2019

# IMPROVED METHODS FOR THE QUANTIFICATION OF VIABLE ASCARIS SUUM AND APPLICATION TO BIOSOLIDS

Tanner Keyzers
*Michigan Technological University*, tgkeyzer@mtu.edu

# IMPROVED METHODS FOR THE QUANTIFICATION OF VIABLE *ASCARIS SUUM* AND APPLICATION TO BIOSOLIDS

By

Tanner G. Keyzers

A THESIS

Submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

In Biological Sciences

MICHIGAN TECHNOLOGICAL UNIVERSITY

2019

This thesis has been approved in partial fulfillment of the requirements for the Degree of MASTER OF SCIENCE in Biological Sciences.

Department of Biological Sciences

| | |
|---|---|
| Thesis Co-Advisor: | *Karyn Fay* |
| Thesis Co-Advisor: | *Jennifer G. Becker* |
| Committee Member: | *Eric A. Seagren* |
| Department Chair: | *Chandrashekhar Joshi* |

# Table of Contents

# List of Figures

# List of Tables

# Author contribution statement

The study reported in this thesis arose out of a much larger pilot-scale study, after it became apparent that existing methods for quantifying viable *Ascaris* ova have a number of limitations when applied to the analysis of biosolids. Thus, the findings reported in this thesis complement and enhance some of the results obtained during the larger study. T.K. designed the experiments described in Chapters 3 and 4 with the assistance of Jennifer Becker and Eric Seagren. T.K. counted the vast majority of ova samples. However, a few samples were counted by Karina Eyre. The ova movement experiments, conducted as part of the development of viable assay were designed by T.K. under the guidance of J.B. and E.S. and conducted by T.K. Experiments designed to compare qPCR- and microscopy-based measures of viable ova were also designed by T.K., who also performed all DNA extractions and qPCR analyses. The chapters of the thesis were all written by T.K.

E.S. and J.B. provided guidance on all aspects of each experiment and the writing of this thesis. They also edited, proofread, and provided comments on each chapter.

# Acknowledgements

**Dr. Jennifer Becker and Dr. Eric Seagren:** For getting me started on this project and the opportunity to obtain my master's degree, as well as the guidance and knowledge that has helped me through my research and writing.

**Karyn Fay:** For all of the academic advice and teachings that gave me a solid base in the laboratory as well as for my future career.

**Karina Eyre and JP Harron:** For the assistance with sampling, experimentation and all of the long sampling and lab days.

**Mark Bowman and Zane Mackenzie:** as well as all of the staff at both GIWA and PLWSA who helped with turning of samples and allowed us to use their laboratories and facilities when needed.

# List of abbreviations

| | |
|---|---|
| CFU | Colony Forming Units |
| LB | Luria-Bertani |
| LCLT | Low-cost low-tech |
| MPN | Most Probable Number |
| PBS | Phosphate-buffered saline |
| PFU | Plaque Forming Unit |
| PFRP | Processes to Further Reduce Pathogens |
| PIOs | Pathogen and indicator organisms |
| TS | Total solids |
| USEPA | United States Environmental Protection Agency |
| VFA | Volatile Fatty Acids |
| VS | Volatile Solids |
| WERF | Water Environment Research Federation |
| WHO | World Health Organization |
| WRRF | Water Resource Recovery Facilities |

# Abstract

The treated solid residuals resulting from wastewater treatment are referred to as biosolids. Biosolids must be monitored during treatment for the presence of pathogens, such as *Ascaris* spp. ova. Unfortunately, the current microscopic method for enumeration of *Ascaris* ova in biosolids is labor and time intensive, and quantifying viable ova that have larvated during treatment is difficult. The goal of this research was to evaluate improved methods for quantification of *Ascaris* ova in biosolids, including a method for quantifying the viability of *Ascaris* ova, and a comparison of the traditional microscopic method with qPCR. Improved methods to promote movement of larvated ova using bleach and heat treatment were tested and shown to increase ova movement when compared with no treatment, or heat or bleach only treatment, thus decreasing the testing time. A comparison of qPCR and microscopic methods using long-term stored biosolids, exhibited a correlation between the qPCR calculated and microscopically counted ova. Propidium monoazide (PMA)-qPCR was also tested as a method for preventing false positives from inactivated but not yet degraded larval ova; however, no decrease in amplification was observed for PMA treated samples. Based on these results, qPCR may be a valid method for quantifying *Ascaris* ova in biosolids.

# 1 Introduction

## 1.1 Background

### 1.1.1 Biosolids

The treated solid residuals resulting from wastewater treatment processes are referred to as biosolids. Management and disposal of biosolids is a major cost and challenge for municipal water resource recovery facilities (WRRFs). Biosolids are an organic, nutrient-rich material that have beneficial effects on soil properties and plant production (NRC, 2002). As a result, of the roughly 6 million dry tons of biosolids annually produced in the U.S., up to 60% are land-applied for a variety of beneficial uses, including as agricultural fertilizers, for degraded land reclamation, and in public parks and golf courses. However, the biosolids also contain pathogens. Therefore, due to the risk of the persistence of pathogens in biosolids, they must be treated to reduce pathogen levels and stabilize the organic matter in the biosolids, thereby reducing their tendency to attract vectors (e.g., rats, etc.) before being land-applied. In 1993, the US Environmental Protection Agency (EPA) set to regulate the application of biosolids and to protect human health under the part 503 rule, which categorized biosolids as either Class A or Class B. The pathogen load resulting from the treatment process used determines the class of biosolids. Class B treatment must reduce the level of fecal coliforms to <2,000,000 colony forming units (CFUs)/g total solids (TS). In comparison, Class A biosolids must have pathogen levels that are near non-detect limits, and therefore meet the following standards: (1) fecal coliforms must be below 1000 most probable number (MPN)/g TS, or the density of *Salmonella* must meet <3 MPN/4g TS; (2) viable helminth ova must be <1 ova /4 g TS, and (3) enteric viruses must be <1 plaque-forming unit (PFU)/4g TS.

There are six alternatives provided in the Part 503 rule for treating biosolids so they can be classified as Class A (EPA, 1994): Alternative 1, thermally-treated

biosolids that are subjected to one of four time-temperature regimes; Alternative 2, biosolids that are treated in a high pH-high temperature process, and meet specific pH, temperature, and air-drying requirements; Alternative 3, biosolids that are treated in other known processes that can reduce enteric viruses and viable helminth ova; Alternative 4, biosolids that treated in unknown processes (biosolids must be tested for *Salmonella* sp. or fecal coliform bacteria, enteric viruses, and viable helminth ova); Alternative 5, biosolids that treated in one of the Processes to Further Reduce Pathogens (PFRP); and Alternative 6, biosolids treated in a process equivalent to a PFRP, i.e., the process can consistently reduce pathogens to levels comparable to those achieved in the PFRPs (EPA, 1994). The PFRPs that can be used to achieve Class A status are composting, heat drying, heat treatment, thermophilic aerobic digestion, beta ray irradiation, gamma ray irradiation, or pasteurization. With the possible exception of composting, these are all expensive, high-maintenance processes, and most are energy-intensive.

The majority of publically owned WRRFs in the U.S. serve small communities, which are defined as having a population of $\leq$ 10,000 people and wastewater flow rates of $< 1 \times 10^6$ gal/day (USEPA, 2012). Unfortunately, these plants often lack the capital resources and personnel and other operating requirements for implementing a PFRP. Fortunately, low-cost, low-technology (LCLT) alternatives are available and being used successfully at WRRFs in the U.S. and elsewhere. A Water Environment Research Foundation (WERF) study evaluated four of these processes: long-term lagooning, air drying, combined lagooning/air drying, and cake storage (Schafer et al., 2004). The process descriptions from that report are briefly summarized below.

*Long-term Lagoon Storage/Treatment:* Liquid and dewatered digested sludge is placed in a lagoon and stored. During storage, biosolids decomposition and pathogen destruction occur. The mechanisms of pathogen inactivation that are active include: predation by other microorganisms, thermal inactivation, being

out-competed by other microorganisms for resources, and chemical inactivation (e.g., due to ammonia or organic acids).

*Air-Drying:* Liquid digested biosolids are placed on a sand drying bed, and digested dewatered sludge is placed on an impervious pad. After the biosolids dry sufficiently, windrows can be formed and turned, which aids in drying, assists in the oxidation of organic matter, and may generate sufficient heat to accelerate pathogen kill. Pathogen kill is a function of desiccation, temperature, and time.

*Cake Storage:* This process is similar to air drying except that larger windrows are used with minimal turning. Pathogen destruction mechanisms are the same as for air drying except that desiccation plays a minor role.

*Coupled Lagoon Storage/Air Drying Systems:* This is a combination of the lagoon storage technique, plus the air drying method. Thus, pathogens are sequentially subjected to the inactivation mechanisms that occur in both the lagoon and air-drying processes.

### 1.1.2 Pathogens

In addition to the types of bacteria, enteric viruses, protozoa, and helminths covered in the Part 503 regulations, biosolids have the potential to include a wide range of other human pathogens and indicator organisms (PIOs). For example, *Salmonella spp.*, *Shigella spp*, *Escherichia coli*, and *Campylobacter jejuni* are pathogenic bacteria found in biosolids, while some of the enteric viruses of concern include Poliovirus, Adenovirus, and Coxsackievirus. Parasites found in biosolids include protozoans like *Giardia spp.*, and *Cryptosporidium parvum*, as well as helminth worms including *Ascaris lumbricoides*, *Trichuris trichiura*, *Toxocara canis*, and *Taenia spp.* (Gerba & Smith, 2005). However, helminth ova and enteric viruses levels in the US are often well below Class A levels, even in Class B biosolids. This is not due to the inactivation processes used for Class B biosolids treatment, but instead is due to the lack of infections in the population of

the United States, as well as other industrialized countries. However, in lower income countries, or countries with poor sanitation, the amount of pathogenic organisms is much greater (Amahmid, Asmama, & Bouhoum, 2002; Pepper, Brooks, Sinclair, Gurian, & Gerba, 2010).

### 1.1.3 *Ascaris* spp.

The PIOs that are the focus of this project are *Ascaris* spp. *Ascaris*, also known as intestinal roundworms, are parasitic helminths that are most commonly found in parts of Asia, Africa and South America (Amahmid, 1999). There are two main human health related species of *Ascaris* that exist: *A. lumbricoides*, and *A. suum*. *A. lumbricoides* is the human pathogen causing ascariasis, while *A. suum* is known as a pig roundworm. Although considered different species, *A. suum* can cross infect humans, and there is debate if they could be considered a single species (Leles, 2012).

*Ascaris* have a very complex life cycle (Figure 1.1). The cycle begins with ingestion of larvated ova from contaminated food, water or soil. The ova travel to the small intestine, where the larvae hatch. Larvae then penetrate the intestinal wall and enter the bloodstream where they travel to the liver, then to the lungs where they leave the bloodstream and enter the alveoli. Once there the larvae will mature for approximately two weeks, before bursting through the alveolar walls and travel up the bronchioles. Larvae are coughed,up and then swallowed in to the pharynx, returning to the stomach and then small intestine. There, larvae mature into adult worms and mate, with females then producing over 200,000 eggs each day (Bethony, 2006). These fertilized ova are then excreted into the environment in human feces. The single celled ova mature into the fully larvated form over the course of 18-28 days, at which point they are then infective, and thus able to start the cycle over when ingested by a human host. Fertilized ova are able to persist in the environment for several years, depending on temperature and moisture of the environment that they are in.

Fertilized ova contain a single cell surrounded by a thick chitin layer that can be either corticated or decorticated. These ova range from 40-75 µm in length and 30-50 µm in width. Unfertilized ova are larger and longer than fertilized ova, stretching 85-95 µm with a width of 38-45 µm. The unembryonated inside of the unfertilized egg contains a shapeless protoplasm, while the shell is a thin chitin layer that is typically corticated. Both male and female adult *Ascaris* worms are a cream white color, with females being slightly larger at 22-35 cm in length, while males growing up to 30 cm. Females are long, thinly shaped while males have an incurved tale (Zeibig, 2003).

