IMPACTS OF ACID CONCENTRATION, CONTACT TIME, TEMPERATURE AND SURFACTANT ON THE ACTIVITIES OF DIFFERENT SHORT-CHAIN & MEDIUM-CHAIN FATTY ACIDS ON *ASCARIS SUUM* EGGS IN SOIL AND WATER

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by

Nuzhat Islam

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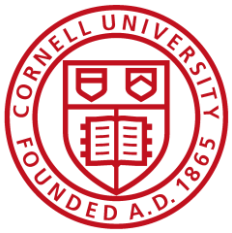

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By Nuzhat Islam

This thesis/dissertation document has been electronically approved by the following individuals:

Tammo S. Steenhuis (Chairperson)

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ABSTRACT

Ascaris suum eggs, due to their inherent environmental indestructability, are routinely used as bio-indicators to test the ovicidal activity of various manure and biosolids disinfection methods. Exposure to organic acid is a possible disinfection method of inactivating *Ascaris* eggs. Previous research has shown the eggs could readily be killed when the pH of the acid solution was below the pK_a of the acid, where most of the acid is in the undissociated form. Expanding on this earlier work, various concentrations and contact times of butanoic, pentanoic, hexanoic and heptanoic acid, with and without 18 mM Tween 20 at pH 4, were examined to determine minimum required times for total inactivation at 22°C and 37°C. Increasing the temperature by 15 degrees caused a significant decrease in the contact time required for total inactivation. The results suggest that higher concentration of pentanoic and hexanoic acids with or without the addition of the surfactant Tween 20 have potential for the rapid inactivation of helminth eggs in soil and water. Treating with acids with added surfactant, Tween 20, did not have any significant effect on egg inactivation in case of both lower and higher concentration and in both temperature.

BIOGRAPHICAL SKETCH

Nuzhat Islam was born in Brahmanbaria, Bangladesh on November 1st 1982 and grew up in Mymensingh, Bangladesh. She attended in high school at K.B. High School in Mymensingh, graduating in 1999, where she became interested in Biology, as she was encouraged by her father, Dr. Md. Nazrul Islam, former professor in Bangladesh Agricultural University, Mymensingh. Before her high school graduation, in 1999, unfortunate demise of her father happened, who was a great influence in all aspects of her life, and this influenced her to study in Bangladesh Agricultural University.

In 2000, although she gained admission in some prestigious universities in Bangladesh as an undergraduate student; but among them, she selected Bangladesh Agricultural University. She graduated in 2004 with her DVM (Doctor of Veterinary Medicine) degree. During her studies, Nuzhat enjoyed spending time in the research laboratory and developed an interest in Parasitology. She was also an active member of student council (BAU), Bangladesh Rover Scout, cultural organization, blood donating organization and within the debate club of her University. Just before graduation, she married Mozammal Hossain in November 2004 and came to the United States with her husband. In 2006, Nuzhat had started a part time job as a research assistant at Cornell University in department of Microbiology & Immunology in Dr. Dwight D. Bowman's laboratory. By this time, she was blessed by Allah to be a mother of a wonderful son, Tirmizi. So there was a gap in her academic carrier.

In 2011, Nuzhat again got a job as a research technician in Dr. Theodore G. Clark laboratory in Department of Microbiology and Immunology at Cornell University. Her interest in Parasitology led her to transfer to the department of Biological and Environmental Engineering at Cornell University to continue her MS studies in 2012, where she has spent another 1 happy year in

laboratory of Dr. Dwight D. Bowman. Upon completion of her MS, Nuzhat has an interest to be a Ph.D. candidate to achieve her and her parent's dream.

Dedicated to

My Mother Nurun Nahar Begum

&

My Father Dr. Md. Nazrul Islam

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First and foremost I thank ALLAH for arranging all the circumstances of my life in such a way that I could meet the wonderful professors, advisors, coworkers, and friends that have made unique and important contributions to the achievement of this goal.

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Most importantly, I am dedicating my thesis to my father, (late) Dr. Md. Nazrul Islam and my mother, Nurun Nahar Begum 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. I extend my thanks to my younger brothers, Md. Minhaz-Ul Islam and Md. Mohshee-Ul Islam from the bottom of my heart. I am deeply indebted to my spouse, M. Mozammal Hossain, for his patience and desultory dedication, his optimism and for making me laugh when I was hopeless throughout my ceaseless academic life, and also to my son, Tirmizi M. Hossain, for the motivation to surpass my limitations. I extend my appreciation to my Aunt, Nazmoon Nahar and my uncle, Delowar Hossain; my friends Araceli Lucio-Forster, Alice Lee and Sheila Saia.

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CHAPTER 1
INTRODUCTION

1.1. Problem Statement

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 use of wastewater sludge in agriculture is definitely an affordable solution for their disposal. Moreover, given the high concentrations of organic matter and some nitrogen and phosphorous in the sludge this method has the advantage of being a rational, cost-cutting method in fertilization and offers a reliable alternative to more costly disposal methods, such as incineration or landfill. More than half of the approximately 6 million tons (dry weight) of biosolids generated annually from municipal wastewater treatment in the United States is applied to agricultural lands. Sewage sludge and wastewater, disposed as fertilizer, on agricultural land and used for irrigation may contain human and animal pathogens (e.g. viruses, bacteria, protozoan cysts and helminth eggs). The spread of enteric diseases is increased through the insanitary disposal of wastewater and sludge and this is particularly true in most developing countries where the level of education, concern for health, and ability to afford hygienic facilities are at a minimum; so poor socio-economic conditions are linked with higher prevalence of ascariasis. The growing world-wide concern about health risks related to the disposal and reuse of wastewater and its

solid residue has stimulated the search for better disinfection processes. Many diseases caused by fecal pathogens are among the main causes of childhood 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 supplies, 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 such associated deaths (99.8%) occur in developing countries, and 90% of them are in 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. The risk of disease transmission depends upon the number and viability of the pathogens in the wastewater and on the opportunity for them to infect an appropriate host. 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). Following the ingestion of *A. suum* egg-contaminated food and water by humans or other mammals, larvae reach the liver and lung alveoli via the bloodstream, resulting in eosinophilic pneumonia, liver lesions, myelitis, and visceral larva migrans, characterized by neurological symptoms, including encephalomyeloradiculoneuropathy. Thus, the inactivation of helminth eggs by any means is critical for minimizing risks to the environment and public health. An adult female *A. suum* worm sheds up to 200,000 eggs daily; these eggs are passed in the feces of the infected individual and are thus present in wastewater, contaminated soil, and, in some cases, contaminated drinking water sources. One criterion for assessing the safe disposal of sludge containing parasites is the viability of *Ascaris* sp. eggs.

1.2. Solutions

Common methods for inactivating *Ascaris* eggs in sludge and fecal matter use high temperature, high pH, or both. Other processes for inactivation include alkaline stabilization, acid treatment, anaerobic digestion, ultraviolet exposure, thermal drying, dehydration, composting and disinfection with metal. Both eggs and unsheathed larvae are remarkably resistant to drying, freezing, other weather conditions and chemicals, and can survive for long periods in soil. Many procedures are available for the decontamination of sludge to be used as manure in agriculture (pasteurization, irradiation, lime stabilization), each characterized by a certain degree of efficiency, but all are limited by specific energetic problems and costs, often in association with reduced agronomic value of the materials due to the treatment itself. Heat treatments are effective but also expensive to produce. Thermophilic aerobic pre-treatment and anaerobic digestions have become quite popular but these plants require a minimum of 4 hours at 60°C in an aerobic pre-treatment reactor and a minimum 20 days at 35°C in an anaerobic digestion reactor. Considering the effect on environment, SCFAs are safe as they can be neutralized either naturally or by spraying with baking soda in soil. Once at a pH above the pKa, they are far less toxic and will be degraded by bacteria. One advantage of the use of SCFA may be their production within the anaerobic digester during the inactivation process. High quantities of fatty acids have been generated during the anaerobic digestion of cattle manure using a ‘carboxylate platform’ to convert waste into bioproducts such as carboxylates. Digesters are maintained at 37°C, and it may be possible to produce excess butanoic acid that could also be sold to the human industry. Thus multiple benefits would be obtained, i.e. during the digestion process SCFA would be generated in levels that would inactivate the *Ascaris* eggs and other pathogens resulting in materials that can be safely applied to land and the production of commercially usable chemical. From a public health perspective, adequate inactivation of fecal pathogens in a sanitation system is essential before any use or disposal of fecal material.

1.3. Overall Objectives

This thesis represents the results of using certain concentration of different SCFAs to reduce egg viability at different temperature. The acid pH was determined following the model of *Ascaris suum* inhibition (IC_{50} moles/liter) as a function of pH and finally the pH was 4. The two different temperatures such as 22°C and 37°C represents the weather condition of temperate and tropical regions. In soil experiments, 4°C is used to observe the eggs viability in cold weather after treating with fatty acids. However, the main objective is to develop a safe method for inactivating parasite eggs in the environment, which will not alter the soil pH or will not cause any harmful effect to other animals. Short-chain and medium-chain fatty acids have the potential to inactivate pathogens in soil and water, including the *Ascaris suum* egg.

1.4. Specific Objectives

1. To describe the morphological changes of *Ascaris suum* eggs 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 acid concentration needed to inactivate *Ascaris suum* eggs in a solution.

3. To determine the inactivation of *Ascaris suum* eggs by short-chain and medium-chain fatty acids added to water and soil in a laboratory environment.

4. To compare the inactivation rate of *Ascaris suum* eggs achieved in water and in soil and also the differences between treating the eggs with or without addition of surfactant with acids.

5. To identify the acid concentration and temperature needed to inactivate *Ascaris suum* eggs in soil.

1.5. Hypotheses

Short-chain and medium chain fatty acids increase the egg inactivation rate with higher acid concentration, inactivation occurs more slowly as the temperature decreases, addition of surfactant Tween 20 lowers the time required for total egg inactivation.

CHAPTER 2
LITERATURE REVIEW

2.1. Sanitation and public health

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). 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. 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. Infectious diarrhea, 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). 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. But in 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

Table 1: Main Fecal Pathogens of Concern for Public Health

Group	Pathogen	Disease and Symptoms
Bacteria	*Aeromonas spp.*	Enteritis
	Campylobacter jejuni/coli	Campylobacteriosis-diarrhea, 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, diarrhea, joint pains, rash.
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 diarrhea
	Ancylostoma duodenale/ Necator Americanus	Itch, rash, cough, anemia, protein deficiency
	Schistosoma spp.	Schistosomiasis, bilharzia
Parasitic protozoa	*Cryptosporidium parvum*	Cryptosporidiosis – watery diarrhea, abdominal cramps and pain
	Cyclospora cayetanensis	Often asymptomatic; diarrhea, abdominal pain
	Entamoeaba histolytica	Amoebiasis – often asymptomatic; dysentery, abdominal discomfort, fever, chills
	Giardia intestinalis	Giardiasis – diarrhea, abdominal cramps, malaise, weight loss

Source: (WHO, 2006b)

living, such as housing and nutrition, improved (Bryant, 2003). 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 million 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* accounts 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.

2.2. Indicator Organism

When monitoring and assessing the behavior of pathogens in the environment, indicator organisms are often used instead of the actual pathogens of concern. This is because pathogens can be hazardous to humans and often appear at low concentrations in natural environments, making them difficult and costly to detect. Indicator organisms are therefore chosen to simulate these hard-to-detect pathogens, making research on microbial behavior more reliable, faster and cost-effective. However, indicator organisms have to meet several conditions in order to be reliable. Such conditions have been widely discussed (Payment and Franco 1993; Mara and Horan 2003; Hach 2000) and include;

- having the same origin as the pathogen it's representing
- always be present when (and only when) the pathogen is present
- exist in high enough numbers to be detected
- be equally persistent or more persistent than the pathogens it is representing

 be non-pathogenic

 be easy to measure in the laboratory

Based on these criteria, several organisms have been chosen frequently as indicators of microbial behavior. The following section provides insight into the strengths and limitations of the indicator organisms chosen in this study; *Ascaris suum,* to emphasize the importance of including multiple indicator organisms in health risk assessments.

2.3. *Ascaris* Eggs

Helminths are worms measuring from 1mm to several meters in length, which come from microscopic eggs (US-EPA, 1992). *Ascaris* eggs have a 3- to 4-µm thick, four-layer shell that consists of an inner lipoprotein layer (ascaroside layer), a thicker chitin/protein layer, a lipoprotein vitelline layer, and an outer acid mucopolysaccharide/protein uterine layer, each of which has a characteristic chemical composition. Two of these, namely the innermost lipoid membrane and the chitinous shell, have been recognized for many years (Chitwood, 1937). Recent evidence, however, supports that the chitinous shell contains both a protein and a chitin layer, and that another protein is to be found either as a separate layer or as a part of the lipoid membrane. The various layers of the primary envelope will be considered in the order in which they are formed, beginning with the first (outermost), and finishing with the lipoid membrane. The outer layer is usually fully formed by the time the egg has traversed one-third the length of the uterus. The formation of the fertilization membrane is followed quickly by the appearance between it and the cytoplasmic surface of a secretion which rapidly hardens to form the second layer. This optically clear layer is about 3 µ thick, consists mainly of chitin, although delicate protein fibrils similar in their properties to those of the fertilization membrane may also be present (Monné and Hönig, 1954b). Yanagisawa, 1955, suggested that a third layer of the *Ascaris* envelope is formed from granules which appear first in the young oocytes and in which

little change was noted until the chitin layer was well-formed. These granules then moved to the protoplasmic surface and coalesced, after which the whole mass was excreted. During the formation of the first three layers of the primary egg envelope, the refringent granules containing ascarosides (or ascaroside esters) are concentrated in the center of the oocyte (Yanagisawa, 1955). These granules move to the periphery where they are discharged to form the fourth layer, which is generally referred to as the lipoid or vitelline membrane. As *Ascaris* eggs progress down the uterus, they acquire on their outer surface a very thick and sticky coat. The dense sticky material secreted by the uterine cells of the female *Ascaris* adheres to the egg, and gradually forms the outer layer of the external coat. This external coat adds considerably to the dimensions of the egg, which have otherwise remained constant since fertilization. Unlike the layers of the primary envelope, it is a product of the uterine secretions, and hence is a secondary envelope. The function of the outer coat is uncertain, but Fairbairn stated that its stickiness has obvious advantages. It may also afford protection against desiccation or solvent action. Monné and Hönig (1954b) observed that in eggs passed naturally in the feces of infected animals the properties of this external coat were completely changed. It was no longer digested by pepsin or papain, nor was it soluble in dilute acid and alkali. It could, however, like the fertilization membrane, be dissolved in hypochlorite solutions. The inner lipoprotein layer consists of a unique mixture of 25% protein and 75% lipid containing ascarosides and is responsible for the impermeability of the shell (Wharton, 1980). The chitinous layer, which provides structural strength, contains chitin spindles in a protein matrix (Gamble, 1995). The compositions of the vitelline and uterine layers are not well characterized, but both contain protein (Wharton, 1980). The vitelline membrane provides the egg with a covering which is extremely resistant to chemical action and is also highly impermeable to all substances except gases and lipoid solvents. The chemical inertness of the vitelline membrane must also be associated with complete impermeability to the multitude of toxic substances which would otherwise destroy the vitellus or embryo. It is, however, permeable to the

respiratory gases and to other gases such as hydrogen cyanide (Huff and Boell, 1936) and hydrazoic acid, ammonia, and carbon monoxide (Passey and Fairbairn, 1955).The shell comprised of protein and chitin, is probably permeable, but provides mechanical strength as well as chemical resistance. On the other hand, it is essentially impermeable to cyanide, azide, or ammonium ions (Resnitschenko, 1927, 1928; Passey and Fairbairn, 1955) as it is to salts in general. These properties suggest that the membrane might also be permeable to water vapor. The perivitelline fluid of embryonated eggs, and possibly of unembryonated eggs as well, contains large amounts of trehalose, which are equivalent osmotically to 1.16 % sodium chloride. If the vitelline membrane were permeable to water vapor, then osmotic phenomena would be expected to appear in hypotonic or hypertonic solutions. The fact that the eggs or embryos survive indefinitely in distilled water could then be explained satisfactorily by assuming that the hard shell has sufficient strength to resist the osmotic stress established under these conditions. On the other hand, hypertonic solutions do not produce plasmolysis of the egg until present in concentrations approximating the osmotic equivalent of 15 % sodium chloride (Bataillon, 1901; Zawadowski, 1928) and sucrose, according to Bataillon, causes no plasmolysis even in the most concentrated solutions. The outer three layers can be removed by soaking the eggs in a solution of hypochlorite, leaving only the inner lipoprotein layer (Barrett, 1976; Kennedy, 1986); this process is referred to as “decortication.”

