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Effects of Solids Retention Time and Feeding Frequency on Performance and Pathogen Fate in Semi-continuous Mesophilic Anaerobic Digesters

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Effects of Solids Retention Time and Feeding Frequency on Performance and Pathogen Fate in Semi-continuous Mesophilic Anaerobic Digesters

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
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Dedication

To my wife Kathryn for steadfast patience and support; and to my grandfather William Denis for his unconditional love for learning.

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Abstract

Anaerobic digestion is a biochemical process in which organic carbon is biodegraded in an oxygen free environment through a microbial consortium. Engineered biological systems used for resource recovery often utilize anaerobic digestion to treat anthropogenic organic wastes by reclaiming the carbon as energy (methane gas) and a soil amendment (biosolids). Small-scale, or household, semi-continuous anaerobic digesters have been used in developed and developing countries for many decades to produce biogas from human and livestock waste, which is used for heating, lighting, and cooking. This application has been shown to improve the quality of life of the user. Although there is great potential for small-scale semi-continuous anaerobic digestion to provide much needed resource recovery functions and quality of life improvements in future development, the manner in which these systems are operated could lead to unintended consequences on human health because human waste often contains resistant pathogens. This paradigm is best demonstrated by soil-transmitted helminths that are known to be highly resilient in mesophilic anaerobic digestion environments and endemic to many developing countries. The idea that soil-transmitted helminths survive mesophilic anaerobic digestion is exacerbated when the biosolids from the digesters are land applied as a soil-amendment because this process fits perfectly into the lifecycle of soil-transmitted helminths that need soil environments to develop into infective larva.

This research was divided into three sections to investigate the fate of human pathogens during semi-continuous anaerobic digestion and investigate techniques to enhance their removal. The sections were: 1) an examination into the fate (embryonation, development, inactivation,

destruction) of *Ascaris suum* ova during mesophilic semi-continuous anaerobic digestion, with an emphasis on increased inactivation, 2) an investigation into the performance (volatile solids (VS) removal, *E. coli* and *Salmonella* destruction, methane production) of semi-continuous mesophilic anaerobic digesters and the effect of variations to solids retention time (SRT) and feeding frequency, and 3) development and application of mathematical models for pathogen inactivation kinetics and typical semi-continuous reactor residence time distributions to predict the removal efficiency of *Ascaris suum* ova during semi-continuous anaerobic digestion under different operating conditions.

Results of these studies showed that during semi-continuous mesophilic anaerobic digestion variations in feeding frequency did not impact the fate of *Ascaris suum* ova or *Salmonella*; however it was observed that better removal of *E. coli* and higher methane production was achieved at the longer feeding interval (weekly). Additional results indicated that embryonated ova were destroyed faster than unembryonated ova under the experimental conditions, which suggests a potential mechanism to enhance removal of this common pathogen. Since an increased feeding interval proved to be beneficial for digester performance our findings suggest that wastes containing *Ascaris suum* ova could be stored in an aerated environment, for a period of time that does not negatively impact resource recovery, to lengthen the time between feedings and promote ova embryonation and ensuing destruction during digestion. Modeling results indicate that under mesophilic conditions (35^{o}C) the ova of *Ascaris suum* could survive for 22 days and will not be completely removed from the effluent under typical feeding frequencies and average SRT were examined. Therefore, the use of anaerobic digestion as a resource recovery technology where soil-transmitted helminths proliferate should be applied with extra operational safeguards or be included as one step of several in a small-scale treatment train.

Chapter 1: Introduction

1.1 Motivation and Significance

Since the initiation of the Millennium Development Goals in 1990 there has been tremendous improvement in sanitation coverage across developing countries (MDG, 2013). During this same time period the popularity of renewable energy technologies to mitigate the effects of climate change and also reduce fossil fuel dependence has increased globally (IEA, 2013). Between 2005 and 2011 the global annual funding for clean-energy based technologies grew from $50 billion to $200 billion, and is estimated to surpass $450 billion by 2030 (Bloomberg, 2011; ADB, 2013). The Water Environment Research Foundation's research on energy neutrality or "net zero" for water resource recovery facilities is aimed at characterizing the energy balances of common wastewater treatment configurations and evaluating the potential for employing best practices and innovative but demonstrated technologies to improve wastewater treatment energy balances.

Mesophilic anaerobic digestion is a type of renewable energy technology that is being promoted worldwide because of the improvement to domestic, industrial and agricultural waste treatment and resource recovery (energy and soil amendment), but also the numerous benefits that can be derived from a well-functioning system (Bruce et al., 2000; Eamens et al., 2006; Katuwal and Bohara, 2009; Gautam et al., 2009; Ferrer et al., 2011; Rowse 2012; Surendra et al., 2014). The use of anaerobic digestion occurs on many different scales across the globe. In the United States, it has been estimated that 1 in 4 wastewater treatment plants (> 1 MGD) utilize anaerobic digestion to process solids, with over 90% of these systems operated in the mesophilic

temperature range (Qi et al., 2013). In Cambodia, the Dutch Ministry of Foreign Affairs, in partnership with the Cambodian Ministry of Agriculture, Forestry and Fishing, have dedicated resources to construct over 20,000 small-scale (< 30 GD) anaerobic digesters as a means to improve access to sanitation systems and improve energy dependence by 2012 (NBP, 2011). This program has already constructed over 10,000 small-scale systems to date. China has similar initiatives aimed at expanding the use of household scale digesters. Overall, one study estimated that anaerobic digesters are currently in-use in nearly 25 million households in developing countries worldwide (NRC, 2007).

Conventional anaerobic digester design is based upon a constant digestion temperature with controlled feeding and mixing strategies that promote consistent organic loading rates that can be translated into average solids retention time (SRT) profiles. This predictable environment allows for optimized performance in terms of volatile solids (VS) reduction and biogas production; however, the problems with this approach when applied to the developing world are twofold. The first is that small-scale anaerobic digesters that are operated by households are typically unmixed and fed inconsistently. This strategy leads to unpredictable performance in terms of the quality and quantity of resources recovered (methane and biosolids).

The second is that small-scale anaerobic digesters that are operated by households are typically controlled by variable ambient temperatures that do not exceed 35 ^{o}C even in the hottest climates (Figure 1.1). Because temperature is the primary mechanism to inactivate human pathogens during anaerobic digestion (Pecson et al., 2007; Nordin et al., 2009, Maya et al., 2012), ambient temperatures that do not exceed mesophilic conditions will present an issue when anaerobic digestion is used in regions that have pathogens such as soil-transmitted helminths in their wastes.

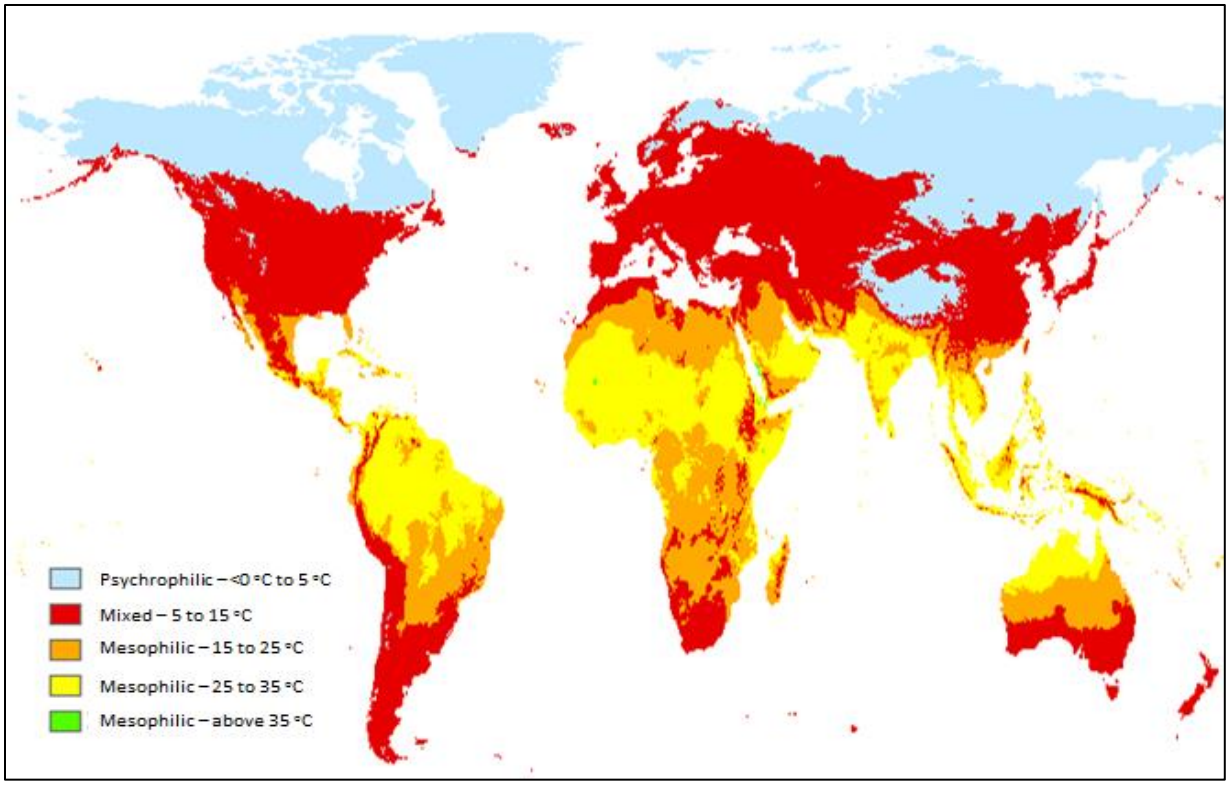

Figure 1.1 – The 12-month average ambient temperature for all global locations imply that all anaerobic digesters operated under ambient conditions will be psychrophilic or mesophilic. Map created using ArcMap10.2, data were obtained from the NASA Langley Research Center Atmospheric Science Data Center.

Soil-transmitted helminths are a relevant organism for this dissertation because the ova of these parasites have the ability to resist many disinfection processes, including inactivation by physical or chemical treatments involved with anaerobic digestion (Ghiglietti et al., 1997). The intestinal roundworm, *Ascaris lumbricoides*, infects nearly 800 million people worldwide, especially in tropical and subtropical regions, and relies on ova deposition to humid soils to spread from host to host (WHO, 2014; Pullan et al., 2014). Figure 1.2 depicts the global prevalence of *Ascaris lumbricoides* related infections.

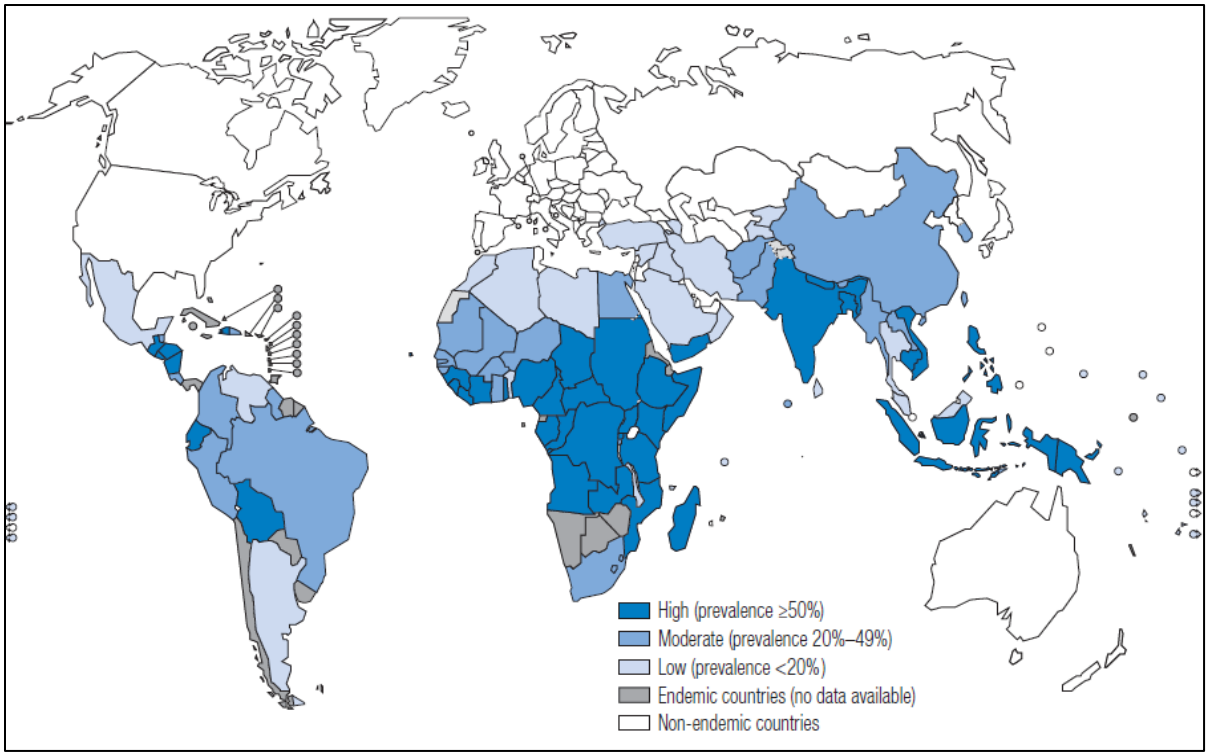

Figure 1.2 – The global prevalence of *Ascaris lumbricoides* related infections. One-sixth of the human population is estimated to be infected by *Ascaris lumbricoides* or another roundworm (WHO, 2014).

1.2 Research Gaps

Prior studies have found that soil-transmitted helminths (and other pathogens) are capable of surviving and re-entering transmission routes to humans when sanitation technologies such as upflow anaerobic sludge blanket reactors and waste stabilization ponds (e.g., Verbyla et al., 2013a; Symonds et al., 2014; Verbyla et al., 2015), composting latrines (Mehl et al., 2011), latrines with urine diversion (Trimmer, 2015), or solar toilets (Cruz-Espinoza et al., 2012a) are applied as means to improve human health. These studies suggest that despite the benefits to human health and the environment gained from the use of these systems, their application may not be entirely safe for human health, which is further demonstrated by Verbyla et al. (2013b) who linked the importance of protecting human health with food production by using recovered resources. This study is focused on whether the same phenomena are occurring with mesophilic

anaerobic digestion systems because the application of small-scale anaerobic digestion could result in unintended consequences with their use in regions where soil-transmitted helminths (and other pathogens) are common and psychrophilic or mesophilic digestion temperatures are used. The implications of this research on public health are large because in many cases when engineered or modern systems are used by the general public, the outcome is assumed by beneficiaries to be safe.

Three research gaps were identified for investigation in this research. First, since anaerobic digestion is often used for co-digestion of human wastes that potentially contain human pathogens with livestock waste, a direct link to human health exists and warrants further investigation. At the moment we lack sufficient understanding of the fate of *Ascaris suum* ova (embryonated and unembryonated) during mesophilic (35^{o}C) anaerobic digestion under semi-continuous and varying average SRT configurations. For example, Pecson et al. (2007) found that the time needed to reach near complete inactivation varied greatly between 30 ^{o}C and 40 ^{o}C, 180 days and 5 days, respectively, at a neutral pH, which brings to question the amount of time needed to inactivate at temperatures that fall within this range, such as 35 ^{o}C. Based upon Figure 1.1, 35 ^{o}C is the maximum temperature that an ambient temperature regulated system would most likely reach in the field; therefore, understanding the best case scenario for *Ascaris suum* inactivation at this temperature is an important boundary to define. In regards to protecting public health, no prior literature was identified for this research that investigates the development of *Ascaris suum* ova during mesophilic anaerobic digestion, or compares the inactivation of embryonated ova to unembryonated ova under these conditions, as well as, no prior published research focusing on the destruction of the ova during digestion. Because ovum development is required to produce an infective larva, the question about whether larva development occurs

during mesophilic anaerobic digestion is also important to understand if mesophilic anaerobic digestion technologies are to provide benefits while still protecting public health.

Second, because typical small-scale anaerobic digesters that are applied in millions of locations globally are operated in a semi-continuous manner, further research is needed to understand how the variation of the feeding frequency and average SRT affect the performance of the system. Performance can be estimated using different metrics, such as methane production, VS or chemical oxygen demand (COD) removal, and the destruction of indicator organisms (*E. coli* and *Salmonella*). There is very limited previously published knowledge pertaining to the effect of feeding frequency and average solids retention time on the methane production and indicator organism removal of semi-continuous digesters operated under mesophilic conditions.

Third, mathematical models have not been developed that allow us to quantify how variations in average SRT and feeding frequency of a mesophilic anaerobic digester will affect the residence time distribution profile of the reactor. This mathematical understanding can also be integrated with information on inactivation kinetics to model the fate of pathogens of concern. This is important to understand because in many applications the reactor will not be operated under continuously-fed conditions, nor will the reactor follow ideal-flow models because of the intermittent feeding pattern that is typical in household applications. This knowledge gap is also important to study because there is no existing literature describing the effect of feeding frequency and solids retention time on the survival of *Ascaris suum* under mesophilic digestion conditions.

1.3 Research Questions

This research study is directed by the following research questions based upon the previously defined research gaps.

1. What happens to relevant microbiological pollutants, e.g., *Ascaris suum, E. coli,* and *Salmonella*, during mesophilic anaerobic digestion at 35 ^{o}C? Mainly, how long (in days) can they survive for?
2. For *Ascaris suum*, do embryonated ova have different survival rates during mesophilic anaerobic digestion when compared to ova that have not embryonated?
3. Also for *Ascaris suum*, during mesophilic anaerobic digestion, does an ovum develop, become inactivated or destroyed?
4. How does the performance of semi-continuous anaerobic digesters change when variations to feeding frequency and solids retention time are made?
5. What mathematical relationship can be developed to describe the fate of *Ascaris suum* ova (and other pathogens) during semi-continuous reactor operation, and can it be used to estimate pathogen survival when the feeding frequency and solids retention time are varied?

In this dissertation, Chapter 2 provides a critical review of the literature. Questions 1 through 3 were experimentally investigated and are discussed in Chapter 3. Questions 3 and 4 were experimentally investigated and are discussed in Chapter 4. Question 5 was investigated by development of a mathematical model and is discussed in Chapter 5. A summary of research and conclusions is provided in Chapter 6.

Chapter 2: Literature Review

2.1 Introduction

Section 2.2 provides an overview of the relevant research related to anaerobic digestion systems and the requirements needed to produce a safe residual (biosolids). Section 2.3 discusses the aspects of soil-transmitted helminths that are related to both the survival and the death of the parasite in the context of an anaerobic environment. Details related to the mechanisms responsible for inactivation and the rate of inactivation are discussed in detail along with a review of a published model that can be used to describe the fate of helminths in biological waste to energy systems when their survival and residence times are known.

2.2 Anaerobic Digestion Overview

Anaerobic digestion is a microbial process where biodegradable organic carbon (VS or substrate) is transformed primarily into the reduced and oxidized forms of carbon, known as methane and carbon dioxide, in addition to some organic residual. This process occurs in four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis, all of which occur given a delicate balance of microbial ecology within the digester. Figure 2.1 illustrates how these steps work together. Equation 2.1 provides overall stoichiometry for the digestion process assuming a typical wastewater with an empirical molecular formula $C_5H_7O_2N$ (Haandel & Lubbe, 2007).

$$C_5H_7O_2N + 4H_2O \rightarrow HCO_3^- + 1.5CO_2 + 2.5CH_4 + NH_4^+ \qquad \text{Equation 2.1}$$

Because the carbon cycle can be controlled in an engineered system, this makes anaerobic digestion a primary candidate for managing anthropogenic organic waste streams in

many industrial and agricultural settings, such as concentrated animal feeding operations (CAFOs). Anaerobic digestion is also popular for the treatment of primary and waste activated sludge at domestic wastewater treatment plants as well as for treatment of the organic fraction of municipal solids waste, such as food and yard waste. The microorganisms that perform the anaerobic digestion include a combination of fermentative, acetogenic and acidogenic bacteria, in addition to methanogenic archaea, all of which require an oxygen free environment to achieve their symbiotic metabolism.

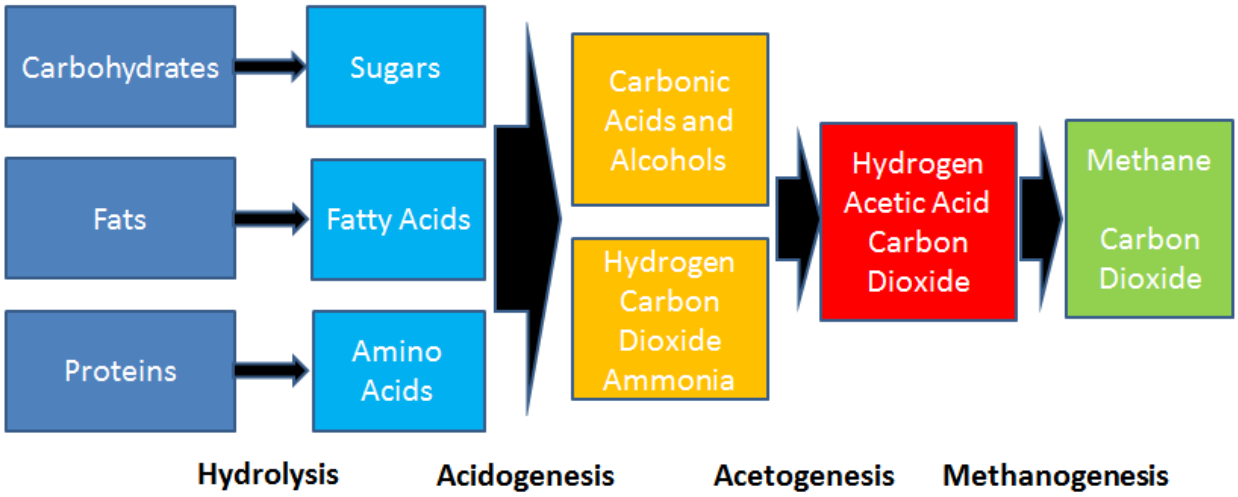

Figure 2.1 – The anaerobic digestion process (Adapted from Rittmann and McCarty, 2001).

One benefit of engineered anaerobic digestion systems is the ability to recover almost all of the carbon from the substrate in the form of methane, carbon dioxide, and organic biosolids that can be land applied under certain conditions. The quantity and quality of the residuals (biosolids) produced is an important topic, and is dependent on the metabolic efficiency of the organisms in the digester and the characteristics of the substrate. The metabolic efficiency is governed by the reactor operating conditions that are tied to the microbial, physical and chemical attributes of the anaerobic digestion system. These factors are discussed in greater detail in the following sections. Nutrients, such as, nitrogen, and phosphorus can also be recovered from the

residuals produced during anaerobic digestion (Amini, 2014). Additional benefits of anaerobic digestion can include odor reduction, solids volume reduction, pathogen removal and greenhouse gas emission mitigation when covered systems are utilized (Mihelcic and Zimmerman, 2014). When anaerobic digestion is used in small-scale applications, such as, households or family farms, benefits that improve the quality of life for the user have been attained (Bruce et al., 2000; Eamens et al., 2006; Katuwal and Bohara, 2009; Gautam et al., 2009; Ferrer et al., 2011; Rowse 2012; Surendra et al., 2014).

2.2.1 Microbial Aspects of Anaerobic Digestion

In a well-functioning anaerobic digester it is common for the bacterial population concentration to be greater than 10^{16} cells/ml (Amani et. al., 2010). This population is typically made of saccharolytic, proteolytic and lipolytic bacteria in addition to methanogens (Gerardi, 2003). Of these four groups of organisms, the methanogens are known to be highly sensitive to their environment in terms of pH, temperature and the concentrations of certain chemical compounds (ammonia, volatile fatty acids (VFAs)); they are also the slowest growing organisms in the anaerobic digestion reactor. Methanogens are completely dependent on the acetogens and acidogens to survive, as these two organisms convert simple monomers formed during the hydrolysis step into VFAs (proteolytic) and then into acetic acid, carbon dioxide and hydrogen (lipolytic) to supply the methane production process. This relationship is symbiotic as methanogens maintain the digester environment by consuming the protons and VFAs formed during acidogenesis and acetogenesis, which otherwise would become inhibitory to the biodegradation process.

According to Gerardi (2003) the supply of hydrogen is often the limiting step in methane production in anaerobic digestion systems, as a result many research projects are currently being

performed to optimize this aspect of anaerobic digestion system design and operation. Another limiting step in the production of methane is the accumulation of VFAs in the reactor produced during the acidogenesis step. This balance can be difficult to manage on a large scale because acidogens and acetogens continuously produce compounds that lower the pH of the system below the preferred range of 6.4 to 8 for methanogens if sufficient buffering capacity is not available (Speece, 1996; Rittmann and McCarty, 2001). This disparity can promote ineffective biogas production in reactors that do not have tight control over the operating environment. Overall, the methanogens sensitivity to the reactor environment also creates an ideal setting for microorganisms, including some pathogens, to survive and possibly multiply during their residence in the system.

2.2.2 Physical Aspects

The physical aspects of the anaerobic digestion process can be characterized into the categories of time, temperature, mixing and the loading rate of the reactor. Variations among these categories can significantly impact system design and performance with respect to biogas yield, pathogen inactivation, influent quality, reactor size, settling properties of the biomass and the amount of energy needed to operate it. The following subsections offer more detail on the physical parameters associated with anaerobic digestion.

2.2.2.1 Solids Retention Time (SRT)

SRT, also known as mean cell residence time (MCRT), is an important operating factor to consider in the anaerobic digestion system as the consumption of the substrate is controlled by the kinetics of the microorganisms. The relationship between the reactor volume (V) and volumetric flow rate (Q) is often used to define the SRT of a completely mixed system, presented in Equation 2.2.

$$SRT = \frac{V}{Q} = \frac{VX}{Q_w X_w + Q_e X_e}$$ Equation 2.2

In some reactor configurations where the effluent suspended solids concentration is decoupled from the mixed liquor concentration, the SRT can further defined by including the recycle (Q_e) and waste (Q_w) volumetric flow rates in combination with the respective biomass concentrations (X_e and X_w). This relationship is also shown in Equation 2.2, where (X) represents the mixed liquor suspended solids concentration. This mathematical expression can be applied to anaerobic digestion systems that are semi-continuously operated or systems that are not completely mixed, much like those found in small-scale household settings.

The optimal value for the SRT ultimately will be a function of the operating temperature, waste composition, reactor type, and other process details (Buekens, 2005). In general it can be assumed that a longer SRT will allow for more degradation and pathogen inactivation of the substrate when compared to a shorter SRT under the same operating conditions. The SRT is a very important design parameter to work with because the bacteria and archaea providing the carbon conversion have an optimum time that they need to be in the reactor for to perform their metabolism and produce methane. If an insufficient SRT is used the microorganisms will wash out of the reactor. A rule of thumb often followed in the design of anaerobic digestion system is the use of SRT that is at least twice the generation time of the methanogens under the digester conditions (Dohányos et. al., 2001). Weimer (1998) reported that slow-growing mesophilic methanogens can require up to a 130 hour generation time, which correlates to 5 days or a minimum SRT of 10 days.

In terms of pathogen reduction, the SRT of the system can be an important ally for the operator. A 2012 report documented that a completely-mixed mesophilic anaerobic digestion process was able to remove *E. coli* and *Salmonella sp*. from the influent with removal

efficiencies of 1.93, 2.98 and 3.01 $\log_{10}$ units for *E. coli* and 1.93, 2.76 to 3.72 $\log_{10}$ units for *Salmonella sp*. (Chen et al., 2012). This improvement occurred as the SRT increased from 11 days to 16 days to 25 days and highlights an aspect of the reactor that can be optimized to kill pathogens. The problem with SRT and pathogen removal is that SRT only represents an average cell residence time, which means that there will be a percentage of cells that are in the digester for periods that are both longer and shorter than this value. If the cell is pathogenic and has a short enough residence time, it may exit the digester in a viable state. This is dependent on the operating parameters of the digester and the inactivation characteristics of the cell.

2.2.2.2 Temperature

Aside from the presence of inhibitory compounds in the substrate, the operating condition that has the greatest influence on the rate of the anaerobic digestion process is the temperature of the environment, with higher temperatures leading to faster and more sanitary biodegradation of the substrate. There are three classes of temperature that define the rate of the anaerobic digestion process; the psychrophilic organisms are able to function at the coldest temperature range (-10 – 15 ^{o}C), while mesophilic microorganisms are most effective at 37 ^{o}C, but can survive between 20 – 40 ^{o}C. Thermophilic microorganisms are most effective at producing methane at > 45 ^{o}C and can withstand temperatures that range from 40 ^{o}C to 70 ^{o}C in most cases (Madigan and Martino, 2006). Some hyperthermophiles can withstand temperatures up to 120 ^{o}C (Takai et al., 2008), however the application of this high temperature is not realistic when treating large volumes of organic waste in anaerobic digesters due to the energy requirements.

There are interesting tradeoffs that exist between the temperature regimes as a psychrophilic system would require less energy to operate than the thermophilic system; however, the colder system would require a much larger reactor volume to achieve complete

digestion and pathogen inactivation of the substrate which would add expenses and additional resources the system. Research has also shown that the lower temperature ranges typically promote more robust organisms that are more able to adapt to fluctuations in the operating temperatures than the thermophilic organisms can indicating the need for precise process control when operating a thermophilic system (Amani et al., 2010). Additional disadvantages to the thermophilic temperature range are the high sensitivity to temperature fluctuations of the microorganisms and the increased production of VFAs, both of which can be disruptive to the anaerobic digestion process (Kim et al., 2002; Gerardi, 2003).