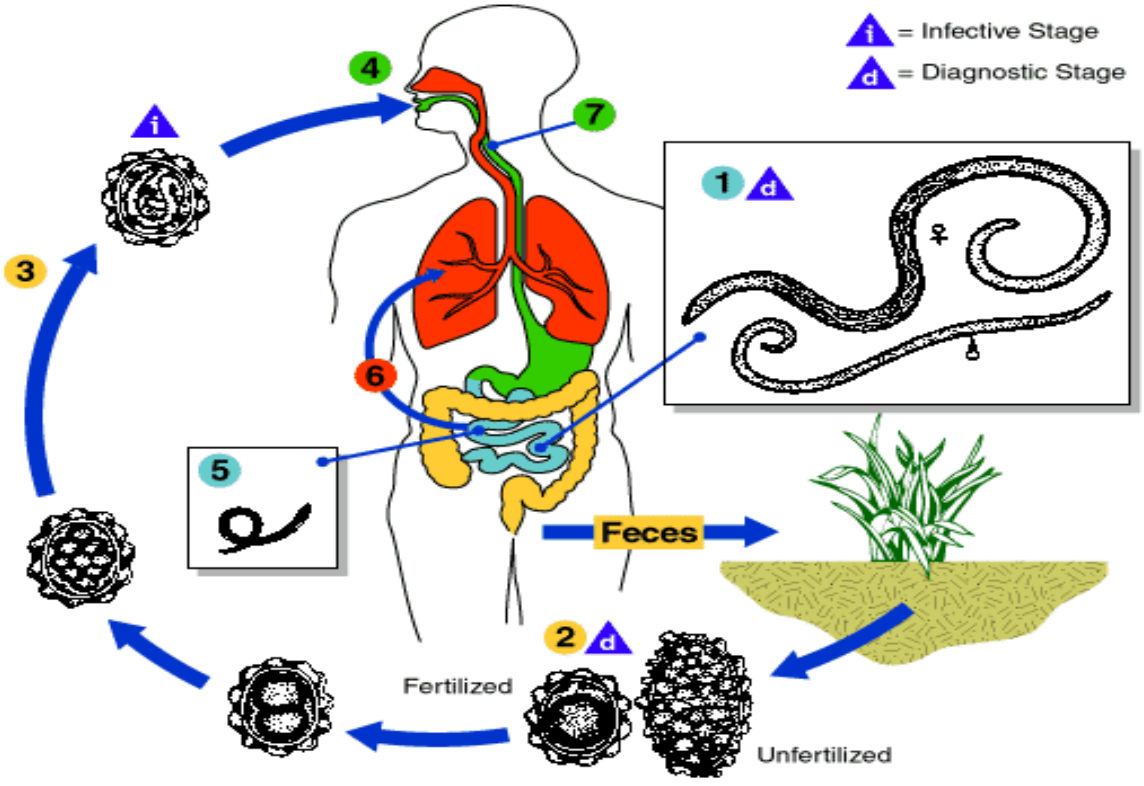



Figure 1.1: Life cycle of *Ascaris* spp. (CDC, 2018)

The maturation of single celled ova into an infective, fully larvated larvae occurs over several weeks once expelled from the host. The optimum environment for ova development is a warm, moist, and shaded area in soil, feces or water (CDC,

2018). The USEPA has 6 stage categories for *Ascaris* including adult, single celled ova, and four larvae stages (L1-L4) as illustrated in Figure 1.2. Ova maturation stages can be divided up even further when observed microscopically into 12 stages including 1-cell, 2-cell, 3-cell, 4-cell, early morula, late morula, blastula, gastrula, pre-lava 1, pre-larva 2, L1, and L2 (Cruz, Allanson, Kwa, Azizan, & Izurleta, 2012). L3-L4 and adult stages occur while inside the host, and are not observed during incubation.

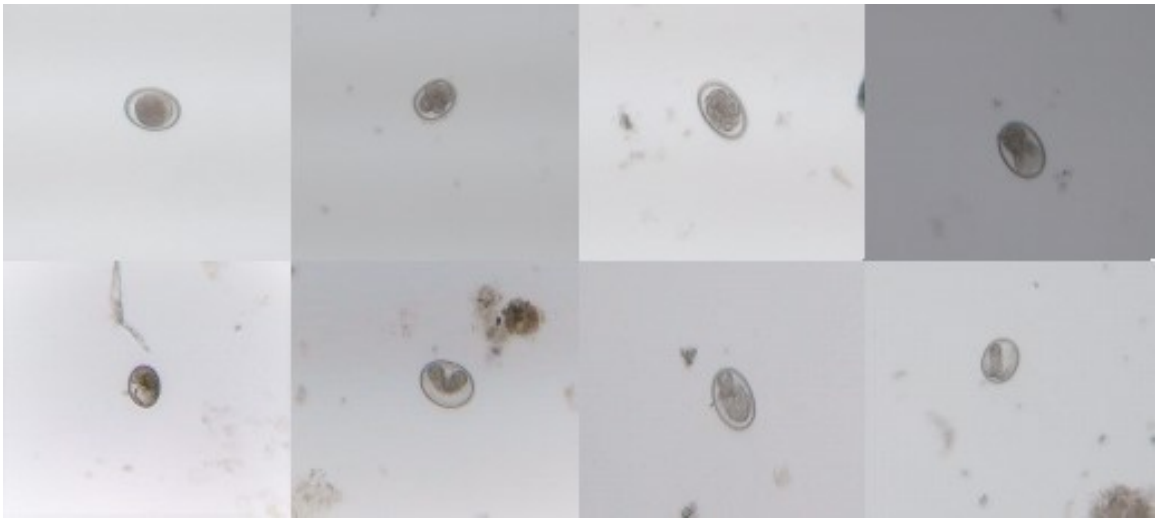


Figure 1.2: Developmental progression of *Ascaris suum* from a single celled ovum to a fully developed larva over the course of ~20 days.

### 1.1.4 Health Concerns and Prevention

Parasitic helminths are of concern because of their human health effects. Worldwide, 1.5 billion people are infected with soil-transmitted helminths, with over 800 million infections caused by *Ascaris* spp. (Fenwick, 2012). Most of these infections occur in warm, tropical environments with high rates of poverty and low hygiene and sanitation standards (Bethony, 2006; EPA, 2018).

Using disability-adjusted life years (DALYs), total helminth infections account for 39 million total life years lost, with 10.5 million years coming strictly from *Ascaris* infections (Stephenson, 2000). While those infected with small numbers of

worms are usually asymptomatic (Dold, 2014), patients infected with a large number of worms may show symptoms of vomiting, fever, distention and abdominal pain. Blockage of worms in the intestine, liver, or pancreas can cause damage and complications. Helminth infections in children can affect both their physical and psychological development (Stephenson, 2000). *Ascaris* infections in young children can also lead to malnutrition, which can lead mental development disabilities (Oberhelman, 1998).

Recommended preventative measures to control ascariasis include washing hands and all food grown in the presence of manure. Children should also be supervised and prevented from eating soils or any possible contaminated materials. If an infection does occur, administration of anti-helminths such as albendazole and mebenazole are common treatment methods (CDC, 2018).

## 1.2 Scope and Objectives of Study

The overall goal for the larger project of which the research reported here is a part is to develop a rational and universal approach for the design of LCLT Class A biosolids treatment processes. To achieve that goal, fundamental information on the impact of key process parameters on the kinetics of inactivation of PIOs is being collected under a wide range of conditions in carefully controlled laboratory studies, and validated in pilot-scale studies conducted at collaborating WRRFs that use different activated sludge configurations and produce Class B biosolids using anaerobic digestion.

Detection of pathogens is critical for achieving the overall project goal of evaluating the inactivation of PIOs, and more generally for monitoring for human and environmental safety during the wastewater treatment process and beneficial reuse of biosolids. Although some of the key PIOs are relatively easy to quantify using conventional culture-based techniques (e.g., fecal coliforms), other organisms such as viruses and helminth ova are much more difficult to quantify via culture-based techniques and require specially trained technicians. For

example, culture-based quantification of *Ascaris* ova takes almost a month of incubation time, and many hours of laboratory effort to interpret a single sample using conventional microscopic counting methods. Therefore, the goal of this specific study was to improve upon the current enumeration methods for quantifying *Ascaris* ova in biosolids, and test a possible new approach for differentiating between live and dead ova using a PMA-qPCR. Specifically, the objectives of this research were as follows:

*Objective #1: Develop an improved method to quantify the viability of Ascaris ova*. To determine the health risk that biosolids or other infective material pose, the viability of PIOs must be determined quickly and efficiently. The ability of *Ascaris* species to survive long periods of time make it an excellent indicator organism, but determination of viability of *Ascaris* ova can be difficult during long term storage, such as occurs in LCLT treatment methods for Class A biosolids. Therefore, the first objective of his study was to work to improve upon existing several methods that are currently used to quantify the viability of *Ascaris* ova in biosolids.

*Objective #2: Compare enumeration of Ascaris ova via qPCR with a conventional microscopic technique*. Molecular methods such as qPCR are potentially capable of analyzing a large number of samples, in less time with greater accuracy than can be achieved with microscopic methods, making them a popular choice for pathogen detection. Although it is not currently an EPA-approved method, several studies have demonstrated the possibility of qPCR as a method to quantify helminths in wastewater and biosolids. If a molecular technique is to become a standard method for helminth quantification, more data are required comparing data obtained using the conventional microscopic technique with qPCR data. In addition, the qPCR techniques require refinement to provide for accurate measurements and prevent false-positives and false-

negatives. Therefore, the second objective of his study was to work to improve upon existing methods that have been used to quantify *Ascaris* ova in biosolids via qPCR, especially coupled with the use of intercalating dyes such as PMA, which may be able to distinguish between living and dead cells.

In the following chapter (Chapter 2), the background literature is reviewed in more detail for the LCLT technique of cake storage. The subsequent chapters are focused on Objective 1, the development of experimental techniques for determining *Ascaris* ova viability through induced motility using a bleach and heat treatment (Chapter 3), and Objective 2, the quantification of *Ascaris* ova using qPCR (Chapter 4). Finally, the conclusions drawn from this research are presented in Chapter 5, along with recommendations for future work.

## 1.3 References

Amahmid, O., Asmama, S., & Bouhoum, K. (1999). The effect of waste water reuse in irrigation on the contamination level of food crops by Giardia cysts and Ascaris eggs. International Journal of Food Microbiology, 49(1-2), 19-26.

Amahmid, O., Asmama, S., & Bouhoum, K. (2002). Urban wastewater treatment in stabilization ponds: occurrence and removal of pathogens. Urban Water, 4, 255-262.

Ayres, R., Lee, D., Mara, D., & Silva, S. (1993). The accumulation, distribution and viability of human parasitic nematode eggs in the sludge of a primary facultative waste stabilization pond. Transactions of the Royal Society of Tropical Medicine and Hygiene, 87(3), 256-258.

Capizzi, S., Chevallier, A., & Schwartzbrod, J. (1999). Destruction of Ascaris ova by accelerated electron. Radiation Physics and Chemistry, 56(5-6), 591-595.

CDC. (2018). Ascariasis Prevention & Control. Retrieved from https://www.cdc.gov/parasites/ascariasis/prevent.html

CDC. (2018). Parasits-Ascariasis: Biology. Retrieved from https://www.cdc.gov/parasites/ascariasis/biology.html

Dold, C., & Holland, C. V. (2011). Ascaris and ascariasis. Microbes and Infection, 13, 632-637.

Gerba, C. P., & Smith, J. E. (2005). Sources of Pathogenic Microorganisms and Their Fate during Land Application of Wastes. Journal of Environmental Quality, 34(1), 42-48.

Leles, D., Gardner, S. L., Reinhard, K., Iñiguez, A., & Araujo, A. (2012). Are Ascaris lumbricoides and Ascaris suum a single species? Parasites & Vectors, 5(42).

Maya, C., Jiménez, B., & Schwartzbrod, J. (2006). Comparison of Techniques for the Detection of Helminth Ova in Drinking Water and Wastewater. Water Environment Research, 78(I), 118-124.

Oberhelman, R. A., Guerrero, E. S., Fernandez, M. L., Silio, M., Mercado, D., Comiskey, N., . . . Mera, R. (1998). CORRELATIONS BETWEEN INTESTINAL PARASITOSIS, PHYSICAL GROWTH, AND PSYCHOMOTOR DEVELOPMENT AMONG INFANTS AND CHILDREN FROM RURAL NICARAGUA. The American Journal of Tropical Medicine and Hygiene, 58(4), 470-475.

Pepper, I. L., Brooks, J. P., & Gerba, C. P. (2006). Pathogens in Biosolids Advances in Agronomy, 90, 1-41.

Ponugoti, P. R., Dahab, M. F., & Surampalli, R. (1997). Effects of different biosolids treatment systems on pathogen and pathogen indicator reducation. Water Environment Research, 69(7), 1195-1206.

Rogers, R. A. (1956). A Study of Eggs of Ascaris Lumbricoides var. suum with the Electron Microscope. The Journal of Parasitology, 42(2), 97-108.

Roussell, D., Gruldl, M., & Bennett, K. (1994). Germ-line Determination in Caenorhabditis and Ascaris: Will a Helicase Begin to unravel the mystery? Parasitology Today, 10(3), 110-113.

Stephenson, L., Latham, M., & Ottesen, E. (2000). Malnutrition and parasitic helminth infections. Parasitology, 121(S1), S23-S38.