The shells of *Ascaris* eggs when first removed from the uterus are essentially colorless, whereas those of fecal eggs are usually yellowish brown. This color development, which has usually been attributed to staining with the bile pigments, may perhaps be associated also with a chemical transformation of the secondary envelope (Monné and Hönig, 1954b). In addition, the outer layers of the primary egg envelope of decoated eggs acquire a brown color in the course of embryonation. There is some evidence that the colored shell protects the egg from the harmful effects of ultraviolet, and possibly also from blue-violet radiation. Nolf (1932) obserbed that water cultures of fecal eggs would embryonate satisfactorily in direct sunlight, whereas

colorless eggs collected from the uterus did not. Nolf also found that *Ascaris* eggs were much more sensitive to the effects of controlled ultraviolet radiation. According to Monné and Hönig (1954a), the color of egg shell is due to the presence of quinone-tanned proteins, which are absent in unembryonated *Ascaris* eggs. Miretski (1952) reported that colorless eggs would embryonate in direct sunlight if protected by glass and a solution of 1% potassium dichromate, whose color approximates that of the naturally colored shell of fecal eggs. Eggs protected from sunlight by glass and a 4 % solution of tetramminecopper sulfate (blue) did not embryonate.

2.4. Organic acid

Organic acids are distinguished from other acids by the functional group COOH to which an organic group or a hydrogen atom may be attached. Common names used to describe this group of organic compounds include fatty, volatile fatty, lipophilic, weak or carboxylic acids. The saturated straight chain organic acids listed in table 2 may be grouped arbitrarily according to their carbon chain length, i,e, short-chain and medium-chain fatty acids, which contain 1-6 and 7-10 carbon atom respectively. The individual acids are named systematically from the normal alkane of the same number of carbon atoms by dropping the final “e” and adding the suffix “oic” (table 2).

2.4.1. Synthesis of SCFA

SCFAs are organic fatty acids with 1 to 6 carbon atoms and are the principle anions which arise from bacterial fermentation of polysaccharide, oligosaccharide, proteins, peptide and glycoprotein precursors in the colon (Miller and Wolin, 1979; Cummings and Macfarlane, 1991). Fermentation involves a variety of reactions and metabolic processes in the anaerobic microbial breakdown of organic matter, yielding metabolizable energy for microbial growth and maintenance and other metabolic end products for host use. The chief end products are SCFAs

together with gases (CO_2, CH_4 and H_2) and heat (Toppings and Clifton, 2001). There was no difference in the concentrations or relative compositions of SCFAs between methane-excreting subjects and non-methane-excreting subjects. SCFAs constitute approximately two-thirds of the colonic anion concentration (70-130 mmol/l), mainly as acetate, propionate, and butyrate. Dietary carbohydrates, specifically resistant starches and dietary fiber, are substrates for fermentation that produce SCFAs, but fiber is less important than other substrate for the bacterial formation of SCFAs in the colon. Approximately 80-90% of SCFAs, which are produced from the breakdown of dietary food, are absorbed in colon while the rest are excreted in feces. Fermentation of proteins and amino acids by proteolytic bacteria yield branched SCFAs, CO_2, CH_4, H_2, phenols and amines (Roberfroid, 2005). The SCFAs formed most likely do not exert any direct laxative effect. SCFA production and absorption are closely related to the nourishment of the colonic mucosa and sodium and water absorption, and mechanisms of diarrhea. SCFA are rapidly absorbed by nonionic diffusion mostly but also by active transport mediated by a sodium-coupled transporter, thereby fostering the absorption of sodium and water. The production of SCFAs in determined by many factors including the numbers and types of microflory present in the colon (Roberfroid, 2005). Studies found significant differences between sexes in the fecal concentrations of SCFAs. The total SCFA concentration and that of the three major acids (acetic, propionic and n-butyric acid) were higher in men compared to women. The concentrations of the minor acids (iso-butyric, iso-valeric, n-valeric and n-caproic) were similar in males and females.

2.4.2. Nutritional implications of short-chain fatty acids

The SCFAs absorbed from the colon can be utilized as an energy source by the host, but they contribute only to a small part *(5*- 10%) of total energy in healthy individuals on Western diets (McNeil, 1984). The colonic mucosa obtains its energy by oxidizing mainly SCFAs in the order of butyric>propionic>acetic acid (Clausen, 1994). The SCFAs that escape metabolism in the

colonocytes enter the hepatic portal blood. Acetic acid is utilized by the liver where it is transferred into Acetyl-CoA, which can act as a precursor for lipogenesis (Remesy, 1992), but also stimulates gluconeogenesis (Wolever, 1995). Low concentrations of acetic acid can also be detected in venous blood in peripheral tissues (Scheppach, 1991). SCFAs formed at fermentation of carbohydrates in the colon have been reported to affect carbohydrate metabolism (Thorburn, 1993). Thus, barley containing high amounts of fermentable carbohydrates improved glucose tolerance in healthy subjects compared to rice with a lower amount of indigestible carbohydrates. Propionic acid is mainly metabolized in the liver and has been shown to inhibit gluconeogenis and increase glycolysis in rat hepatocytes (Anderson, 1984). It has also been proposed that propionic acid may lower plasma cholesterol concentrations by inhibiting hepatic cholesterogenesis (Chen, 1984). In humans, however, the synthesis of cholesterol from acetic acid decreased when propionate is infused rectally (Wolever, 1991), suggesting that the ratio of SCFAs formed upon colonic fermentation may also be of physiological importance. Butyric acid is the main energy substrate for the colonocytes (Roediger, 1982) and is metabolized by the cells in preference to glucose or glutamine, accounting for 70% of the total energy demand of the colonic mucosa (Scheppach, 1994). Butyric acid has been reported to be important in the prevention and treatment of diseases of the colonic mucosa, such as distal ulcerative colitis (Cummings, 1997) and cancer (Scheppach, 1995). Although butyric acid serves as the primary energy source for the normal colonic epithelium and stimulates growth of colonic mucosa, the growth of colonic tumor cell lines has been reported to be slowed down by butyrate (Whitehead, 1986). Butyrate also appears to reduce cell differentiation (Barnard, 1993) and stimulate apoptosis (Hague, 1995) in tumor cell lines. In addition to serving as a nutrient to the mucosa cells, SCFAs have other specific colonic effects such as increasing mucosal blood flow (Mortensen, 1990). Further, the decreased luminal pH induced by SCFA production may stimulate mineral absorption through increased mineral solubility (Coudray, 1997; Younes, 1996).

2.4.3. Antimicrobial properties of organic acids

Although the antibacterial mechanism(s) for organic acids are not fully understood, they are capable of exhibiting bacteriostatic and bactericidal properties depending on the physiological status of the organism and the physicochemical characteristics of the external environment. Organic acids are more effective than mineral acids as antimicrobial agents, although they exhibit broad-spectrum antibacterial activity, the antibacterial efficiency of individual acids varies (Goepfert and Hicks, 1969). Salsali (2006) investigated the influence of organic acids on the inactivation of Salmonella spp. over a range of digestion temperatures, organic acids concentrations, and pH. The reduction of Salmonella spp. in digester effluents, when dosed with volatile organic acids, was found to depend on pH, temperature, the chain-length of the acids, and the concentration and composition of the acids present. For many years microbial growth inhibition by organic acids was explained by the ability of these acids to pass across the cell membrane, dissociate in the more alkaline interior and acidify the cell cytoplasm (Kashket, 1987). In addition, it was assumed that bacteria maintained a slightly alkaline intracellular pH, but this assumption was largely based on work with laboratory cultures of *E. coli* (Padan, 1981). Less direct antibacterial activities have also been attributed to organic acids and include interference with nutrient transport, cytoplasmic membrane damage resulting in leakage, disruption of outer membrane permeability, and influencing macromolecular synthesis (Cherrington, 1991; Davidson, 2001). Consequently, bactericidal concentrations of organic acids may be due to the combination of dissipation of proton-motive force and inability to maintain internal pH followed by denaturation of acid-sensitive proteins and DNA. Sublethal concentrations may elicit their effects on overall cell physiology and lead to responses such as enlargement of bacterial cell size (Thompson and Hinton, 1996). In the food animal industry, organic acids were originally added to animal feeds to serve as fungistats (Paster, 1979; Dixon and Hamilton, 1981), but in the past 30 years, formic and propionic acids and various combinations have also been examined for potential bactericidal activity in feeds and feed

ingredients contaminated with foodborne pathogens, particularly *Salmonella* spp. (Khan and Katamay, 1969). Bacteria can utilize acetic acid as a carbon and energy source by inducing enzymes of the glyoxylate pathway, isocitrate lyase and malate synthase which allows net assimilation of carbon (Nunn, 1987). Gram-negative bacteria are relatively resistant to medium-chain fatty acid and long-chain fatty acid, while the ability of the organism to metabolize short-chain fatty acid may affect the antibacterial activity of the acid. The addition of formic acid to the feed of laying hens lowered the incidence of salmonella infections in their newly hatched progeny.

CHAPTER 3

ASCARIS SUUM EGG INACTIVATION USING DIFFERENT SHORT-CHAIN AND MEDIUM-CHAIN FATTY ACIDS: ACID CONCENTRATION, CONTACT TIME, TEMPERATURE AND SURFACTANT FACTS

3.1. Abstract

Ascaris lumbricoides is the most prevalent of the parasitic intestinal worms; an estimated 1.4 billion people are infected worldwide, mostly in developing countries. Helminth infections like ascariasis lead to a host of physical and mental disabilities, including cognitive and societal impairment, higher susceptibility to infection, decreased responsiveness to vaccination, and malnutrition, which impair the development of several hundred million children in developing countries. In the United States and other developed countries, the swine parasite *Ascaris suum* eggs, which are ubiquitous and relatively resistant to most forms of treatment, is routinely used as a surrogate for the human parasite for monitoring the ability of different processes to destroy helminth eggs for the purpose of sanitation to protect public health. Like other natural and chemical processes, exposure to organic acids is another possible method for inactivating *Ascaris* eggs. The objective of our research is to determine the most effective of the different SCFAs to utilize in disinfection process and to decrease the viability of *Ascaris* eggs within shortest period of time in the environment and eventually in soil and in waste treatment plants, thereby creating safer water sources and soil. In my experiment, *Ascaris* eggs were first collected from feces of naturally infected pigs and then exposed to different molarities of butanoic, pentanoic, hexanoic and heptanoic acids (all in upper limits of solubility in water and more than upper limits) with and without Tween 20 and all at pH 4 at 22°C & 37°C for different period of times. In each case, a surfactant, Tween 20, was added with acid solution to make the

higher acid molarities soluble in water. Increasing the temperature by 15 degrees and higher concentration of each acid caused a significant decrease in the contact time required for total inactivation. Inactivation rate is also influenced by increasing contact time, and ultimately, 100% inactivation can be achieved. The results suggest that higher concentration of pentanoic and hexanoic acids with or without the addition of the surfactant Tween 20 have potential for the rapid inactivation of helminth eggs in water within minutes and within 4 hours in soil at 37°C. No significant difference was observed when using the surfactant Tween 20 either at higher temperatures or at the different acid concentrations tests. *Ascaris* eggs are clearly susceptible to inactivation by short-chain and medium-chain fatty acids in both water and soil.

3.2. Introduction

Ascaris suum (Goeze, 1782), a parasite of swine, with the transmission mechanism (fecal/oral), affects millions of pigs and is responsible for substantial economic losses in many countries (O'Lorcain and Holland, 2000). Cross-infection of people with the porcine species, *Ascaris suum*, and with the human species, *Ascaris lumbricoides* appears uncommon but may occur upon occasion (Lysek, 1967; Galvin, 1968; Beaver, Jung & Cupp, 1984; Anderson, 1995), particularly in endemic regions where pigs and humans live in close proximity to one another, or where the excreta of both humans and pigs are used as a fertilizer (Peng, 1996). Evidence from Central and North America indicates that, genetically, the two parasite populations appear to be reproductively isolated (Anderson, Romero- Abal & Jaenike, 1993). In contrast, (Peng, 1998) found no significant heterogeneity in the genetic composition of the *Ascaris* infra-populations in both humans and pigs, perhaps because of agricultural practices in China, which include the use of nightsoil (human feces and urine) as fertilizer on food crops that have resulted in a random distribution of alleles within the parasite populations over time. Both species of *Ascaris*

are transmitted through the ingestion of crops polluted with wastewater or sludge. Although there are several examples of the use of untreated wastewater for agricultural irrigation, in the case of sludge there are no reports of its untreated use. In the United States around 41% of treated sludge (biosolids) is used for agriculture (US-EPA, 1999), while in Europe the practice varies among countries (10-80%). In contrast, in developing countries where sludge production is still low due to the low level of wastewater treatment, most of the sludge produced is discharged into rivers, sewers or simply abandoned in the soil. Helminth eggs are the most resistant to many types of inactivation and eggs of the genus *Ascaris* have the highest resistance and survive under numerous treatment conditions (Feachem, 1983; Gaasenbeek and Borgsteede, 1998; Reimers, 1986b). *Ascaris suum* eggs are resistant towards most disinfection treatments; in sewage sludge, a treatment lasting 2 months with an initial pH of 12.5 was required to obtain no viable organisms (Gaspard, 1995). A pH over 10 at temperatures above 10°C was sufficient for inactivation of bacteria (Allievi, 1994) but *Ascaris* eggs can be inactivated in minutes by temperatures above 60°C, but they can survive for more than 1 year at 40°C (Feachem, 1980). In the case of *Ascaris* eggs held for 21 days at 38°C in sludge from various sewage treatment works in municipal Chicago (USA), with the exception of one sample, at least 46 percent of the eggs remained viable, and some were then found to be infective to pigs (Fitzgerald and Ashley, 1977). 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°C) increase pathogen survival, while most of them are inactivated at high temperatures (> 40°C) showing an exponential decline in population (Feachem, 1983).