2.2.2.3 Organic Loading Rate (OLR)

The organic loading rate (OLR), presented in Equation 2.3, is the amount of biodegradable VS that are applied to one cubic meter of digester volume per day (Zaher et al., 2007). An optimum range for the organic loading rate (OLR) is 2.5-3.5 kg VS/m^3-day and will vary depending on the SRT and hydraulic retention time of the reactor (Kinyua, 2013). The OLR is also an indicator of the expected methane yield for the reactor where yield is expressed as m^3/kg VS-day. For example, observations made of biogas yield from swine manure ranged between 0.34-0.55 m^3/kg VS-day under batch mesophilic conditions (Chynoweth et al., 1999).

$$OLR = \frac{kg\ Volatile\ Suspended\ Solids}{m^3-day} \qquad \text{Equation 2.3}$$

2.2.2.4 Mixing

Reactor mixing is an interesting operational characteristic for anaerobic digesters. There are three typical mixing strategies used in anaerobic digestion systems: continuous, intermittent and minimal; however, within current publications there are conflicting opinions about which method is best in terms of biogas production. Inadequate mixing leads to the non-uniform distribution of substrates, enzymes and microorganisms, incomplete stabilization of the waste, a

decrease in methane production and pathogen destruction (Kaparaju et al., 2008; Karim et al., 2005). However, observations made by Kinyua (2015) regarding unmixed digesters in Costa Rica showed that biogas production and effluent quality was adequate. Chen et al. (1990) suggests that minimal mixing is needed to promote the symbiotic lifestyle between the methanogens and the acetogens, which is enhanced by their close spatial proximity to each other. This may be disrupted by over-mixing, which can also damage the cell walls of the microorganisms (Kaparaju et al., 2008). On the other side, continuous mixing has been shown to improve biogas production when compared to unmixed scenarios (Ho et al., 1985; Karim et al., 2005). Additional research in needed to better understand how anaerobic systems that are unmixed perform compared to mixed systems, as eliminating the need to mix will reduce the energy demand of the anaerobic digester.

2.2.3 Chemical Aspects

2.2.3.1 Nutrients

The soluble forms of nitrogen and phosphorus are important to the function of all microorganisms involved with the anaerobic digestion process; and other nutrients such sulfur, cobalt, iron, selenium, and sodium are considered to be micronutrients vital to organism health (Speece, 1996; Amani et al., 2010). A well-functioning anaerobic digestion reactor processing low-loading wastes should have a COD:N:P mass ratio of 350:7:1 in the substrate, while a waste consisting of only vegetable biomass requires more nitrogen and phosphorus to be effectively digested, with an optimal mass ratio of 125:4:1 (Bouallagui, 2003). This is probably due to the greater difficulty associated with digesting cellulose rich materials and the fact that nitrogen and phosphorus are not a major component of plant cells, which both lead to a smaller ratio. Nitrogen is a complicated nutrient in anaerobic systems because it is needed for biomass growth,

but at high concentrations and particular reactor environments (high temperature and pH), the speciation of nitrogen can be present in the form of free ammonia, which has lethal effects on most microorganisms, including those responsible for anaerobic digestion. This is discussed in greater detail in section 2.2.3.3. Because many anaerobic digester influents contain high amounts of nitrogen because of the waste being fed or their position in the wastewater treatment process, careful control over reactor pH is necessary.

2.2.3.2 pH and Alkalinity

The optimum pH range for methanogens function is 6.8 to 8.4, while the fermentative bacteria associated with the first three steps in the anaerobic digestion process can function in the pH range of 4.0 to 8.5 (Hwang et al., 2004). The pH balance of the anaerobic system is determined mostly by the influent quality (i.e., nitrogen and organic carbon concentrations) and impacts factors such as the microbial activity and the speciation of inhibitory compounds (Rittmann and McCarty, 2001). One of the constituents that contribute to pH change are VFAs that are produced during the acidogenesis phase of the anaerobic digestion process. The accumulation of VFAs in the digester can be traced to the OLR of the system, where higher relative OLRs can cause VFA accumulation. As VFAs accumulate in the reactor, the pH will decrease placing stress on the biogas producing organisms, while conversely, as nitrogen is liberated from the influent it will increase the pH of the system as it consumes a proton. Because methanogens are sensitive to changes in pH, alkalinity can be used to buffer rapid pH fluctuations during reactor operation. The recommended alkalinity in an anaerobic digestion system is 7 mg of calcium carbonate for every one milligram of biochemical oxygen demand (Amani et al., 2010).

2.2.3.3 Inhibitory Compounds

The final chemical aspect of concern for anaerobic digestion is the presence of inhibitory compounds that may form, enter or accumulate in the reactor. Ammonia and hydrogen sulfide can be disruptive to the anaerobic digestion process. Nitrogen, for example, will be in the neutral ionized (NH_4^+) form at the pH where methanogens thrive (pKa is 9.3 for NH_4^+/NH_3) (Mihelcic and Zimmerman, 2014), while the lethal form of nitrogen, free ammonia (NH_3), which is toxic to many microorganisms will be present at a higher pH and higher temperature. Hydrogen sulfide is believed to be able to diffuse into cell membranes of microorganisms and denature proteins, rendering the cell useless if prolonged exposure occurs (Conn et al., 1987). The effect of VFAs on the pH of the reactor has been previously discussed and its ability to change the pH to levels that harm methanogens confirms its role in inhibition for the anaerobic digestion process. At a neutral pH though, the methanogens have been found to be tolerable of volatile fatty acid concentrations up to 10,000 mg/L (Amani et al., 2010).

2.2.4 Reactor Configurations

The type of reactor used to perform the anaerobic digestion process varies from site to site and mainly depends on the scale of the operation. In the developed world it is typical for anaerobic digestion reactors treating municipal, industrial and agricultural waste to function in a completely mixed continuous flow environment. In the developing world simpler designs are used such as, the fixed-dome and tubular digester configurations (Figure 2.2). In these simpler designs the influent is added semi-continuously with little mixing occurring. The fixed-dome reactor is one configuration that has proliferated in the clean energy sector and has been installed throughout the developing world due to it simple design and maintenance requirements (ADB, 2013). Globally, an estimated 35 million household-scale (3 to 10 m^3) fixed-dome digesters are

currently in use (Bruun et al., 2014), while China plans to install up to 80 million digesters by 2020 (NDC, 2007).

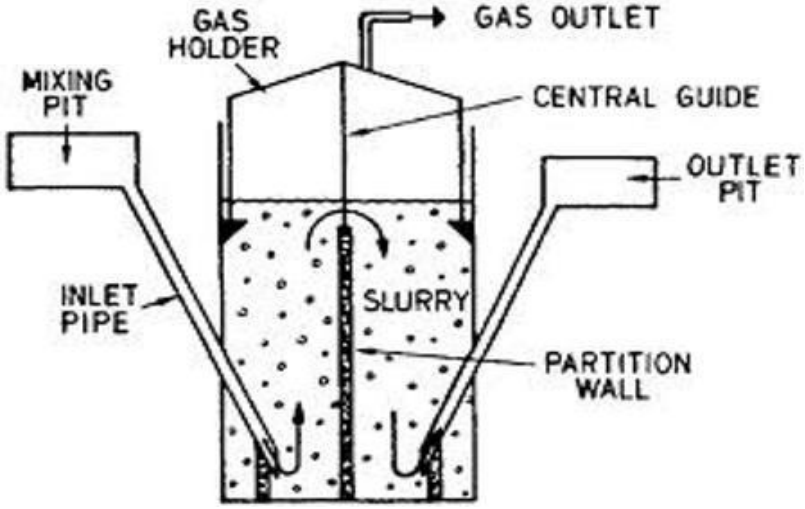

Figure 2.2 – A schematic view of a typical semi-continuous anaerobic digester configuration. This style is similar to the fixed-dome or floating-drum arrangement. Image by Permaculture Science (Public Domain).

2.2.5 Pathogen Reduction during Mesophilic Anaerobic Digestion

Human pathogenic organisms present in feces can be bacterial (*E. coli, Salmonella, Clostridium)*, protozoan (*Cryptosporidium, Giardia)*, helminth (*Ascaris lumbricoides, Taenia solium*) or viral (Hepatitis A, Rotavirus). In terms of anaerobic digestion under mesophilic conditions, the survival of these pathogens is an important question to understand because of the preference for many disease causing agents to be transmitted by the fecal to oral route. The removal potential for each class of organism is not similar, and differences within the classes have also been demonstrated.

Recent studies (e.g., Figure 2.3) have concluded that there is some pathogen reduction ability of mesophilic anaerobic systems (Olsen and Larsen, 1987); however, results vary relating to effectiveness among the pathogens and operating conditions observed. A study on the effects of temperature on pathogen fate in anaerobic digestion found a 1 to 2 log inactivation of *E. coli*

under mesophilic temperatures compared to a 2 to 5 log inactivation under thermophilic temperatures (Ziemba & Peccia, 2012). A 2010 study of mesophilic anaerobic digestion found a 1.9 log reduction of *Salmonella* at a SRT of 12 days, while a 3.75 log reduction occurred at an SRT of 25 days at the same temperature (Chen et. al., 2010). Masse et al. (2011) observed almost no removal of *Clostridium* or *Enterococcus spp.* after two weeks of retention time at 24 ^{o}C in an anaerobic sequencing batch reactor. Additional results related to *E. coli* and *Salmonella* survival with variations to SRT and feeding frequency at 35 ^{o}C can be found in Chapter 4.

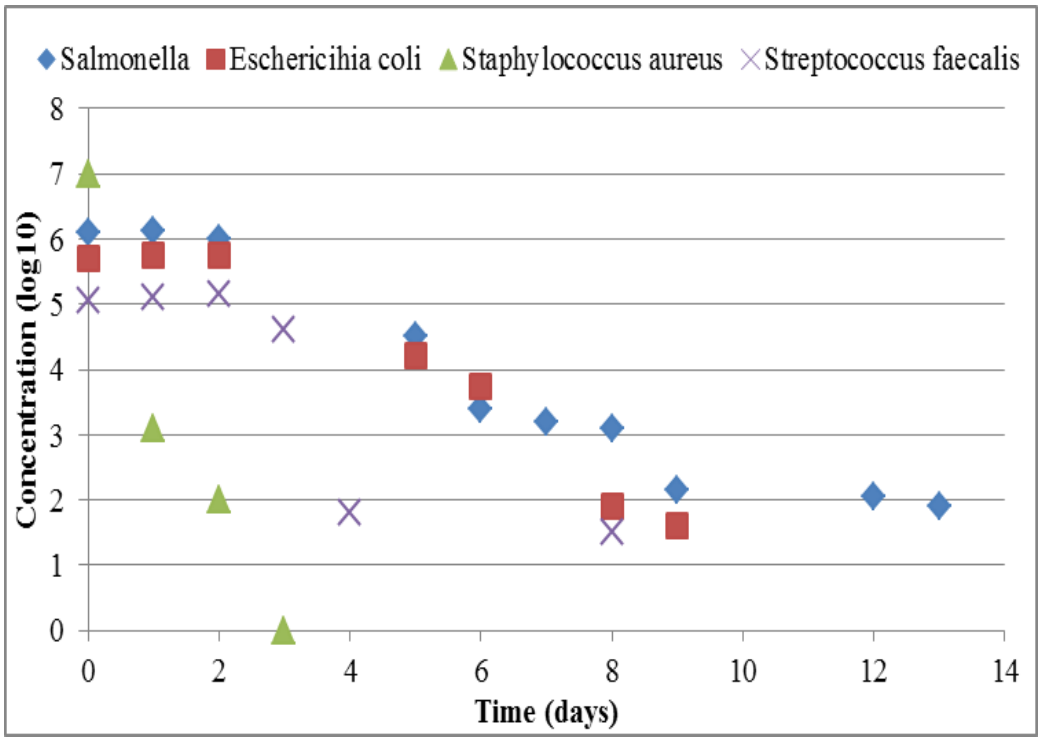

Figure 2.3 – Examples of survival curves for *Salmonella typhimurium, Escherichia coli, Staphylococcus aureus* and *Streptococcus faecalis.* Results taken from a CSTR anaerobic digestion system operated at 35^{o}C. Data from Olsen and Larsen (1987).

Survival of soil-transmitted helminths has also been investigated. Johnson et al. (1998) revealed that at least half of the population of *Ascaris suum* ova that entered a mesophilic anaerobic digestion process where alive after 5 weeks of residence time and that a similar number of ova survived for at least 29 weeks in a sludge storage lagoon. Pecson et al. (2007)

demonstrated that *Ascaris suum* ovum could withstand mesophilic temperatures for lengthy periods of time, e.g., 450 days at 20 ^{o}C, 180 days at 30 ^{o}C, and 4 days at 40 ^{o}C when free ammonia concentrations remained below 80 mg NH_3-N/L. The thermophilic temperature range of the anaerobic digestion process is known to be more effective at pathogen reduction because the kinetics of desiccation and protein denaturation occur more rapidly at higher temperatures. Pecson et al. (2007) reported inactivation of *Ascaris suum* ova in the thermophilic range as the ova were found to be 100 percent in non-viable after 110 minutes at 50 ^{o}C. Additional results related to *Ascaris suum* survival with variations to SRT and feeding frequency at 35 ^{o}C can be found in Chapter 3.

Overall the anaerobic digestion process does have the ability to reduce the concentrations of many pathogenic microorganisms in the influent, as was shown in Figure 2.3. However, if lower temperatures are used, the engineered design volume should promote the longest SRT feasible for the site to ensure a well-aged effluent to maximize the pathogen inactivation. Table 2.1 summarizes some of the challenges associated with anaerobic digestion in terms of pathogen destruction, time and temperature are the only operating factors that both promote good reactor operation and pathogen removal during anaerobic digestion.

Table 2.1 – Summary of the operating factors used to design anaerobic digestion systems that efficiently produce biogas and how they affect pathogen removal. A combination of time and temperature may be the best option to promote anaerobic digestion and obtain safe residuals.

Operating Factor	AD Reactor Operation	Pathogen Inactivation
Time	Long	Very Long
Temperature	High	High
VFA	Low	Very High
pH	Neutral	Low or High
Free Ammonia	Low	High

2.2.6 Biosolids Classifications

The safe use of residuals (i.e., biosolids) for land application will become a more pressing issue in today's society as human feces and animal manures are anaerobically digested. The Environmental Protection Agency (EPA) has developed a two tier classification system to rate biosolids, Class A and Class B (USEPA, 2000). Class A sludge is typically dried and pasteurized while Class B sludge is typically "undigested" and is volatile. Since Class A is pasteurized, the microbial content is probably minimal, however; Class B residuals potentially pose a greater risk to human health as they may contain human pathogens. Despite the fact that these regulations are developed for municipal wastewater sludge, their application to livestock waste is appropriate because certain human pathogens can be transmitted through animal waste.

For example, a 2007 survey of residences living near land applied biosolids concluded that the frequency of reported occurrence of bronchitis, upper respiratory infection, and giardiasis were statistically significantly elevated (Khuder et. al., 2007). These findings suggest an increased risk for certain respiratory, gastrointestinal and other diseases among residents living near farm fields on which the use of biosolids was possible. Furthermore, a 2006 study found that concentrations of common enteric pathogens were often well above soil background levels at 6 months, and in some cases 11-12 months, after land application of the residuals (Eamens et. al., 2006). In digested biosolids *Ascaris*, *Trichuris*, *Taenia* and *Toxocara* numbers have varied from 0 to 9 ova/g of biosolid (Sidhu et al., 2007; Barbier et al., 1990; Straub et al., 1993) with *Ascaris* being more common (Jimenez et al., 2000; Schwartzbrod et al., 1987). From 1988 to 2006, Class B biosolids in Pima County Arizona, were reported to have pathogen concentrations as follows: total coliforms (2-4 x 10^7 CFU/g), fecal coliforms (1-6 x 10^7 CFU/g),

salmonella (40 CFU/4g) and enteric viruses were measured to be 0.6-2 x 10^7 CFU/g (Pepper et. al., 2010).

The presence of human pathogens alone emphasizes the importance of producing a sanitary anaerobic digestion effluent to protect human health. The literature further indicates that Class B biosolids are potentially threatening to human health and one could infer that most biosolids produced in the developing world would fall into this category. Further research is needed to identify the operating conditions in these settings and also to determine what kinds of pathogens might be present in sludge with varying sources like humans or farm animals.

2.3 *Ascaris suum*

2.3.1 Model Organism

Since the risk to public health associated with anaerobic digestion residual reuse is not well understood it is important to develop and conduct experiments that better define the fate of pathogens in anaerobic digestion systems. A suitable surrogate pathogen for this type of work would need to be a robust organism that has shown previous resistance to anaerobic digestion treatment processes and overall resilience to disinfection. The ova of the soil-transmitted helminth, *Ascaris suum*, fit into this category, their size of 40 μm also allows for an easy viability evaluation via light microscopy. Table 2.2 summarizes some of the current studies pertaining to this issue when *Ascaris suum* ova is the focus. Ova resiliency has also been demonstrated by Johnson et al. (1998) who found infective *Ascaris suum* ova after 29 weeks in anaerobically digested and stored biosolids while Sanguinetti et al. (2005) tested the viability of *Ascaris suum* ova in pond stored biosolids and found that ova remained viable up to 6 years. A 2011 study found that pathogens, such as helminth ova, were not being destroyed completely in composting latrines after months of exposure resulting in transmission of infective ova via the soil (Mehl et

al., 2011). Matthews (1985) found that osmotic stress was shown to have no effect on the fate of the *Ascaris* ova.

Table 2.2 – Current inactivation studies identified that focus on *Ascaris suum*.

Reference	Matrix Type	Process Type	Methods	Parameters
Chen et al. (2012)	Sludge	Mesophilic Anaerobic Digestion (CSTR)	Non-mesh bag, MPN/DS readings from sludge samples every 2 days.	SRT, Volatile Suspended Solids, Total Solids, VFA, NH_4^+-N
Popat et al. (2010)	Primary Sludge	Mesophilic Anaerobic Digestion (CSTR)	Non-mesh bag, duplicate spikes for each time step, Viability count performed by ova supplier.	Temperature (50-55 ^oC, Time
Eriksen et al. (1996)	Sludge	Sludge Cake (pH>12)	Mesh bags pressed into 10 locations of the sludge cake, embryonation read every 2 weeks for 12 weeks.	pH, Temperature, Time
Konate et al. (2013)	Pond Sludge	Stabilization Ponds	Sludge sampled at discharge of each pond every 2 weeks for the presence of parasites.	Hydraulic Retention Time
Cruz-Espinoza et al. (2012a)	Human feces	Solar Toilet	Triplicate samples at each time step, two samples used to determine viability of the parasite.	Temperature, pH, Nitrogen, Time
Ghiglietti et al. (1997)	Sludge	Sludge Decontamination	Six samples at varying NH_4OH concentrations were analyzed for ova viability.	Nitrogen, pH
Nordin et al. (2009)	Urine	Liquid-Solid Separating Latrine	*Ascaris* mixed directly with urine, and then separated by sieving to read viability.	Ambient Temperature, Nitrogen, Time
Papajova et al. (2008)	Sludge	Field Composting Factory-Anaerobic Stabilization	*Ascaris* placed into polyurethane carriers to expose the ova to the composting system. Samples done in triplicate.	pH, Dry Matter, Organic Matter, Inert Matter, NH_4^+, N, P
Buitron et al. (1998)	Sludge	Electricity Generation w/ WWTP effluent	Ova separated from WW sample. Viability checked via duplicates.	Compression and Decompression at the Turbine
Kone et al. (2007)	Fecal Sludge	Sludge Dewatering-Composting	Compost pile sampled in the center and outside and then combined.	Windrow turning frequency

2.3.2 Ova Properties

The ova of *Ascaris suum* are able to survive extreme environments when they are outside of the mammalian host. At the time of fertilization, the ova are believed to possess all of the nutrients and vital amino acids that the single cell will need to develop into a larval stage worm. All of this occurring without secretion of waste products from the ova, with the exception of oxygen and carbon dioxide (Wharton, 1980). This reveals the efficient and independent nature of helminth development that can occur without dependency on the environment. Another characteristic of this ova is that after embryonation the amount of oxygen consumed by the larva decreases significantly indicating that after only 18 days of incubation the resistance capabilities to anaerobic conditions will increase and therefore their long-term survivability, evidence perhaps for what was observed by Johnson et al. (1998) and Sanguinetti et al. (2005). This idea that the ova will not interact with its surroundings by taking in or even emitting compounds exemplifies why the organism is difficult to kill and prepared to survive psychrophilic or mesophilic temperatures in anaerobic digestion systems that are similar to the host environment.

The ova can be divided into two regions, the ova-shell and the developmental envelop (Rogers, 1956). The ova-shell of *Ascaris suum* ova is probably where most of its durability and resilience is derived. This structure is made up of four distinct layers that each have a unique composition and function in the overall survival of the ova (Wharton, 1980). Starting with the outer most layer there is a combination of mucopolysaccharide and uterine proteins that form an uneven protective coating to the ova that is not critical in the overall survival of the ova. In fact, it was common in this study's preliminary experiments to observe ova during viability counts that were missing this layer, they are called decorticated. This sticky layer is deposited on the ova as it exits the adult female worm and probably offers some adhesive properties to the ova.

The next layer is a lipoprotein known as vitelline that provides protection against some acids, alkalis, and corrosive materials (Rogers, 1956). This layer is a rather weak barrier against organic acids and is permeable to gaseous and aqueous ammonia, it also has been measured to have melting point of 70 ^{o}C, which may explain the thermal resistivity of *the Ascaris suum* ova in temperatures that are below thermophilic. The third layer from the outside is a hard chitin/protein arrangement that provides limited chemical resistance and is probably for structural strength. The most innermost ova-shell layer is also a lipoprotein (ascaroside) that is unique to the *Ascaris* family. This layer also provides impermeability for the ova but some exceptions to the impermeability would be to certain gases like NH_3 or most organic solvents (Gamble et al., 1995).

2.3.3 Inactivation Mechanisms of *Ascaris suum*

The anaerobic digestion reactor environment can be a harsh environment for any microorganism to survive in, given the numerous mechanisms of inactivation that are possibly in effect. Table 2.3 organizes the mechanisms for pathogen inactivation into categories of physical, chemical and microbial nature. These mechanisms are further described in section 2.3.3.

Table 2.3 – Inactivation mechanisms that could influence the fate of an organism inside an anaerobic digestion system.

Physical	Chemical	Microbial
Temperature Time Desiccation (Solid State AD)	Volatile Fatty Acids Free Ammonia Osmotic Pressure Alkalinity	Competition Predation

Regardless of the mechanism that is responsible for pathogen inactivation or the speed at which it occurs, the process of transforming from a viable to a nonviable state for most microorganisms is an irreversible procedure that renders the organism harmless to human or animal health. On the cellular level the response to the mechanism may be traceable to

denatured proteins within the ova shell or developmental cells of the roundworm. Denaturation can disrupt cell activity and lead to death, it is a process in which proteins lose their type-II and type-III structures through the application of heat, strong acids and bases, or organic solvents (Voet et al., 1990). Type-II structures involve the H-bonds that hold together the non-polar and polar side chains of the protein which will weaken under stress and allow for the protein to unfold an become non-functional. Type-III structures are lower level H-bonds within the side chains that will also weaken under environmental stress allowing for further denaturation (Alberts et al., 1989). When the structure of these proteins weakens and change shape, the associated enzymes with not bind to the site because their shape determines their use. Components like DNA will separate into two strands as well. At some point in the denaturation process the protein unfolds in a manner or to an extent that exposes the hydrophobic portion of the molecule. These exposed portions will migrate towards each other and essentially bond together is what is referred to as a communal aggregation (Voet et al., 1990).

2.3.3.1 Chemical

Free ammonia can be detrimental to the survivability of many microorganism including pathogens (Madigan et al., 2012). Ghiglietti et al. (1997) found that *Ascaris* ova stored in untreated sludge were only 2% inactivated after 90 days while complete inactivation after 21 days was observed when the sludge was mixed with NH_4OH at 4%. Denaturation by urea, which can also be in the form of free ammonia at the appropriate pH, has been researched as well, with similar conclusions made about is impact to protein structure. Camilloni et al. (2008) decided that urea can disrupt the bonding to the β-sheet, which is responsible for maintaining protein structure within its side chains. A 2003 study concluded that there are both direct and indirect effects of urea to protein structure. The direct interaction involves the urea increasing the

solubility of the protein by bonding to it which will change the balance of forces on the molecule causing bond breakage. The indirect impact of urea is the change in solvency of the water envelope surrounding the molecule, having a similar impact to the structure of the molecule (Bennion and Dagget, 2003).

VFAs are also present in the reactor as part of the anaerobic digestion process, recalling that they are produced during the acidogenesis phase. Propionate may be the most toxic of the VFAs and may exert toxicity at concentrations less than 5 mg/L (Pullammanappallil et al., 2001). This toxicity is due to an increase in the non-dissociated form of the VFAs, which at high enough concentration gradients, can flow freely through the cell membrane of microorganisms where they dissociate, reduce the pH and disrupt the homoeostasis of the cell (Appels et al., 2008). Butkus et al. (2011) concluded that butanoic and pentanioc acids can be added to the sludge to reduce the survival of *Ascaris* ova prior to land application. Kinyua et al. (2014) did not measure the concentration of propionate; however, they did measure the concentration of VFAs as acetic acid in a mixed semi-continuous mesophilic anaerobic digester and observed an influent concentration of nearly 6,000 mg acetate/L and an effluent concentration of 2,100 mg acetate/L.

2.3.3.2 Physical

Denaturation by heat happens two ways. The first involves a decrease in viscosity of the water network that surrounds the proteins creating less support to the molecule due the higher temperature. This force imbalance will allow the protein structure to move and eventually break at the H-bonds holding it together (Brovchenko et. al., 2005). The second mechanism of heat denaturation involves an increase in kinetic energy that heat adds to the system which causes a vibration throughout the protein until it breaks apart exposing the hydrophobic ends of the molecule (Koizumi et. al., 2007). This change in the protein then results in communal

aggregation as the molecules are attracted to each other, which is typically an irreversible process that inactivates the ova.

Numerous studies have published results that confirm the effect of temperature on the fate of helminths ova. Pecson et al. (2007) found that at neutral pH the concentration of viable *Ascaris* ova was reduced by nearly 2 $\log_{10}$ units after 12 days at 40 ^{o}C while the same level of removal was achieved after 100 minutes at 50 ^{o}C. The general conclusion made by these researchers is that as the temperature of the system increases the rate of inactivation will also increase. The first observation made from Figure 2.4 is the effect of temperature on the death kinetics of *Ascaris* ova. Looking at the two data series from Pecson et al. (2007), which represent two different concentrations of free ammonia (130 mg free ammonia/L compared to 1,130 mg NH_3-N/L), it can be seen that higher temperatures inactivate the ova faster. This was verified by Cruz-Espinoza et al. (2012a) who showed a faster time to total inactivation as the temperature increases when ova mixed with human feces was examined (Figure 2.4). In the case of Cruz-Espinoza et al. (2012a), where inactivation at 40 ^{o}C occurred in only one day as compared to 14 and 3.4 days for the Pecson study. The increased rate can be attributed to the significantly lower moisture content of the sludge used during the experiment (23% compared to 95%, Cruz-Espinoza et al., 2012a). This indicates that the moisture content of the system can have a significant effect on helminth inactivation too and may shed light onto a key parameter that anaerobic digestion systems should be designed around to protect human health.

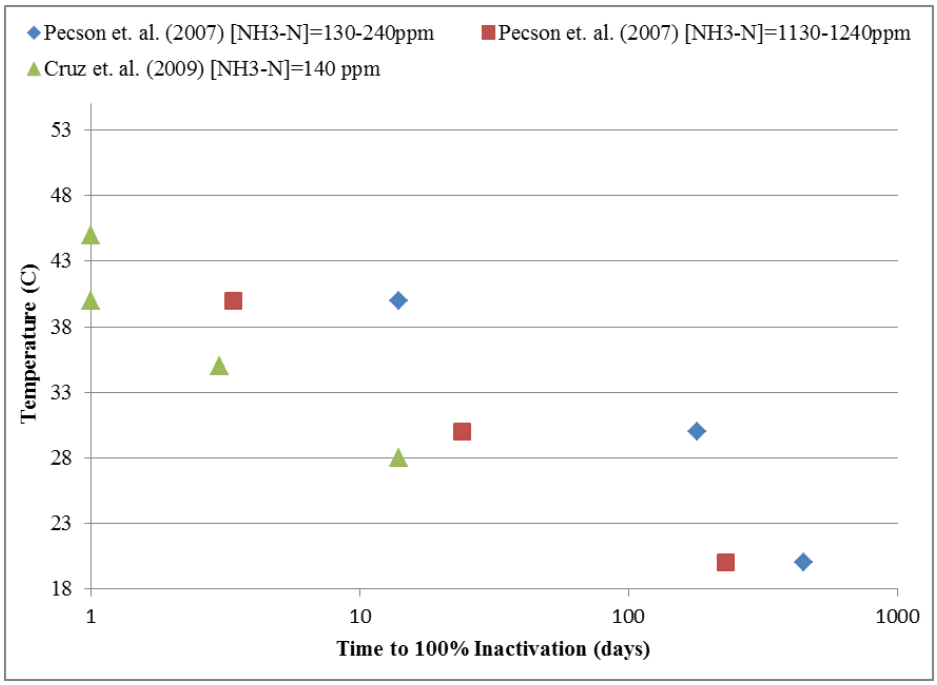

Figure 2.4 – A comparison of two studies that looked at the inactivation of *Ascaris suum* ova. The studies used variations in temperature and ammonia concentrations. In both cases, inactivation increased as the concentration and temperature increased.

The first observation made from Figure 2.4 is the effect of temperature on the death kinetics of *Ascaris* ova. Looking at the two data series from Pecson et al. (2007), which represent two different concentrations of free ammonia (130 ppm compared to 1,130 ppm), it can be seen that higher temperatures inactivate the ova faster. This was verified by Cruz-Espinoza et al. (2012a) who showed a faster time to total inactivation as the temperature increases when ova mixed with human feces was examined. In the case of Cruz-Espinoza et al. (2012a), where inactivation at 40^{o}C occurred in only one day as compared to 14 and 3.4 days for the Pecson study. The increased rate can be attributed to the significantly lower moisture content of the sludge used during the experiment (23% compared to 95%, Cruz-Espinoza et al., 2012a). This indicates that the moisture content of the system can have a significant effect on helminth inactivation too and may shed light onto a key parameter that anaerobic digestion systems should be designed around to protect human health. In many cases, anaerobic digesters are operated

with total solids concentrations that are very low (2 to 5%) indicating that desiccation is not a primary inactivation mechanism. An exception to this might be with solid-state digesters that have a much higher total solids concentration.