# 2 Literature Review of Cake Storage

## 2.1 Background

As noted above, most small- and mid-sized water resource recovery facilities (WRRFs), do not have the resources to implement higher-technology processes for producing Class A biosolids and are in need of low-cost, low-technology alternatives (Farrell et al. 2004). Fortunately, such processes are available and being used successfully at WRRFs in the U.S. and elsewhere. A Water Environment Research Foundation (WERF) study evaluated four of these processes: long-term lagooning, air-drying, combined lagooning/air drying, and cake storage (Farrell et al. 2004). The goal of this chapter is to review the cake storage process in more detail.

## 2.2 Process Description

Cake storage of biosolids is a similar process to an air-drying system, which can be applied to dewatered biosolids that have previously been aerobically or anaerobically digested (Figure 2.1). After dewatering, as in air-drying, the dewatered cake biosolids are stacked in either a windrow, or large pile, and stored for an extended period to achieve pathogen and indicator organism (PIO) inactivation (Farrell et al., 2004). Despite the similarities with air drying, several key differences between cake storage and air-drying exist. For example, in cake storage, piles and

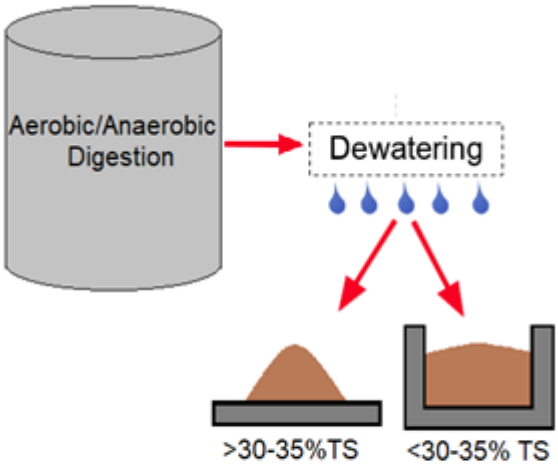



Figure 2.1: Diagram of the cake storage process from digestion to pile formation

windrows can be higher and much larger than air-drying windrows. Typical pile height during cake storage are greater than four feet, whereas in air drying windrow heights of two feet are more typical. In addition, air-drying requires the frequent turning of windrows, whereas, cake storage, involves infrequent turning or zero turning of the piles. To be able to store the cake in windrows, the total solids content needs to exceed 30-35% total solids. If cake storage is to be applied to biosolids with total solids less than 30-35%, it will probably be necessary to use a trench or retaining walls to contain the cake. Drying of solids occurs during cake storage, mainly to the solids being exposed to the environment. However, whereas drying is a major goal of the air-drying process, it is not the goal of the cake storage method to produce dried biosolids, nor is drying considered as a method for pathogen reduction.

If the material being treated via cake storage receives little or no mechanical mixing, the process and final product will not be uniform (Farrell et al. 2004). Any mixing will help ensure that the inside and outside of the windrow are treated similarly and will help with oxygen supply, which will in turn increase the oxidation of organic matter and the accompanying heat generation and temperature increase. The latter is important because in the absence of mixing, the transport of air and water is by diffusion only, which is very slow. Loss of moisture from the outer surface of the pile is a concern, but can be mitigated by covering the cake storage pile with polyethylene sheeting, which will reduce evaporation from the pile and associated cooling as well as maintaining of the pile exterior. Covering may also assist in the increasing above ambient temperatures, which may contribute to pathogen destruction. Because little drying will occur with covering, the material will have to be used as is or treated further in other processes to decrease the moisture content.

## 2.3 Mechanisms of Pathogen Destruction

For biosolids to achieve Class A status, a significant reduction in the number of PIOs must be achieved, as discussed in Chapter 1. The inactivation of PIOs is due to several different physical, chemical, and biological stressors, such as storage time and drying, thermal effects, bacterial competition, and chemical levels, as discussed further below. The mechanisms of pathogen destruction in cake storage are generally similar to those described as being important for air drying (i.e., desiccation, chemical reactions, thermal effects, retention time, competition from non-pathogenic bacteria), except that desiccation is unlikely to play a major role in cake storage (Farrell et al, 2004).

### 2.3.1 Storage Time and Drying

Although drying is not a main goal of the cake storage process, it can occur, mainly on the external surfaces of a storage pile, and can contribute to the inactivation of PIOs. For example, Rouch et al. (2011) looked at the inactivation of *E. coli* and *Salmonella* via the effects of drying during cake storage under ambient temperatures. During the 4 week testing period of this study, biosolids were dried to a total of 15-20% moisture, resulting in a >4 log/g reduction in the amount of *E. coli*, with a final concentration of less than 10 cfu/g for *E. coli*. Dried biosolids also showed a significant (3-5 log ) decrease in *Salmonella* spp., with 5 out of 6 samples showing no quantifiable amount. In comparison, in samples that were kept moist, several boxes had only slight decreases of 0.5 log, while several others showed a 2-4 log reduction over 2 weeks, followed by a slight increase by week 4. Similarly, Yeager et al. (1981) found a correlation between moisture content and bacterial levels in stored sludge that was seeded with *Streptococcus faecalis, Proteus mirabilis, and Salmonella typhimurium* and monitored over 100 days at 21$^{O}$C. While the bacterial levels were stable at 5% solids, there was prompt inactivation at less than 20% moisture.

Desiccation can also lead to inactivation of helminth ova if total solids (TS%) reach 95% or higher. In fact, desiccation is one method that has been found to consistently inactivate helminth ova. In tests performed in a tropical climate (Brazil), inactivation of *Ascaris* below Class A standards was achieved in piles stored for 2-years, when sufficient drying occurred. Biosolids that had been stored underneath a covered patio reached less than 1 viable ova/ 4 grams while ova in boxes that were exposed to sun and rain remained above Class A standards. The total solids in covered boxes reached at least 70%, while uncovered boxes reached a little over 60%. Covered boxes did not reach Class A status until the 2-year mark, while the uncovered boxes were trending toward Class A and may have reached the threshold with several more months of storage. The temperature of the biosolids was not measured, and heat may have been another variable aiding inactivation (Pompeo et al. 2016).

### 2.3.2 Thermal Effects

Temperature is considered a well-known and useful stressor that leads to rapid inactivation of pathogens and other microorganisms. Many studies have looked at the effects of temperature during cake storage including Ahmed and Sorensen (1995, 1997). In the initial study, indicator organisms including *S. typhimurium, Campylobacter jejuni, Yersinia enterocolitica,* bacteriophage f2, and poliovirus were subjected to different temperatures over several testing runs in a laboratory setting. Higher temperatures were demonstrated in these sudies to result in greater PIO destruction than lower temperatures. For example, at 38$^{o}$C, *C. jejuni*, reached below detection limits after 50-100 days, while at 49$^{o}$C, pathogen levels were below detection limits within one day. Nevertheless, different microorganisms were observed to be more resistant to heat than others. During the trial at 22$^{o}$C, *C. jejuni* levels dropped below the detection limit after 10 days, while *S. typhimurium*, and *Y. enterocolitica* took around 30 days to drop below detection limits. Similarly, while *C. jejuni* was inactivated very quickly at 38$^{o}$C, *S.*

*typhimurium* did not reach detection limits until 20 days, and 7 days at 49°C. In comparison, at 5°C, there was little reduction in *C. jejuni*, and no reduction in the levels of *S. typhimurium* and *Y. enterocolitica*.

Ahmed and Sorensen (1997) also performed a field study of cake storage at five WRRFs located throughout Utah, using tubes of spiked biosolids, suspended in 5 ft. tall windrows. In the study, temperatures were recorded over a 90 day period during the summer. At two plants the biosolids temperatures closely resembled the ambient temperature, while several plants had significantly higher biosolids temperatures than ambient temperatures. Bacterial pathogen die off was rapid at all of the plants. *Salmonella* levels were reduced to below detection limits within the first 90 days, in both turned and unturned piles. Similarly, concentrations of *C. jejuni* were decreased to below the detection limit within 50 days in all of the turned piles, although it took around 80 days to drop below the detection limit in unturned piles at several of the WRRFs. In two of the plants, *C. jejuni* levels in the unturned piles were not decreased until after 148 days sampling. This suggests that *C. jejuni* levels are able to survive 5 months or longer under some storage conditions. Most plants also showed a decrease in fecal coliforms to below the detection limit within 100 days, while one plant took 150 days to reach detection limit. Interestingly, *S. typhimurium* and *Y. enterocolitica* deactivation rates were similar between the laboratory and field studies, the deactivation rates for *C. jejuni* in the field study were very different than found in the laboratory study, indicating some microorganism survival rates can vary greatly between laboratory and field studies.

Similar studies have also looked at temperature as a deactivating agent of bacterial pathogens during cake storage and have derived similar conclusions. Al-Ghazali and Al-Azawi (1988) found that *Listeria monocytogenes* was reduced in 6 weeks to non-detectable levels on the surface of the biosolids piles at a temperatures of 28-32 °C. They also found that internal temperatures of the piles had reached 48-54 °C and *L. monocytogenes* levels had declined to below

detectable levels by week 8. Another stressor that could have affected pathogen inactivation in this study was the moisture content, which fell from 35% to 8% on the pile surface, and from 43% to 11% in the middle of the pile.

Other cake storage studies have looked at the inactivation of viruses due to temperature effects in a broad range of mediums. Hurst et al. (1988) looked at several viruses and the effect that different environmental variables had on them. In wetted soil samples, poliovirus concentrations were tested at temperatures of 1, 23, and 37 $^{o}$C. At 1 $^{o}$C, virus levels remained fairly constant and decreased less than 1-log over 75 days. In comparison, at 23$^{o}$C, there was a gradual decline in poliovirus that resulted in a 3-log decrease over 75 days, while the 37$^{o}$C sample had a much more rapid decline than the cooler samples, reaching a 3-log reduction in around 10 days. Similarly, Casanova et al. (2015) used a bacteriophage as a substitute, and found a 2 log reduction over 24 hours and a >7 log reduction after 3 days at 30 $^{O}$C, while samples at 22 $^{O}$C decreased by 0.14-log after 24 hours and 5-log after 6 days.

Temperature of the environment has also been demonstrated to be a stressor that can decrease the numbers of viable helminth ova in biosolids. Specifically, to inactivate helminth ova requires temperatures above 40 $^{o}$C, with a contact time for 10-20 hours (Jimenez-Cisneros 2006). These conditions may be difficult to achieve with cake storage, but temperature effects been demonstrated in several different studies. Pecson et al. (2007) found that temperature has a direct effect on ova inactivation. In samples kept at a temperature of 20 $^{o}$C, the inactivation period was considered to be several hundred days; however, the inactivation period was found to decrease significantly with every 10 $^{o}$C increase in temperature, with samples at 50 $^{o}$C becoming inactivated within several hours (Pecson 2007). Maya et al. (2012) obtained similar results for a wide variety of helminth species, with a distinct relationship between increasing inactivation and increasing temperatures. The study also showed that a decrease in moisture, coupled with an increase in temperature, as well as low pH, could decrease the

contact time to achieve 100% inactivation. In addition, Maya et al. (2006) also found that ova in a larval stage are more susceptible to inactivation than non-larvated eggs. Nevertheless, because it is difficult to reach sufficiently high temperatures or ova inactivation in temperate climates using the cake storage method, it is suggested that storage time is the controlling factor in the inactivation of ova. When Ahmed and Sorensen (1997) tested for *Ascaris* ova inactivation, they found a steady decrease of ova but needed at least 330-400 days before Class A status was achieved. Similarly, a study in Australia found a less than 1-log reduction of *Ascaris* ova over a 21 week stored biosolids laboratory study (Smart Water Fund 2012).

### 2.3.3 Competition with Non-Pathogenic Bacteria

The presence of native non-pathogenic microflora can also contribute to a decrease in the number of pathogenic bacteria due to predation and competition for nutrients, space and other resources. In a study performed by Hussong et al. (1985), *Salmonella* were introduced into two sets of composted sewage sludge, one that was sterilized and one that contained natural microflora. The samples were incubated at 36$^{o}$C and room temperature, and with and without moisture. *Salmonella* was able to grow and survive in the sterilized moist samples as well as the dried samples, at 36$^{o}$C and room temperature, although there was large inactivation in subsequent weeks. However, the non-sterilized samples exhibited much lower growth of *Salmonella* in both the moist and dry samples. Mondal et al (2015) observed similar results for the decay of *Salmonella* in sterilized and unsterilized sewage sludge.

Viral pathogens can also be deactivated through the presence of other microorganisms. In a study performed by Hurst et al. (1980) on soil, poliovirus was spiked into sterile and non-sterile samples, which were kept under either anaerobic or aerobic conditions, as well as different temperatures. Hurst et al. (1980) found that in both the anaerobic and aerobic samples, viral inactivation

was significantly higher in the non-sterile groups compared to the sterile samples at every temperature. Furthermore, the aerobic samples showed a much higher inactivation rate than the anaerobic samples, suggesting that aerobic microorganisms are better at deactivating viruses than the microorganisms present under anaerobic conditions. Finally, similar to most other studies, Hurst et al. (1980) found that increasing the temperature resulted in a much faster decay rate for polioviruses.