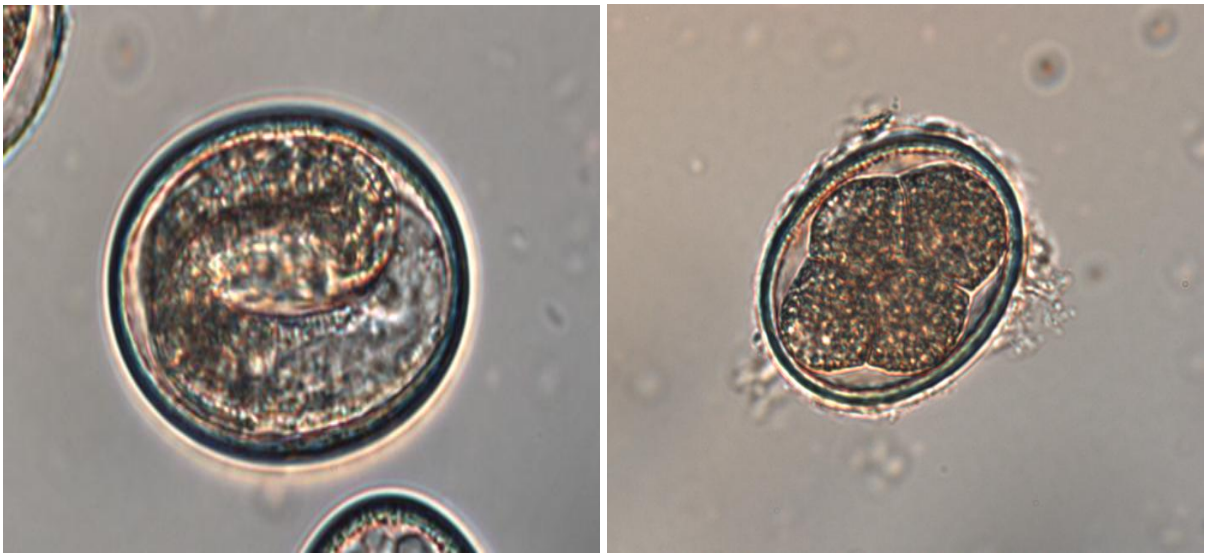


Figure 1: An embryonated *Ascaris suum* egg (left). Unembryonated *Ascaris suum* egg (right).

The impermeability of the inner ascaroside membrane also protects the eggs from a variety of strong acids, strong bases, oxidants, reductants, protein-disrupting agents, and surface-active agents (Barrett, 1976). It has also been established that the vitelline membrane is primarily responsible for the impermeability effect. Moreover, eggs encased only in the vitelline membrane (Fairbairn and Passey, 1955) or in which the hard shell is cracked (Zawadowski, 1928) appear to be fully resistant to a wide variety of solutions. The lipoid nature of this membrane, on the other hand, renders it sensitive to the action of many organic solvents. Among the solvents which dissolve it may be mentioned chloroform, ethyl ether, alcohols, phenols and cresols. Surface active agents (Jaskoski, 1954) and noxious gases such as methyl bromide (Enigk, 1953) may damage or penetrate it. Izumi (1952) tabulated the relative destructive action of a number of such compounds, some of which have been proposed as disinfectants for contaminated areas. The vitelline membrane melts at approximately 70°C. If the vitelline membrane were permeable to water vapor, then osmotic phenomena would be expected to appear in hypotonic or hypertonic solutions. The fact that the eggs or embryos

survive indefinitely in distilled water could then be explained satisfactorily by assuming that the hard shell has sufficient strength to resist the osmotic stress established under these conditions. On the other hand, hypertonic solutions do not produce plasmolysis of the egg until present in concentrations approximating the osmotic equivalent of 15 % sodium chloride (Bataillon, 1901; Zawadowski, 1928) and sucrose, according to Bataillon, causes no plasmolysis even in the most concentrated solutions. The chitin is soluble in hot, concentrated mineral acids and in sodium hypochlorite, but is insoluble in hot alkali. The eggs are oval-shaped, heavy and often found in sludges. Eggs contained in sludge are not always viable and infective. They need to develop larvae to be infective, for which a certain temperature, moisture and the availability of oxygen are required (26°C and 1 month in laboratory conditions). These conditions can be found in soil, crops and sludge storage systems where eggs can develop larvae in 10 days. Helminth eggs can live in water, soil and crops for several months or years (Feachem, 1983). Naturally, the eggs can be rendered non-viable by using extreme heat (>40°C) or with UV radiation (as from sunlight). the contribution of UV to the overall removal and inactivation of *Ascaris* eggs in a treatment process may or may not be significant, depending on the influent concentrations and the desired effluent quality (e.g., the World Health Organization recommendations for reuse of wastewater for agricultural irrigation require less than 0.1 or 1 intestinal nematode egg/liter, depending on the application [World Health Organization. 1989]). *Ascaris* eggs are more resistant to external conditions because of their structural composition of cell membrane and are permeable only to organic solvents and lipid soluble gases (Fairbairn, 1957). Exposure of deshelled or decorticated eggs to a variety of proteolytic, amylolytic and lipolytic enzymes had no detectable effect on permeability. The resistance of the eggs to many treatment factors and disinfectants makes *Ascaris* eggs a conservative indicator organism for environmental pollution and treatment efficiency (O'Lorcain and Holland, 2000).

Organic acids have a long history of being utilized as food additives and preservatives for preventing food deterioration and extending the shelf life of perishable food ingredients. As a group these compounds primarily include the saturated straight-chain monocarboxylic acids and their respective derivatives (unsaturated, hydroxylic, phenolic, and multicarboxylic versions) and are often generically referred to as fatty acids, volatile fatty acids, or weak or carboxylic acids (Cherrington, 1991). Several of these organic acid compounds are used as direct additives incorporated into human foods or can accumulate over time as a consequence of the fermentation activity of indigenous or starter cultures added to certain dairy, vegetable, and meat products. In addition, acid sprays have been incorporated as sanitizers during meat processing (Acuff, 1987; Cherrington, 1991; Dickson, 1992; Hardin, 1995; Dorsa, 1997). Some organic acids, particularly the short-chain fatty acids (SCFA), acetate, propionate, and butyrate (table 2), are produced in millimolar quantities in the gastrointestinal tracts of food animals and humans and characteristically occur in high concentrations in regions where strictly anaerobic microflora are predominant. In the food animal industry, organic acids were originally added to animal feeds to serve as fungistats (Paster, 1979; Dixon and Hamilton, 1981). Organic acids are more toxic than mineral ones because they interfere with cellular reactions (El-Ziney, 1997). Sulphuric, hydrochloric, propionic, acetic, and peracetic acid are used to inactivate pathogens, the latter two being the most effective. A 550 ppm concentration of peracetic acid in physicochemical sludge with a higher number of pathogen content can inactivate helminth eggs, the latter in only 10 minutes (Barrios, 2004). The toxicity of fatty acids to prokaryotes (Cherrington, 1991) and some eukaryotic organisms (House, 1967; Donaldson, 1970) has been reported and the toxicity also has been attributed to acidification of the cytoplasm, disruption of the cell membrane and/or its functioning, interference with metabolism (catabolic and anabolic) and damaging DNA (Cherrington, 1991). It has been assumed that undissociated forms of organic acids can easily penetrate the lipid membrane and once internalized into the neutral pH of the cell cytoplasm dissociate into anions and protons (Eklund, 1983, 1985; Salmond, 1984;

Cherrington, 1990, 1991; Davidson, 2001). Russell (1992) has hypothesized that anion accumulation is the primary toxic effect of organic acids and that some organisms are more resistant to organic acids because they are capable of allowing their internal pH to decline. Acid pH is considered a primary determinant of effectiveness because it affects the concentration of undissociated acid formed (Davidson, 2001). At pHs below the pKa values, the concentration of the undissociated form of the organic acids increases and therefore under these conditions greater toxicity is expected (Taherzadeh, 1997). Export of excess protons requires consumption of cellular adenosine triphosphate (ATP) and may result in depletion of cellular energy (Davidson, 2001). Other toxicity mechanisms have been proposed that attribute membrane uncoupling capabilities for organic acids. It has been speculated that organic acids interfere with cytoplasmic membrane structure and membrane proteins such that electron transport is uncoupled and subsequent ATP production is reduced or that organic acids serve as uncouplers that generally dissipate pH and electrical gradients across cell membranes (Sheu and Freese, 1972; Sheu, 1972, 1975; Freese, 1973; Salmond, 1984; Russell, 1992; Axe and Bailey, 1995; Davidson, 2001). The effectiveness of organic acids is believe to be dependent on concentration, pH, temperature, exposure time and the degree of sensitivity of specific types of pathogens (Goepfert and Hicks, 1969; Abdul and Lloyd, 1985; Fukushi, 2003) and also dependent on carbon chain length, concentration, pH and K_{ow} (hydrophobicity) (Butkus, 2011). Butanoic acid and other lower molecular weight acids are liquid at room temperature and miscible with water (Cherrington, 1991), pentanoic acid is slightly soluble in water but soluble in alcohol and ether, heptanoic and hexanoic acids have much lower aqueous solubility at room temperature (Ralston and Hoerr, 1942).

Table 2: Nomenclature of organic acids (after Streitwieser and Heathcock 1981)

Compound	Common name	Systematic name
Short chain fatty acid		
C_1 HCOOH	**Formic**	**Methanoic**
C_2 CH_3COOH	**Acetic**	**Ethanoic**
C_3 CH_3CH_2COOH	**Propionic**	**Propanoic**
C_4 $CH_3(CH_2)_2COOH$	**Butyric**	**Butanoic**
C_5 $CH_3(CH_2)_3COOH$	**Valeric**	**Pentanoic**
C_6 $CH_3(CH_2)_4COOH$	**Caproic**	**Hexanoic**
Medium-chain Fatty acid		
C_7 $CH_3(CH_2)_5COOH$	**Enanthic**	**Heptanoic**
C_8 $CH_3(CH_2)_6COOH$	**Caprylic**	**Octanoic**
C_9 $CH_3(CH_2)_7COOH$	**Pelargonic**	**Nonanoic**
C_{10} $CH_3(CH_2)_8COOH$	**Capric**	**Decanoic**

In addition to simple presence of the organic acids, temperature has an impact on the composition of the cell membrane. Cell components, such as proteins, lipids, and carbohydrates, are responsible for transport phenomena into the cell and can withstand a narrow range of temperatures. An increase in temperature beyond what the membrane is accustomed to can result in changes in molecular structure of the membrane. The fluidity of the cellular membrane may increase, thereby allowing more rapid diffusion of organic acids into the cytoplasm (Demeyer and Henderickx, 1967). Higher temperatures also may cause the lipid layer in the eggshell wall to become more fluid, allowing more diffusion of organic acids into the cytoplasm (Salsali, 2006). There is a reversible change in permeability between 44 and 65°C. Above 65°C, the ascaroside membrane becomes permanently disorganized and this approaches the melting point of isolated ascarosides which is about 82°C (Fairbairn, 1970).

Inactivation rates of *Ascaris* eggs vary widely over the temperature range used for sludge treatment (20–80°C), so the maximum temperature attained and the temperature profile during treatment are critically important (Feachem, 1983). As *Ascaris* eggs are highly resistant, they can survive in a variety of settings including aerobic and anaerobic environments. Under the absence of other factors, temperatures less than 40°C are usually not effective at inactivating *Ascaris* eggs except over very long time periods (>1 year). During a biological degradation, such as an anaerobic or aerobic digestion, many factors may affect the degree of ascarid egg inactivation, however, temperature is still the dominant factor (Feachem, 1983). Thus, at mesophilic temperatures (<40°C), both anaerobic and aerobic environments are only partially effective at inactivating *Ascaris* eggs, whereas the thermophilic temperatures (>50°C) can inactivate eggs to under the detectable limits (Pecson and Nelson, 2005).

In the present paper, *Ascaris suum* eggs in water and soil were treated with short-chain and medium-chain fatty acids with and without the surfactant Tween 20 at pH 4. The soil samples containing added eggs were treated with higher concentration of pentanoic and hexanoic acids integrated with Tween 20.

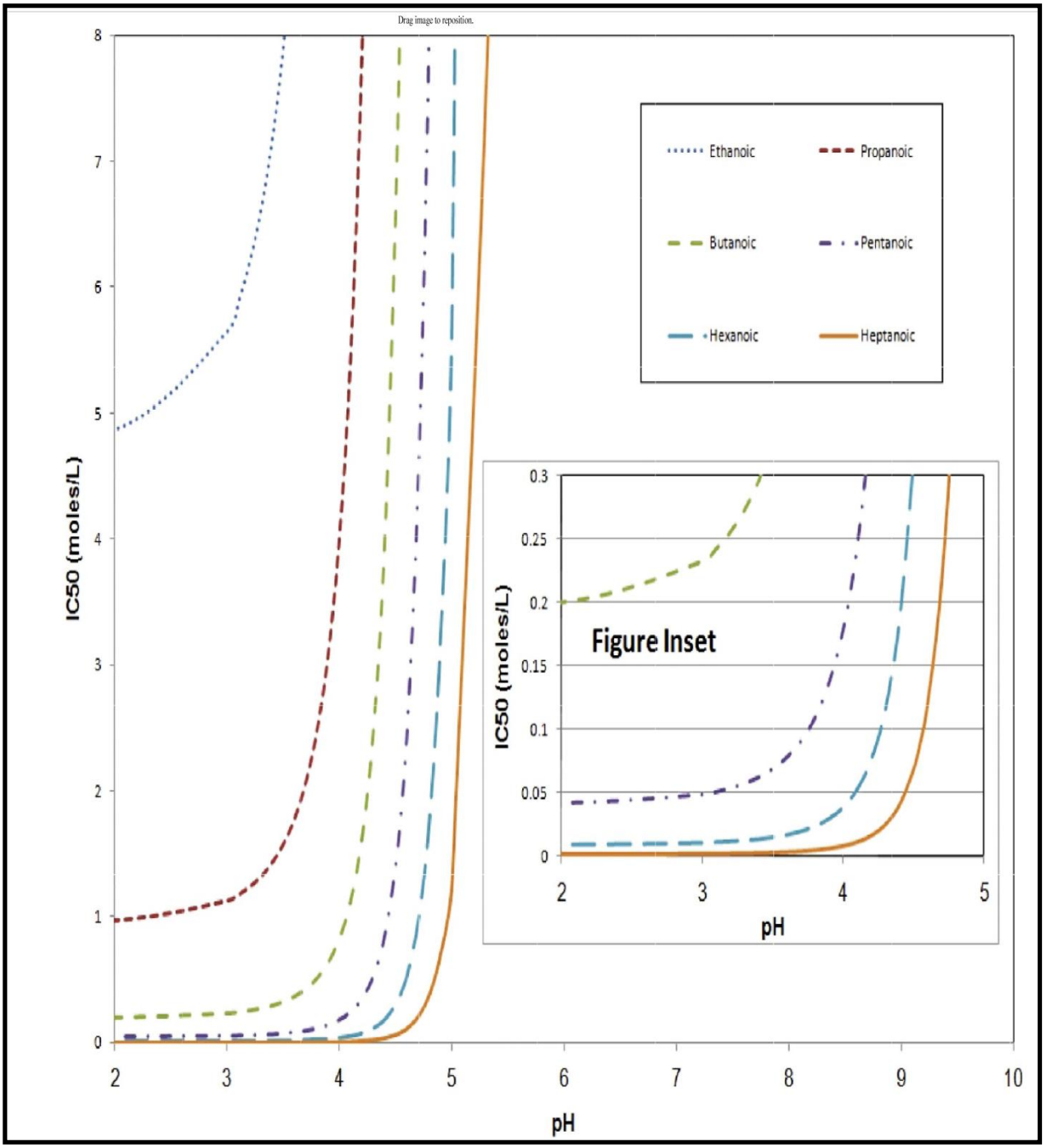


Figure 2: Model of *Ascaris suum* inhibition (IC_{50} moles/liter) as a function of pH. Model predictions were based on a quantitative structure–activity model (QSAR) according to a relationship reported by Kamlet et al. (18).