Overall, the addition of moisture to the system, perhaps for ease of manure handling or even organic loading, actually promotes pathogen survival in the system by maintaining a high humidity. Adobe bricks are known for their ability to wick moisture and could serve as a suitable building material for vault latrines or low moisture systems. Systems that use desiccation or urine separation would also be successful at accomplishing this.

2.3.4 Inactivation Kinetics of *Ascaris suum*

The rate at which a microorganism dies over time is the kinetics of inactivation and is the best measure that engineers have to be able to design effective systems that protect human health. Fundamentally the fate kinetics for pathogens are a function of both a concentration of some inactivation mechanism, such as heat or free ammonia, and a sufficient amount of exposure time to achieve a satisfactory percent of death for the organism(s). Much like the variation in removal potential for different organisms, the inactivation kinetics also vary from pathogen to pathogen and between combinations of concentration and exposure time. In particular we are concerned with the fate kinetics of the *Ascaris* family to better understand how helminthes survive inside anaerobic digestion processes. Two recent studies have performed research in the area of time to inactivation while adjusting operating parameters, such as temperature and free ammonia concentration. Figure 2.4 compares these two studies and illustrates several important concepts related to helminth destruction.

The first observation made from Figure 2.4 is the effect of temperature on the death kinetics of *Ascaris* ova. Looking at the two data series from Pecson et al. (2007), which

represent two different concentrations of free ammonia (130 ppm compared to 1,130 ppm), it can be seen that higher temperatures inactivate the ova faster. This was verified by Cruz-Espinoza et al. (2012a) who showed a faster time to total inactivation as the temperature increases when ova mixed with human feces was examined. In the case of Cruz-Espinoza et al. (2012a), where inactivation at 40 ^{o}C occurred in only one day as compared to 14 and 3.4 days for the Pecson study. The increased rate can be attributed to the significantly lower moisture content of the sludge used during the experiment (23% compared to 95%, Cruz-Espinoza et al., 2012a). This indicates that the moisture content of the system can have a significant effect on helminth inactivation too and may shed light onto a key parameter that anaerobic digestion systems should be designed around to protect human health.

Assuming that first order rate kinetics are followed during ova inactivation, Popat et al. (2010) and Aitken et al. (2005) attempted to determine the first-order rate constant (k [d^{-1}]) that represents the inactivation of *Ascaris* ova at different temperatures. Popat et al. found values ranging from 1.2 to 3 d^{-1} within a temperature range of 51 ^{o}C to 55 ^{o}C when five data points were used, while the Aitken paper published rate constants between 4 and 13 d^{-1} over the same temperature range. They were both looking at first stage activated sludge at a similar pH range. Comments from Popat et al. (2010) indicate that the rate constants determined in Aitken et al. (2005) were based upon a linear regression containing only three points so those reported values may not be accurate. A different study modeled the inactivation kinetics in sludge from a primary settling tank by also assuming first order kinetics and found a rate constant of 0.20 d^{-1} (pH = 12.7, moisture content = 90%). This value changed to 0.70 d^{-1} when the pH was lowered to 5.3, indicating that a lower pH is better at removing pathogens when the humidity is high (Maya et al., 2012). The difference between inactivation rates in different environments from

these studies highlights the gap in knowledge for understanding helminth inactivation kinetics, especially in well-functioning anaerobic digestion systems when factors like pH, temperature and contact time can vary spatially and temporally in the system.

2.3.5 Inactivation Models of Microorganisms

The fate of any microorganism in an anaerobic digestion system is a function of many variables that are simultaneously in effect. Selleck et al. (1970) published a mathematical model (Equation 2.4) to estimate the concentration of a microorganism in the reactor effluent that is based upon both the inactivation kinetics of each organism [R(θ)] and the residence time distribution [E(θ)] of the reactor. This model is based on a segregated flow assumption that simulates the effects of non-ideal mixing buy assuming that the microorganism travels through the reactor in a discrete element and reacts with the bulk solution during its residence time (Crittenden et al., 2005).

$$\frac{N}{N_o} = \int_{\theta=0}^{\theta=\infty} R[\theta]E[\theta]d\theta \qquad \text{Equation 2.4}$$

In Equation 2.4, theta (θ) represents the normalized time of the reactor, which is the observed time period divided by the average hydraulic residence time. Ideally this model can be used to find the optimum residence time that promotes pathogen inactivation for any species and could be very powerful in the protection of human health as region specific parameters could easily be manipulated. The drawback to this model is the need for an accurate residence time distribution [E (θ)] for a non-ideal reactor which may not be easy to come by depending on site limitations.

Chapter 3: The Survival of *Ascaris Suum* Ova during Semi-continuous Mesophilic Anaerobic Digestion[1]

3.1 Introduction

Over the past several decades, single-stage small-scale mesophilic (operated between 20 ^{o}C and 45 ^{o}C) anaerobic digestion has been deployed throughout the world as a means to provide energy to rural communities that are often not served by conventional energy infrastructure. Globally, an estimated 35 million household-scale (3 to 10 m^3) fixed-dome digesters are currently in use (Bruun et al., 2014), while China plans to install up to 80 million digesters by 2020 (NDC, 2007). As small-scale anaerobic digestion is promoted, one priority must be to protect at-risk human populations from geographically specific pathogens that have been shown to survive waste treatment processes (Symonds et al., 2014). Much of the prior research on anaerobic digester design and operation has focused on optimizing the digestion of the substrate and subsequent gas production instead of investigating pathogen removal (Eamens et al., 2006; Chen et al., 2012). In the field these digesters are often operated passively, which means that the digestion temperature will be regulated by local ambient conditions that are often insufficient to destroy geographically prominent pathogens. Environmental and human health problems may also be exacerbated when the digestate is promoted for immediate land application to recover

[1] Adapted with permission from "Assessing the Fate of *Ascaris suum* Ova during Mesophilic Anaerobic Digestion" in *Environmental Science and Technology*, Volume 49, Number 5, pages 3128-3135. Copyright 2015 American Chemical Society.

valued nutrients for crop production, especially for specific pathogen life-cycles that use the soil as a transmission vector.

Geohelminths, or soil-transmitted helminths, are parasites that cause neglected tropical disease, are transmitted through the fecal-oral pathway, and inflict severe morbidity on 1.5 billion people globally (24%) (WHO, 2014). The intestinal roundworm, *Ascaris lumbricoides*, is one type of geohelminth with global health concerns because it infects nearly 800 million people, especially in tropical and subtropical regions (WHO 2014; Pullan et al., 2014). It is also the most persistent pathogen found in human waste in many parts of the world (WHO, 1989). The negative health outcomes of infection are numerous (Gibson, 2014) and include morbidity due to nutritional impairment, impairment of the growth and physical development of children, cognitive and intellectual impairment, and increased susceptibility to other diseases (WHO, 2010; Bethony et al., 2006).

As part of its lifecycle, *Ascaris* spp. requires deposition of ova to the soil to develop into motile larva. Therefore, biosolids management strategies for small scale anaerobic digesters may enhance ova survival because the conditions encountered during digestion are not lethal enough to inactivate the ova. Although prior studies exist related to the fate of *Ascaris* spp. ova in engineered waste management systems such as waste stabilization ponds (Verbyla et al, 2013) and composting latrines (Mehl et al., 2011), limited peer-reviewed studies exist on the fate of embryonated and un-embryonated *Ascaris* spp. ova during mesophilic anaerobic digestion. For example, *Ascaris suum* ova were found to retain their ability to develop into motile larva after 10 days of exposure to batch mesophilic (37^{o}C) anaerobic conditions (Johansen et al., 2013). Another investigation examining the survival rate of *Ascaris suum* ova in a mesophilic anaerobic digester (32 – 35 ^{o}C) found that nearly 50% of the ova remained viable after 30 days of residence

time (Johnson et al., 1998). Other indicator organisms also have shown resistance to the digestion process; for example, one study observed that as the SRT of a mesophilic anaerobic digester processing swine manure was increased, bacterial populations of *Salmonella* and *E. coli* were reduced, but not more than a 3-$\log_{10}$ removal at a 25 day SRT (Chen et al., 2012).

These studies demonstrate how engineered technology promoted and used in the northern hemisphere may not be sufficient for the specific pathogens, management strategies, and type of substrate encountered in some developing world locations. This is especially important considering the current emphasis of promoting the benefits of resource recovery when managing anthropogenic wastes (Mihelcic et al., 2011; Verbyla et al., 2013b, 2015; Guest et al., 2009; Cornejo et al., 2013; Symonds et al., 2014; Verbyla et al., 2015). Accordingly, the objective of this study was to investigate the effect of operational characteristics (i.e., SRT and feeding frequency) on the fate of *Ascaris suum* ova in mesophilic anaerobic digesters operated at 35 ^{o}C. *Ascaris suum* was used as a model for *Ascaris lumbricoides* because it is not as infectious to humans and *Ascaris lumbricoides* and *Ascaris suum* are closely related and may represent a single species (Leles et al., 2012). This study is important because it builds upon prior knowledge that ova of soil-transmitted geohelminths can survive in mesophilic anaerobic digesters by demonstrating that the ova actually undergo enhanced survival under the environmental conditions in the reactors. This study also presents a mechanism to improve the destruction of the ova during mesophilic anaerobic digestion by utilizing the physiological response of the ova to the presence of oxygen, which causes the pathogen to become more vulnerable to its surroundings.

3.2 Materials and Methods

3.2.1 Anaerobic Digester Set-up

Six bench-scale 900-ml anaerobic digesters processing swine manure (labeled as E15, E30, E45, W15, W30, W45) were operated at a feeding frequency of every other day (E) or weekly (W) and varying SRT (number refers to the average SRT in days) for ten months. The digesters were initially inoculated with 5.4 L (900 ml each) of active anaerobic biomass from a pilot-scale mesophilic (35 ^{o}C) anaerobic digester treating swine manure.

The influent slurry (feed) was prepared by blending 280 g of fresh swine manure from the Twenty-Four Rivers Family Farm (Plant City, Florida) with 1500 ml of local groundwater (University of South Florida Botanical Gardens) in addition to sufficient urea to maintain approximately 1000 mg/L NH_3-N in the digester. Each 900-ml digester was fed the influent slurry at the exchange volume stated in Table B1, every other day or weekly, after an equivalent volume of digester contents were removed as effluent. Each digester was maintained and operated at 35 ^{o}C with no mechanical mixing, which is similar to a previous bench-scale experiment that optimized the digestion of swine manure (Kinyua et al, 2014). The top of each digester was fitted with an air-lock to maintain anaerobic conditions while allowing biogas to escape, the lock was removed quickly only to perform sampling and feeding. The exception to this was during biogas production measurements when the biogas was routed to a wet-tip gas meter, as described in Section 3.2.2.

Three phosphate buffered (Fisher 7447-40-7, 0.01M phosphate buffer, 0.0027M KCl, and 0.137M NaCl, pH 7.4 at 25 °C) control reactors (labeled as Ar_{28}, $Ar_{35,}$ and An) were employed. Control (Ar_{35}) was operated aerobically at 35 ^{o}C and control (An) was operated anaerobically at 35^{o}C during both the ova inactivation experiment (Section 3.2.4) and the ova development

experiment (Section 3.2.5). Control (Ar_{28}) was operated aerobically at 28 ^{o}C to simulate an ideal ova development environment (Section 3.2.5) and was used solely during the ova development experiment. To mimic the average concentrations observed in the digesters during the study, 1.9 g/L of urea (Fisher 57-13-6) and 0.6 g/L of sodium acetate (Fisher 127-09-3) were added to the buffered controls. A detailed explanation of the anaerobic digester and control set-up is provided in supporting information Table B1.

3.2.2 Anaerobic Digester Monitoring Program

Digester influent and effluent were monitored weekly throughout the experimental period for: chemical oxygen demand (COD) through oxidization by potassium dichromate (Hach TNT825), oxidation reduction potential (ORP: Oakton Instruments, Vernon Hills (IL), WD-35650-10), VFAs measured as mg HOAc/L by diol reaction and reduction with Fe^{3+} (Hach TNT872), ammonia through reaction with ions of hypochlorite and salicylate in sodium nitroprusside to form indophenol blue (Hach TNT832), and total VS (ASTM 2540E). The volume (ml) of biogas produced was measured by using a wet tip gas meter (Archae Press) for 21 days for each digester and then normalized by the amount of substrate added to the digester. The methane fraction of the biogas was measured by dissolving the carbon dioxide portion of 10 ml of biogas into 1% v/v potassium hydroxide and measuring the displaced volume (ASTM D1827).

3.2.3 *Ascaris suum* Ova Mesh Bag Preparation

A 9-µl (approximately 900 ova) spike of viable (78%) *Ascaris suum* (Excelsior Sentinel Inc.) ova recovered from the feces of naturally infected pigs was pipetted into a single nylon mesh bag with 30-µm openings (Small Parts, Inc.). At the time of preparation the ova were two months old as stated by the supplier. The initial viability of the *Ascaris* ova stock was determined

by aerobically incubating an aliquot of approximately 1000 ova for 15 days at 28 ^{o}C in 0.1N sulfuric acid in triplicate. These triplicates were then transferred to a single glass-slide and analyzed at a 100x magnification for the total number of observed ova and the number of ova containing motel larva (described in Section 3.2.5). The mesh bag retains the ova but allows the digester's contents to exchange across the bag's openings. The mesh was cut, folded and closed with a thermo-sealer (A. Daigger & Company) to create 2.5 cm × 2.5 cm sized bags. After preparation the bags were stored in phosphate buffered saline at 4 °C for no more than 3 months before use, which is similar to the method used by others (Cruz-Espinoza et al., 2012a; Cruz-Espinoza et al., 2012b).

3.2.4 Un-embryonated Ova Recovery and Inactivation Method

Fifty bags were placed into each of the digesters and controls. Each day the digesters and controls were agitated by hand-shaking the bottle for 10 seconds to ensure complete mixing of the digester (or control) contents with the nylon mesh bags. Triplicate bags were randomly removed from each digester every other day for 24 days. Upon removal, the bags were rinsed with sterile 0.1N sulfuric acid and then immediately incubated aerobically at 28 ^{o}C in a new sterile 0.1N sulfuric acid solution using a Gyromax 727 Incubator (Amerex Instruments) for an additional 15 days. The aerobic controls (Ar_{28} and Ar_{35}) were the exception, their bags were removed, rinsed and incubated for a period of 15 days minus the number of experimental days the bag was in the control. Bags exposed to the control for longer than 15 days were not incubated. After incubation, the mesh bag was rinsed with sterile 0.1N sulfuric acid to prepare a clean cutting line along one edge of the bag. Once cut open, the entire contents of the bag were pipetted into a 0.5-ml micro centrifuge tube. The inside of the bag was then rinsed with sufficient 0.1N sulfuric acid to dislodge any visible remnants into the tubes. The tubes were then

centrifuged in an Eppendorf 5424 centrifuge for 10 minutes at 1000 rpm (1 rcf). Upon completion the entire pellet volume, typically 10 µl, was transferred by pipette onto a single microscope slide.

Slides were analyzed for the number of recovered ova by microscopy at 40x magnification. Samples recovering less than 360 ova (900 initial) were not analyzed for ova inactivation in order to maintain a 4% confidence interval ($\alpha = 0.05$); however, all sample bags were included in the ova recovery calculation where the number of recovered ova was divided by 900. A baseline recovery (% ova) was established by repeating the above recovery procedure for ten repetitions before the experimental work began.

Recovered ova were inspected for viability by microscopy at 100x magnification (Nikon E200 microscope). This method is described in detail elsewhere (Cruz-Espinoza et al., 2012a; Cruz-Espinoza et al., 2012b). The motile forms (life stages referred to as J, K and L) were considered to be viable and are discussed in more detail in Section 3.2.5. The percentage of viability was calculated using Equation 3.1 where the number of viable ova observed per slide was divided by the total number of ova observed per slide. This percentage was then divided by the initial stock viability (78%) to normalize the data to 100%.

$$\%\, Viable\ Ova = \left(\frac{\#\ of\ Viable\ Ova\ Observed}{Total\ \#\ of\ Ova\ Observed}\right) \qquad \text{Equation 3.1}$$

3.2.5 Ova Development Method

From the same set of fifty bags, a single bag was also removed from digesters and controls (E15, W45, Ar_{28}, Ar_{35}, and An) every third day. These bags were not incubated after removal but were transferred directly to a microscope slide to observe the progress of ova development at 100x magnification. Twelve ova life stages are given alphabetic notation (A to L) to signify which stage was observed during the experiment, or NV to represent a non-viable

ova (Cruz-Espinoza et al., 2012b). Stages A through D represent the transition from the one cell embryo to a clearly divided four cell ova. Stage E (early morula) contains 5 to 10 cells, stage F (late morula) contains ≥ 11 cells, and stage G (blastula) the embryo loses the visible cell divisions and is replaced by a spherical layer of cells surrounding a pseudo fluid-filled cavity. Stages H (gastrula) and I (pre-larva) appear as a kidney shaped structure that begins to lengthen and narrow into concentric coils inside the ovum. The motile form of *Ascaris suum* ova was considered to be developmental stages J, K and L which appear as a well-defined motile worm larva within the ovum that responds to light from the microscope. This means that an ovum observed to be in stage A-I would not be immediately infective if discharged to environment. However, if provided suitable conditions (e.g. soil type, temperature, high humidity, and time) the ovum could probably develop into an infective larva in the soil environment.

3.2.6 Embryonated Ova Inactivation Method

An ovum inactivation experiment using embryonated ova was performed using anaerobic digester E15 simultaneously with the unembryonated inactivation experiment (Section 2.4). The results are reported as E15* in Section 3.4. In this experiment, 21 nylon mesh bags were prepared as described previously (Section 2.3). They were then exposed to an optimal development environment, which is, incubated in an aerated phosphate buffered solution (Fisher, 7447-40-7) at 28^{o}C for 15 days. On day 16, the bags were removed from the incubator, marked with a black dot and transferred to digester E15. Marked triplicate bags were then removed and inspected for viability every fourth day, similar to the method described in Section 2.4 with the exception that motility resulting from light stimulus was used to ascertain the difference between viable and non-viable.

3.2.7 Statistical Methods

Using Minitab 16 (State College, PA) the one-way normal analysis of variance (ANOVA, $\alpha = 0.05$) procedure was used to detect differences in the characteristics of digester effluent from the six combinations of SRT and feeding frequency used in this study. The ANOVA procedure was also applied to the inactivation data (Table B3) to examine for significant differences in viability between the embryonated and unembryonated ova each day in the anaerobic digesters and buffered controls. Comparisons were made between groups based upon: SRT, feeding frequency, or the combination of SRT and feeding frequency.

3.3 Results

3.3.1 Anaerobic Digester Performance

The results of the anaerobic digester monitoring program (Table B2) demonstrated that the six digesters were functioning well as verified by the average twenty-one day biogas production rate (525 ml/g-VS added), methane content of the produced biogas (59%), and the average VS removal (45%). A complete list of performance metrics is provided in the supporting information (Table B2). There were no significant differences in effluent ammonia concentration between the controls and digesters (mean of 960 mg NH_3-N/L) when SRT and cycle frequency were varied. From Table B2, there was a decrease in effluent fatty acid concentration as the SRT of the digester was increased in digesters that were fed weekly and every-other-day, however the difference was not significant.

3.3.2 Ova Inactivation and Recovery

Seven bags were rejected during the experiment because less than 360 ova were recovered. Since 50 bags were seeded, additional bags were available to replace the rejected bags and maintain the triple replicates in all seven events. Results of the ANOVA analysis of the *Ascaris suum* ova inactivation data (Table B3) from the six digesters showed that the only

statistical difference observed between them was on day 20 ($F_{value} = 7.4$; $F_{critical} = 4.3$; $\alpha = 0.05$; $p = 0.01$) when the data were grouped by average SRT or feeding frequency. This implies there was no significant difference in inactivation of *Ascaris suum* ova when the average SRT or feeding frequency of the digester were varied. The fact that no significant difference in the inactivation results was detected between the six digesters also suggests that the effects of feeding the experimental digesters with different volumes of slurry stored at 4 ^{o}C had little to no impact on the operating temperature of the systems. Thus, if the operating temperature was consistently lowered for a sufficient period of time during feeding, then longer survival times would be expected.

The results from the inactivation study with both digesters and controls are presented in Figure 3.1. A large percent (65-70%) of the ova exposed to the anaerobic digesters at 35 ^{o}C (shown as AVE in Figure 3.1) and the anaerobic control (An) retained their viability for up to 16 days; after which an increased inactivation rate was observed until day 24 when near complete inactivation of all ova was observed. Comparatively, only 5% the ova exposed to the 35 ^{o}C aerobic control (Ar_{35}) retained their viability for up to 14 days of residence time, when on day 16 the ANOVA analysis detected significant difference in the average viability of the *Ascaris suum* ova between the average of the six digesters (65%) and the aerobic control (< 1%), on day 16, where $F_{value} = 46.8$; $F_{critical} = 5.6$; $\alpha = 0.05$; $p = 0.0002$. However, statistical differences were not detected between the average of the six digesters and the buffered anaerobic control (An), with the exception of days 12, 14 and 16, when slightly better *Ascaris suum* ova inactivation was observed in the anaerobic buffered control (Ar_{35}).

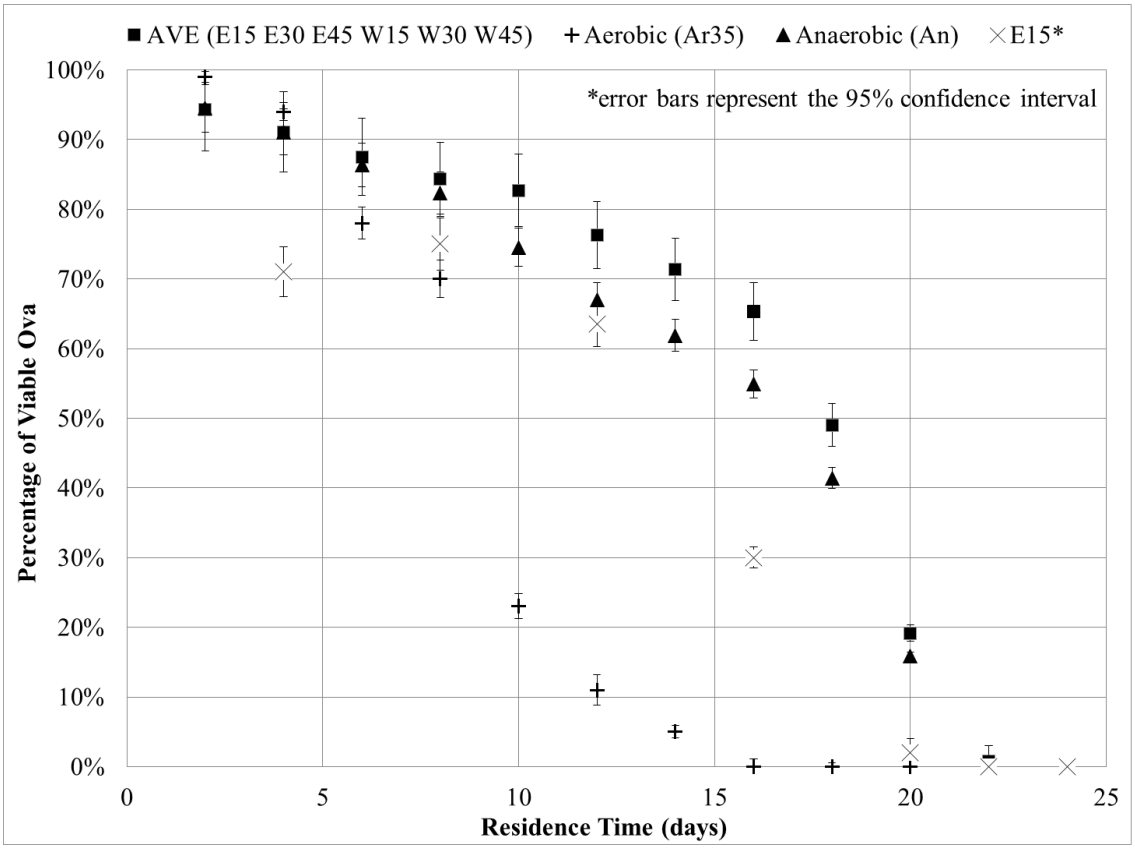

Figure 3.1 - The average percentage of *Ascaris suum* ova that remain motile overtime in the anaerobic digesters (AVE). Results are compared with the aerobic control Ar_{35} (black crosses) and anaerobic control (An). E15* and shows that embryonated ova inactivate faster than unembryonated ova do (Section 3.3.4).

The results of the ova recovery experiment are shown in Figure 3.2. The baseline recovery determined prior to the experiment (55%) was compared with the average experimental recovery over the study period, where the average recovery from the digesters is the total number of viable and non-viable ova counted from each sample bag divided by the initial seed population of 900 ova. The results indicate that fewer ova were recovered over time, implying that some ova were being completely destroyed in the digester. A typical observation of an *Ascaris suum* ova becoming vacuolated as the contents exit the shell leaving an empty case behind is provided in Appendix B (Figure B1).

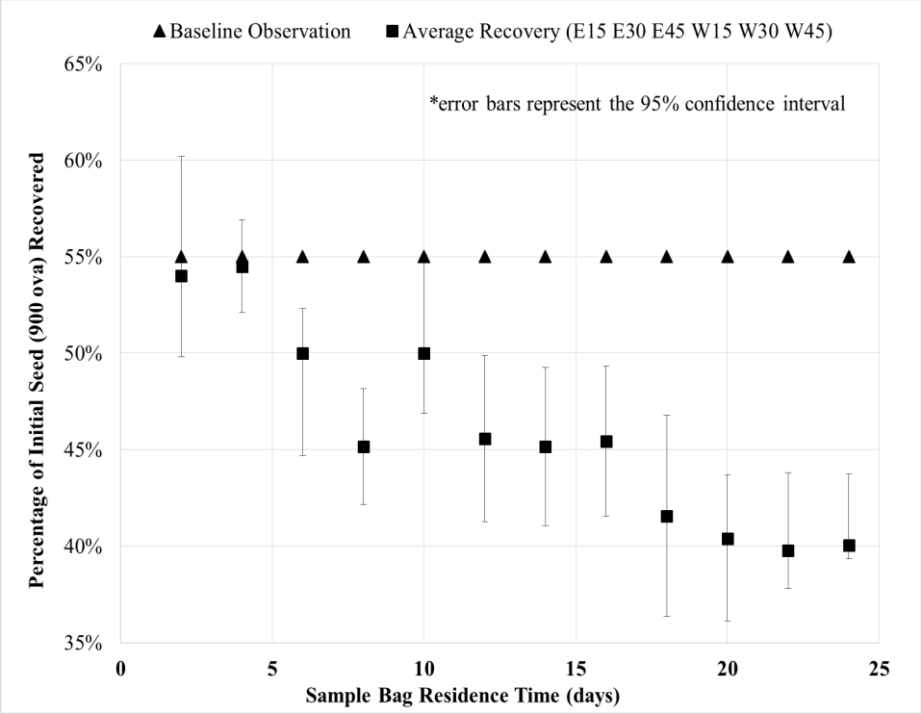

Figure 3.2 - The average percentage of viable and non-viable ova recovered from all of the anaerobic digesters during the experiment compared to a baseline established through a repetitive recovery procedure.

3.3.3 Ova Development

The results of the ova development experiment are shown in Figure 3.3. Four bar graphs (A-D) are shown; each representing the amount of time that nylon bags containing viable *Ascaris suum* ova were exposed to the anaerobic digesters and controls. Within each bar graph the frequency of observed development stages (A-L) reached by the ova during the experiment are shown. Figure 3.3A (three days) shows that in the anaerobic control (An) 36 observations of viable unembryonated ova and four observations of non-viable ova were made. This result was similar to observations made in the other anaerobic digesters and controls (E15, W45, An) and demonstrates that in the anaerobic environment, the *Ascaris suum* ova remained unembryonated during their entire residence time, showing no indication of development inside the anaerobic environment. In contrast, Figure 3.3D (18 days residence) shows results from the ideal 28 ^{o}C aerobic control (Ar_{28}). Figure 3.3D also shows that 35 motile ova (ova life stages K and L) and

three non-viable ova were observed after 18 days in the aerobic control. Conversely, Figures 3.3A-D show that the ova exposed to the aerobic conditions (Ar_{28} and Ar_{35}) both progressed through the developmental stages over time. This is most likely because of the presence of oxygen in the system; however, the ova exposed to the 28^{o}C aerobic environment (Ar_{28}) reached motility by day 15 (ova development stages J, K and L). Comparatively, the 35 ^{o}C aerobic environment (Ar_{35}) promoted similar development up to day 9, after which, ova fail to progress to motility and became mostly non-viable as early as day 16 (as was shown in Figure 3.1).