## 2.4 Treatment Considerations

Based on the literature, there are a number of treatment guidelines that should be considered when attempting to achieve Class A biosolids production with cake storage. These treatment considerations fall into the categories of pretreatment requirements, potential for pathogen regrowth, climate effects, and the impact of mixing.

### 2.4.1 Pretreatment

Pretreatment of biosolids can have an effect on the characteristics of biosolids and their pathogens levels. For example, mesophilic digestion (37$^{o}$C) and thermophilic digestion (55$^{o}$C) result in similar levels of pH, TS, VS, and COD in the effluent, and comparable biogas production (Gavala et al. 2003), but differ in their effluent pathogen levels. If the retention time is sufficiently long, thermophilic digestion is capable of producing Class A quality biosolids, while mesophilic is unable to reach this quality.

Similarly, the dewatering method used prior to cake storage can cause a difference in the number of pathogens and the consistency of biosolids. Erkan and Sanin (2013) found that in belt-pressed dried solids there was no spike in the growth of fecal coliforms after transitioning to cake storage, whereas centrifugation of mesophilically-digested anaerobic sludge resulted in a spike in PIOs quickly after dewatering. It is thought that the slight heating that occurs

during centrifugation, or the shearing that occurs to the biosolids during the process, results in the slight bump in microorganisms.

### 2.4.2 Pathogen Regrowth

Helminth ova, other parasites, and enteric viruses do not regrow during or after long-term storage because of their need for a host for replication. However, bacterial pathogens do have the potential for regrowth in stored biosolids even after long periods of dry, hot weather, in particular when storage is followed by a wet period. This is demonstrated by the data of Gibbs et al. (1997) who monitored fecal coliforms, fecal streptococci and *Salmonella* levels in 1 m tall biosolids piles during their study. During the hotter, dryer summer months, bacterial levels were below or near detection limits for most of the sampling events. Nevertheless, the bacterial levels increased during the beginning of the cooler, wetter winter, with the fecal coliform levels even increasing above the levels present at the beginning of the study. In one trial that were presented, there was  a span of 50 weeks during which fecal coliforms and salmonella were undetectable, which was followed by regrowth when the conditions improved. Similarly, Zaleski et al. (2005) observed increases in the numbers of *Salmonella* and fecal coliforms following rainfall events in dried biosolids. In this case, the authors attributed the increase to the possibility of recolonization from an external source instead of regrowth. Potential external sources of PIOs include fecal contamination from vectors such as rats, birds or insects around the biosolids.

### 2.4.3 Climate

As discussed above, thermal effects are one of the most effective treatment methods for the inactivation of pathogens and indicator organisms, and climates that are hotter and drier have a much more significant impact on these organisms. A study by Jepsen et al. (1997) in Denmark found similar results as studies discussed above. During warmer summer months, *Fecal Streptococus*

(FS) dropped from $10^6$ FS/g to $10^4$ FS/g within 2 months, while *Salmonella* levels were decreased to below detection limits after 1 month. In winter months, FS concentrations decreased less than 1-log, while *Salmonella* levels were still detectable after 6 months of storage. The decay rates of these organisms are similar to those found in the study performed by Ahmed and Sorensen (1995 and 1997) in Utah. Thus, climates with longer, hotter summers would be much more effective for cake storage than treatment in colder climates with shorter summers.

### 2.4.4 Mixing

The results to date regarding the impact of mixing on the efficacy of cake storage for PIO inactivation are inconsistent. A study of *Ascaris* levels in stored biosolids by Pompeo et al. (2016) did not find any significant differences in *Ascaris* ova inactivation between boxes that were turned twice a month compared to those that were not turned at all. They did, however, determine that placing the boxes under a covered patio resulted in an increase in the inactivation of ova, compared with boxes that were not under a cover. Conversely, Ahmed et al. (Ahmed & Sorensen), found that increasing the mixing frequency in cake storage piles resulted in increased peak pile temperatures. Piles that were turned once per month had higher recorded temperatures than static piles at every sampling period. One plant where separate piles were turned once or twice a month showed a significant impact of turning on pile temperature, with piles subjected to one turning per month having an average temperature of 50.4 $^oC$, while piles turned twice per month reached 57.4 $^oC$. In comparison, the temperature in static piles was closely correlated with the ambient air temperature, and well below the temperatures reached by the turned piles.

## 2.5 References

Ahmed, A.U. and Sorensen, D.L. (1995) Kinetics of Pathogen Destruction during Storage of Dewatered Biosolids. Water Environment Research 67(2), 143-150.

Ahmed, A.U. and Sorensen, D.L. (1997) Autoheating and Pathogen Destruction During Storage of Dewatered Biosolids with Minimal Mixing. Water Environment Research 69(1), 81-94.

Casanova, L.M. and Weaver, S.R. (2015) Inactivation of an Enveloped Surrogate Virus in Human Sewage. Environment Science & Technology Letters 2(3), 76-78.

Erkan, M. and Sanin, F.D. (2013) Can Sludge Dewatering Reactivate Microorganisms in Mesophilically Digested Anaerobic Sludge? Case of Belt Filter versus Centrifuge. Water Research 47, 428-438.

Farrell, J.B., Merrill, D.T. and Schafer, P.L. (2004) Producing Class A Biosolids With Low-Cost, Low-Technology Treatment Processes. Water Environment Research Foundation.

Flemming, C.A., Simhon, A. and Odumeru, J.A. Pathogen Characterization of Fresh and Stored Mesophilic Anaerobically Digested Biosolids. Water Environment Research 89(11).

Fu, B., Jiang, Q., Liu, H.B. and Liu, H. (2015) Quantification of Viable but Nonculturable *Salmonella* spp. and *Shigella* spp. During Sludge Anaerobic Digestion and their Reactivation During Cake Storage. Journal of Applied Microbiology 119(4), 1138-1147.

Garrec, N., Picard-Bonnaud, F. and Pourcher, A.M. (2003) Occurrence of Listeria sp. and L. monocytogenes in sewage sludge used for land application: effect of dewatering, liming and storage in tank on survival of *Listeria* species. FEMS Immunology and Medical Microbiology 35, 275-283.

Gavala, H., N., Yenal, U., Skiadas, I.V., Westermann, P. and Ahring, B.K. (2003) Mesophilic and Thermophilic Anaerobic Digestion of Primary and Secondary Sludge. Effect of Pre-treatment at Elevated Temperature. Water Research 37, 4561-4572.

Gibbs, R.A., Hu, C.J., Ho, G.E. and Unkovich, I. (1997) Regrowth of Faecal Colifroms and Salmonellae in Stored Biosolids and Soil Amended with Biosolids. Water Science & Technology 35(11), 269-275.

Higgins, M.J., Chen, Y.-C., Murthy, S.N., Hendrickson, D., Farrell, J. and Perry, S. (2007) Reactivation and Growth of Non-culturable Indicator Bacteria in Anaerobically Digested Biosolids after Centrifuge Dewatering. 2007 41, 665-673.

Hurst, C.J. (1988) Influence of Aerobic Microorganisms upon Virus Survival in Soil. Canadian Journal of Microbiology 34, 696-699.

Hurst, C.J., Gerba, C.P. and Cech, I. (1980) Effects of Environmental Variables and Soil Characteristics on Virus Survival in Soil. Applied and Environmental Microbiology 40(6), 1067-1079.

Hussong, D., Burge, W.D. and Enkiri, N.K. (1985) Occurrence, Growth, and Suppression of *Salmonellae* in Composted Sewage Sludge. Applied and Environmental Microbiology 50(4), 887-893.

Jepsen, S.-E., Krause, M. and Grüttner, H. (1997) Reduction of Fecal *Streptococcus* and *Salmonella* by Selected Treatment Methods for Sludge and Organic Waste. Water Science & Technology 36(11), 203-210.

Kim, M., O'Shaughnessy, S.A. and Choi, C.Y. (2001) Prediction of Microbial Inactivation in Biosolids as a Function of Moisture Content and Temperature using Artificial Neural Networks. Journal of Residuals Science and Technology 8(1), 1-8.

Koné, D., Cofie, O., Zurbrügg, C., Gallizzi, K., Moser, D., Drescher, S. and Strauss, M. (2007) Helminth Eggs Inactivation Efficiency by Faecal Sludge Dewatering and Co-composting in Tropical Climates. Water Research 41, 4397-4402.

Maya, C., Torner-Morales, F.J., Lucario, E.S., Hernández, E. and Jiménez, 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.

Mondal, T., Rouch, D.A., Thurbon, N., Smith, S.R. and Deighton, M. (2015) Factors Affecting Decay of *Salmonella birkenhead* and Coliphage MS2 during Mesophilic Anaerobic Digestion and Air Drying of Sewage Sludge. Journal of Water and Health 13(2), 459-472.

O'Connor, N.A., Surapaneni, A., Smith, D. and Stevens, D. (2017) Occurrence and fate of *Ascaris lumbricoides* Ova in Biosolids in Victoria, Australia: a Human Health Risk Assessment of Biosolids Storage Periods. Water Science & Technology 76(6), 1332-1346.

Pompeo, R.P., Andreoli, C.V., de Castro, E.A. and Aisse, M.M. (2016) Influence of Long-Term Storage Operating Conditions on the Reduction of Viable *Ascaris* Eggs in Sewage Sludge for Agricultural Reuse. Water Air Soil Pollution 227.

Qi, Y., Dentel, S.K. and Herson, D.S. (2007) Increases in Fecal Coliform Bacteria Resulting from Centrifugal Dewatering of Digested Biosolids. Water Research 41, 571-580.

Qi, Y., Dentel, S.K. and Herson, D.S. (2008) Effect of Total Solids on Fecal Colifrom Regrowth in Anaerobically Digested Biosolids. Water Research 42, 3817-3825.

Rouch, D.A., Mondal, T., Pai, S., Glauche, F., Fleming, V.A., Thurbon, N., Blackbeard, J., Smith, S.R. and Deighton, M. (2011) Microbial Safety of Air-dried and Rewetted Biosolids. Journal of Water and Health 9(2), 403-414.

Sidhu, J., Gibbs, R.A., Ho, G.E. and Unkovich, I. (2001) The Role of Indigenous Microorganisms in Suppression of *Salmonella* Regrowth in Composted Biosolids. Water Research 35(4), 913-920.

Spillmann, S.K., Traub, F., Schwyzer, M. and Wyler, R. (1987) Inactivation of Animal Viruses during Sewage Sludge Treatment. Applied and Environmental Microbiology 53(9), 2077-2081.

Wéry, N., Lhoutellier, C., Ducray, F., Delgenés, J.-P. and Godon, J.-J. (2008) Behavior of Pathogenic Indicator Bacteria During Urban Wastewater Treatment and Sludge Composting, as revealed by Quantitative PCR. Water Research 42, 53-62.

Yeager, J.G. and Ward, R.L. (1981) Effects of Moisture Content on Long-Term Survival and Regrowth of

Bacteria in Wastewater Sludge. Applied and Environmental Microbiology 41(5), 1117-1122.

Zaleski, K.J., Josephson, K.L., Gerba, C.P. and Pepper, I.L. (2005a) Potential Regrowth and Recolonization of *Salmonellae* and Indicators in Biosolids and Biosolid-Amended Soil. Applied and Environmental Microbiology 71(7), 3701-3708.

Zaleski, K.J., Josephson, K.L., Gerba, C.P. and Pepper, I.L. (2005b) Survival, Growth, and Regrowth of Enteric Indicator and Pathogenic Bacteria in Biosolids, Compost, Soil, and Land Applied Biosolids. Journal of Residuals Science and Technology 2(1), 49-63.

# 3 A Comparison of Methods for Inducing Viable Larvated *Ascaris suum* Ova Motility Using Heating and Bleach Treatments

## 3.1 Introduction

Worldwide, 1.5 billion people are infected with soil-transmitted helminths, with over 800 million infections caused by *Ascaris* spp. (Fenwick, 2012). Most of these infections occur in warm, tropical environments with high rates of poverty and low hygiene and sanitation standards (Bethony, 2006; EPA, 2018). *Ascaris* infections occur from ingesting produce, water or soil that has been contaminated with fertilized ova.