3.3. Materials and Methods

3.3.1. Collection & cleaning of *Ascaris suum* eggs:

Unembryonated *Ascaris suum* eggs were obtained from farm raised pigs at a slaughter house in Pennsylvania. The contents of intestinal tracts identified as containing adult *A. suum*, was diluted in water and passed through a series of sieves to remove particulates, and ultimately the eggs were collected on a 500 mesh sieve. The eggs and similar sized particulates were transferred in a slurry to 4.5 liter buckets to about a depth of 3 cm, and the buckets were filled with deionized water containing 0.1N H_2SO_4. The eggs are stored at 4°C, and the acidic water is changed regularly.

At the time of use for this work, the eggs in the sediment were further cleaned by centrifugal flotation in a $MgSO_4$ solution at specific gravity 1.2. The floated eggs were poured over a 500 mesh sieve, and then washed back into a 15 ml conical centrifuge tube. The volume of eggs to be utilized in any given study was determined by a dilution egg count method. We identified two eggs populations in the counting cell in accordance with morphological criteria: eggs that had only a cell wall (composed of an inner lipid layer, an intermediary chitinous layer and an outer vitellin membrane) and eggs that had both a cell wall and an outer layer. The outer layer is made up of secretions deposited as the egg passes through the uterus. However, it is not evenly distributed, so that the surface may be divided into depressions and ridges, or it may be absent from some eggs. It is responsible for the resistant properties of the eggs. Both populations are normally present in newly laid eggs.

3.3.2. Selection of acid concentrations used in assays and utilization of Tween 20:

The solubility of the different acids at 25°C are reported as butanoic acid being miscible with water; 4.97 g/100 mL (0.487 M) for pentanoic acid; and 1.082 g/100 mL (0.0932 M) for hexanoic acid. The maximum solubility value for heptanoic acid (at 15°C) is given as 0.2419 g/100 mL (0.0185813 M). However, empirically, when trying to make acids at maximum solubility two phases would form at lower concentrations than the reported maximum solubility, e.g., for pentanoic acid the maximum obtainable solubility was 0.2 M; thus it was decided that all assays would be run at a maximum of concentration of 1.5 M. Tween 20 is usually used for solubilizing the membrane protein during isolation of membrane-protein complexes. Previous studies suggest that the Tween 20 alone has no effect on egg inactivation process, but in our experiment this was used to make the acid more soluble in water.

Table 3: Acid preparation for a total volume of 250 ml (all volumes in milliliters)

ACID	MOLARITY	18 mM TWEEN 20	VOLUME ACID	VOLUME TWEEN 20	VOLUME WATER
BUTANOIC	1.0		**22.85**		**227.15**
	1.36		**31.2**		**218.8**
	1.5	yes	**34.28**	**5**	**210.72**
PENTANOIC	0.2		**5.44**		**244.56**
	0.2	yes	**5.44**	**5**	**239.56**
	1.5		**40.8**		**209.2**
	1.5	yes	**40.8**	**5**	**204.2**
HEXANOIC	0.038		**1.2**		**248.8**
	0.038	yes	**1.2**	**5**	**243.8**
	1.5		**47.3**		**202.7**
	1.5	yes	**47.3**	**5**	**197.7**
HEPTANOIC	1.5	yes	**53.7**	**5**	**191.3**

3.3.3. Exposure of *A. suum* eggs to fatty acids in water samples:

1000 eggs were added to acid solution for a final volume of 1 ml in 1.5 ml microfuge tube, vortexed for 3 seconds and placed in a heating block at 37°C or 22°C in static condition. At various sampling times, the tubes were removed and centrifuged at centrifugal force 1200 g for 2 minutes to pellet the eggs. The acid was suctioned off without disturbing the egg pellet, and the eggs were washed 3 times with phosphate buffer (10 mM, pH 7.0). After washing, the eggs were transferred to 24 well culture plates with 0.5% formalin solution to retard mold growth during incubation. The plate, wrapped in a wet paper towel in a plastic bag, was statically incubated at 28°C for 20 days. Unless otherwise stated all experiments were carried out in triplicate for each exposure time/temperature/acid concentration/acid with surfactant.

Table 4: Water solubility of different fatty acids

Solubility of fatty acids		
	Carbon #	Solubility (M)
Acetic	2	>1.5
Propanoic	3	>1.5
Butanoic	4	1.36
Pentanoic	5	0.2
Hexanoic	6	0.038
Heptanoic	7	0.0019
Octanoic	8	0.00047
Nonanoic	9	0.0001

3.3.4. Assessing percentage of eggs successfully embryonating:

After 20 days, 300 ul of 6% sodium hypochlorite (Clorox) was added to each well to remove the outer albuminous layer from the eggs, and after 10 minutes later eggs were then examined microscopically. Eggs were scored as larvated (viable) or non-larvated (nonviable). The percent of non-viable eggs was calculated as the number of non-viable eggs divided by total number of eggs counted. If an initial quick examination revealed that very few eggs were kept from embryonating, only 100 eggs were scored. If more than 5 to 10% of the eggs appeared not to have embryonated, i.e., if there appeared to have been some effect on egg viability, 300 eggs from each well were scored. If initial observations made it appear that all or nearly all eggs were dead, then the 700 or so eggs in each well were all examined, typically around 700 eggs per well. The data represented on the graphs represents the total number of viable eggs counted in the three replicates, i.e., percentage viable out of 300 total eggs, 900 total eggs, or approximately 2,100 total eggs.

3.3.5. Application of SCFA to soil containing *A. suum* eggs:

To wet the soil, a kg of Hyponex ® potting soil (determined by dry-weight calculations to contain 13% water by weight) was mixed with 1000 ml water and allowed to stand in a closed container for 2 days. Then, the soil was mixed using a Kitchen Aid Classic Plus Stand Mixer at speed 6 for 30 minutes. After the mixing, 1.5 X 10^5 unembryonated eggs of *A. suum* (3 ml with 50,000 *A. suum* eggs per ml) were added to the soil that was then mixed for another 30 minutes. The soil was then transferred to 75 20-ml glass vials with screw-top lids and placed in a refrigerator at 4°C; each vial of soil contained about 900 eggs. In these two experiments, there was only one vial examined for each time (0, 0.33, 0.50, 0.9, 1.5, 2.5, 4, and 6.7 hours) and temperature (4°C, 22°C, and 37°C) for each acid.

Three ml of the acid-Tween 20 solution at concentration 1.5 M was added to each 12 grams of the wet soil containing eggs. In the case of samples to be held at 4°C, the vials were removed from the refrigerator, the acid added, the vials placed in a zippered plastic bag, and returned to the refrigerator. For samples to be held at 22°C samples, the vials were allowed to warm to room temperature before the acid was added; after its addition, the vials were placed in a sealed box that was held in a chamber at 22°C. For the eggs at 37°C, the vials were allowed to reach room temperature, and then transferred to a 37°C incubator for at least 10 minutes before the acid was added. After the acid was added, the vials were placed in a zippered plastic bag in a 37°C incubator. The pH of the samples was taken at the longest exposure time point verifying that the pH did not change during the exposure and remained at the starting pH of 4.

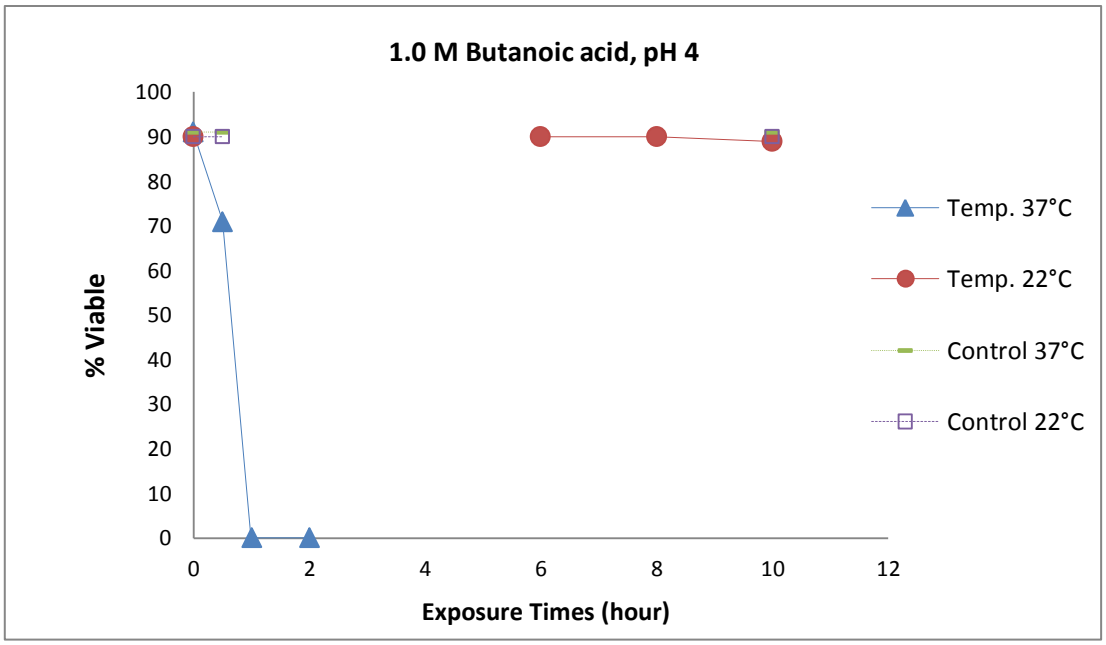
After exposure, the soil was transferred to a 50 ml tube that was then filled with 10 mM sodium phosphate buffer to neutralize the acid. After centrifugation at full speed for 5 minutes, the supernatant was removed and the sediment resuspended again in the buffer, and centrifugation repeated and supernatant discarded. After the second centrifugation, the sediment resuspended in 1.2 spg $MgSO_4$ solution, centrifuged for 5 minutes, and then the top 15 or so ml were transferred to a clean 50 ml tube that was filled with water and again centrifuged for 5 minutes. Then, all but the bottom 5 ml was removed from the tubes by aspiration, and the 5 ml were transferred to a well in a 12 well plate and 0.5% formalin solution was added to prevent mold growth. The plates were statically incubated at 28°C for 20 days with each plate being wrapped in a paper towel in a zippered plastic bag. The viability of the eggs from soil was examined again as for eggs treated with aqueous solutions of water.

Figure 3: Daily development of *Ascaris suum* eggs culture at 28°C.

3.4. Results

All the four acids appear to be an effective way for inactivating *Ascaris suum* in water and soil.

A



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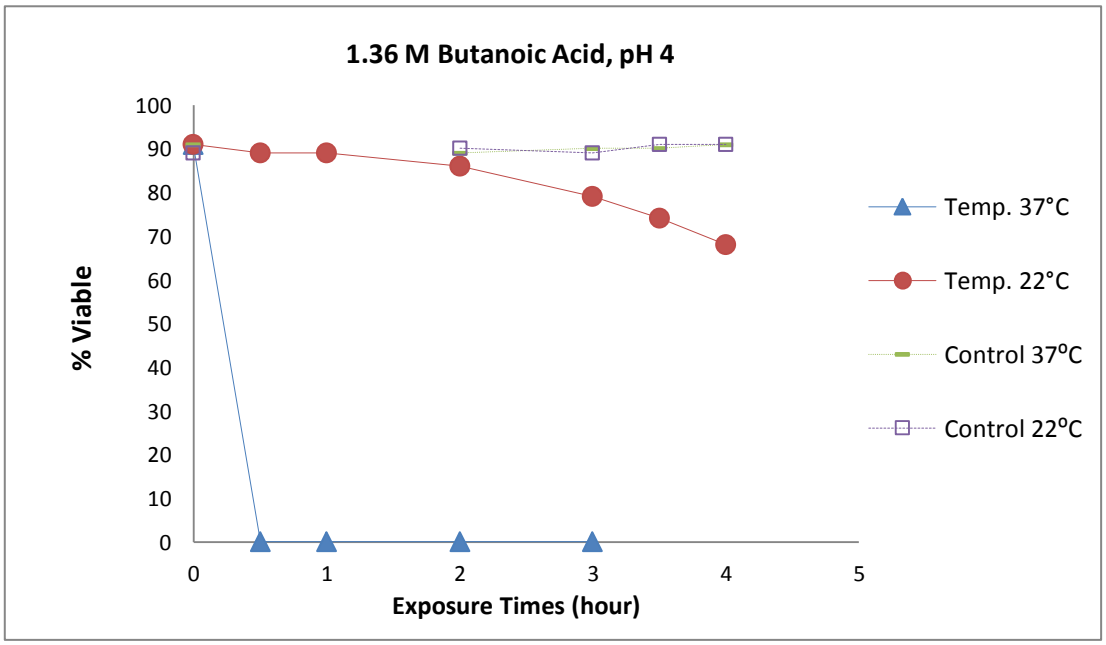


Figure 4 (a): Inactivation of *Ascaris suum* eggs by butanoic acid. A. 1.0 M butanoic acid at pH 4; B. 1.36 M butanoic acid, pH 4.

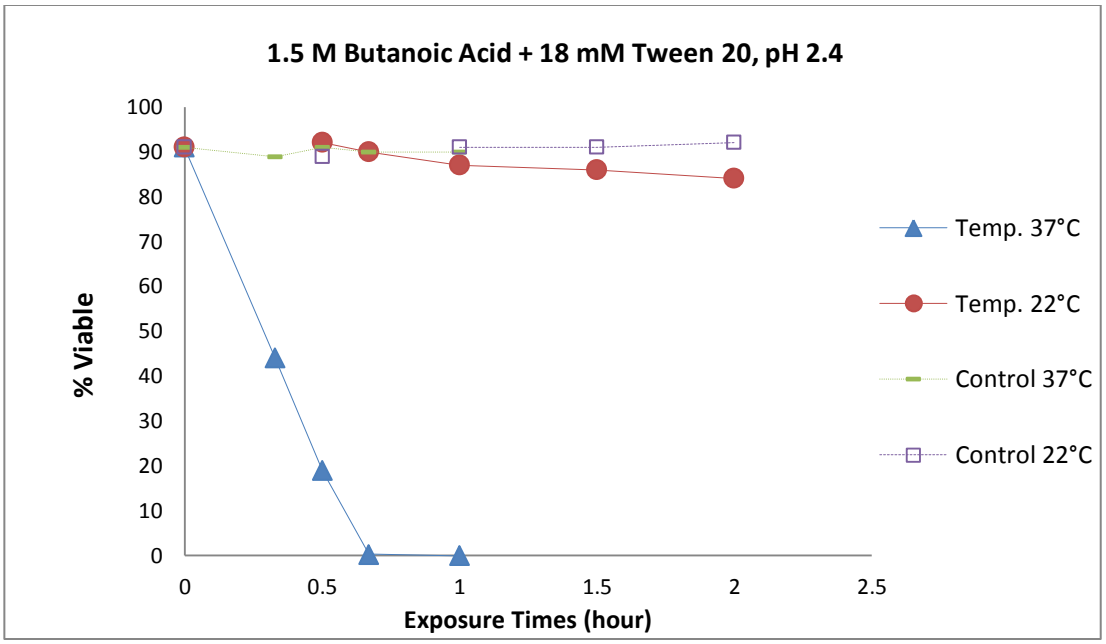
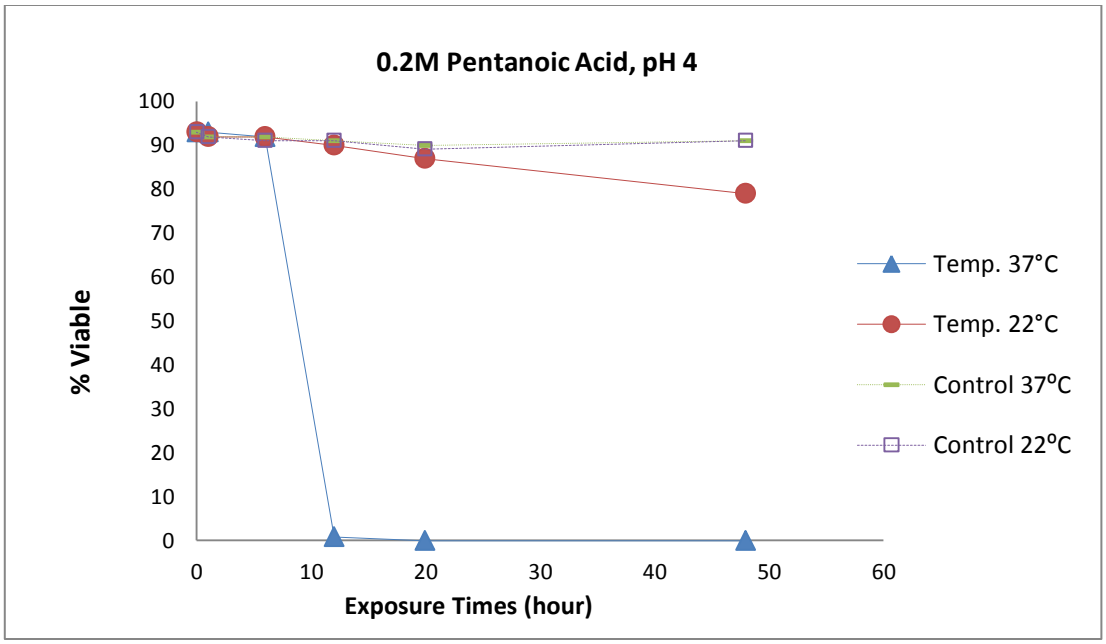


Figure 4 (b): Inactivation of *Ascaris suum* eggs by butanoic acid using 1.5 M butanoic acid with 18 mM Tween 20 at pH 2.4.