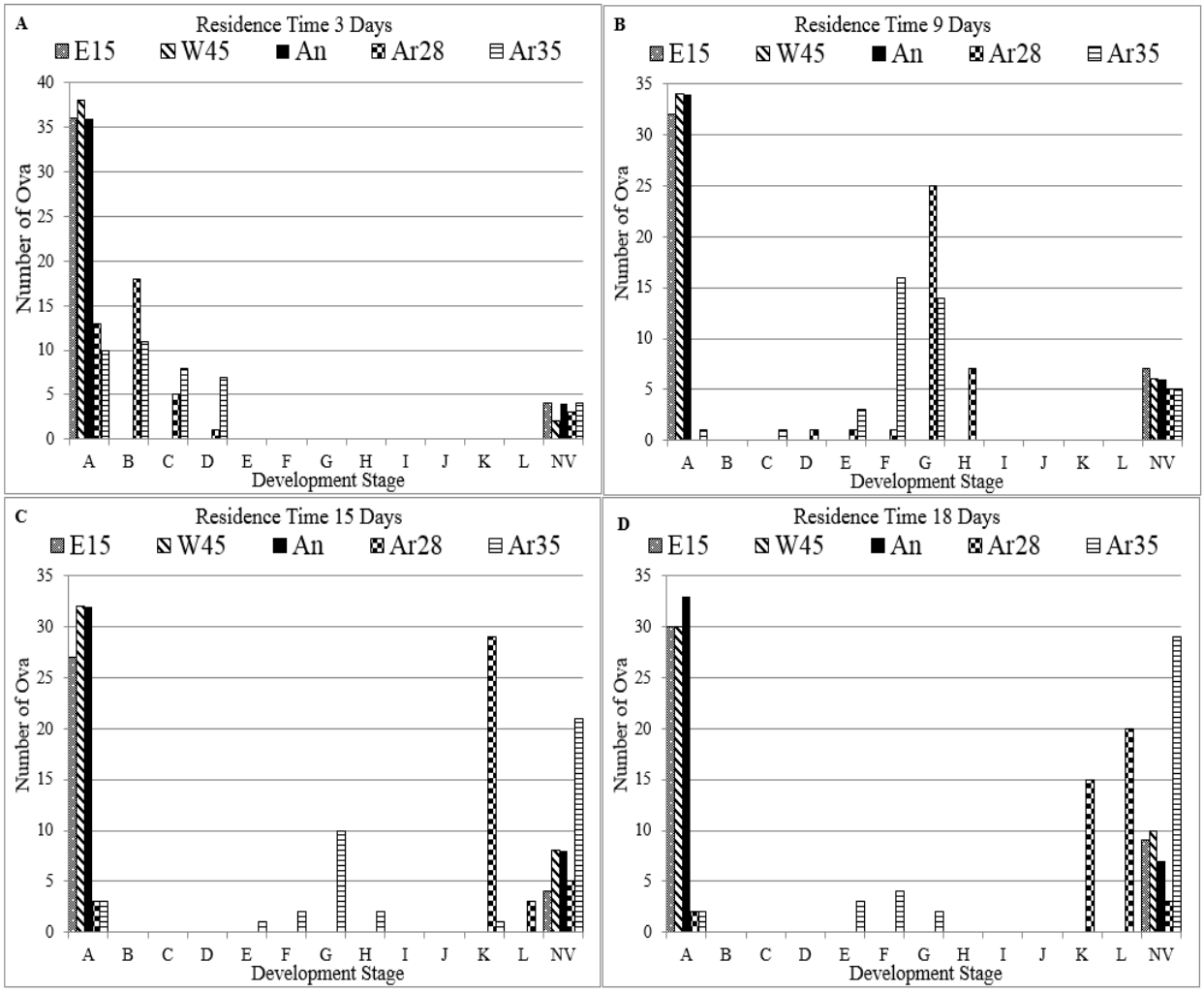

Figure 3.3 - Ova development graphs for representative digesters and controls. Results show that the anaerobic environments (E15, W45 and An) did not promote *Ascaris suum* ova development over time regardless of SRT or feeding frequency ova residence time, or feeding frequency while the aerobic controls did.

Microscopic photos of *Ascaris suum* ova collected over time in the controls and digesters are shown in Figure 3.4. A motile larva can be seen within the ova (T = 15 days, control Ar_{28}) and another hatching from its ovum (T = 18 days, control Ar_{28}). In contrast, the anaerobic digesters (E15 and W45) and anaerobic controls (An) show that ova remain unembryonated ova for the duration of the experiment.

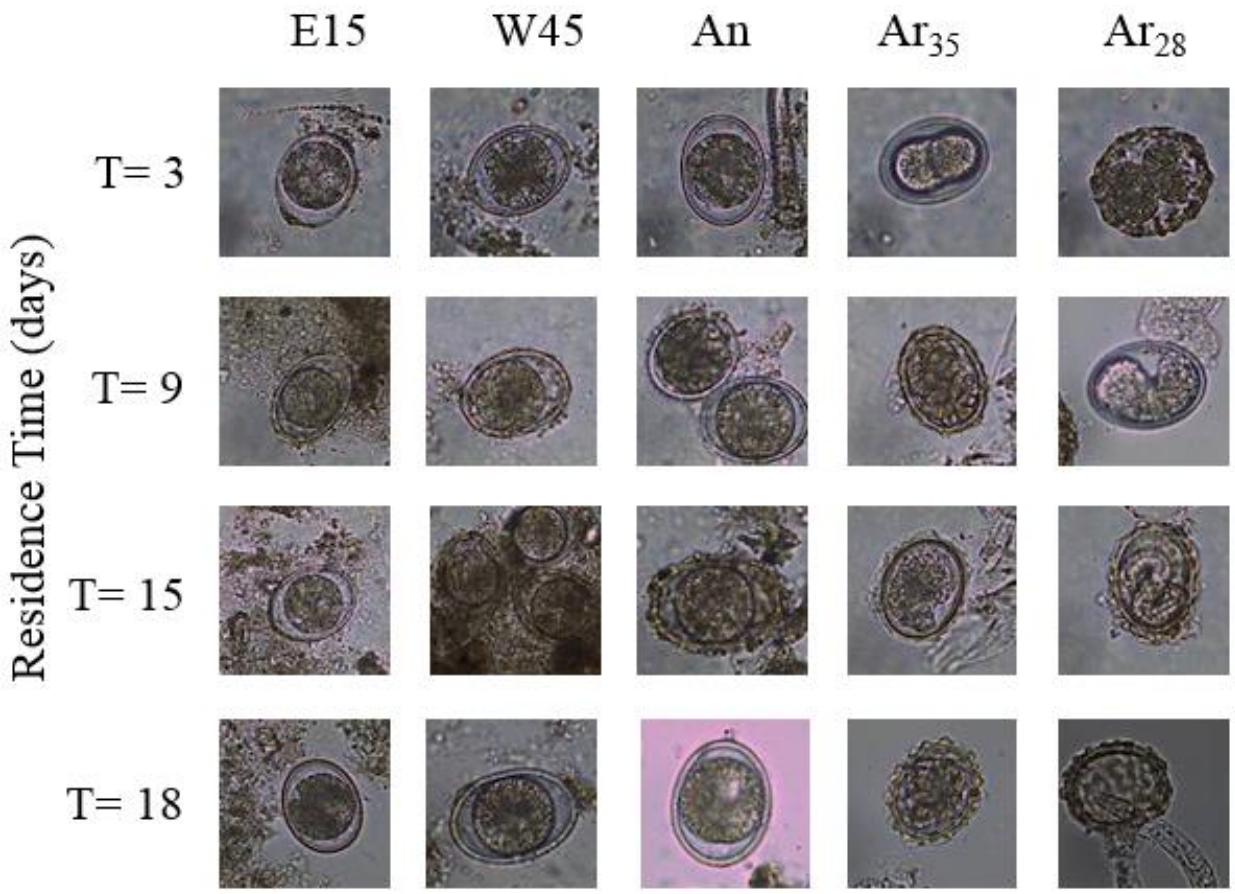

Figure 3.4 - Photographs of selected ova from the development study. The difference on day 18 between Ar_{28} and Ar_{35} demonstrates the lower resistance to temperature that *Ascaris suum* ova have when they are more developed.

3.3.4 Embryonated Ova Inactivation Experiment

The results of the embryonated ova inactivation experiment (labelled experiment E15*) were provided in Figure 3.1. Ova that were allowed to develop into a motile form under ideal conditions for 16 days were inactivated faster inside the digester when compared to ova that had

not developed past the unembryonated stage (labelled as AVE). This significant difference was observed after 8 days of residence time in the digester, where undeveloped ova retained 85% viability while developed ova viability was observed to be 75% ($F_{value} = 20.5$; $F_{critical} = 5.6$; $\alpha = 0.05$; $p = 0.002$). This result is further demonstrated eight days later (day 16 of the experiment), where undeveloped ova retained 65% viability while developed ova viability was reduced to 30% ($F_{value} = 46.8$; $F_{critical} = 5.6$; $\alpha = 0.05$; $p = 0.0002$). In addition, the embryonated ova were inactivated approximately three days faster than the unembryonated ova were (digesters E15 and W45, and control An). When comparing the viability of the ova in experiment E15* to the 35 ^{o}C aerobic control (Ar_{35}) on day 16 it can be seen that a significant difference also exists, 30% viability versus less than 1% viability in the control ($F_{value} = 27$; $F_{critical} = 7.7$; $\alpha = 0.05$; $p = 0.006$).

3.4 Discussion

The inactivation of unembryonated ova experiments showed that variations in SRT and feeding frequency of the anaerobic digester did not promote an environment where significant differences in the survival of *Ascaris suum* ova could be observed. Measured VS removal and biogas production were similar to values observed in other studies. Normally operating anaerobic digesters achieve 30 to 50% removal of VS and a biogas methane concentration greater than 50% (Rittmann and McCarty, 2001). Other studies anaerobically digesting swine manure at 35 ^{o}C demonstrated a wide range of performance, with 20 and 60% VS removal and average biogas methane concentrations greater than 50% (Amani et al., 2010).

The lack of inactivation can be explained by the non-lethal concentrations of ammonia and VFAs measured in the digesters (Table B2). Aqueous phase free ammonia is lethal to *Ascaris suum* ova above 80 mg NH_3/L (Cruz-Espinoza et al., 2014a; Pecson et al., 2007; Nordin

et al., 2009). However, based upon the concentrations of total ammonia nitrogen in the digesters (E15: 1,000 mg NH_3-N/L +/- 164; W45: 960 mg/L +/- 82), temperature (35 ^oC) and near-neutral pH (7.2 - 7.3) the calculated concentration of aqueous free ammonia was below (17.5 mg NH_3/L in E15 and 16.8 mg NH_3/L in digester W45) the inhibitory level.

VFAs, such as propanoic and butanoic acid have been shown to be lethal to *Ascaris suum* ova at concentrations greater than 1,000 mg/L (Butkus et al., 2012); however, observed concentrations of VFAs (measured as acetic acid in mg HOAc) were much lower (E15: 440 mg/L +/- 50.1; W45: 390 mg/L +/- 44.8). The effluent fatty acid results were similar to observations made by Ndegwa et al., who used an anaerobic sequencing batch reactor to treat swine manure at 35 ^oC. VFA (mg HOAc/L) concentrations were higher as the SRT decreased from 45 days (375 mg/L HOAc) to 15 days (435 mg/L HOAc) when compared to the mean of 420 mg/L HOAc (Ngedwa et al., 2008). Our resulting HOAc concentrations also decreased as SRT increased, and can be explained by the higher organic loading rate of the digesters with a shorter SRT, 3.6 kg VS/m^3-day at 15 days compared to 0.8 at 45 days.

An important result of the unembryonated inactivation study was the observed 24 days required to reach near complete inactivation at 35 ^oC. This number represents only the amount of time required to inactivate an *Ascaris* spp. ovum in a batch reactor under the experimental conditions. To better understand the implications of this result, a mathematical model of the residence time distribution in the digester is required. However, because residence time distributions are unique to the digester configuration, the implications on reactor design to attain a minimum residence time of 24 days would be different for every digester (e.g., semi-continuous, sequencing batch reactor, continuous, plug-flow). Our observation of 24 days at 35^oC, fits within the observations from batch studies determining *Ascaris suum* ova inactivation

with temperature; that is, 450 days was required to inactivate ova at 20 ^{o}C, 180 days at 30 ^{o}C, 14 days at 40 ^{o}C and 110 minutes at 50 ^{o}C (Nordin et al., 2009). This result demonstrates increased inactivation can be achieved by increasing the operating temperature of the digester by just a few degrees. This is especially true in the upper range of mesophilic anaerobic digestion (above 30 ^{o}C) when the time to inactivate is reduced from hundreds of days to a few weeks. The implications of this result are important because it highlights unintended consequences associated with using biotechnology to recover energy from pathogen containing wastes when ample system design is not incorporated. When managed in the right way, anaerobic digestion can help sanitation of infected remains from humans and animals to increase health of people living in rural communities by working in conjunction with other methods of control.

The results of the inactivation experiments comparing ova survival in anaerobic environments (E15, W45, An) with an aerobic environment (Ar_{35}) at 35 ^{o}C (Figure 3.1) revealed that ova exposed to an aerobic environment will inactivate much faster than ova in an anaerobic digester. This result has also been reported for other pathogens (Maya et al., 2012), although that study did not specifically look at mesophilic anaerobic digestion. More importantly, our results demonstrate that ova exposed to anaerobic environments (digester or buffered control) can survive longer than ova exposed to the aerobic buffered control at the same temperature. An explanation for this observation is that the ova shell is weaker in developed ova, in preparation for hatching when compared to an undeveloped ova. This leaves the ova more susceptible to environmental inactivation mechanisms; such as free ammonia, VFAs or temperature (Wharton, 1980; Brown, 1928).

In response to this observation, the pretreatment inactivation experiment of our study examined the fate of *Ascaris suum* ova containing motile larva and identified a potentially

successful mechanism to enhance *Ascaris suum* ova inactivation during mesophilic anaerobic digestion. This observation could lead to potential mitigation strategies in regions where soil-transmitted helminths are prevalent because aerobic pre-treatment could be used to trigger ova development before feeding the digester. A quantitative study of the influence of oxygen and temperature on the embryonic development of the *Ascaris suum* ova found that only 2×10^{-6} g of oxygen are needed to fully develop a single ovum from a single-cell embryo (stage A) to the motile forms (J, K, and L) (Brown, 1928), meaning that a pretreatment step that aerates the substrate might be sufficient to trigger development before entering the digester. Based upon the ova development experiment, approximately 15 days would be needed to promote full development of the ova at 28 ^{o}C, which is similar to ambient conditions and provides insight into how long the ova-laden waste should be pretreated. The Food and Agricultural Organization (Rome, Italy) recognizes that composting waste in piped-windrows is effective to achieve large scale passive aeration (FAO, 2006). Furthermore, a study examining the viability of *Parascaris equorum* ova found that windrow composting was effective at rendering the ova nonviable when tested under conditions at a working farm (Gould et al., 2013). The potential drawback of piped-windrow aeration would be the subsequent reduction in biogas production during anaerobic digestion from increased conversion of VS to carbon dioxide during the windrowing process.

The ova development experiment was performed to investigate whether the ova developed over time in the anaerobic digester. The results showed that *Ascaris suum* ova introduced to the digester at 28 or 35 ^{o}C will remain unembryonated during their entire residence time due to the lack of oxygen maintained by the digester's COD (effluent average of 2160 mg COD/L), resembling a dormancy stage. Conversely, when provided with an aerobic environment at 28 ^{o}C (Ar_{28}) the ova progressed through its development stages and reached a motile form that

hatches from the ovum upon stimulation from light microscopy and isolation on the glass slide. At 35°C (Ar_{35}) the embryos progressed through half of the developmental stages before being destroyed as shown in Figures 3.3C and 3.3D where all of the observations associated with aerobic control (Ar_{35}) were either non-viable (life stage NV) or non-motile (life stages below J). Kim et al. (2012) examined ova development in an environmental chamber maintained under aerobic conditions at similar temperatures and humidity to our study (control An), and observed similar patterns of accelerated development at higher temperatures that ultimately fail to reach full infectivity. Our finding that ova remain unembryonated during mesophilic anaerobic digestion is important because the survival strategy of dormancy has been described with bacteria (Colwell et al., 1985) and in plant nematodes (Perry, 1989), but has never been previously described in relation with soil-transmitted helminths, such as *Ascaris spp*.

The destruction of *Ascaris suum* ova, investigated by measuring the total number of ova recovered from each sample bag, revealed that fewer ova were recovered from each bag over time. This result implies that either ova destruction or hatching was occurring. However, because only unembryonated ova were observed in the digesters (Figures 3.3A-3.3D), ova destruction must be the only fate for the ova, as motile larva were not observed to form under these conditions. This previously unconsidered inactivation mechanism ultimately will influence the results of experiments utilizing *Ascaris suum* ova in a conservative manner, because non-viable ova cannot be accounted for at all times nor are they included in accepted counting procedures. This result is important because it suggests that the fate of an *Ascaris suum* ovum in an anaerobic digester is not just viable or non-viable, as most studies typically monitor. It also suggests the results of previous studies could be misleading if proper control over the number of ova used in

the experiment is not used. This level of sensitivity is needed because the infective dose of *Ascaris suum* is only one ovum.

3.5 Conclusions

Future research is needed to develop engineered systems that passively promote ova development without reducing the resource recovery potential of technologies, such as anaerobic digestion. Our results show that when a technology such as mesophilic anaerobic digestion is promoted to the developing world as a method to improve access to waste management while recovering valuable resources, the technology transfer strategy needs to take into account local climatic and health conditions so mutually beneficial outcomes can be attained (Mihelcic et al., 2007).

Chapter 4: The Effects of Solids Retention Time and Feeding Frequency on the Performance of Semi-continuous Mesophilic Anaerobic Digestion[2]

4.1 Introduction

As household-scale anaerobic digestion is being actively promoted in developing countries, research is needed to better understand the performance of these systems and their potential impact on environmental health. Household scale anaerobic digestion has been promoted for several decades to provide renewable energy production in rural areas of China, India, Sub-Saharan Africa and the Americas (Perez et al., 2014). The most widely used household systems are fixed dome, floating drum, and plastic tubular digesters. These are considered to be semi-continuous reactors because substrate addition occurs on an interval that is intermittent. They are used to treat agricultural residues (e.g. animal manure, crop residuals) and sometimes human wastewater. Benefits of household anaerobic digestion can include access to improved sanitation, decreased demand for woody biomass for heating and cooking, and production of an effluent that can be used as a soil amendment (Rowse, 2012). Globally, an estimated 35 million household-scale (3 to 10 m^3) fixed-dome digesters are currently in use (Bruun et al., 2014), while China plans to install up to 80 million digesters by 2020 (NDC, 2007). Since many household-scale as well as large-scale anaerobic digesters are operated in a

[2] This chapter was published as "Semi-continuous mesophilic anaerobic digester performance under variations in solids retention time and feeding frequency" in *Bioresource Technology*, Volume 190, pages 359-366. Copyright 2015 Elsevier.

semi-continuous manner, more research is needed to understand how traditional operating parameters affect the performance of these systems.

A large number of prior studies on anaerobic digestion performance have mainly been focused on optimizing biochemical methane production and VS destruction through manipulations in operating strategies in anaerobic digesters operated as continuously mixed flow reactors (CMFRs) (e.g., Lee et al., 2011; Chen et al., 2012). However limited knowledge exists pertaining to the performance of semi-continuous and/or unmixed systems. Lee et al. (2011) showed that removal efficiencies for chemical oxygen demand (COD) and VS in bench-scale CMFR mesophilic anaerobic digesters decreased with a reduction in SRT from 20 to 4 days. That study also reported that a reduction in SRT caused significant shifts in the microbial population as bacteria and archaea present became less diverse. This observation infers that the physiochemical characteristics of the biomass change with variations in SRT in CMFRs. This is hypothesized to influence the biochemical performance of the system when coupled with longer or shorter feeding frequencies such as those found in semi-continuous systems. A comparison between continuous influent additions and semi-continuous additions in anaerobic bioreactors was conducted by Li et al. (2014a) using chicken manure and corn stover. They compared the mesophilic anaerobic co-digestion in semi-continuous and continuous systems and found that 25% more methane was produced in the semi-continuous configuration, 281 ml/g-VS_{added} compared to 223 ml/g-VS_{added} when the influent was added constantly. Their results indicate a biochemical advantage to feeding the system less often without changing the organic loading rate. Other studies have evaluated continuous against semi-continuous mesophilic anaerobic digestion under varying organic loading rates. These studies concluded that biochemical methane production was slightly better in the semi-continuous systems, and also increased in

both types as the organic loading rate increased up to 6 kg/m^3/day, after which methanogenesis inhibition was likely due to volatile fatty acid accumulation that occurred (Escudero et al., 2014; Li et al., 2014b). Wang et al. (2014) presented a feeding strategy that alternated the substrate composition (i.e. food waste, chicken manure) of a semi-continuous mixed mesophilic anaerobic digester, and found that biogas production was improved when food waste was fed more often than manure. This finding has a useful application to anaerobic digester use at the household scale because of the blend of domestic and agricultural wastes that are commonly found there; however the effect of SRT or the length of time between feedings (i.e., feeding frequency) on system performance was not investigated, as was true for the other publications reviewed.

The performance of an anaerobic system can also be measured in terms of pathogen removal instead of biochemical methane production. As demonstrated by recent investigations (Chen et al., 2012; Huong et al., 2014; Manser et al., 2015a), the survival of indicator bacteria and human pathogens during mesophilic anaerobic digestion is common. The US Environmental Protection Agency recommends that sludge digested anaerobically under mesophilic temperatures should be kept out of human and animal contact for at least one year after digestion (USEPA, 2000). Further investigation is needed to find operational strategies for household scale anaerobic systems that enhance pathogen removal because the biosolids are typically promoted for immediate land application. Recalling that changes to the SRT of continuous anaerobic systems may cause shifts in the microbial populations (Lee et al., 2011) through different methane production values, it is important to investigate if this finding holds true for other microorganisms, such as *E. coli* or *Salmonella*, in semi-continuous systems because of the threat to public health that mesophilic sludge can present. Furthermore, the yet undefined

influence of varying feeding frequencies on the semi-continuous system could be used to enhance pathogen removal and warrants investigation.

The objective of this research was to investigate how variations in SRT and feeding frequency both influence biochemical processes of mesophilic anaerobic digesters operated semi-continuously in an unmixed reactor. This configuration was selected to mimic how millions of anaerobic household digesters are operated in the field. Performance metrics used in this study include: the biochemical methane formation potential assay (BMP), the specific methanogenic activity assay (SMA), biogas production and methane content, VS removal, soluble chemical oxygen demand (sCOD) removal, *E. coli* removal, and *Salmonella* removal. To our knowledge this is the first study to also investigate the fate of indicator bacteria and human pathogens during semi-continuous anaerobic digestion under different feeding frequencies under conditions that would be encountered in the field at millions of existing or planned household systems.

4.2 Material and Methods

4.2.1 Anaerobic Digester Set-up

A pilot-scale (21 L) semi-continuous anaerobic digester that was operated for more than one year with an influent swine waste feed and an SRT of 21 days prior to the beginning of this experiment. The pilot-scale digester was initially inoculated with seed sludge from an anaerobic digester treating food waste in the laboratory of Dr. Ann Wilkie in the Department of Soil and Water Science at the University of Florida in Gainesville, FL (Kinyua et al., 2014). The inoculum biomass for the small-scale experimental digesters used in this study was harvested from the pilot-scale system. Influent for the experimental systems consisted of 280 grams of

fresh manure (Twenty-Four Rivers Family Farm, Plant City, Florida) and 1,500 ml of local groundwater (University of South Florida Botanical Gardens).

As described by Manser et al. (2015a), 900-ml of the inoculum biomass from the pilot-scale system was transferred into six unique one-liter glass bottles to represent bench-scale anaerobic digesters. These were labeled as E15, E30, E45, W15, W30, and W45 (Table B1). The label identification represents the feeding frequency (E – every other day; W – weekly) and the average SRT (15, 30 or 45 days). The six experimental digesters were consistently fed the same swine manure slurry, as described previously, throughout the start-up, operational and experimental phases. However, it should be noted that due to daily variations in the manure source some variations in the feed composition were observed during the study. The detailed values reported in Tables 4.1 (influent) and B.2 (effluent) are averages of at least 10 weeks of measurements. Urea was also added to simulate the presence of urine and to maintain the total nitrogen in the system to 1,000 mg/L. Each digester was operated at 35 ^{o}C with no mixing, which is similar to a previous bench-scale experiment optimizing digestion of swine manure (Kinyua et al., 2014). The top of each reactor was fitted with a three piece air-lock (Northern Brewer #7010) to maintain anaerobic conditions while allowing biogas to escape; the lock was removed quickly only to perform sampling, feeding, and wasting. The exception to this was during biogas production measurements when the biogas was routed to a wet-tip gas meter, as described in Section 4.2.3.

Table 4.1 – Summary of the swine manure slurry influent key parameters. The data shown are the averages of weekly measurements collected for 10 months. Standard deviations are shown in parentheses.

Parameter (units)	Swine Manure Slurry Influent Parameter (standard deviation)
pH	8.4 (0.2)
Total Solids (g/L)	68 (19.6)
Volatile Solids (g/L)	46 (13.7)
Soluble Chemical Oxygen Demand (mg /L)	5,290 (460)
Ammonia Nitrogen (mg/L NH_3-N)	434 (133)
Volatile Fatty Acids (mg/L HOAc)	1,130 (330)

4.2.2 Analytical Methods

Anaerobic digester performance was measured by collecting 50-ml grab samples during the feeding (influent) and wasting (effluent) exchange steps, with the exception of digester E45 as the influent/effluent volume was limited to 40 ml for this system. Samples were collected every other Monday for 10 months. The grab samples were divided, with one sample used to measure total VS (ASTM 2540E). The other sample was centrifuged at 10,000 rpm (10 rcf) for 10 minutes. The supernatant was analyzed colorimetrically for the following: sCOD by oxidization by potassium dichromate (Hach TNT825), volatile fatty acid (VFA) by diol reaction and reduction with Fe^{3+} (Hach TNT872) and reported as mg HOAc/L, and ammonia by reaction with ions of hypochlorite and salicylate in sodium nitroprusside to form indophenol blue (Hach TNT832).

4.2.3 Biogas Quality and Quantity

The volume (ml) of biogas produced was measured at 35 ^{o}C using a Wet Tip gas meter (Wayne, PA) over 21 days for each digester and then normalized by the amount of substrate (VS) added to the digester. The methane fraction of the biogas was measured by dissolving the carbon dioxide portion of a 10-ml of biogas sample into 1% v/v potassium hydroxide solution and measuring the displaced liquid volume (ASTM D1827). The methane produced for each

digester was determined from the average methane concentration of the biogas and the amount of biogas produced during the twenty-one day measurement period. The calculated volume was then corrected to STP conditions by a factor of 1.12, assuming constant pressure.

4.2.4 Indicator Organism Assays

The densities of *E. coli* and *Salmonella* were measured by collecting 50-ml grab samples (40-ml from digester E45) during random feeding (influent) and wasting (effluent) maintenance steps. Samples from the influent and effluent were collected on three separate occasions at least ten days apart. Samples for *E. coli* analysis were immediately processed according to the IDEXX Colilert system (Westbrook, ME) manufacturer instructions. *Salmonella* samples were maintained at 4 ^{o}C during transportation to EMSL Analytical Laboratories in Orlando, FL, where the samples were processed according to *Standard Method* 9260B.

4.2.5 BMP and SMA Assays

After eight months of operation BMP and SMA assays were conducted. The BMP assay was used to assess the robustness of the biomass from each digester. The assay was performed according to the method of Lisboa and Lansing (2013), with the exception that no additional nutrients were added because swine manure has been shown to contain sufficient nutrients for the test (Garcia et al., 2010). Triplicate 150-ml glass septum sealed bottles were used for the assay. For each bottle, 40 ml of biomass (approximately 30 g/L) was extracted from each digester and centrifuged at 10,000 rpm (10 rcf) for 10 minutes. The supernatant was discarded and the centrate was suspended in a phosphate buffered saline solution in sufficient volume to reach 40 ml. This suspension was added to a bottle containing 60 ml of sterilized substrate (4 g/L glucose), capped and placed on a shaking table (85 rpm) at 35 ^{o}C for 14 days. The volume of biogas produced was measured daily by using a gas-tight 50-ml glass syringe. The methane

content of the biogas was measured three times during the 14 days as described previously. The SMA assay was used to assess the functionality of the biomass. The experimental set-up and procedure for the SMA assay was identical to the BMP assay with one exception, the quantity and quality of the biogas generated was only measured for the first 72 hours (3 days versus 14) of the experiment. This method was similar to that presented by Jimenez et al. (2015).

4.2.6 Statistical Methods

The one-way normal analysis of means (ANOM) procedure in Minitab 16 was used to compare anaerobic digester operation parameters and pathogen removal within a 95% confidence interval. To complete the analysis, groupings were created for the respective parameters based upon: average SRT, feeding frequency and individual digesters. For example, statistical differences resulting from variations to feeding frequency were detected by grouping digesters E15, E30 and E45 and comparing the results with the group W15, W30, and W45.

4.3 Results and Discussion

4.3.1 Digester Performance

The six anaerobic digesters in this study were monitored for the quantity of biogas produced (Figure 4.1) and methane content (Table B2) during the experiment. After multiplying the quantity by the methane content, the value was corrected to STP and normalized by the organic loading rate to obtain a methane yield (Figure 4.1). Methane yields ranged from 0.15 to 0.23 m^3 CH_4/kg-VS_{added}, which were similar to values reported by Kinyua et al. (2014) while semi-continuously digesting swine manure at 35 ^oC in reactors fed three times per week at SRTs ranging from 15 to 42 days. The best performances in that study were observed at SRTs of 21 and 28 days, which supports our observations that an SRT of 30 days was more productive than 15 or 45 days. Digester W30 had the highest methane yield (0.23 m^3 CH_4/kg-VS_{added}), while

digester E45 had the lowest (0.15 m^3 CH_4/kg-VS_{added}). When grouped by SRT, the 15 and 30 day digesters had a higher methane yield than the 45 day groups. When grouped by feeding frequency, the digesters fed weekly produced 10% more biogas than those fed every other day (0.20 compared with 0.18 m^3 CH_4/kg-VS_{added}).