In many parts of the world, a key strategy for mitigating threats to water supply and the lack of fertilizers is the beneficial reuse of wastewater and sludges. However, the pathogens, including *Ascaris* spp., associated with these medias is a major concern with respect to human health that must be eliminated via treatment prior to use. Unfortunately, appropriate sanitation is often lacking, and the proper precautions are not taken before these materials are applied to fields (Jensen, 2009). To protect human health, it is important to be able to monitor for the pathogens potentially present in wastes, wastewater and the solid residuals from treatment. *Ascaris,* ova comprise the strictest constraint on beneficial reuse because of their resistance to treatment, persistence in the soil, and public health risks (Fidjeland et al, 2015, Chaona et al, 2018). The World Health Organization (WHO) designates that < viable 1 ova per liter of wastewater is the maximum concentrations to eliminate risk of ascariasis (Ashbolt, 2004). In the United States, the Environmental Protection Agency (USEPA) requires wastewater solids be treated to reduce the level of enteric indicator organisms and pathogens. Only solids that have been treated in this manner may be land applied and are known as biosolids. Two categories of biosolids, Class A and Class B, exist. Class A biosolids, unlike Class B biosolids, can be land applied

without restrictions because they must meet strict limits on the number of pathogenic bacteria, viruses, and helminths. For example, it must be demonstrated that Class A biosolids contain <1 viable helminth ova per 4 g total solids of biosolids.

Methods used to enumerate viable helminth ova recommended by the USEPA (USEPA, 2003), and other commonly used methods (Bowman et al 2003), include incubation of samples for 28 or more days at 26$^{o}$C, after which ova are inspected using phase-contrast microscopy and ova and categorized as unfertilized, fertilized first-stage, or fertilized second-stage based on their development (USEPA 2003). The premise of this test is that ova that have developed to a fully larvated stage are viable, whereas ova that do not develop are non-viable. However, under some conditions, fertilized ova may develop to larval stages during wastewater pr biosolids treatment, and subsequently be inactivated. The EPA method is ineffective at distinguishing larvated ova that have been inactivated, but are still intact, from those that develop to mature stages during incubation in the laboratory. Thus, improved methods for quantifying viable helminth ova are needed.

One potential basis for distinguishing viable and nonviable *Ascaris* larvae is using motility (Cruz, 2012; Manser, 2015). These methods require 5 to 10 minutes of observation per ova to determine if motility is occurring (Schmitz, 2016). However, Smith (1991) found that suspending *Ascaris* ova in a 1% sodium hypochlorite solution caused ova to move vigorously, and as a result, evidence of motility was generally observed within ova. No attempt was made to optimize the treatment used to induce motility in this earlier study. The current study aimed to determine the optimal treatment for increasing the motility of *Ascaris* ova during viability testing by comparing the effectiveness of bleach treatment, heating the ova, or a combination of the two treatments.

## 3.2 Methods

*Ascaris suum* ova were obtained from Dwight Bowman (Cornell University). A 0.1 N sulfuric acid solution (50 mL) contained $10^6$ ova and was stored at 4 $^oC$ until used. The initial viability of the ova was characterized by removing 200-µL aliquot and transferring it to 20 mL of DI water. Formalin was added to the sample at a concentration of 0.5% to prevent fungal and bacterial growth, before incubating the subsample at 26 $^oC$ for 28 days. The ova were then inspected microscopically to determine the number that matured to the second stage, after which the ova stock solution was aliquoted into ten 2-mL aliquots. Aliquoted samples were then stored in at 4 $^oC$ refrigerator until the experiments described below were performed.

Initially, a preliminary experiment was performed to screen for the possible effects of different heating times and the presence of bleach on the movement of ova movement. The treatments that were tested in the preliminary experiment included: no heat, 5, 10, 20, or 30 minutes of incubation at 40 $^oC$, bleach with no heating, and bleach (1%) with incubation at 40 $^oC$ for 20 minutes. Each of these treatments was tested on 10 second-stage ova.

To more carefully test for any synergistic or antagonistic effects between bleach and incubation at 40 $^oC$ for 20 minutes (heat) on the movement of larvated ova, a two-factor (heat and bleach), two-level (low and high) factorial experiment was conducted. Based on the results of the preliminary experiment, four different combinations of treatments were tested: (1) no bleach and no heat, (2) bleach and no heat, (3) no bleach and heat, and (4) bleach plus heat. Each test was run in duplicate and in a random order. A total of 30 ova were selected at random and counted for each trial of the full factorial experiment.

For all movement trials, a 2-mL aliquot of the ova stock was vortexed for 5 seconds to create a homogenized mixture. 700 µL was aseptically pipetted onto a Sedgewick-Rafter counting chamber and covered with a cover slip. If the effect

of heating was being tested, the aliquot was vortexed to homogenize, placed in a hot water bath at 40 +/- 2 $^{o}$C for the selected time period, and then transferred to the counting chamber. If the effect of bleach was being tested, a 75 µL solution of 10% bleach was pipetted into the counting chamber after the 700 µL ova sample was loaded into the counting chamber. The ova and bleach solution was then mixed for 1 minute by gently swirling the chamber by hand. The sample was then placed under a microscope at low light settings to observe ova movement using the 40X objective. Only ova that had fully larvated were observed for a maximum of 1 minute. Any movement during the 1-minute observation period was considered a positive result, while those ova that did not move were counted as negative.

## 3.3 Results

The preliminary experiment demonstrated clear differences in ova movement for different heating times, and due to the addition of bleach to heated and non-heated samples. Based on these results, heating for at least 20 minutes was required to maximize ova movement. There was no additional improvement observed for longer heating times (30 minutes). The highest ova movement count was observed with bleach and heat for 20 minutes, while surprisingly, bleach by itself did not produce any movement. Based on these results, the conditions for the full factorial experiment were selected.

| Treatment | Number of second stage ova (out of 10) exhibiting movement within 1 minute |
|---|---|
| No Heat | 0 |
| 5 min at 40 ºC | 0 |
| 10 min at 40 ºC | 2 |
| 20 min at 40 ºC | 5 |
| 30 min at 40 ºC | 4 |
| Bleach + 20 min at 40 ºC | 7 |
| Bleach + No Heat | 0 |

Table 3.1: Results from initial, small-scale study to determine the optimum combination of heating times and bleach needed to induce movement of viable second-stage *Ascaris suum* ova during microscopic inspection.

The results of the two-factor, two-level factorial experiment are summarized in Table 3.2. Differences in the motility counts were observed in the samples treated with bleach, heat, or a combination of the two. For example, when the ova were pipetted directly on to the counting chamber without prior treatment, the number of ova displaying motility averaged 6 out of thirty for Trials 1 and 2. However, when bleach was added to the slide before observation under the microscope, the average motility counts increased to 19.5 viable ova per 30 ova counted. In comparison, when 20 minutes of heat was applied to each sample before counting without bleach, the average number of viable ova was 18.5. Finally, the trials that combined the 1% bleach treatment and heating to 40 ºC for 20 minutes produced the highest average count of 22.5 viable ova.

| | Number of ova out of 30 exhibiting movement within 1 minute | | |
|---|---|---|---|
| Treatment | Replicate 1 | Replicate 2 | Total |
| No Bleach No Heat | 7 | 5 | 12 |
| Bleach No Heat | 19 | 20 | 39 |
| No Bleach Heat | 17 | 20 | 37 |
| Bleach Heat | 26 | 19 | 45 |
| Total | 69 | 64 | 133 |

Table 3.2: Results of the factorial experiment to determine the optimum combination of heating to 40 $^{o}$C and application of bleach (1%) to induce movement of viable second-stage *Ascaris suum* ova during microscopic inspection.

To determine the significance of each of the treatment as well as synergistic and antagonistic effects, an analysis of variance (ANOVA) was performed (Table 3.3). The main effect of bleach treatment was calculated to be 7.75, the main effect of heat was calculated to be 8.75, and the interaction effect of bleach and heat was calculated at -4.75. The resulting sum of squares calculated for the bleach effect and heat effect were 120.125 and 153.125, respectively, while the sum of squares for the two-factor interaction was 45.125. Based on these values, the F value was calculated for each treatment as summarized in Table 3.3. These F values were then compared to the F value for 4 degrees of freedom, with $p < 0.05$, which is 7.71. Accordingly, the main effect of bleach on the movement of ova is significant (15.254 > 7.71), as was the effect of heat (19.444 > 7.71). However, the interaction between bleach and heat on ova motility is not

significant (5.730 < 7.71), i.e., there is no synergistic or antagonistic effect between heat and bleach. These results indicate that the main effects of heat and bleach are additive. Therefore, increasing both bleach and heat from low to high values increases ova movement by their main effects, 7.75 and 8.75 respectively, for a total increase of 16.5 over the baseline condition of no bleach and no heat. This is consistent with the results of the preliminary study and indicates that the combination of heat and bleach gives the highest viable ova count.

| Experimental Treatment | Sum of Squares | Degrees of Freedom | Mean Square | F |
|---|---|---|---|---|
| Heat | 153.125 | 1 | 153.125 | 19.444 > F0.05(1,4) |
| Bleach | 120.125 | 1 | 120.125 | 15.254 > F0.05(1,4) |
| Heat + Bleach | 45.125 | 1 | 45.125 | 5.730 < F0.05(1,4) |
| Error | 31.5 | 4 | 7.875 | |
| Total | 349.875 | 7 | | |
| F0.05(1,4) | 7.71 | | | |

Table 3.3: Analysis of variance for the two-factor (heat and bleach), two-level (low and high) factorial experiment designed to identify the optimum treatment for inducing movement of viable second-stage *Ascaris suum* ova during microscopic inspection.

## 3.4 Conclusions

The natural development of *Ascaris* ova to larvated forms in the environment can make it impossible to determine viability using only incubation development methods. Methods such as observation for motility offer a reliable way to quantify viable *Ascaris* ova. Due to the long observation time required for each ova, we developed a method to promote ova movement using a bleach and heating

treatment before counting. The application of bleach plus heat increased the total number of ova that exhibited motility when compared with no treatment, bleach alone or heat alone.

## 3.5 References


Ashbolt, N. J. (2004). Microbial contamination of drinking water and disease outcomes in developing regions. *Toxicology, 198*, 229-238.

Bowman, D. D., Little, M. D., & Reimers, R. S. (2003). Precision and Accuracy of an Assay for Detecting *Ascaris* eggs in Various Biosolids Matrices. *Water Research, 37*, 2063-2072.

Cruz, L. M., Allanson, M., Kwa, B., Azizan, A., & Izurleta, R. (2012). Morphological Changes of *Ascaris* Spp. Eggs During Their Development Outside the Host. *Journal of Parasitology, 98*(1), 63-68.

Dąbrowska, J., Zdybel, J., Karamon, J., Kochanowski, M., Stojeckl, K., Cencek, T., & Kłapeć, T. (2014). Assessment of viability of the nematode eggs (*Ascaris, Toxocara, Trichuris*) in sewage sludge with the use of LIVE/DEAD Bacterial Viability Kit. *Annals of Agricultural and Environmental Medicine, 21*(1), 35-41.

Drake, L., Jukes, M., Stemberg, R., & DAP, B. (2000). Geohelminth infections (ascariasis, trichuriasis, and hookworm): Cognitive and developmental impacts. *Seminars in Pediatric Infectious Diseases, 11*(4), 245-251.

Fenwick, A. (2012). The global burden of neglected tropical diseases. *Public Health, 126*, 233-236.

Jensen, P. K., Phuc, P. D., Konradsen, F., Klank, L. T., & Dalsgaard, A. (Environmental Health). Survival of *Ascaris* eggs and hygienic quality of human excreta in Vietnamese composting latrines. *2009, 8*(57), 9.

Manser, N. D., Wald, I., Ergas, S. J., Izurieta, R., & Mihelcic, J. R. (2015). Assessing the Fate of *Ascaris suum* Ova during Mesophilic Anaerobic Digestion. *Environment Science & Technology, 49*, 3128-3135.

Organization, W. H. (2018). Soil-transmitted helminth infections. Retrieved from http://www.who.int/en/news-room/fact-sheets/detail/soil-transmitted-helminth-infections

Palmer, P. E. S., & Reeder, M. M. (2001). *The Imaging of Tropical Diseases* (Vol. 2). Germany: Springer-Verlag Berlin Heidelberg.

Schmitz, B., Pearce-Walker, J., Gerba, C. P., & Pepper, I. L. (2016). A Method for Determining *Ascaris* Viability Based on Early-to-Late Stage In-Vitro Ova Development. *Journal of Residuals Science and Technology, 13*(4), 275-286.

Smith, C. (1991). Induced Larval Motility: A Possible Viability Test for Embryonated *Ascaris lumbricoides* Ova. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 85*, 760.

# 4 Use of Molecular Methods for the detection and quantification of *Ascaris* Ova During Long-term Biosolids Storage

## 4.1 Introduction

In many parts of the developing world, ascariasis, which is caused by infection with the parasitic helminth *Ascaris lumbricoides* is rampant. It is estimated that 890 million people worldwide are infected with *Ascaris*, typically caused by inadequate sanitation (Pullan, 2014). Infection with *Ascaris* or other parasitic worms is not endemic in the U.S. Nevertheless, wastewater treatment utilities that wish to distribute wastewater treatment solids without restriction for land application or other beneficial reuses must ensure that the solids meet strict standards for pathogenic bacteria, enteric viruses and helminth ova by treating the solids with established methods. The Class A biosolids standards require that biosolids contain less than 1 viable helminth ovum per 4 grams of total solids at the time of distribution (EPA, 1993).

