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Inactivation of *Ascaris suum* by Ammonia in Feces Simulating the Physical-
Chemical Parameters of the Solar Toilet under Laboratory Conditions

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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Date of Approval:
November 10, 2010

Keywords: *Ascaris*, inactivation, urea, ammonia, feces, solar toilet

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DEDICATION

I dedicate this Doctoral dissertation to my loving Mom, Dr. Ligia Espinoza Aguirre, for instilling the importance of hard work, tenacity and higher education. There is no doubt in my mind that without her continued support and counsel I could not have completed this process.

ACKNOWLEDGEMENTS

First and foremost I thank GOD for arranging all the circumstances of my life in such a way that I could meet the wonderful professors, advisors, co-workers, and friends that have made unique and important contributions to the achievement of this goal. I want to solemnly express my deep gratitude to my major professor, Dr. Ricardo Izurieta, who offered me knowledge and support through-out my entire academic program and research. I thank my committee members and Dr. Bjorn Vinneras for their insight and guidance. I would also like to thank Dr. Lakshminarayan Rajaram for his assistance in data management and statistical analysis. I acknowledge the support provided by the entire Department of Global Health: they created the working conditions that were essential to the accomplishment of this goal. Very special thanks to my colleagues who helped me in the lab.

My dearest friend and roommate Ellen McCreedy, she was always there supporting and cheering me up and stood by me through the good and bad times. I will always appreciate her many hours of proofreading and her belief in me. My boyfriend Max J. Moreno, I am grateful for his encouragement, understanding, patience and love in the past few years.

Most importantly, I thank my family, for their faith in me and allowing me to be as ambitious as I wanted. It was under their watchful eye that I gained so much drive and an ability to tackle challenges head on.

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ABSTRACT

Access to sustainable sanitation systems is a determining factor in human health and economic development. However, more than a third of the world's population lives without access to improved sanitation facilities. To meet the sanitation United Nations Millennium Development target, "*halve, by 2015, the proportion of people without sustainable access to safe drinking water and basic sanitation*", a wide range of non conventional sanitation technologies have been implemented in developing countries, including waterless systems. These systems function by diverting urine away from feces and collecting, storing, and dehydrating the fecal material in watertight dehydration vaults. From a public health perspective, adequate inactivation of fecal pathogens in a sanitation system is essential before any use or disposal of fecal material. In rural areas of El Salvador, the solar toilet is capable of inactivating fecal pathogens and reducing the prevalence of parasitic infections in its users when compared to other waterless systems. Nevertheless, not all solar toilets are able to inactivate completely *Ascaris* spp. ova after the recommended storage period. Un-ionized ammonia (NH₃) has the potential to inactivate pathogens in solutions and sludge, including *Ascaris* spp. ova. This study hypothesized that adding ammonia to the solar toilet

will improve the technology since pathogen inactivation with ammonia could be potentiated by the alkaline medium and high temperatures achieved inside the toilet vaults.

To evaluate this approach, a series of experiments in solution and biosolid were performed in a laboratory environment using physical and chemical parameters similar to those achieved by the solar toilet. Eggs of the swine *Ascaris* species, *Ascaris suum*, were used as model in all experiments. In ammonia solution, the parasite ova were stored for a period of three days and; in biosolid, the parasite ova were stored for two months. Urea was used as the source of ammonia in biosolid. In addition to the experiments with ammonia, normal viability and morphological changes within the parasite ova during incubation *in vitro* at 28 C° were investigated and described to complement current literature published.

Results from the experiments in ammonia solution indicated that addition of ammonia (1% and 2%) could improve the system since the critical parameters that significantly reduced *A. suum* ova viability to zero in three days could be achieved by the solar toilet: temperature of 35°C or higher and pH value of 9.3. Results from the experiments in biosolid further showed that inactivation of *A. suum* ova was faster in samples exposed to urea and to temperatures higher than 28°C. All samples exposed to urea achieved 100% inactivation after 14 days (28°C), 3 days (35°C) and 24 hours (40°C and 45°C). Survival analysis of the data showed that there was a significant difference (p value <.0001) between the inactivation achieved in the samples exposed to urea (1% and 2%)

and the samples not exposed to urea. A logistic regression analysis estimated the effect of Urea (Treatment, OR: 25.9), Temperature (OR: 1.8), and Storage (OR: 1.17) on inactivation.

Results from the experiment with *A. suum* ova in normal incubation solution showed that the ova went through clearly identified morphological changes at different speed of development. Two new additional stages of development were identified (Pre-larva 1 and Pre-larva 2) and no significant statistical difference was observed among the viability reported early in incubation and the one reported after three weeks of *in vitro* incubation, indicating that early stages of development may be use as an alternative to reduce the time to report viability.

The results of this study suggest that inactivation of *Ascaris* spp ova by ammonia is possible in fecal material stored in the solar toilet or any other dry toilet, if the following physical and chemical conditions are met: a closed vault with a minimum temperature of 28°C; an initial pH of 8.3, minimum moisture of 27.5%, and addition of 1% urea to the biosolid. At 28°C longer storage time would be required for 100% inactivation while at higher temperatures less time of storage would be necessary. A community intervention is recommended to include field conditions and human behavior as other predictors for *Ascaris* spp. inactivation by ammonia.

CHAPTER 1.

INTRODUCTION

1.1 Purpose of the Study

Access to adequate and safe sanitation is a precondition to improve the health status of populations, especially children. Annually an estimated 5.4 billion cases of diarrhoea and 1.6 million deaths could be reduced worldwide in children less than five years of age mainly, by improving access to safe water and sanitation (Hutton & Haller, 2004). To meet the sanitation United Nations Millennium Development Goal at least 1.4 billion people must gain access to improved sanitation by the year 2015 (United Nations, 2008). A wide range of sanitation technologies are available in developing countries to help meet this challenge (Nelson & Murray, 2008). Urine diverting dry toilet (UDDT) is a dry sanitation technology that does not use water and diverts urine away from feces; it is especially useful in deficient water regions, in areas with a high groundwater table and in rocky areas where is difficult to dig (Tilley, Luthi, Morel, Zurbrugg, & Schertenleib, 2008).

The solar toilet is a UDDT sanitation technology developed for rural areas of El Salvador. It has been evolving since its introduction in 1994 and uses high pH additives, a simple solar panel to increase temperature inside the chamber,

and six to eight weeks of storage to generate a product that meets the World Health Organization and the USEPA fecal coliforms health target and standard: < 1000 Fecal Coliforms per gram (U.S Environmental Protection Agency, 1994; World Health Organization, 2006a). Communities using the solar toilet have the lowest prevalence of enteric parasitic infections, when compared with other communities using other sanitation systems (Corrales, Izurieta, & Moe, 2006). However, the solar toilet is not able to inactivate 100% the ova of *A. lumbricoides*, which are considered among the most environmental resistant pathogens excreted in human feces (Moe & Izurieta, 2003; World Health Organization, 2006a).

Adequate treatment of waste in a sanitation system is important for public health. It guarantees that fecal pathogens do not contaminate the environment and that transmission of many infectious diseases that contributes to the main killers of children under five year of age, including diarrhoea, pneumonia, neonatal disorders and undernutrition, is broken (UNICEF, 2006). Un-ionized ammonia (NH₃) has the potential to inactivate pathogens in solutions and sludge, including the *Ascaris* spp. ova (Bujoczek, 2001; Chefranova lu, 1977; Chefranova lu, Oshevskaya, & Gushchin, 1984; Chefranova lu, Petranovskaia, & Khodakova, 1978; Ghiglietti, Genchi, Di Matteo, Calcaterra, & Colombi, 1997; Ghiglietti, Rossi, Ramsan, & Colombi, 1995; Mendez, Jimenez, & Barrios, 2002; Pecson, Barrios, Jiménez, & Nelson, 2007; Pecson & Nelson, 2005). Studies in human feces are limited, but the results also indicate the potential of ammonia to

inactivate *Ascaris* spp. ova (Nordin, Nyberg, & Vinnerås, 2009; Vinnerås, Holmqvist, Bagge, Albihn, & Jönsson, 2003).

Adding ammonia to the solar toilet could result in total inactivation of the *Ascaris* spp. ova and others pathogens excreted in human feces by the potentiation of the effect of ammonia by the physical and chemical parameters achieved in the solar toilet. The purpose of the present research is to evaluate this approach, in a lab setting, by simulating the conditions and material present in the solar toilet and assessing the effect of ammonia on the inactivation of *A. suum* ova.

This dissertation has been divided in complementary subtopics: Chapter 1 describes the purpose of the study and objectives; Chapter 2 includes the literature review; and Chapter 3 to 5 covers the steps taken to complete this research.

1.2 Hypothesis

Ammonia (1% and 2% Urea) inactivates *Ascaris suum* ova in biosolid at the chemical and physical parameters present in the solar toilet

1.3 Overall Objective

To assess the inactivation of *Ascaris suum* ova by un-ionized ammonia in biosolid under laboratory conditions, using the chemical and physical parameters present in the solar toilet: alkaline pH, temperature equal or greater than 28°C, and low moisture content.

1.4 Specific Objective

1. To describe the morphological changes of *Ascaris suum* ova observed during *in vitro* incubation for a minimum period of three weeks and to explore if there are differences between viability proportions reported before and after three weeks of incubation.
2. To determine the ammonia concentration needed to inactivate *Ascaris suum* ova in a solution simulating the physical-chemical parameters achieved in the solar toilet
3. To determine the inactivation of *Ascaris suum* ova by urea added to feces simulating the physical and chemical parameters of the solar toilet in a laboratory environment
4. To compare the inactivation rate of *Ascaris suum* ova achieved in ammonia solution and in feces.
5. To identify the ammonia concentration (1% vs. 2%) needed to inactivate *Ascaris suum* ova in a mixture of material (feces + additive).

1.5 Research Question

We hypothesized that adding urea (1% and 2% urea) to the solar toilet will inactivate the resistant *Ascaris suum* ova. In addition, we hypothesized that the high temperature and pH of the solar toilet and the lower moisture content will potentiate the inactivation of this parasite by ammonia.

CHAPTER 2.

LITERATURE REVIEW

2.1 Sanitation and Health

During the 19th century the leading causes of morbidity and death in Europe and North America were attributed to lack of safe of water and sanitary conditions. Large population density, accumulation of garbage and waste, and crowding conditions characterized urban areas. Cholera outbreaks, diarrhea, and typhoid were common diseases affecting negatively the health status of populations, especially children. These negative effects were evidenced in the United States by a decline in height average in those born in the 1820's and 1830's and, in Britain, by a decline in life expectancy at birth during the first half of the 19th century. It was until the later part of the 19th century that a rise in the health status of populations was observed as public health practices, such as access to safe water and sewage disposal, and standards of living, such as housing and nutrition, improved (Bryant, 2003). Nowadays, industrialized countries have access to safe water and adequate sanitation coverage (99%) with very low under-five mortality rate¹ (6/1,000 live birth), when compared to developing countries (Figure 2.1) (United Nations Children's Fund, 2009; World

¹ Under-five mortality rate – Probability of dying between birth and exactly five years of age, expressed per 1,000 live births.

Health Organization and United Nations Children's Fund Joint Monitoring Programme for Water Supply and Sanitation (JMP), 2008).

Thus, it is not surprising that access to safe water and sanitation was ranked as the greatest medical advance of the last century and half by readers of the *British Medical Journal* in 2007 (Kamerow, 2007). Unfortunately, 2.5 billion people continue to lack access to adequate sanitation in developing countries at the present time. Similar to the 19th century, children are the most vulnerable age group to suffer from the burden of sanitation related disease (JMP, 2008).

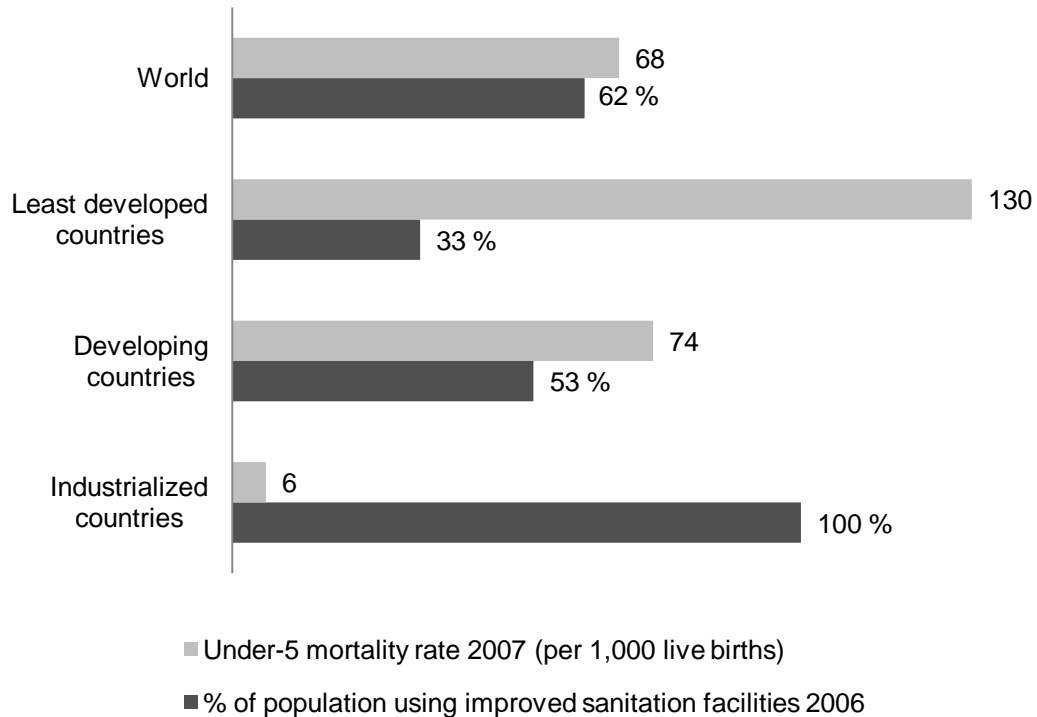


Figure 2.1 Under Five Mortality Rate and Sanitation Coverage

2.1.1 Millennium Development Goals and target ten. The importance of adequate sanitation to individual’s development, dignity and health is recognized in the Millennium Development Goals (MDG). These goals were adopted at the United Nations (UN) Millennium Summit in September 2000 and included a set of

time-bound targets to address the most pressing development issues in the world (Table 2.1). Sanitation, Target 10, was included in the MDG in 2002 at the UN World Summit on Sustainable Development (UN Millennium Project, 2005). With this resolution, water and sanitation were recognized as essential for success in the fight against poverty and hunger, morbidity and mortality, gender inequity and lack of protection of natural resources (UN Millennium Project, 2005).

The Joint Monitoring Programme for Water Supply and Sanitation (JMP) is the entity responsible of monitoring progress on target ten worldwide. For this purpose, the JMP defines an improved sanitation facility “*as one that hygienically separates human excreta from human contact*” (WHO/UNICEF, 2010). These facilities should not be shared or public and include:

- flush or pour-flush toilets connected to piped sewer system, septic tank, or pit latrine;
- ventilated improved pit (VIP) latrine;
- pit latrine with slab; and
- composting toilet

Although this definition facilitates monitoring global sanitation coverage using national surveys and other data sources, it does not consider health impact; individual safety; social and cultural acceptability of the facility; safe disposal or discharge of excreta; adequate operation and maintenance of the system; efficacy of the system to inactivate pathogens; installation longevity, functionality and sustainability (Rosemarin et al., 2008).

Table 2.1

United Nations Millennium Development Goals and Targets

Target	Goal
	Goal 1. Eradicate Extreme Hunger and Poverty
Target 1	Halve, between 1990 and 2015, the proportion of people whose income is less than \$1 a day
Target 2	Halve, between 1990 and 2015, the proportion of people who suffer from hunger
	Goal 2. Achieve Universal Primary Education
Target 3	Ensure that, by 2015, children everywhere, boys and girls alike, will be able to complete a full course of primary schooling
	Goal 3. Promote Gender Equality and Empower Women
Target 4	Eliminate gender disparity in primary and secondary education, preferably by 2005, and in all levels of education no later than 2015
	Goal 4. Reduce Child Mortality
Target 5	Reduce by two-thirds, between 1990 and 2015, the under-five mortality rate
	Goal 5. Improve Maternal Health
Target 6	Reduce by three-quarters, between 1990 and 2015, the maternal mortality ratio
	Goal 6. Combat HIV/AIDS, Malaria and other diseases
Target 7	Have halted by 2015 and begun to reverse the spread of HIV/AIDS
	Goal 7. Ensure Environmental Sustainability
Target 9	Integrate the principles of sustainable development into country policies and programs and reverse the loss of environmental resources
Target 10	Halve, by 2015, the proportion of people without sustainable access to safe drinking water and basic sanitation
Target 11	Have achieved by 2020 a significant improvement in the lives of at least 100 million slum dwellers
	Goal 8. Develop a Global Partnership for Development
Target 12	Develop further an open, rule-based, predictable, nondiscriminatory trading and financial system
Target 13	Address the special needs of the Least Developed Countries
Target 14	Address the special needs of landlocked developing countries and small island developing states
Target 15	Deal comprehensively with the debt problems of developing countries through national and international measures in order to make debt sustainable in the long term
Target 16	In cooperation with developing countries, develop and implement strategies for decent and productive work for youth
Target 17	In cooperation with pharmaceutical companies, provide access to affordable essential drugs in developing countries
Target 18	In cooperation with the private sector, make available the benefits of new technologies, especially information and communications technologies

*Source: UN Millennium Development, 2005

These elements are very difficult to evaluate and require a more rigorous definition of adequate sanitation. However, in the last few years the simplicity of the JMP standards in sanitation is being recognized and the need for more appropriate indicators and targets for sanitation has been placed (Bartram, 2008). The last JMP report on water and sanitation presents sanitation as a four step ladder, which allows observing sanitation trends globally. It continues using the same definitions but it gives the percentage of people (a) practicing open defecation, (b) using unimproved sanitation facilities; (c) shared sanitation facilities, and (d) improve sanitation facilities (JMP, 2008). The four sanitation ladder does not include elements of sustainability and health impact; nevertheless it represents the first changes to improve the sanitation standards.

The last JMP report on the progress on sanitation showed that 61% of the world's population was using an improved sanitation facility by 2006 (WHO/UNICEF, 2010). This represented an 8% increase from 1990, which is the base line year to monitor progress on sanitation (UN Millennium Project, 2005). The other 38% of the population, 2.5 billion, reported sharing a sanitation facility with one or more households (8%), using an unimproved sanitation facility (12%) or practicing open defecation (18%) (JMP, 2008).

In order to achieve Target 10, 77% sanitation coverage must be reached by 2015. According to the JMP data, developed regions are on track to meet the MDG target since they have kept 99% sanitation coverage since 1990. Developing regions would need 71% sanitation coverage by 2015 to meet the target. Unfortunately the progress achieved so far (53% of the population in

developing regions reported using an improved sanitation facility by 2006) is insufficient to meet the target. Sub-Saharan Africa and Southern Asia are of special concern as they had the highest percentage of people practicing open defecation (28% and 48% respectively) and the lowest sanitation coverage recorded in 2006 (31% and 33% respectively). Of the 2.5 billion people without access to sanitation, almost 1.8 billion live in Asia and 546 million people live in Sub-Saharan Africa (JMP, 2008). In addition, there is large disparity among rural and urban population sanitation coverage. In developing countries 57% of the population lives in rural areas and only 39% of the rural population reported having access to an improved sanitation facility in 2006. In developed regions, both urban and rural populations have access to improved sanitation facilities (100% and 96% respectively).

2.1.2 Influence of sanitation in other MDGs. Sanitation, which is part of goal seven-target 10, is vital to advance in the other goals (Table 2.2) and to reduce the psychological burden on the mental health of parents due to the loss of a child; and the discomfort on disable people, pregnant women, and sick individuals lacking access to a toilet (UN Millennium Project, 2005). The United Nations estimated that solving the problems of water and sanitation will make a 30% contribution, in average, towards meeting the other Millennium Development Goals (MDG) including child survival, improved maternal health, primary and secondary education, gender equality, environment, and hunger alleviation (United Nations Secretary-General's Advisory Board on Water and Sanitation, 2007).

Table 2.2

Contribution of Access to Domestic Water supply and Sanitation to other MDGs

Goal	Contribution
Poverty	<ul style="list-style-type: none"> • Household livelihood security rests on the health of its members; adults who are ill themselves or who must care for sick children are less productive. • Illnesses caused by unsafe drinking water and inadequate sanitation generate health costs that can claim a large share of poor households' income. • Time spent collecting water cannot be used for other livelihood activities.
Hunger	<ul style="list-style-type: none"> • Healthy people are better able to absorb the nutrients in food than those suffering from water-related diseases, particularly worms, which rob their hosts of calories.
Education	<ul style="list-style-type: none"> • Improved water supply and sanitation services relieve girls from water-fetching duties, allowing them to attend school. • Reducing illness related to water and sanitation, including injuries from water-carrying, improves school attendance, especially for girls. • Having separate sanitation facilities for girls in schools increases their school attendance, especially after menarche.
Gender equality	<ul style="list-style-type: none"> • Community-based organizations for water supply and sanitation can improve social capital of women. • Reduced time, health, and care-giving burdens from improved water services give women more time for productive endeavors, adult education, empowerment activities, and leisure. • Water sources and sanitation facilities closer to home put women and girls at less risk for sexual harassment and assault while gathering water and searching for privacy. • Higher rates of child survival are a precursor to the demographic transition to lower fertility rates; having fewer children reduces women's domestic responsibilities.
Child mortality	<ul style="list-style-type: none"> • Improved sanitation, safe drinking water sources, and greater quantities of domestic water for washing reduce infant and child morbidity and mortality. • Sanitation and safe water in health-care facilities help ensure clean delivery and reduce neonatal deaths. • Mothers with improved water supply and sanitation services are better able to care for their children, both because they have fewer illnesses and because they devote less time to water-fetching and seeking privacy for defecation.
Maternal mortality	<ul style="list-style-type: none"> • Accessible sources of water reduce labor burdens and health problems resulting from water portage, reducing maternal mortality risks. • Improved health and nutrition reduce susceptibility to anemia and other conditions that affect maternal mortality. • Safe drinking water and basic sanitation are needed in health-care facilities to ensure basic hygiene practices following delivery. • Higher rates of child survival are a precursor to the demographic transition toward lower fertility rates, and fewer pregnancies per woman reduce maternal mortality.
Major diseases	<ul style="list-style-type: none"> • Safe drinking water and basic sanitation help prevent water-related diseases, including diarrheal diseases, schistosomiasis, filariasis, trachoma, and helminthes. 1.6 million deaths per year are attributed to unsafe water, poor sanitation, and lack of hygiene. • Improved water supply reduces diarrhea morbidity by 21 percent; improved sanitation reduces diarrhea morbidity by 37.5 percent; hand washing can reduce the number of diarrheal cases by up to 35 percent; additional improvements in drinking water quality, such as point-of-use disinfection, would reduce diarrheal episodes by 45 percent.
Environmental sustainability	<ul style="list-style-type: none"> • Adequate treatment and disposal of excreta and wastewater contributes to better ecosystem management and less pressure on freshwater resources. • Improved sanitation reduces flows of human excreta into waterways, helping to protect human and environmental health. • Inadequate access to safe water and inadequate access to sanitation and other infrastructure are two of the five defining

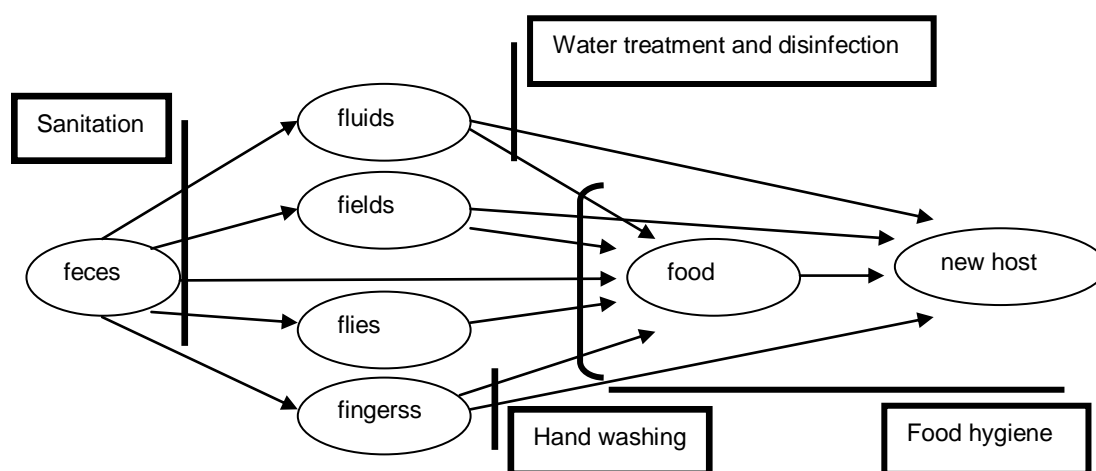
Source: The UN Millennium Project Task Force on Water and Sanitation

It is estimated that 1.3 million women and girls have no access to a toilet. Looking for some privacy, women usually wait until night or before dawn to relieve themselves, increasing health problems such urinary tract infections and chronic constipation, and the risk of violence and rape. Girls also have to drop out of school when they start menstruating due to lack of sanitation facilities in schools (UN Millennium Project, 2005).

2.1.3 Monetary benefits of improved sanitation. Hygiene and sanitation are among the most effective public health interventions; it reduces morbidity and mortality, especially child mortality; it reduces health related costs; results in higher productivity and school attendance, more leisure time, convenience and well being, advance in gender equality, and has a positive impact on economic and poverty reduction. Every \$1 dollar invested in sanitation would yield an economic return of between \$3 and \$34, depending on the region (WHO & UNICEF, 2004)

2.1.4 Effects of sanitation in health. Adequate collection, treatment, and disposal of human excreta combined with hygiene practices are important to break the transmission and the contamination of the environment with pathogens present in human excreta. The main routes of pathogen transmission from excreta to human are presented in Figure 2.2. In areas where sanitation systems are inadequate fecal pathogens are distributed in the environment (groundwater, surface water and soil). Once the environment is contaminated, individuals are infected by ingesting contaminated food or water, through contact with contaminated fingers and, in some cases (as it is the case of hookworm), through

the skin. Cockroaches and flies can also carry fecal pathogens and contaminate food, but washing of fruits and vegetables as well as adequate food cooking can block this transmission pathway.



Source: Adapted from World Bank (2008).

Figure 2.2 The F-Diagram

Many diseases caused by fecal pathogens are among the main causes of children morbidity and mortality in developing countries and result in poor health, impaired ability to learn and work, and high health costs. According to the World Health Organization, unsafe water supply, sanitation and hygiene rank third among the 10 most significant risk factors for poor health in developing countries. Approximately 3.1% of annual deaths (1.7 million) and 3.7% of DALYs (54.2 million) worldwide are attributed to unsafe water supply, sanitation and hygiene. Almost all deaths associated with it (99.8%) occur in developing countries and 90% of them are children (SIWI & WHO, 2005; WHO, 2002b). The first risk factor for poor health is malnutrition, which is geographically associated with poor environmental conditions such as lack of safe water and adequate sanitation.

Infectious diarrhoea, schistosomiasis, ascariasis, trichuriasis, and hookworms are among the main diseases contributing to the burden associated with unsafe water, sanitation and hygiene (WHO, 2002b). These diseases affect close to half the people at any given time and cause the occupancy of more than half the hospital beds in the developing world (UN Millennium Project, 2005).

2.1.4.1 Diarrhea. Worldwide, there are 4 billion cases of diarrhea each year causing 4% of all deaths and 5% of health loss to disability (WHO, 2000). Of the billions of diarrhea cases, 88% are attributed to unsafe water supply or inadequate sanitation and hygiene; and, of the millions of deaths, 90% are children under five living in developing countries (UN Millennium Project, 2005; UNWater, 2008). According to the World Health Organization global mortality report, there were 2.2 million deaths due to diarrhea in 1998, 2 million deaths in 2001 and 1.8 million deaths in 2002. This data shows that the number of diarrhea cases is decreasing, nevertheless, this disease continues to be the second largest killer of children worldwide and poor sanitation and hygiene are either the chief or the underlying cause (WHO, 2000, 2004a, 2004b, 2008b).

Pathogens causing diarrhea are excreta related and improving sanitation reduces diarrhea morbidity by 37.5% (UN Millennium Project, 2005; WHO, 2008b). The health consequences of this disease are particularly severe in children. In addition of the 5000 children dying every day due to diarrhea, those who survive and suffer persistent diarrhea are affected by malnutrition (associated with more than half of all under five deaths), wasting and stunting of growth, and have an increase in their susceptibility to infectious respiratory

disease (pneumonia), malaria and measles (UNICEF, 2006). Malnutrition is an underlying cause in 52% of pneumonia deaths and 60% of diarrhoea deaths. Both diseases are the main causes of child mortality worldwide.

2.1.4.2 Schistosomiasis and soil-transmitted helminthes. Some 200 million people are infected with schistosomiasis resulting in 1.7 million DALYs and 200,000 deaths each year. Of the people infected, 20 million have severe disease and 120 million have symptoms. This disease is endemic in 76 countries and around 600 million people are at risk of infection making it the second most important parasitic infection after malaria in terms of public health and economic impact (WHO, 2001c, 2003, 2006c).

Schistosomiasis is related to lack of basic sanitation and sources of safe water. It is estimated that access to basic sanitation reduces the disease by up to 77 % (UN Millennium Project, 2005). The disease mainly affect children from 5 to 15 years old and the health consequences vary, depending of the worm load and the amount of time a person is infected (WHO, 2001c, 2003). In children the disease contributes to retarded growth, affect cognitive development, and aggravate malnutrition and anemia. If the disease is not treated and becomes chronic and irreversible, it results in enlarged spleen, bladder and kidney failure, liver fibrosis, bladder cancer, and nervous system pathology such as seizures, paralysis or spinal cord inflammation (WHO, 2001c, 2003, 2006c).

Soil transmitted helminthes produce the most common infections worldwide and the causal agents are *Ascaris lumbricoides*, *T. trichiura*, and hookworms (*Ancylostoma duodenale* and *Necator americanus*). Around 2 billion

people are infected by these helminthes, 133 millions suffer from high-intensity intestinal infections and 135,000 people are estimated to die every year from these infections (UNICEF, 2006; WHO, 2003). The World Health Organization estimates that *A. lumbricoides* infects over 1 billion people, *T. trichiura* infects 795 million, and hookworms 740 million individuals (WHO, 2008c). *Ascaris* account for most of these infections and it is more common in children between 3 and 8 years old. It causes 60,000 deaths per year, especially in children (WHO, 2001b).

Morbidity by *Ascaris lumbricoides* can be reduced by 29% by providing access to safe water, sanitation and hygiene. The health consequences of this infection are many and depend on the worm load. They range from diarrhea, abdominal pain, malaise and weakness to reduced cognitive capacity and impair physical growth. A child infected by *Ascaris* diverts a third of the food he/she consumes resulting in malnutrition and a cycle of infectious diseases (UN Millennium Project, 2005; WHO, 2008b, 2008c). Hookworms cause intestinal blood loss and produce anemia; trichuriasis cause chronic colitis (WHO, 2008b).

Once more, the population most vulnerable to these preventable sanitation related diseases is children. Without adequate access to improved sanitation and treatment they become physically and intellectually compromised by anemia and malnutrition, resulting in attention deficits, learning disabilities, school absenteeism and higher dropout rates (WHO, 2006c).

2.2 Excreta and Pathogens

Excreta refer to human feces and urine (WHO, 2006a). Exposure to untreated excreta is always considered unsafe due to the presence of a high variety of pathogens. Many of these pathogens are transmitted when excreta of an infected individual enter in contact with the mouth of a new host by a variety of routes (see Figure 2). This is known as the fecal oral cycle of transmission. Adequate sanitation facilities could block these routes at their point of origin and avoid the transmission of diseases related to excreted pathogens (Feachem, Bradley, Garelick, & Mara, 1983; WHO, 2006b).

2.2.1 Characteristics of excreta. The frequency of defecation, volume, consistency, moisture content and composition of feces vary among populations and it is influenced by factors such as diet, age and climate. The volume of wet excreted feces may vary from 20 grams to 1.5 kilograms per day, with lower volume produced by children, adolescents and the elderly. The amount of urine produce by an individual per day varies from 1 to 1.3 kg. The moisture content for a wet fecal volume of 100 to 150 grams per day is around 75%, increasing with higher fecal volume. The normal frequency of defecation is 1 per day, but it also may increase to 3 per day for vegetarian individuals (Feachem et al., 1983). The chemical constituents of excreta and urine are presented in Table 2.3.

2.2.2 Types of pathogen in feces. Four groups of pathogens are found in excreta: viruses, bacteria, protozoa and helminthes. These pathogens are associated with gastrointestinal diseases giving rise to symptoms such as diarrhea, dysentery, vomiting, and stomach cramps but could also affect other

organs and cause severe health consequences such as malnutrition (Droste, 1997). Table 2.4 describes the main pathogens of concern.

Table 2.3

Composition of Urine and Feces Expressed as g/day and mg/day for Metals

Parameter	Urine	Feces
H ₂ O	1487	109.6
TS	20	30.1
TSS	0.76	25
VS	7.4	23.9
COD _{tot}	8.5	37.4
BOD	5	22.6
N _{tot}	11	1.5
NNH ₃ /NH ₄	10.3	0.3
P _{tot}	0.9	0.5
S _{tot}	0.70	0.162
K _{tot}	2.4	0.9
Pb	0.012	0.038
Cd	0.0005	0.010
Hg	0.00082	0.009
Cu	0.10	1.00
Cr	0.010	0.124
Ni	0.011	0.188
Zn	0.3	10.7

Source: (Jonsson, Baky, Jeppsson, Hellstrom, & Karrman, 2005)

2.2.2.1 Bacteria. Enteric bacteria are the most numerous living organisms found in human feces. They are part of the human intestinal flora and they are routinely excreted. The average concentration of bacteria in 1 g of feces of a healthy individual is equivalent to 1.8×10^{12} Escherichia coli cells (Droste, 1997).