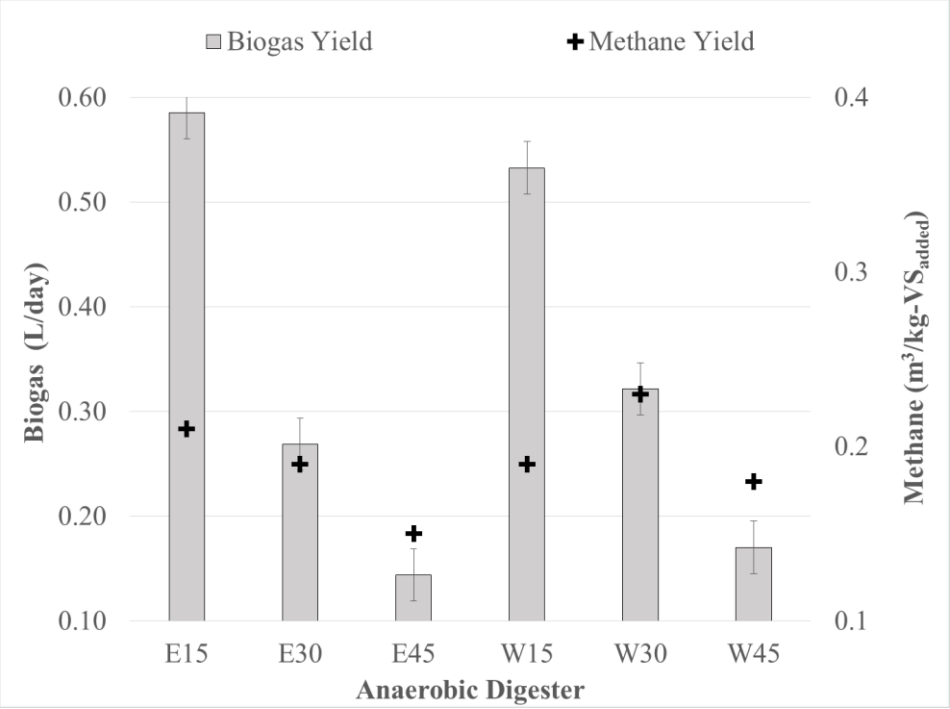

Figure 4.1 – The yields of the biogas and methane produced by the unmixed semi-continuous mesophilic anaerobic digesters in this study, corrected to STP.

Methane contents for the six digesters are shown in Table B2. The average value of 60% +/- 1.2% falls into the range reported in a review of continuous anaerobic digestion (Amani et. al. 2010). Methane content of the biogas increased with increasing SRT; however no difference in methane content was observed with variation in the feeding frequency. Our results suggest that a semi-continuous anaerobic digester that is fed with a longer interval between feedings is capable of producing more methane and if a longer feeding frequency is combined with an SRT of 30 days this effect is enhanced. Therefore this research suggests that household scale digesters should be fed less often and infrastructure should be improved to include a substrate

storage area to accommodate the longer time between feeding. Note that the ability of farmers to operate digesters at longer feeding frequencies might be limited by the frequency of cleaning livestock pens, veterinary health considerations, availability of storage tanks, labor availability, etc.

VS removal among the six digesters was not significantly different (Table B2), with the exception of E15, which had the lowest removal of the group (38%) compared with the average (44%) and best (47%) of all six digesters. The ANOM statistical procedure indicated that digesters with a higher SRT (E45 and W45) had higher VS removal compared to the average VS removal of the other digesters (45% compared to 41%). A higher VS removal associated with higher SRT was expected because a longer retention time provides more opportunity for volatile compounds to be degraded by the microbial community. In a similar experiment investigating the effect of SRT on the bioavailability of organic carbon in anaerobically digested swine waste, VS removals improved slightly (50 to 52%) when the SRT of the digester was increased from 14 to 28 days (Kinyua et al., 2014). No effect on VS removal was observed between the digesters fed every other day or weekly. Considering that the digesters fed weekly produced more biogas than those fed every other day, it is interesting that the VS removal in the weekly fed systems was not better.

The average effluent sCOD concentration in the six digester effluents was 2,160 mg/L, which corresponds to an average removal of 59% (from Table 4.1, influent sCOD was 5,290 mg/L). The highest sCOD removal was observed in digesters E45 (62%) and W45 (61%) compared to the lowest removal in digester E15 (56%). Similar to VS removal, removal of sCOD improved with a longer SRT; however, variations in the feeding frequency did not affect sCOD removal (59% for groups E and W). Kinyua et al. (2014) also observed better sCOD

removal with a longer SRT, 71% removal at a 14 day SRT up to 75% removal at 42 days in a semi-continuous operating scheme. The one-way normal ANOM procedure verified that there were no significant differences in the sCOD removal among the combinations of SRT or feeding frequency. In terms of practical implementation at the household scale, recovering resources from the sCOD and VS should be optimized by designing for a digester volume that can support an SRT of 30 to 45 days.

4.3.2 Biochemical Methane Formation Potential Assay

The BMP assay was used to measure the biomass robustness of the six digesters. Six different methane production trends were observed over the fourteen day experiment (Figure 4.2). Digester E45 had the greatest methane production (320 ml CH_4), while digesters E30 and W15 produced at least 33% less methane (both < 215 ml CH_4). Digester E30 and W15 also stopped producing biogas after eight days in the limited substrate environment compared to digester E45. This reduction is demonstrated by the slope of the BMP curve for each digester on days 10 through 14, 10.4 ml CH_4/day in the gas productive digester E45 compared to 2.2 ml CH_4/day in digester W15. Because the amount of available food is fixed, the biomasses that were able to produce biogas consistently during the test period can be considered to be more robust because they were able to adapt and thrive in the new environment more readily than the other biomasses (Elbeshbishy et al., 2012). Molecular qPCR studies carried out by St. Pierre et al. (2013) showed that an increase in SRT from 21 to 30 days resulted in a more diverse methanogenic population. Our observations also suggest that anaerobic digesters with a longer time between feeding (i.e., longer feeding frequency) produce methane faster than those fed more often, implying the presence of a robust and diverse microbial population. This finding provides support for operating a household digester with a longer interval between substrate

additions to select for a biomass that is capable of withstanding process changes and interruptions.

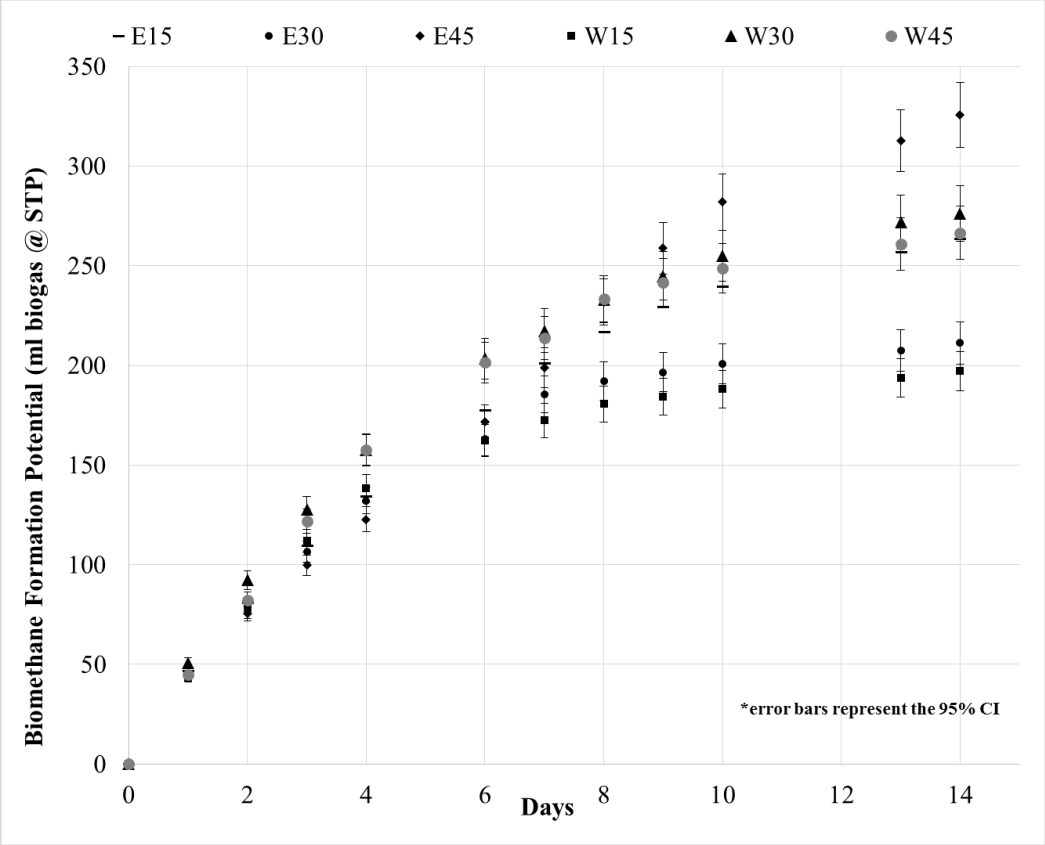

Figure 4.2 – The biochemical methane formation potential of the six experimental digesters in this study.

4.3.3 Specific Methanogenic Activity Assay

The SMA assay was used to observe the functionality of the biomass, which was defined in this study as the ability of the biomass to adapt or transition to a new process environment without sacrificing biogas production (Sorenson and Ahring, 1993). The SMA assay used the amount of biogas (or methane) produced during the first three days of the BMP assay and is represented by the slope of a line from day zero to day three in Figure 4.2. Figure 4.3A presents the results of that analysis and shows that digester W30 had the highest SMA (42 ml CH_4/day) and that digester E45 had the lowest (33 ml CH_4/day) in the limited substrate environment. This

result is interesting because the biomass from digester E45 was the most robust in terms of surviving in the limited substrate environment used in the assay, but it was the slowest to acclimate to the limited substrate environment. This implies that a combination of shorter intervals between feedings and a longer SRT will promote a more reliable anaerobic digestion biomass that is resistant to system shock. Reliability is important at the house-hold scale because resources to re-establish a functioning anaerobic biomass may be limited.

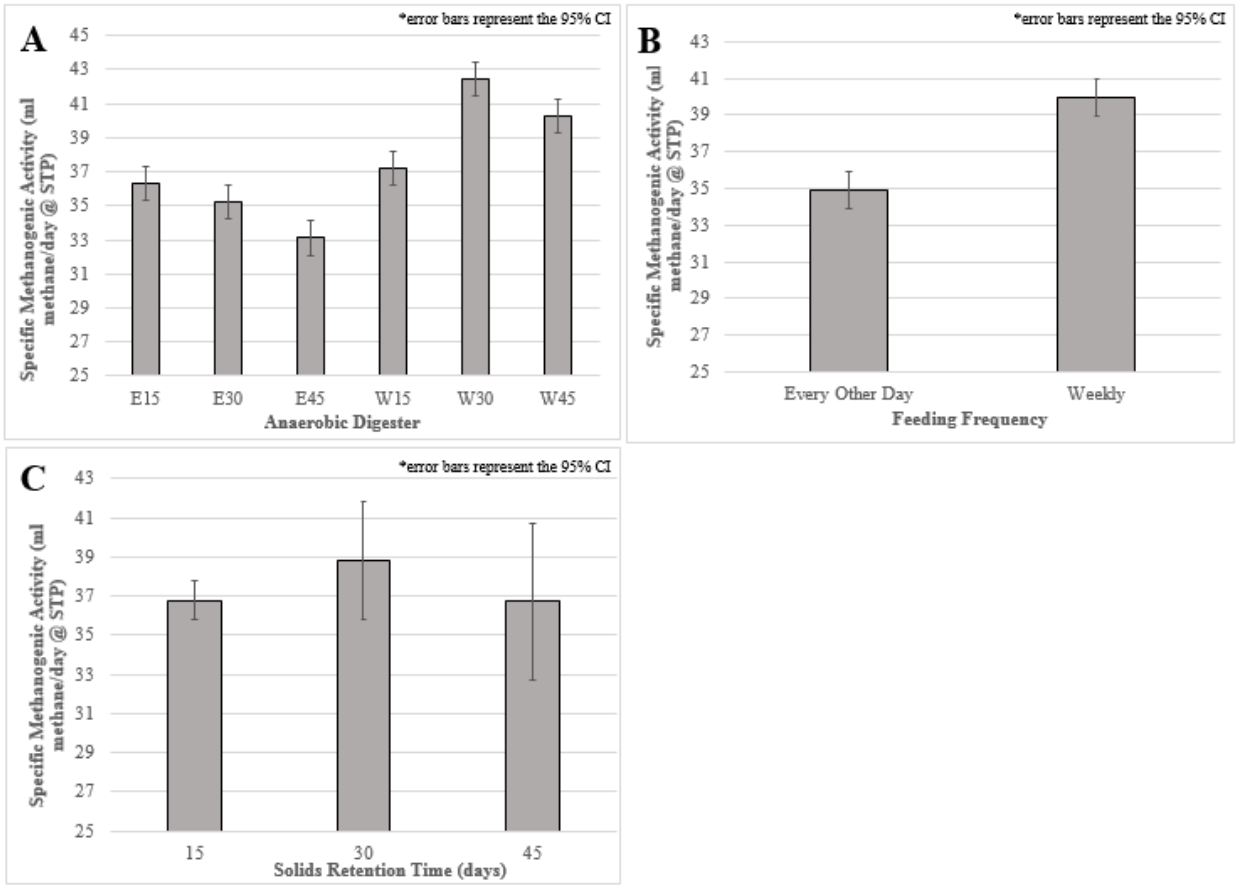

Figure 4.3 – The specific methanogenic activity of the six experimental digesters are shown in (A). The same parameter is shown in (B) for digesters grouped by feeding frequency and digesters grouped by solids retention time are shown in (C).

Figure 4.3B shows that the digesters that were fed less frequently had significantly higher SMA assay scores (40 ml CH_4/day) when compared to the more frequently fed systems (35 ml CH_4/day). One reason for this observation is because the longer cycled biomass is accustomed to

periods of starvation so the balance of microbial activity favors the production of storage polymers instead of immediate biogas production (Hwang et al., 2010). This makes it possible for the biomass from these longer cycled digesters to produce biogas faster during a SMA because they have brought bioavailable substrate with them in their storage polymers. These results suggest that process stability can be improved by feeding the digester less frequently.

4.3.4 *E. coli* and *Salmonella* Removal

It is important to understand how indicator bacteria and human pathogens interact with an anaerobic microbial population that has adapted to a variable feeding environment because these microorganisms, and other human pathogens present in livestock waste, have been shown to survive the mesophilic anaerobic digestion process (Johnson, et al., 1998; Chen et al., 2012; Manser et al., 2015a). The removal of *E. coli* was measured in the six digesters. The fecal indicator bacteria *E. coli* was selected because it sometimes can be pathogenic to humans and allows for comparison with previously published research. Measured concentrations of *E. coli* (MPN/g biosolids) in the influent and effluent of the six digesters are shown in Figure 4.4A. A removal of $\geq$ 2-$\log_{10}$ reduction in the *E. coli* was observed in all digesters, digester W30 nearly achieved a 3-$\log_{10}$ reduction (99.8%) and digester E45 barely reached the 2-$\log_{10}$ (99.1%) level. Similar results were observed by Smith et al. (2005), who observed a 2-$\log_{10}$ reduction in *E. coli* after 20 days at 30 ^{o}C during the mesophilic digestion of municipal sludge. As shown in Table B2, the concentrations of total ammonia nitrogen did not exceed 1,000 mg/L (NH_3-N), which when coupled with the observed temperature (35 ^{o}C) and pH of the systems (average of 7.26) does not allow for the formation of free ammonia and limits the level of *E. coli* removal that can be achieved. Vinnerås et al. (2008) reported an inactivation threshold concentration of ammonia for indicator bacteria to be approximately 40 mM free ammonia (e.g. 2,100 mg/L NH_3-N and pH

8.9 at 24 °C). The volatile fatty acid concentrations reported in Table B2 also remained below lethal levels for many microorganisms, including fermentative bacteria such as *E. coli* (3,600 mg/L as acetate; Wolin, 1969) and methanogenic bacteria (10,000 mg/L as acetate, Amani et al., 2010). The performance results in Table 4.2 are useful because they show that the experimental digesters were functioning well below inhibitory levels for methanogenesis despite the lack of disinfection occurring within them. This exemplifies a common trade-off that must be made when mesophilic anaerobic digesters are used at the household scale, especially when fed with substrates containing human pathogens.

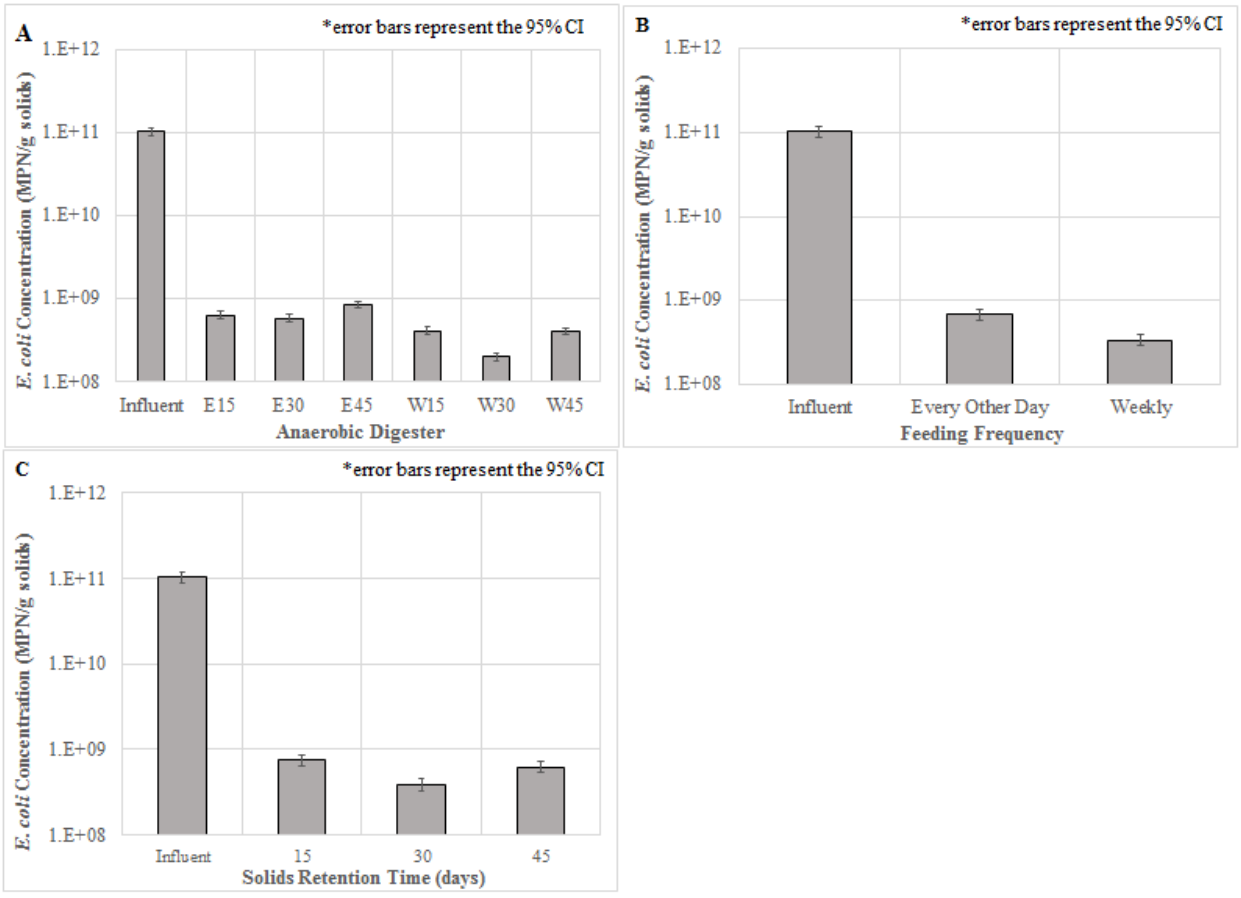

Figure 4.4 – The *E. coli* concentrations of the influent and six experimental digesters are shown in (A). The same parameter is shown in (B) for digesters grouped by feeding frequency and digesters grouped by solids retention time are shown in (C).

Using a 95% confidence interval, the one-way normal ANOM procedure showed that when grouped by feeding frequency, there was greater removal of *E. coli* in the digesters fed weekly (99.7%) compared to the digesters fed every-other-day (99.4%). This result is shown in Figure 4.4B and was verified by single factor ANOVA (95% confidence interval) where the F_{value} (6.1) exceeded the $F_{critical}$ of 4.5 ($\alpha = 0.05$, $p = 0.02$). *E. coli* serves as an indicator bacteria for many other human pathogens that survive the digestion process. Based upon this finding, it may be possible to operate household digesters with a longer interval between feedings to encourage pathogen destruction.

The different *E.coli* removals could be due to the effect of the indigenous microbial community structure of each digester, which was adapted to thrive in the anaerobic environment though mechanisms of predation and competition. Stolpovsky et al. (2011) modeled microbial population shifts of two competing species exposed to periodic feeding and used the model to infer that microorganisms with an effective dormancy-reactivation strategy developed through long periods between feeding cycles may have a competitive advantage over microorganisms that have not acclimated to the starvation period. That is, organisms that can switch rapidly in response to fluctuations in external conditions may outcompete fast-growing organisms. The higher nutrient (N and P) level of the swine manure influent may also increase the activity of native bacterial predators such as ciliated protozoa (Garcia et al., 2010). Protozoa have been shown to prey upon *E. coli* in activated sludge systems (Curds and Fey, 1969); a higher number of protozoa also have been correlated with higher biogas production during anaerobic wastewater treatment (Pyria et al., 2007). Our results show that digesters with higher biogas production had better *E. coli* removal. Digester W30 produced more biogas (715-ml) and had significantly higher *E. coli* removal (99.9%) compared to digester E45 (472-ml, 99.3%). Referring to Figures

4.3B and 4.4B, it can be seen that a longer interval between feedings promoted a higher SMA assay score and better removal of *E. coli*. Our observation supports the results of Choi et al. (2009) who found that the increased activity of the native microbial population (in our case measured by SMA assay) is capable of outcompeting other microorganisms like *E. coli* in anaerobic wastewater treatment systems. This finding is important for the operation and performance of household anaerobic digesters because effluents may be used to fertilize food crops.

Measured concentrations of *Salmonella* (MPN/4g biosolids) in the influent and effluent of the six digesters are shown in Figure 4.5A. The range of influent *Salmonella* concentrations was highly variable; however, consistent removal was achieved by the six digesters with an average removal of 86%. Although not significant, removal was better when the SRT and feeding frequency of the digesters were increased simultaneously. These results are reasonable, as 0.5 to 4-$\log_{10}$ *Salmonella* destruction during mesophilic anaerobic digestion has been observed previously (USEPA, 2013); and can be partially explained by the non-lethal concentrations of ammonia and VFAs observed in the digesters during the experiment (Table 4.2). Huong et al. (2014) studied biogas plants on swine farms in Vietnam and found that of the 96 farms surveyed, almost 80% discharged effluent containing pathogens, such as *Salmonella,* and/or fecal indicator bacteria. Certain strains of *Salmonella,* such as *S. Typhimurium,* have been shown to be more resistant to environmental stresses and survive for longer periods of time when compared to *E. coli* (Franz et al., 2005).

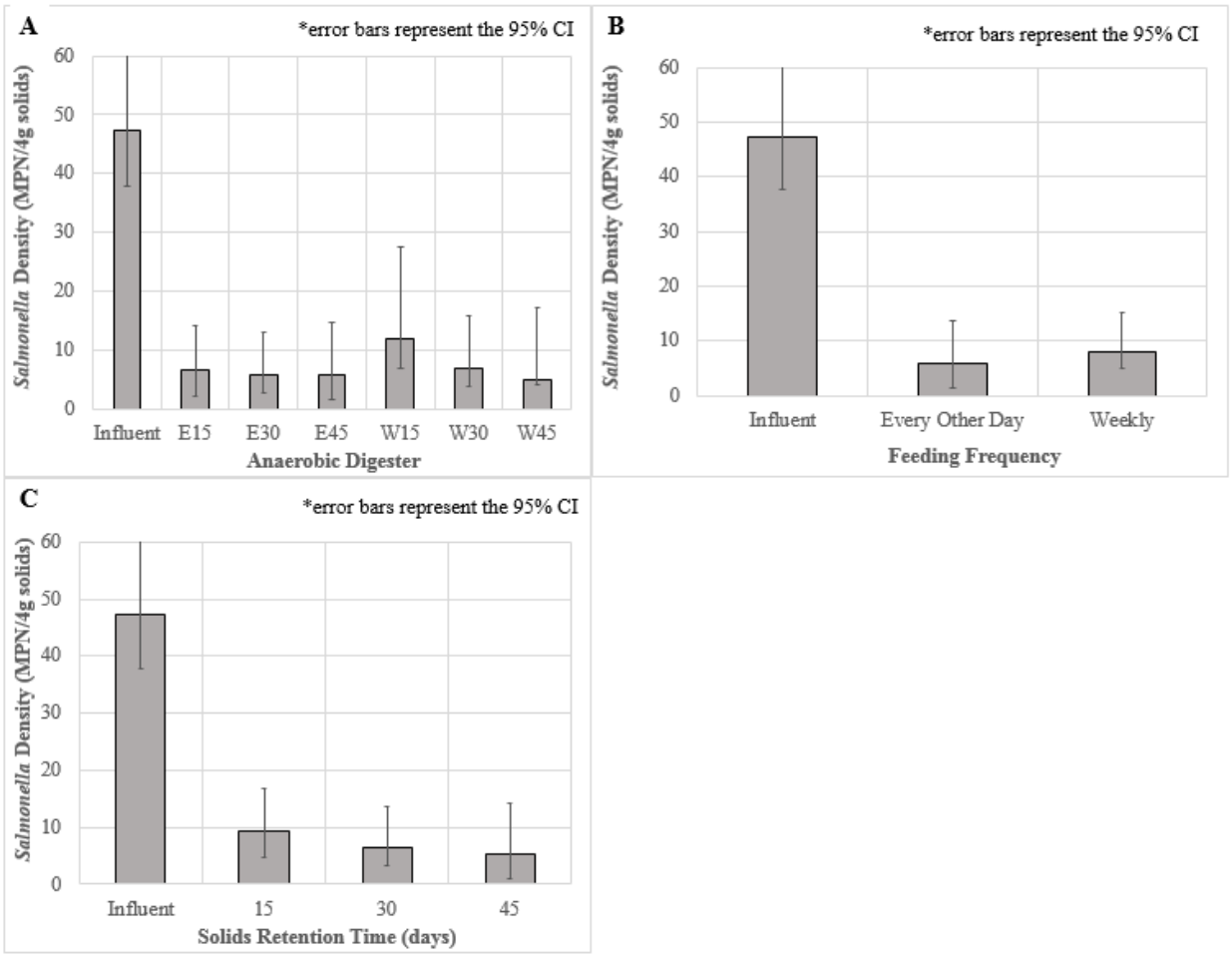

Figure 4.5 –The *Salmonella* concentrations of the influent and six experimental digesters are shown in (A). The same parameter is shown in (B) for digesters grouped by feeding frequency and digesters grouped by solids retention time are shown in (C).

Understanding the fate of *Salmonella* during mesophilic anaerobic digestion is important because it is a human pathogen that can be derived from the handling of agricultural wastes. Our results demonstrate the difficulty in predicting the removal of pathogens, such as *Salmonella* from the effluent of anaerobic digestion systems. This issue could be exacerbated when other human pathogens (i.e. roundworms, viruses, protozoa) are considered because their removals may not correlate with *E. coli*.

4.4 Conclusions

This chapter demonstrated that semi-continuous mesophilic anaerobic digesters operated with longer feeding frequencies will have higher average methane yields, better specific methanogenic activities, and improved fecal indicator bacteria destruction than those fed on a shorter interval. These results have direct implications on how household- and large-scale biological waste-to-energy systems can be operated to improve resource recovery and protect public health. Furthermore, based upon the range of SRTs tested in our study the digesters operated at the highest SRT resulted in the better performance when coupled with a longer feeding frequency.

Chapter 5: Modeling *Ascaris Suum* Survival in Semi-continuous Anaerobic Digesters

5.1 Introduction

Anaerobic digestion of carbon-rich anthropogenic wastes has been established globally as an important biotechnology for resource recovery and sustainable energy generation. When applied on a small scale, the benefits of anaerobic digestion are often recognized directly and indirectly in several areas (Surendra et al., 2014). For example, the most direct benefit is the provision of biogas that can be used as an energy source and a stabilized nutrient-rich slurry that can be used as a fertilizer or soil amendment (Eamens et al., 2006). Indirect benefits include improved economic advantages (Ferrer et al., 2011), reduced environmental impacts (Gautam et al., 2009; Rowse, 2012), better human health outcomes resulting from less solid biomass combustion (Bruce et al., 2000; Rowse, 2012), and social enhancements that improve gender equity (Katuwal and Bohara, 2009). In all, the combined effect of these benefits is an improved quality of life for the user, which makes the application of anaerobic digestion at the household scale a promising technology for communities that are distant from centralized wastewater treatment and energy infrastructure.

The most commonly applied anaerobic digester configurations in the developing world are the fixed-dome or tubular digester, with an estimated 100 million systems planned to be in service globally by 2020 (NDC, 2007). These systems typically have a working volume of 3 to 10 m^3 (Bruun et al., 2014). Unlike large-scale continuously–fed anaerobic digesters that treat municipal, industrial and agricultural waste, the smaller digesters utilized at the household level

are operated in a semi-continuous manner, meaning discrete influent additions. This characteristic of small-scale anaerobic digesters is important because it changes the relationship of the mixed liquor residence time for the reactor, which can have direct implications on how the digester performs and how long certain constituents (e.g. pathogens, volatile organics) are exposed to the reactor conditions. Chapter 4 (Manser et al., 2015b) showed that when the discrete feeding interval of a semi-continuous digester was increased from 2 days to 7 days, better methane production and *E. coli* destruction was achieved. However, the same study did not observe any improved removal of *Salmonella* when the feeding interval or SRT were varied. These results show that the influence of discrete feedings on pathogen removal are not consistent for different pathogen types during anaerobic digestion; however, the improved removal of *E. coli* in the weekly-fed digesters suggests that the feeding frequency may have some effect on pathogen fate and warrants further investigation..