Wastewater treatment utilities may also seek regulatory approval for the use of alternative solids treatment methods by demonstrating that they consistently achieve adequate reduction of pathogens that are equivalent to the “Processes to Further Reduce Pathogens” (PFRP). Demonstrating PFRP equivalency requires at least a two-log reduction in the abundance of viable *Ascaris* ova. The U.S. Environmental Protection Agency (EPA) method for quantifying viable helminth ova uses flotation and filtration to elute ova from solids, followed by incubation and then enumeration of ova at different developmental stages via microscopic inspection (Bowman, 2003). Unfortunately, this method results in variable and often low ova recovery rates (Alum, 2019; Steinbaum, 2017), and it requires over one month to complete.

In addition, under some conditions, helminth ova may develop into advanced larval stages and subsequently be inactivated prior to the incubation stage. This

can make it difficult to assess viability via microscopic methods. Thus, there is an urgent need for methods that can be used rapidly and accurately to quantify viable helminth ova at low concentrations in both developed countries and in global water and sanitation hygiene (WASH) applications. We recently reported on a variation of the EPA's method for quantifying helminth ova that incorporates assessment of motility to distinguish viable and non-viable helminths in advanced larval stages.

qPCR-based methods offer an alternative to lengthy culture- and microscopy-based methods, and previous studies have shown that a qPCR-based method targeting the internal transcribed spacer 1 (ITS-1) region could be used to quantify *Ascaris* in water samples. Pecson et al. (2006) showed that ITS-1 copies increased with incubation of viable ova, while a constant qPCR signal or cycle threshold (Ct) value resulted when inactivated ova were incubated (Pecson, 2006). In a related study, Raynal et al (2012) showed that when fully larvated ova were inactivated via several disinfecting procedures, the qPCR signal decreased (Raynal, 2012).

However, several complications can reduce the accuracy of qPCR-based methods when they are applied to environmental samples. For example, environment inhibitors such as humic acids, polysaccharides, or bile may be present and reduce amplification and contribute to false-negative results or low estimates of population abundance (Schrader, 2012). Another challenge is that qPCR may amplify target DNA sequences that have been released to the environment but have not degraded, as well as target DNA within intact but non-viable cells. This may lead to false-positive results and/or overestimation of the abundance of the number of copies of target DNA in environmental samples (Chaiyanan, 2001; Wang, 2006).

One promising strategy for limiting amplification of target DNA derived from non-viable cells is to treat samples with a non-specific DNA intercalating dye such as

propidium monoazide (PMA) (Li, 2014; Parshionikar, 2010; Bae, 2009; Taskin, 2011; Alonso, 2014; Karim, 2015). The intercalating dye binds with extracellular DNA and DNA in weakened, permeable non-viable cells. The dye-bound DNA is then not available for amplification via qPCR. Theoretically, only DNA extracted from viable cells, which are generally not permeable by PMA, is thus amplified via qPCR. However, very few studies have evaluated the use of PMA-qPCR to limit amplification of target DNA from soil-transmitted helminths. Treatment of inactivated *Ancylostoma caninum* ova with 100 µM PMA reduced the qPCR signal 4 orders of magnitude compared to a sample containing 100% viable ova (Gyawali, 2017). The qPCR signal for a PMA-treated sample containing a mixture of 50% viable and 50% non-viable *A. caninum* was reduced one order of magnitude compared to the 100% viable sample. In contrast, amplification of the 100% viable and 100% non-viable samples treated with PMA yielded very different results, with the 100% non-viable *A. caninum* being reduced 4 orders of magnitude compared to the 100% viable sample.

The overall goals of this research were to: (1) optimize a PMA-qPCR method for differentiation of DNA derived from viable and nonviable *Ascaris* (roundworm) ova eluted from biosolids, and (2) apply this method to quantify inactivation of viable *Ascaris* ova during long term storage and air-drying of seeded wastewater treatment biosolids.

## 4.2 Methods

### 4.2.1 Biosolids Spiking/Sampling

*Ascaris suum* ova were harvested by Dwight Bowman (Cornell University) and shipped in a 0.1 N sulfuric acid solution. The ova had a viability of 90.87% at the time of shipment and were stored at 4 $^{o}$C until needed.

Freshly pressed biosolids were collected from both GIWA and PLWSA. Three grams of the biosolids were added to each containment pouch by adding 150 µL

of an *Ascaris* ova stock solution containing 200 ova/µL. This yielded a concentration of 10,000 ova per gram wet weight biosolids, or 30,000 ova per bag. Each bag was then heat-sealed three times across the top with a heat sealer. Importantly, the pore size of the bags was 25 µm. *Ascaris* ova are 30 to 50 µm. Therefore, the bags served as sentinel chambers that exposed ova to the physical and chemical conditions in the biosolids, but did not allow them to be released into the pilot-scale test beds.

The integrity of the bags was tested using the following procedure. A bag seeded with ova was placed in a biosolids pile for one week, and then stirred in a beaker of water overnight using a magnetic stirrer. The water was subjected to a double-centrifugation elution method using water and magnesium sulfate to recover any ova that might have escaped. Microscopic examination of the eluent revealed no eggs.

Ova-seeded bags for a given test bed were gathered into a nylon mesh bag (25 µm pore size, 12 in x 18 in, filterbags.com) and suspended in a square plastic stack as previously described (Becker, 2018). On day 300 at PLWSA and day 335 at GIWA, additional ova bags were prepared. Aged biosolids (3 g) from the appropriate test bed were placed into each bag, 30,000 ova were added, and the bags were sealed as described above. The sealed ova bags were placed in suet feeders, which were subsequently packed with aged biosolids obtained from the test beds. One suet feeder was buried in each test bed at the same depth as the original set of bags, and chains were attached to the suet feeders to facilitate their retrieval at sampling events.

Ova-seeded bags were collected from both the stack and the suet feeder (after day 300) from each test bed at every sampling event at PLWSA, whereas at GIWA, ova-seeded bags the stacks and suet feeders (after day 335) were collected on alternating sampling dates until air-drying was initiated. During air-

drying, the bags were collected from both stacks and suet feeders at each sampling event.

At GIWA, when the biosolids were transferred from the test beds to air-drying piles, all of the remaining ova-seeded bags for a given treatment (inside or outside storage) were gathered into a single nylon bag and placed in a short stack, which was inserted in the pile. Likewise, all of the ova-seeded bags in the suet feeders were consolidated into a single suet feeder for a given treatment and placed in the appropriate pile. The air-drying phase at GIWA also included a third windrow formed with fresh digested and dewatered biosolids. Inside the freshly dewatered biosolids, a suet feeder containing newly made sentinel bags spiked with 30,000 ova was. This pile, known as the direct air-drying pile, was sampled at each sampling event.

Sentinel bags from each test bed were transported on ice until they could be stored at 4 $^{o}$C for up to one week before the ova were eluted.

### 4.2.2 Ova Elution

Ova were eluted from biosolids as described by Bowman et al. (2003) with the following modifications: All glassware and containers were treated with organosilane prior to elutions. Ova bags spiked with *Ascaris* ova were used instead of freshly pressed biosolids cake. First, the top of the ova bag was cut open, and then the two sides were cut open to form a long rectangular sheet, which was placed into a 600-mL beaker. The scissors were washed into the beaker using a wash bottle containing DI water. A stir bar was added to mix the solids (instead of blending the sample), and the beaker was covered with a layer of Parafilm™. Four sieves with increasingly small openings (#20, 60, 80, and 100) were stacked and placed on a tight-fitting bucket and used to clear larger particles from the mixture. The sample was rinsed with a hand-held pressure washer through the sieve stack.

After settling overnight, the sediment was distributed evenly between six 50-mL centrifuge tubes. After the wash steps, the pellet was broken up via vortexing, or a wooden stick to help completely break up the pellet. the stick was washed into the tube using deionized water. A water aspiration vacuum filtration apparatus incorporating a #400 sieve was prepared by covering the sieve with Parafilm™ to increase suction when pouring solution through sieve The supernatant was decanted and discarded, and the pellet was washed into a 15-mL screw top vial (Thermo Scientific; Model 2116-0015PK) using a 0.5% formalin solution.

During enumeration, a 700-µL sample was observed under 100X magnification in a Sedgwick-Rafter counting chamber with cover slip. Different stages of ova were counted and categorized based on their development. Duplicate 700 µL samples were enumerated microscopically. Ova were not only categorized based on first and second stages, but also as being viable or non-viable, based on the larval movement, as described above.

### 4.2.3 qPCR Standards Preparation

A Topo 2.1-cloned bacterial plasmid containing the ITS-1 sequence target was obtained from Kara Nelson (University of California, Berkley). To prepare the aliquoted samples, LB broth was inoculated from a stock of ITS-1 *E. coli* and grown overnight at 37 $^{o}$C. A LB agar plate was then streaked using the *E. coli* culture grown overnight, and then the plate was incubated overnight. A single colony was used to inoculate another flask of LB broth, incubated overnight, and then aliquoted in to 2-mL freezer vials and stored at -80 $^{o}$C in a 20% glycerol solution. To extract the ITS-1 plasmid, an aliquot was taken from the freezer and inoculated in LB broth overnight. The plasmid was extracted and purified using a Zyppy Plasmid Miniprep kit according to the manufacturer's instructions. The abundance and purity of the plasmid extract were quantified spectrophotometrically. Linearized plasmid was prepared by incubating 1 µg of DNA with 10 units of HindIII and 5 µL of NEB buffer in a 50 µL total reaction

volume at 37 ºC for 1 hour, followed by incubation at 80 ºC for 20 minutes to inactivate the restriction enzyme. Cut plasmids were visualized on 1% agarose gel to ensure that they were of the correct length and that there was a singular product. The purity and size of the linearized pCR 2.1-TOPO plasmid containing the 201-bp ITS-1 insert were inspected using gel electrophoresis. The linearized plasmid copy concentration was quantified using a Nanodrop spectrophotometer (Marshall Scientific; Hampton, NH)..The total mass of DNA (in ng) was then divided by the weight of a single plasmid, calculated using the plasmid size of 4100 base pairs (bp) and the mass of a single bp (650 Da). The stock solution ($2.2 \times 10^{11}$ copies/µL) was serially diluted to produce standards containing $10^7$, $10^5$, $10^3$, $10^2$, and $10^1$ copies/µL. Standards and a negative control were amplified in triplicate along with each set of samples, which were also analyzed in triplicate (Yun et al. 2006; Ahmed et al. 2014).

### 4.2.4 DNA Extraction

DNA was extracted from biosolids eluent using a DNeasy Powersoil kit (MoBio; Carlsbad, CA) according to the manufacturer's instructions (Appendix B), with slight modifications. Initially, a 100 µL ova suspension was added to the tubes containing beads, instead of adding 0.25 g of sample, as specified in the manufacturer's protocol. Tubes were homogenized using a Mini-BeadBeater-8 (BioSpec Products; Bartlesville, OK) for 3 minutes on the highest setting. For the final DNA elution from the microcentrifuge spin column, 50 µL of solution C6 was used instead of the 100 µL specified in the original protocol.

qPCR was performed using 8-tube strips and a StepOnePlus qPCR thermocycler using Forward Primer (TGCACATAAGTACTATTTGCGCGTAT), 0.25 µL 10 µM ITS0-1 Reverse Primer (TGATGTAATAGCAGTCGGCGG), 0.37 µL 10 µM ITS-1 Taqman Probe (FAM-CGTGAGCCACATAGTAAATTGCACACAAATG-TAMRA). Each reaction tube contained 10 µL of Applied Biosystems 2X Fast Master mix, 0.25 µL of forward and reverse primers (10 µM), 0.37 µL Probe (10 µM), 2 µL of

extracted sample DNA or plasmid control, and sufficient PCR-grade water to bring the volume to 20 µL. The temperature cycle parameters were as follows: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 seconds and 59 °C for 1 minute.

### 4.2.5 DNA Recovery From Ova and Inhibition Testing

Biosolids were spiked with the plasmids containing the ITS-1 gene and amplification of the extracted DNA in several dilutions was performed to determine if the DNA extracts contained any PCR inhibitors derived from the biosolids. Linearized ITS-1 plasmids ($10^6$) were spiked into 0.25 g of fresh biosolids and mixed in a microcentrifuge tube. The samples were extracted in triplicate, using the PowerSoil extraction kit as described above, except that the sample was vortexed instead of being subjected to bead-beating. Negative biosolids controls that were not spiked with ITS-1 plasmid were run in triplicate with the spiked samples.