Table 2.4

Main Fecal Pathogens of Concern for Public Health

Group	Pathogen	Disease and Symptoms
Bacteria	Aeromonas spp.	Enteritis
	Campylobacter jejuni/coli	Campylobacteriosis-diarrhoea, cramps, abdominal pain, fever nausea, arthritis; Guillain-Barre syndrome
	Escherichia coli (EIEC, EPEC, ETEC, EHEC)	Enteritis
	Plesiomonas shigelloides	Enteritis
	Salmonella typhi/paratyphi	Typhoid/paratyphoid fever – headache, fever, malaise, anorexia, bradycardia, splenomegally, cough
	Salmonella spp.	Salmonellosis – diarrhea, fever, abdominal cramps
	Shigella spp.	Shigellosis – dysentery, vomiting, cramps, fever; Reiter's syndrome
	Vibrio cholera	Cholera – watery diarrhea, lethal if severe and untreated
	Yersinia spp.	Yersiniosis – fever, abdominal pain, diarrhoea, joint pains, rash.
Viruses	Enteric adenovirus 40 & 41	Enteritis
	Astrovirus	Enteritis
	Calicivirus (including norovirus)	Enteritis
	Coxsackievirus	Various; respiratory illness, enteritis; viral meningitis
	Echovirus	Aseptic meningitis; encephalitis; often asymptomatic
	Enterovirus types 68-71	Meningitis; encephalitis; paralysis
	Hepatitis A virus	Hepatitis – fever, malaise, anorexia, nausea, abdominal discomfort, jaundice
	Hepatitis E virus	Hepatitis
	Poliovirus	Poliomyelitis – often asymptomatic, fever, nausea, vomiting, headache, paralysis
	Rotavirus	Enteritis
Parasitic protozoa	Cryptosporidium parvum	Cryptosporidiosis – watery diarrhoea, abdominal cramps and pain
	Cyclospora cayetanensis	Often asymptomatic; diarrhoea, abdominal pain
	Entamoeba histolytica	Amoebiasis – often asymptomatic; dysentery, abdominal discomfort, fever, chills
	Giardia intestinalis	Giardiasis – diarrhoea, abdominal cramps, malaise, weight loss
Helminths	Ascaris lumbricoides	Ascariasis – generally no or few symptoms; wheezing, coughing, fever, enteritis, pulmonary eosinophilia
	Taenia solium/saginata	Taeniasis
	Trichuris trichiura	Trichuriasis – unapparent through vague digestive tract distress to emaciation with dry skin and diarrhoea
	Ancylostoma duodenale/ Necator Americanus	Itch, rash, cough, anemia, protein deficiency
	Schistosoma spp.	Schistosomiasis, bilharzia

Source: (WHO, 2006b)

The main groups of non-pathogenic bacteria are Enterobacteria, Streptococci, Lactobacilli, Clostridia, Bacteroides, Bifidobacteria and Eubacteria; some of them are used as indicator of fecal contamination (Metcalf & Eddy, 2003). Those bacteria that are not part of the normal fecal flora are classified as pathogenic and many of them are associated with diarrhea or dysentery. Among the pathogenic bacteria of major concern are *Salmonella typhi*, *Vibrio cholera*, *Shigella*, *Campylobacter* and enterohaemorrhagic *E. coli* (EHEC)(WHO, 2006a) Bacterial diarrheas are classified into toxigenic and invasive. In toxigenic diarrheas an enterotoxin produced by the bacteria is the main pathogenic mechanism. In invasive diarrheas the bacteria itself penetrate the mucosa surface (Hamer & Gorbach, 2002).

Toxigenic diarrhea is produced by *V. cholera* and enterotoxigenic *Escherichia coli* (ETEC). The enterotoxins, from both microorganisms, have their greatest activity in the upper small bowel and produce a watery and voluminous fecal effluent which quickly leads to dehydration characteristic of this type of diarrheas (Hamer & Gorbach, 2002).

In 2007, 177,963 cholera cases and 4,031 deaths were reported to the World Health Organization from 53 countries; 93.6% of global cases and 99% of global deaths were reported from Africa. Asia ranks second in cholera cases and deaths. Cases did not include cases labeled as acute watery diarrhea and the true figures are likely to be higher. The overall case fatality rate (CFR) was 2.3%; however, groups living in high risk areas reported a 35% CFR (WHO, 2008a).

The clinical manifestations of cholera vary from asymptomatic carrier (75%) to mild and even severe diarrhea with dehydration, renal failure and hypovolemic shock (WHO, 2008b). In those that develop the disease, 20% or less present signs of moderate or severe dehydration. Vomiting and abdominal distention may be present at the initial stage followed by large volume of rice water stools. Transmission occurs through direct fecal-oral contamination or through ingestion of fecal contaminated water and food. A large dose (10^6 - 10^9) is required to cause infection but individuals with low immunity (malnourished children and people affected with HIV) are more easily infected and at greater risk of death (Hamer & Gorbach, 2002; WHO, 2008b).

Poor environmental conditions, lack of safe water, and lack of adequate sanitation are closely linked to the diseases as the *V. cholera* uses brackish water and estuaries as reservoir in addition to humans (WHO, 2008b). The *Vibrio* is capable of surviving in aquatic environments for long periods of time, been considered an autochthonous specie in estuarine and brackish waters (Tamplin, Gauzens, Huq, Sack, & Colwell, 1990). In developing countries, cholera is a threat especially in countries where the minimum hygiene standards are not met. Peri-urban slums, refugee camps and areas of disaster are at increased risk of the disease for the same reason (WHO, 2008b). Infected individuals can be treated adequately through the administration of oral and intravenous rehydration but the access to safe water and sanitation remains an important factor in the control and prevention of cholera outbreaks.

Similar to cholera, enterotoxigenic *Escherichia coli* is acquired by consuming contaminated food and liquids and produces an enterotoxin that acts pathophysiologically like the cholera toxin. It activates adenylate cyclase and cause secretion of fluid and electrolytes into the intestinal lumen. Infants and young children are the most affected by this disease and the highest incidence is observed in the tropics. The disease starts with upper intestinal distress followed with watery diarrhea after 24 to 48 hours of incubation. In severe cases the disease is similar to cholera and can be misdiagnose (Hamer & Gorbach, 2002).

The disease is spread through food and water contaminated with human feces. Similar to cholera, ETEC has been found to survive in surface waters and studies from endemic countries, such as Bangladesh and Bolivia, revealed a high prevalence of ETEC in their local rivers and sources of surface water (Ohno et al., 1997; Qadri, Svennerholm, Faruque, & Sack, 2005). In areas where drinking water and sanitation are inadequate, ETEC is an important cause of diarrheal diseases (Qadri et al., 2005) even though individuals living in endemic areas seem to develop immunity to ETEC over time (Hamer & Gorbach, 2002). Asymptomatic carriers are also frequent in areas of poor sanitation (Qadri et al., 2005). An extensive review carried by Qadri et al (2005) found that ETEC is an important cause of diarrhea in children in developing countries and that clean water and adequate sanitation are essential to prevent the disease.

Invasive diarrhea is caused by bacterial pathogens that invade the intestinal epithelium producing ulceration and an acute inflammatory reaction. This group of pathogens target the lower bowel and include *Salmonella*, *Shigella*,

invasive *E. coli* (EIEC), *Campylobacter*, and *Yersinia* (Hamer & Gorbach, 2002). The mechanism by which these pathogens produced diarrhea is not well understood yet, but it is suggested that an enterotoxin is responsible for the fluid production at the beginning of the disease. Once the pathogen invades the cell, the local synthesis of prostaglandins is increased further stimulating fluid secretion. The damage caused to the epithelium by the invasion of the pathogen prevents reabsorption of fluids from the lumen resulting in a net accumulation of luminal fluid and diarrhea(Hamer & Gorbach, 2002).

Shigella is one of the main pathogens causing diarrhea and dysentery worldwide (WHO, 2009a). Dysentery refers to stool with blood and mucus that is related to the invasion of the intestinal epithelium. Ten to twenty percent of diarrhea cases are shigellosis cases and children 6 months to 5 years of age are the most affected by the disease (Hamer & Gorbach, 2002). According to the Diseases of the Most Impoverished (DOMI) Programme of the International Vaccine Institute (IVI), the annual shigellosis incidence rate in children under five from 2000 to 2004 were: 46/1,000 in Dhaka, Bangladesh; 19/1,000 in Hebei Province, China; 16-17/1,000 in the North Jakarta, Indonesia and Pakistan; and 4-5/1,000 in the coastal city of Nha Trang, Vietnam and in Saraburi Province in Thailand. Eighty percent of all cases found were children three years or younger and the second population group most affected were individuals 70 year old or over (IVI, 2007). In Asia 414,000 annual deaths are estimated although the true figures are likely to be higher (Legros, 2004).

The bacteria can be transmitted through person to person contact or through ingestion of contaminated food and water (WHO, 2009a) and an individual could develop dysentery with as few as 200 bacteria. The disease is characterized by lower abdominal pain, fever, rectal burning and dysentery stools. Intestinal perforation and severe protein loss are complications of the disease. The bacterium does not invade the bloodstream but malnourished children and immunocompromised individuals are at higher risk of bacteremia. Some individuals become chronic carriers and could pass the bacteria in their feces for 1 year or more suffering intermittent attacks of the disease (Hamer & Gorbach, 2002).

Disease outbreaks are related to poor environmental conditions (overcrowding, lack of safe water and adequate sanitation facilities and poor hygiene) and improvement of these conditions is as important as adequate case management to prevent the disease and control outbreaks (Legros, 2004).

Salmonella is responsible for nontyphoidal salmonellosis and typhoid and paratyphoid fever (Hamer & Gorbach, 2002). The disease mainly affects children younger than five years and young adults and it is an important public-health problem in developing countries including south-central and southeast Asia, the middle east, Africa, and South America (Bhan, Bahl, & Bhatnagar, 2005; Graham, 2002). In developed countries, salmonella is a problem for travelers going to endemic areas.

Nontyphoidal salmonella is a form of gastroenteritis produced by nontyphoidal strains of *Salmonella* (Hamer & Gorbach, 2002). The bacterium is a

major cause of food borne illness and the symptoms of the infection include fever, abdominal pain, diarrhea, nausea and sometimes vomiting (WHO, 2009b). Immunity to the bacterium is related to age and children less than 1 year of age have the highest attack rate (Hamer & Gorbach, 2002).

Typhoid and paratyphoid fever is caused by *S. typhi* and *S. paratyphi* and has more systemic than intestinal symptoms (Hamer & Gorbach, 2002). They include sustained high fever, malaise, headache, constipation or diarrhoea, rose-colored spots on the chest, and hepatosplenomegaly. Approximately 50% of infected individuals are shedding the bacteria in their feces after 6 weeks and 1 to 3% become chronic carriers after 1 year (Hamer & Gorbach, 2002). The disease is acquired through consumption of water or food that has been contaminated by feces of an acutely infected or convalescent individual or a chronic asymptomatic carrier.

The World Health Organization estimated that typhoid fever produced 21,650,974 illnesses and 216,510 deaths during 2000 and that paratyphoid fever caused 5,412,744 illnesses worldwide (Crump, Luby, & Mintz, 2004). Vaccination in high-risk areas is the control strategy recommended by WHO to prevent and control the disease. However improved water quality and sanitation are the long-term solutions to control the transmission of the disease (WHO, 2003). Bhan et al (2005) suggest that *S. typhi* could be eradicated as this *Salmonella* strain is restricted to human beings, but lack of sanitation and increasing multidrug resistance organisms remain an obstacle to achieve this goal.

Campylobacter species are another major cause of diarrheal diseases in developing and developed countries. According to WHO *Campylobacter* spp. are the most common bacterial cause of gastroenteritis worldwide (WHO, 2000a). In the United States 4% to 11% of diarrheal cases are caused by this bacterium (Hamer & Gorbach, 2002). In developing countries, it is an important cause of morbidity and even mortality especially in infants and children when they are exposed to contaminated water and farm animals. The estimated case-fatality rate 30 days post-infection is estimated 4 deaths per 1000 infections. The true figures are likely to be higher, however (WHO, 2000b)

The reservoir includes many animals and transmission occurs from infected animals and their food products to humans or through contaminated water. The symptoms of the disease usually starts two to five days after infection and include diarrhea, fever, abdominal pain, dysentery, headache, anorexia, nausea and/or vomiting. Bacteremia, hepatitis, pancreatitis, abortion, reactive arthritis and neurological disorders are complications of the disease reported with various degrees of frequency and approximately 16% of patients become asymptomatic carriers for 2 to 10 weeks after the disease (WHO, 2000a).

Aeromonas spp. are common bacteria in the environment found in soil, fresh and brackish water, sewage, wastewater and foods, particularly meat and milk. The distribution of the bacteria in the environment is significantly related with fecal pollution in waters (Araujo, Arribas, & Pares, 1991). According to the WHO guidelines for drinking water, these bacteria have been associated with gastrointestinal diseases but there is not consistency in the epidemiological

evidence (WHO, 2008c). However, *Aeromonas* is described in the literature as a diarrhea causing agent (Hamer & Gorbach, 2002).

Symptoms vary from mild diarrhea to severe illness with blood and mucus in the stools. It affects children and adults and immunocompromised individuals are at higher risk of bacteremia (Hamer & Gorbach, 2002). The bacteria is also related with wound and respiratory tract infections (World Health Organization, 2008c).

The prevalence and asymptomatic carriers of *Aeromonas* spp. varies among communities. Studies of acute gastroenteritis in different areas of the world report a prevalence of *Aeromonas* spp. ranging from 2.5% to 6.6% in Brazil (Guerra et al., 2007), 11.83% in Merida, Venezuela (Longa et al., 2005); 4.5% in Tehran, Iran (Soltan Dallal & Moezardalan, 2004); 9% in Dhaka, Bangladesh (Haque et al., 2003); 9% in Peru (Pazzaglia et al., 1991); 2.26% in Lagos, Nigeria (Alabi & Odugbemi, 1990); and 9% in urban Somalia (Casalino et al., 1988). Some of these studies include adults but even in these studies the most affected population is children. The percent of asymptomatic carriers could be as high as 27% in Thailand (Hamer & Gorbach, 2002).

Because the previous bacterial diseases involve human contact with feces, the provision of adequate sanitation facilities, safe water and hygiene would highly reduce their transmission.

2.2.2.2 Viruses. Viruses excreted in feces cannot multiply outside of an adequate host cell, but they can survive for many weeks in the environment, especially in temperature less than 15 °C. They can be isolated from water and

soil contaminated with human excreta and the usual load of viruses is 10^9 infectious virus particles per gram of human feces, regardless of whether the individual is experiencing any disease (Feachem et al., 1983). Viral pathogens excreted in human feces are a major cause of gastroenteritis worldwide causing acute disease with a short incubation period (WHO, 2008c).

Rotavirus alone is a leading cause of severe diarrheal disease in infants and children (Hamer & Gorbach, 2002) and the most important single cause of infant death worldwide (WHO, 2008c). In 2003, it was estimated that annually rotavirus causes 111 million episodes of diarrheal requiring home care, 25 million clinic visits, 2 million hospitalization and about 352,000 – 592,000 deaths in children less than five years of age. Eighty two percent of death cases occurred in poor countries (Parashar, Hummelman, Breeze, Miller, & Glass, 2003).

The virus is transmitted by the fecal-oral route. Children aged 3 to 15 months are the most affected, while older children and adults can pass the virus in their feces asymptotically (Hamer & Gorbach, 2002). An infected individual excrete up to 10^{11} rotaviruses per gram of feces for about 8 days; for this reason areas with poor environmental conditions (lack of sanitation and safe water) are at higher risk of transmission. Waterborne outbreaks have also been described (China 1982-1983) (WHO, 2008c).

Symptoms of the infection start after 1 to 3 days of incubation and include severe watery diarrhea, fever, abdominal pain and vomiting. They last 5 to 7 days and complications such as severe dehydration, metabolic acidosis, and death can occur if patient is not treated adequately (Hamer & Gorbach, 2002).

Control measure to prevent the disease include avoiding source water contamination by human waste and protection of drinking-water supplies from contamination during its distribution (WHO, 2008c).

Caliciviruses are single-stranded RNA viruses that include the genera Norovirus (Norwalk-like virus) and cause gastroenteritis in all age groups. The morphologic and physiologic abnormalities caused by these viruses reverse in 1 to 2 weeks. Symptoms last for 24 to 48 hours and include diarrhea, nausea, abdominal cramps, vomiting, and muscle aches (Hamer & Gorbach, 2002).

A systematic review estimated that norovirus infections causes 900,000 clinic visits and 64,000 hospitalizations each year in industrialized countries, and up to 200,000 deaths in children less than 5 years old in developing countries (Patel et al., 2008). During 2002, CDC received reports of 21 outbreaks of gastroenteritis in cruise ships; four were confirmed to be associated with noroviruses (CDC, 2002). Transmission occurs by the fecal-oral route and therefore, the virus is passed in human feces and can pollute the environment if adequate sanitation and safe water are lacking. Asymptomatic carriers can shed the virus for up to two weeks, and the virus survive chlorinated water, freezing temperature and heating temperature (60° C) as well. Person to person contact, inhalation of contaminated aerosols and dust particles, and airborne particles of vomitus are also common routes of transmission. Outbreaks of the disease have been linked to contaminated water supply. Control measure to prevent the disease include avoiding source water contamination by human waste and

protection of drinking-water supplies from contamination during its distribution (WHO, 2008c).

Adenovirus (double-stranded DNA) serotypes 40 and 41 are responsible for gastroenteritis in children less than 2 years old. The incubation period for these viruses is of 8 to 10 days and the disease can last for up to 2 weeks (Hamer & Gorbach, 2002). These viruses are excreted in large number in human feces and they have been found in sewage, raw water sources and treated drinking water supplies worldwide. However, the information regarding these viruses is limited because they are not detectable by conventional lab methods. Contaminated drinking water could be a source of infections and because they are resistant to water treatment and disinfection processes, *E. coli* is not an adequate indicator of their presence or absence from drinking water supplies (WHO, 2008c).

Astroviruses are single-stranded RNA viruses responsible of diarrheal diseases in children under five years of age. The disease is self limited and the symptoms include watery or mucoid stools, nausea, vomiting, and fever. Approximately 6% of the infected children develop dehydration and underestimation of the cases is likely since the disease is usually mild (Hamer & Gorbach, 2002). Outbreaks of diarrhea caused by this virus have been reported in families, schools and day care centers (WHO, 2008c).

The virus is transmitted by the fecal oral route. Infected individuals pass a large number of viruses in their feces and contamination of the environment is possible in areas where sanitation is lacking. It has been detected in contaminated water

sources and drinking water supplies but transmission by drinking water has not been confirmed (WHO, 2008c).

The genus *Enterovirus* is a group of single-stranded RNA viruses that includes 69 serotypes that infect humans: poliovirus type 1-3, Coxsackievirus types A1-A24, echovirus type 1-33 and Enterovirus types EV68-EV73 (WHO, 2008c). Humans, especially children, are commonly infected by these viruses which are the cause of a variety of illnesses including poliomyelitis, nonspecific febrile illnesses, herpangina, uncomplicated hand-food-and-mouth disease, respiratory tract infections, and complications, such as meningitis, encephalitis, paralytic disease, myocarditis, chronic or disseminated infection in immunocompromised individuals and newborns with neonatal sepsis (Abzug, 2004).

The majority of infections are asymptomatic but in those that become ill, severity and incidence is inversely related to age (Abzug, 2004). Infected individual excrete the viruses in their feces for many weeks and transmission occur mainly by the fecal-oral or by the respiratory route if a respiratory illness is associated (WHO, 2002a). Viruses have been detected in soil, sewage, water sources, treated drinking water supplies and food (WHO, 2008c).

Poliovirus is transmitted through contaminated food and water and affects mainly children under five years of age. One of 200 infections leads to irreversible paralysis and 5% to 10% of those paralyzed die. The number of polio cases have decreased with vaccination but still there are four endemic countries: Afghanistan, India, Nigeria and Pakistan (WHO, 2008a). A total of 839 cases of

polio have been reported to the WHO during the first 7 months of year 2009. These cases include outbreaks in endemic (621 polio cases) and non-endemic countries (218). The last outbreak of polio was reported in July 2009, in the northern state of Nigeria with 363 cases of wild poliovirus type. Poor sanitation and hygiene conditions facilitate the entrance of the virus in the environment and transmission of the disease to susceptible populations (Global Polio Eradication Initiative, 2009).

Hepatitis refers to inflammation of the liver and hepatitis A and E virus are two of the causal agents of the disease. The transmission is from person to person and by the fecal oral route through ingestion of contaminated water or food. Therefore, the disease is associated with poor sanitation and hygiene and lack of safe water. In areas where hepatitis A virus is endemic, the disease occurs in early childhood without long term-consequences and a low mortality (0.2%). In adults the disease is severe with a mortality of 70 to 90% and uncommon survival in patients 50 years of age and over. Symptoms of the disease include fever, weakness, anorexia, nausea, abdominal discomfort and jaundice. In severe cases fulminant hepatitis leads to death (WHO, 2001a).

Hepatitis A virus is distributed worldwide and, in areas where sanitation lacks, children become immune early in life. In areas where sanitation is adequate, the infection occur later in life (WHO, 2008c). Hepatitis E virus has a longer incubation period (average 40 days) than Hepatitis A virus and, it affects young adults rather than young children. This virus is also an important cause of viral hepatitis in developing countries including India, Nepal, central Asia, Mexico

and parts of Africa. Similar to Hepatitis A virus, fecal polluted water is an important route in the transmission of the disease (WHO, 2008c).

2.2.2.3 Protozoa. Protozoa and helminthes cause a significant amount of morbidity and mortality worldwide and many of them are transmitted by ingestion of contaminated water and food with feces of infected humans and animals. Their resistance to different treatment and environmental conditions (including water treatment by wastewater treatment plants) make them more prevalent in areas where access to safe water and adequate sanitation are limited. However, in developed areas protozoa represent a health risk in immunocompromised individuals and travelers going to developing countries (WHO, 2008c) .

Protozoa infections caused by *Giardia* spp., *Cryptosporidium parvum*, *Entamoeba histolytica* and *Cyclospora cayetanensis* account for the majority of infections caused by these group of parasites (Pierce & Kirkpatrick, 2009). Many infected individuals remain asymptomatic and those who become ill present dysentery and diarrhea as their main symptom (Feachem et al., 1983).

Cryptosporidium parvum is a very resistant protozoa that can survive for weeks and even months in fresh water. It is transmitted by fecal-oral route through contact with contaminated water (drinking or recreational water) or food. The oocysts of the parasite are excreted in feces of infected individuals and animals and are highly resistant to oxidizing disinfectants such as chlorine. In addition the oocysts are very difficult to remove by conventional filtration processes (WHO, 2008c).

The parasite can infect individuals with as few as 1 to 10 oocysts causing self-limited diarrhea of 10-14 days of duration in immunocompetent individuals. In immunocompromised individuals, the clinical presentation of diarrhea is similar to that of cholera and can be fatal (Hutson & Guerrant, 2002). In developed countries *C. parvum* is a cause of diarrhea outbreaks in hospitals and day care centers. The most known outbreak is the waterborne outbreak in Milwaukee, USA, in 1993, where more than 400,000 people were infected (Hutson & Guerrant, 2002). In developing countries, *C. parvum* is a common cause of acute and persistent diarrhea in children and immunocompromised individuals (Huang, Chappell, & Okhuysen, 2004).

The prevalence of *Cryptosporidium* in the developing world had been reported to be 29.6% among children with diarrhea episodes (Miller et al., 1994), and 8.5% among children with no symptoms of the disease (Tumwine et al., 2003). The parasite is found more frequently in children with persistent diarrhea than in those with acute or no diarrhea and it has been significantly associated with malnutrition (Newman et al., 1999; Tumwine et al., 2003). Children with severe dehydration, persistent diarrhea, stunted or underweight children have higher mortality rates than those without these conditions (Tumwine et al., 2003).

Entamoeba histolytica is distributed worldwide and, as *C. parvum*, the cysts are passed in the feces, survive in the environment for weeks and are very resistant to chlorination and desiccation. It is transmitted by the fecal-oral route mainly, although sexual transmission, particularly among male having sex with men, has been documented (WHO, 2008c). Approximately 90 % of infected

individuals are asymptomatic carriers. The other 10% of infected individuals develop dysentery and a small percentage extra intestinal disease (e.g. amebic liver, pericarditis, lung abscess, brain abscess). Other symptoms include abdominal pain, tenesmus and fever. Acute necrotizing colitis is a lethal complication in 0.5% of cases if no treatment is provided (Hutson & Guerrant, 2002).

The non pathogenic *E. dispar* is similar in appearance to *E. histolytica* and for this reason, there are large differences in the reported numbers of carriers or infected individuals among countries (Gendrel, Treluyer, & Richard-Lenoble, 2003). The incidence is higher in developing countries where lack of sanitation and safe water is limited. The contribution of the parasite to the burden of diarrheal diseases in children in developing areas is substantial (Haque et al., 2006). The prevalence in developed countries may be less than 10% (WHO, 2008c). Studies of *E. histolytica* in the developing world revealed a prevalence of 11.2% (Vietnam) to 13.8% (Mexico) among asymptomatic individuals (Blessmann et al., 2003; Ramos et al., 2005). Non symptomatic carriers were 2.7 times more likely to re-infection than the general population (Blessmann et al., 2003).

Giardia lamblia is another protozoon that can survive for weeks and months in fresh water, very resistant to dehydration, extreme temperature and chlorination. It is also known as *Giardia intestinalis* or *G. duodenalis* (WHO, 2008c). It is excreted in large numbers in feces and transmitted by the fecal oral route. Ingestion of as few as 10 cysts can result in infection. The majority of

infections occur in infants and essentially all children have been infected by 2 to 3 years of age. Asymptomatic infections are common in children and adults. *Giardia* causes a variety of symptoms, from asymptomatic infections to severe chronic diarrhea with malabsorption. Symptoms may include fatigue, abdominal cramps, bloating, malodorous stool, flatulence, weight loss, fever and vomiting. Immunocompromised individuals and children less than two years old are at higher risk of severe diarrhea and malabsorption (Hutson & Guerrant, 2002).

The parasite is distributed worldwide and is an important cause of chronic diarrhea, malnutrition and cognitive impairment in children in developing countries (Ali & Hill, 2003). In the United State, *Giardia* is a frequently identified intestinal parasite associated with waterborne and food borne outbreaks (WHO, 2008c). In developing countries *Giardia* is endemic and affects children mainly. Lack of sanitation and safe water have been described as risk factors for infection (Prado et al., 2003; Redlinger et al., 2002) and vulnerable population for the disease include children with underweight, stunting and wasting (Al-Mekhlafi et al., 2005). Persistent diarrhea and recurrent or relapsing infections are common among symptomatic infected children (Robert et al., 2001).

Cyclospora cayetanensis has a clinical presentation similar to *Cryptosporidium* but it differs in that the parasite is not immediately infective after being passed in the feces (Hutson & Guerrant, 2002). *Cyclospora* requires 7 to 12 days in the environment to become infective and the oocysts are resistant to disinfection and chlorine. It is transmitted by the fecal-oral route and the primary source of exposure is contaminated water or food. Symptoms of the disease

include watery diarrhea, abdominal cramps, weight loss, anorexia, myalgia, vomiting and/or fever, and relapse is common (WHO, 2008c).

In the United States and Canada, *C. cayetanensis* has been associated to food borne outbreaks (Hutson & Guerrant, 2002). In the developing world Cyclospora infections had been found to be relatively common, especially among children 1.5 to 9 years old, and often asymptomatic. Unsafe water and contact with soil have been found significantly associated with the disease in children (Bern & Hernandez, 1999). A suburban community in Venezuela showed a Cyclospora prevalence of 6.1%. Of those infected, 84.6% were asymptomatic (Chacin-Bonilla, De Young, & Estevez, 2003). Another study in Jordan identified a 6% prevalence of Cyclospora among symptomatic cases (Nimri, 2003).

2.2.2.4 Helminthes. Helminthes infect a large number of people and animals. Soil transmitted helminths (*Ascaris lumbricoides*, *Trichuris trichiura*, *Ancylostoma duodenale* and *Necator americanus*) and schistosomiasis are the most common helminth infections around the world. These parasites cause a high burden of disease and health consequences, especially in children in developing countries (Feachem et al., 1983). They are concentrated in tropical and subtropical areas and mainly affect poor populations which lack of adequate housing, sanitation and safe water. More than 2 billion people are affected by these parasites and co-infection with more than one parasite is not unusual (WHO, 2009c). Between 3.9 and 4.5 million DALYs are estimated to be lost to soil-transmitted helminthes and schistosoma respectively; and children,

adolescents and women of reproductive age are at the highest risk to suffer these illnesses (WHO, 2001).

Infections with these parasites are a major public health concern since the poorest people are the most affected and cost-effective solutions to reduce morbidity are available (WHO, 2001). Although, these parasites rarely cause death they have an important health impact on those chronically affected. Chronic and high intensity infections can result in malnutrition; stunting and cognitive impairment (Hotez et al., 2006). Repeated infections can also lead to macronutrient and micronutrient deficiencies with adverse consequences that can perpetuate from one generation to the next. Infants born from pregnant undernourished women have an increase risk of been born with prematurity, low birth weight and early death (Weigel et al., 1996). If they survive, they also have an increased risk of poor growth and development in childhood and adolescence (Steketee, 2003). People infected pass the parasite eggs in their feces and, in about 2 to 3 weeks of development in the environment, the eggs are ready to infect a new host. The transmission is by the fecal-oral route, in the case of soil-transmitted helminthes, and by direct contact of the skin with contaminated soil, in the case of hookworms.

A. lumbricoides infects more than one 1 billion people worldwide with 350 million cases presenting morbidity and 60,000 dying each year (WHO, 2001). It does not produce symptoms unless an individual carries large number of worms. Children become infected by eating dirt or by placing dirty fingers and toys in their mouth. Once ingested, eggs hatch in the duodenum and the worm

penetrates the mucosa producing little damage. Larva migrates through the lymphatics or venules to the lungs. Transplacental migration has been also documented. The larva molts twice in the lungs, breaks into the alveoli and ascends to the pharynx where it is swallowed and passes through the stomach to the small intestine, where it matures. In the small intestine the worm feeds on the liquid content of the intestinal lumen and in moderate and severe infections can cause malnutrition, underdevelopment and cognitive impairment in small children. Abdominal pain, asthma, rashes, eye pain, insomnia, and restlessness, could result from the allergic responses to metabolites liberated by the worm. Intestinal obstruction is the most common complication and fatality could result from obstruction, intussusceptions, or volvulus, due to intestinal necrosis. Because worms are highly motile, they could also cause biliary duct obstruction, liver abscesses, ascending cholangitis, acalculous cholecystitis or acute pancreatitis (Elliott, 2002; Schmidt & Roberts, 2000). Adult females may produce 200,000 ova per day which are passed in the feces and, under favorable conditions (warm, moist, shaded soil), may become infective in 2 to 3 weeks, completing the life cycle (John & Petri, 2006).

T. trichiura infects approximately 795 million people and its transmission is similar to that of *A. lumbricoides*. Once an infective ovum is ingested, it hatches in the intestine and the larva migrates to the cecum, mature, mate, and lay eggs (8-12 weeks). If the individual harbor small number of worm (less than 15), the parasite acts as commensal. Small children are predisposed to heavy infections (200 to 1,000 worms), which could result in dysentery, anemia, growth

retardation, finger clubbing (odd thickening of the ends of the digits) and rectal prolapse. Moderate to heavy infection also affect cognitive function of children. As the anterior end of the parasite remains embedded in the colon mucosa hemorrhage could occur resulting in anemia. Trichuriasis mimics inflammatory bowel diseases, but is readily curable. The adult female passes 3,000 to 7,000 eggs daily in the feces; under adequate environmental conditions (warm, moist, shaded soil), they become infective in approximately 10 days (Elliott, 2002; John & Petri, 2006; Schmidt & Roberts, 2000).

Ancylostoma duodenale and *Necator americanus* (hookworms) infect approximately 740 million people worldwide, with the majority of infections occurring in sub-Saharan Africa (WHO, 2001). The infective stage of the parasite is the third-stage larvae which penetrate the intact skin after exposure to contaminated soil. Their manifestation and pathogenesis follows the migration path in an individual through the skin, lungs, and intestine. In the cutaneous phase the larva can produce a pruritic serpiginous rash. Once the larva enters the blood vessels, it migrates to the right heart and the lungs. When it breaks into the alveoli each site hemorrhages with serious consequences in massive infections but usually the lung phase is asymptomatic. Then, the larva migrates up the pulmonary tree and is swallowed maturing into the small intestine. The intestinal phase is the most important period of pathogenesis as the worm attach to the mucosa and start feeding on blood. The worm changes attaching at multiple places in the mucosa exacerbating bleeding and blood loss. Calculated

blood loss is about 0.03 ml per day for *N. americanus* and 0.26 ml per day for *A. duodenale* (Schmidt & Roberts, 2000).

Heavy infections may cause an individual to lose up to 200ml of blood per day producing an iron-deficiency anemia as the body iron reserves are lost. Abdominal pain, loss of appetite, and geophagy are common symptoms of moderate infection. Heavy infections result in severe protein deficiency, edema and potbelly in children with delayed puberty, mental dullness, heart failure and death. The disease is usually manifested in the presence of malnutrition and complicated with other parasites. If the young do not have an adequate diet, losing protein and iron, causes stunted growth and below-average intelligence. Treatment can substantially increase fitness, appetite and growth. To complete the life cycle, adult females pass up to 10,000 (*N. americanus*) and 30,000 (*A. duodenale*) eggs per day. These eggs developed to infective filariform larvae, under adequate environmental conditions (warm, moist, shaded soil), in about 5 to 10 days (Elliott, 2002; John & Petri, 2006; Schmidt & Roberts, 2000).

Schistosoma spp. (*Schistosoma haematobium*, *S. japonicum*, and *S. mansoni*) infect about 187 million people worldwide. This parasite requires a water source and a snail to develop in the environment. In the body the damage is caused primarily by the eggs of the parasite. In general, eggs are eliminated with feces or urine and under optimal conditions they hatch and release a miracidia. This miracidia swims and penetrates a specific snail (intermediate host) and develops to a cercaria, which penetrates the skin of the human host after being released from the snail. During the skin penetration cercaria loses its

forked tail and becomes schistosomulae. Dermatitis may develop if the individual has been sensitized by earlier infections. The schistosomulae migrates through several tissues to their residence in the veins. The migration phase of this parasite is usually asymptomatic. Adult worms of *S. japonicum* are frequently found in the superior mesenteric veins draining the small intestine, and *S. mansoni* in the superior mesenteric veins draining the large intestine. *S. haematobium* most often occurs in the venous plexus of bladder, but it can also be found in the rectal venules. Females parasites deposit the eggs in the small venules of the portal and perivesical systems moving progressively towards the lumen of the intestine (*S. mansoni* and *S. japonicum*) and of the bladder and ureters (*S. haematobium*), eliminated with feces or urine, respectively (Centers for Disease Control & Prevention, 2009). The acute phase of the disease (Katayama fever) occurs when the parasite start laying eggs and it is characterized by chills and fever, fatigue, headache, malaise, muscle aches, lymphadenopathy and gastrointestinal discomfort. When the eggs of the parasite remain in the gut or bladder wall a delayed type hypersensitivity reaction occur around the eggs. In the chronic phase, mild, chronic, bloody diarrhea with mild abdominal pain and lethargy develop. Those affected by *S. haematobium* may suffer from painful urination and blood in the urine (Schmidt & Roberts, 2000).

Those affected by *S. japonicum* and *S. mansoni* may develop egg granulomas and fibrosis in the liver limiting the portal blood flow. As the fibrotic reactions continue, periportal cirrhosis, portal hypertension, splenomegaly and ascites develop. Some eggs produce pseudotubercles in the lungs, nervous

system and other organs. *S. japonicum* is the most severe of the three species and 60% of all neurological disease and almost all brain lesions are due to this specie. *S. haematobium* is considered the least serious, but the casual relation with bladder cancer is strong. Invasion of the female reproductive tract is also common (Schmidt & Roberts, 2000).