3.4.1. Butanoic Acid (Figure 4)**:** At 37°C, all eggs subjected to 1.0 M or 1.36 M butanoic acid at pH 4 or 1.5 M butanoic acid in 18 mM Tween 20 at pH 2.4 were fully inactivated by a little over a half hour. However, at 22°C, with all other conditions being the same, there was no marked difference between the eggs in acid with or without Tween 20 and the eggs maintained at 22°C as controls in water.

A



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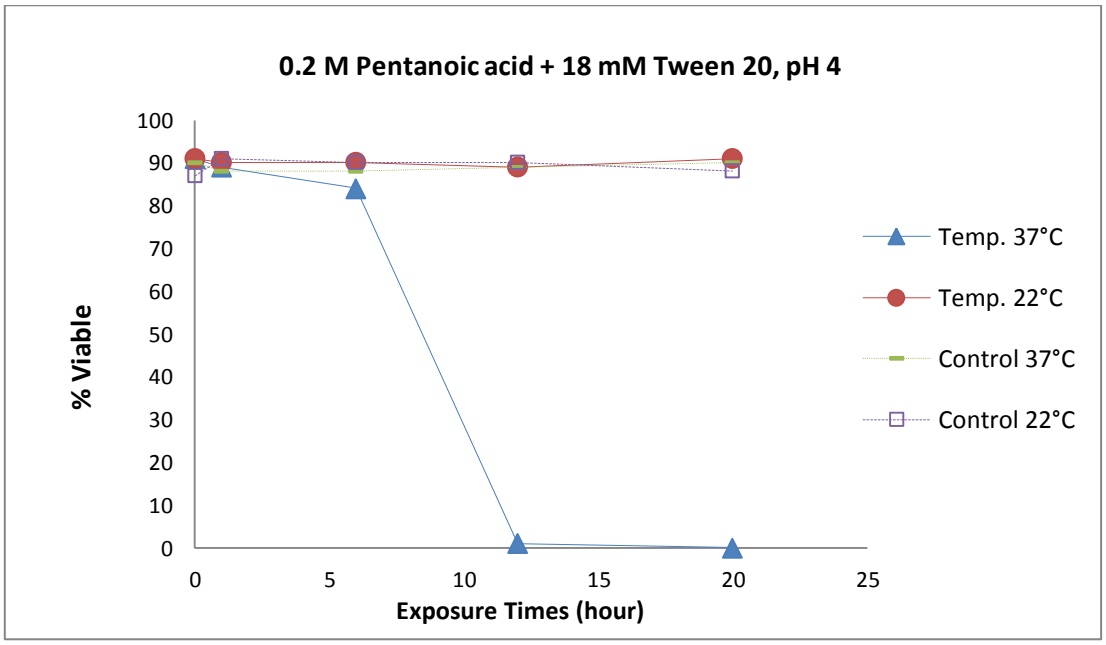
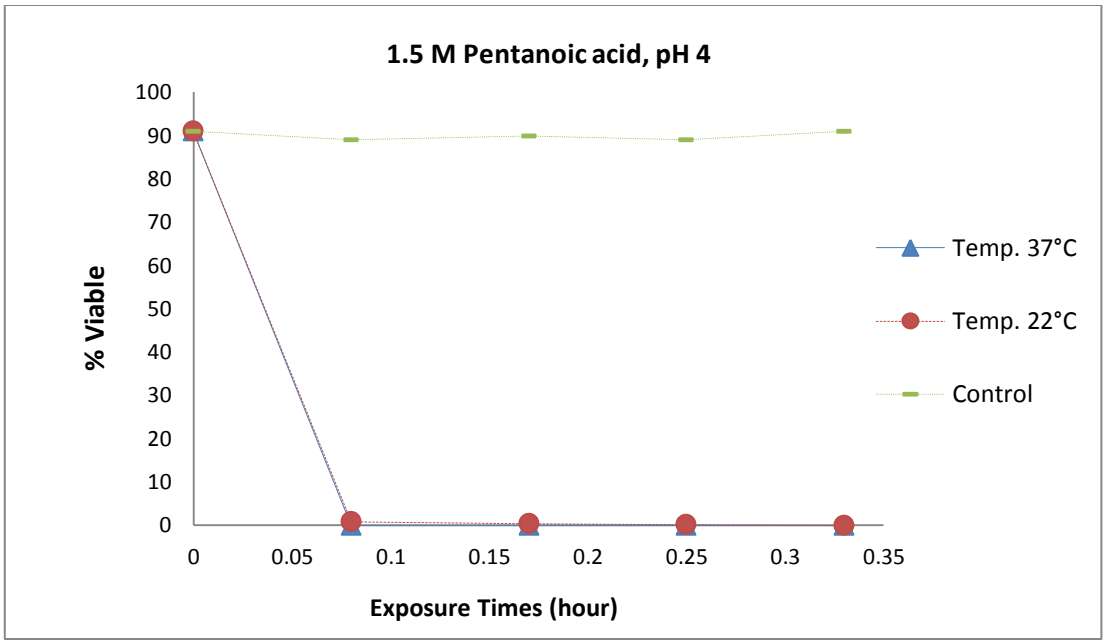


Figure 5 (a): Inactivation of *Ascaris suum* eggs by pentanoic acid. A. 0.2 M pentanoic acid at pH 4; B. 0.2 M pentanoic acid with 18 mM Tween 20 at pH 4.

A



B

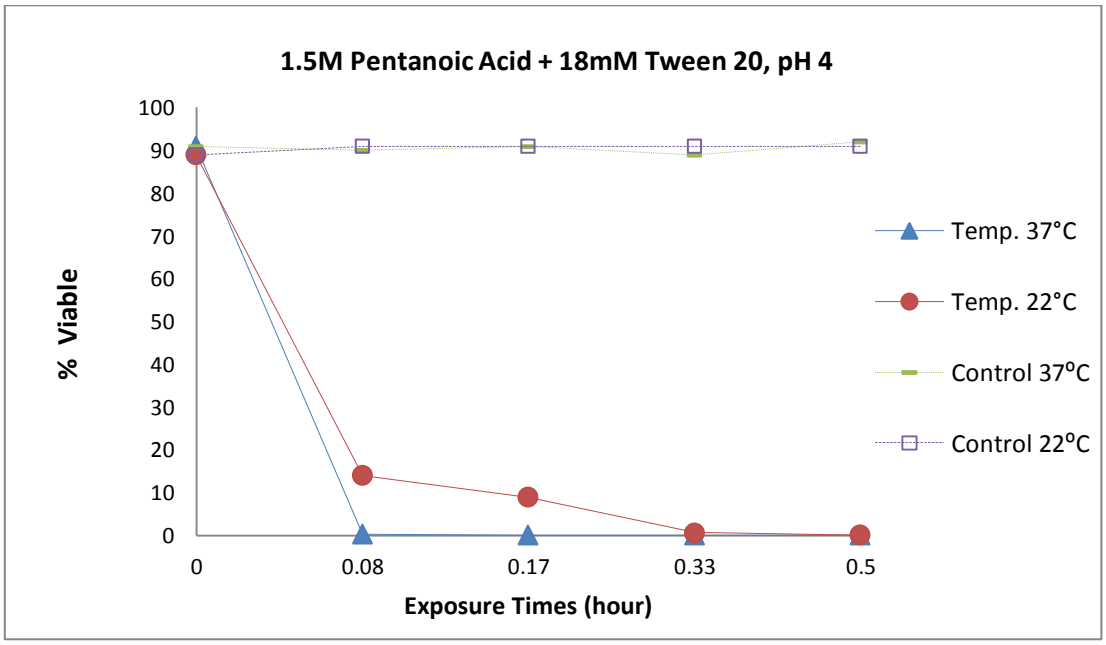
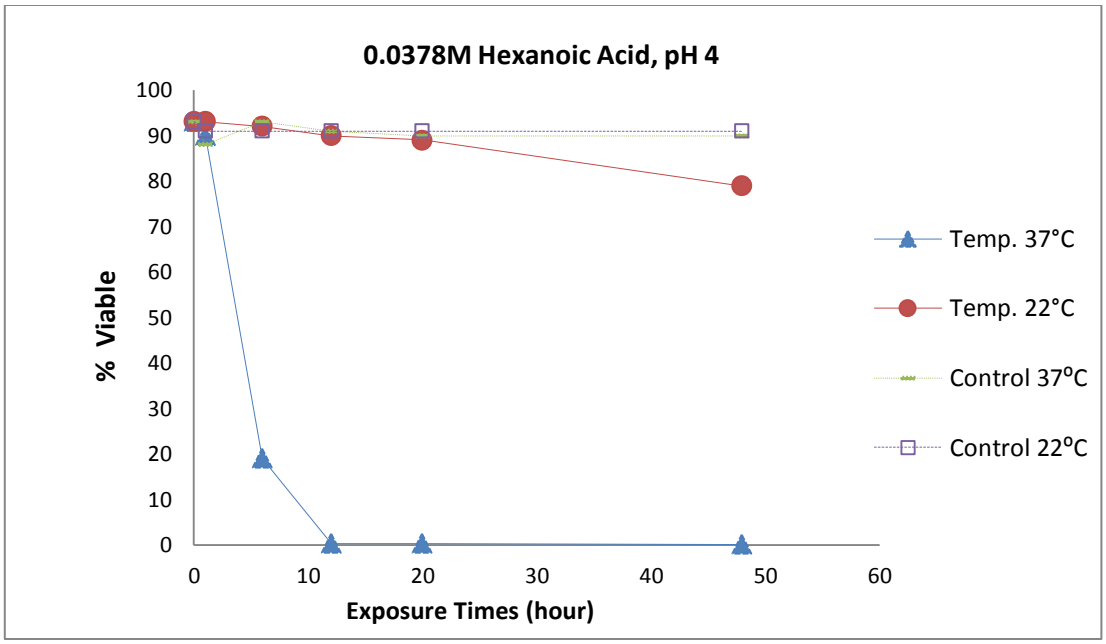


Figure 5 (b): Inactivation of *Ascaris suum* eggs by pentanoic acid. A. 1.5 pentanoic acid at pH 4; B. 1.5 M pentanoic acid with 18 mM Tween 20 at pH 4.

3.4.2. Pentanoic acid (Figure 5)**:** In the case of 0.2 M pentanoic acid at pH 4, with or without the presence of Tween 20, at 37°C, all eggs were inactivated after about 12 hours of exposure. At 22°C without the presence of Tween 20, there was an apparent increase in egg activation over time, but even at 48 hours, only about 79% of the eggs were inactivated; the eggs in the presence of Tween 20 with pentanoic acid at 0.2 M were not examined for more than 20 hours. Increasing the concentration of pentanoic acid to 1.5 M caused the complete inactivation of the eggs within less than 0.1 hour at both 22°C and 37°C, and when Tween 20 was added, the apparent rate of inactivation at 22°C seem slightly reduced; however all eggs were inactivated within 0.5 hours.

3.4.3. Hexanoic acid (Figure 6)**:** Hexanoic acid at a concentration of 0.0378 M at pH 4 caused the inactivation of all eggs at 37°C within slightly over 10 hours and only about a 21% inactivation of eggs at 22°C after 48 hours; the results with the addition of Tween 20 were basically equivalent to the results without Tween 20, although there did appear to be a quicker inactivation of eggs by 20 hours at 22°C. When the concentration of hexanoic acid was increased to 1.5 M with or without Tween 20, all eggs were inactivated by less than 0.1 hour at both 37°C and 22°C.