The survival of soil-transmitted helminth ova, such as *Ascaris suum*, during anaerobic digestion has been determined to be exceptional due to the fact that a well-functioning digester environment is not lethal enough to promote their destruction (Chen et al., 2012). Table 5.1 reports the time required to inactivate 99% of *Ascaris suum* ova (t_{99}) under different temperature conditions from several different studies. From the information in Table 5.1 it can be seen that anaerobic digestion that occurs below 35 ^{o}C will not be sufficient to achieve rapid ova inactivation, and at digestion temperatures below 20 ^{o}C, a significant percentage of ova were predicted to remain viable for several years. This observation is important because small-scale anaerobic digesters are often operated at ambient temperature conditions, which do not exceed 35^{o}C in many places where these digesters are in service. This means that the primary inactivation mechanism (temperature) in a well-functioning digester can only be utilized by

retaining the ova in the system for as long as possible through manipulation of the average SRT, or possibly by changing the time between feedings. The issue is further exacerbated because the prevalence of *Ascaris* related infections currently impacts 1 in 4 people globally and the parasite can survive many household anaerobic digestion strategies (Pullan et al., 2014; Manser et al., 2015c), placing great need for improved understanding about how operation of small-scale anaerobic reactors may help to address issues of food security and energy demand, while also harming human health in some regions of the world (Verbyla et al., 2013b).

Table 5.1 – Values of the time needed to reach 99% (t_{99}) inactivation of *Ascaris suum* ova under various temperatures reported in literature. The reported values are from controlled experiments at a pH of 7 and relative humidity of 100%.

Temperature	t_{99} (days)	Reference(s)
10 ^{o}C	> 2,555	Sanguinetti et al., 2005
20 ^{o}C	450	Pecson et al., 2007
30 ^{o}C	180	Pecson et al., 2007
35 ^{o}C	22	Manser et al., 2015a (Chapter 3)
40 ^{o}C	5	Pecson et al., 2007
50 ^{o}C	110 min to 4 days	Pecson et al., 2007; Hawksworth et al., 2010

There currently is limited knowledge related to the residence time distribution profiles for small-scale semi-continuous anaerobic digesters, especially when combined with variations in the feeding frequency (Escudero et al., 2014; Li et al., 2014b; Wang et al., 2014). The primary research objective is to develop a mathematical model that incorporates semi-continuous reactor residence time distribution theory and inactivation kinetics to predict the survival of *Ascaris suum* ova in discretely-fed semi-continuous digesters. This model will be useful to determine if the feeding frequency or SRT of the reactor is more important in pathogen removal.

5.2 Materials and Methods

This study will relate three inactivation modeling approaches to current inactivation data to determine which provides the best fit and is the most appropriate for use in the main

expression. An additional model that describes the residence time distribution of semi-continuous reactors will also be developed. The expressions will then be used with Equation 2.4 to estimate the concentration of viable ova in the digester effluent.

5.2.1 Experimental Anaerobic Digesters

Six bench-scale semi-continuous anaerobic digesters were inoculated and operated as described in Chapters 3 and 4 (Manser et al., 2015a and 2015b). The digesters were maintained at a mesophilic temperature of 35 ^{o}C with no mixing and were operated with average SRTs of either 15, 30 or 45 days. The semi-continuous configuration was sustained by influent additions and effluent removals occurring every-other day or weekly. The six digesters were identified as E15, E30, E45, W15, W30 and W45, which corresponds to a feeding frequency (E: every-other day; W: weekly) and the average SRT (15, 30, or 45 days). The digester influent and effluent were monitored weekly for various chemical and physical parameters to ensure that well-functioning systems were present. The volume of biogas and methane produced by each digester was also monitored to ensure anaerobic conditions were maintained. A complete discussion of performance metrics and related results from the six digesters is available in Chapters 3 and 4.

5.2.2 Inactivation Data Acquisition

Following the detailed methodology presented in Chapter 3 (Manser et al., 2015a), viable ova of *Ascaris suum* were introduced into each of the six digesters using 36 nylon mesh (35µm) bags to enclose the ova during the experiment, but also allow for interaction with the digester environment. Each bag contained approximately 900 ova. Over the following 24 days the bags were removed in triplicate every-other day and rinsed with deionized water. The bags were then incubated aerobically for 15 days at 28 ^{o}C in a 0.2M phosphate buffered solution with hand-mixing each day to ensure an aerobic environment was maintained. After incubation the bags were rinsed with deionized water and then opened; the ova were then extracted by pipette and

placed upon a glass microscope slide for viability measurements. Using a light microscope with a magnification of 100, a minimum of 360 ova were observed to determine the percentage of the initial 900 ova that remained viable. The results of the inactivation experiment are provided in Table 5.2.

Table 5.2 - The percent viability of *Ascaris suum* ova for anaerobic reactors and controls. The viability percentage is the average of triplicate measurements of at least 360 ova observations after a 15day aerobic incubation at 28 ^{o}C. Viable ova were considered to be motile larva.

Day	E15	E30	E45	W15	W30	W45	AVE
2	98%	97%	93%	92%	93%	93%	94%
4	94%	92%	89%	89%	93%	90%	91%
6	86%	89%	87%	87%	88%	88%	88%
8	84%	86%	85%	84%	85%	82%	84%
10	80%	81%	87%	82%	84%	82%	83%
12	76%	80%	77%	75%	74%	76%	76%
14	71%	72%	69%	72%	74%	70%	71%
16	73%	62%	71%	65%	64%	57%	65%
18	53%	53%	44%	41%	48%	57%	49%
20	23%	19%	22%	18%	12%	21%	19%
22	1%	3%	1%	1%	3%	1%	2%
24	0%	0%	0%	0%	0%	0%	0%

The symbols in Table 5.2 correspond to different experimental reactors and controls. Reactors, denoted as E15, E30, E45, W15, W30, and W45, represent the feeding frequency (E for every other day, W for weekly) and the average SRT (15, 30 or 45 days) used to investigate the fate of unembryonated ova during anaerobic digestion.

5.3 Modeling Approach

Three model types were examined in this study. The first model type (Equation 5.1) combined residence time distribution models with pathogen survival models into a mathematical relationship that can be used to predict the effluent concentration for the constituent of concern, in this case viable *Ascaris suum* ova. The second represented the residence time distribution of the semi-continuous anaerobic digesters based upon the average SRT and feeding frequency (Equation 5.3). The third were models fitted to the inactivation data (Equations 5.6 through 5.8), which in this study were represented by three unique tactics: 1) a two-stage linear approach, 2) a sudden die-off approach, and 3) an error function approach.

5.3.1 Estimation Model

Selleck et al. (1970) first published a model (shown in Equation 5.1) to estimate the concentration of a microorganism in reactor effluent that is based upon both the inactivation kinetics of each organism [$R(t_m)$] and the residence time distribution [$E(t_m)$] of the reactor. This model is based on a segregated flow assumption that simulates the effects of non-ideal mixing by assuming that the microorganism travels through the reactor in a discrete element and reacts with the bulk solution during its residence time (Crittenden et al., 2005). The variable (t_m) is the residence time of a fluid parcel or *Ascaris suum* ovum that is in the reactor for (m) feeding intervals.

$$\frac{N}{N_o} = \sum_{m=1}^{\infty} E(t_m)\, R(t_m),\ where\ t_m = m \times n \qquad \text{Equation 5.1}$$

5.3.2 Semi-continuous Residence Time Distribution Model for $E(t_m)$

For a semi-continuous reactor three variables are defined: the time interval (usually reported in days) between successive reactor feedings (n), the average hydraulic retention time (usually reported in days) in the reactor, and the fraction of reactor fluid volume displaced during each feeding (p). These variables are related by Equation 5.2. The average SRT (mathematically expressed as τ in this chapter), in days, can also be represented by the relationship shown in Equation 5.2, where Q is the time-averaged flow rate into and out of the reactor, V corresponds to the working volume of the reactor, and V_f corresponds to the volume of each feeding.

$$p = \frac{n}{\tau} = \frac{nQ}{V} = \frac{V_f}{V} \qquad \text{Equation 5.2}$$

Accordingly, the discrete residence time distribution, $E(t_m)$, for a semi-continuous reactor is represented by Equation 5.3.

$$E(t_m) = p(1-p)^{m-1} \qquad \text{Equation 5.3}$$

For Equation 5.4 to be true two conditions must be satisfied. The first is that the sum of all E(t_m) fractions for a system must be equal to 1 (Equation 5.4). The second is that the average time the fluid is in the reactor is equal to τ (Equation 5.5).

$$\sum_{m=1}^{\infty} E(t_m) = 1 \qquad \text{Equation 5.4}$$

$$\sum_{m=1}^{\infty} t_m E(t_m) = \tau \qquad \text{Equation 5.5}$$

5.3.3 Pathogen Survival Models for R(t)

Based upon the inactivation data provided in Table 5.2, three inactivation models, known as R(t), were fit to the data using Microsoft Excel. R(t) is the fraction of ova surviving after a time t in the reactor. Therefore as t increases, R(t) decreases, because over time, fewer ova survive.

The two-stage linear approach utilized Equation 5.6; where the variable (t) represents the number of days that viable ova were exposed to the reactor conditions, and the variable (k) represents the slope of the inactivation data with units of 1/day. The variable (b) is the y-intercept for the inactivation model, and the value t_Δ is the day where the slope of the inactivation data changes. This value is determined experimentally.

$$R(t) = k_1 t + b_1, \ when\ 0 < t < t_\Delta \qquad \text{Equation 5.6}$$

$$R(t) = k_2 t + b_2, \qquad when\ t > t_\Delta$$

A corrected error function approach was also utilized, Equation 5.7, were variables (α) and (β) represent fitting parameters with units of days, variable (t) represents the number of days that the ova were exposed to the reactor conditions, and variable ($t_{critical}$) is estimated based upon the time when 50% viability of the ova remained by microscopic observation. In this study $t_{critical}$ was estimated to be 18 days based upon the conclusions of Chapter 3 (Manser et al., 2015a).

$$R(t) = \frac{ERFC\left(\frac{t - t_{critical}}{\alpha}\right) \times e^{\left(\frac{-t}{\beta}\right)}}{2} \qquad \text{Equation 5.7}$$

The final inactivation model approach, the sudden die-off, is represented by Equation 5.8, where the variable (H) denotes a Heaviside step function, (t) represents the number of days that the ova were exposed to the reactor conditions, and the variable (t_{99}) is estimated to be 22 days based upon the time when 0% viability remains by microscopic observation (Chapter 3, Manser et al., 2015a). Equation 5.8 works as follows; if the ova are exposed to the reactor for less than 22 days ($t \leq t_{99}$), then the value of H is zero meaning that the function value is 1 and all ova survive. However, when the exposure time is longer than 22 days ($t > t_{99}$), then the value of H is 1 and therefore no ova survive. This corresponds to a situation where ova that are in the reactor for less than some critical time are all considered to be viable. In contrast, when exposure time is greater than $t_{critical}$ then all ova are considered to be non-viable.

$$R(t) = 1 - H(t - t_{99}) \qquad \text{Equation 5.8}$$

The quality of fitness for each model will be determined by calculating the total variance between the twelve observed data points (y_o) in Table 5.2 and the associated predicted results of the model (y_m). A positive variance indicates that the model over estimates the percent of viable ova overall, and the smallest total variance amongst the models will indicate the closet fit. The total variance of each model can be compared to see which provides the closest fit. This calculation is shown in Equation 5.9.

$$Total\ Variance = \sum_{n=1}^{12}(y_m - y_o) \qquad \text{Equation 5.9}$$

5.3.4 Scenario Description

Using the functions defined previously (Equations 5.3, and 5.6 to 5.8), several scenarios were modelled with Equation 5.1 to estimate the fraction of *Ascaris suum* ova that remain viable. The four scenarios used to represent different inactivation kinetics for *Ascaris suum* ova examined in this Chapter are presented in Table 5.3. The results of these scenarios were then

compared to predict which combination of feeding frequency and average SRT produced effluent with the lowest amount of viable ova. Also listed in Table 5.3 (scenario 4), the t_{99} values reported in Table 5.1 for various temperature conditions were also modelled with Equation 5.1 using the sudden die-off inactivation approach (Equation 5.8).

Table 5.3 – A summary of the scenarios modeled. Modeling was done using the residence time distribution of semi-continuous anaerobic digesters and several inactivation kinetic approaches.

Scenario	Discrete Residence Time Distribution	Inactivation Model	Explanation
1	Equation 5.3	Two-Stage Linear (Equations 5.6a,b)	This model assumes that *Ascaris suum* ova have two distinct inactivation trends that follow a linear relationship.
2		Corrected Error Function (Equation 5.7)	This model assumes that *Ascaris suum* ova follow a non-linear inactivation trend, which is best represented as a corrected error function.
3		Sudden Die-Off (35 ^{o}C) (Equation 5.8)	This model assumes that *Ascaris suum* will remain viable until some point in time where complete inactivation occurs at 35 ^{o}C.
4		Sudden Die-Off (Table 5.1) (Equation 5.8)	This model assumes that *Ascaris suum* will remain viable until some point in time where complete inactivation occurs at the times listed in Table 5.1.

5.4 Results

5.4.1 Semi-continuous Residence Time Distribution

Using Equation 5.3, the results for the semi-continuous residence time distributions of the six anaerobic digesters in this study are presented in Figures 5.1 (n=2) and 5.2 (n=7). Figures 5.1 and 5.2 can be interpreted as the percentage of reactor contents (or effluent) with a particular age. For example in digester E30 (n=2, τ =30) the x-axis value of 4 days corresponds to

approximately 6.2% of the reactor contents, as seen from the y-axis. This means that 6.2% of the effluent was added to the reactor 2 feedings ago (4/n = 2). By adding the discrete values of 2, 4, 6 and 8 in digester E30 (6.7%, 6.2%, 5.8% and 5.4%), nearly 24% of the effluent has a residence time of ≤ 8 days. Comparatively, over 46% of the digester W15 effluent has an age of 7 days (Figure 5.2, discrete point 1 for n=7, τ =15 series).

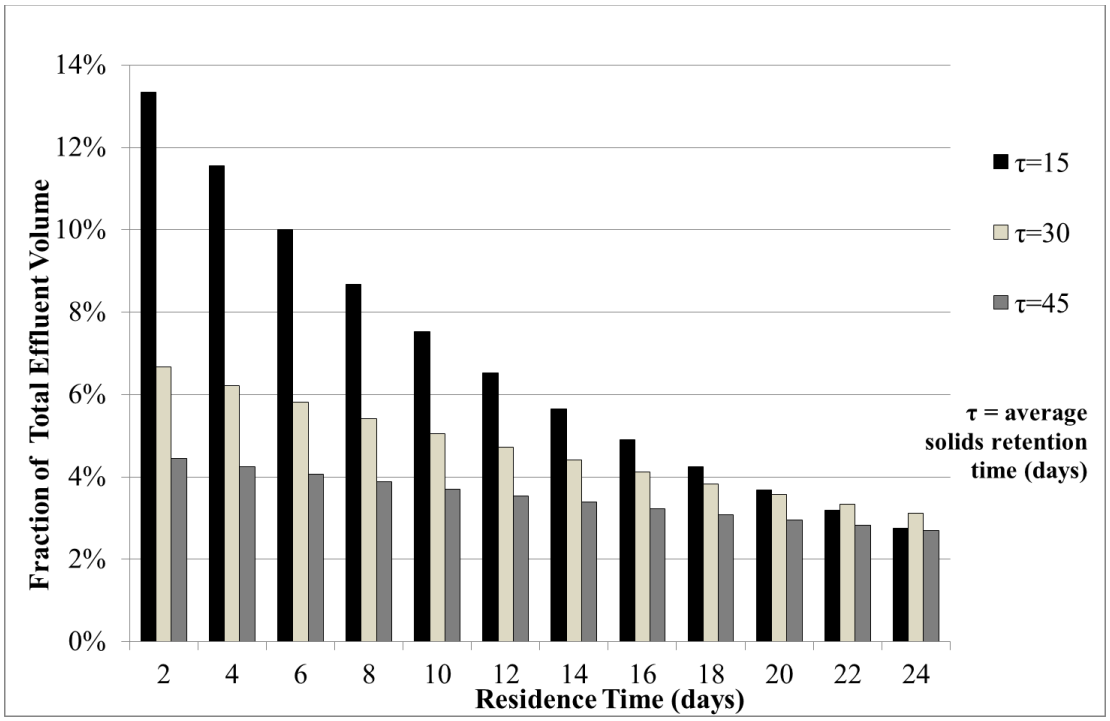

Figure 5.1 – Mathematical residence time distributions of the semi-continuous anaerobic digesters from this study when fed every-other-day (n=2).

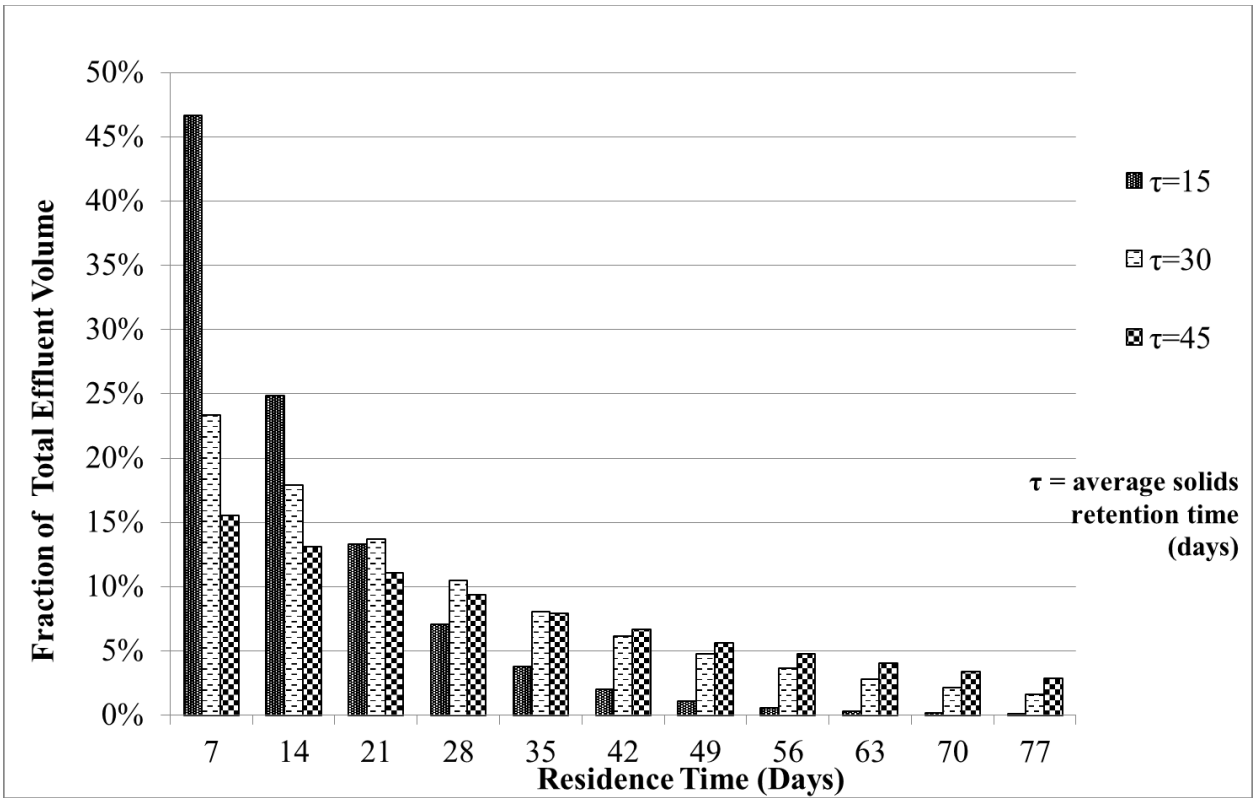

Figure 5.2 – The mathematical residence time distributions of the semi-continuous anaerobic digesters from this study when fed weekly (n=7).

Chapter 3 (Manser et al., 2015a) concluded that 22 days were required to inactivate the ova of *Ascaris suum* at 35 ^{o}C. Therefore, Figures 5.1 and 5.2 can be further interpreted by summing the percentages (y-axis) that correspond to each discrete x-axis value up to 22 days. For example, in Figure 5.1 where the feeding frequency was every other day (n=2), the first eleven values of the residence time distribution are less than 22 days old. Hence, the summation of these eleven terms represents the percentage of digester effluent that contains a viable *Ascaris suum* ova. In Figure 5.2, where the feeding frequency was weekly (n=7), the first 3 terms represent the percentage of effluent younger than 22 days. This analysis assumes that the same influent concentration of *Ascaris suum* ova would be entering the system during each feeding event and the digester is well-mixed prior to feeding.

5.4.2 Pathogen Survival Models

Table 5.2 provides the complete set of inactivation data for the six experimental digesters in this study. Based upon a conclusion of Chapter 3 (Manser et al., 2015a) that no statistical difference could be detected between the six data sets on each sample day, the remainder of this chapter, will use the average inactivation values reported in Table 5.2 for the inactivation modeling result that follows.

5.4.2.1 Two-stage Linear Model

Using Equation 5.6 and the average inactivation results (Table 5.2), a two-stage linear die-off model was fit to the experimental data. This is shown in Figure 5.3. Two stages were selected because of the clear difference in the rate of change that occurred before and after day 16 (t_{Δ}=16 for Equation 5.6). Based upon the mathematical expressions for the two relationships, it can be inferred that after day 16 the inactivation rate increased by 9% from approximately 2% per day to over 11% per day until day 22 when near complete inactivation was observed; this represents a rate increase of over five times. The R^2 values for the two linear expressions were 0.9823 and 0.9783 as shown on the figure. When comparing the predicted values to the observed values, the average variance shown in Figure 5.3 was 0.2% for the two-stage linear approach.

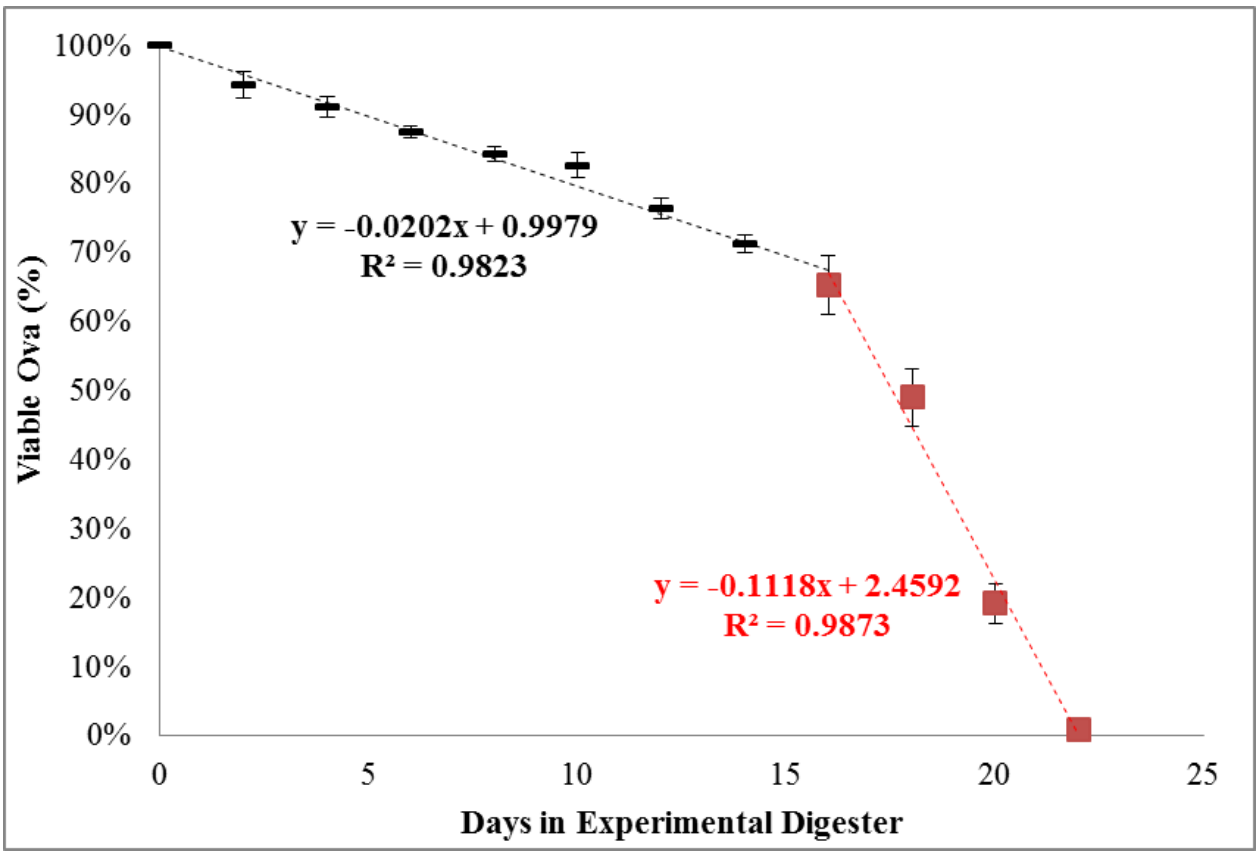

Figure 5.3 – Two-stage linear die-off model of *Ascaris suum* ova in an anaerobic digester for the inactivation data provided in Table 5.2.

5.4.2.2 Corrected Error Function Model

Using Equation 5.7 and the average inactivation results (Table 5.2), a corrected error function was fit to the data. This is shown in Figure 5.4. For this particular expression variable values were estimated to be: $t_{critical}$ = 19d, α = 2d, and β = 44d, which provided for a close fit to the observed data. The largest deviation of the model from observed data occurred on days 16, 18 and 20 where an average departure of 3% to 4% were found using the values previously identified. The remaining model predictions were within 2% of the observed data, with many deviations less than 1%. When comparing the predicted values to the observed values, the average variance shown in Figure 5.4 was 0.7% for the corrected error function approach.

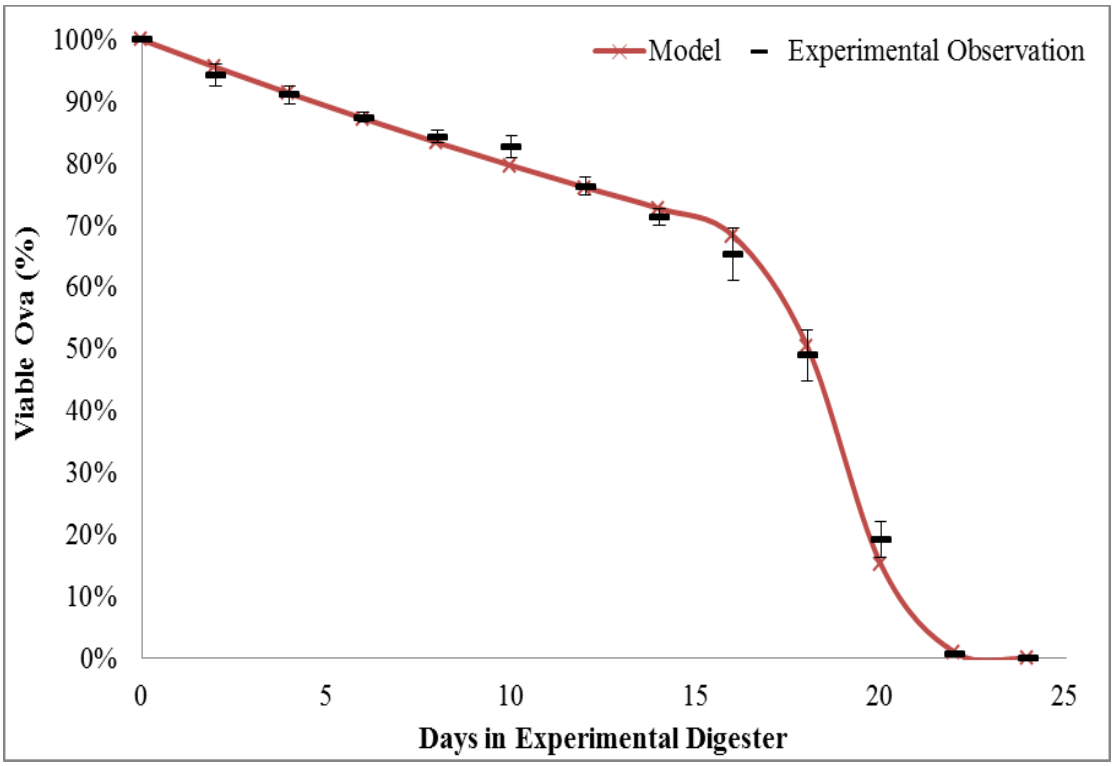

Figure 5.4 – The corrected error function die-off model for *Ascaris suum* ova in an anaerobic digester for the inactivation data listed in Table 5.2.

5.4.2.3 Sudden Die-off Model

Using Equation 5.8 and the average inactivation results (Table 5.2), a conceptual representation of the sudden die-off approach is shown in Figure 5.5. In this approach the assumption carries that all ova present in the effluent up until t_{99} are viable, which in this experiment was determined to be up to 22 days of exposure at 35 ^{o}C (Chapter 3, Manser et al., 2015a). After day 22 there are no infective ova present in the effluent. When comparing the predicted values to the observed values, the average variance shown in Figure 5.4 was 25% for the sudden die-off approach, which is a significant over-estimate. This value is much greater than the average variances calculated for the two-stage linear (0.2%) and corrected error function (0.7%) models, and demonstrates how this modeling approach is more conservative and less accurate.