The main strategy recommended by WHO to control and prevent these helminthes infections is administration of anthelmintic drugs. This strategy reduces morbidity in intensely infected populations in the short term (Hotez et al., 2006). Access to adequate and safe sanitation facilities is the most important intervention to ensure sustained reduction and elimination of these parasitic diseases in the long term (WHO, 2006c).

2.2.3 Categories of excreta related pathogens. In order for an infection to be transmitted, a pathogen excreted (from a patient, carrier or reservoir) needs to enter the susceptible host by the mouth or some other route. There are several factors influencing this pathogen transmission route (WHO, 2006b):

- Epidemiologic characteristics (infectious dose, latency, intermediate host, etc.)
- Persistence in different environment outside the human body
- Major transmission routes
- Relative susceptibility to different treatment techniques
- Management control measures

The excreted load², latency³, persistence⁴, and multiplication of a pathogen will influence the infective dose or dose required to infect a new host (Feachem et al., 1983). The excreted load is determined by the infectious prevalence and volume of feces excreted (WHO, 2006b). The latent period depends of the organism; viruses, bacteria and the majority of protozoa have no latent period and become infectious immediately after being excreted; soil transmitted helminthes require completing a stage of their life cycle in the soil before becoming infectious (Feachem et al., 1983).

The persistence of the pathogens in the environment will depend on the receiving environment. The risk of infection will depend of the infective dose and the vulnerability of new host (WHO, 2006b). Individuals infected with *Ascaris* spp. may develop little immunity, while infections with virus or bacteria may produce long-lasting immunity (Feachem et al., 1983). Feachem et al. (1983), created VI categories of excreta related pathogens based on the environmental or epidemiological characteristics: latency, infective dose, persistence, multiplication and transmission (Table 2.5).

Category I have the lowest median infective dose ($ID_{50} < 10^2$), spread easily from person to person and become infective immediately after excretion. Category II includes persistent bacteria which are less likely to spread person to person and have a medium to high median infective dose ($ID_{50} \geq 10^4$). Parasites are included in Category III, IV and V. They are highly persistent and require

² Excreted load: concentration of pathogens passed in the feces of an infected person.

³ Latency: the interval between the excretion of a pathogen and its becoming infective to a new host.

⁴ Persistence: viability of the pathogen in the environment.

completing a stage of their life cycle out of the host to become infective. Category VI does not really refer to pathogens, instead it refers to insect vectors of diseases such as flies and cockroaches, which are vehicles to spread excreta related pathogens diseases.

Table 2.5

Categories of Excreta Related Pathogens

Pathogen	Latency (days)	Persistence	Multiplication Outside Human	Median Infective Dose (ID50)	Intermediate Host
CATEGORY I					
Enteroviruses ¹	0	3 months	No	L	None
Hepatitis A virus	0	?	No	L	None
Rotavirus	0	?	No	L	None
Entamoeba histolytica	0	25 days	No	L	None
Giardia lamblia	0	25 days	No	L	None
CATEGORY II					
Campylobacter jejuni	0	7 days	Yes ²	H	None
Escherichia coli ³	0	3 months	Yes ^b	H	None
Salmonella					
S. typhi	0	2 months	Yes	H	None
Other salmonellae	0	3 months	Yes	H	None
Shigella spp.	0	1 month	Yes	M	None
Vibrio Cholerae	0	1 month	Yes	H	None
Yersinia enterocolitica	0	3 month	Yes	H	None
CATEGORY III					
Ascaris lumbricoides	10	Several months	No	L	None
Hookworms ⁴	7	3 months	No	L	None
Trichuris Trichiura	20	9 months	No	L	None
CATEGORY IV					
Taenia saginata/solium	60	9 months	No	L	None
CATEGORY V					
S. haematobium	35	2 days	Yes	L	Snail
S. japonicum	49	2 days	Yes	L	Snail
S. mansoni	28	2 days	Yes	L	Snail

¹ Includes poliovirus, echovirus and coxsackieviruses

² Multiplication takes place predominantly on food

³ EIEC, EPEC, ETEC, EHEC

⁴ *Ancylostoma duodenale* and *Necator americanus*

Source:(Feachem et al., 1983)

Adequate sanitation facilities will create a barrier to the transmission of these diseases, but the highest control and effectiveness would be achieved for

diseases in categories III, IV and V (Feachem et al., 1983). Nevertheless, discharge of wastewater into rivers or its use in agriculture facilitate the transmission of all fecal pathogens if the wastewater is not adequately treated (Schonning & Stenstrom, 2004).

Providing access to sanitation facilities as well as assuring their use by all members of the household is important to control these diseases. Most of the excreta related diseases affect children and teenagers, who usually do not use the toilets because it is located away from the house or because of fear of falling down the toilet. These behaviors increase their own health risk and the risk of the neighbor. Therefore, health education is as important as an adequate sanitation facility.

2.2.4 Pathogen survival. The main natural environmental factors limiting pathogen survival in feces are time and temperature. After excretion, viruses and protozoa start decreasing in number, bacteria may multiply if the environment conditions are adequate and rich in nutrient, and helminthes may survive for long periods of time. Low temperatures ($< 5\text{ }^{\circ}\text{C}$) increase pathogen survival, while most of them are inactivated at high temperatures ($> 40\text{ }^{\circ}\text{C}$) showing an exponential decline in population (Feachem et al., 1983).

2.2.4.1 Survival in feces and soil. The natural die-off of pathogens in feces depends on time, type of organism and storage conditions such as temperature, pH, moisture, and biological competition. Storage conditions and type of pathogen influence their inactivation. Table 2.6 shows the survival time of

pathogens in feces and soil, considering storage and no additional treatment (Schonning & Stenstrom, 2004).

The soil factors affecting survival time of bacteria are moisture content, organic matter, pH, sunlight and temperature. Survival time is increased by moist soil, the presence of organic matter in soil, alkaline pH, low sunlight exposure and low temperature. Viral survival is increased at low temperatures; protozoa cysts are unlikely to survive more than 10 days, and helminthes eggs could survive for several years (Feachem et al., 1983). When feces are applied to crops, pathogen inactivation increases due to temperature, exposure to sun, and low air humidity.

Table 2.6

Survival Times of Excreted Pathogens at 20-30 °C and Decimal Reduction Values during Storage

Pathogen	Survival Time in Feces and Sludge (days)	Faeces T90 ~ 20°C ^b	Survival Time in Soil (days)	Survival Time on Crops (days)
Viruses				
Enteroviruses	<100	*	<100	<60
Rotavirus	*	20-100		
Hepatitis A	*	20-50		
Bacteria				
Fecal coliforms	<90	15-35 (E. coli)	<70	<30
Salmonella spp.	<60	10-50	<70	<30
V. cholera	<30		<20	<5
Protozoa				
E. histolytica cysts	<30	*	<20	<10
Giardia	*	5-50		
Cryptosporidium	*	20-120		
Helminths				
A. lumbricoides ova	Several months	50-200	Several months	<60

* data missing Source: Adapted from Feachem et al. (1983) and Caroline Schonning et al. (2004).

2.2.4.2 Indicators of fecal contamination. The indicator used in water to determine water fecal contamination is the non-pathogenic fecal coliform

bacteria. This indicator is made up two groups: total coliforms, which includes coliform bacteria from feces and also from unpolluted soil and water; and fecal coliforms, which originate exclusively from human and warm-blooded animal feces (Viessman & Hammer, 2005). There is not a specific indicator to determine pathogen inactivation in feces.

According to the United States Environmental Protection Agency (USEPA) human excreta is considered meeting Class A biosolids⁵ requirements if:

- They do not contain detectible levels of pathogens
- They meet the vector attraction reduction requirements
- They have low levels of metals contents

Only after meeting pathogen reduction requirements (Table 2.7), Class A biosolids can be recycled and used as fertilizers in the field (U.S Environmental Protection Agency, 2008).

Table 2.7

Detection Limits of Pathogens for Class A Biosolids

Pathogens/Indicator	Limit (dry-weight basis)
Fecal coliform	< 1000 fecal coliform per gram of total solids
Salmonella spp.	< 3 MPN Salmonella sp. /4 g. total solids
Enteric virus	< 1 PFU/4 g. total solids
Viable helminth ova	< 1 viable ova/4 g total solids

Source: (U.S Environmental Protection Agency, 1994)

MPN: most probable number

PFU: plaque forming unit

⁵ Biosolids: According to USEPA, they are nutrient-rich organic materials resulting from the treatment of domestic sewage in a treatment facility. When treated and processed, these residuals can be recycled and applied as fertilizer to improve and maintain productive soils and stimulate plant growth.

The treatment alternatives to achieve Class A biosolids set by USEPA are practiced in developed countries. In developing countries, sanitation alternatives are different and present a challenge to public health. With the adoption of the MDG, decentralized sanitation systems are in expansion and, other treatment alternatives to meet Class A biosolids are being developed. To assure that these new systems produce safe biosolids the World Health Organization developed the “Guidelines for the safe use of wastewater, excreta and greywater” (World Health Organization, 2006a).

The necessity of assuring a safe end-product comes from the opportunity and value of using excreta as a fertilizer, mainly in developing countries. If adequately managed, excreta could have a positive health impact increasing food security, nutritional status, and income (WHO, 2006b). The next section will describe Dry Sanitation as one of the options to on-site treatment of excreta.

2.3 Sanitation Systems

A sanitation system could be defined as the combination of technologies involved to manage wastes from the point of generation to the point of use or ultimate disposal. Management, operation and maintenance are important components of any sanitation system since these components assure the sustainability and functionality of the system (Tilley et al., 2008).

Eight sanitation system templates or models have been described by the Compendium of Sanitation Systems and Technologies published by the Swiss Federal Institute of Aquatic Science and Technology:

System 1: Single Pit System

System 2: Waterless System with Alternating Pits

System 3: Pour Flush System with Twin Pits

System 4: Waterless System with Urine Diversion

System 5: Blackwater Treatment System with Infiltration

System 6: Blackwater Treatment System with Sewerage

System 7: (Semi-) Centralized Treatment System

System 8: Sewerage System with Urine Diversion

The sanitation system models range from simple (System 1) to complex (System 8) and each of them describes a group of compatible technology combinations that could be used to design a sanitation system (Tilley et al., 2008). System 4, waterless system with urine diversion, describe those technologies that do not use water to collect and transport feces and urine and separates urine from feces at the source point. This system embraces dry toilet technologies and will be described further in this chapter. Please refer to the Compendium of Sanitation Systems and Technologies for more information about other systems (Tilley et al., 2008).

2.3.1 General description of waterless system with urine diversion.

Waterless systems with urine diversion divert urine away from feces. It does not use water and it is especially useful in deficient water regions, in areas with a high groundwater table and in rocky areas where is difficult to dig. The devices used by individuals in this type of system include a urine diverting dry toilet (UDDT) or/and a urinal. The culture of the target population (sitters, squatters,

washers, wipers, etc) determines the products flowing into the system and the number of diversions in the device. The products could be excreta (feces and urine), anal cleansing water⁶ and dry cleansing materials⁷; the devices include a toilet seat, a 3-hole squat slab or a 2-hole squat slab (Figure 3) (Tilley et al., 2008).

Feces are collected, stored and dehydrated in watertight dehydration vaults. To function adequately the vaults are kept as dry as possible and additives such as ash, lime, dry earth, etc., are used to minimize odors and vectors. The additives increase the pH of the material, potentiating inactivation of pathogen. Once feces complete the storage time recommended, the user usually removes and transports the material for use or disposal (Tilley et al., 2008).

Urine is collected, stored and treated in sealed storage tanks. Human urine usually does not contain pathogens that could represent a health risk to the environment, and contains nutrients (N, P and K) that make it a valuable resource to fertilize crops (Schonning & Stenstrom, 2004). For this reason urine could be diverted directed to the ground or use in the land (Tilley et al., 2008). Nevertheless, urine storage is recommended since cross-contamination with feces is possible. At 4°C - 20°C, the recommended storage time varies from one to six months in large scale systems depending on the type of crop to be fertilized. For single household, direct urine application could be practiced as long as one month passes between fertilization and harvest (Schonning &

⁶ Anal cleansing water refers to the water use to clean oneself after defecating and/or urinating.

⁷ Dry cleansing material refers to paper, corncobs, stones or other dry material used for anal cleansing.

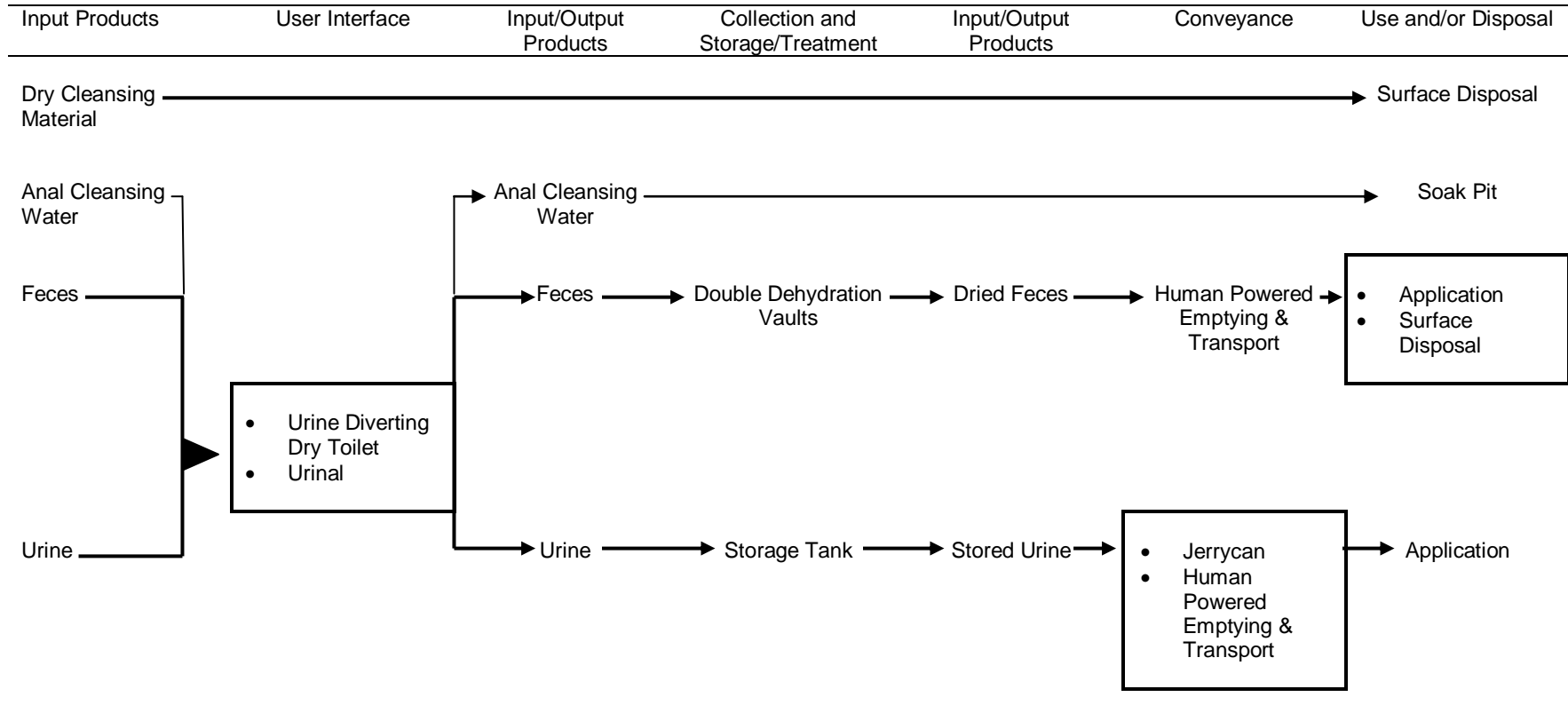
Stenstrom, 2004). The benefits of separating urine and feces are known in the areas of the world where this practice is a tradition; they include (Schonning & Stenstrom, 2004):

- Reducing the volume of the material. Dehydration decreases the fecal material volume and the vault can be used for longer periods of time.
- Reducing smell. This increase convenience and acceptability of the toilet and handling of excreta.
- Reducing human and environmental risk of contamination with fresh excreta. Groundwater, environmental, and human contamination is avoided by preventing leaching, dispersion of potential pathogens present in excreta, and direct contact with human respectively.
- Reduction of pathogen in excreta and facilitates manipulation of excreta. Drying the feces allows some pathogen inactivation, adding other chemical materials to achieve further inactivation and facilitates handling urine and feces separately.

These benefits apply to the system if proper operation and management is practiced. The adequate performance of the system, from the technological point of view, will depend on the effective separation of feces and urine, the use of additives, and the environment temperature contributing to the dehydration of feces. User's preferences should be taken in account at the moment of deciding agricultural use of urine. When anal cleansing water is used, it could be mixed with urine if no application to agriculture is planned; otherwise anal cleansing water should be treated as greywater (Tilley et al., 2008).

Table 2.8

System Template for Waterless System with Urine Diversion



Adapted from Tilley et al., 2008

2.3.2 Urine dry diverting toilet and Eco-san. A dry urine diverting toilet (UDDT) is a sanitation device that does not use water and separates feces and urine. In populations where squatting defecation posture and anal cleansing water is practiced the UDDT is designed with three divisions (Figure 2.3); in population where sitting defecation posture and dry cleansing material are used the UDDT is designed with two divisions (Figure 2.4).

This sanitation device and its waterless technology is promoted by ecological groups since it does not use water and therefore, does not pollute or waste water and human waste become a resource for soil conditioning and/or fertilizer that can be used if sanitized appropriately. This approach is also known as “ecological sanitation” or “eco-san” (Winblad & Simpson-Hebert, 2004).

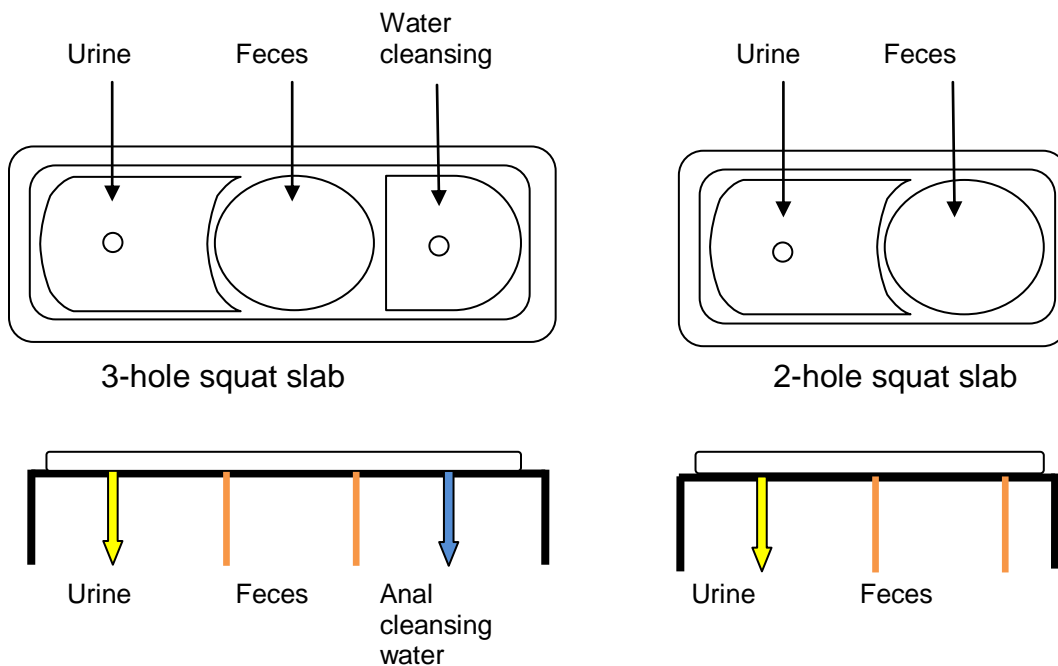


Figure 2.3 UDDT: Squat Slab



Figure 2.4 UDDT: Toilet Seat

In addition to the health benefits of having adequate sanitation, this technology brings other benefits that impact positively human health. First, reduction in the contamination of water sources from inadequate sewage discharge and conventional pit latrines that store feces. This problem occurs in developing countries mainly since water treatment plants do not have the technology to optimally reduce fecal pathogens to a safe level before discharge and, there is no enforcement of environmental standards, if any exist (WHO, 2006a). By contaminating water sources a route of transmission of fecal pathogens is created increasing the health risk of individuals that come in contact with and/or ingest this water.

Second, reduction in household water usage. Sewage sanitation systems use large amounts of water and economic resources to transport human waste to centralized treatment plants. This aspect is important as population is growing in cities and urban slums become more common. Third, usage of sanitized excreta for agricultural purposes. This is an advantage since fertilizers prices are rising

and some components of current fertilizers are non-renewable and their sources are been depleted.

For any of these advantages and benefits to be tangible, certain elements must exist such as user acceptability and education, economic accessibility, proper construction, adequate management, operation and maintenance; effective sanitation of material; protection of the environment, and a plan developed on the disposal and/or use of the sanitized products. Mistakes made during the construction of the toilet and lack of understanding of its maintenance and use could lead to inadequate functioning of the system. This is important as acceptability and satisfaction of users is related to adequate performance of the system and support services (Cordova & Knuth, 2005).

The inactivation of pathogens present in excreta in this type of eco-san system is achieved in two steps. The first part of the process occurs in the chambers below the toilet. During this period feces are stored and fecal pathogens are reduced by storage time, decomposition, dehydration, competition of nutrient among microorganisms and increased pH (addition of alkaline additives). This is known as primary treatments. If needed, a secondary treatments (further storage time; high temperature; composting and alkaline pH) could take place out of the toilet chamber (off site) (Winblad & Simpson-Hebert, 2004). The combination of treatments will depend on the prevalence of enteric pathogens in each community. Helminth ova are very resistant to treatment and may requires further treatment (Winblad & Simpson-Hebert, 2004).

2.3.3 Solar toilet prototype IV. There are a variety of toilets functioning today that use the eco-san approach. The solar toilets built in El Salvador are among them and use a solar heater to increase the evaporation, temperature and dehydration of the material contained inside the chambers. These toilets were introduced in 1994 to El Salvador as part of a sanitation program supported by Ministry of Public Health, UNICEF and different non-governmental organizations. Since their introduction the solar toilets have been evolving to increase the storage capacity and improve the physical and chemical parameters used to sanitize the material.

The first prototype was known as Solar Toilet Prototype 1 (Tecpan) which had a single vault. The localization of the toilet in the shadow of bushes and trees limited its capacity to increase the temperature and inactivate pathogens, although the toilet achieved high pH (average pH=10.4). The second prototype was built in 1995 and was known as Tecpan II. In this model the chamber had a partition separating fresh from stored fecal material. Despite these improvements the overall size of the chamber was too small to allow adequate storage time and the peak temperature (34°C) and pH (8.2) were relatively low. The third prototype, built in 1999, had a larger chamber, allowing longer storage time, and good solar exposure resulting in an average pH and peak temperature of 10.3 and 42°C respectively (Moe & Izurieta, 2003).

Microbiological analysis from the three solar toilets showed their higher pathogen inactivation capacity and effectiveness at reducing the prevalence of enteric parasitic infections when compared to population using double-vault urine

diverting toilets (DVUD⁸), pit latrines and open defecation (Corrales et al., 2006). Nevertheless, longer storage time were suggested as higher concentrations of fecal coliforms, *C. perfringens* and coliphage were found when compared to DVUD toilets which usually allows an average storage time of 306 days (Moe & Izurieta, 2003).

Given this suggestion and the positive health impact in the communities using solar toilets, the Ministry of Public Health of El Salvador developed the solar toilet prototype IV in 2003 (Figure 2.5). This model had a larger chamber that allows storage of fecal material for approximately 60 days with better solar exposure and achieved a peak temperature of 41.5°C , an average pH of 9.8, a average moisture content of 15% (after 60 days of storage), and met the USEPA fecal coliforms standards (Izurieta et al., 2006).

A total of 23 toilets were built in a community in El Salvador. Ventilation tubes were added to five of them and one model was built with antisismic design. The option of placing the toilet next to the household was given to users since these toilets are not associated to bad odors and flies when adequately built and maintained. The chambers and solar panel were oriented to the south to assure the best sun exposure (Sorto, 2004).

⁸ The Spanish acronym for DVUD is LASF which stands for Letrine Abonera Seca Familiar or “dry fertilizer family latrine”.



Source: (Sorto, 2004)

Figure 2.5 Solar Toilet Prototype IV

In regards to the design, the solar toilet prototype IV did not include a hand washing device, a urinal, and a toilet seat cover for the cement toilet. These components are important incentive to use the toilet since the urinal decreases the chances of contaminating the fecal chamber with urine avoiding odor problems; the hand washing device facilitates hand washing after using the toilet and therefore, decrease the risk of disease transmission by contaminated hands; and a seat cover increase the comfort of the user.

Despite the advantages of the new prototype, in regards to the improved physical and chemical parameters to inactivate fecal pathogens, viable *Ascaris* ova were found in the stored material after 60 days of storage. The combination of dehydration, storage time, high temperature, alkaline pH achieved in the solar toilet prototype IV decrease the number of viable *Ascaris* ova but do not meet the USEPA standard to consider the fecal material safe from a public health perspective.

Ammonia is another chemical that may be used to reduce fecal pathogens in eco-san systems (Winblad & Simpson-Hebert, 2004) . In the last few years, many studies have been done to assess the capacity of ammonia as an agent for pathogen inactivation and the results have shown that un-ionized ammonia (NH₃) could potentially inactivate pathogens in solutions and sludge, including the *Ascaris spp.* eggs (Bujoczek, 2001; Chefranova lu, 1977; Chefranova lu et al., 1984; Chefranova lu et al., 1978; Ghiglietti et al., 1997; Ghiglietti et al., 1995; Mendez et al., 2002; Pecson et al., 2007; Pecson & Nelson, 2005). Studies in human feces are limited, but the results also indicate a high pathogen inactivation rate with un-ionized ammonia (Nordin et al., 2009; Vinnerås et al., 2003). The capacity of ammonia to inactivate pathogens is described in chapter four.

2.3.4 Sustainable sanitation systems. According to the Sustainable Sanitation Alliance (SuSanA) (2008) *“the main objective of a sanitation system is to protect and promote human health by providing a clean environment and breaking the cycle of disease. In order to be sustainable a sanitation system has to be not only economically viable, socially acceptable, and technically and institutionally appropriate, it should also protect the environment and the natural resources.”* This definition of sanitation includes different criteria important to make a sanitation system sustainable. One of these elements, *“socially acceptable”*, refers to the socio-cultural context that needs to be taken into account when planning to introduce any sanitation system to a community.

The success of a sanitation project depends not only on the sanitation system but also on changes in the hygiene behaviors of the community (Yacooob & Whiteford, 1994). As hygiene behavior is a key determinant of the effectiveness, use, and sustainability of a sanitation system, the World Health Organization (WHO) recommends performing a detailed assessment of the local social and cultural aspects of the community which vary in different parts of the world and directly impact behavior and practices (WHO, 2006a). A sanitation solution is not a “one-size fits all” since a system accepted in one culture does not guaranty that it could, or will, work in another one (Rosemarin et al., 2008). One of the basic principles to planning and implementing a sanitation system, is to involve participation of all stakeholders, especially consumers and providers of services (WSSCC/Sandec, 2000). Participatory community approaches have been used with the purposes of promoting hygiene behaviors, improving sanitation and fostering community management of water and sanitation facilities. PHAST, (Participatory Hygiene and Sanitation Transformation), is recommended by WHO to achieve this purpose (WHO, 2000c).

Although, there is a need for good quality evaluations of the effectiveness of this approach in large scale projects (World Bank, 2005) , the small field projects documented have been successful in empowering communities to manage and control sanitation related-diseases and improve their behavior and environmental conditions (WHO, 2000c). PHAST is a methodology of participatory learning adapted from the SARAR (Self-esteem, Associative strengths, Resourcefulness, Action-planning, and Responsibility) methodology. It

stimulates the participation of women, men and children and promotes the capacity of individuals to be creative and to be leaders of change (WHO, 2000c).

An experience from Mexico, in the peri-urban community of San Juan Tlacontenco, Cuernavaca, Morelos, with 30 families attributes the success of the sanitation project to the SARAR methodology (Sawyer, 2007). This 2,000 inhabitants community is located in a mountain with volcanic soil characteristics, practices low scale agriculture, has limited access to potable water, and uses pit latrines and pour flush toilets. In 2005, 30 urine diverting dry toilets (UDDT) were installed; the unit included a sink for hand washing and a waterless urinal for men. The UDDT were chosen by the community and a non-governmental organization (NGO) provided the technical assistance for their construction. This system benefitted the community as it was waterless, allowed for biosolids to be used in their agricultural activities, and did not contaminated the environment, which was a community concern expressed through SARAR methodology. Two years later, 28 families (90%) were still using their toilets and they expressed their satisfaction with the system, saying they would not change the UDDT for another system if given the chance. Although the NGO, SARAR transformacion S.C., provided ongoing advice for the operation and maintenance of the toilets, one family that moved into the area after the implementation period had difficulties maintaining the toilet, emphasizing the importance of participation in the process. After the success of this small pilot project, 126 families from the same community requested the training to use and construct UDDT in their

houses (personal observation during training in Ecological alternatives in Tepoztlan, Mexico, March 2009).

On the other hand, sanitation projects that have failed lacked the community participation needed during planning and implementation. An experience from a rural community in Bolivia, where UDDT were constructed, was unsuccessful even though they were offered the same benefits previously mentioned, including hygiene education and training on the use and maintenance of the toilet. The difference in outcomes is a result of culture. The Bolivian communities are part of the Quechuas and Aymaras cultures; they are an agriculturally based society but they do not use human feces or urine as fertilizer. An evaluation of the project revealed that 23% of the families were using the system frequently and correctly; 30% of the families were using the latrine for storage; and, unfortunately, open defecation was still preferred. Operation and maintenance of the units were inadequate as the community members were disgusted by dealing with feces. The community did not use the biosolids in their crops because the latrines took too long to fill up (1.5 years). They perceived that the use of feces in crops could be dangerous for their health and they could affect the equilibrium of the "Pachamama" (mother earth) (United Nations Children's Fund, 2008).

The previous experience exemplifies the different problems that a sanitation project could encounter if the sociocultural aspects of the users are not included in the planning and implementation. This also applies to the implementation of the solar toilet in a community, as hygiene behavior, cultural

beliefs, and preferences are important determinants in the success of any sanitation system.

2.5 Description of the Community Using the Solar Toilet Prototype IV.

Twenty-five Prototype IV Solar toilets were constructed in the community “Vista Hermosa” in El Majahual, located 1.5 miles from the Pacific coast of El Salvador. This community has about 60 families, with an average of 6 members per household. Most adults in the community are either fishermen or masons. Living conditions in Vista Hermosa are very poor. There is a lack of basic services such as electricity and potable water, and some households have community pit latrines which are shared by 3 – 4 households. Drinking water is supplied by a water truck that is paid for by the community.

Vista Hermosa was chosen by the Ministry of Public Health for the solar toilet intervention because of the poor socio-economic and sanitary conditions in this community. Improved sanitary facilities were urgently needed, and the whole community had expressed their support to the construction of the solar toilets. In addition, the geographic and ecological characteristics of the village (dry area with scarce vegetation) made it ideal for the construction of the toilets. Treated biosolids from the solar toilets could be used as soil conditioner for home gardens.

A survey was carried out in the community using the solar toilets prototype IV. This survey describes the demographic characteristics, sanitation knowledge, attitudes and practices of the population, including some of the households using the toilet. Since use and maintenance are important components in the adequate

functioning of a sanitation system, a description of these practices among households using the solar toilet prototype IV are included below.

Ash, lime, and soil are the additives users add to the toilet. Some users add one to four cups every time after using the toilet. Other users add one to four cups once every week. All users reported not placing toilet paper in the chamber. Tierney (2002) found that households with previous training were more likely to properly use and maintain the toilet, including mixing the vault content (Tierney, A. 2002, not published).

According to the UNICEF training manual for solar latrines, users should use ash only or a mixture of one part of lime and five part of dirt as additives every time the toilet is used (Landin, Carbonell, & Soderberg, 1997). A new manual updated in 2004 by the Ministry of Health of El Salvador, advice the users to cover the fecal material with a cup of lime (approximately 100 grams) after defecation (Ministerio de Salud Publica y Asistencia Social, 2004). Previous to the last manual, it was reported that 40% of users added ash and lime to the toilet, and approximately 29% used ash only (Tierney, A. 2002, not published).

2.4 Ammonia

Ammonia was first synthesized in 1908 by a German chemist of Jewish origins. During this time, a rapid increase in population in Europe necessitated a corresponding increase in food production and chemists needed to find a method of forming nitrogen compounds to be used as fertilizers to help feeding the world. Fritz Haber discovered the ammonia synthesis process (Haber-Bosh process) in

1908 and for his discovery he was awarded the Nobel Prize for chemistry in 1918 (Petrucci & Harwood, 1993).

Chemically, ammonia is described as a colorless, poisonous gas that is able to dissolve in water and that has a very strong characteristic smell (Rayner-Canham, 1996). In nature, ammonia is naturally found in water and soil and it is the initial product of decay of nitrogenous organic wastes (Manahan, 2000). In industry, ammonia is mainly used as a fertilizer for crop production (Petrucci & Harwood, 1993). The different properties of ammonia make this compound of interest to investigators since it could be use as a source of Nitrogen for plants in fertilizers, in cleaning products and to inactivate microorganisms (Warren, 1962).

The main objective of this document is to present the published evidence regarding the toxic effect of ammonia in fecal pathogens. The use of Urea as a source of ammonia and the chemical processes involved in its transformation are also described.

2.4.1 Urea as a Source of Ammonia. Urea is a fertilizer widely use in agriculture as a source of Nitrogen (N). It contains 46% N and it is rapidly hydrolyzed in the soil to ammonium and bicarbonate ions (Figure 2.6) (FIFA Ed, 2006).

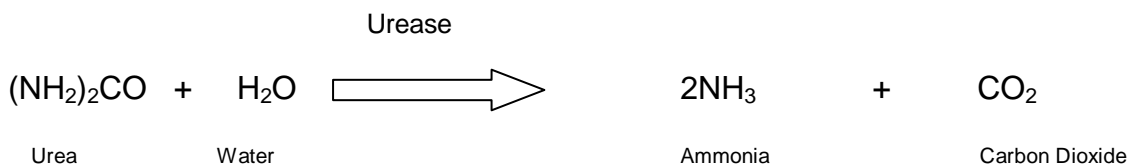


Figure 2.6 Hydrolysis of Urea

Urease is the enzyme that catalyses the hydrolysis of urea to carbon dioxide and ammonia affecting the fate and performance of urea. Its presence in soil was first indicated by Rotini in 1935 (Burns, 1978). Once urea is hydrolyzed, significant quantities of ammonia may be lost by volatilization (FIFA Ed, 2006).