A



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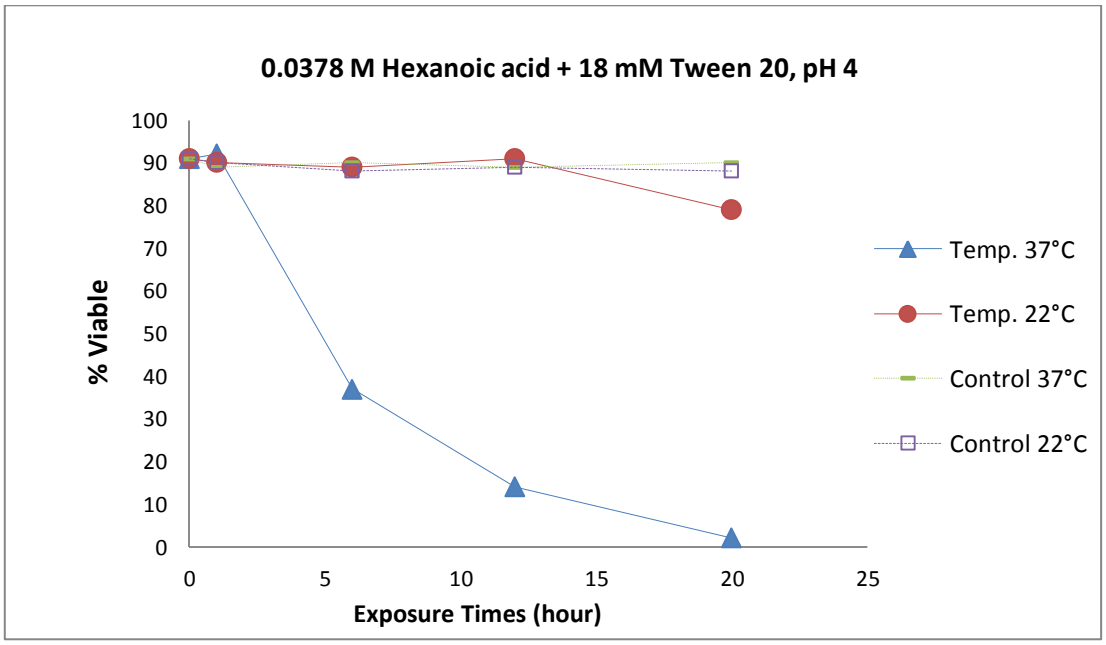
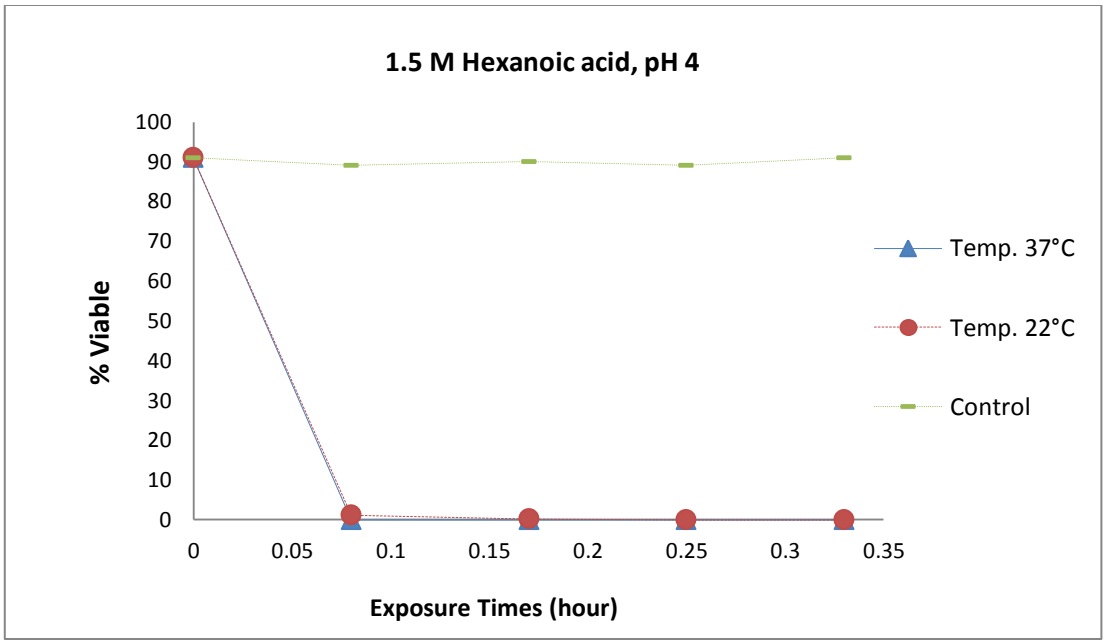


Figure 6 (a): Inactivation of *Ascaris suum* eggs by hexanoic acid. A. 0.0378 M hexanoic acid at pH 4; B. 0.0378 M hexanoic acid with 18 mM Tween 20 at pH 4.

A



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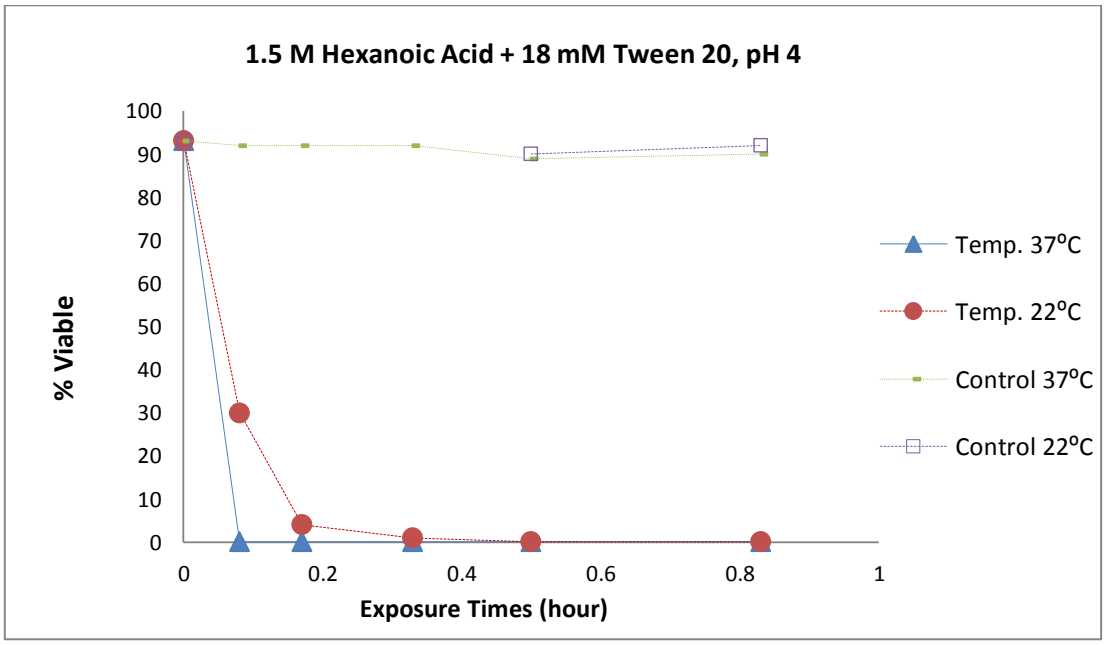

Figure 6 (b): Inactivation of *Ascaris suum* eggs by hexanoic acid. A. 1.5 hexanoic acid at pH 4; B. 1.5 M hexanoic acid with 18 mM Tween 20 at pH 4.

3.4.4. Heptanoic acid (Figure 7)**:** Heptanoic acid was only examined in one experiment at 1.5 M with 18 mM Tween 20 at pH 4. At 37°C, 100% of the eggs were inactivated at the first time point examined – 1 hour after exposure. As all the eggs were inactivated at the first time point, and further experiments were not done in less than 1 hour, it is not sure that at 37°C, the contact time point less that 1 hour might be able to inactivate eggs up to 100%. At 22°C, complete inactivation with heptanoic acid and Tween 20 did not occur until after 7 hours of exposure.

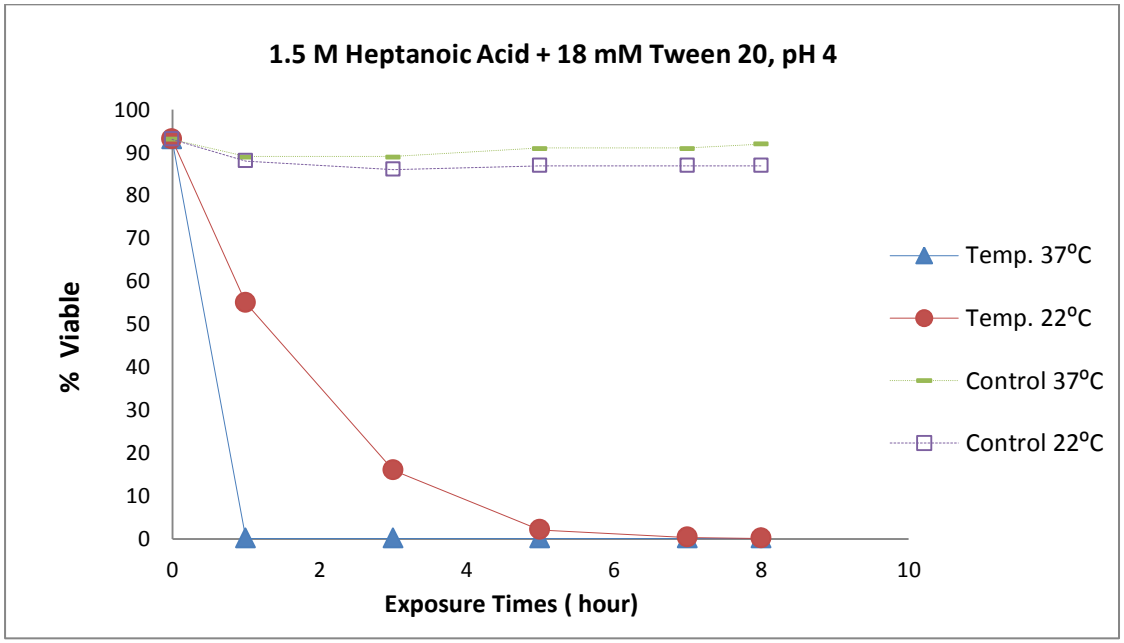

Figure 7: Inactivation of *Ascaris suum* eggs by 1.5 M heptanoic acid with 18 mM Tween 20 at pH 4.

3.4.5. Eggs in soil (Figure 8)**:** The treatment of eggs in soil at 4°C, 22°C and 37°C were done in the presence of 18 mM Tween 20 at pH 4 using 1.5 M pentanoic and 1.5 M hexanoic acid. There was no inactivation in either acid at 4°C or 22°C after the last time point at 6.7 hours. At 37°C, in the case of pentanoic acid, there was a 50% inactivation by 2 hours with all eggs being inactivated after 4 hours of exposure. With hexanoic acid, there was no inactivation at 2 hours, but all eggs were similarly inactivated after 4 hours of exposure.

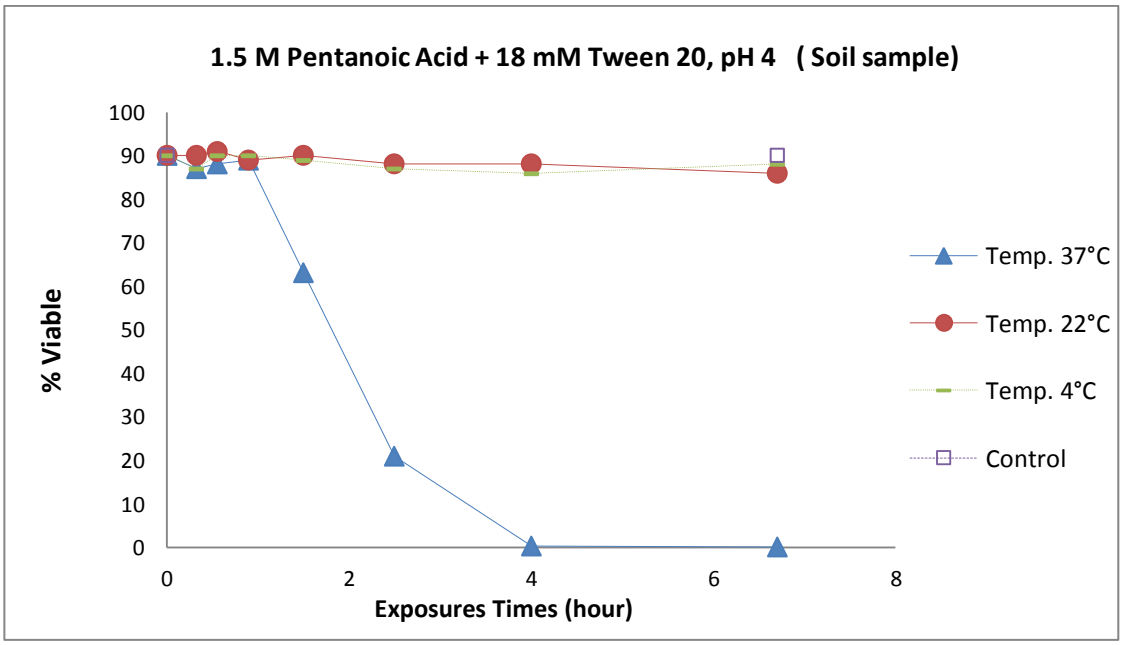

Figure 8 (a): Inactivation of *Ascaris suum* eggs with the addition of 1.5 M pentanoic acid with 18 mM Tween 20 at pH 4.

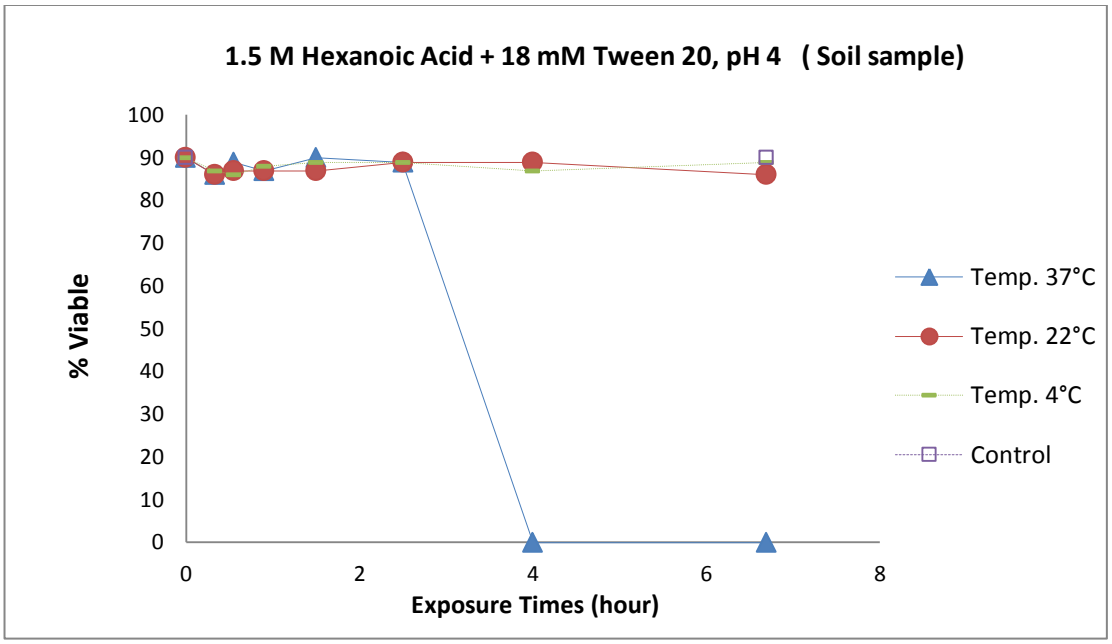

Figure 8 (b): Inactivation of *Ascaris suum* eggs with the addition of 1.5 M hexanoic acid with 18 mM Tween 20 at pH 4.

3.4.6. Appearance of eggs after treatments:

In case of all acids, treatment with lower concentration did not cause any significant damage to outer albuminous layer of the egg. Moreover, after 20 or more days of culture when the eggs were being scored for viability, 6% sodium hypochlorite (Clorox) was added to remove the outer coating for easier visibility of the egg's contents. The inside of the eggs remained intact either contained a developing larva or a single undeveloped cell or in some cases bubbly egg contents (Figure 9 left). However, during the examination of eggs treated with the higher acid concentrations, with or without Tween 20, even when examined almost immediately after acid addition, if the eggs were subjected to the same sodium hypochlorite treatment, the zygote within the egg would immediately become clear, or the egg would rupture and rapidly release its

contents (Figure 9 right). In soil sample, the eggs were although treated with higher concentrated acids, the appearance did not change, but they were dead.