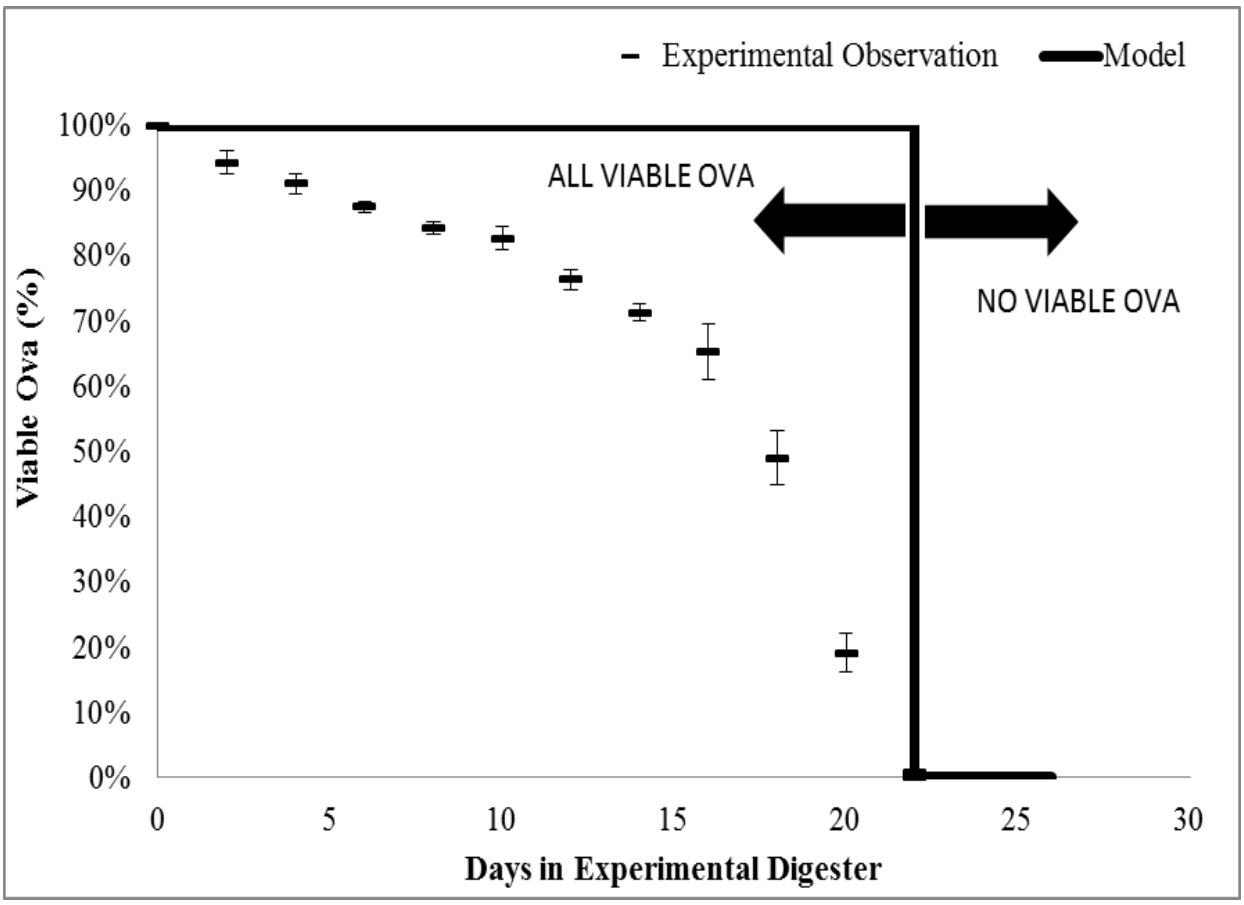

Figure 5.5 – The conceptual representation of the sudden die-off model for *Ascaris suum* ova in an anaerobic digester for the average inactivation data listed in Table 5.2. A residence time less than 22 days corresponds to all ova in the effluent being viable, while no viable ova are present after 22 days.

5.4.3 Estimation of Scenarios

Using the three models described previously in conjunction with the residence time distributions of the six experimental digesters shown in Figures 5.1 and 5.2, the estimation of the fraction (N/N_o) of ova remaining viable was executed for the scenarios outlined in Table 5.3 by applying Equation 5.1. Numerical integration was performed using the trapezoidal rule. A summary of the estimations is provided in Figure 5.6.

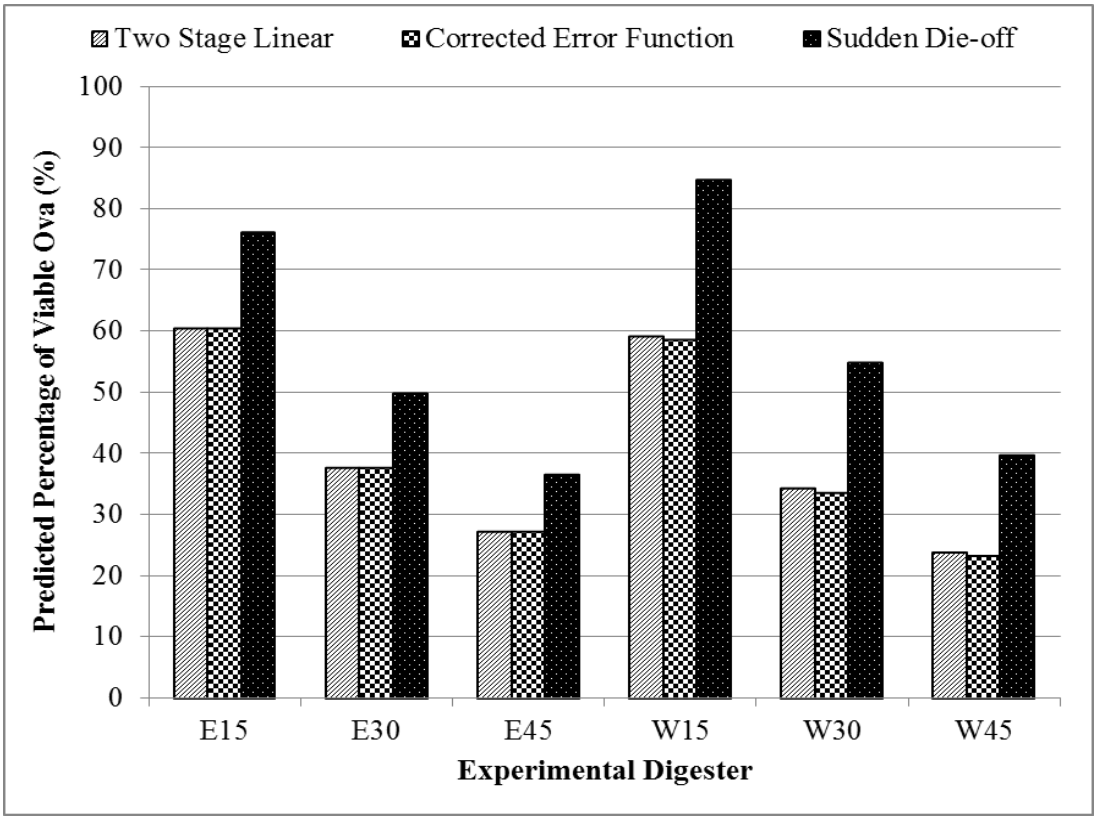

Figure 5.6 – The estimation of the fraction of viable *Ascaris suum* ova exiting the digester under three different inactivation modeling approaches using a semi-continuous residence time distribution. The sudden die-off approach yields the largest estimation because it assumes that at all ova retained less than 22 days are viable. Symbols on the x-axis represent the feeding frequency (every other day (E), weekly (W)) and the SRT (15, 30, or 45 days) of the experimental digesters.

The results presented in Figure 5.6 indicate that the sudden die-off approach provides estimates that are much larger than the other two inactivation models. For example, comparing the predicted percentage of ova survival for digester E30 ($n=2$, $\tau=30$) across the three scenarios, nearly 50% of ova are estimated to remain viable ova using the sudden die-off approach while approximately 38% is estimated by the linear and corrected error function approaches. In almost all cases the predictions made by the linear and corrected error function models were in close agreement to each other (estimated ova viability agrees to within 1%), as compared to the sudden die-off model where differences as large as 25% exist.

Figure 5.7 presents the results of scenario 4 (Table 5.3) where the t_{99} values for different digestion temperatures identified from the literature were used as the $t_{critical}$ values for the sudden die-off approach. The values at 35 ^{o}C are also included for direct comparison. From Figure 5.7 an interesting result is that unless a weekly feeding frequency is used in combination with a digestion temperature of 40 ^{o}C, there was some portion of the ova entering the system that remain viable upon exit. In cases where the average ambient temperature (often < 30 ^{o}C) dictates the digestion temperature, there is very little ova inactivation expected under the three SRTs or two feeding frequencies analyzed here.

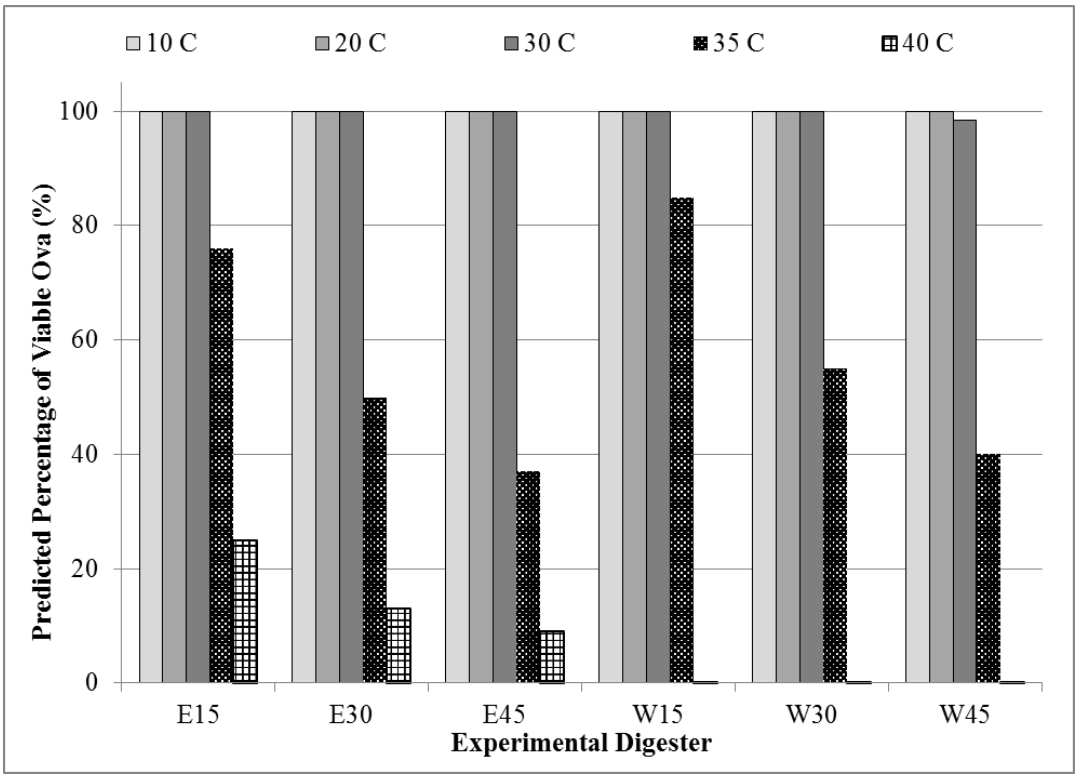

Figure 5.7 – Using t_{99} values from Table 5.1, the estimation of viable ova percentages in the effluent are determined for different digestion temperatures. Based upon the semi-continuous residence time distributions for the experimental digesters, only systems operated above 40 ^{o}C and fed weekly are capable of removing 100% of viable ova.

5.5 Discussion

The residence time distributions shown in Figure 5.1 for the six semi-continuous digesters in this study depict the fundamental issue that these systems have in terms of inactivation of resilient pathogens, such as the ova of *Ascaris suum.* That is, regardless of the feeding frequency and average SRT combination selected, there will be some portion of the digester effluent that has been in the reactor for less than the amount of time required to inactivate the ova. From Chapter 3 (Manser et al., 2015a), 22 days at 35 ^{o}C was required to inactivate 100% of ova, Table 5.1 listed other inactivation times reported in the literature for other digestion temperatures. This phenomenon has negative implications for the use of the semi-continuous anaerobic digester configuration at the household scale when wastes containing human or animal pathogens are included as influent. In the case of soil-transmitted helminths, such as *Ascaris suum,* this situation is worsened because the effluent is typically promoted to be land applied as a soil amendment or fertilizer (NBP, 2011), which places the inactivated ova directly into its preferred environment for development and transmission (Hagel and Giusti, 2010). Future work is needed to compare the residence time distributions of other reactor configurations, such as the use of baffles or the plug-flow concept to identify ways to ensure that the 100% of the effluent age is greater than the time needed to inactivate the most resilient pathogen present in the influent. In the broader scope, the implications of these results for engineered resource recovery systems indicate that achieving human health protection must be a primary objective when these methods are utilized. Prior studies have also demonstrated this conflicting message persists when the treatment of anthropogenic waste is attempted with low-impact development technologies, such as anaerobic digesters or composting latrines (Guest et al., 2009; Mehl et al., 2011; Cornejo et al., 2013).

Three approaches were considered to represent the inactivation kinetics data of the *Ascaris suum* ova at 35 ^{o}C from Chapter 3 (Manser et al., 2015a): a two-stage linear (Figure 5.3), a corrected error function (Figure 5.4), and a sudden die-off model (Figure 5.5). The predictive ability of the two-stage linear and the corrected error function approaches were both similar to each other in terms of matching the observed data to the modeled expectations (Figure 5.5), meaning that either could be suitable for future work in modeling inactivation kinetics of *Ascaris suum* ova. Popat et al. (2010) also selected a linear modeling approach when measuring the inactivation of *Ascaris suum* ova; however, that study was performed under thermophilic temperatures (50 ^{o}C) so only a single-stage linear function was used as inactivation occurs quickly at the temperature (Table 5.1). In the case of mesophilic temperatures, the emergence of a two-stage linear relationship is interesting because it implies that ova are resilient for some time (2% inactivation per day for 16 days), but then after some point they become less resilient and the start dying rapidly (11% inactivation per day after 16 days). Section 2.3.3 described how the denaturation of cellular components can occur over time when environmental conditions are correct, and possibly explain the 550% increase in ova inactivation observed in this study. Because the concentration of free ammonia in the experimental digesters was shown to be non-lethal and the digestion temperature of 35 ^{o}C did not promote rapid inactivation (Chapter 3, Manser et al., 2015a), these conditions must be suitable enough to affect ova survival during their residence time if sufficient contact time is provided.

The results of the sudden die-off inactivation approach (Figure 5.6) did not fit the data as well the other two modeling concepts because it assumes that all ova are viable up until t_{99}. This suggests that this model represents a conservative or worst-case scenario. However, the practicality of the sudden die-off model may be more pertinent than the other two pathogen

survival models when soil-transmitted helminths are of concern. This is true because most previous studies (Table 5.1) investigated the time to 100% inactivation instead of the inactivation kinetics study done for Chapter 3 (Manser et al., 2015a), meaning the application of the sudden die-off model with other published data is more practical and more protective for human health.

The results shown in Figure 5.7 exemplify the trade-off between effluent safety and ease-of-use design that is occurring within the millions of household scale anaerobic digesters currently operated at ambient temperatures. Using the sudden die-off approach for t_{99} values (Table 5.1) at different digestion temperatures this modeling exercise estimated that at least one infective ovum will be in the effluent of all digesters operated at or below 35 ^{o}C regardless of feeding frequency or SRT. This outcome is improved when the feeding frequency of the digester is increased from every-other day to weekly if the average temperature in the digester is maintained at 40 ^{o}C because it is estimated that only 5 days is needed to inactivate *Ascaris suum* ova at that temperature, meaning that influent added one week prior has had sufficient contact time to inactivate all ova when influent is added and effluent removed the following week.

Also shown in Figure 5.7 is the concept that removal changes with feeding frequency, that is, it decreases from 25% to 15% as (n) is increased from 2 to 7 in reactor E15. However, an interesting connection that can be made is that as the feeding frequency (n) exceeds the value of $t_{critical}$ in the sudden die-off model there will be no fraction of the reactor volume that is less than $t_{critical}$. This relationship is shown in Equation 5.9, and implies that feeding frequency (n) is important for controlling the removal of *Ascaris suum* ovum.

$$When\ n \geq t_{critical}, then\ \frac{N}{N_o} = 0.$$

$$When\ n < t_{critical}, then\ \frac{N}{N_o} > 0. \qquad \text{Equation 5.9}$$

This mathematical modeling result is key because Chapter 4 (Manser et al., 2015b) concluded that the removal of *E. coli* and the production of methane could also be enhanced by increasing the feeding frequency from every other day to weekly implying that feeding frequency may be more important to improving pathogen removal, in addition to digester performance, than the average SRT of the system. This connection has implications for operating semi-continuous systems at the household or farm scale. The first is that increasing the feeding interval increases the minimal contact time achieved in the system, regardless of the digestion temperature or average solids retention time. This situation allows the user to maximize the removal of constituents such as helminth ova or even volatile solids that may enter the system.

The second implication is that designing the digester to maintain a higher digestion temperature will obviously benefit the pathogen removal of the system, but even a small increase has significant benefits. For example, increasing the digestion temperature from 30 ^{o}C to 35 ^{o}C can reduce the percentage of effluent containing a viable ovum by over 60% (digester E45, Figure 5.6). In the field it may be possible to construct the digester in a location that receives the most sunlight to enhance the heating of the system. It is also possible to construct the digester above ground to increase the surface area of the reactor that is in contact with the sun, as opposed to configurations that are installed below or partially below ground. Finally, the digester could be painted black or another dark color to increase the amount of solar energy that it absorbs during the day light hours. In the case of tubular digesters that are often constructed with a plastic cover (Figure 5.8), a darker plastic material could be used in place of clear or grey colors. This technique could transfer some of the captured heat into the digester and increase the digestion temperature. Galvin (2013) found that the embodied energy of resource recovery

systems could be “net-positive” if methane capture or biosolids salvage was performed, so enhancing the digestion temperature could also improve the overall function and appropriateness of the system. Future work is needed to understand if these modifications can lead to measurable benefits in the field application of semi-continuous anaerobic digesters.

Figure 5.8 – A common tubular digester configuration with a clear plastic cover used in South America (Galvin, 2013).

Chapter 6: Conclusions and Recommendations for Future Research

This research examined: 1) the fate of *Ascaris suum* ova during semi-continuous mesophilic anaerobic digestion of swine manure under varying feeding frequency and SRT configurations, 2) the performance of the semi-continuous digester in terms of methane production and indicator organism removal under the experimental conditions, 3) the mathematical relationship between the average SRT and feeding frequency and their effect on the residence time distribution of the semi-continuous digesters, and 4) the combined effect of inactivation kinetics of *Ascaris suum* ova and residence time distribution of the reactors on the removal of the ova. This research study was directed by the following research questions based upon the previously defined research gaps.

1. What happens to relevant microbiological pollutants, e.g., *Ascaris suum, E. coli*, and *Salmonella*, during mesophilic anaerobic digestion at 35 ^{o}C? Mainly, how long (in days) can they survive for?

   Based upon the results shown in Table B3 and Figure 3.1, the ova were 100% inactivated by day 24 of the experiment. Based upon the results shown in Figures 4.4A-C, the average *E coli r*emoval for the six digesters was 2-$\log_{10;}$ however, the digesters fed weekly had statistically better average removal (99.7%) compared to the digesters fed every-other day (99.4%). Based upon the results shown in Figures 4.5A-C, there was no significant difference in *Salmonella* removals in the six experimental digesters.

2. For *Ascaris suum*, do embryonated ova have different survival rates during mesophilic anaerobic digestion when compared to ova that have not embryonated?

   Based upon the results shown in Table B3 and Figure 3.1, the ova that were embryonated prior to exposure to the experimental conditions were inactivated faster than unembryonated ova were. This was demonstrated on day 16 where only 30% of embryonated ova remained viable (E15*) compared to nearly 65% of unembryonated ova (AVE). This result has a strong impact on public health as it implies that mesophilic anaerobic digestion actually protects some the most prominent pathogens that burden the developing world.

3. Also for *Ascaris suum*, during mesophilic anaerobic digestion, does an ovum develop, become inactivated or destroyed?

   Based upon the results shown in Figures 3.3 and 3.4, unembryonated ova remained unembryonated during the experiment indicating that mesophilic anaerobic environments will not promote ova development due to the lack of oxygen. Based upon the results shown in Figure 3.2, the average percentage of viable and non-viable ova recovered from all the anaerobic digesters during the experiment compared to a baseline percentage was lower over the course of the experiment, 55% at time zero compared to 41% by day 24. This implies ova destruction which is an alternative fate for ova that is not typically measured in published research. This evidence could suggest that inactivation studies, including this one, may be under reporting the actual kinetics of removal for *Ascaris suum* and other organisms that use a similar method for enumeration.

4. How does the performance of semi-continuous anaerobic digesters change when variations to feeding frequency and solids retention time are made?

Based upon the results shown in Figure 4.1, the average methane yield was 10% higher in the digesters fed weekly compared to those fed every-other day, 0.20 compared with 0.18 m^3 CH_4/kg-VS_{added}.

5. What mathematical relationship can be developed to describe the fate of *Ascaris suum* ova during semi-continuous reactor operation, and can it be used to estimate pathogen survival when the feeding frequency and solids retention time are varied?

Equation 5.1 represents the mathematical relationship used to estimate the pathogen fate during semi-continuous reactor operation. Equation 5.3 is the actual residence time distribution for a semi-continuous digester that is needed to account for variations to feeding frequency. As shown in Figure 5.7, there was a limited effect on pathogen survival by the SRT of the digester; however, there was an effect caused by feeding frequency variation. It was found from the model that when the feeding frequency of the digester was greater or equal to the time needed to completely inactivate *Ascaris suum* ova, then zero viable ovum would exit the reactor. This modeling result suggests that the feeding frequency is possibly more important in removing viable *Ascaris suum* from the reactor effluent than the SRT is.

In conclusion, it is important to understand that the application of partial waste management technologies to resource limited environments can have unwanted consequences on human health. Mesophilic anaerobic digestion is successful in developed nations because it is applied as part of a larger treatment scheme, which may include post-treatment and monitoring of effluent quality and enforcement of standards. Potentially pathogenic organisms are either removed during these enhanced processes or additional barriers may be required, such as preventing land application of effluents to food crops. Therefore, the application of only

mesophilic anaerobic digestion to provide waste-to-energy capabilities and improvements to sanitation may not provide sufficient removal of pathogens, such as *Ascaris*, which are prevalent in many developing countries. This has been demonstrated with composting latrines (Mehl et al., 2011), solar toilets (Cruz-Espinoza et al., 2012a), stabilization ponds (Verbyla et al., 2013a; Symonds et al., 2014; Verbyla et al., 2015), and double-vault urine-diverting composting latrines (Gibson, 2014; Trimmer, 2015). The broader implications of this research indicate that when biological resource recovery systems are used as the sole method for treatment, additional steps should be taken to ensure the protection of human health. This study suggests improving the digester design and placement to enhance the digestion temperature of the system, providing an organic waste storage area to provide aeration time to enhance soil-transmitted helminth ovum development, and increasing the feeding frequency of the digester to a value that approaches the minimum time needed to inactivate the pathogen of concern for a particular geographic context will improve soil-transmitted helminth removal. Of course, the context specific characteristics of political, social and economic realities will ultimately influence how these suggestions are able to be implemented.

Based upon the findings of this research there are two important future research directions that have been identified. The first involves improving the quality of existing data related to prevalence and operating conditions for household anaerobic digesters globally. The effectiveness of this study would be improved if accurate data were available regarding the actual environmental conditions (e.g. temperature, pH, TAN and VFA concentrations) and average SRT of the systems that are currently in use. The ability to predict pathogen survival would also be enhanced with this information. The second research direction involves using molecular tools to investigate how the community structure dynamics of the anaerobic digester

are affected by variations to feeding frequency and average SRT. Several results from this research imply that changes to these parameters influence the behavior of the system in terms of performance and pathogen removal, so linking these behaviors to differences in microbial community structure could lead to a more thorough understanding of what is driving these differences.

References

[1] ADB (2002). Report and Recommendation of the President to the Board of Directors on a Proposed Loan to the Efficient Utilization of Agricultural Waste Projects. http://www2.adb.org/Documents/RRPs/PRC/rrp_33443.pdf. Accessed on June 7, 2015.

[2] Aitken, M.D., Sobsey, M.D., Blauth, K.E., Shehee, M., Crunk, P.L. & Walters, G.W. (2005). Inactivation of *Ascaris suum* and poliovirus in biosolids under thermophilic anaerobic digestion conditions. *Environmental Science & Technology*, 39, 5804-5809.

[3] Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. & Watson, D. (1989). Molecular Biology of the Cell. Second Edition. Garland Publishing, New York.

[4] Amani, T., Nosrati, M. & Sreekrishnan, T.R. (2010). Anaerobic Digestion from the Viewpoint of Microbiological, Chemical, and Operational Aspects – a Review. *Environmental Reviews*, 18, 255–278.

[5] Amini, A. (2014). Sustainable Energy and Nutrient Recovery from Swine Waste. Graduate Thesis. Department of Civil and Environmental Engineering, University of South Florida. http://scholarcommons.usf.edu/etd/4977. Accessed on June 7, 2015.

[6] Appels, L., Baeyens, J., Degrève, J., & Dewil R. (2008). Principles and potential of the anaerobic digestion of waste-activated sludge. *Progress in Energy and Combustion Science*, 34, 755–781.

[7] Barbier, D., Perrine, D., Duhamel, C., Doublet, R., & Georges, P. (1990). Parasitic hazard with sewage sludge applied to land. *Applied Environmental Microbiology*, 56(5), 1420–1422.

[8] Bennion, B.J. & Dagget, V. (2003). The molecular basis for the chemical denaturation of proteins by urea. *Proceedings of the National Academy of Science,* 100(9), 5142-5147.

[9] Bethony, J., Brooker, S., Albonico, M., Geiger, S. M., Loukas, A., Diemert, D., & Hotez, P.J. (2006). Soil-transmitted helminth infections: Ascariasis, trichuriasis, and hookworm. *The Lancet*, 367. 1521-32.

[10] Bloomberg (2011). Global Renewable Energy Market Outlook. Bloomberg New Energy Finance Report.

[11] Bouallagui, H., Ben Cheikh, R., Marouani, L., & Hamdi, M.C. (2003). Mesophilic biogas production from fruit and vegetable waste in a tubular digester. *Bioresource Technology*, 86(1): 85–89

[12] Brovchenko, I., Krukau, A. Smolin, N., Oleinikova, A., Geiger, A., & Winter, R. (2005). Thermal breaking of spanning water networks in the hydration shell of proteins. *Journal of Chemical Physics*, 123, 2249-05.

[13] Brown, H.W. (1928). A quantitative study of the influence of oxygen and temperature on the embryonic development of the eggs of the pig ascarid (*Ascaris suum* goeze). *Journal of Parisitology*, 14(3):141-160.

[14] Bruce N., Perez-Padilla R., & Albalak, R. (2000). Indoor air pollution in developing countries: a major environmental and public health challenge. *Bulletin World Health Organization*, 78, 1078–1092.

[15] Bruun, S., Stoumann Jensesn, L., Vu, V.T.K., & Sommer, S. (2014). Small-scale household biogas digesters: An option for global warming mitigation or a potential climate bomb? *Renewable and Sustainable Energy Reviews.* 33, 736-741.

[16] Buekens, A. (2005). Energy recovery from residual waste by means of anaerobic digestion technologies. In Proceedings of the Conference: The future of residual waste management in Europe. November 17-18, 2005, Luxembourg.

[17] Buitron G., & Matilde G. (1998). Effects of Compression-Decompression on Helminth Eggs Present in Sludge of a Settling Tank. *Water Research*, 32(5), 1708-1712.

[18] Butkus, M.A., Hughes, K.T., Bowman, D.D., Liotta, J.L., Jenkins, M.B., & Labare, M.P. (2011). Inactivation of *Ascaris suum* by short-chain fatty acids. *Applied and Environmental Microbiology*, 77(1), 363-366.

[19] Camilloni, C., Rocco, A.G., Gianazza, E., Broglia, A. & Tiana, G. (2008). Urea and guanidium chloride denature protein L in different ways in molecular dynamic structures. *Biophysical Journal.* 94, 4654-4661.

[20] Chen, T., Fu, B., Wang, Y., Jiang, Q. & Liu, H. (2012). Reactor performance and bacterial pathogen removal in response to sludge retention time in a mesophilic anaerobic digester treating sewage sludge. *Bioresource Technology*, 106, 20-26.

[21] Chen, T.H., Chynoweth, P., & Biljetina, R. (1990). Anaerobic digestion of municipal solid waste in a non-mixed solids concentrating digester. *Applied Biochemical Biotechnology*, 24–25(1), 533–544.

[22] Chynoweth, D. P., Wilkie, A. C., & Owens, J. M. (1999). Anaerobic treatment of piggery slurry - review. *Asian-Australasian Journal of Animal Sciences*, 12(4), 607-628.

[23] Choi, S.K., Park, S.Y., Kim, R., Kim, S.B., Lee, C.H., & Kim, J.F. (2009). Identification of a polymyxin synthetase gene cluster of Paenibacillus polymyxa and heterologous expression of the gene in *Bacillus subtilis*. *Journal of Bacteriology*, 191, 3350–3358.

[24] Colwell, R.R., Brayton, P.R., Grimes. D.J., Roszak, D.B., Huq, S.A., & Palmer, M. (1985). Viable but non-culturable *Vibrio cholera* and related pathogens in the environments: Implications for the release for genetically engineered microorganisms. *Nature Biotechnology*, 3, 817-820.