Several factors affect the hydrolysis of urea and the rate of ammonia volatilization in soil:

- Temperature: increasing soil temperature increases the rate of hydrolysis (Fisher & Parks, 1958) while colder soil slows the hydrolysis of urea (FIFA Ed, 2006).
- Other chemicals: Some chemicals inhibit the action of the urease enzyme and prevent urea hydrolysis when applied along with the urea: KCl fertilizer is one of them (Troeh & Thompson, 1993).
- Urea concentration: High concentrations of urea increase the rate of hydrolysis until the amount of urea added is sufficient to saturate the enzyme with substrate (Burns, 1978).
- Urea application: Ammonia losses decrease when urea is mixed with soil or covered with a layer of soil during application while it increases if left on soil surface.
- pH: The soil close to the area where urea is applied could reach pH values as high as 9.2, however, hydrolysis of urea decreases with increasing soil pH. The main reason is that activity of urease declines with increasing pH. The optimum pH for its activity is around 6 to 8 (Burns, 1978; Overren & Moe, 1967).

2.4.2 Ammonia Chemistry in Water. When ammonia reacts with water it is partially ionized into ammonium (NH_4^+) and hydroxide ions (OH^-); this reaction is a reversible reaction that reaches a condition of equilibrium between the ionized form (NH_4^+) and the un-ionized form ($\text{NH}_{3\text{aq}}$) (Figure 2.7) (Manahan, 2000):

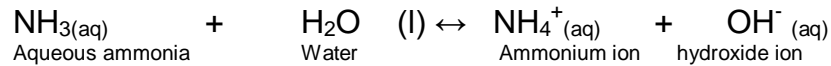


Figure 2.7 Reversible Reaction Between Ammonia and Ammonium Ion

In this reversible reaction aqueous ammonia behaves as a base and water as an acid; while the ammonium ion behaves as an acid and the hydroxide ion as a base (Petrucci & Harwood, 1993). The equilibrium between the two species ($\text{NH}_{3(\text{aq})}$ and $\text{NH}_4^+(\text{aq})$) is governed by the equilibrium constant pKa (Ka is the acid dissociation constant of the $\text{NH}_4^+(\text{aq})$ ion)(Emerson, Russo, Lund, & Thurston, 1975).

$$K_a = \frac{[\text{NH}_3][\text{H}^+]}{[\text{NH}_4^+]}$$

Acid dissociation constant

$$\text{pKa} = -\text{Log}_{10}K_a$$

The pKa is determined by temperature and as temperature increases the pKa decreases, shifting the equilibrium toward un-ionized ammonia ($\text{NH}_{3(\text{aq})}$) (Figure 2.8) (Emerson et al., 1975; Rayner-Canham, 1996).

The added concentration of these two chemical species ($\text{NH}_{3(\text{aq})}$ and $\text{NH}_4^+(\text{aq})$) in a solution is known as total ammonia and their individual concentrations (speciation) depends mainly of temperature, pH and ionic

strength of the solution (Emerson et al., 1975; U.S Environmental Protection Agency (USEPA), 1979).

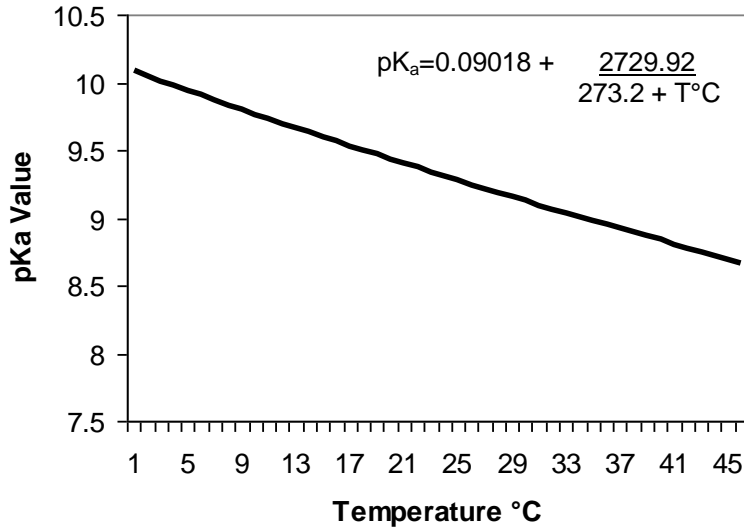


Figure 2.8 pKa Values as a Function of Temperature (0-45°C)

Using the formula of Emerson (1975) the relative amounts of $\text{NH}_3(\text{aq})$ expressed as percentage of total ammonia can be calculated as a function of temperature and pH (Emerson et al., 1975). An increment of pH is correlated with an increase in the aqueous ammonia concentration and, theoretically, it is observed that at a pH lower than 7 the ammonium ion (NH_4^+) is the main chemical specie present in the solution regardless of temperature (Figure 2.9).

Other important factor affecting the ammonia-water equilibrium in a lesser extent is the ionic strength; as the ionic strength increases the pKa increases reducing the percentage of unionized ammonia (Bower & Bidwell, 1978). However, the $\text{NH}_3(\text{aq})$ reduction is insignificant in waters with up to 200-300 mg/liter dissolved solids (U.S Environmental Protection Agency, 1979 EPA-600/3-79-091).

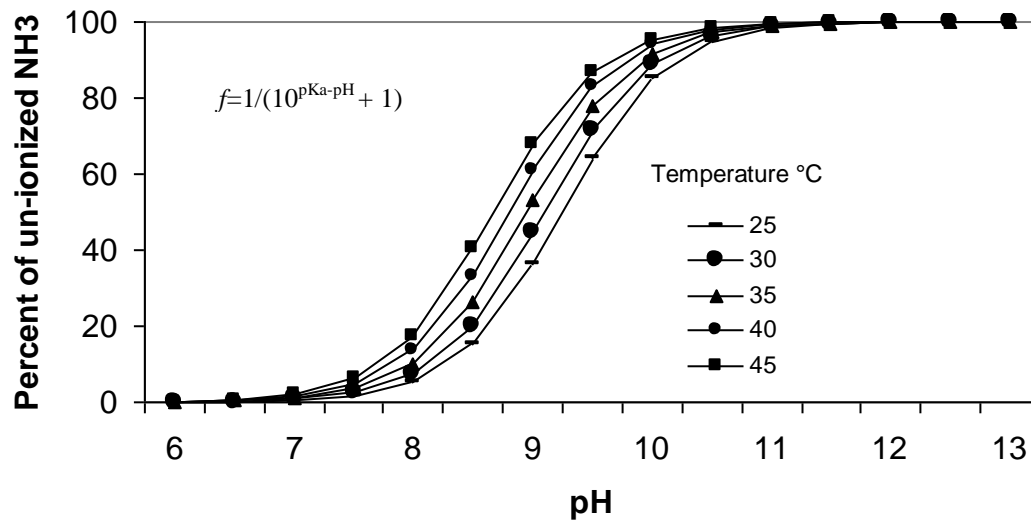
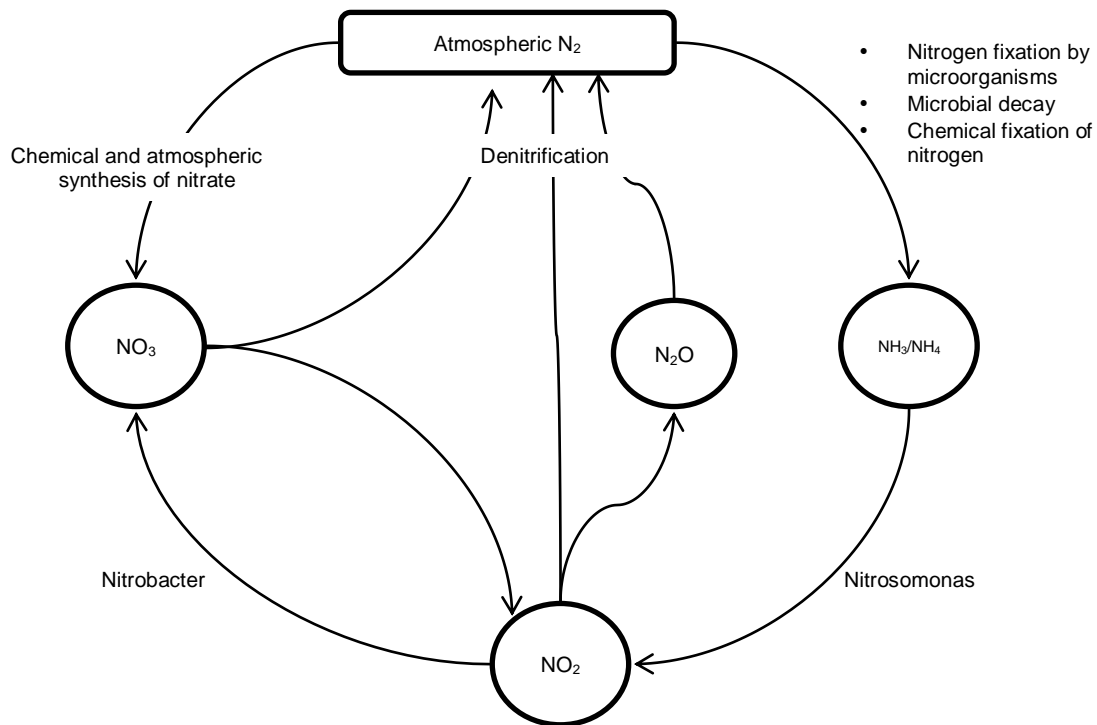


Figure 2.9 Percent of NH_{3(aq)} in Aqueous Solution as a Function of pH and Temperature

2.4.3 Ammonia in Wastewater. In wastewater engineering, ammonia (NH₃) and the ammonium ion (NH₄⁺) are considered a fraction of the different nitrogen compounds normally present in wastewater. The added concentrations of both chemical species are expressed as Nitrogen and known as Total Ammonia Nitrogen (TAN). Different from water solutions, wastewater is characterized by containing a variety of solid and organic materials that react and affect nitrogen compounds present in this type of solution (Metcalf & Eddy, 2003).

The natural pathways of nitrogen in the aquatic and soil environment, known as Nitrogen cycle, influence the concentration of ammonia as it is an intermediate compound in the cycle (Figure 2.10). The atmosphere is 78% elemental nitrogen (N₂) that can not be used by living microorganisms unless it is transformed to nitrate (NO₃⁻) and ammonium (NH₄⁺) ions through the nitrogen

cycle. The processes involved in the nitrogen cycle are: fixation (molecular nitrogen is fixed as organic nitrogen); ammonification (the release of ammonium ions from decomposing organic materials); nitrification (ammonia is oxidized to nitrate); nitrate reduction (nitrate ion is reduced to form compounds having nitrogen in lower oxidation state); and denitrification (the reduction of nitrate and nitrite to elemental nitrogen gas). These natural processes are mediated by microorganism present in soil and water (Manahan, 2000; Troeh & Thompson, 1993).



Source: (Manahan, 2000)

Figure 2.10 The Nitrogen Cycle

Typically, untreated domestic wastewater contain between 20 mg/L and 70mg/L of total nitrogen, of which 60% is in the form of ammonia and 40% from

organic matter (Viessman & Hammer, 2005). This nitrogen concentration, however, varies as wastewater is treated by different processes (Table 2.9).

The potential toxicity of ammonia to living organisms of receiving water is one of the reasons to remove nitrogen from wastewater. Some of the natural processes observed in the nitrogen cycle, ammonification-nitrification - denitrification, are used by wastewater engineers to achieve this (Metcalf & Eddy, 2003).

Table 2.9

Nitrogen Concentration of Sludge as Percentage of Total Solids

Biosolid	TS ¹ , %	Nitrogen, % of total solid
Untreated primary sludge	6 (5-9)	2.5 (1.5-4)
Digested primary sludge	4 (2-5)	3 (1.6 -3)
Untreated activated sludge	0.8 -1.2	3.7 (2.4-5)

¹ TS: total dry solids
Adapted from Metcalf and Eddy, Wastewater Engineering 2003

2.4.4 Ammonia in Human Feces. Human excreta results from the elimination of indigestible parts of ingested food, digestive juices and cells shed by the mucous membrane lining the digestive canal (Mamoru Nishimuta, 2006). The physical and chemical characteristics of excreta vary according to diet, climate and state of health of individuals (Feachem, 1984). An analysis of the different wastewater fractions in Sweden presented data of the mass and composition of feces, including Nitrogen content (Table 2.10)(Jonsson et al., 2005).

Table 2.10

Nitrogen Content in Human feces Excreted by Individual per Day

Nitrogen	g per day
Total Nitrogen	1.5
Ammonia/ammonium Nitrogen	0.3
Nitrate-Nitrogen	0.0
Soluble & Organic Nitrogen	0.45
Particulate & Organic Nitrogen	0.75

Adapted from H. Jonsson (2005)

The decomposition of organic matter is the source of nitrogen in human feces. The ammonia present in feces is produced by either aerobic or anaerobic processes.

2.4.5 Ammonia toxicity. The toxicity of ammonia to living organisms has been attributed to the un-ionized form (NH_3) in the aqueous as well as in the gas state (Himathongkham & Riemann, 1999; Rayner-Canham, 1996; United State Environmental Protection Agency (USEPA), 1999; Viessman & Hammer, 2005; Warren, 1962). The un-ionized form of ammonia is lipid soluble and able to penetrate, in aquatic organism, the cell membranes at the gill surface by passive diffusion (Environmental Protection Agency (EPA), 1999). In animals, high levels of ammonia are toxic and can cause functional disturbances of the central nervous system, leading to coma and death (Monfort, Kosenko, Erceg, Canales, & Felipo, 2002).

In sludge and human feces, the ammonia toxicity to pathogens varies among the main groups (bacteria, virus, helminthes and protozoa) and the mechanisms by which ammonia inactivates them are not well understood yet. It

has been observed that elevated concentrations of un-ionized ammonia are associated with faster inactivation of fecal pathogens; however, adequate inactivation depends on pathogen sensitivity to ammonia, pH levels, temperature, treatment duration, total solids, and concentration of un-ionized ammonia.

The published literature describes the tolerance of different fecal pathogens to ammonia toxicity (Table 2.11). Bacteria have shown to be the most sensitive microorganisms to ammonia toxicity, while some enteric viruses, especially dsRNA and DNA viruses, have shown to be very resistant.

The experimental conditions used to assess ammonia as sanitizing agent vary greatly in the literature published. Data related with ammonia toxicity tested in solutions is the most abundant, followed by ammonia tested in sludge and human feces; most of the experiments have been performed under laboratory conditions. Sludge and human feces have multiple compounds that could affect pathogen inactivation under experimental conditions. Carbonate and ammonia are among the molecules contributing to microorganism inactivation in alkaline treatments (Park & Diez-Gonzalez, 2003). To control for the effects of ammonia while doing experiments in sludge, free-ammonia sludge would be needed to observe the effects of ammonia in both groups.

Removing ammonia from sludge without changing the properties of sludge is very difficult and for this reason most of the published literature report experiments in solution, where other factors affecting inactivation could be controlled. The tolerance and specific conditions in which fecal pathogens have been inactivated by ammonia are described below.

Table 2.11

Tolerance of Fecal Pathogens to Ammonia Toxicity

Pathogen	Tolerance to ammonia	Reference (table footnote)
BACTERIA		
<i>Enterococcus faecalis</i>	No	2, 3, 13
<i>Salmonella Typhimurium</i>	No	2, 3, 6, 7, 25
<i>Escherichia coli</i>	No	4
<i>Salmonella spp.</i>	No	4, 5, 11
<i>Enterococcus spp.</i>	No	4
<i>Clostridia spp.</i>	Yes	4, 21
<i>E. coli</i> O157:H7	No	6, 7
<i>E. coli</i> O124	No	25
<i>Listeria monocytogenes</i>	No	6
<i>Fecal coliforms</i>	No	11, 13
VIRUS		
ΦX174 (ssDNA)	Yes	2
MS2 (ssRNA)	Yes	2
<i>Salmonella</i> phage 28B (dsDNA)	No	2, 4
Poliovirus type 1 strain Sabin (ssRNA)	No	12, 26
Poliovirus type 1 strain CHAT (ssRNA)	No	14, 16, 19
Poliovirus type 1 strain Mahoney (ssRNA)	No	14
Poliovirus type 2 strain 712 (ssRNA)	No	14
Coxsackievirus A13 (ssRNA)	No	14
Coxsackievirus B1 (ssRNA)	No	14
Coxsackievirus B4 (ssRNA)	No	12
Echovirus 11 (ssRNA)	No	14
Echovirus 6 (ssRNA)	No	12
Reovirus type 3	Yes	14, 15
Bacteriophage f2 (ssRNA)	No	16, 19
F-specific RNA bacteriophages (ssRNA)	No	20
HELMINTHS		
<i>Ascaris suum</i>	No	1, 4, 9, 10, 11, 17, 18, 21, 23, 24, 25, 28
<i>Ascaris lumbricoides</i>	No	9, 11
<i>Trichuris muris</i>	No	9, 11
<i>Hymenolepis</i> spp	No	11
<i>Toxocara</i> spp	No	11
<i>Trichocephalus muris</i>	No	25
<i>Diphyllobothrium latum</i>	No	25
PROTOZOA		
<i>Cryptosporidium parvum</i> Oocyst	No	8
<i>Entamoeba histolytica</i>	No	22
<i>Giardia lamblia</i>	No	22

1=Nordin, Nyberg, & Vinnerås, 2009; 2=Ottoson, Schnurer, & Vinneras, 2008; 3=Ottoson, Nordin, von Rosen, & Vinnerås, 2008; 4=Vinnerås, Holmqvist, Bagge, Albiñ, & Jönsson, 2003; 5=Vinnerås, 2007; 6=Himathongkham & Riemann, 1999; 7=Park & Diez-Gonzalez, 2003; 8=Jenkins, Bowman, & Ghiorse, 1998; 9=Ghiglietti, Rossi, Ramsan, & Colombi, 1995; 10=Ghiglietti, Genchi, Di Matteo, Calcaterra, & Colombi, 1997; 11=Mendez, Jimenez, & Barrios, 2002; 12=Fenters, Reed, Lue Hing, & Bertucci, 1979; 13=Allievi, Colombi, Calcaterra, & Ferrari, 1994; 14=Ward & Ashley, Apr 1977; 15=Ward & Ashley, Dec 1977; 16=Cramer, Burge, & Kawata, 1983; 17=Pecson & Nelson, 2005; 18=Pecson, Barrios, Jiménez, & Nelson, 2007; 19=Burge, Cramer, & Kawata, 1983; 20=Schaper, Duran, & Jofre, 2002; 21=Bujoczek, 2001; 22=Chefranova & Gordeeva, 1977; 23=Chefranova lu, Oshevskaia, & Gushchin, 1984; 24=Chefranova, 1977; 25=Chefranova lu, Petranovskaia, & Khodakova, 1978; 26=Reed, Fenters, & Lue, 1975; 27=Kato, Reimers, Fogarty, & Bowman, 2001; 28=Seamster, 1950

2.4.5.1 Ammonia toxicity to fecal bacteria. Enteric bacteria are the most numerous living organisms found in human feces. They are part of the human intestinal flora and they are routinely excreted. The average concentration of bacteria in 1 g of feces of a healthy individual is equivalent to 1.8×10^{12} *Escherichia coli* cells (Droste, 1997). The main groups of non-pathogenic bacteria are Enterobacteria, Streptococci, Lactobacilli, Clostridia, Bacteroides, Bifidobacteria and Eubacteria; some of them are used as indicators of fecal contamination (Metcalf & Eddy, 2003). Those bacteria that are not part of the normal fecal flora are classified as pathogenic. Among the pathogenic bacteria of major concern are *Salmonella typhi*, *Vibrio cholera*, *Shigella*, *Campylobacter* and enterohaemorrhagic *E. coli* (EHEC)(WHO, 2006a).

Different bacteria indicators are used to test for fecal contamination, but only one is specific for human pathogens. Total coliforms bacteria include coliforms bacteria from soil, feces or other origin. Fecal coliforms include coliforms bacteria from human and warm blooded animals. *Escherichia coli* (*E. coli*), normally excreted in human feces, is considered (by USEPA), as the specific indicator of human fecal contamination and possible presence of enteric pathogens (Metcalf & Eddy, 2003; Viessman & Hammer, 2005). To consider any biosolid safe from a health perspective (Class A biosolid), the USEPA standards requires less than 1000 MPN of fecal coliforms per 1 g of total sludge biosolids (dry – weight) and less than 3 MPN of *Salmonella* spp. in 4 g of total sludge biosolids (dry-weight) (U.S Environmental Protection Agency, 1994).

The literature reviewed indicates that ammonia increases the inactivation rate of bacteria when added to different material such as manure, sludge, human feces and solutions (Table 2.12). *Enterococcus faecalis* and *Salmonella* spp. are among the more sensitive bacteria to ammonia toxicity, even at very low temperatures (4°C) (J. Ottoson, Nordin, von Rosen, & Vinnerås, 2008). Other fecal indicators and bacteria (*Enterococcus faecalis*, *Salmonella* spp., *E. coli* O157:H7, *Clostridia* spp., and *L. monocytogenes*) have also been inactivated by un-ionized ammonia (Allievi, Colombi, Calcaterra, & Ferrari, 1994; Himathongkham & Riemann, 1999; Mendez, Jimenez, & Maya, 2004; Mendez et al., 2002; J. Ottoson et al., 2008; J. R. Ottoson, Schnurer, & Vinneras, 2008; Park & Diez-Gonzalez, 2003; Vinnerås et al., 2003).

The temperature at which ammonia toxicity to bacteria has been tested range from 10°C to 37°C; the pH values from 7.9-10.5; the NH₃ concentration from 782 mg/l to 36 g/kg of feces, and the time of exposure to un-ionized ammonia from 2 hours to 41 days. Most researchers reported moisture levels of 88% (sludge, human feces, etc.), but even those testing the effect of ammonia in very dry conditions (90% TS) have reported bacteria inactivation in less than three days (Himathongkham & Riemann, 1999). Fecal coliforms and salmonella spp. have been the bacteria more frequently used in the experiments and no re-growth of any bacteria have been reported after using ammonia (Mendez et al., 2002; Vinnerås et al., 2003)..

Table 2.12

Summary of Published Research on Bacteria Inactivation by Ammonia

Source	Medium	Pathogen	Chemical agent	Dose	Pathogen concentration before experiments	Moisture Dry solid	pH	T°	Inactivation Achieved	Sampling Time (days)
Ottoson et al., 2008	Cattle manure	<i>Enterococcus faecalis</i>	Urea	2% (11.2 g/kg NH3-N)	10 ⁸ CFU/g	12%	9.15	14	10 ⁴ CFU/g	≈ 41 days
			Aq. NH3	0.5% (4.97 g/kg NH3-N)	10 ⁸ CFU/g	12%	9.64	14	<10 ³ CFU/g	≈ 27 days
		<i>S. Typhimurim (phage type 178)</i>	Urea	2% (11.2 g/kg NH3-N)	109 CFU/g	12%	9.15	14	102 CFU/g	≈ 11 days
			Aq. NH3	0.5% (4.97 g/kg NH3-N)	109 CFU/g	12%	9.64	14	102 CFU/g	≈ 2 days
Park et al., 2003	Cattle manure (faeces+urine)	<i>Total coliforms</i>	Urea	100 mmol/l	108 cells/g	NR	8.5	-- ⁱ	100%	7 days
		<i>Escherichia coli O157:H7</i>	Urea	300 mmol/l	107 to 108 cells/g	NR	8.5	--a	100%	4 days
		<i>Salmonella Typhimurium DT104</i>	Urea	300 mmol/l	107 to 108 cells/g	NR	8.5	--a	100%	4 days
Ottoson et al., 2008	Material from mesophilic reactor	<i>S. Typhimurim (phage type 178)</i>	NH3	46mmol/l	106 to 108 CFU/g	NR	7.9	37	6 log reduction	48h
		<i>Enterococcus faecalis</i>	NH3	46mmol/l	106 to 108 CFU/g	NR	7.9	37	5 log reduction	96h
Allievi 1994	Anaerobic sludge	<i>Fecal coliforms</i>	NH4OH	20 g NH3/kg of sludge	10 ⁷ cells/g	10%	10.5	20-25	100%	< 1 day ⁱ
			<i>Fecal streptococci</i>	NH4OH	20 g NH3/kg of sludge	10 ⁵ cells/g	10%	10.5	20-25	100%
		<i>Fecal streptococci</i>	NH4OH	4 gNH3/Kg sludge	NR	NR	10	10 o 20	100%	≈ 39 days
			NH4OH	6.7gNH3/Kg sludge	NR	NR	10.2	10 o 20	100%	≈12 days
			NH4OH	9.7gNH3/Kg sludge	NR	NR	10.5	10 o 20	100%	≈ 15 days
			NH4OH	14gNH3/Kg sludge	NR	NR	10.6	10 o 20	100%	≈ 12 days
			NH4OH	6.7gNH3/Kg sludge	10 ^{4-4.5} cells/g	NR	10.2	20	100%	20
		Mixed Sludge ^l	<i>Fecal streptococci</i>	NH4OH	6.7gNH3/Kg sludge	10 ^{3-3.5} cells/g	NR	9.8	20	100%

ⁱ Temperature described as room temperature. Temperature value not reported.^j Sampling time performed at the end of addition of NH4OH to the sample. Not specific time value reported.^k Mixed aerobic and anaerobic sludge in equal parts.^l Mixed sludge + saline solution ((NH4)2SO4 + KCl) 4:1 sludge/solution ratio(w/w)

Table 2.12

Summary of Published Research on Bacteria Inactivation by Ammonia (continuation...)

Source	Medium	Pathogen	Chemical agent	Dose	Pathogen concentration before experiments	Moisture Dry solid	pH	T°	Inactivation Achieved	Sampling Time (days)
Mendez et al., 2002	Sludge	<i>Fecal Coliforms</i>	NH ₄ OH	20% w/w NH ₃	107 to 108 MPN/g	5.40%	10.3	20	< 10 PMN/g TS (SD 0.8)	2 hours
		<i>Salmonella spp.</i>	NH ₄ OH	20% w/w NH ₃	106 MPN/g	5.40%	10.3	20	< 10 ¹ PMN/g TS	2 hours
Mendez et al 2004	Sludge	<i>Fecal coliform</i>	NH ₄ OH	20% w/w NH ₃	107 to 108.2 MPN/g TS	5.40%	10.3	20	10 ⁻⁷ MPN/g TS	2 hours
		<i>Salmonella spp.</i>	NH ₄ OH	20% w/w NH ₃	105.4 to 106.8 MPN/g TS	5.40%	10.3	20	10 ⁻⁵ MPN/g TS	2 hours
Vinneras et al., 2003	Human Feces	<i>Escherichia coli</i>	urea	30g N/kg of feces	107	10%	9.2-9.3	20	100%	5
		<i>Salmonella spp.</i>	urea	30g N/kg of feces	107 CFU/ml	10%	9.2-9.3	20	100%	5
		<i>Clostridia spp.</i>	urea	30g N/kg of feces	104	10%	9.2-9.3	20	No Effect ^m	50
		<i>Enterococcus spp.</i>	urea	30g N/kg of feces	107	10%	9.2-9.3	20	< 2 log CFU/ ml	21
Himathongkham et al., 1999	Chicken manure	<i>E. coli O157:H7</i>	NH ₃ gas	1%	109 CFU/g	90%	NR	20	8 log reduction	3 days
		<i>S. typhimurium</i>	NH ₃ gas	1%	109 CFU/g	90%	NR	20	8 log reduction	3 days
		<i>L. monocytogenes</i>	NH ₃ gas	1%	109 CFU/g	90%	NR	20	4 log reduction	3 days

ⁱ Temperature described as room temperature. Temperature value not reported.

^j Sampling time performed at the end of addition of NH₄OH to the sample. Not specific time value reported.

^k Mixed aerobic and anaerobic sludge in equal parts.

^l Mixed sludge + saline solution ((NH₄)₂SO₄ + KCl) 4:1 sludge/solution ratio(w/w)

^m No reduction was observed after 50 days

Ammonia inactivates bacteria by crossing the membrane and causing damage to the cell as a result of rapid alkalinization of the cytoplasm or through a decrease in intracellular K⁺ concentration (Kadam & Boone, 1996; Park & Diez-Gonzalez, 2003).

2.4.5.2 Ammonia toxicity to viruses. Information regarding the virucidal activity of ammonia found date back to the 70s. One of the oldest papers found indicated that intrinsic ammonia in anaerobic digestion of sewage sludge was able to inactivate viruses after 6 to 7 days (Reed, Fenters, & Lue Hing, 1975). In 1976, R. L Ward et al, suspected that a component present in anaerobic digested sludge was causing an irreversible inactivation of polioviruses (Ward & Ashley, 1976). In 1977, R. L Ward and C.S. Ashley observed that ammonia, in its uncharged state, was the virucidal component present in sludge (Ward & Ashley, 1977b). Many studies have been done to evaluate ammonia as a virucidal agent since then (Cramer, Burge, & Kawata, 1983; Fenters, Reed, Lue Hing, & Bertucci, 1979; J. R. Ottoson et al., 2008; Reed et al., 1975; Schaper, Duran, & Jofre, 2002; Vinnerås et al., 2003; Ward, 1978; Ward & Ashley, 1977a, 1977b).

There are more than 100 different viruses excreted in human feces in concentrations as high as 10⁶ viruses per gram of feces (Droste, 1997). Enteroviruses, Norwalk viruses, rotaviruses, reoviruses, caliciviruses, adenoviruses and hepatitis A are among the most important from a health perspective. Phages, viruses than infect bacteria, are used as indicator of viruses in the environment (Metcalf & Eddy, 2003).

The capacity of ammonia to inactivate viruses excreted in feces has been measured in several types of viruses and phages, although the parameters used in the published research vary greatly (Table 2.13). The temperatures range from 10 to 60°C; the pH values range from 7 to 10, the ammonia concentration from 40 to 8500 mg/L and the duration of treatment from hours to days. Anaerobic digested sludge, material from biogas plants, ammonia solution, and human feces have been used in these experiments. Enteroviruses have been the most studied viruses and, within this group, Poliovirus has been the more frequently used.

Table 2.13

Viruses in Which Ammonia Toxicity Has Been Evaluated

Main Group	Subtype
Enteroviruses	Poliovirus
	Poliovirus type 1 strain CHAT
	Poliovirus type 1 strain CHAT
	Poliovirus type 1 strain Mahoney
	Poliovirus type 1 strain Sabin
	Poliovirus type 2 strain 712
	Echoviruses
	Echovirus 11
	Echovirus 6
	Coxsackie viruses
	Coxsackie virus A13
	Coxsackie virus B1
	Coxsackie virus B4
Reoviruses	Reovirus type 3
Bacteriophage	Single-stranded RNA
	MS2
	F2
	F-specific RNA
	Genotypes I, II, III, IV
	Single-stranded DNA phage
	ΦX174
Double-stranded DNA phage	
Salmonella phage 28B	

The literature reviewed indicates that ammonia toxicity varies among and within viruses groups. Polioviruses have been inactivated by ammonia at temperatures as low as 20°C and pH values of 8 (Cramer et al., 1983), while reoviruses shown resistance to ammonia at temperatures as high as 47°C and pH level of 8.5 (Ward & Ashley, 1977a).

Within the enterovirus group, some have been inactivated at a faster rate than others. This variability seems to be related to the concentration of ammonia, time of exposure to the chemical, and the type of virus (Table 2.14). The lowest effective un-ionized ammonia concentration reported is 5.5 mg/l at pH 7 and 35°C, which inactivated Poliovirus 1 in a solution after seven days of treatment (Fenters et al., 1979). The highest un-ionized ammonia concentration reported is 6882 mg/l at pH 10 and at 21°C, which reduced poliovirus virus to <500 PFU/ml from an initial titer of 3.1×10^8 PFU/ml, after 4 hours of treatment (Ward & Ashley, 1977b). These data suggest that the lower the un-ionized ammonia concentration, the higher the time of inactivation.

The type of virus also influences its tolerance to ammonia. The literature reviewed suggests that single-stranded RNA (ssRNA) viruses are more easily inactivated by ammonia than double-stranded RNA (dsRNA) viruses (Ward & Ashley, 1977b) . Reoviruses and some phages (Φ X174 and Salmonella phage 28B) are not ssRNA viruses and have shown resistance to ammonia toxicity (J. R. Ottoson et al., 2008; Ward & Ashley, 1977a).

Table 2.14

Summary of Published Research on Virus Inactivation by Ammonia

Reference	Material	Virus	Chemical agent	Dose	Ammonia as NH ₃ mg/l	Pathogen concentration before experiments	pH	T°	NH ₃ mg/L (available) ⁿ	Inactivation Achieved	Sampling Time
Fenters et al., 1979	Supernatant of anaerobically digested sludge (ADS)	<i>P-1 (Sabin)^m</i>	Intrinsic ammonia	900 mg/l as NH ₃ -N	1093	10 ^{2.3} to 10 ^{5.3} TCID ₅₀	7.4-7.6	35	--	100%	3 days
				900 mg/l as NH ₃ -N	1093	10 ^{2.3} to 10 ^{5.3} TCID ₅₀	7.4-7.6	20	--	100%	4 days
		<i>Coxsackie B4</i>	Intrinsic ammonia	900 mg/l as NH ₃ -N	1093	10 ^{2.3} to 10 ^{5.3} TCID ₅₀	7.4-7.6	20	--	100%	4 days
								25	--	100%	4 days
								30	--	100%	5 days
	PBS solution	<i>P-1 (Sabin)^a</i>	NH ₄ OH + NH ₄ Cl	500 mg/l NH ₃	500	NR ^o	≈ 7	35	5.5	100%	7 days
				750 mg/l NH ₃	750	NR	≈ 7	35	8.3	100%	7 days
				1000 mg/l NH ₃	1000	NR	≈ 7	35	11	100%	7 days
				1500 mg/l NH ₃	1500	NR	≈ 7	35	16.5	100%	7 days

^m P-1, Poliovirus type 1; Strain of the virus in parenthesisⁿ pH values were not measured at sampling. Un-ionized ammonia cannot be calculated.^o NR: not reported

Table 2.14

Summary of Published Research on Virus Inactivation by Ammonia (continuation...)