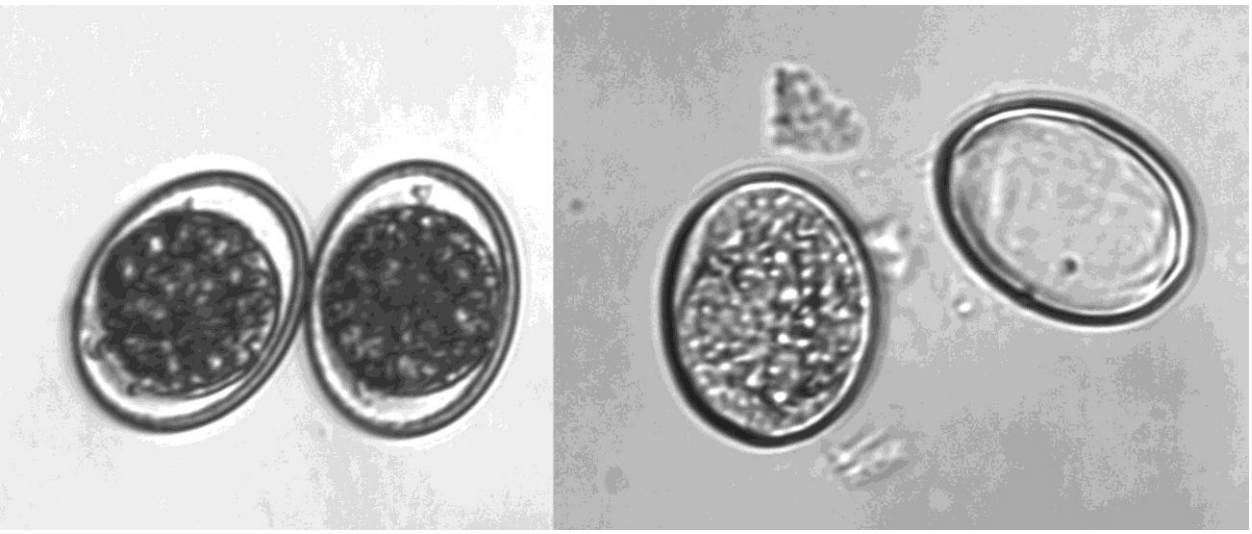

Figure 9: Effects of sodium hypochlorite on the appearance of non-viable *Ascaris suum* eggs after treatment with differing concentrations of pentanoic acid. Sodium hypochlorite treated eggs from 20-day culture after treatment with 0.2 M pentanoic acid at pH 4 at 37°C (Left picture). Sodium hypochlorite treated eggs from 20-day culture after treatment with 1.5 M pentanoic acid with Tween 20 at pH 4 at 22°C (Right picture). The image on the right shows how the internal contents of the eggs are destroyed within a minute after the addition of sodium hypochlorite for the purpose of removing any remaining portions of the albuminous layer.

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3.5. Discussions

According to Feachem (1983) and Hays (1997), to inactivate helminth eggs the temperature must be raised above 40°C for 10 to 20 days or moisture must be reduced below 5% (i.e. a TS. 95%). The results of our research showed that the effect of SCFAs on the viability of *A. suum* eggs was dependent on acid concentration and the temperature at which the exposure occurs. An elevation of 15°C, from 22°C to 37°C, had a marked increase on the killing of the eggs by the acids. At 37°C, with eggs in water with 1.5 M butanoic, pentanoic, or hexanoic acids, all eggs were inactivated within less than 1 hour of contact, and under these conditions both pentanoic and hexanoic acids inactivated the eggs after only 5 minutes of contact. At 22°C, only the 5- and 6-chain acids had a rate of inactivation that was similar to that observed at 37°C. Addition of Tween 20 to the 1.5 M solutions of the 4-6 chain fatty acids had very little effect on the rate of kill, but did slightly lower the rate of inactivation in the case of 1.5 M pentanoic acid. The effects of heptanoic acid were only examined at 1.5 M in the presence of Tween 20, and like the effects of the 5- and 6-chain fatty acids, all eggs were 100% inactivated within an hour at 37°C, but unlike hexanoic acid at 22°C, 8 hours, rather than minutes, were required for complete inactivation of all exposed eggs. Overall in aqueous solutions, 100% of the eggs treated with the SCFAs, with or without Tween 20, were killed in less than 15 hours. At 37°C, 100% of eggs treated with 1.5 M pentanoic or hexanoic acid, with or without Tween 20 were killed in less than 10 minutes. The uses of lower concentrations of acid (0.2 M pentanoic and 0.0378 M hexanoic) significantly increased the time needed for exposure from minutes to more than 10 hours.

When the eggs were added to soil, the speed of kill of both the 1.5 M pentanoic and hexanoic acids at pH 4 were reduced from several minutes to around 4 hours at 37°C. At both 22°C and also at 4°C, there was no inactivating effect of the two acids observed within the maximum exposure time of 6.7 hours. Although the final concentration of acid in the soil was probably

only around 0.5 M since the pre-wetted soil contained about 50% water, this is still near the published maximal solubility of the acid in water, i.e., 0.487 M. It is impossible at this time to sort out if the difference was due to the lowered concentration of the acids caused by interaction with the soil particulates or due to static hindrances caused by these particles. Thus, it might be of interest to determine if higher application rates could still cause faster inactivation in pre-wetted soil or whether longer contact times would show an increased inactivation at the lower temperatures that were tested.

Treating *A. suum* in water samples with lower acid concentrations in lower temperature (22°) used in this study did not reduce significantly the egg viability, while the highest temperature (37°) in same acid concentrations inactivated all eggs after hours. Although soil samples were treated with higher concentrated acids, same results were observed in case of both pentanoic and hexanoic treatment i,e, higher temperature (37°C) was able to inactivate eggs quickly compared to lower temperature (22°C). This suggests that, under laboratory conditions, 37°C is the minimum temperature required to achieve total inactivation with no other agent or chemical added to the acid in lower concentration in aqueous solution and higher acid concentration with Tween 20 in soil matters. 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* eggs under similar experimental conditions (Ghiglietti, 1995; Pecson & Nelson, 2005). Temperature, by itself, is able to inactivate the parasite (a minimum of 45°C for a period of 1 year) (Feachem, 1983). With a sufficiently long exposure to heat, the resilient lipid membrane of the egg begins to lose its integrity, rendering the egg vulnerable to osmotic effects or chemicals present in the external milieu (Barrett, 1976). Our results also demonstrated the increased temperature like 37°C is enough to inactivate *Ascaris* eggs even in lower acid concentration, but in this case more contact time is required compared to higher acid concentration. At low temperature, time of exposure becomes an important factor to inactivate

the parasite. At 22°C, Ghiglietti (1997) observed 100% *Ascaris* spp. ova inactivation in sludge (2%v/w, pH=12) in 40-60 days. The steep relationship between inactivation rate and temperature is reflected in the Arrhenius correlations for *Ascaris suum* over the temperature range 49-53°C. Kato (2003) observed high reductions of *Ascaris suum* (four-log) within 1 hour of incubation in anaerobically digesting biosolids at 55°C. The EPA time-temperature relationships are partly justified as being consistent with requirements for pasteurization of eggnog under U.S. Food and Drug Administration (FDA) regulations (EPA/625/R-92/013). Inspection reveals that the slope of the time-temperature relationship in the FDA regulations is dominated by extrapolation from high-temperature/ short-time pasteurization conditions (83°C for 15 s or 80°C for 25 s) to a single data point: 30 minutes pasteurization at 69°C (U. S. Code of Federal Regulations). We also question the relevance of extrapolating thermal inactivation parameters from eggnog to sludge because of the substantial differences in the matrices, which can influence microbial inactivation kinetics. The proteins, fats, and carbohydrates found in milk products can increase the heat resistance of microbes (Jay, 2000), and the nature of these constituents is likely to differ substantially in biosolids.

Organic acids are weakly acidic since they do not readily donate protons in aqueous solution. The relative strength of an acid is reflected in its dissociation constant K_a, or pK_a (-log K_a). The acid (HA) dissociates in water to the proton (H^+) and anion (A^-) such that at equilibrium $[H^+]\,[A^-] / [HA] = K_a$, the dissociation constant which is dependent on acid concentration. Dissociation of weak acids is also pH dependent and increases as the pH values approached neutrality (Cherrington, 1991). The antimicrobial activity of organic acids increases with decreasing pH value, and since a greater proportion of undissociated molecule exist as the pH value decreases (Freese, 1973).

The changes in the eggshell observed in the presence of the higher concentration of acid is most likely due to the damage of the protective ascaroside layer, this is the thin, clear, internal

portion of the eggshell that has been described in detail (Fairbairn and Passey, 1955). This condition was also observed when James P. Lahnert used phenol as an ovicide. Phenol alone acts slowly, embryonated eggs being killed in 5 min, showing swollen and thickened shells after 9 min. The eggs are nearly cleared after 20 min and later cleared to a colorless condition with the embryo destroyed (Lehnert, 1972). Yoshida (1920) illustrated the function of the shell in protecting the vitellus or embryo from acids, alkalis, or corrosive chemicals. It has also been established that the vitelline membrane is primarily responsible for this effect. Thus, acid and alkali dissolve the external coat, and hypochlorite dissolve the hard shell as well, without making the embryo more susceptible to damage by these agents. Using decorticated embryonated eggs, it has been shown that the embryos within the eggshell are unaffected by placing the eggs in 2 N hydrochloric acid, 2 N nitric acid, 2 N sodium hydroxide, 0.5 ammonium hydroxide, 3.3 N formaldehyde, and 4 N sodium chloride, but if the eggs are slightly crushed when in these solutions the larva inside is rapidly dissolved (Fairbairn and Passey, 1955). It was also shown by these same authors that organic solvents, e.g., chloroform rapidly dissolve the membrane and kill the larva inside, and that the membrane be melted with heat at 75°C. The membrane is formed of unsaponifiable lipids with ascaryl alcohol making up 77% of these lipids. The lipoid nature of this membrane renders it sensitive to the action of many organic solvents. Among the solvents which dissolve it may be mentioned chloroform, ethyl ether, alcohols, phenols and cresols. The SCFAs might be capable of inactivating *Ascaris* eggs was first suggested in 1951 (Takeyama, 1951). In this early and brief discussion of the effects of SCFAs, it was stated that butanoic acid killed *Ascaris* eggs in 15 to 25 days at 17°C and in 50 to 60 days at 3-7°C. More recently, butanoic, pentanoic, and hexanoic have been shown to be toxic to *Ascaris* eggs with the effect being most dramatic when the solution is maintained at a pH below the pKa of the SCFA; these studies were all performed at 37°C. In the studies reported here, the work has examined the effects of a reduced temperature, and found that at 22°C, saturated pentanoic and hexanoic acid (aqueous acid 1.5 M at pH 4) could inactivate eggs within very short periods

as at 37°C. In soil, the 1.5 M pentanoic and hexanoic acids inactivated the eggs within 4 hours at 37°C. Also, the examination of the eggshell and its response to treatment with the usually impermeable sodium hydroxide indicates that the SCFA must be interacting to cause fatal alterations in the ascaroside membrane making the egg permeable to many agents that are liable to kill the egg inside. The next step for direct applicability will be to those conditions that alter the eggshell the most rapidly under different temperature conditions. However, in the case relative to treating pathogens in biosolids within anaerobic digesters, lagoons, or septic systems, the detention times are often days and typically several to many weeks. Thus, further work needs to define minimum levels that are 100% efficacious in periods such as one or two weeks.

Using the SCFAs, it may be possible to develop means for the rapid damage of the ascaroside layer that would allow the easy penetration of other agents into the eggshell. Also, since most nematodes have similar layers protecting the zygote and developing larva inside the eggshell, this suggests that these SCFAs could be developed in a staging manner for treating nematodes in the environment, such as with plant parasitic nematodes or soil-transmitted pathogens, where the SCFA could be used alone or simply as an initial agent make the membrane permeable. It has been observed that when the eggs are placed in the highly lethal SCFAs, that from almost immediately to within minutes after application the ascaroside layer is sufficiently damaged to allow the dissolution of the zygote by sodium hypochlorite which is usually not at all harmful to the egg. Also, it should be reiterated that in the work reported here, the eggs being used are fully corticated eggs with the complete albuminous, protein, and chitinous layers over the internal ascaroside layer.

APPENDIX

Experiment using 1.5 M butanoic acid with 18 mM Tween 20, pH 2.4

Experiment Start Date	Incubation Time	Incubation Set Temparature	Date after 20 days	Sample Viable/ Non Viable	Sample % Viable	Control Viable/ Non Viable	Control % Viable
Mar.05.13	30 min	22°C	Mar.25.13	124/11	92%	105/13	89%
Mar.05.13	40 min	22°C	Mar.25.13	102/11	90%		
Mar.05.13	60 min	22°C	Mar.25.13	107/16	87%	116/12	91%
Mar.05.13	90 min	22°C	Mar.25.13	119/20	86%	102/10	91%
Mar.05.13	120 min	22°C	Mar.25.13	136/26	84%	113/10	92%
Mar.06.13	20 min	37°C	Mar.26.13	267/336	44%	109/13	89%
Mar.06.13	30 min	37°C	Mar.26.13	53/219	19%	107/10	91%
Mar.06.13	40 min	37°C	Mar.26.13	2/600+	0.3%	115/13	90%
Mar.06.13	60 min	37°C	Mar.26.13	0/700+	0%	106/12	90%

Experiment using 0.2 M pentanoic acid, pH 4

Experiment Start Date	Incubation Time	Incubation Set Temparature	Date after 20 days	Sample Viable/ Non Viable	Sample % Viable	Control Viable/ Non Viable	Control % Viable
Dec.20.12	1 hour	22°C	Jan.10.13	98/9	92%	103/9	92%
Dec.20.12	6 hour	22°C	Jan.10.13	120/1	92%	97/10	91%
Dec.19.12	12 hour	22°C	Jan.09.13	114/12	90%	101/10	91%
Jan.14.13	20 hour	22°C	Feb.04.13	119/17	87%	129/16	89%
Jan.14.13	48 hour	22°C	Feb.06.13	172/45	79%	119/12	91%
Dec.13.12	1 hour	37°C	Jan.02.13	192/15	93%	113/10	92%
Dec.13.12	6 hour	37°C	Jan.02.13	183/16	92%	98/9	92%
Dec.13.12	12 hour	37°C	Jan.03.13	2/255	0.8%	107/11	91%
Dec.13.12	20 hour	37°C	Jan.03.13	1/768	0.1%	114/12	90%
Dec.13.12	48 hour	37°C	Jan.03.13	0/700+	0%	108/11	91%

Experiment using 0.2 M pentanoic acid with 18 mM Tween 20, pH 4

Experiment Start Date	Incubation Time	Incubation Set Temparature	Date after 20 days	Sample Viable/ Non Viable	Sample % Viable	Control Viable/ Non Viable	Control % Viable
May.28.13	5 min	22°C	June.17.13	116/14	89%	101/10	91%
May.28.13	15 min	22°C	June.17.13	125/15	89%	119/13	90%
May.28.13	30 min	22°C	June.17.13	109/15	88%	99/12	89%
May.30.13	1 hour	22°C	June.19.13	103/11	90%	106/10	91%
May.30.13	6 hour	22°C	June.19.13	99/11	90%	112/12	90%
May.30.13	12 hour	22°C	June.19.13	101/12	89%	103/11	90%
May.30.13	20 hour	22°C	June.19.13	110/11	91%	107/14	88%
May.28.13	5 min	37°C	June.17.13	105/14	88%	110/14	89%
May.28.13	15 min	37°C	June.17.13	103/16	87%	118/13	90%
May.28.13	30 min	37°C	June.17.13	121/18	87%	101/10	91%
June.05.13	1 hour	37°C	June.25.13	126/15	89%	113/15	88%
June.05.13	6 hour	37°C	June.25.13	228/43	84%	105/14	88%
June.05.13	12 hour	37°C	June.25.13	1/294	1%	102/12	89%
May.28.13	20 hour	37°C	June.17.13	0/700+	0%	108/12	90%

Experiment using 1.5 M pentanoic acid, pH 4

Experiment Start Date	Incubation Time	Incubation Set Temparature	Date after 20 days	Sample Viable/ Non Viable	Sample % Viable	Control Viable/ Non Viable	Control % Viable
May.15.13	5 min	22°C	June.04.13	3/356	0.8%	117/15	89%
May.15.13	10 min	22°C	June.04.13	1/348	0.3%	101/11	90%
May.15.13	15 min	22°C	June.04.13	1/700+	0.1%	103/13	89%
May.15.13	20 min	22°C	June.04.13	0/1300+	0%	108/11	91%
May.16.13	5 min	37°C	June.05.13	0/1300+	0%	117/15	89%
May.16.13	10 min	37°C	June.05.13	0/1300+	0%	101/11	90%
May.16.13	15 min	37°C	June.05.13	0/1300+	0%	103/13	89%
May.16.13	20 min	37°C	June.05.13	0/1300+	0%	108/11	91%