To quantify the DNA extraction efficiency from ova that were eluted from biosolids, triplicate 100 µL samples of eluent from control samples containing viable ova were extracted using the bead-beating PowerSoil extraction protocol. The percent recovery was calculated according to:

$$\%\,\text{Recovery} = \frac{\text{ITS}-1\,\text{copies}}{\text{number of ova} * \frac{600\ \text{cells}}{2^{\text{nd}}\,\text{stage ovum}} * \frac{33\,\text{ITS}-1\,\text{copies}}{\text{cell}}} * 100\% \qquad [4\text{-}1]$$

where the number of ova was determined microscopically.

### 4.2.6 PMA Treatment

Propidium monoazide (1 mg) was dissolved in 980 µL of 20% dimethyl sulfoxide solution (Fisher Biotech) to obtain a 2 mM working stock solution and then stored in the dark at -20 °C. To treat each sample, 100 µL of an eluted ova suspension

was added to a clear microcentrifuge tube, and the PMA working stock was added to achieve the desired final concentration. Samples containing PMA were incubated in the dark at room temperature for 5 minutes, before being exposed to a 650 W continuous-beam lamp (SP12-001, Fovitec, Irvine, CA) for 15 minutes to promote photoactivation of PMA and binding of DNA. During the photoactivation step, the light source was held in place 20 cm above the samples using a ring stand. To prevent sample evaporation or overheating, the samples were placed on top of a layer of ice in a plastic tub that was lined with aluminum foil. After being exposed to light, the samples were centrifuged for 5 minutes at 2500 x g, decanted to remove PMA, and resuspended in PBS.

Ideally, the concentration of PMA used to treat samples will bind DNA derived from non-viable *Ascaris* ova without inhibiting qPCR amplification of DNA derived from viable ova. To determine the optimal PMA concentration, biosolids were seeded with *Ascaris* ova and stored at 4 ˚C until being incubated and subjected to the elution process described above. Triplicate samples of viable second-stage ova were then exposed to 0, 25, 50, 100, and 200 µM PMA. The optimal PMA dose was then applied to second-stage ova that were treated with 10% bleach for 15 minutes, incubated at 70 $^{o}$C for 15 minutes and at 80 $^{o}$C for 5 minutes in block heater (Torrey Pines Scientific, Carlsbad, CA) to determine if PMA could distinguish between living and inactivated *Ascaris* ova.

### 4.2.7 Statistical Analysis

All statistical analyses were performed using Microsoft Excel 2016. Correlation between microscopic ova counts and those that were calculated via qPCR was calculated using the Spearman's rank correlation. t-tests were used to compare the number of ova in samples treated via PMA-qPCR and qPCR, and in samples treated with different physical-chemical inactivation methods performed prior to applying PMA-qPCR.

## 4.3 Results and Discussion

### 4.3.1 qPCR Standards and Recovery

The qPCR standard curves for the ITS-1 plasmid gene had correlation coefficients ($R^2$) > 0.99 (Appendix C). qPCR amplification efficiencies ranged from 90% to 103%. The standard curve had a slope of -3.381. Amplification of the diluted plasmid DNA indicated that the biosolids caused insignificant inhibition because serial the serial dilutions all yielded ITS-1 sequence copy numbers that were less than 8% lower than the theoretical number of ITS-1 sequence copies added to the samples. The biosolids matrices that were not spiked with ITS-1 plasmid were all negative for ITS-1 sequence copies, indicating that there was no native *Ascaris* ova in the biosolids and no contamination of samples with *Ascaris* DNA.

ITS-1 sequence copy numbers were converted to viable second stage ova concentrations by dividing the qPCR-based ITS-1 copy numbers by the number of ITS-1 sequences per second-stage ovum, which was experimentally determined. To determine this value, triplicate biosolids samples (3 g) were spiked with 30,000 ova, which were subsequently eluted, incubated, and enumerated microscopically. The mean number of second-stage viable ova recovered was 77. The corresponding numbers of ITS-1 sequence copies in the samples determined using qPCR were 275905, 298921, and 215546, respectively. The geometric mean of the ITS-1 copy number was divided by the mean number of ova (260,986.5 ITS-1 sequence copies ÷ 77 ova) to obtain a value of 3,389 ITS-1 sequences per second-stage ovum.

### 4.3.2 qPCR enumeration of ova in biosolids during long-term storage and air drying

Initially, single-celled ova were seeded at a target concentration of approximately 30,000 fertilized ova per 3 g biosolids wet weight. An average of 12,696 ova per 3 g biosolids weight were recovered (Figure 4.1). This corresponds to a recovery

of 42.3% of total ova, which is within the range of recoveries 33.3 to 81.5% of *Ascaris* ova from sludge, biosolids, and wastewater reported in previous studies (Alum, 2014; Karkashan, 2015; Maya, 2006; Ravindran, 2019).

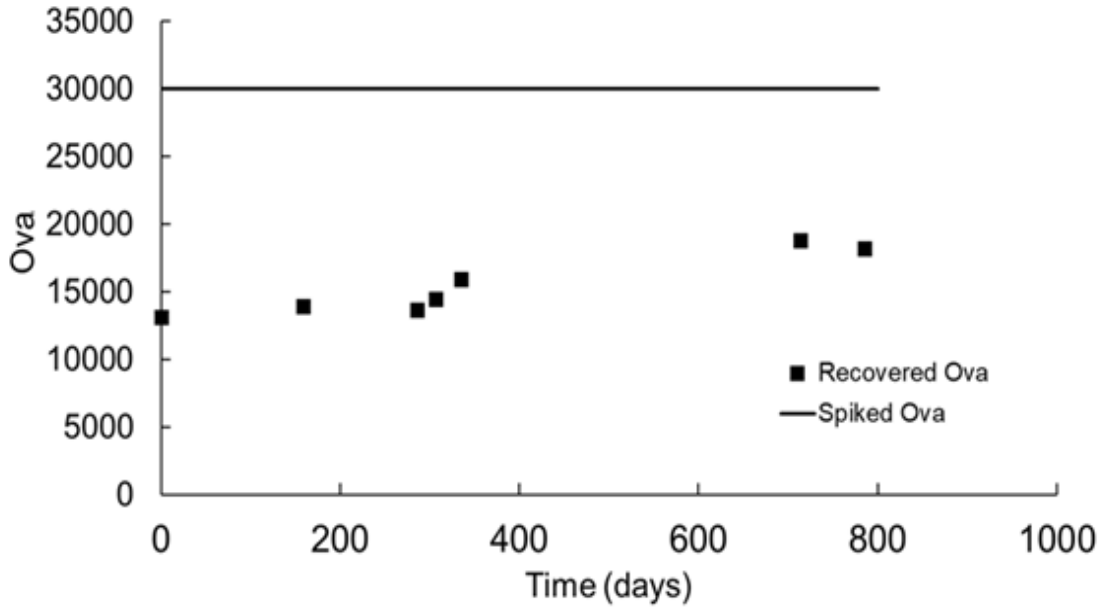



Figure 4.1: Number of *Ascaris* ova recovered from seeded biosolid samples (3 g wet weight) stored at 4 ˚C.

Microscopic enumeration of *Ascaris* ova following incubation indicated that the total second-stage and viable second-stage ova in GIWA inside test bed #5 followed similar trends over a two-year period (Figure 4.2).

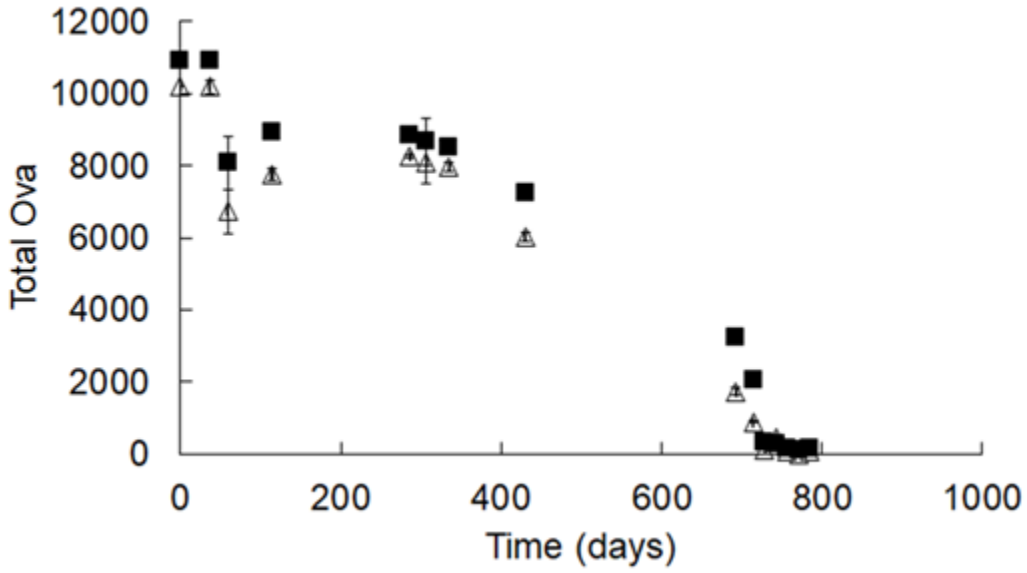



Figure 4.2: Total (■) and viable (Δ) second-stage *Ascaris suum* ova counted microscopically in test bed 5 at GIWA. Error bars represent 95% confidence intervals.

Total second-stage ova decreased from 10929 on day 0 to 2395 ± 65 on day 714, the last day of long-term storage, and 809 ± 54 on day 785, the last day of air drying. During the same period, viable second-stage ova decreased from 10201 on day 0 to 1038 ± 28 on day 714, and 351 ± 24 on day 785. The similar patterns in the total and viable second-stage ova suggest that as viable second-stage ova are inactivated in the biosolids, the outer shell, as well as the larvae inside the ova, degrade. The PLWSA long-term storage test bed 2 results followed trends similar to those observed in the GIWA test bed (Figure 4.3). Total second-stage ova decreased from 7879 on day 0 to 1220.57 ± 1424 on day 393, and viable second-stage ova decreased from 7194 to 651 ± 108 during this period. Finally, in the freshly digested and dewatered biosolids that were treated directly using air drying, the total second-stage and viable second-stage ova decreased from 18069 ± 738 on day 15 to 470 ± 50 on day 71 of air-drying, respectively (Figure 4.4). During microscopic observation of several of the direct

air drying samples, *Ascaris* worms were detected following sample incubation, as discussed below.

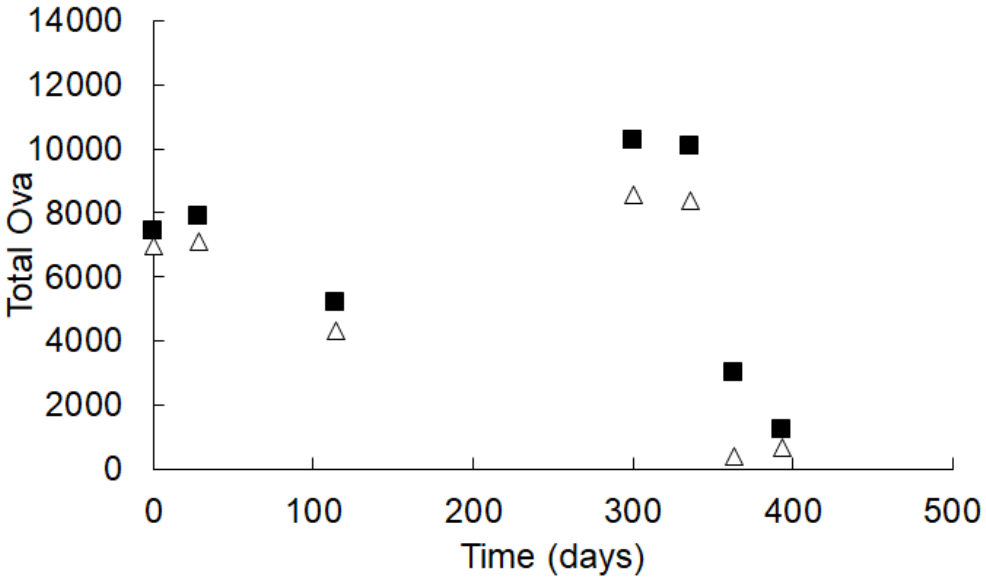



Figure 4.3: Total (■) and viable (Δ) second-stage *Ascaris suum* ova counted microscopically in test bed 2 at PLWSA.