Reference	Material	Virus	Chemical agent	Dose	Ammonia as NH ₃ mg/l	Pathogen concentration before experiments	pH	T°	NH ₃ mg/L (available)	Inactivation Achieved	Sampling Time
Cramer et al., 1983	Solution	Phage f2	NH ₄ CL	0.25M NH ₄ CL	4250	2.1 x 10 ⁶ to 2.0 x 10 ⁷ PFU/ml	8.6	20	578	0.035% ^p	6.2 days
		Phage f2	NH ₄ CL	0.25M NH ₄ CL	4250	2.1 x 10 ⁶ to 2.0 x 10 ⁷ PFU/ml	8	20	162	0.001%	7.2-8.3 days
		P-1 (CHAT) ^q	NH ₄ CL	0.25M NH ₄ CL	4250	2.1 x 10 ⁶ to 2.0 x 10 ⁷ PFU/ml	8	20	162	0.001%	35-40h
Schaper et al., 2002	Solution	Genotype I ^f	NH ₃	40 mg/L	40 mg/L	10 ⁶ PFU/ml	10	20	32	0.2 (0.3) ^s	8 h
		Genotype II	NH ₃	40 mg/L	40 mg/L	10 ⁶ PFU/ml	10	20	32	1.8 (0.8)	8 h
		Genotype III	NH ₃	40 mg/L	40 mg/L	10 ⁶ PFU/ml	10	20	32	1.9 (0.7)	8 h
		Genotype IV	NH ₃	40 mg/L	40 mg/L	10 ⁶ PFU/ml	10	20	32	2.3 (0.7)	8 h
Ottoson et al., 2008	Material from a mesophilic reactor	MS2	NH ₃	46mmol/l	782	10 ⁸ PFU/g	7.9	37	74	no effect	9 days
		Salmonella phage 28B	NH ₃	46mmol/l	782	10 ⁸ PFU/g	7.9	37	74	no effect	9 days
		ΦX174	NH ₃	46mmol/l	782	10 ⁴ PFU/g	7.9	37	74	no effect	9 days
Ward et al., Dec 1977	Solution	R- 3(Dearing) ^t	NH ₄ Cl	0.5M	8500	6 x 10 ⁷ PFU	8.5	47	3680	4.7x10 ⁷ PFU/ml ^u	10 minutes

^p Virus survival (%)^q P-1, Poliovirus type 1; Strain of the virus in parenthesis.^f F-specific RNA bacteriophage (ssRNA)^s Log₁₀ reduction (Standard Deviation) in phage numbers^t R-3, Reovirus type 3; strain of the virus in parenthesis.^u Reovirus recovery

Table 2.14

Summary of Published Research on Virus Inactivation by Ammonia (continuation...)

Reference	Material	Virus	Chemical agent	Dose	Ammonia as NH ₃ mg/l	Pathogen concentration before experiments	pH	T°	NH ₃ mg/L (available)	Inactivation Achieved	Sampling Time
Vinneras et al., 2003	Human feces ^v	Salmonella phage 28B	Urea	30g N/Kg of feces	36.4 g NH ₃ /Kg	10 ⁴ ova in each nylon bag	9.2-9.3	20	8000	< 1 log ₁₀ PFU/ml	50 days
Ward et al., Apr 1977	ADS	P-1 (CHAT) ^a	Intrinsic ammonia	0.475M as NH ₃	8075	NR	≈ 9.5	21	4633	<10 ⁻² pfu ^w	1 day
	Solution	P-1 (CHAT) ^a	NH ₄ Cl	0.475M as NH ₃	8075	NR	≈ 9.5	21	4633	<10 ⁻⁵ pfu	1 day
	Solution	P-1 (CHAT) ^a	NH ₄ Cl	0.5M as NH ₃	8500	NR	8	21	347	^x 1.4 x 10 ⁸	4h
						NR	9	21	2537	3.8 x 10 ⁴	4h
						NR	10	21	6882	<5 x 10 ²	4h
						NR	9.5	21	4876	< 0.000035	3 days
	Solution	P-1 (CHAT) ^a	NH ₄ Cl	0.5 M	8500	NR	9.5	21	4876	< 0.000014	3 days
						NR	9.5	21	4876	< 0.000044	3 days
						NR	9.5	21	4876	< 0.00012	3 days
						NR	9.5	21	4876	< 0.000046	3 days
						NR	9.5	21	4876	< 0.00015	3 days
	R- 3 ^y	NH ₄ Cl	0.5 M	8500	NR	9.5	21	4876	3.1	3 days	

^v Moisture Dry Solids, 10%^w Surviving fraction of PFU^x Recovery of PFU/ml^y R-3, Reovirus Type 3

Ammonia inactivates enteroviruses (Polioviruses) by inducing cleavage of viral RNA and, bacteriophage f2, by breakage of nucleic acid chains (Burge, Cramer, & Kawata, 1983; Ward, 1978). More research is needed before ammonia could be ruled out as a virucidal agent for Reoviruses and other enteric viruses.

2.4.5.3 Ammonia Toxicity to Helminthes. *Ascaris lumbricoides*, may survive wastewater and sludge disinfection procedures and environmental stresses for many years (Metcalf & Eddy, 2003; WHO, 2006a). Due to its resistance to different treatments, *Ascaris lumbricoides*, has been suggested as an index of hygiene quality (WHO, 2006a).

An infected individual could excrete up to 300,000 *Ascaris lumbricoides* ova per gram of feces depending on the worm load (Feachem et al., 1983; John & Petri, 2006). According to the United States Environmental Protection Agency (USEPA), a density of <1 viable ova/4 g total solids (dry-weight) is required to consider any biosolid or sludge safe for application in agriculture, either as fertilizer or soil conditioner (U.S. Environmental Protection Agency, 1994). The World Health Organization guidelines recommend a $\log_{10} 6^{10}$ reduction in pathogen concentration to ensure safe reuse of biosolids (WHO, 2006a).

The literature reviewed regarding the effect of ammonia on helminthes eggs present in solid biological waste, especially *Ascaris* spp. ova, date back to the 1970's. In 1977, a researcher from Russia, observed that ammonia inactivated *Ascaris suum* eggs in solid and liquid biological wastes at 18-22°C in less than 15 days (Chefranova et al., 1977). In subsequent studies the author reported that

other helminthes (*Diphyllobothrium latum* and *Trichocephalus muris*) were also inactivated by ammonia and that they were even more sensitive than *Ascaris ova* to ammonia toxicity (Chefranova et al., 1978).

From the 1990's until today the literature published in the use of ammonia as an ovicidal agent has increased. Most of the researchers have used sludge and ammonia solution, and few have tested ammonia in human feces collected from urine-diverting toilets. The total solid content of the material used in the experiments ranged from 5 to 17%; the temperature at which an effective treatment has been observed varied from 20 to 50°C; the pH values from 8.9 to 12.7; duration of treatment from 2 hours to 87 days; and the ammonia concentrations from 0.6 g/l to 36.4 g/kg as NH₃ (Table 2.15). These parameters not only influence the final un-ionized ammonia concentration available in the material but also the permeability of the cell-membrane of the parasite ova to the un-ionized ammonia (Wharton, 1980).

Temperature, by itself, is able to inactivate the parasite (a minimum of 45°C for a period of 1 year) (Feachem et al., 1983). Adding ammonia to different material seems to decrease the temperature required for inactivation. Pecson et al., (2007) observed a 99% inactivation of *Ascaris suum* in three days, by adding ammonia (5500 ppm as N) to an aqueous solution at 34°C and pH 11 (Pecson & Nelson, 2005). At low temperature, time of exposure becomes an important factor to inactivate the parasite. At 22°C, Ghiglietti et al., (1997) observed 100% *Ascaris* spp. ova inactivation in sludge (2%v/w, pH=12) in 40-60 days (Ghiglietti et al., 1997). At 20°C, Mendez et al., (2002) achieved 94% *Ascaris ova*

inactivation in two hours in sludge using very high ammonia concentration (50% w/w, pH=10.74) (Mendez et al., 2002).

pH values greater than 8 are needed to have available un-ionized ammonia in the material, but it does not guarantee complete inactivation if temperature, time and ammonia concentration are not adequate. Ghiglietti et al., (1997) did not observe any effect on *Ascaris ova* after 90 days of treatment with ammonia at pH values of 12.3 and a temperature of 22°C (Ghiglietti et al., 1997). A difference in inactivation time between solution and sludge treated with ammonia has been observed. Ghiglietti et al., (1997) reported that *Ascaris ova* were inactivated in less time in solution samples than in sludge samples. The author suggested that this difference was due to a reduction on the permeability of the cell-membrane caused by a low concentration of oxygen in sludge. This lack of permeability of the membrane protected the *Ascaris suum* egg from ammonia penetration into the cell and its subsequent inactivation (Ghiglietti et al., 1997). Pecson et al., (2007) observed the opposite result with *Ascaris lumbricoides* ova; inactivation by ammonia was faster in sludge samples compared to buffered solution samples. The authors suggested that this difference could be related to the presence of other compounds in sludge increasing the inactivation of the parasite or, to a higher susceptibility of *Ascaris lumbricoides* eggs to ammonia toxicity than *Ascaris suum* ova, which is the parasite usually used as a model for the human parasite *Ascaris lumbricoides* (Pecson et al., 2007). Other researchers have used ammonia fumes/gas and have also reported *Ascaris* spp. ova inactivation (Seamster, 1950).

Ammonia has shown to effectively inactivate other parasites as well. The literature published report that ammonia is toxic to ova of *Trichuris* spp., *Hymenolepis* spp., *Toxocara* spp., *Diphyllobothrium latum* and *Trichocephalus muris* (Chefranova et al., 1978; Ghiglietti et al., 1995; Mendez et al., 2002). These parasites have shown less tolerance to ammonia toxicity than *Ascaris* spp.

The mechanism by which ammonia inactivates *Ascaris* spp. ova is not well understood. Pecson et al., (2005) suggested that temperature increases the permeability of the lipid membrane of the egg-shell allowing the diffusion of unionized ammonia into the shell, with the subsequent increase of intracellular pH and inactivation of the parasite (Pecson & Nelson, 2005). This hypothesis, in turn, explained the author's observation regarding the need of lower NH₃ concentration to inactivate *Ascaris* spp. ova as temperature increases. Nordin et al., (2007) also suggest that ammonia inactivation is temperature dependent as it influences the egg-shell permeability (Nordin, 2007). Therefore, temperature plays an important role as it may potentiate inactivation.

Table 2.15

Summary of Published Research on Helminth Inactivation by Ammonia

Source	Medium	Pathogen	Chemical agent	Dose	Ammonia as NH ₃ g/l	Initial pathogen concentration	pH	T°	NH ₃ mg/L (available)	Inactivation Achieved	Sampling Time (days)
Ghiglietti 1995	Water solution	A. suum	NH ₄ OH	0.6%	2.9	1000 ova/10ml	11.9	30		98%	14
				0.6%	2.9	1000 ova/10ml		40	100%	7	
		Trichuris muris	NH ₄ OH	0.6%	2.9	1000 ova/10ml	11.9	30		99%	14
				0.6%	2.9	1000 ova/10ml		40	100%	7	
				0.6%	2.9	1000 ova/10ml	11.9	22		97%	21
				0.6%	2.9	1000 ova/10ml		30	100%	7	
Ghiglietti 1997	distilled water	A. suum	NH ₄ OH	0.5% v/w	2.4	100 ova/ml	12.3	22		no effect after 90 days	90
				1% v/w	4.8	100 ova/ml	12.5	22	100%	40	
				1.5% v/w	7.3	100 ova/ml	12.7	22	98%	21	
				2% v/w	9.7	100 ova/ml	12.7	22	100%	21	
				2% v/w	9.7	100 ova/ml	12.7	22	100%	21	
	sludge, 10% TS	A. suum	NH ₄ OH	0.50%	2.4	100 ova/ml	9.1	22		no effect after 90 days	90
				2%	9.7	100 ova/ml	12	22	100%	60	
				2.50%	12	100 ova/ml	12.7	22	100%	40	
				4%	19.4	100 ova/ml	12.7	22	100%	21	
				4%	19.4	100 ova/ml	12.7	22	100%	21	
Mendez 2002	Sludge, 5.4% TS	Trichuris spp.	NH ₄ OH	20% w/w NH ₃		7 ova/g TS	10.3	20		100%	2 hours
		Hymenolepis spp.		20% w/w NH ₃		5 ova/g TS	10.3	20		100%	2 hours
		Toxocara spp.		20% w/w NH ₃		1 ova/g TS	10.3	20		100%	2 hours
		Ascaris spp.		50% w/w NH ₃		93 ova/g TS	10.74	20		94%	2 hours

Table 2.15

Summary of Published Research on Helminth Inactivation by Ammonia (continuation...)

Source	Medium	Pathogen	Chemical agent	Dose	Ammonia as NH ₃ g/l	Initial pathogen concentration	pH	T°	NH ₃ mg/L (available)	Inactivation Achieved	Sampling Time (days)
Vinneras 2003	Human Feces 10% TS	Ascaris suum	urea	30g N/kg of feces	36.4g NH ₃ /kg	10 ⁴ eggs/bag	9.2-9.3	20	8000ppm	100%	50 days
Pecson 2005	Solution	Ascaris suum	NH ₄ Cl (ppm as N)	5500	6.6	2000	11	34		99%	3
				3500	4.5	2000	11	36	99%	3	
				2500	3	2000	11	38	99%	3	
				2000	2.4	2000	11	40	99%	3	
				1000	1.2	2000	11	42	99%	3	
				1000	1.2	2000	11	44	99%	3	
				500	0.6	2000	11	46	99%	3	
				0		2000	11	48	99%	3	
				7500	9.1	2000	9	36	99%	3	
				5000	6	2000	9	38	99%	3	
				3000	3.6	2000	9	40	99%	3	
				2000	2.4	2000	9	42	99%	3	
				1500	1.8	2000	9	44	99%	3	
				1000	1.2	2000	9	46	99%	3	
0		2000	9	48	99%	3					
Pecson 2007	Sludge 5% TS	Ascaris spp.	NH ₄ Cl	1000 NH ₃ -N mg/l	1.2	105.4 ova/g TS	12	20			87
				1000 NH ₃ -N mg/l	1.2	105.4 ova/g TS	12	30		16	
				5000 NH ₃ -N mg/l	6	105.4 ova/g TS	12	20		25	
				5000 NH ₃ -N mg/l	6	105.4 ova/g TS	12	30		5 days	
				5000 NH ₃ -N mg/l	6	54.7 ova/g TS	12	40		0.39	

Table 2.15

Summary of published research on helminth inactivation by ammonia (continuation...)

Source	Medium	Pathogen	Chemical agent	Dose	Ammonia as NH ₃ g/l	Initial pathogen concentration	pH	T°	NH ₃ mg/L (available)	Inactivation Achieved	Sampling Time (days)
Mendez 2004	Sludge 5.4%	Ascaris	NH ₄ OH	20% w/w NH ₃		98 viable ova/g TS	10.3	50	9118mg/l	100%	2hours
Annika 2007	human feces 17% TS	Ascaris suum	Urea	Ash + 1%	8.8	104 egg/bag	10	24			22
				1%	14.9	104 egg/bag	8.9	24		47	
				2%	22.9	104 egg/bag	9	24		35	
				Ash + 1%	2.4	104 egg/bag	12.8	34		4	
				1%	18.3	104 egg/bag	8.9	34		10	
				2%	29.3	104 egg/bag	9	34		4	

CHAPTER 3.
**MORPHOLOGICAL CHANGES OF ASCARIS SPP. OVA DURING ITS
DEVELOPMENT OUT OF THE HUMAN HOST**

3.1 Abstract

Background: *Ascaris lumbricoides* is of particular importance to public health as it causes a great burden of disease in children in developing countries. Information on the infective stage and the pathological damage caused by the parasite is widely available in the literature; while information about early embryonic development and its life cycle out of the host is limited.

Objective: The purpose of this study was to register and describe the morphological changes within the parasite ova during incubation *in vitro* at 28 C°.

Methods: *A. suum* ova (4000 ova/ml), used as a model for *A. lumbricoides*, were placed for incubation in 0.1N H₂SO₄, at 28 C°, in the dark, for 21 days. Every day, subsamples of approximately 100 *Ascaris suum* ova were taken from the incubation solution for microscopic evaluation. Development, morphological changes and viability of the first 40 ova observed were registered in a log sheet and documented with photos.

Results: Twelve stages of development were identified within the ova: 1-Cell, 2-Cell, 3-Cell, 4-Cell, Early Morula, Late Morula, Blastula, Gastrula, Pre-

larva 1, Pre-larva 2, Larva 1, and Larva 2. Each stage was observed for at least three continuous days. By the end of the first week most ova observed were in Late Morula stage (72.5%); on day 14 of incubation, 90% had developed to Larva-1 stage and by day 16, 62.5% had developed to Larva-2 stage. At the end of incubation, 100% ova were in Larva 2 stage. No difference was found between viability recorded from day 5 to 20 of incubation, and viability reported after three weeks of incubation (Chi-Square, $p > 0.05$).

Conclusions: *A. suum* ova went through clearly identified morphological changes at different speed of development. Two new additional stages of development were identified: Pre-larva 1 (larva coiled creating no more than one concentric ring) and Pre-larva 2 (larva coiled creating at least one and a half concentric ring). No significant statistical difference was observed among the viability reported early in incubation and the one reported after three weeks of *in vitro* incubation. If viability of *Ascaris* spp. ova is defined as a healthy developing ovum that will continue growing to become an infective larva under constant *in vitro* incubation conditions then, observing the ova under the microscope early during incubation, instead of waiting up to three-four weeks for the development of a larva, could be an alternative to reduce the time to report viability.

Keywords: development, *Ascaris*, incubation, viability

3.2 Introduction

Ascaris lumbricoides causes a great burden of disease in the tropics and subtropics, especially in areas with inadequate sanitation systems (World Health Organization, 2009c). Around 1 billion people are infected with the parasite but school age children (5-15 years old) are the most affected and carry the highest intensity of infection. As a consequence of infection, infected school age children experience a negative impact on growth, nutrition status, physical fitness and capacity to learn in school (Hotez et al., 2006; O'Lorcain & Holland, 2000).

The parasite's ova are excreted in feces of infected individuals. Under adequate environmental conditions, the ova developed from one cell ova to larva stage in approximately two to three weeks and become infective to a new host if ingested via the fecal-oral route of transmission (Centers for Disease Control & Prevention, 2009). The ingestion of only one embryonated ovum is enough to cause the disease (World Health Organization, 2004). When the ova of this parasite are present in the environment, they become very resistant to hard environmental conditions and wastewater treatment (Metcalf & Eddy, 2003). For this reason, detecting the presence and viability status of the parasite ova in wastewater and excreta reused in agriculture or disposed in water surfaces is of particular importance to public health, mainly in the developing world where Ascariasis is highly prevalent (World Health Organization, 2006a).

Several techniques have been described in the literature to determine viability of *Ascaris* spp. ova including morphological criteria; incubation and flotation techniques; use of dyes; larva motility induction; infectivity animal

models and PCR (Pecson, Barrios, Johnson, & Nelson, 2006; World Health Organization, 2004). A combination of two or more of these techniques is currently used to ascertain the presence and viability of these ova in wastewater, sludge, compost, and biosolid. The most accepted procedures are the United States Environmental Protection Agency (USEPA) method and the Tulane method. The two methods require up to three to four weeks of incubation respectively to report whether the parasite ova, if present, are viable or not (Bowman, Little, & Reimers, 2003; USEPA, 2003). At the end of incubation, the USEPA method requires reporting number of unembryonated *Ascaris* ova, and the number of various larvae per gram of dry weight. The Tulane method considers viable an ovum with a developed larva inside, and non-viable an ovum with no larva developed within. None of the methods consider an ovum in early stages of development as a potential infective/viable ovum.

The published literature on the early developmental stages of the parasite from fertilized ova to infective ova is limited (Chitwood & Chitwood, 1977; Christenson, 1935; Jaskoski & Egan, 1953; Johnson, Dixon, & Ross, 1998; Seamster, 1950). Most parasitology books describe nematode embryology in general and most published articles focus on the infective stages of the parasite to the host and the life cycle of the parasite within the host (Christenson, 1935; Douvres, Tromba, & Malakatis, 1969; Fagerholm, Nansen, Roepstorff, Frandsen, & Eriksen, 2000; Geenen et al., 1999; Grady & Harpur, 1985; Malla, Sofi, Ganguly, & Mahajan, 1991; Schmidt & Roberts, 2000). The few articles describing parasite ova development outside of the host, consider some stages

and the periods of time required for development of those specific stages but they fail to present comprehensive and detail information of the morphological changes of the parasite ova from one cell to larva stages; the time at which those changes are expected to occur during incubation; the speed of development, and viability during incubation on a continuum.

The objective of this study was to register and describe the morphological changes observed during three weeks of incubation *in vitro* at 28 C° within the parasite ova and to explore if there were differences between viability reported before and at completion of three weeks of incubation. Early development stages were considered viable as they potentially could become an infective ovum.

3.3 Materials and Method

A. suum ova, obtained from the intestinal content of pigs, were used as a model for *A. lumbricoides* (World Health Organization, 2004). They were purchased from Excelsior Sentinel (Ithaca, NY) and stored in 0.5% formalin at 4 C° until used. The ova were placed in 50 ml centrifuge containing 5 milliliter of 0.1 N H₂SO₄ (Fisher). The final ova concentration in the tube was approximately 4000 ova/ml. The level of the solution was marked in the centrifuge tube with a fine tip pen as a reference and de-ionized water was added if evaporation was observed. The tube was placed in an incubator at 28 C°, open, in the dark, for 21 days. Every day the incubator's temperature was monitored and registered and the samples were mixed by hand to allow oxygenation.

Subsamples of approximately 100 *Ascaris suum* ova were taken daily and placed in a microscope slide with a cover glass for microscopic evaluation. The

slide was sealed with wax on the edges to avoid evaporation during evaluation. The development and morphological changes and viability of the first 40 ova observed were documented with photos and registered in a log sheet. Artificial hatching of the ova was performed according to Eriksen (1981) on incubation day 15 and 16 to facilitate identification of molt and Larva 2 stage. For these samples, wax was not used to allow evaporation of solution and hatching of larva after 5 to 10 minutes under the microscope.

The set of criteria used during microscopic examination to classify the stages of development and viability of the ova were created using the work of Christenson (1935) and Caceres et al (1987) (Table 3.1). For the purpose of this experiment any ovum found to meet any of the viability criteria was considered viable before completing 21 days of incubation. After completing 3 weeks of incubation, viability was calculated based on larva stages only.

For data analysis and creation of graphs, a database was created in Microsoft Excel 2007. To test whether the proportion of viable ova observed from day 5 to day 20 of incubation differed significantly from the proportion of viable ova observed at three weeks of incubation (87.5%), a Chi-Square test for proportions was used using the PROC FREQ procedure in SAS (SAS 9.2, SAS Institute Inc.; Cary, NC). The 95 % confidence limits of the proportion were calculated using the Binomial test.

Table 3.1

Criteria to Identify Stage of Development and Viability of Ascaris suum Ova by Microscopy

Non viable ova*	Viable ova
<ul style="list-style-type: none"> ➤ Poorly defined structures ➤ Contraction, rupture and loss of membrane continuity. ➤ No larva movement observed by microscope light stimulation. ➤ Vacuolization of cytoplasm and cellular condensation ova in unicellular stage with granulated and vacuolated cytoplasm 	<ul style="list-style-type: none"> ➤ <i>One to Four Cell</i> : Observation of 1, 2, 3, or 4 clearly well defined cells within the ovum ➤ <i>Early morula</i>: Observation of 5 to 10 cells within the ovum. ➤ <i>Late morula</i>: Observation of 11 or more cells within the ovum. ➤ <i>Blastula</i>: Observation of a spherical layer of cells surrounding a fluid-filled cavity. ➤ <i>Gastrula</i>: Observation of a layer of cells surrounding the embryo, plus a kidney shape invagination in one side of the embryo. ➤ <i>Larva 1</i>: Observation of a larva structure within the ovum, no molt and motility in response to light stimulation. ➤ <i>Larva 2</i>: Observation of larva structure within the ovum, a molt, and motility in response to light stimulation.

* Adapted from Caceres et al, 1987

3.4 Results

Twelve stages of development were identified during microscopic evaluation: 1-Cell, 2-Cell, 3-Cell, 4-Cell, Early Morula, Late Morula, Blastula, Gastrula, Pre-larva 1, Pre-larva 2, Larva 1, and Larva 2. Each stage was observed for at least three days, except 3-Cell stage. The viability registered at the end of experiment (day 21) was 87.5% (N=100).

3.4.1 Stages of Development. The first two days of incubation (day 0 and day 1) no cell division was observed within the ova. On day 2, 5% of the ova had experienced the first cleavage and, by day 3, 15% of the ova were at 1-Cell

stage; 47.5% were at 2-Cell stage; 10% were at 3-Cell stage and 17.5% were at 4-Cell stage. The development from 2 to 4-Cell ova was fast and reduced the number of days at which 3-Cell stage was observed to two (day 2-3) (Figure 3.1).

On day 4, 45% of ova were at the Early Morula stage and 27.5% were at 4-Cell stage; the others were in 1-2 Cell stage. Early Morula stage achieved its maximum development (peak) on day five (82.5%) and Late Morula stage on day six (80%). On day 7 the majority of ova observed were in Late Morula stage (72.5%) and typical Blastula stage started to appear (7.5%).

On day 8, 1-Cell stage was observed for the last time (2.5%), Blastula stage peaked (60%), and Gastrula stage ova appeared (20%). On day 9 Gastrula stage peaked (57.5%) and few Blastula stage ova were observed (25%). On day 10, movements were observed within the ova after light stimulation in the microscope. In addition, two different larva were noticed and identified as Pre-larva stage 1 (37.5%) and Pre-larva stage 2 (27.5%). Both stages were observed for at least three days continually and were considered as stages preliminary to larva 1 (Figure 3.1).

On day 12, Pre-larva stage 2 (75%) peaked and Larva 1 (7.5%) stage was observed for the first time. Most ova were in Larva 1 stage by day 13 (85%) and day 14 (90%). On day 15 and 16 artificial hatching was performed to facilitate mold identification; no Larva 2 was observed on day 15 while most ova observed on day 16 were in Larva 2 stage (62.5%). By day 17, 95% of the ova were in Larva 2 stage (artificial hatching performed). On day 18, all observed ova were in larva 2 stage (92.5%) or hatching naturally (5%).

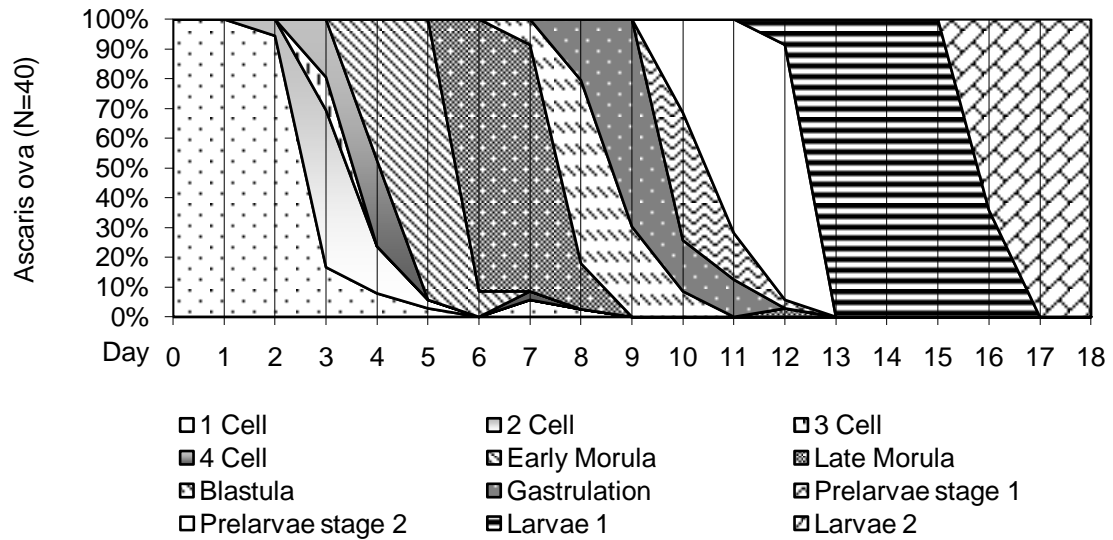


Figure 3.1 Development of *A. suum* Ova during Incubation at 28°C

Based on these results, a table and a timeline, describing the stages of development observed and the time at which these changes occurred during this experiment, was created (Table 3.2, Figure 3.2). Each stage included in the table was observed for at least three days continually, except for 3-Cell stage. A photographic documentation of the different stages observed is included in Figure 3.3.

Each stage was typically observed for approximately three to five days, with a peak of development around the second to third day of observation. Overlapping of each stage with the previous and the following stage of development was also typical; except for 1-Cell and Larva 2 stage.

Table 3.2

Developmental Stages of A. suum Ova Observed during Incubation at 28°C

Stage of development	Description
	Intact ovum in any of the following stages:
One to Four Cell	Observation of 1, 2, 3, or 4 clearly well defined cells within the ovum by microscopy
Early morula	Observation of 5 to 10 cells within the ovum
Late morula	Observation of 11 or more cells within the ovum
Blastula	Observation of a spherical layer of cells surrounding a pseudo fluid-filled cavity
Gastrula	Observation of a layer of cells surrounding the embryo, plus a kidney shape invagination in one side of the embryo.
Pre-larva 1	Observation of a larva like structure, coiled, creating no more than one concentric ring inside the ovum
Pre-larva 2	Observation of a larva like structure, coiled, creating at least one and a half concentric ring inside the ovum
Larva 1	Observation of a larva, without well defined structures inside the larvae body wall, absence of molt and intense motility in response to light
Larva 2	Observation of a larva with more defined structures, a molt, and a delayed motility response to light stimulation

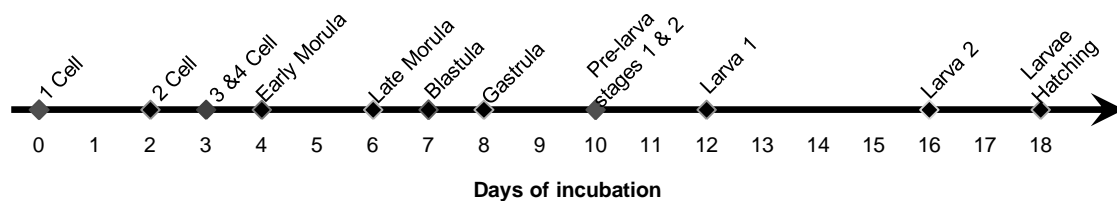


Figure 3.2 Timeline of *A. suum* Ova Development when incubated *in vitro* at 28°C°



Figure 3.3 Photographic Documentation of *A. suum* Ova Development

3.4.2 Viability. The viability of the *A. suum* batch used in this experiment at day 21 of incubation was 87.5% (No. =100 *A. suum* ova). A Chi-Square test for specific proportions (Table A1 in Appendix A) performed between the proportions of viable ova observed each day (from day 5 to 20) and the proportion of viable ova registered after three weeks of incubation showed no significant differences during any day of incubation. The viability proportion with the confidence limits (95%) per day is presented in Figure 3.4. This suggests that the identification of

early stages of development during incubation could potentially be used as an indicator of viability during *A. suum* ova incubation under the conditions used in this study.

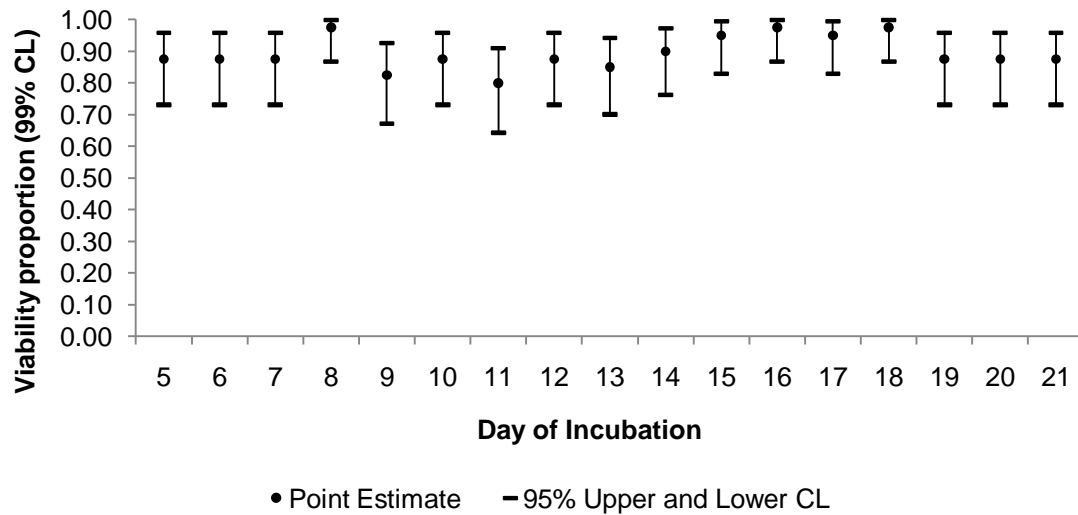


Figure 3.4 Viability Proportions and 95% Confidence Limits from Day 5 to Day 21 of Incubation

3.5 Discussion

3.5.1 Development stages. During 21 days of incubation, this study identified twelve stages of development in the *A. suum* ova life cycle completed outside of the host. Other authors describe five to ten stages of development with some variations in the nomenclature used, but most of them mention Two to Four Cell, Morula, and Larva stages (Bassiouni, Soliman, Mahmoud, & Bassiouni, 1990; Brown, 1927; Chitwood & Chitwood, 1977; Christenson, 1935; Jaskoski & Egan, 1953). The visual representations of the morphological changes from the book “*Introduction to Nematology*” by Chitwood (1977) are very similar to the developmental stages observed during this experiment, however, the stages

presented in the book are limited to eight with no information regarding speed of development and viability (Chitwood & Chitwood, 1977). The two additional stages preliminary to Larva 1, Pre-larva 1 and Pre-larva 2, were considered after observing in the microscope that each one persisted continually for at least three days and presented unique features that differentiate them from other stages. Both stages were observed the same day (day=10) in the microscope. It is possible that Pre-larva 1 preceded Pre-larva 2 on day nine of experiment, not being noticed by the authors due to small numbers of ova observed daily. Larva 2 stage, on the other hand, was observed four days after Larva 1 stage. In this case, it is also possible that Larva 2 developed before day 16 and could have been missed due to the small number of ova observed (N=40/day).

There is increasing evidence of an additional development stage, Larva 3, identified by electron microscope (Fagerholm et al., 2000; Geenen et al., 1999; Kirchgäßner, Schmahl, Al-Quraishy, Ghaffar, & Mehlhorn, 2008; Maung, 1978). This stage could be considered as another stage of development or the infective stage to the host in the future. In the present study, Larva 3 stage was not reported since it is very difficult to observe by standard microscopy and longer time of incubation may be required for its development.

3.5.2 Speed of Development. The speed of change from one stage to another varied during incubation. It took 48 hours to observe 2-Cell ova and less than 24 hours to notice the development to 4-Cell ova. The continuing stages, (Early and Late Morula, Blastula and Gastrula) were observed at an approximate constant rate of development until larvae stages were noticed.

There is a gap of three days between Larva 1 and Larva 2 development. As mentioned before, Larva 2 could have passed unnoticed before day 16. This suggests that *A. suum* ova incubated at 28°C require a minimum of 48 hours to start cleavage but, once they reach this point, development initiates. This development is fast at the beginning, slows down and become constant in the middle, and move more slowly at the end, during larvae development.

Figure 3.1 presents the changes in the speed of development while Figure 3.2 presents the point in time at which each stage was first observed. Both figures were used as a tool to guide microscopy assessment of *A. suum* ova viability in other studies done by the author. It is important to mention that different factors could affect development during incubation under conditions similar to this study; that is *A. suum* ova incubated *in vitro* with sulfuric acid. Temperature is one of the most important factors influencing the speed of development (Arene, 1986). Development takes longer at lower temperatures and it is faster at higher temperatures, achieving a maximum rate of development at $31 \pm 1^\circ\text{C}$ (Arene, 1986; Seamster, 1950). Geene et al. (1999), observed Larva 1 after 17 days of incubation at 18-22°C; this study observed Larva 1 stages at 12 days of incubation. Johnson et al. (1998), reported ova with two or more cells at 48 hours of incubation at 32.5 C°, while this study reported Two cell ova after 72 hours of incubation. Similar to the results presented, Pecson et al., reported the development of Larva stages at 12 days of incubation at 28°C (Pecson et al., 2006).