Experiment using 1.5 M pentanoic acid with 18 mM Tween 20, pH 4

Experiment Start Date	Incubation Time	Incubation Set Temparature	Date after 20 days	Sample Viable/ Non Viable	Sample % Viable	Control Viable/ Non Viable	Control % Viable
Dec.28.12	5 min	22°C	Jan.17.13	52/320	14%	98/10	91%
Dec.28.12	10 min	22°C	Jan.17.13	29/279	9%	110/11	91%
Dec.28.12	20 min	22°C	Jan.17.13	2/330	0.6%	101/10	91%
Dec.28.12	30 min	22°C	Jan.17.13	0/723	0%	109/11	91%
Jan.11.13	5 min	37°C	Jan.31.13	1/368	0.27%	96/11	90%
Jan.11.13	10 min	37°C	Jan.31.13	0/700+	0%	96/9	91%
Jan.11.13	20 min	37°C	Jan.31.13	0/700+	0%	92/11	89%
Jan.11.13	30 min	37°C	Jan.31.13	0/700+	0%	102/9	92%

Experiment using 0.0378 M hexanoic acid, pH 4

Experiment Start Date	Incubation Time	Incubation Set Temparature	Date after 20 days	Sample Viable/ Non Viable	Sample % Viable	Control Viable/ Non Viable	Control % Viable
Dec.28.12	1 hour	22°C	Jan.17.13	104/7	94%	107/11	91%
Dec.26.12	6 hour	22°C	Jan.15.13	109/9	92%	101/10	91%
Dec.19.12	12 hour	22°C	Jan.09.13	117/13	90%	112/11	91%
Jan.14.13	20 hour	22°C	Feb.04.13	112/13	89%	109/11	91%
Jan.14.13	48 hour	22°C	Feb.06.13	154/42	79%	106/12	91%
Jan.02.13	1 hour	37°C	Jan.23.13	106/12	90%	119/17	88%
Jan.02.13	6 hour	37°C	Jan.23.13	57/337	19%	99/8	93%
Jan.02.13	12 hour	37°C	Jan.23.13	1/341	0.29%	107/10	91%
Jan.02.13	20 hour	37°C	Jan.23.13	1/326	0.3%	112/13	90%
Jan.15.13	48 hour	37°C	Feb.07.13	0/700+	0%	116/13	90%

Experiment using 0.0378 M hexanoic acid with 18 mM Tween 20, pH 4

Experiment Start Date	Incubation Time	Incubation Set Temparature	Date after 20 days	Sample Viable/ Non Viable	Sample % Viable	Control Viable/ Non Viable	Control % Viable
May.28.13	5 min	22°C	June.17.13	108/14	89%	104/11	90%
May.28.13	15 min	22°C	June.17.13	103/14	88%	121/15	89%
May.28.13	30 min	22°C	June.17.13	98/11	90%	116/13	90%
May.30.13	1 hour	22°C	June.19.13	110/12	90%	128/15	90%
May.30.13	6 hour	22°C	June.19.13	101/12	89%	103/14	88%
May.30.13	12 hour	22°C	June.19.13	108/11	91%	110/13	89%
May.30.13	20 hour	22°C	June.19.13	136/37	79%	105/14	88%
May.28.13	5 min	37°C	June.17.13	113/14	89%	117/13	90%
May.28.13	15 min	37°C	June.17.13	97/13	88%	105/9	92%
May.28.13	30 min	37°C	June.17.13	106/13	89%	101/14	88%
June.05.13	1 hour	37°C	June.25.13	113/10	92%	112/14	89%
June.05.13	6 hour	37°C	June.25.13	128/219	37%	104/12	90%
June.05.13	12 hour	37°C	June.25.13	29/172	14%	101/13	89%
May.28.13	20 hour	37°C	June.17.13	6/343	2%	107/12	90%

Experiment using 1.5 M hexanoic acid, pH 4

Experiment Start Date	Incubation Time	Incubation Set Temparature	Date after 20 days	Sample Viable/ Non Viable	Sample % Viable	Control Viable/ Non Viable	Control % Viable
May.15.13	5 min	22°C	June.04.13	4/303	1.2%	117/15	89%
May.15.13	10 min	22°C	June.04.13	2/700+	0.3%	101/11	90%
May.15.13	15 min	22°C	June.04.13	1/900+	0.1%	103/13	89%
May.15.13	20 min	22°C	June.04.13	0/900+	0%	108/11	91%
May.16.13	5 min	37°C	June.05.13	0/700+	0%	117/15	89%
May.16.13	10 min	37°C	June.05.13	0/700+	0%	101/11	90%
May.16.13	15 min	37°C	June.05.13	0/700+	0%	103/13	89%
May.16.13	20 min	37°C	June.05.13	0/700+	0%	108/11	91%

Experiment using 1.5 M hexanoic acid with 18 mM Tween 20, pH 4

Experiment Start Date	Incubation Time	Incubation Set Temparature	Date after 20 days	Sample Viable/ Non Viable	Sample % Viable	Control Viable/ Non Viable	Control % Viable
Jan.15.13	30 min	22°C	Feb.04.13	2/1407	0.1%	116/12	91%
Jan.15.13	50 min	22°C	Feb.04.13	0/700+	0%	105/9	92%
Jan.15.13	60 min	22°C	Feb.04.13	0/700+	0%	109/10	92%
Jan.16.13	70 min	22°C	Feb.05.13	0/700+	0%	116/12	91%
Jan.16.13	80 min	22°C	Feb.05.13	0/700+	0%	124/15	89%
Jan.29.13	30 min	37°C	Feb.18.13	0/1300+	0%	118/15	89%
Jan.29.13	50 min	37°C	Feb.18.13	0/1300+	0%	123/14	90%
Jan.29.13	60 min	37°C	Feb.18.13	0/1300+	0%	108/9	92%
Jan.29.13	70 min	37°C	Feb.18.13	0/1300+	0%	112/10	92%
Jan.29.13	80 min	37°C	Feb.18.13	0/1300+	0%	107/10	91%
May.17.13	5 min	22°C	June.06.13	44/103	30%	101/11	90%
May.17.13	10 min	22°C	June.06.13	5/108	4%	101/11	90%
May.17.13	20 min	22°C	June.06.13	3/346	0.9%	101/11	90%
May.09.13	5 min	37°C	May.29.13	0/700+	0%	124/11	92%
May.09.13	10 min	37°C	May.29.13	0/700+	0%	124/11	92%
May.09.13	20 min	37°C	May.29.13	0/700+	0%	124/11	92%

Experiment using 1.5 M heptanoic acid with 18 mM Tween 20, pH 4

Experiment Start Date	Incubation Time	Incubation Set Temparature	Date after 20 days	Sample Viable/ Non Viable	Sample % Viable	Control Viable/ Non Viable	Control % Viable
Feb.08.13	1 hour	22°C	Feb.28.13	129/105	55%	124/17	88%
Feb.08.13	3 hour	22°C	Feb.28.13	36/192	16%	121/20	86%
Feb.08.13	5 hour	22°C	Feb.28.13	12/600+	2%	116/17	87%
Feb.08.13	7 hour	22°C	Feb.28.13	3/1300+	0.2%	126/19	87%
Feb.08.13	8 hour	22°C	Feb.28.13	2/1600+	0.1%	134/20	87%
Feb.16.13	1 hour	37°C	Mar.08.13	1/900+	0.1%	119/17	89%
Feb.16.13	3 hour	37°C	Mar.08.13	0/900+	0%	111/13	89%
Feb.16.13	5 hour	37°C	Mar.08.13	0/1200+	0%	106/11	91%
Feb.18.13	7 hour	37°C	Mar.10.13	0/1200+	0%	127/12	91%
Feb.18.13	8 hour	37°C	Mar.10.13	0/1200+	0%	113/10	92%

Experiment using 1.5 M pentanoic acid with 18 mM Tween 20, pH 4

(Soil Sample)

Experiment Start Date	Incubation Time	Incubation Set Temparature	Date after 20 days	Sample Viable/ Non Viable	Sample % Viable	Control Viable/ Non Viable	Control % Viable
Apr.09.13	1.65 min	22°C	Apr.29.13	110/14	89%	109/12	90%
Apr.09.13	2.72 min	22°C	Apr.29.13	117/13	90%		
Apr.09.13	4.48 min	22°C	Apr.29.13	124/20	86%		
Apr.09.13	7.39 min	22°C	Apr.29.13	108/15	88%		
Apr.12.13	12.18 min	22°C	May.02.13	115/19	86%		
Apr.12.13	20.09 min	22°C	May.02.13	113/12	90%		
Apr.16.13	33.12 min	22°C	May.06.13	127/12	91%		
Apr.16.13	54.6 min	22°C	May.06.13	122/15	89%		
Apr.16.13	90.02 min	22°C	May.06.13	118/13	90%		
Apr.16.13	148.4 min	22°C	May.06.13	116/16	88%		
Apr.22.13	244.7 min	22°C	May.12.13	124/17	88%		
Apr.22.13	403.4 min	22°C	May.12.13	107/13	89%	116/13	90%

Experiment using 1.5 M pentanoic acid with 18 mM Tween 20, pH 4

(Soil Sample)

Experiment Start Date	Incubation Time	Incubation Set Temparature	Date after 20 days	Sample Viable/ Non Viable	Sample % Viable	Control Viable/ Non Viable	Control % Viable
Apr.19.13	1.65 min	37°C	May.09.13	121/13	90%	127/14	90%
Apr.19.13	2.72 min	37°C	May.09.13	118/16	88%		
Apr.19.13	4.48 min	37°C	May.09.13	113/14	89%		
Apr.19.13	7.39 min	37°C	May.09.13	126/13	91%		
Apr.19.13	12.18 min	37°C	May.09.13	117/15	89%		
Apr.19.13	20.09 min	37°C	May.09.13	107/18	87%		
Apr.21.13	33.12 min	37°C	May.11.13	116/16	88%		
Apr.21.13	54.6 min	37°C	May.11.13	125/15	89%		
Apr.22.13	90.02 min	37°C	May.12.13	184/106	63%		
Apr.22.13	148.4 min	37°C	May.12.13	53/196	21%		
Apr.22.13	244.7 min	37°C	May.12.13	2/457	0.4%		
Apr.22.13	403.4 min	37°C	May.12.13	0/700+	0%	121/13	90%

Experiment using 1.5 M pentanoic acid with 18 mM Tween 20, pH 4

(Soil Sample)

Experiment Start Date	Incubation Time	Incubation Set Temparature	Date after 20 days	Sample Viable/ Non Viable	Sample % Viable	Control Viable/ Non Viable	Control % Viable
Apr.10.13	1.65 min	4°C	Apr.30.13	113/15	88%	129/15	90%
Apr.10.13	2.72 min	4°C	Apr.30.13	119/15	89%		
Apr.10.13	4.48 min	4°C	Apr.30.13	124/16	89%		
Apr.10.13	7.39 min	4°C	Apr.30.13	124/21	86%		
Apr.12.13	12.18 min	4°C	May.02.13	127/16	89%		
Apr.12.13	20.09 min	4°C	May.02.13	121/18	87%		
Apr.16.13	33.12 min	4°C	May.06.13	117/13	90%		
Apr.16.13	54.6 min	4°C	May.06.13	110/12	90%		
Apr.16.13	90.02 min	4°C	May.06.13	112/14	89%		
Apr.16.13	148.4 min	4°C	May.06.13	126/19	87%		
Apr.22.13	244.7 min	4°C	May.12.13	123/20	86%		
Apr.22.13	403.4 min	4°C	May.12.13	124/17	88%	125/14	90%

Experiment using 1.5 M hexanoic acid with 18 mM Tween 20, pH 4

(Soil Sample)

Experiment Start Date	Incubation Time	Incubation Set Temparature	Date after 20 days	Sample Viable/ Non Viable	Sample % Viable	Control Viable/ Non Viable	Control % Viable
Apr.25.13	1.65 min	22°C	May.15.13	111/14	87%	109/12	90%
Apr.25.13	2.72 min	22°C	May.15.13	115/16	88%		
Apr.25.13	4.48 min	22°C	May.15.13	125/18	87%		
Apr.25.13	7.39 min	22°C	May.15.13	117/14	89%		
May.04.13	12.18 min	22°C	May.24.13	125/20	86%		
May.04.13	20.09 min	22°C	May.24.13	120/19	86%		
May.04.13	33.12 min	22°C	May.24.13	117/18	87%		
May.04.13	54.6 min	22°C	May.24.13	112/17	87%		
May.04.13	90.02 min	22°C	May.24.13	126/19	87%		
May.04.13	148.4 min	22°C	May.24.13	121/15	89%		
June.06.13	244.7 min	22°C	June.26.13	118/16	88%		
June.06.13	403.4 min	22°C	June.26.13	124/16	89%	116/13	90%

Experiment using 1.5 M hexanoic acid with 18 mM Tween 20, pH 4

(Soil Sample)

Experiment Start Date	Incubation Time	Incubation Set Temparature	Date after 20 days	Sample Viable/ Non Viable	Sample % Viable	Control Viable/ Non Viable	Control % Viable
Apr.19.13	1.65 min	37°C	May.09.13	109/14	89%	127/14	90%
Apr.19.13	2.72 min	37°C	May.09.13	118/12	91%		
Apr.19.13	4.48 min	37°C	May.09.13	120/19	86%		
Apr.19.13	7.39 min	37°C	May.09.13	123/18	87%		
Apr.19.13	12.18 min	37°C	May.09.13	125/18	87%		
Apr.19.13	20.09 min	37°C	May.09.13	121/19	86%		
Apr.21.13	33.12 min	37°C	May.11.13	125/16	89%		
Apr.21.13	54.6 min	37°C	May.11.13	127/19	87%		
June.06.13	90.02 min	37°C	June.26.13	117/13	90%		
June.06.13	148.4 min	37°C	June.26.13	119/14	89%		
June.06.13	244.7 min	37°C	June.26.13	0/700+	0%		
June.06.13	403.4 min	37°C	June.26.13	0/700+	0%	121/13	90%

Experiment using 1.5 M hexanoic acid with 18 mM Tween 20, pH 4

(Soil Sample)

Experiment Start Date	Incubation Time	Incubation Set Temparature	Date after 20 days	Sample Viable/ Non Viable	Sample % Viable	Control Viable/ Non Viable	Control % Viable
Apr.30.13	1.65 min	4°C	May.20.13	128/14	90%	129/15	90%
Apr.30.13	2.72 min	4°C	May.20.13	119/14	89%		
Apr.30.13	4.48 min	4°C	May.20.13	111/12	90%		
Apr.30.13	7.39 min	4°C	May.20.13	122/18	87%		
Apr.30.13	12.18 min	4°C	May.20.13	117/19	86%		
Apr.30.13	20.09 min	4°C	May.20.13	114/17	87%		
Apr.30.13	33.12 min	4°C	May.20.13	125/20	86%		
Apr.30.13	54.6 min	4°C	May.20.13	127/18	88%		
June.06.13	90.02 min	4°C	June.26.13	110/14	89%		
June.06.13	148.4 min	4°C	June.26.13	124/15	89%		
June.06.13	244.7 min	4°C	June.26.13	121/18	87%		
June.06.13	403.4 min	4°C	June.26.13	113/14	89%	125/14	90%

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