.1E-05 | 6.7E-07 |
| 1 | 0.0257 | 30 | 2.4E-05 | 8.0E-07 |
| 1 | 0.0257 | 65 | 5.3E-05 | 1.7E-06 |
| 1 | 0.0257 | 100 | 8.1E-05 | 2.7E-06 |
| 4 | 0.0959 | 30 | 9.1E-05 | 3.0E-06 |
| 4 | 0.0959 | 65 | 2.0E-04 | 6.5E-06 |
| 4 | 0.0959 | 100 | 3.0E-04 | 9.9E-06 |

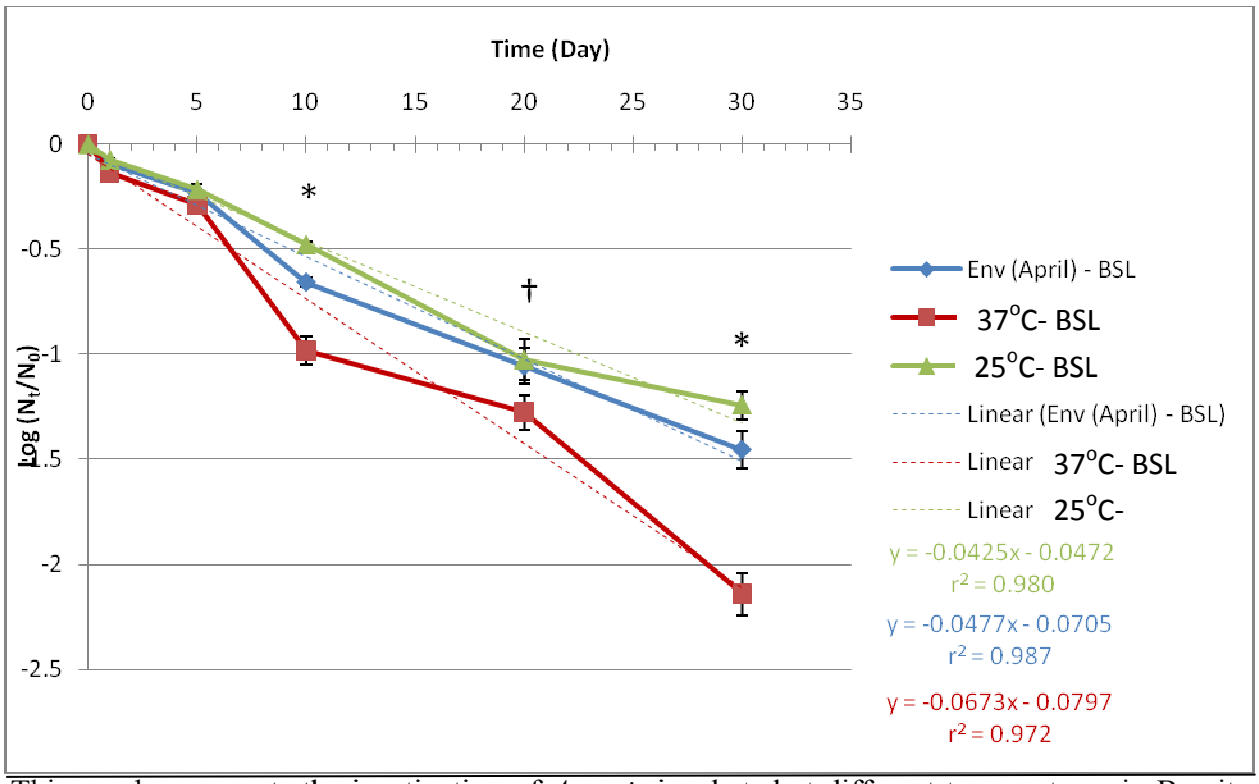
The log reductions represent the log inactivation obtained from experimentation plus 1 $\log_{10}$ for loss during washing (Total log removal due to inactivation and loss after crop washing).

**Figure 1. Effect of soil type on *Ascaris* survival at 37 $^{o}$C**




This graph represents the inactivation of *Ascaris* incubated at 37$^{o}$C in either Brazito sandy loam (BSL) or clay loam (CL). Inactivation was greatest for Brazito sandy loam and rates are given by the equation of the best fit line as given by linear regression and the $r^2$ value. * = $p < 0.05$ for BSL vs. CL.

**Figure 2. Effect of temperature on *Ascaris* survival in Brazito sandy loam**




This graph represents the inactivation of *Ascaris* incubated at different temperatures in Brazito sandy loam (BSL). Inactivation was greatest at 37°C and rates are given by the equation of the best fit line as given by linear regression and the $r^2$ value. * = $p < 0.05$ for 37°C vs 25°C vs Environmental (Env). † = $p < 0.05$ for 37°C vs 25°C and Environmental (Env).