3.5.3 Larva Motility Induction. According to the literature Larva motility with light stimulation is an indication of viability (Caceres et al., 1987; USEPA, 2003). The microscopic evaluation during this experiment revealed different motility intensity not only in larva but also in pre-larvae stages. Pre-Larva 1 and 2 responded to light stimulation with limited intensity of motility; Larva 1 was very sensitive to light as it showed active motility immediately after placing the slide in the microscope. Larva 2, on the other hand, required 5 to 10 minutes in the microscope before any movement could be noticed. These observations are consistent with previous research. Johnson et al., reported that some larvae moved within few minutes of observation in the microscope while others required 5 to 10 minutes (Johnson et al., 1998). Bassiouni et al., observed continuous motility of first larva stages of *Neoascaris vitulorum*, a cattle parasite, and dormant or slight movement in the advanced larvae stages (Bassiouni et al., 1990).

Larva motility induction is a criteria continuously used over the years to evaluate larva of *Ascaris* spp ova viability. Observations in this and other studies are consistent in the time required (5 to 10 minutes) to induce motility in advanced larva stages. This is a time consuming criteria during microscopic evaluation. It could be possible not to use this criterion to determine ova viability during microscopic evaluation if the following requirements are met: the ova have not been exposed to any treatment before *in vitro* incubation; and the shell and larva inside the ovum are intact and complete.

3.5.4 Viability. USEPA standards require a minimum of three weeks of incubation to report viability. Following this standard, the viability of the *A. suum* batch used in this study was of 87.5%. This percentage takes in account larva stages only. Statistical analysis showed no significant difference when early stages of development were considered as potentially viable. One study described similar results after comparing viability at 48 hours of incubation versus thirty days of incubation (Johnson et al., 1998). No other studies were found comparing early versus late viability and this is the first one using statistical analysis to report no significant differences when considering early stages of development observed continuously during incubation.

During microscopic evaluation, correct identification of Late Morula and Blastula stages required more time and effort from the researchers. Early stages and larvae stages were identified easily by microscopy at 100X magnification, while Late Morula and Blastula stages required more time and higher magnification as details became more important to classify the ova in the correct stage. This is one of the limitations of using standard microscopy and a possible reason to require at least three weeks of incubation with development of larvae to report *Ascaris* spp. viability from environmental samples. Pecson et al., (2006) described similar difficulties. As a consequence of these difficulties, he reported five stages of development only in his study: 1 cell, 2-3 cell, 4-6 cell, 7 or more cells, and fully larvated ova (Pecson et al., 2006).

We consider reporting viability early during incubation as a potential alternative based on the statistical analysis results found in this study: no

significant difference between the viability proportions from day 5 to day 21. Nevertheless, the potential use of early stages of development as viability indicator would be applicable to *in vitro* incubation only with ova no previously exposed to any treatment.

Faster methods, such as PCR, have been proposed as an alternative to the incubation technique used in the USEPA and Tulane method, since the use of *Ascaris* spp. ova to monitor pathogen inactivation in biosolids and wastewater has increased. The concern with this method is that material and laboratory equipment to implement it are expensive and the assay requires 10 days of ova incubation prior to application (Pecson et al., 2006). According to our results, viability could be established before 10 days of incubation. In addition, PCR has not been considered as standard procedure by USEPA. In developing countries, where Ascariasis is highly prevalent and access to sanitation is limited, this may be a costly alternative for testing viability in environmental samples.

During the experiment, few ova observed presented morphological abnormalities. These abnormal ova were observed sporadically from day 8-9 until the end of the study. It is presumed that exposure of ova to refrigerated conditions may be related to the presence of abnormal ova that do not develop to fully larva stages (Johnson et al., 1998). The ova used in this experiment were stored at 4°C before utilization.

3.6 Conclusion

A. suum ova went through clearly identified morphological changes at different speed of development. A total of 12 stages were identified including two

additional stages: Pre-larva 1 (larva coiled creating no more than one concentric ring) and Pre-larva 2 (larva coiled creating at least one and a half concentric ring).

No significant statistical difference was observed among the viability reported early in incubation and the one reported after three weeks of *in vitro* incubation. If viability of *Ascaris* spp. ova is defined as a healthy ovum that will continue its development to become an infective larva under constant *in vitro* incubation conditions then, observing the ova under the microscope early during incubation, instead of waiting up to three-four weeks for the development of a larva, could be an alternative to reduce the time to report viability.

CHAPTER 4.

**INACTIVATION OF *ASCARIS SUUM* BY AMMONIA IN WATER SOLUTION
UNDER LABORATORY CONDITIONS**

4.1 Abstract

Background: A wide range of nonconventional sanitation technologies have been implemented in developing countries to help achieving the sanitation United Nations Millennium Development Goal. In rural areas of El Salvador, a waterless sanitation technology, the solar toilet, is a promising system accepted by the people and national authorities. Analysis suggests that its pathogen inactivation capacity needs to be improved. Ammonia could be added to the toilet to improve pathogen inactivation.

Objective: This study evaluated the potential capacity of ammonia to inactivate a resistant fecal pathogen, *Ascaris* spp. ova, in a water solution with temperature and pH values achieved in the solar toilet.

Methods: Permeable Nylon bags with *Ascaris suum* eggs were exposed, in triplicates samples, to different ammonia concentrations (0%, 1%, 2%) in water, pH (7.7, 9.3) and temperatures (28°C, 35°C, 40°C, and 45°C) for a period of three days. After the treatment period pH and ammonia value were measured and *Ascaris suum* ova were incubated for 21 days in 0.1N sulfuric acid at 28°C.

Ova viability was assessed at the end of incubation by microscopy. A minimum of 200 *A. suum* ova were observed and the percentage of viable ova were calculated.

Results: The critical parameters identified to significantly reduce *A. suum* ova viability to zero in three days were: temperature of 35°C or higher and 1% to 2% ammonia concentrations with a pH value of 9.3. At 28°C, not significant (χ^2 , $p > 0.01$) reduction in viability was observed and, at 45°C all *A. suum* ova were inactivated independently of pH and ammonia value. A logistic regression analysis indicates that temperature (OR=1.438) and treatment with ammonia (OR=2.7322) increase inactivation of the parasite ova.

Conclusions: The results of this study suggest that addition of ammonia to the solar toilet may increase the pathogen inactivation capacity of the system. For future studies, it is recommended to evaluate this approach in fecal material with a larger sample size incorporating other parameters found in the solar toilet (low moisture and a mixture of soil and lime as additive) before field application is considered.

Keywords : Sanitation, *Ascaris*, inactivation, ammonia

4.2 Introduction

Access to sustainable sanitation systems is a determining factor in human health and economic development. It has been estimated that improving access to safe water and sanitation would reduce, annually, 5.4 billion cases of diarrhoea and 1.6 million deaths worldwide, mostly, in children less than five years of age (Hutton & Haller, 2004). However, more than a third of the world's population live without access to improved sanitation facilities (WHO/UNICEF, 2010). To meet the sanitation United Nations Millennium Development Goal, "*halve, by 2015, the proportion of people without sustainable access to safe drinking water and basic sanitation*", at least 1.4 billion people must gain access to basic sanitation by the year 2015 (United Nations, 2008).

In an effort to provide this basic service, a wide range of non conventional sanitation technologies have been implemented in developing countries, including waterless systems (Nelson & Murray, 2008). These systems present a re-use oriented approach that considers human waste as a resource, instead of a waste. Water is not used to transport waste and the technology can be built and implemented in areas lacking sewage systems, deficient in water, or with a high ground table. The systems functions by diverting urine away from feces and collecting, storing, and dehydrating the fecal material in watertight dehydration vaults. Subsequently, the material is removed and transported for use or disposal by the user (Tilley et al., 2008).

From a public health perspective, adequate inactivation of fecal pathogens in a sanitation system is essential before any use or disposal of fecal material.

Inadequately sanitized fecal material contributes to the spread of fecal pathogens in the environment and causes a great burden of disease in developing countries, especially in children (Hutton & Haller, 2004). In rural areas of El Salvador, two different waterless sanitation technologies have been implemented and evaluated: double-vault urine diverting toilets (DVUD) and solar toilets (Moe & Izurieta, 2003). Analysis showed the higher capacity of the solar toilet at inactivating fecal pathogens and, at reducing the prevalence of parasitic infections in its users when compared to DVUD toilets and pit latrines (Corrales et al., 2006). Nevertheless, not all solar toilets are able to inactivate completely soil transmitted helminthes ova after the recommended storage period, limiting the use of the treated fecal material as fertilizer/soil conditioner and, probably, increasing the risk of environmental contamination when disposed of (Moe & Izurieta, 2003). Increasing the pathogen inactivation capacity of the solar toilet would improve the technology and the community public health safety.

Un-ionized ammonia (NH_3) has the potential to inactivate pathogens in solutions and sludge, including *Ascaris* spp. ova, which is considered the most resistant pathogen in fecal material and a good index of hygiene quality (Bujoczek, 2001; Chefranová et al., 1977; Chefranová et al., 1984; Chefranová et al., 1978; Ghiglietti et al., 1997; Ghiglietti et al., 1995; Mendez et al., 2002; Metcalf & Eddy, 2003; Pecson et al., 2007; Pecson & Nelson, 2005; World Health Organization, 2006a). Studies on the inactivation of this parasite ova by ammonia in human feces are limited, but results from laboratory experiments suggest a positive inactivation potential (Nordin et al., 2009; Vinnerås et al., 2003).

Adding ammonia to the solar toilet could be considered as a potential approach to improve the technology since the pathogen inactivation effect of this chemical agent could be potentiated by the alkaline medium and high temperatures achieved inside the toilet vaults. To evaluate this approach, in the absence of other chemical compounds present in human feces, a series of experiments in solution were performed. This study presents the results of the laboratory experiments using temperature and pH values similar to those achieved by the solar toilet.

4.3 Methods

4.3.1 Materials. Eggs of the swine *Ascaris* species, *Ascaris suum*, bought from Excelsior Sentinel Inc. (New York), were used as model of the human species, *Ascaris lumbricoides*. A stock of 10,000 ova per ml, with an initial viability of 87.5% (N=200), was prepared from the initial solution and stored at 4°C. The viability of the stock was determined by incubating the ova for a minimum of three weeks in 0.1N sulfuric acid at 28°C. Nylon bags were created and used to avoid dispersion of the ova in the media and to facilitate their collection at the end of the experiments. The nylon mesh, with a 30 micron opening (CMN-0030-D, Small Parts, Inc.), was cut (1.5" x 3"), folded and sealed in the parallel open sides with a bag sealer (EF28264, A. Daigger & Company). After placing one milliliter of the *A. suum* ova stock solution inside the bag, the open side was completely sealed and the bag was stored in de-ionized (DI) water until used.

Two ammonia solutions, 1% and 2%, were prepared with Ammonium hydroxide solution 28% in H₂O (338818-100ML, Sigma-Aldrich) and DI water. Buffers, HEPES and CHES (Fisher Scientific), were used to hold pH values at 7.7 and 9.3 respectively. Sodium hydroxide was used in the control samples to raise pH to 7.7 and 9.3. Ammonia and pH values were measured at room temperature (26-28 °C) using an ammonia electrode (Ion Selective, Ammonia Gas-Sensing Combination Electrode; Fisher Scientific); a pH electrode (AccuCap, pH Combination Electrode; Fisher Scientific); and a meter (Accumet excel, Dual Channel pH/ion/conductivity/DO meter XL 60, Fisher Scientific). In this study, those samples exposed to either 1% or 2% ammonia solution are referred to as treated samples.

4.3.2 Experiment set up. *A. suum* nylon bags were distributed in centrifuge tubes (capacity of 15ml) with 14 ml of ammonia or control solution (Table 4.1). At a pH of 7.7, samples with 1% ammonia would have a very small quantity of ammonia available (3.3% to 9.6%) to inactivate *A. suum* ova (Pecson & Nelson, 2005). For this reason, samples with 1% ammonia were not included within the group with a pH of 7.7.

Table 4.1

*Summary of Experimental Design**

Incubator T°C	pH 7.7		pH 9.3		
	0% NH ₃	2% NH ₃	0% NH ₃	1% NH ₃	2% NH ₃
28 °C	Control	Treated Sample	Control	Treated Sample	Treated Sample
35°C	Control	Treated Sample	Control	Treated Sample	Treated Sample
40°C	Control	Treated Sample	Control	Treated Sample	Treated Sample
45°C	Control	Treated Sample	Control	Treated Sample	Treated Sample

*Each cell/batch had three samples or triplicates

Samples, prepared in triplicates, were kept closed with o-ring screw caps, in dark incubators at four different temperatures (28°C, 35°C, 40°C, and 45°C) for 72 hours (treatment period). When the treatment period was completed, pH and ammonia values were measured at room temperature and registered in a log sheet. Nylon bags were washed in DI water and placed in new tubes with 0.1N sulfuric acid (H₂SO₄) for incubation.

The nylon bag from the third replicate of each batch was rinsed in distilled water and cut. The ova were washed directly into Petri dishes containing 4 ml of the incubation solution (0.1 N H₂SO₄) and subsamples were taken at day 0, 3, 7 and 14 to assess *Ascaris suum* viability under the microscope. The other replicates samples were incubated at 28°C with 0.1N sulfuric acid, in open containers, for 21 days (incubation period); no sampling took place until the incubation period was completed. Adequate aeration and incubation solution level were monitored. DI water was added when solution evaporation was observed. At the end of the incubation period, the percentage of viable *Ascaris* ova in triplicate one and two was assessed by microscopic examination. A minimum of 100 *A. suum* ova were observed to determine viability. To calculate viability proportions, only larva stages were considered in the numerator. Table 4.2 describes the viability vs. non viability criteria used (Cruz, Allanson, & Izurieta, 2009).

Table 4.2

Criteria to Identify Stage of Development and Viability of Ascaris suum Ova by Microscopy

Non viable ova*	Viable ova
<p>Ovum with:</p> <ul style="list-style-type: none"> ➤ Contraction, rupture and loss of the ovum membrane continuity. ➤ Poorly defined structures ➤ Cellular condensation ➤ Granulated or vacuolated cytoplasm ➤ No larvae movement observed by microscope light stimulation 	<p>Intact ovum in any of the following stages:</p> <ul style="list-style-type: none"> ➤ One to Four Cell : Observation of 1, 2, 3, or 4 clearly well defined cells within the ovum ➤ Early morula: Observation of 5 to 10 cells within the ovum. ➤ Late morula: Observation of 11 or more cells within the ovum. ➤ Blastula: Observation of a spherical layer of cells surrounding a pseudo fluid-filled cavity. ➤ Gastrula: Observation of a layer of cells surrounding the embryo, plus a kidney shape invagination in one side of the embryo. ➤ Pre-larva 1: Observation of a larva like structure, coiled, creating no more than one concentric ring inside the ovum. ➤ Pre-larva 2: Observation of a larva like structure, coiled, creating at least one and a half concentric ring inside the ovum. ➤ Larvae 1: Observation of a larvae structure within the ovum, without well defined structures inside the larvae wall, absence of molt and intense motility in response to light. ➤ Larva 2: Observation of more defined structures within the larva, a molt, and a delayed motility response to light stimulation.

* Adapted from Caceres et al, 1987

4.3.3 Statistical analysis. The results of the experiments were introduced in Excel 2007 and imported to SAS system software (version 9.2, SAS Institute Inc., Cary, NC, USA) for analysis. Descriptive statistics (mean and standard

deviation) of ammonia, pH and temperature measurements were calculated. Chi-square test for proportions was used to test viability proportions differences (p value < 0.01) between samples. A logistic regression analysis, relating *A. suum* inactivation (ovum inactivated /ovum viable) to the independent predictors, was developed. The dependent variable, *A. suum* inactivation, was defined as not inactivated when viability was greater than 0% and, inactivated, when viability was equal to 0%. Predictor variables included Temperature (28°C, 35°C, 40°C, and 45°C), pH (7.7, 9.3) and Treatment (0% ammonia, 1% ammonia, 2% ammonia). Univariate and logistic regression analysis were performed and the decision to include terms in the final model was based on the likelihood ratio test and the adequacy of the model using the Hosmer and Lemeshow and Deviance Goodness of Fit tests.

4.4 Results

4.4.1 Monitoring ammonia and pH. Ammonia concentration of solutions were measured after preparing and placing the samples in the incubators. The values registered with the ammonia probe were: 252.9 ppm for 2% NH₃ solution (pH of 7.7); 4,360.1 ppm for 1% NH₃ solution (pH=9.3); and 8,392.8 ppm for 2% NH₃ solution (pH=9.3). These initial measurements were taken at room temperature (26-28°C). After 72 hours of treatment, samples were open and ammonia solution was measured again along with the pH value (Table 4.3). Before the second measurement, samples were left outside the incubator to adjust to room temperature (26.3°C ± 1.42°C).

After three days of treatment the pH value of the control samples placed at a pH=7.7 and pH=9.3, decreased to a mean value of 5.3 (± 0.47) and 5.4

(± 0.55), while the pH of exposed samples increased to 7.8 (± 0.05) and 9.4 (± 0.07) respectively.

Table 4.3

Aqueous Ammonia in Samples after 72 Hours of Storage

Sample group	Ammonia (ppm)							
	28 °C		35°C		40°C		45°C	
	M*	SD*	M*	SD*	M*	SD*	M*	SD*
2% NH ₃ pH7.7	182.0	5	176.0	3.5	188.6	28	183.3	17
1% NH ₃ pH9.3	3886.6	15	3993.3	172	3133.3	274	3326.6	275
2% NH ₃ pH9.3	6046.6	40.4	5310	410.7	4916	135	4810	275

*M=mean; SD=standard deviation

The final concentration of NH₃ in water depends on temperature and pH of the solution. Initial and final measurements of ammonia registered in this study were lower than theoretical values expected. The discrepancy on the values may be related to different causes. Ammonia present in the gas form was not determined and it may have been lost quickly when the samples were open after the treatment period. The ammonia present in the solution (aqueous ammonia) was the only form of ammonia measured. To minimize losing ammonia as gas, we measured ammonia and pH as soon as samples were open after the three days of treatment. However, since alkaline pH increases the concentration of un-ionized ammonia, it is suspected that ammonia was lost as gas continuously during measurement procedures. As part of laboratory safety requirements, the room where the experiments took place had exhaust systems, which have been reported to influence negatively ammonia measurements (Lefcourt, 2002).

4.4.2 A. *suum* viability. Different level of reductions in the viability of *A. suum* ova were observed among samples: control samples versus treated samples (Table 4.4). Viability of ova in samples placed at 28°C did not differ significantly from the initial viability of the batch (87.5%) and, within each pH group, from the control vs. treated samples (Chi-square, $p > 0.01$). At 35°C, a significant ($p < 0.01$) reduction in ova viability from the initial viability was observed in all samples. Although complete inactivation in samples within pH group 7.7 was not achieved, the viability reduction in the sample exposed to 2% ammonia solution (59%) was significantly different (Chi-square=10.79, $p = 0.001$) to its control (73.5%). Samples exposed to ammonia in the pH group of 9.3 achieved total inactivation, while its corresponding control did not.

Table 4.4

Viability of A. suum Ova After 21 Days of Incubation in vitro

Incubator T°C	pH 7.7		pH 9.3		
	0% NH3	2% NH3	0% NH3	1% NH3	2% NH3
28 °C	82%	73.5%	80%	89.5%	84.5%
35°C	73.5%	59%	76%	0%	0%
40°C	75.5%	69%	78%	1%	0%
45°C	0%	0%	0%	0%	0%

At 40°C, the reduction in viability of all samples was significant when compared to initial viability of 87.5% (p value < 0.01). The difference between control and treated samples was also significant within each pH group (p value < 0.01). Similar to results at 35°C, ova viability of samples exposed to 1% and 2% ammonia within the pH group of 9.3 was reduced to 1% and 0% respectively. At

45°C, the viability of all samples was reduced to 0% independently of pH value and ammonia concentration in the solution.

4.4.3 Probability model of *A. suum* inactivation with ammonia. The logistic regression model included Temperature (28°C, 35°C, 40°C, and 45°C), pH (7.7, 9.3) and Treatment (0%, 1%, 2% ammonia) as potential predictors. Treatment and pH, were first removed from the model ($p > 0.05$). In theory, pH and treatment are correlated since pH influences the amount of un-ionized ammonia available (Emerson et al., 1975). Nevertheless, our analysis found that the correlation between these two variables was not significant ($p=0.09$). This finding may have been due to the small sample size of this study (N=20). When each ovum observed in the microscope was considered as a single event in the database, the correlation between the variables was highly significant ($p < 0.0001$). In spite of this, the small database (N=20) was used to estimate the effect of each variable in the logistic regression model, since initial viability of the *A. suum* batch (87.5%) evidenced the possibility of observing inactivated ova without exposing them to any treatment.

The predictor Treatment was added back into the model after assessing the goodness of fit test which showed a better fitted model (Deviance, $p = 0.37$; Hosmer and Lemeshow Test, $p=0.95$) when this variable was included along with Temperature as predictors (Table 4.5).

According to the model increasing temperature increases the probability of inactivating 100% ova by 43%, holding other predictors constant. For treatment, adding ammonia to the solution more than double the probability of inactivating

100% ova, holding other predictors constant. The effect of treatment on inactivation is higher, although not significant.

Table 4.5

Logistic Regression Analysis of A. suum Inactivation in Solution by Ammonia

Physical and Chemical Factors	Parameters Estimate	OR*	95% CI	p-value
Temperature	0.3630	1.438	[1.046, 1.976]	0.02
Treatment	1.0049	2.732	[0.579, 12.88]	0.20

*OR: Odds Ratio

Figure 4.1 and 4.2 presents the estimated probability of total inactivation when all the parameters are included in the model.

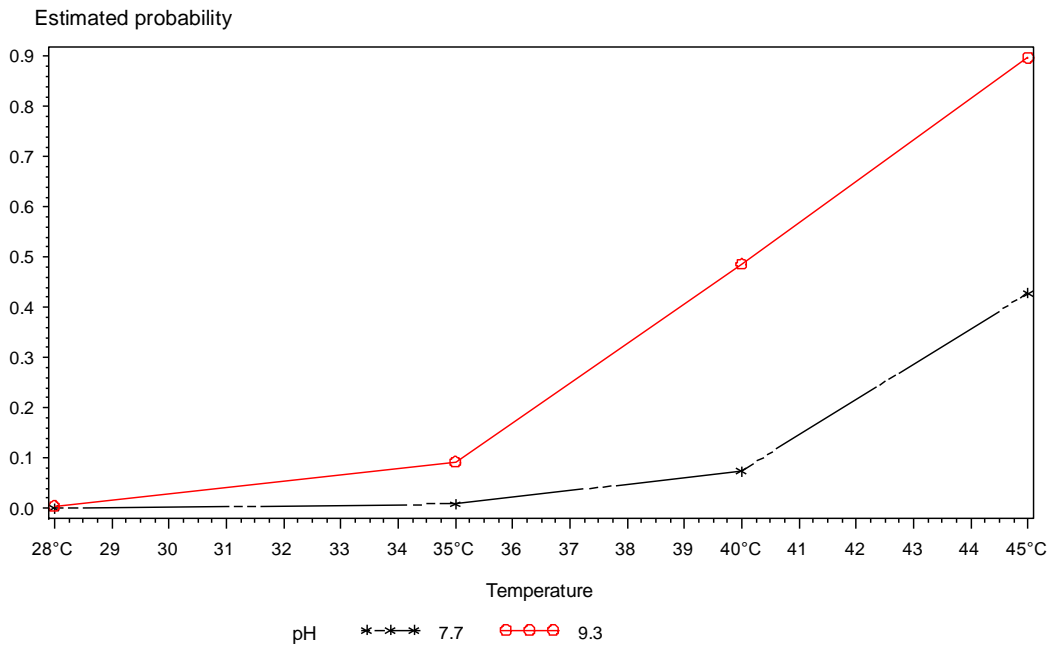


Figure 4.1 Probability of 100% *A. suum* ova Inactivation in Solution after Three Days of Storage with 0% Ammonia

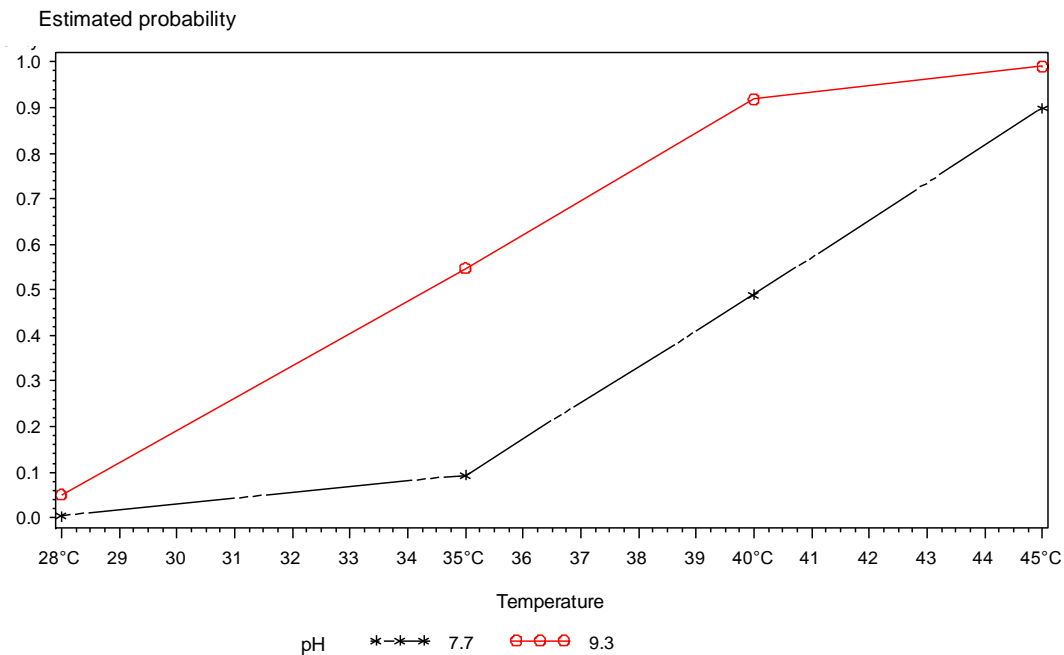


Figure 4.2 Probability of 100% *A. suum* ova Inactivation in Solution after Three Days of Storage with 2% Ammonia

4.5 Discussion

The lowest temperature (28°) used in this study did not reduce significantly the initial viability of *A. suum* ova, while the highest temperature (45°) inactivated all ova after three days, independently of pH and ammonia concentration. This suggests that, under laboratory conditions, 45°C is the minimum temperature required to achieve total inactivation with no other agent or chemical added to the solution. Other researchers have reported a minimum temperature of 48°C after 72 hours, 40°C after 7 days and even 37.8°C after 8 days, to successfully inactivate *A. suum* ova under similar experimental conditions (Ghiglietti et al., 1995; Pecson & Nelson, 2005; Seamster, 1950). These observations make visible the dependence of ova inactivation to temperature and storage time. The optimum temperature of *Ascaris* spp. ova

development under laboratory conditions reported in the literature is 31.1°C, while the maximum temperature at which *Ascaris* spp. ova have been observed to develop is 37.2°C (Arene, 1986; Seamster, 1950)

In our study, total inactivation was achieved at 35° and 40°C in less than three days in those samples exposed to 1% to 2% ammonia with a pH of 9.3, with the exception of the sample exposed to 1% ammonia at 40°C. The 1% viability registered in this sample, could have resulted from contamination when opening the nylon bag since two of the three replicates did achieved 100% inactivation. Samples exposed to lower pH values (7.7) but to the same temperature and ammonia concentration (2%) did not accomplish the same inactivation level. The pH value of the solution influences the concentration of the ionized form (NH_4^+) and the un-ionized form (NH_3) of ammonia available. Un-ionized ammonia is the toxic form of ammonia to the parasite ova. An increment of pH is correlated with an increase of the un-ionized form and, theoretically, pH values greater than 8 are needed to have substantial amounts of un-ionized ammonia (Emerson et al., 1975; Rayner-Canham, 1996). This indicate that samples exposed to a pH of 7.7 and 2% ammonia had lower concentrations of ammonia compared to samples with pH values of 9.3 and, therefore, less capacity to inactivate the parasite ova. These observations are consistent with the theory and with previous publications. Pecson and Nelson (2005) reported increasing inactivation by ammonia in solution with increasing pH values from 7 to 11.

This is the first attempt to model inactivation of *A. suum* ova in solution by ammonia, using temperature (28°C to 45°), pH (7.7, 9.3) and different ammonia concentrations (0%, 1%, and 2%). There are some models reported in the literature of *Ascaris* spp ova inactivation in sludge and biosolids (Nordin et al., 2009). Still, only few authors have published about inactivation of *Ascaris* spp. ova in solution by ammonia but no model of inactivation has been reported (Ghiglietti et al., 1995; Pecson & Nelson, 2005). The model created in this study, indicates that ammonia treatment and temperature have an important influence in the probability of total inactivation of *A. suum* ova in solutions. The effect of ammonia (OR = 2.7) doubles the effect of temperature (OR=1.4). As mentioned previously, the variable pH was excluded from the model because it is correlated with the variable Treatment. For future studies, a larger sample size is recommended to confirm the effect of both variables in the inactivation process and to show the co-linearity of pH and treatment with ammonia.

The mechanism by which ammonia inactivates *Ascaris* spp. ova is not well understood yet. Pecson et al., (2005) suggested that temperature increases the permeability of the lipid membrane of the egg-shell allowing the diffusion of un-ionized ammonia into the ovum, with the subsequent increase of intracellular pH and inactivation of the parasite (Pecson & Nelson, 2005). Nordin et al., (2007) also suggested that ammonia inactivation depends on shell permeability influenced by temperature. This hypothesis is consistent with our observations of greater ova inactivation by ammonia with increasing temperature.

A limitation of the study is the difference found between expected and measured ammonia values. As mentioned before, this could have been related to different causes. The increased pH values registered in the second measurement suggest that ammonia was not lost during the treatment period. The amount of ammonia entering the ova parasite has not been estimated yet, but it may have also influenced the differences in ammonia value found. For future research, measuring ammonia as gas, in addition to measuring the concentration in water, is recommended to obtain a better estimation of ammonia concentration.

In this study, the critical parameters identified to significantly reduce *A. suum* ova viability to 0% in three days were: temperature of 35°C or higher and 1% to 2% ammonia concentrations with a pH value of 9.3. Inside the solar toilets vaults temperatures as high as 41.5°C have been registered with pH values of the fecal material ranging from 8.9 to 12.4 (Izurieta et al., 2006; Moe & Izurieta, 2003). This indicates that addition of ammonia to the solar toilet may increase the pathogen inactivation capacity of the system to a point, that even the most resistant fecal pathogens could be inactivated before fecal material is removed from the toilet vault. This would result in a reduction of environmental contamination, better public health safety and the possibility to use the final material as fertilizer or soil conditioner by the user. However, these experiments were done in water solution and the fecal material present in the solar toilet have moisture less than 52% (wet based). Using the critical parameters found in this study, a small pilot test (results not shown) with fecal material (moisture < 30%

and urea as source of ammonia), resulted in total inactivation of *A. suum* ova.

For future studies, it is recommended to evaluate this approach in fecal material with a larger sample size incorporating other parameters found in the solar toilet (low moisture and a mixture of soil and lime as additive) before field application is considered.

CHAPTER 5.

INACTIVATION OF *A. SUUM* OVA BY AMMONIA IN FECES UNDER LABORATORY CONDITIONS

5.1 Abstract

Background: A variety of non conventional sanitation systems, with a re-use oriented approach, have been implemented in developing countries. A main safety requirement of these technologies is the fecal pathogen inactivation. In rural-agricultural areas of El Salvador, the solar toilet has shown the effectiveness of this non conventional system at inactivating fecal pathogens and reducing the prevalence of parasitic infections in its users but; not all solar toilets units are able to inactivate completely *Ascaris* spp. ova. Addition of ammonia could improve the system since previous laboratory work has shown its potential when exposed to the physical and chemical conditions achieved in the solar toilet.

Objective: The present study evaluated the use of ammonia to sanitize fecal material in laboratory conditions incorporating all physical and chemical parameters of the solar toilet: low moisture; temperature values ranging from

28°C to 45°C; alkaline pH; additive created from a mixture of dirt and lime; 56 days of storage; and 1%-2% urea as source of ammonia.

Methods: To simulate the material inside the toilet's vault (biosolid), human feces were collected and mixed with the equivalent of a cup of additive. Eggs of the swine *Ascaris* species, *Ascaris suum*, were used as model and urea was used as the source of ammonia. Three groups of samples (biosolid with 0% urea, biosolid with 1% urea, and biosolid with 2% urea) were placed in duplicates, closed, at four different temperatures (28°C, 35°C, 40°C, and 45°C). Samples were removed on day 1, 3, 7, 14, 21, 28, 35, 42, 49 and 56. pH, ammonia and moisture were measured from each sample removed from the incubator and, each *A. suum* ova nylon bag was placed for incubation in sulfuric acid (0.1 N) for 21 days. After incubation, parasite ova viability was evaluated in the microscope (minimum of 100 ova). Survival and logistic regression analysis were performed in SAS 9.1.3.

Results: The inactivation of *A. suum* ova was faster in samples exposed to urea and to temperatures higher than 28°C. Samples without urea never reached a 100% inactivation at 28°C within the 56 days of storage. All samples exposed to urea achieved 100% inactivation after 14 days (28°C), 3 days (35°C) and 24 hours (40°C and 45°C). Survival analysis of the data showed that there was a significant difference (p value $<.0001$) between the inactivation achieved in the samples exposed to urea (1% and 2%) and the samples not exposed to urea. The logistic regression model was significant ($p<0.0001$) and included the predictors: Temperature (OR: 1.8), Treatment (OR: 25.9), and Storage (OR:1.17). The

Goodness-of-Fit tests showed that the model fitted the data (Deviance = 1.0000; Hosmer and Lemeshow = 0.9868).

Conclusion: The results of this study suggest that inactivation of *Ascaris* spp ova by ammonia is possible in fecal material stored in the solar toilet or any other dry toilet, if the following physical and chemical conditions are met: a closed vault with a minimum temperature of 28°C; an initial pH of 8.3, minimum moisture of 27.5%, and addition of 1% urea to the biosolid. At 28°C longer storage time would be required for 100% inactivation while at higher temperatures less time of storage would be necessary. A community intervention is recommended to include field conditions and human behavior as other predictors for *Ascaris* spp. inactivation.