[25] Conn, E.E., Stumpf, P.K., Bruening, G., & Doi, R.H. (1987). In Outlines of Biochemistry. Edited by E.E. Conn and P.K. Stumpf. John Wiley & Sons, New York

[26] Cornejo, P., Zhang, Q., & Mihelcic, J.R. (2013). Quantifying benefits of resource recovery from sanitation provision in a developing world setting. *Journal of Environmental Management*, 131, 7-15.

[27] Crittenden, J., Trussel, R., Hand, D., Howe, K., & Tchobanoglous, G. (2005) Water Treatment: Principles and Design; John Wiley & Sons, Inc., Hoboken, New Jersey.

[28] Cruz-Espinoza, L.M., Yeh, D., Vinneras, B., Rajaram, L., Whiteford, L., Corvin, J. & Izurieta, R. (2012a). Inactivation of *Ascaris suum* by ammonia in feces simulating the parameters of the solar toilet. *Journal of Applied Sciences in Environmental Sanitation*, 7(3), 173-182.

[29] Cruz-Espinoza, L.M., Allanson, M., Kwa, B., Azizan, A., & Izurieta, R. (2012b). Morphological changes of *Ascaris* spp. eggs during their development outside of the host. *Journal of Parasitology*, 98(1), 63-68.

[30] Curds, C.R., & Fey G.J. (1969). The effect of ciliated protozoa on the fate of *Escherichia coli* in the activated-sludge process. *Water Research*, 3, 853-867.

[31] Dohányos, M., & Zábranská, J. (2001). Sludge into biosolids: processing, disposal, and utilization. IWA Publishing, London.

[32] Eamens, G.J., Waldron, A.M., &Nicholls, P.J. (2006). Survival of pathogenic and indicator bacteria in biosolids applied to agricultural land. *Australian Journal of Soil Research*, 44 (7), 647–59.

[33] Elbeshbishy, E., Nakhla, G., & Hafez, H. (2012). Biochemical methane potential (BMP) of food waste and primary sludge: Influence of inoculum pre-incubation and inoculum source. *Bioresource Technology*, 110, 18-25.

[34] Eriksen L., Andreasen P, & Ilsoe B. (1996). Inactivation of *Ascaris Suum* eggs during storage of lime treated sewage sludge. *Water Research*, 30(4), 1026-1029.

[35] Escudero, A., Lacalle, A., Blanco, F., Pinto, M., & Diaz, I. (2014). Semi-continuous anaerobic digestion of solid slaughterhouse waste. *Journal of Environmental Chemical Engineering*, 2, 819-825.

[36] FAO. (2006). On-farm composting methods: large scale passive aeration: Technologies and practices for small agricultural producers. Food and Agricultural Organization of the United Nations. http://teca.fao.org/read/4295. Accessed on June 8, 2015.

[37] Ferrer, I., Garfí, M., Uggetti, E., Ferrer-Martí, L., Calderon, A., & Velo, E. (2011). Biogas production in low-cost household digesters at the Peruvian Andes. *Biomass and Bioenergy*, 35, 1668-1674.

[38] Franz, E., van Diepeningen, A.D., de Vos, O.J., & van Bruggen, A.H.C. (2005). Effects of cattle feeding regimen and soil management type on the fate of *Escherichia coli* O157: H7 and *Salmonella enterica serovar Typhimurium* in manure, manure amended soil, and lettuce. *Applied and Environmental Microbiology*, 71, 6165–6174.

[39] Galvin, C.M. (2013). Embodied energy and carbon footprint of household latrines in rural Peru: the impact of integrating resource recovery. Graduate Thesis. Department of Civil and Environmental Engineering. http://scholarcommons.usf.edu/etd/4489/. Accessed on June 8, 2015.

[40] Gamble, H.R., Fetterer, R.H., & Urban, J.J.F. (1995). Biochemistry and Molecular Biology of Parasites. First Edition. Academic Press, London, United Kingdom.

[41] Garcia, R., Baelum, J., Fredslund, L., Santorum, P., & Jacobsen, C.S., (2010). Influence of temperature and predation on survival of *Salmonella enterica serovar Typhimurium* and expression of invA in soil and manure-amended soil. *Applied and Environmental Microbiology*, 76, 5025–5031.

[42] Gautam R., Baral, S., & Herat, S. (2009). Biogas as a sustainable energy source in Nepal: present status and future challenges. *Renewable and Sustainable Energy Reviews*, 13, 248–252.

[43] Gerardi, M.H. (2003). Wastewater microbiology series: The microbiology of anaerobic digesters. John Wiley & Sons Inc., New York.

[44] Ghiglietti, R., Genchi C., Di Matteo L., Calcaterra E. & Colombi A. (1997). Survival of *Ascaris suum* eggs in ammonia-treated wastewater sludges. *Bioresource Technology*, 59, 195-198.

[45] Gibson, D. (2014). Inactivation of *Ascaris* in Double-Vault Urine-Diverting Composting Latrines in Panama: Methods and Environmental Health Engineering Field Applications. Graduate Thesis. Department of Global Health, University of South Florida. http://scholarcommons.usf.edu/etd/5224/. Accessed on June 7, 2015.

[46] Gould, J.C., Rassano, M.G., Lawrence, L.M., Burk, S.V., Ennis, R.B., & Lyons, E.T. (2013). The effects of windrow composting on the viability of *Parascaris equorum* eggs. *Veterinary Parasitology*, 191(1-2), 73-80.

[47] GTZ (2010). Installation manual for Low-cost polyethylene tube digesters. https://energypedia.info/images/1/19/Low_cost_polyethylene_tube_installation.pdf. Accessed on June 9, 2015.

[48] Guest, J.S., Skerlos, S.J., Barnard, J.L., Beck, M.B., Daigger, G.T., Hilger, H., Jackson, S.J., Karvazy, K., Kelly, L., Macpherson, L., Mihelcic, J.R., Pramanik, A., Raskin, L., van Loosdrecht, M.C.C., Yeh, D.H., & Love, N.G. (2009). A New Planning and Design Paradigm to Achieve Sustainable Resource Recovery from Wastewater. *Environmental Science & Technology*, 43, 6126–6130.

[49] Guyatt, H.L., & Bundy, D.A.P. (1991). Estimating prevalence of community morbidity due to intestinal helminths: prevalence of infection as an indicator of prevalence of disease. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 85, 778-782.

[50] Haandel, A.V., & Lubbe, J.V.D. (2007). Handbook biological waste water treatment: Design and optimisation of activated sludge systems. Quist Publishing, Leidschendam, The Netherlands.

[51] Hagel, I., & Giusti, T. (2010). *Ascaris lumbricoides*: an overview of therapeutic targets. *Infectious Disorders Drug Targets*, 10, 349–67.

[52] Hawksworth, D., Archer, C., Rajcoomar, K., Buckley, C., & Stenstrom, T.A. (2010). The Effect of Temperature and Relative Humidity on the Viability of *Ascaris* Ova In Urine Diversion Waste. WISA 2010 Biennial Conference, April 18-22, Durban, South Africa.

[53] Ho, C.C., & Tan, Y.K. (1985). Anaerobic treatment of palm oil mill effluent by tank digesters. *Journal of Chemical Technology Biotechnology*, 35(2): 155–164.

[54] Hwang, M.H., Jang, N.J., Hyun, S.H., & Kim, I.S. (2004). Anaerobic bio-hydrogen production from ethanol fermentation: the role of pH. *Journal of Biote*chnology, 111(3), 297–309.

[55] Hwang, K., Song, M., Kim, W., Kim, N., & Hwang, S. (2010). Effects of prolonged starvation on methanogenic population dynamics in anaerobic digestion of swine wastewater. *Bioresource Technology*, 101.

[56] Huong L.Q., Forslund, A., Madsen, H., & Dalsgaard, A. (2014). Survival of *Salmonella* spp. and fecal indicator bacteria in Vietnamese biogas digesters receiving pig slurry. *International Journal of Hygiene and Environmental Health*, 217(7), 785-795.

[57] IEA. (2013). Medium-Term Renewable Energy Market Report 2013. International Energy Agency, Paris.

[58] Jimenez, B., Barrios, J.A., & Maya, C. (2000). Class B biosolids production from wastewater sludge with high pathogenic content generated in an advanced primary treatment. *Water Science Technology*, 42(9), 103–110.

[59] Jimenez, J., Guardia-Puebla, Y., Cisneros-Ortiz, M.E., Morgan-Sagastume J.M., Guerra, G., & Noyola, A. (2015). Optimization of the specific methanogenic activity during the anaerobic co-digestion of pig manure and rice straw, using industrial clay residues as inorganic additive. *Chemical Engineering Journal*, 259, 703–714.

[60] Johansen, A., Nielsen, H.B., Hansen, C.M., Andreasen, C., Carlsgart, J., Hauggard-Nielsen G., & Roepstorff, A. (2013). Survival of weed seeds and animal parasites as affected by anaerobic digestion at mesophilic and thermophilic conditions. *Waste Management*, 33, 807–812.

[61] Johnson, P.W., Dixon, R., & Ross, A.D. (1998). An in-vitro test for assessing the viability of *Ascaris suum* eggs exposed to various sewage treatment processes. *International Journal for Parasitology*, 28, 627–33.

[62] Katuwal H., & Bohara A.K. (2009). Biogas: a promising renewable technology and its impact on rural households in Nepal. *Renewable and Sustainable Energy Reviews*, 13, 2668–2674.

[63] Kaparaju, P., Buendia, I., Ellegaard, L., & Angelidakia, I. (2008). Effects of mixing on methane production during thermophilic anaerobic digestion of manure: lab-scale and pilot-scale studies. *Bioresource Technology*, 99(11), 4919–4928.

[64] Karim, K., Hoffmann, R., Thomas Klasson, K., & Al-Dahhan, M.H. (2005). Anaerobic digestion of animal waste: effect of mode of mixing. *Water Research*. 39(15): 3597–3606.

[65] Khuder, S., Milz, S.A., Bisesi, M., Vincent, R., McNulty, W., & Czajkowski, K. (2007). Health Survey of Residents Living Near Farm Fields Permitted to Receive Biosolids. *Archives of Environmental & Occupational Health*, 62(1), 5–11.

[66] Kim, M., Ahn, Y.H., & Speece, R.E. (2002). Comparative process stability and efficiency of anaerobic digestion; mesophilic vs. thermophilic. *Water Research*, 36(17), 4369–4385

[67] Kim M.K., Pyo, K.H., Hwang, Y.S., Park, K.H., Hwang, I.G., Chai, J.Y., & Shin, E.H. (2012). Effect of temperature on embryonation of *Ascaris suum* eggs in an environmental chamber. *The Korean Journal of Parasitology*, 50(3), 239-242.

[68] Kinyua, M.N. (2013). Effect of Solids Retention Time on the Denitrification Potential of Anaerobically Digested Swine Waste. Graduate Thesis. Department of Civil and Environmental Engineering, University of South Florida. http:// scholarcommons.usf.edu/etd/4520. Accessed on June 7, 2015.

[69] Kinyua, M.N., Cunningham, J.A., & Ergas, S.J. (2014). Effect of solids retention time on the bioavailability of organic carbon in anaerobically digested swine waste. *Bioresource Technology*, 162, 14-20.

[70] Koizumi, M., Hirai, H., Onai, T., Inoue, K., & Hirai, M. (2007). Collapse of the hydration shell of a protein prior to thermal unfolding, *Journal of Applied Crystallography*. 40, 175-178.

[71] Konate Y., Maiga A.H., Basset D., Casellas C., & Picot B. (2013). Parasite removal by waste stabilization ponds in Burkina Faso, accumulation and inactivation in sludge. *Ecological Engineering*, 50, 101-106.

[72] Kone D., Cofie O., Zurbrugg C., Gallizza K., Moser D., Drescher S., & Strauss M. (2007). Helminth eggs inactivation by faecal sludge dewatering and co-composting in tropical climate. *Water Research*, 44, 4397-4402.

[73] Lee, I., Parameswaran, P., & Rittmann, B.E. (2011). Effects of solids retention time on methanogenesis in anaerobic digestion of thickened mixed sludge. *Bioresource Technology*, 102, 10266-10272.

[74] Leles, D., Gardner, S.L., Reinhard, K., Iniguez, A., & Araujo, A. (2012). Are *Ascaris lumbricoides* and *Ascaris suum* a single species? *Parasites and Vectors*, 5, 42-48.

[75] Li, Y., Zhang, R., He, Y., Zhang, C., Liu, X., & Chen, C. (2014a). Anaerobic co-digestion of chicken manure and corn stover in batch and continuously stirred tank reactor (CSTR). *Bioresource Technology*, 156, 342-347.

[76] Li, J., Wei, L., Duan, Q., Hu, G., Zhang, G. (2014b). Semi-continuous anaerobic co-digestion of dairy manure with three crop residues for biogas production. *Bioresource Technology*, 156, 307-313.

[77] Lisboa, M.S., & Lansing, S. (2013). Characterizing food waste substrates for co-digestions through biochemical methane potential (BMP) experiments. *Waste Management*, 33, 2664-2669.

[78] Madigan, M.T., Martinko, J.M., Stahl, D.A. & Clarck, D.P. (2012). Brock Biology of Microorganisms, 13th Edition. Pearson Education Inc., San Francisco.

[79] Manser, N.D., Wald, I., Ergas, S.J., Izurieta, R., & Mihelcic, J.R. (2015a). Assessing the fate of *Ascaris suum* ova during mesophilic anaerobic digestion. *Environmental Science and Technology*, 49 (5), 3128-3135.

[80] Manser, N.D., Mihelcic, J.R. & Ergas, S.J. (2015b). Semi-continuous mesophilic anaerobic digester performance under variations in solids retention time and feeding frequency. *Bioresource Technology*, 190, 359-366.

[81] Manser, N.D., Wald, I., Gibson, D.A., Izurieta, R., & Mihelcic, J.R. (2015c). Health concerns transferring waste to energy technologies in the Americas: soil-transmitted helminths. Proceedings from Water and Energy 2015: Opportunities for Energy and Resource Recovery in the Changing World. Washington D.C., June 8-10, 2015. *The Water Environment Federation*.

[82] Masse D., Gilbert Y. & Topp E. (2011). Pathogen removal in farm-scale psychrophilic anaerobic digesters processing swine manure. *Bioresource Technology*, 102, 641-646.

[83] Matthews, B.E. (1985). The influence of temperature and osmotic stress on the development and eclosion of hookworm eggs. *Journal of Helminthology*. 59, 217-224.

[84] Maya, C., Torner-Morales, F.J., Lucario, E.S., Hernandez, E., & Jimenez, B. (2012). Viability of six species of larval and non-larval helminth eggs for different conditions of temperature, pH, and dryness. *Water Research*, 46, 4770-4782.

[85] MDG. (2013). The millennium development goals report 2013. United Nations Development Programme. http://www.un.org/millenniumgoals/pdf/report-2013/mdg-report-2013-english.pdf. Accessed on June 9, 2015.

[86] Mehl, J., Kaiser. J., Hurtado, D., Gibson, D.A., Izurieta, R., & Mihelcic, J.R. (2011). Pathogen destruction and solids decomposition in composting latrines: study of fundamental mechanisms and user operation in rural Panama. *Journal of Water and Health*, 9, 187-199.

[87] Mihelcic, J.R., Zimmerman, J.B., & Ramaswami, A. (2007). Integrating developed and developing world knowledge into global discussions and strategies for sustainability. Part 1: Science and technology. *Environmental Science & Technology*, 41, 3415-3421.

[88] Mihelcic, J.R., Fry, L.M., Shaw, R. (2011). Global potential of phosphorus recovery from human urine and feces. *Chemosphere,* 84(6), 832-839.

[89] Mihelcic, J.R., & Zimmerman, J.B. (2014). Environmental engineering: Fundamentals, sustainability, design. 2nd Edition, John Wiley & Sons, Hoboken, N. J.

[90] NBP (2011). Monitoring report (period I) for the verification of national biodigester programme. National Biodigester Program. http://www.snvworld.org/en/countries/cambodia/publications?v=all&page=1. Accessed on June 8, 2015.

[91] NDC (2007). Medium and long-term development plan for renewable energy in China. National Development and Reform Commission, Beijing. http://www.martinot.info/China_RE_Plan_to_2020_Sep-2007.pdf. Accessed on June 8, 2015.

[92] Ndegwa, P.M., Hamilton, D.W., Lalman, J.A., & Cumba, H.J. (2008). Effects of cycle-frequency and temperature on the performance of anaerobic sequencing batch reactors (ASBRs) treating swine waste. *Bioresource Technology*, 99, 1972-980.

[93] Nordin, A., Nyberg, K., & Vinneras, B. (2009). Inactivation of *Ascaris* eggs in source-separated urine and feces by ammonia at ambient temperatures. *Applied Environmental Microbiology*, 75(3), 662-667.

[94] Olsen, J.E. & Larsen, M.E. (1987). Bacterial decimation times in anaerobic digestion of Animal Slurries. *Biological Wastes,* 21, 153-168.

[95] Papajova I., Juris P., Szabova E., Venglovsky J., Saskova N., Sefcikova H., Martinez J., & Gabon T. (2008). Decontamination by anaerobic stabilization of the environment contaminated with enteronematode eggs *Toxocara canis* and *Ascaris Suum*. *Bioresource Technology*, 99, 4966-4971.

[96] Pecson, B.M., Barrios, J.A., Jimenez, B.E. & Nelson, K.L. (2007). The effects of temperature, pH, and ammonia concentration on the inactivation of *Ascaris* eggs in sewage sludge. *Water Research*, 41(13), 2893-2902.

[97] Pepper, I.L., Brooks, J.P., Sinclair R.G., Gurian, P.L. & Gerba, C.P. (2010). Pathogens and Indicators in United States Class B Biosolids: National and Historical Distributions. *Journal of Environmental Quality: Technical Report on Waste Management,* 39.

[98] Perez, I., Garfi, M., Cadena, E., & Ferrer, I. (2014). Technical, economic and environmental assessment of household biogas digesters for rural communities. *Renewable Energy*, 62, 313-318.

[99] Perry, R.N. (1989). Dormancy and hatching of nematode eggs. *Parasitology Today*, 5(12), 377-383.

[100] Popat S.C., Yates M.V., & Deshusses M.A. (2010). Kinetics of inactivation of indicator pathogens during thermophillic anaerobic digestion. *Water Research*, 44, 5965-5972.

[101] Pullammanappallil, P.C., Chyoneth, D.P. Lyberators, G., & Svoronos, S.A. (2001). Stable performance of anaerobic digestion in the presence of high concentration of propionic acid. *Bioresource Technology*, 78(2), 165-169.

[102] Pullan, R.L., Smith, J.L., Jasrasaria, R., & Brooker, S.J. (2014). Global numbers of infection and disease burden of soil transmitted helminth infections in 2010. *Parasites & Vectors*, 7, 37.

[103] Pyria, M., Haridas, A., & Manilal, V.B. (2007). Involvement of protozoa in anaerobic wastewater treatment process. *Water Research*, 41, 4639-4645.

[104] Rittmann, B.E., & McCarty, P.L. (2001). Environmental biotechnology: Principles and applications. McGraw-Hill, Boston, MA.

[105] Rogers, R.A. (1956). A Study of Eggs of *Ascaris lumbricoides* var. *suum* with the Electron Microscope. *Journal of Parasitology Archives.* Paper 618.

[106] Rowse, L.E. (2012). Design of Small Scale Anaerobic Digesters for Application in Rural Developing Countries. Graduate Thesis. Department of Civil and Environmental Engineering, University of South Florida. http://scholarcommons.usf.edu/etd/3324. Accessed on June 8, 2015.

[107] St. Pierre, B., & Wright, A.G. (2013). Metagenomic analysis of methanogen populations in three full-scale mesophilic anaerobic manure digesters operated on dairy farms in Vermont, USA. *Bioresource Technology*, 138, 277-284.

[108] Sanguinetti, G.S., Tortul, C., Garcia, M.C., Ferrer, V., Montangero, A., & Strauss, M. (2005). Investigating helminth eggs and *Salmonella spp*. in stabilization ponds treating septage. *Water Science Technology*, 51(12), 239–47.

[109] Schwartzbrod, J., Mathieu, C., Thevenot, M.T., Baradel, J,M., & Schwartzbrod, L. (1987). Wastewater sludge: parasitological and virological contamination. *Water Science Technology*, 19(8), 33–40.

[110] Selleck, R., Collins, H., & White, G. (1970). Kinetics of wastewater chlorination in a continuous flow process. International Water Pollution Research Conference, San Francisco, CA.

[111] Sewell, D.L. (1995). Laboratory-associated infections and biosafety. *Clinical Microbiology Reviews*, 8, 389-405.

[112] Sidhu, J.P.S., & Toze, S.G. (2009). Human Pathogens and their Indicators in Biosolids: A Literature Review. *Environment International.* 35, 187-201.

[113] Smith, S.R., Lang, N.L., Cheung, K.H.M., & Spanoudaki, K. (2005). Factors controlling pathogen destruction during anaerobic digestion of biowastes. *Waste Management,* 25, 417-425.

[114] Sorenson, A.H., Ahring, B.K. (1993). Measurements of the specific methanogenic activity of anaerobic digester biomass. *Applied Microbiology and Biotechnology*, 40, 427-431.

[115] Speece, R.E. (1996). Anaerobic biotechnology for industrial wastewater. Archaea Press, Nashville, TN.

[116] Straub, T.M., Pepper, I.L., & Gerba, C.P. (1993). Hazards from pathogenic microorganisms in land disposed sewage sludge. *Reviews of Environmental Contamination and Toxicology*, 132, 55–91.

[117] Stolpovsky, K., Martinez-Lavanchy, P., Heipieper, H.J., Van Cappellen, P., & Thullner, M. (2011). Incorporating dormancy in dynamic microbial community models. *Ecological Modelling*, 222, 3092-3102.

[118] Surendra, K.C., Takara, D., Hashimoto, A.G., & Khanal, S.K. (2014). Biogas as a sustainable energy source for developing countries: Opportunities and challenges. *Renewable and Sustainable Energy Reviews*, 31, 846-859.

[119] Symonds, E.M., Verbyla, M.E., Lukasik, J.O., Kafle, R.C., Breitbart, M., & Mihelcic, J.R. (2014). A case study of enteric virus removal and insights into the associated risk of water reuse for two wastewater treatment pond systems in Bolivia. *Water Research*, 65, 257-270.

[120] Tchobanoglous, G., Burton, F.L., & Stensel, H.D. (2003). Wastewater Engineering: Treatment and reuse (4th ed. / revised by George Tchobanoglous, Franklin L. Burton, H., David, Stensel.), McGraw-Hill, Boston, MA.

[121] Trimmer, J. (2015). Ecological Sanitation in Uganda: Promotion through Demonstration Facilities and Potential for Ascaris Reduction by Free Ammonia Inactivation Using Stored Urine. Graduate Thesis. Department of Civil and Environmental Engineering, University of South Florida. http://scholarcommons.usf.edu/etd/. Accessed on June 25, 2015.

[122] Qi, Y., Beecher, N., & Stone, L. (2013). A National Survey of Biogas Use at Wastewater Treatment Plants in the United States: The Results. *Proceedings of the Water Environment Federation,* 2013 (5), 655-666.

[123] USEPA (2000). A guide to field storage of biosolids and the organic by-products used in agriculture and for soil resource management. EPA/832-B-00-007. USEPA, Office of Wastewater Management, Washington, DC. http://water.epa.gov/scitech/wastetech/biosolids/guide.cfm. Accesses on June 8, 2015.

[124] Verbyla, M.W., Oakley, S.M., Lizima, L.A., Zhang, J., Iriarte, M., Tejada-Martinez, A.E., & Mihelcic, J.R. (2013a). *Taenia* eggs in a stabilization pond system with poor hydraulics: Concern for human cysticercosis? *Water Science & Technology*, 68, 2698-2703.

[125] Verbyla, M.E., Oakley, S.M., & Mihelcic, J.R. (2013b). Wastewater infrastructure for small cities in an urbanizing world: Integrating the protection of human health and the environment with resource recovery and food security, *Environmental Science & Technology*, 47(8), 3598–3605.

[126] Verbyla, M.E., Mihelcic, J.R. (2015). A review of virus removal in wastewater treatment pond systems, *Water Research*, 71, 107-124.

[127] Vinnerås, B., Nordin, A., Niwagaba, C., & Nyberg, K. (2008). Inactivation of bacteria and viruses in human urine depending on temperature and dilution rate. *Water Research*, 42 (15), 4067-4074.

[128] Voet, D., & Voet, J.G. (1990). Biochemistry. Jon & Wiley Sons, Inc., New York.

[129] Wang, M., Sun, X., Li, P., Yin, L., Liu, D., Zhang, Y., Li, W., & Zheng, G. (2014). A novel alternate feeding mode for semi-continuous anaerobic co-digestion of food waste with chicken manure. *Bioresource Technology*, 164, 309-314.

[130] Wharton, D. (1980). Nematode egg-shells. *Parasitology*, 81, 447–463.

[131] Weimer, P.J. (1998). Manipulating ruminal fermentation: a microbial ecological perspective. *Journal of Animal Science*, 76 (12), 3114–3122.

[132] WHO (1989). Health guidelines for the use of wastewater in agriculture and aquaculture. World Health Organization, Geneva. http://apps.who.int/iris/handle/10665/39401. Accessed on June 8, 2015.

[133] WHO (2010). Working to overcome the global impact of neglected tropical diseases: First WHO report on neglected tropical diseases. World Health Organization, Geneva. http://www.who.int/neglected_diseases/2010report/en/. Accessed on June 8, 2015.

[134] WHO (2014). Soil-transmitted helminth infections Factsheet N˚366. World Health Organization, Geneva. http://www.who.int/mediacentre/factsheets/fs366/en/#. Accesses on June 8, 2015.

[135] Wolin, M. (1969). Volatile fatty acids and the inhibition of *Escherichia coli* growth by rumen fluid. *Applied Microbiology*, 17 (1), 83-87.

[136] Zaher, U., Cheong, D.Y., Wu, B., & Chen, S. (2007). Producing energy and fertilizer from organic municipal solid waste. *Ecology Publication*, No. 07–07–024. Department of Biological Systems Engineering Washington State University.

[137] Ziemba, C., & Peccia, J. (2012). Fecal coliform population dynamics associated with the thermophilic stabilization of treated sewage sludge. *Journal of Environmental Monitoring*, 14(10), 2755-2761.

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Appendix B: Supporting Information[3]

Table B1 - Details of operational characteristics of experimental reactors and controls.

ID	Type	Temperature	Feeding Frequency	Solids Retention Time (days)	Exchange Volume (ml)	Organic Loading Rate (kg VS/m^3-day)
E15	900 ml Anaerobic Reactor	35 °C	every other day	15	120	3.6
E30				30	60	1.2
E45				45	40	0.8
W15			weekly	15	420	3.6
W30				30	210	1.2
W45				45	140	0.8
An	Buffered Control		n/a			
Ar_{35}						
Ar_{28}		28 °C				

Table B2 - Key parameters monitored during the study. The tabled values represent an average of all observations made during the experimental period, standard deviations are listed in parentheses when needed.

Parameter	E15 (E15*)	E30	E45	W15	W30	W45	An_{35}	Ar	Ar_{28}
pH	7.2	7.3	7.3	7.2	7.2	7.3	7.1	7.2	7.1
Volatile Solids Removal (%)	38	47	47	45	43	44	not measured		
Chemical Oxygen Demand (mg COD/L)	2,260 (434)	2,190 (392)	2,010 (280)	2,200 (530)	2,240 (374)	2,080 (254)	not measured		
Ammonium (mg/L NH_3-N)	1,000 (165)	970 (122)	1,000 (101)	950 (126)	910 (133)	960 (82)	980 (45)	995 (65)	1,010 (38)
Volatile Fatty Acid (mg/L HOAc)	430 (50)	415 (49)	360 (42)	440 (67)	470 (60)	390 (44)	450 (61)	420 (40)	440 (44)
21-Day Biogas Production (ml/g-VS added)	567	520	418	516	634	495	not measured		
Methane Content (%)	56%	60%	61%	57%	58%	60%	not measured		

[3] This section was published as supporting information for "Assessing the Fate of *Ascaris suum* Ova during Mesophilic Anaerobic Digestion" in Environmental Science and Technology journal, Volume 49, Number 5, pages 3128-3135. Copyright 2015 American Chemical Society.

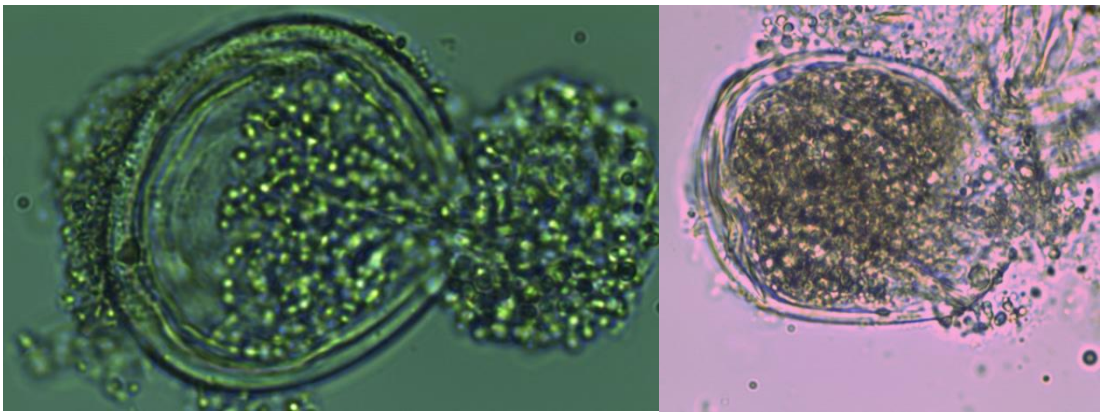

Figure B1 - *Ascaris suum* ova exposed to the reactors are seen in both photographs to be exhibiting morphological damage to the interior cells, in addition to being vacuolated, or destroyed, during their residence.