Keywords: ammonia, inactivation, *Ascaris*, sanitation, dry toilet

5.2 Introduction

Providing basic sanitation continues to be a challenge in the 21st century, despite the United Nations Millennium Development Goal (MDG) of reducing by half the proportion of people without sustainable access to safe drinking water and basic sanitation by 2015. About 2.6 billion people lack access to basic sanitation and 1.1 billion still practice open defecation. This situation contributes to the spread of human pathogens in soil and water and increases the risk of transmission of many infectious diseases (WHO/UNICEF, 2010). Among the main diseases are water and sanitation related illnesses which account for 80% of diseases in developing countries (Water Advocates, 2009). Diarrhoea is directly caused by fecal pathogens and results in 4 billion cases and 1.8 million deaths annually, most of them (90%) among children under five (UNICEF, 2005). Fifty percent of malnutrition is associated with repeated diarrhoea episodes and intestinal nematode infections, illnesses linked to inadequate sanitation (World Health Organization, 2006b). In addition, the United Nations estimated that solving the problems of water and sanitation will make a 30% contribution, on average, towards meeting the other Millennium Development Goals (MDG) including child survival, improved maternal health, primary and secondary education, gender equality, environment, and hunger alleviation (United Nations Secretary-General's Advisory Board on Water and Sanitation, 2007).

The implementation of conventional sanitation systems represent an expensive and inappropriate option in developing countries where seven out of 10 people without improved sanitation live in rural areas (WHO/UNICEF, 2010).

As an alternative, a variety of non conventional sanitation systems (NCSS), with a reuse-oriented approach, have been implemented. Many of the NCSS offer more environmental and long-lasting technologies, align with the main objective of sustainable sanitation, “*to protect and promote human health by providing a clean environment and breaking the cycle of disease*”(Rosemarin et al., 2008). The use of this type of technology is also driven by other issues such as increasing water scarcity; degradation of freshwater resources; environmental sustainability; and the potential use of biosolid nutrient content for food production (IWMI, 2010; Rosemarin et al., 2008; World Health Organization, 2006a).

One of the main requirements of a sustainable sanitation system is the risk reduction to fecal pathogens exposure from the point of collection of fecal material to the point of reuse or disposal (Rosemarin et al., 2008). This criterion is of extreme importance to public health to break the fecal-oral transmission of diseases. In rural-agricultural areas of El Salvador, analysis of the inactivation capacity of a waterless sanitation system, the solar toilet, have shown the effectiveness of the toilet at inactivating fecal pathogens and reducing the prevalence of parasitic infections in its users when compared to double-vault urine diverting toilets (DVUD) toilets and pit latrines (Corrales et al., 2006). The solar toilet is an improved dry toilet design that uses high pH additives, a simple solar panel, and six weeks of storage to generate a product that meets the United States Environmental Protection Agency fecal coliforms standard (< 1000 Fecal Coliforms per gram). Nevertheless, not all solar toilets are able to

inactivate completely *Ascaris* spp. ova after the recommended storage period, limiting the use of the treated fecal material as fertilizer/soil conditioner and, probably, increasing the risk of environmental contamination if disposed of incorrectly (Moe & Izurieta, 2003). *Ascaris* spp. ova are one of the most resistant pathogens present in feces (Feachem et al., 1983). This unique characteristic has made this parasite an indicator and index of hygiene quality (World Health Organization, 2006a).

In an effort to improve this technology, the addition of a new additive to the solar toilet has been proposed (not published, Cruz et al., 2010). This additive, ammonia, would allow the use of the sanitized fecal material after treated as a soil conditioner. Ammonia (NH₃) has the potential to inactivate pathogens in solutions and sludge, including *Ascaris* spp. ova (Bujoczek, 2001; Chefranova Iu, 1977; Chefranova Iu et al., 1984; Chefranova Iu et al., 1978; Ghiglietti et al., 1997; Ghiglietti et al., 1995; Mendez et al., 2002; Metcalf & Eddy, 2003; Pecson et al., 2007; Pecson & Nelson, 2005; World Health Organization, 2006a). Studies on the inactivation of this parasite by ammonia in human feces are limited, but results from laboratory experiments suggest a positive inactivation potential (Nordin et al., 2009; Vinnerås et al., 2003).

An evaluation of the inactivation capacity of this additive in solution, using *Ascaris suum* ova and temperatures and pH values of the solar toilet, resulted in 100% inactivation suggesting that adding ammonia to the solar toilet may improve the technology. An additional small pilot test, carried out by our team, (three samples placed at 35°C for three days) with fecal material (moisture <

30% and urea as source of ammonia), provided further evidence of the potential used of ammonia to inactivate *A. suum* ova (not published, Cruz et al., 2010).

The present study evaluated the use of ammonia to sanitize fecal material in laboratory conditions with a larger sample size and incorporating all physical and chemical parameters of the solar toilet: low moisture; temperature values ranging from 28°C to 45°C; alkaline pH; additive created from a mixture of dirt and lime; 56 days of storage; and urea (as source of ammonia) at 1% and 2% concentration.

5.3 Methods

5.3.1 Materials. To simulate the material inside the toilet's vault, human feces were collected in a stool container (Commode Specimen Collection System, Fisher Scientific 02-544-208) from healthy volunteers between 21 and 60 years old. Following the practice of the solar toilet users in El Salvador, the equivalent of a cup of additive was added by the volunteer to the container after defecation (Ministerio de Salud Publica y Asistencia Social, 2004). Dirt and lime (Certified Calcium Hydroxide, Fisher Scientific) were used to prepare the additive (1:60 volume, lime to dirt). Using try and error, the combination of 1 part lime volume to 60 part dirt volume was chosen from different combinations based on the final pH produced after mixing the additive with fecal material: pH of 8.3 when a cup of additive is mixed with 520 g of feces, and pH of 9.8 when a cup of additive is mixed with 130 g of fecal material. A maximum pH of 10 has been reported to be the breaking point for urea transformation to ammonia in feces (Nordin et al., 2009). In the laboratory, the material of each stool container was

mixed to homogenize it for five minutes, clockwise, and stored at room temperature, closed, until used. In this study we used the term biosolid to refer to the fecal material mixed with additive.

Eggs of the swine *Ascaris* species, *Ascaris suum*, bought from Excelsior Sentinel Inc. (New York), were used as model of *Ascaris lumbricoides*. The initial viability of the ova stock, determined by incubating the ova for a minimum of three weeks in 0.1N sulfuric acid at 28°C, was 70% (N=600). Nylon bags were created and used to avoid dispersion of the ova in the biosolid and to facilitate their collection at the end of the experiments. The nylon mesh (20 micron opening; NC9252629, Fisher Scientific) was cut (1.5" x 3"), folded, and sealed in the parallel open sides with a bag sealer (EF28264, A. Daigger & Company). After placing 200 µl of the *A. suum* ova stock solution inside the nylon bag (1ml ≈ 60,000 ova), the open side was completely sealed and the bag was stored in de-ionized (DI) water at 4°C until used.

Biosolid (17,231 grams of feces mixed with additive) were placed in one storage bin, mixed for 10 minutes with a wood stick, clockwise and distributed in three storage bins. Urea (Certified Urea, U15500, Fisher Scientific) was added to two of them at a concentration of 1% and 2% (weight/weight), mixed for five minutes clockwise with a stick, and distributed in the sample containers (I-CHEM Short wide-mouth clear Type III glass jar with Teflon®-lined, Polypropylene closure) immediately after prepared. Each sample was prepared by adding 70 grams of biosolid (biosolid with 0% urea, biosolid with 1% urea, or biosolid with

2% urea), and one *A. suum* nylon bag, placed in the middle of the container. All containers were immediately closed with a PTFE-lined polypropylene closure.

5.3.2 Experiment set up. Three groups of samples (biosolid with 0% urea, biosolid with 1% urea, and biosolid with 2% urea) were placed in duplicates at four different temperatures (28°C, 35°C, 40°C , and 45°C), in the dark for a period of 8 weeks (Table 5.1). These eight weeks correspond to the amount of time feces are left for pathogen inactivation in the solar toilet by recommendation of the Ministry of Health of El Salvador (Ministerio de Salud Publica y Asistencia Social, 2004). During these 8 weeks, samples were removed in duplicates from the incubator at ten different times: 1, 3, 7, 14, 21, 28, 35, 42, 49 and 56 days.

Table 5.1

Summary of Experimental Design

Incubator Temperature	Groups		
	Biosolid + 0% urea	Biosolid + 1% urea	Biosolid + 2% urea
28 °C	Control sample	Treated sample	Treated sample
35°C	Control sample	Treated sample	Treated sample
40°C	Control sample	Treated sample	Treated sample
45°C	Control sample	Treated sample	Treated sample

Each sample removed from the incubator was left to adjust to room temperature before opened. pH and ammonia (gas and soil fraction) were measured using an ammonia electrode (Ion Selective, Ammonia Gas-Sensing Combination Electrode; Fisher Scientific); a pH electrode (AccuCap, pH Combination Electrode; Fisher Scientific); and a meter (Accumet excel, Dual Channel pH/ion/conductivity/DO meter XL 60, Fisher Scientific). Moisture was

measured using the oven drying method (Canadian Society of Soil Science, 1993); and temperature was monitored daily during treatment and incubation period. To analyze pH and ammonia in soil, biosolids were mixed with DI water (1:9 dilution), covered with parafilm, stirred continuously for 10 minutes and left stand for 5 minutes before the pH and ammonia meter electrode were immersed into the clear supernatant (Canadian Society of Soil Science, 1993). For ammonia gas measurement, the ammonia electrode was placed inside the container immediately after opening and covered with parafilm to minimize ammonia gas losses.

The *Ascaris suum* nylon bags from the duplicates samples removed were washed twice in DI water and placed for incubation in a centrifuge tube with 20 milliliter of sulfuric acid (0.1 N) for 21 days. After 21 days of incubation the nylon bags were opened directly into a Petri dish with 2ml of 0.1N sulfuric acid. A sample (25 μ l) was taken to examine viability in the microscope at 40x and 100x magnification (Table 5.2). A minimum of 100 ova were observed from each sample and only larva stages were used to calculate final viability.

5.3.3 Statistical analysis. A database was created in Microsoft Excel 2007 and imported to SAS 9.1.3, (SAS Institute Inc.; Cary, NC) to perform statistical analysis: Survival and logistic regression analysis were performed. For survival analysis the sensor variable was viability greater than 0%. For a logistic regression model the dependent variable, *A. suum* inactivation, was coded 1 (not inactivated) when viability was greater than 0% and 0 (inactivated) when viability was equal to 0%.

Table 5.2

Criteria to Identify Stage of Development and Viability of A.suum Ova by Microscopy

Non viable ova*	Viable ova
<p>Ovum with:</p> <ul style="list-style-type: none"> ➤ Contraction, rupture and loss of the ovum membrane continuity ➤ Cellular condensation ➤ Granulated or vacuolated cytoplasm ➤ Poorly defined structures ➤ No larvae movement observed by microscope light stimulation 	<p>Intact ovum in any of the following stages:</p> <ul style="list-style-type: none"> ➤ <i>One to Four Cell</i> : Observation of 1, 2, 3, or 4 clearly well defined cells within the ovum ➤ <i>Early morula</i>: Observation of 5 to 10 cells within the ovum ➤ <i>Late morula</i>: Observation of 11 or more cells within the ovum ➤ <i>Blastula</i>: Observation of a spherical layer of cells surrounding a pseudo fluid-filled cavity ➤ <i>Gastrula</i>: Observation of a layer of cells surrounding the embryo, plus a kidney shape invagination in one side of the embryo ➤ <i>Pre-larva 1</i>: Observation of a larva like structure, coiled, creating no more than one concentric ring inside the ovum ➤ <i>Pre-larva 2</i>: Observation of a larva like structure, coiled, creating at least one and a half concentric ring inside the ovum ➤ <i>Larva 1</i>: Observation of a larva structure within the ovum, without well defined structures inside the larvae wall, absence of molt and intense motility in response to light ➤ <i>Larva 2</i>: Observation of more defined structures within the larva, a molt, and a delayed motility response to light stimulation

*Adapted from Caceres et al, 1987

Predictor variables included temperature (28°C, 35°C, 40°C, and 45°C), pH, moisture, days of storage (storage), and treatment (0% urea, 1% urea, 2% urea). Variables were selected using the stepwise procedure and the adequacy

of the model was tested using the Hosmer and Lemeshow and Deviance Goodness of Fit tests.

5.4 Results

5.4.1 Monitoring physical and chemical parameters. The physical and chemical parameters measured during the experiment are described in Table 5.3. Before adding urea, the biosolid had a pH value of 8.35, moisture of 27.5% (wet based), and an ammonia soil content of 20.5 ppm. During the experiments variations in the moisture content (range 23.2%-31%) of the material did not differ significantly from initial measurement (Mean=27.2662, 99% CL 26.9380 – 27.5944, $p > 0.05$).

Table 5.3

Descriptive Statistics of Physical and Chemical Parameters

Parameter	Mean	95% CL	Minimum-Maximum
Biosolid + 0% urea			
Ammonia*	139.72	[124.08, 155.35]	55.65 - 280.85
pH	8.30	[8.26, 8.34]	7.98 - 8.51
Moisture	26.41	[25.97, 26.84]	23.20 - 29.70
Biosolid + 1% urea			
Ammonia	1287.30	[1216.98, 1357.75]	825.30 –
pH	8.62	[8.59, 8.65]	1512.50
Moisture	27.79	[27.39, 28.18]	8.47 – 8.83
			25.85 - 31.05
Biosolid + 2% urea			
Ammonia	2286.44	[2087.64, 2485.2]	1539.0 -
pH	8.85	[8.84, 8.87]	3162.50
Moisture	27.59	[27.24, 27.95]	8.74 -8.94
			25.15 -30.90

*Ammonia=ammonia gas + ammonia water

The ammonia content of samples with 1% and 2% urea, increased during the first two weeks of the experiments, reached a high point, and decreased continuously until the end of the experiments (Figure 5.1). pH value increased

in the first 24 hours of experiment and experienced very little variation during the following 56 days of storage (Figure 5.2).

The increasing values of both parameters were expected but the reduction of ammonia was not. It is believed that ammonia gas escaped from the containers after the second week of the experiments since changes in the lid of containers and an ammonia smell was noticed. Reductions in the ammonia measurements in the soil were observed after the fourth week of the experiments.

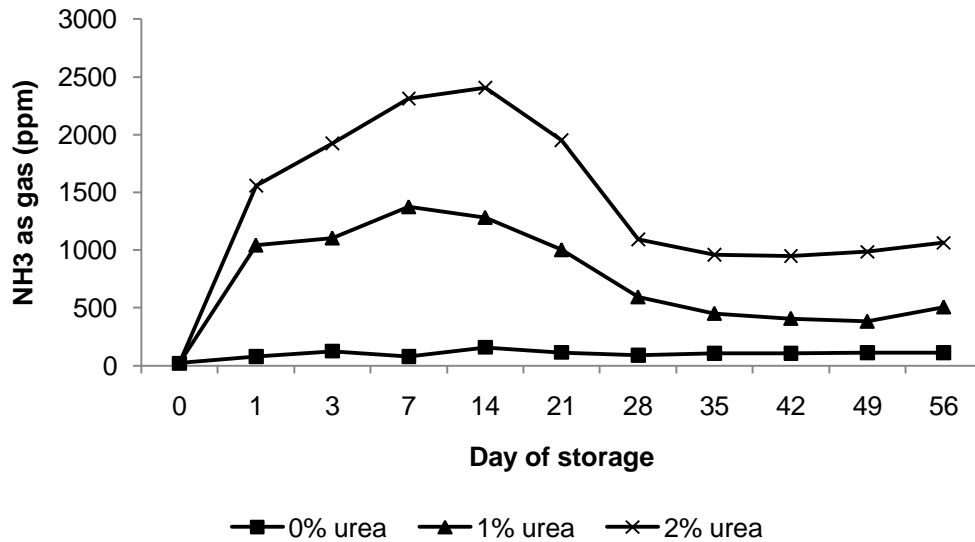


Figure 5.1 Ammonia Gas Variations in Biosolid during Storage

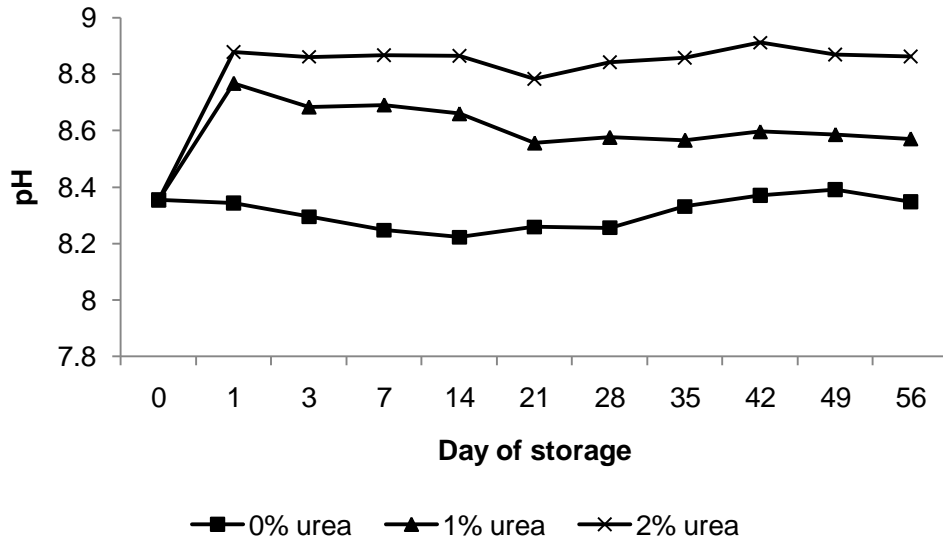


Figure 5.2 pH Variations in Biosolid during Storage

The increase on the pH values in the samples with 1% and 2% urea was very similar within each treatment group, independently of incubator temperature. In the first 24 hours the pH of the samples increased to 8.76 (samples exposed to 1% urea) and 8.88 (samples exposed to 2% urea). After 56 days of treatment, their pH value decreased by approximately 0.3 and 0.1 units respectively. The pH of the control samples (0% urea) was reduced from an initial value of 8.37 to a value of 8.2 by the end of the experiments.

5.4.2 A. suum inactivation. The inactivation of *A. suum* ova was faster in samples exposed to urea and to temperatures higher than 28°C. Samples without urea never reached a 100% inactivation at 28°C within the 56 days of storage, while the samples exposed to 35°C, 40°C, and 45°C achieved total inactivation after 21 days, 7 days and 24 hours of storage respectively.

All samples exposed to urea achieved 100% inactivation after 14 days (28°C), 3 days (35°C) and 24 hours (40°C and 45°C). Ammonia leakage could

not have affected the speed of inactivation since it started after the second week of storage and by this time those samples exposed to urea had already completed 100% ova inactivation.

The inactivation achieved at the end of the experiment in the samples exposed to 28°C and 0% urea was high (6 viable larvae out of 855 ova). Within this group, a higher inactivation rate was noticed after the third week of storage. This increase seems to be related with the period at which ammonia leakage from the containers with urea was observed. Control (0% urea) and treated (1%-2% urea) samples were placed in the same incubator during the experiments and, there probably was a leak of ammonia gas into the control samples influencing the inactivation proportion observed in these samples.

Survival analysis of the data showed that there was a significant difference (p value $<.0001$) between the inactivation achieved in the samples exposed to urea (1% and 2%) and the samples without urea (Figure 5.3). However, adjusted paired comparisons showed no significant difference (p value = 1) in the inactivation of samples exposed to 1% urea from samples exposed to 2% urea. This result suggests that inactivation of *A. suum* ova may not be increased by adding urea concentrations greater than 1% under laboratory conditions. Within temperature groups, a significant difference ($p < 0.001$) in the inactivation achieved between control samples (0% urea) and treated samples (1-2% urea) was observed, except at 45°C. At the highest temperature (45°C), all samples achieved a 100% inactivation in less than 24 hours independently of urea addition.

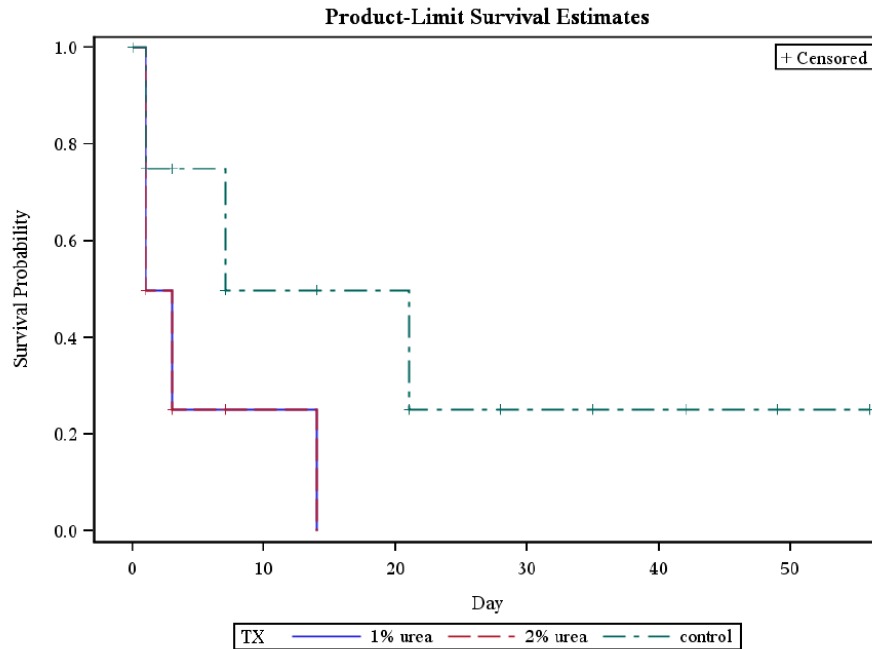


Figure 5.3 The survival curves show a significant difference in survival between the control group (0% urea) and the group with urea (1% and 2% urea), but it does not evidence difference in the survival probability between 1% and 2% group. This survival curves include the effect of temperature (28°C to 45°C).

5.4.3 Model of *A. suum* ova inactivation. The logistic regression model included temperature, storage, treatment, pH and moisture. Using a stepwise procedure, moisture ($p = 0.1947$) and pH ($p = 0.4147$) were removed from the full model. The predictor pH was found highly correlated with Treatment (p value <0.0001) as suggested by the theory (Emerson et al., 1975). The final model therefore included temperature (25°C, 35°C, 40°C, 45°C), Storage (1, 3, 7, 14, 21, 28, 35, 42, 49, 56 days), and Treatment (0%, 1%, 2% urea). The results are summarized in Table 5.4.

Table 5.4

Logistic Regression Analysis of A. suum ova Inactivation in Biosolid

Physical and chemical factors	Parameters			
	Estimate	OR*	95% CL	p-value
Temperature	0.6096	1.840	[1.352, 2.504]	0.0001
Treatment	3.2565	25.959	[4.043, 166.694]	0.0006
Storage	0.1637	1.178	[1.078, 1.287]	0.0003

*OR: Odds Ratio

The Goodness-of-Fit tests showed that the model fitted the data (Deviance = 1.0000; Hosmer and Lemeshow = 0.9868). The fitted logistic response function is in Figure 5.4, 5.5, and 5.6. According to the model, increasing storage day increases the probability of 100% inactivation by 17.8% holding temperature and treatment constant; increasing temperature increases the probability of 100% inactivation by 84%, holding treatment and storage constant; and addition of urea treatment to the biosolid increases the odds of inactivating 100% of the parasite ova more than 25 times, holding temperature and storage constant. These odds estimation show the inactivation potential of ammonia when used at chemical and physical parameters found in the solar toilet.

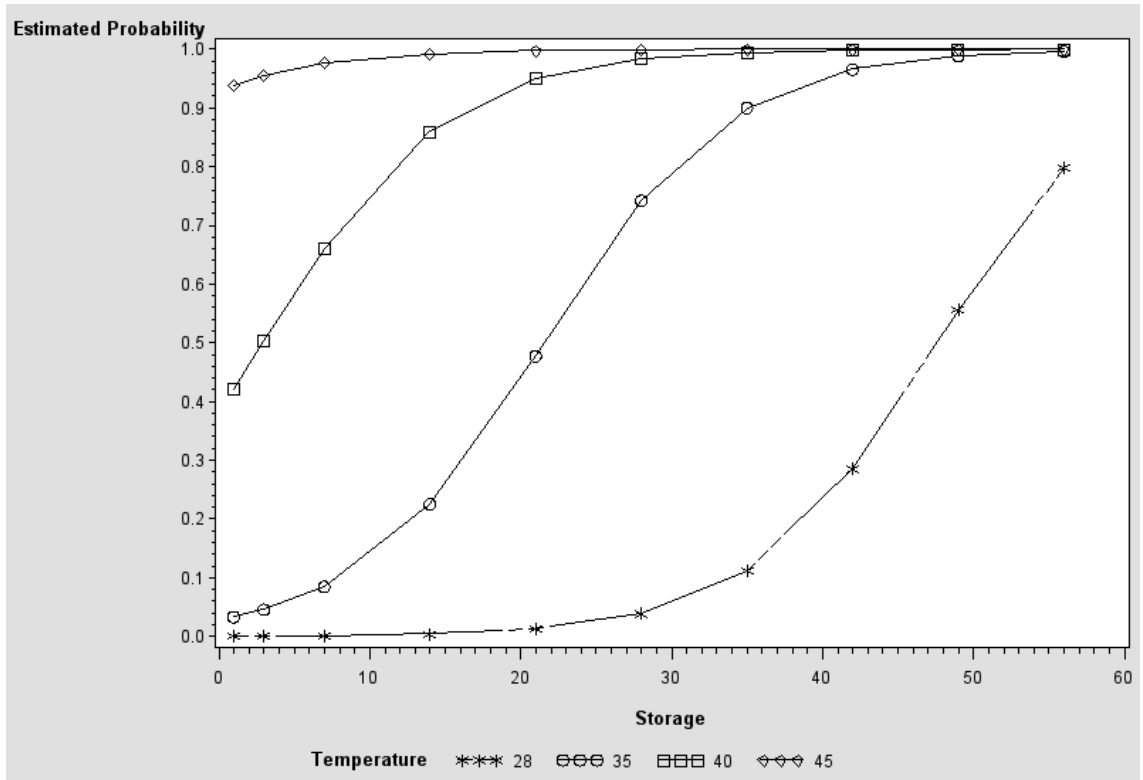


Figure 5.4 Estimated probabilities of *A. suum* ova inactivation in biosolid (feces + additive) with 0% urea in laboratory conditions

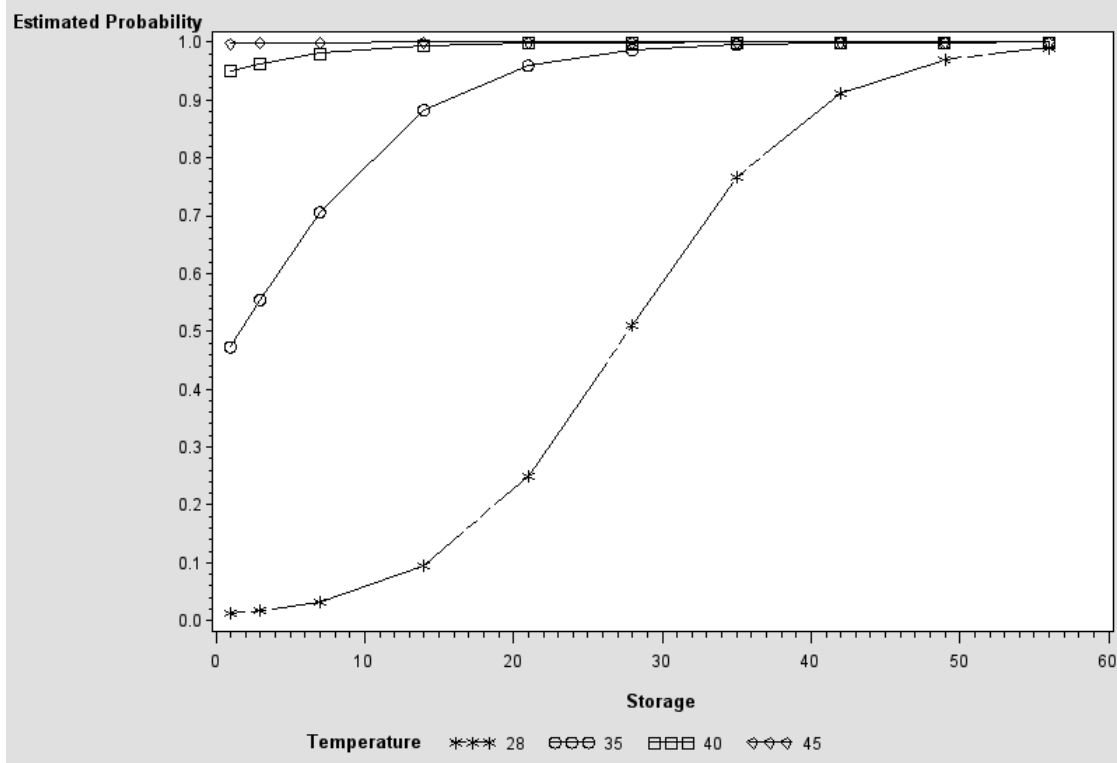


Figure 5.5 Estimated probabilities of *A. suum* ova inactivation in biosolid (feces + additive) treated with 1% urea in laboratory conditions

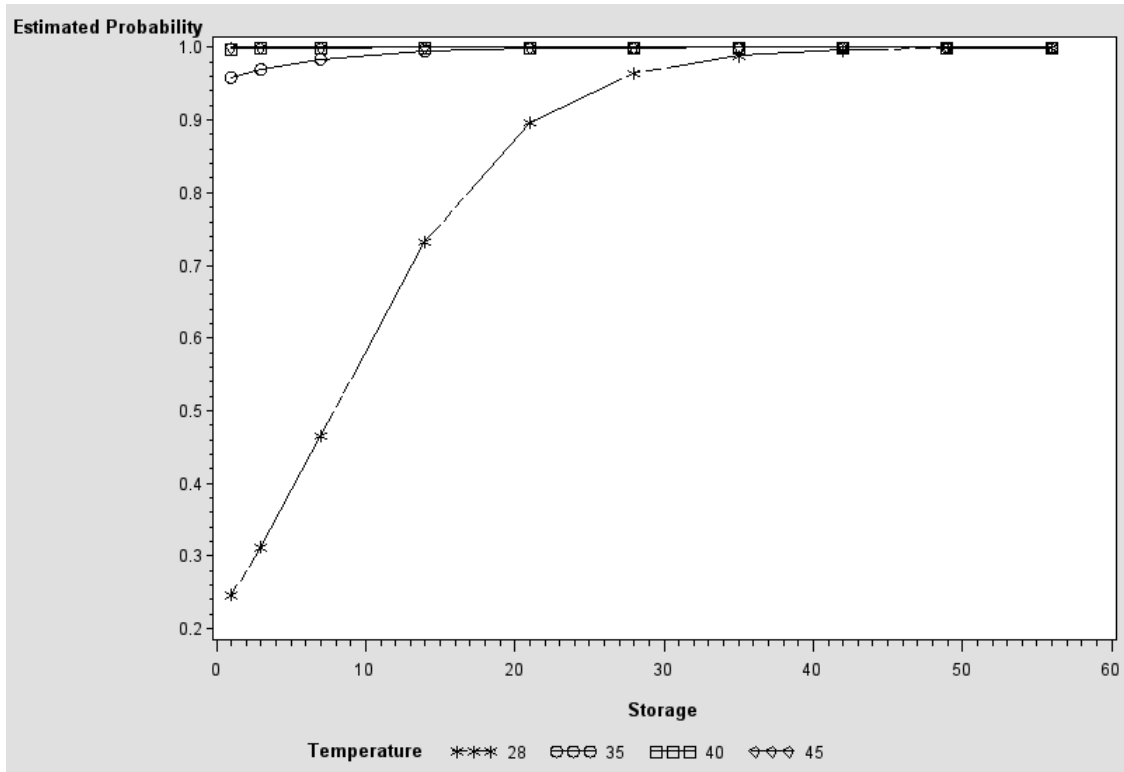


Figure 5.6 Estimated probabilities of *A. suum* ova inactivation in biosolid (feces + additive) treated with 2% urea in laboratory conditions.

5.6 Discussion

The results of this study suggest that inactivation of *Ascaris* spp ova by ammonia is possible in fecal material stored in the solar toilet or any other dry toilet, if the following physical and chemical conditions are met: a closed vault with a minimum temperature of 28°C; an initial pH of 8.3, minimum moisture of 27.5%, and addition of 1% urea to the biosolid. At 28°C longer storage time would be required for 100% inactivation while at higher temperatures less time of storage would be necessary. Similar temperature and time dependent inactivation was observed by Nordin et al., (2009) after adding 1% and 2% urea to fecal material at lower temperatures (24°C and 34°C). At 24°C they reported viable ova in both treatments after 35 days of storage, with a higher percentage of viable ova (78% ± 6.5%) in biosolid exposed to 1% urea compared to biosolid exposed to 2% urea (1 larva stage ova out of 806 inactivated ova). At 34°C they reported 100% *A. suum* ova inactivation after 10 days of storage with 1% urea and, after 4 days of storage with 2% urea (Nordin et al., 2009). In our study, 14 days, 3 days, and 1 day were required to achieve 100% *A. suum* ova inactivation in biosolid treated with 1% and 2% urea at 28°C, 35°C, and 40°C respectively. At 28°C we observed that some samples exposed to 2% urea had a higher number of inactivated ova when compared to 1% urea before total inactivation was registered at day 14 of storage. At 35°C the number of inactivated ova in treated samples (1% versus 2% urea) was very similar (44% versus 47.5% respectively) and, at 40°C and 45°C was the same.

Both results evidence the effect of increasing temperature at reducing the number of days required for *A. suum* ova inactivation when using 1% and 2% urea. They also show that as concentrations of urea increase (from 1% to 2%) in biosolid, the time required for inactivation decrease at temperatures equal or lower than $\leq 34^{\circ}\text{C}$. At 35°C or higher, according to our results, increasing concentrations of urea ($>1\%$) do not reduce significantly this time.

pH levels achieved in this experiment were very similar to those achieved by Nordin et al.,(2009) even though they did not add any additive to the samples mixed with urea and adjusted the moisture content to 83% (wet based). In a dry toilet, the use of additives after each defecation contributes to pathogen inactivation by increasing dryness and pH of the fecal material (Winblad & Simpson-Hebert, 2004). By adding the equivalent to a cup of additive to each stool sample collected, the moisture of the biosolid used in our study was reduced to 27.5% (wet based), which is very dry compared to the moisture level (83%-95%, wet based) reported previously (Nordin, 2007; Pecson et al., 2007; Vinnerås et al., 2003). In these very dry conditions, the increase of ammonia observed during the first two weeks of the experiments indicates that urea was chemically transformed to ammonia by the enzyme urease in our biosolid and, that ammonia was continuously available inside the containers. Nevertheless, the mean value of ammonia calculated (taking in account the 56 days of storage) was low when compared to the theoretical values expected at room temperature (26°C). Nordin et al., (2009) also registered lower ammonia measurements at the end of their experiments. In our study, the peak ammonia values achieved during

the second week of storage were very close to the expected theoretical values. Therefore, it is clear that ammonia losses after the second week of storage influenced the mean ammonia value reported, but did not influence *Ascaris suum* ova inactivation. The peak ammonia value (day 14 at 28°C and day 3 at 35°C) corresponded with 100% inactivation of the parasite ova in samples with urea placed to 28°C and 35°C; while samples placed at 40°C achieved total inactivation before the peak ammonia value was observed (day 7).

Pecson et al., (2005) reported that the minimum ammonia concentration to achieved 99% inactivation in 72 hours at 38°C in solution was 2992ppm of NH₃ (pH=11). A similar study revealed that inactivation was faster in sludge (Pecson et al., 2007). Nordin et al., (2009), reported 99% ova inactivation in 4 days (34°C) with approximately 3814ppm of ammonia in feces. Our results showed that the minimum ammonia concentration (as NH₃) required to inactivate 100% of *Ascaris suum* ova in feces were around 1350 ppm at 28°C and 35°C; and 794 ppm at 40°C. Cruz et al., (not published, 2009), did not find a significant inactivation of the parasite ova after three days of storage in ammonia solution (2%) using temperature and pH values similar to those achieved in the solar toilet (28°C, pH 9.3) (not published, Cruz et al., 2010). In this study there was a significant increase in the inactivation of the parasite at the same temperature and pH in feces amended with 2% urea after three days of storage. This data suggests that inactivation in our samples treated with 1% and 2% urea were faster and required less ammonia concentration than previously reported. It is suspected that the lower moisture level of the biosolid used in this study facilitated the

movement of ammonia gas inside the material, promoting a faster inactivation with less ammonia concentration. A faster bacterial inactivation by ammonia gas in chicken manure has been reported in previous literature (Himathongkham & Riemann, 1999).

Inactivation of the parasite ova in samples with 0% urea was achieved at 35°C and 40°C, at 21 and 7 days of storage respectively. Nordin et al., (2009) reported a 50% ova viability reduction in feces stored for 35 days and no viability reduction in ova placed in NaCl at the same temperature. While addition of ammonia clearly reduces the storage time needed to achieve total *Ascaris* spp. ova inactivation it is suggested that intrinsic ammonia and other compounds naturally present in feces and sludge may influence inactivation of the parasite ova in a closed system when ammonia is not added to the material (Nordin, 2007; Pecson et al., 2007).

The implications of these results are the potential use of ammonia in the solar toilet in the field as a sanitizing agent. *Ascaris suum* ova were used to evaluate this approach, but no other microbiological indicator was included in the experiments. Previous literature reports that the solar toilet has been effective at inactivating Fecal Coliforms (< 1000 Fecal Coliforms per gram total solid) in less than 55 days without ammonia (Izurieta, Karpur, Moe, Cruz, & Corrales, 2008). Studies in sludge and feces report that bacterial inactivation by ammonia has been achieved in less than 1 week at temperatures of 37°C or lower (Allievi et al., 1994; Mendez et al., 2004; Mendez et al., 2002; J. Ottoson et al., 2008; J. R. Ottoson et al., 2008; Park & Diez-Gonzalez, 2003; Vinnerås et al., 2003). Since

the solar toilet reaches temperatures as high as 41°C it is expected that bacterial inactivation will be achieved in less time than usual by adding ammonia without risk of bacterial regrowth.

In regards to the virucidal effect of the solar toilet, the published literature indicates that it is not able to inactivate somatic coliphage (Moe & Izurieta, 2003). Studies on sludge and solution have shown the capacity of ammonia to inactivate enteroviruses and some phages at different temperatures (20°C, 35°C, 37°C and 47°C) in less than 9 days (Cramer et al., 1983; Fenters et al., 1979; J. R. Ottoson et al., 2008; Schaper et al., 2002; Ward & Ashley, 1977a, 1977b). The parameters at which ammonia has been reported to inactivate viruses are similar to the solar toilet parameters, suggesting a good probability that viruses may also be inactivated inside the toilet with the addition of ammonia.

Some solar toilets units have shown not to be effective at inactivating all parasites (Moe & Izurieta, 2003). Our results suggest that the toilet, by itself, would be able to inactivate the parasite ova if temperatures of 35°C or higher were achieved inside the vault for at least 21 days continuously. If urea is added to the vault and proper closure is secured, then, this time would be reduced to three days. Ammonia has also been effective at inactivating other parasites ova (*Trichuris* spp., *Hymenolepis* spp., *Toxocara* spp., *Diphyllobothrium latum* and *Trichocephalus muris*) and protozoa at a faster rate than *Ascaris* spp. ova, suggesting that by inactivating *Ascaris* spp. ova, inactivation of other parasites ova may be achieved (Chefranov Lu et al., 1978; Ghiglietti et al., 1995; Jenkins, Bowman, & Ghiorse, 1998; Mendez et al., 2002).

The logistic regression model of inactivation presented here works well within the physical and chemical parameters used in this study. It is consistent with a model previously reported by the authors using a smaller sample size (n=20) (not published, Cruz et al., 2010). The model presented here further confirms a significant correlation between pH and treatment (urea), the effect of increasing temperature on inactivation; the importance of time to achieve inactivation at low temperatures and the powerful influence of adding urea to the feces to reduce the time required to achieve total *Ascaris suum* ova inactivation. Other authors have used linear regression models or shouldered inactivation based on exponential decay to estimate the time required to achieve 99% inactivation (Nordin, 2007; Pecson et al., 2007). In our study we wanted to determine if the final parasite viability was 0% (total inactivation) or not and logistic regression better estimated this dichotomous result. Probability estimations presented in Figure 4-6 created by the model were highly significant and showed well the effect of urea on inactivation when added to this type of biosolid.

Therefore, although these results support the potential use of urea as a source of ammonia to sanitize the fecal material in the solar toilet or any dry toilet that meet the requirement mentioned at the beginning, a community intervention is recommended to include field conditions and human behavior as other predictors for *Ascaris* spp. inactivation.

5.7 Limitations of the study

There are some limitations based on the methodology of the study. Leakage of ammonia after day 14 of storage is one of these limitations. The containers used in the experiments ((I-CHEM Short wide-mouth clear Type III glass jar with Teflon®-lined, Polypropylene closure) are used to collect soil samples with semi-volatile materials; nevertheless, addition of urea could have caused an increase in internal pressure subsequently cracking the containers' lid and producing an ammonia leakage to the environment.

The use of nylon bags is another limitation of the study that may influence the generalizability of the results. Nylon bags concentrate the parasite ova in one location and facilitate recuperating the ova from biosolid for analysis. In the field, the parasite ovum is found mixed with biosolid in different locations and this environment may or may not protect the ovum from contact with ammonia. In our results, we observed that ova inactivation was faster in biosolid with low moisture content when compared to material with high moisture content. This may be related to the ability of ammonia gas to penetrate biosolid easier in dry material than in wet material. Nevertheless, until similar results are observed in the field, we considered the use of nylon bags as a limitation that should be taken into account in the translation of the results of this study to practice.

5.8 Public health implications and challenges

The result of this study supports the application of urea as a sanitizing agent in dry toilets. However, many challenges will be faced during the translation of the results into a community setting. From a public health

perspective, it is important to have the community approval and understanding of the use of urea in the toilet before any application starts. Also, urea should be available to the community's members participating in the project. An educational intervention should be introduced into the community to facilitate the transition from current to new procedures for using the toilet with urea. Including local authorities, community leaders and community health workers in the educational process would be key for the acceptance of the community.

Human behavior should also be taken into account when planning the implementation of the technology. A baseline practices, knowledge and attitudes survey would collect the information required to address common mistakes in the educational intervention. In addition, posters with instructions on the proper use and management of the technology could be distributed and placed in the toilet for the user to refer when needed. A local leader or community health worker, willing to help in the process, could also provide support to the users when needed.

Expected challenges for the acceptance will be the strong smell that could result from the use of urea. This fact should be addressed in the educational intervention. In addition proper protection equipment must be provided to the person in charge of handling and applying urea within the toilet. Procedures to minimize exposure must be taught and given in writing to this person. Proper mixing procedures when applying urea should be emphasized to the user since this step is important for the success of the intervention.

5.9 Alternative laboratory methods

In this study microscopy was used as the main method to determine parasite viability. This method is considered the standard method currently recommended by the United States Environmental Protection Agency (USEPA, 2003). The advantage of this method is the ability to use it in less developed countries. The disadvantage is the training required to adequately identify the ova in the microscope.

PCR is another laboratory assay that could be used as an alternative to microscopy. This is a rapid diagnostic test to detect the presence of *Ascaris* spp. ova in biosolid. It could be used to separate the toilets with parasite ova from those toilets without parasite ova, facilitating the identification of toilets that would require a microbiological evaluation. The disadvantage of this method is the cost of the equipment and the time of incubation of the ova needed to obtain DNA material for the assay. In addition, this method is not considered as a standard method by USEPA yet, limiting its use.

Development of faster and low cost laboratory techniques to determine ova viability of the ova in the environment is needed.

5.10 General Conclusions and Recommendations

- The results of this study suggest that inactivation of *Ascaris* spp ova by ammonia is possible in fecal material stored in the solar toilet or any other dry toilet, if the following physical and chemical conditions are met: a closed vault with a minimum temperature of 28°C; an initial pH of 8.3, minimum moisture of 27.5%, and addition of 1% urea to the biosolid.

- The solar toilet, by itself, would be able to inactivate the parasite ova if temperatures of 35°C or higher were achieved inside the vault for at least 21 days continuously. If urea is added to the vault and proper closure is secured, then, this time would be reduced to three days.
- There will be many challenges during the translation of the results into practice, including but not limited to:
 - Continuous access of urea for the users.
 - Community approval and understanding of the procedures.
 - Support available in the community
 - Proper use of protecting equipment when handling biosolid with urea
 - Tolerance of smell of urea
- To address some of these challenges it is recommended:
 - Creation of partnerships with local authorities and community leaders to secure availability of urea
 - Educational interventions
 - Adequate training of supporting individuals living in the community
 - Delivery of written instructions on the procedures to mix and handle biosolids
 - Adequate use of protecting equipment
 - Continuous evaluation of the performance of the technology with the input of users

- Development of sensible indirect measurements techniques, such as ammonia probe to use in the field, easy enough for the local leaders to implement on a regular basis.

CHAPTER 6.

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APPENDICES

Appendix A: Additional Tables

Table A1

Chi-Square and [95% Confidence Intervals] of Proportions Estimated from A. suum Ova during Incubation in vitro

Day of incubation	Ova		Viability Proportion	Chi-Square Test for Specified Proportion (0.875)*	95% CL	
	Viable	No Viable			LL	UL
5	35	5	0.875	0.00	[0.73]	[0.95]
6	35	5	0.875	0.00	[0.73]	[0.95]
7	35	5	0.875	0.00	[0.73]	[0.95]
8	39	1	0.975	3.65	[0.86]	[0.99]
9	33	7	0.825	0.91	[0.67]	[0.92]
10	35	5	0.875	0.00	[0.73]	[0.95]
11	32	8	0.800	2.05	[0.64]	[0.90]
12	35	5	0.875	0.00	[0.73]	[0.95]
13	34	6	0.850	0.22	[0.70]	[0.94]
14	36	4	0.900	0.22	[0.76]	[0.97]
15	38	2	0.950	2.05	[0.83]	[0.99]
16	39	1	0.975	3.65	[0.86]	[0.99]
17	38	2	0.950	2.05	[0.83]	[0.99]
18	39	1	0.975	3.65	[0.86]	[0.99]
19	35	5	0.875	0.00	[0.73]	[0.95]
20	35	5	0.875	0.00	[0.73]	[0.95]
21	35	5	0.875	0.00	[0.73]	[0.95]

*p >.05

Table A2

pH Values of Samples after 72 Hours of Storage in Solution

Sample Group	pH							
	28 °C		35°C		40°C		45°C	
	M*	(SD)*	M*	(SD)*	M*	(SD)*	M*	(SD)*
pH 7.7								
0% NH3	5.3	(.32)	6.0	(.36)	5.2	(0.0)	4.9	(.20)
2% NH3	7.9	(.01)	7.9	(0.0)	7.8	(0.0)	7.8	(.05)
pH 9.3								
0% NH3	6.2	(.07)	5.6	(.10)	5.3	(.05)	4.8	(.10)
1% NH3	9.4	(.05)	9.4	(0.0)	9.3	(.05)	9.3	(.05)
2% NH3	9.4	(.05)	9.4	(.05)	9.3	(.05)	9.5	(0.0)

Table A3

Logistic Regression Model from Data Collected during Inactivation of A. suum Ova in Solution in Three Days

Analysis of Maximum Likelihood Estimates				
Parameter	DF	Estimate	OR	Pr > ChiSq
Intercept	1	-15.3564		0.0234
Temperature	1	0.363	1.438	0.0253
Treatment	1	1.0049	2.732	0.2042

Table A4

Logistic Regression Model from Data Collected from A. suum Ova Inactivation in Biosolid during 56 Days of Storage

Parameter	DF	Estimate	OR	Pr > ChiSq
Intercept	1	-24.8643		0.0001
Temperature	1	0.6096	1.840	0.0001
Treatment	1	3.2565	25.959	0.0006
Storage	1	0.1637	1.178	0.0003

**Appendix B: Additional Figures of the Physical-Chemical Parameters and
A. *suum* Ova Inactivation in Biosolid during 56 Days of Storage**

Figure B1 Moisture Variations of Samples Stored at 28°C

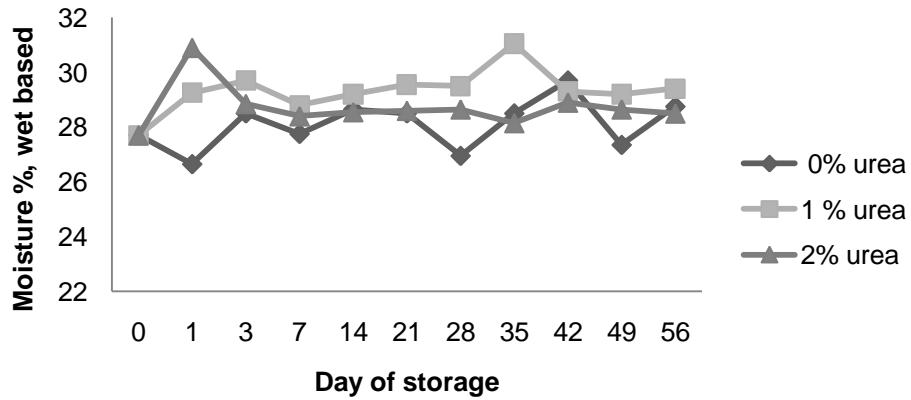


Figure B2 Ammonia Gas (ppm) Variations of Samples Stored at 28°C

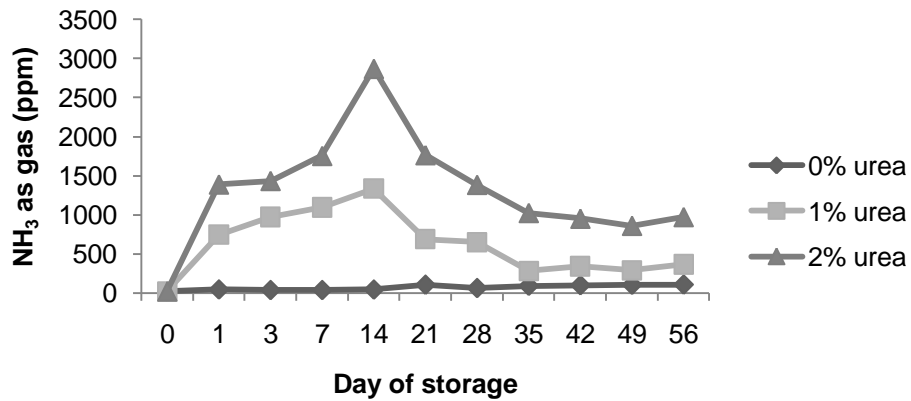


Figure B3 pH Variations of Samples Stored at 28°C

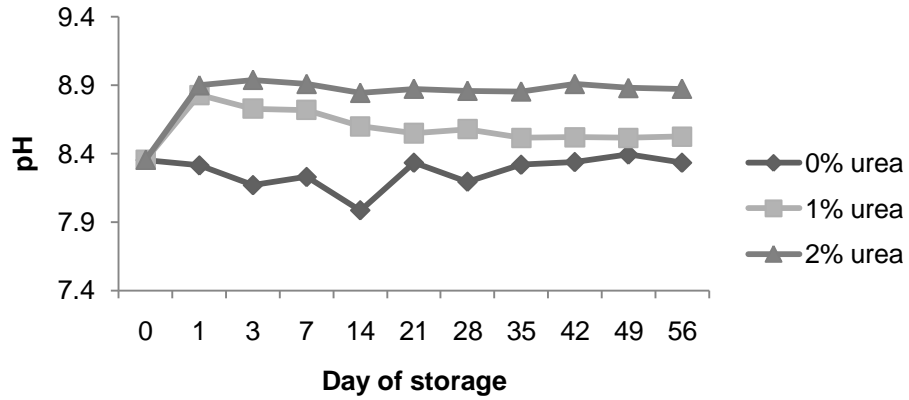


Figure B4 *Ascaris suum* Ova Inactivation (%) in Samples Stored at 28°C

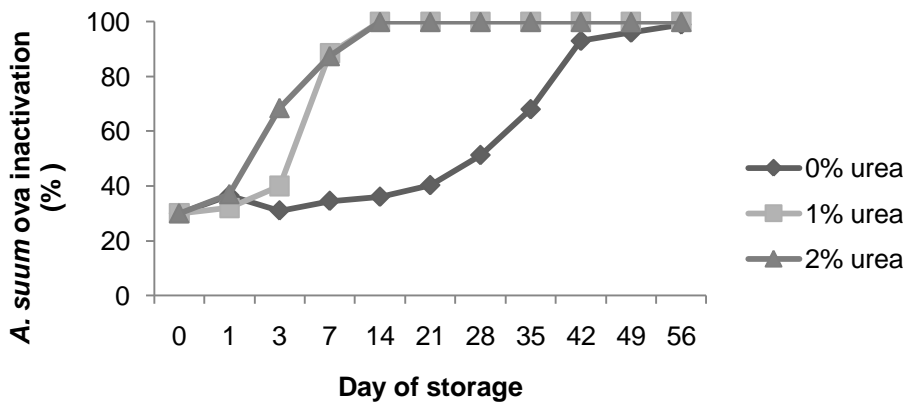


Figure B5 Moisture Variations of Samples Stored at 35°C

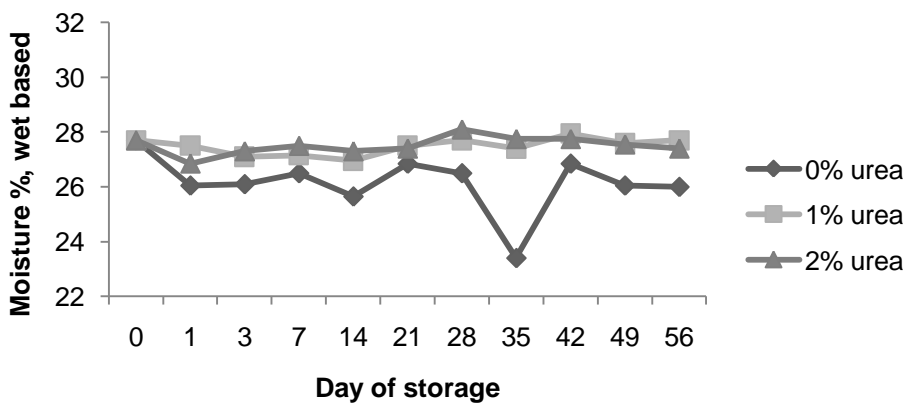


Figure B6 Ammonia Gas (ppm) Variations of Samples Stored at 35°C

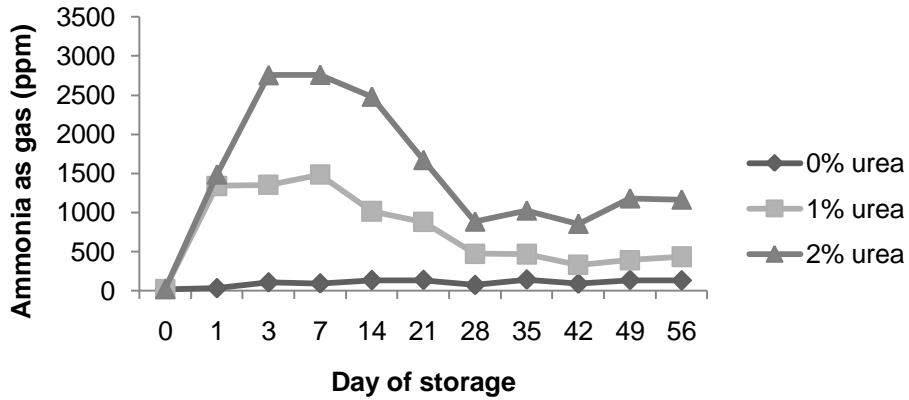


Figure B7 pH Variations of Samples Stored at 35°C

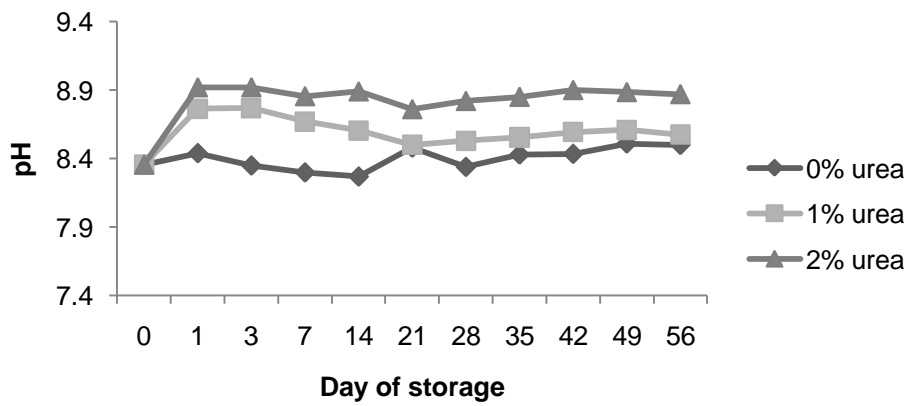


Figure B8 *Ascaris suum* Ova Inactivation (%) in Samples Stored at 35°C

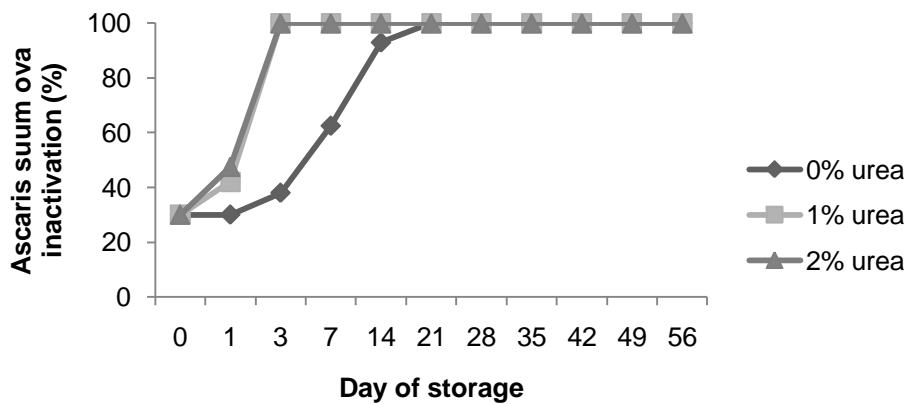


Figure B9 Moisture Variations of Samples Stored at 40°C

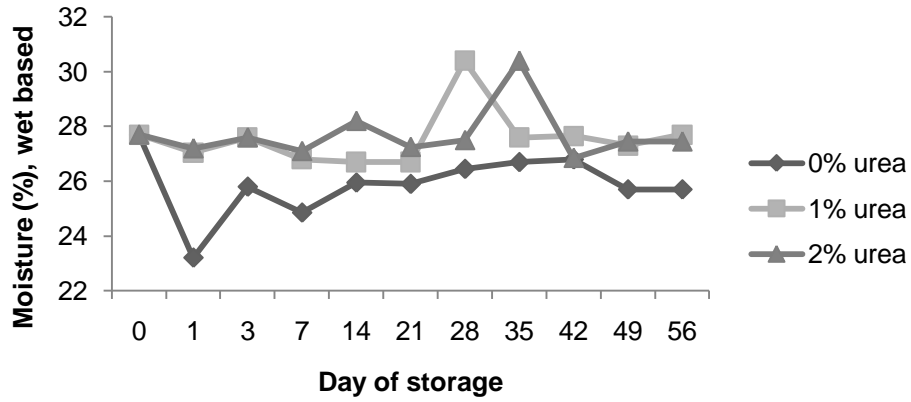


Figure B10 Ammonia Gas (ppm) Variations of Samples Stored at 40°C

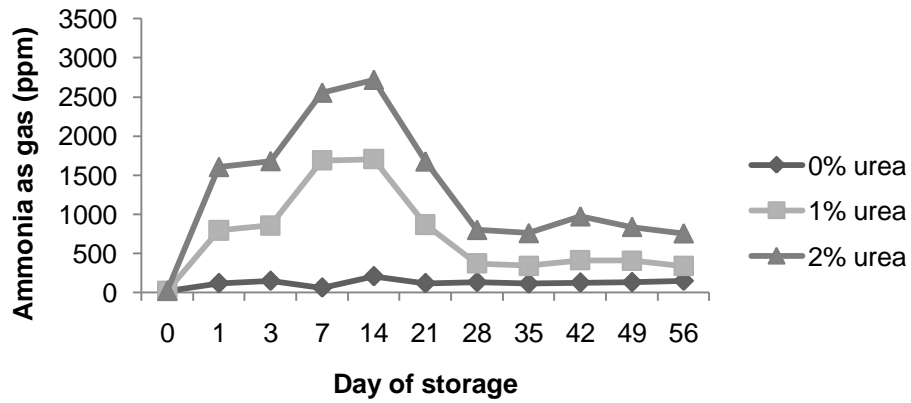


Figure B11 pH Variations of Samples Stored at 40°C

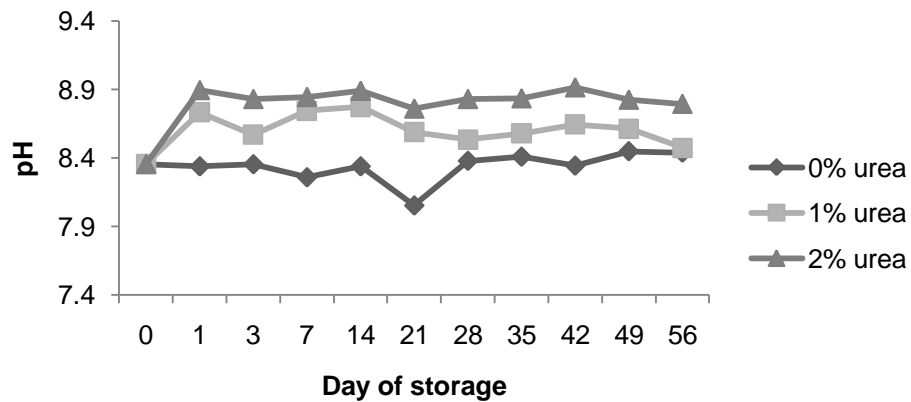


Figure B12 *Ascaris suum* Ova Inactivation (%) in Samples Stored at 40°C

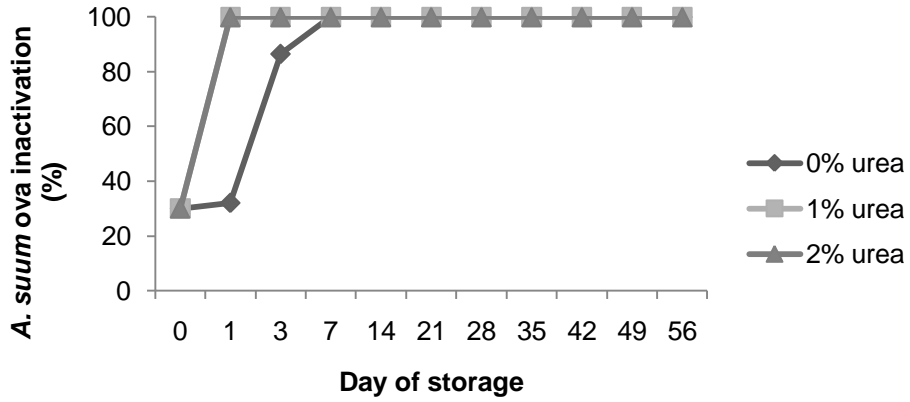


Figure B13 Moisture Variations of Samples Stored at 45°C

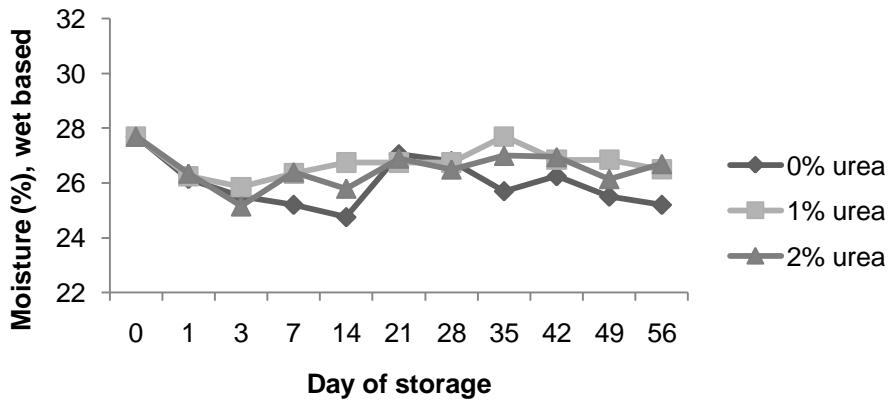


Figure B14 Ammonia Gas (ppm) Variations of Samples Stored at 45°C

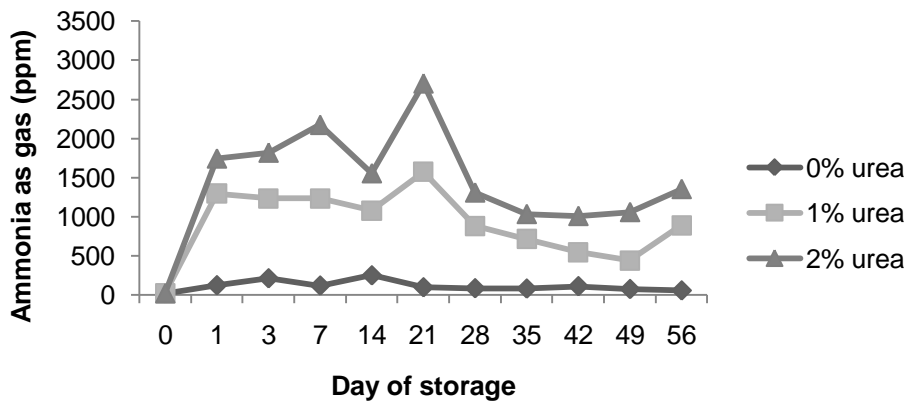


Figure B15 pH Variations of Samples Stored at 45°C

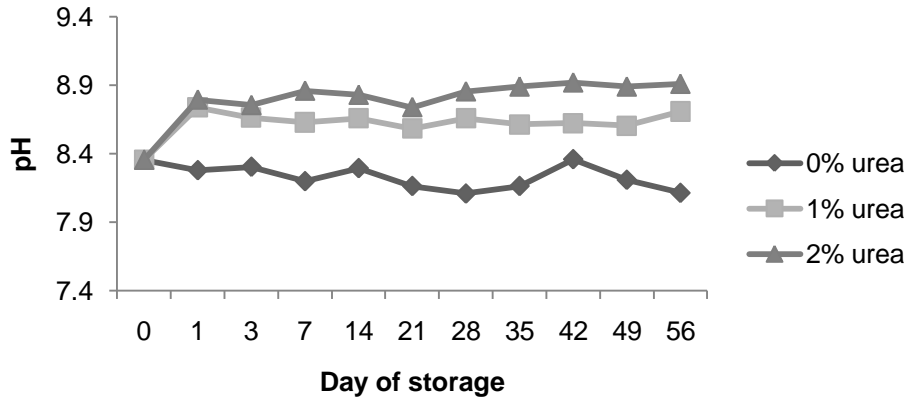
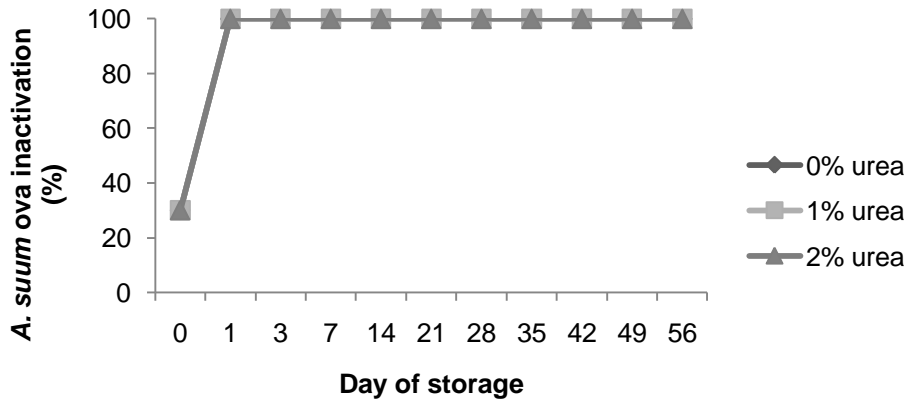


Figure B16 *Ascaris suum* ova Inactivation (%) in Samples Stored at 45°C



ABOUT THE AUTHOR

Ligia María Cruz Espinoza is originally from León, Nicaragua. She graduated from Pureza de Maria High School in 1995 as the Valedictorian of her class and completed Medical studies at the “Universidad Nacional Autónoma de Nicaragua”, located in León city, Nicaragua, in 2004. During her medical studies she received a Nuffic Scholarship to study Public Health in an International Perspective at the University of Nijmegen, in the Netherlands. After this experience, she worked and volunteer in rural areas of León city in a program for adolescents in danger, providing education and medical assistance. She also participated in projects evaluating the effectiveness of vaccines at reducing diarrheal diseases in children.

In 2006, she received the Latin American and Caribbean Scholarship to complete a Master of Public Health with a concentration in Global Health at the University of South Florida, Tampa, USA. During this period, she volunteered in the Tobacco Research and Intervention Program at the H. Lee Moffitt Cancer Center and Research Institute, developing and adapting health education materials to prevent smoking relapse in pregnant and postpartum Hispanic women. In 2007, she was awarded with the Graduate Multidisciplinary Scholarship to continue with a PhD program in Global Communicable Disease at the same university. Her research focuses in improving sustainable sanitation technologies implemented in El Salvador to reduce environmental health risks in local communities and the degradation of the environment. In 2008, she

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In 2009, she worked in Suriname as a consultant for the Pan American Health Organization in the area of neglected diseases providing technical assistance to the Bureau of Public Health of Suriname in the planning and implementation of a national schistosomiasis and soil transmitted helminthes school survey. Next year she will continue with a Postdoctoral program to implement the results of her study in rural communities of El Salvador using solar dry toilets.