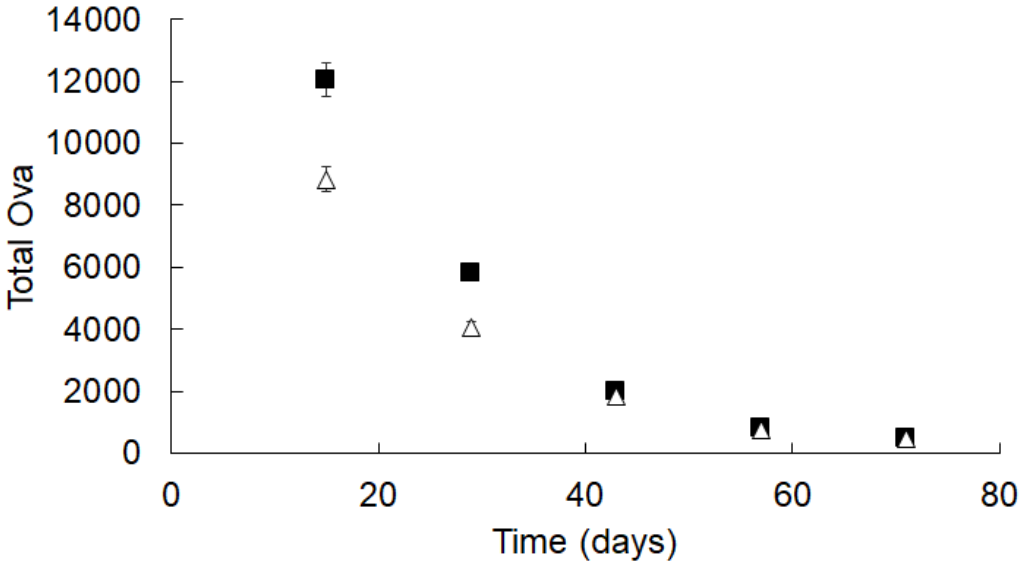



Figure 4.4: Total (■) and viable (Δ) second-stage *Ascaris suum* ova counted microscopically in the air drying pile at GIWA. Error bars represent 95% confidence intervals.

The number of cells enumerated via qPCR followed the same qualitative trends as the microscopic counts of total second stage larvae. In general, the number of total second stage ova in the GIWA test bed 5 samples determined via qPCR was within 50% of the values measured microscopically (Figure 4.5). The only exceptions were days 286 and 430 when the percent difference between the qPCR- and microscopy-based counts was 58% and 51%, respectively.

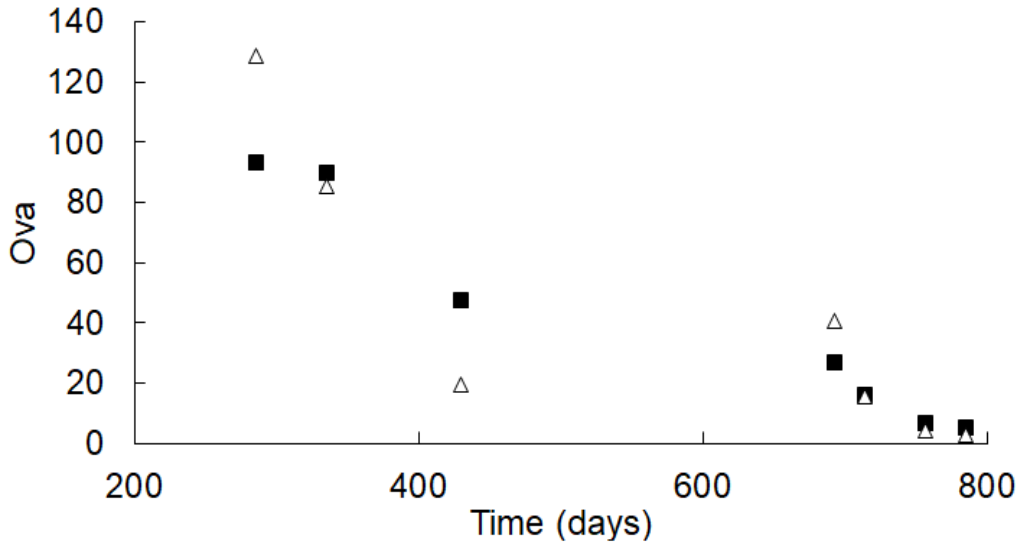



Figure 4.5: Viable second-stage *Ascaris* ova enumerated in GIWA test bed 5 via microscopy (■) and qPCR (Δ), assuming 3389 ITS-1 sequence copies per ovum.

Similarly, the qPCR-based counts of viable second stage ova in PLWSA test bed 2 on days 300, 393, and 421 differed from the microscopy-based counts by 10, 49, and 200%, respectively (Figure 4.6). However, it is important to note that the total number of ova that were counted in test bed 2 after day 300 were quite low, and thus any differences in the qPCR and microscopic counts resulted in relatively large error values when calculated on a percentage basis. On an absolute basis, the differences in the number of viable second-stage ova enumerated via qPCR and microscopic inspection on days 300, 393, and 421 were just 6.7, <1, and 2.7, respectively. In contrast, qPCR-based measures of

viable second-stage ova in the direct air-drying pile at GIWA were higher than the microscopy-based counts in all of the samples (Figure 4.7). In particular, on days 15 and 29, over the qPCR-based counts exceeded the microscopy-based counts by 1300 and 2500%, respectively. On days 43, 57, and 71, qPCR-based counts exceeded the microscopy-based measures by 600, 362, and 256%, respectively, but it is important to note that these values represent small absolute differences in the counts due to the low numbers of ova in the samples.

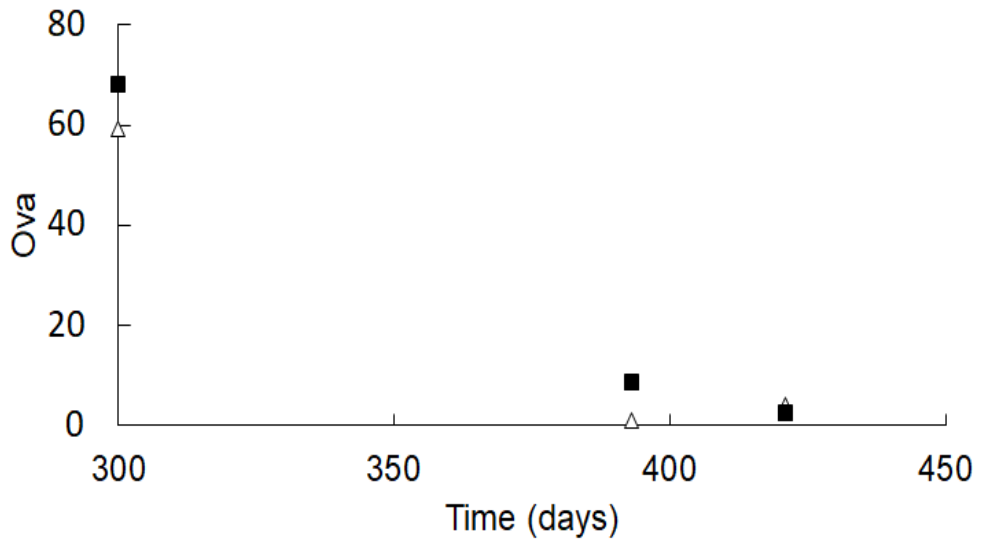



Figure 4.6: Viable second-stage *Ascaris* ova enumerated in PLWSA test bed 2 via microscopy (■) and qPCR (Δ), assuming 3389 ITS-1 sequence copies per ovum.

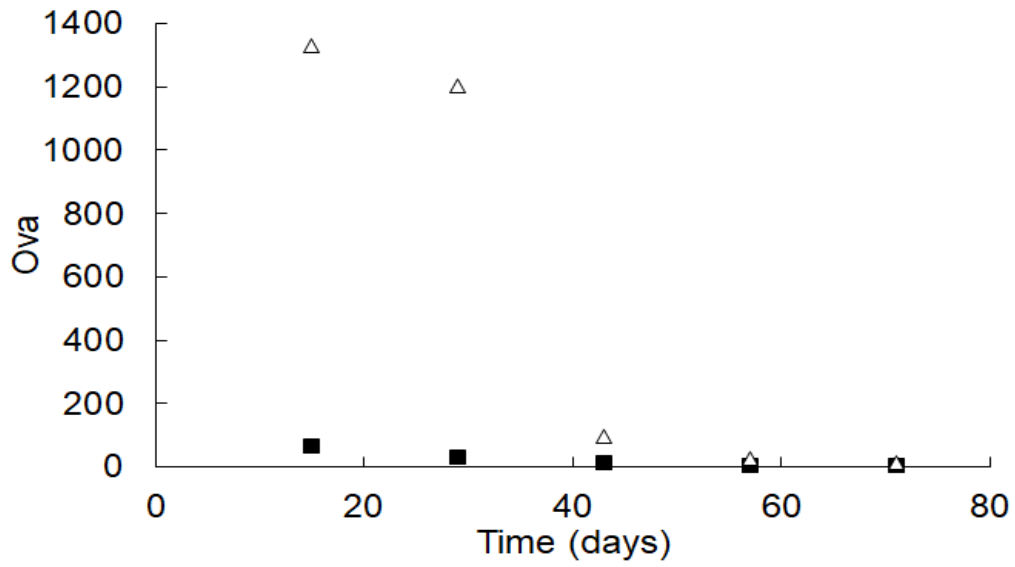



Figure 4.7: Viable second-stage *Ascaris* ova enumerated in the direct air drying pile at GIWA via microscopy (■) and qPCR (Δ), assuming 3389 ITS-1 sequence copies per ovum.

Several aspects of ova development and inactivation in the freshly digested and dewatered biosolids that were directly subjected to air-drying presumably contributed to the differences observed in the qPCR- and microscopy-based ova estimates shown in Figure 4.7. First, on days 15 and 29, three and two worms, respectively, were observed in the eluted ova samples. Although it is not a normal step in their lifecycle, other studies have noted hatching of ova in anaerobic and aerobic digesters (Manser, 2015) as well as in bile (Jaskoski, 1964). Several factors have been linked to the in vitro hatching of *Ascaris* ova, including temperatures near human homeostasis, higher gas-phase $CO_2$ concentrations, the chemical composition of the aqueous environment, and pH (Rodgers, 1958; Fairbairn, 1961). These ejected worms are larger than second stage larvae (Figure 4.8), and therefore presumably have more cells. After hatching, growth may have continued, resulting in further increases in the total cell numbers. The higher cell numbers in emergent worms is important because it means that a single *Ascaris* worm will generate a higher qPCR signal

compared to a single *Ascaris* larva, resulting in an overestimate of ove numbers based on ITS-1 copies. This may explain why the two counts of total ova counts measured using qPCR were orders of magnitude higher than those counted via microscopy on days 15 and 29. In addition, over the course of the 71 day air drying treatment, conditions in the pile became very dry and hot ($T \geq 40$ ˚C), and therefore, fewer first-stage ova could develop into second-stage ova (in the biosolids or during laboratory incubation), i.e., the ova were inactivated. As a result, the number of second-stage ova observed microscopically dropped significantly at each sampling event, while the number of first-stage ova increased (Figure 4.9). Target DNA within these first-stage ova presumably was amplified via qPCR. Thus, the contributions of ITS-1 DNA derived from first-stage ova could potentially explain, at least in part, why the qPCR-based estimates of viable second-stage ova exceeded the microscopic counts on days 43, 57, and 71.

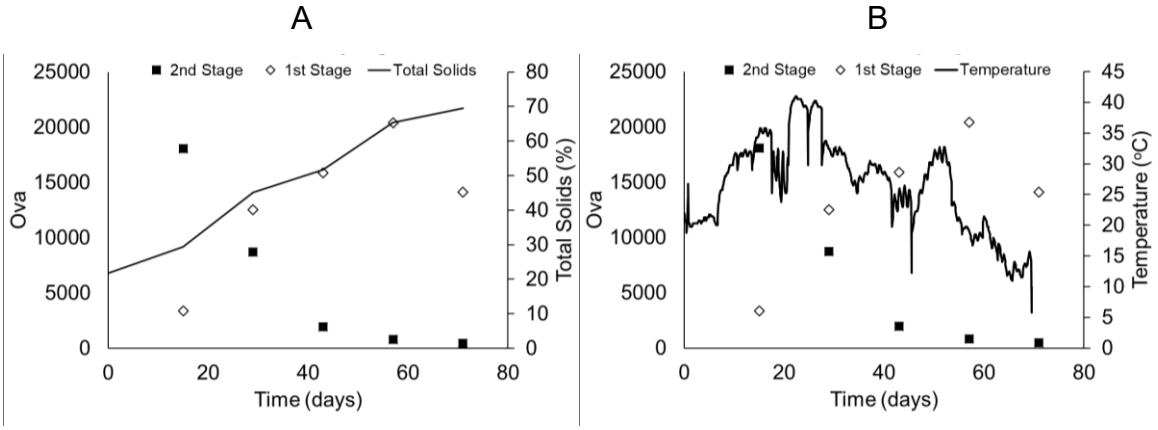



Figure 4.9: The total number of second stage ova, first stage ova, and (A) % total solids and (B) temperature in the direct air drying pile at GIWA.

The viable second-stage ova data shown in Figures 4.2, 4.3, and 4.4 were pooled after omitting the data obtained for the day 15 and day 20 samples from the direct air drying pile, and the qPCR-based ova estimates were plotted as a function of the microscopy based ova numbers in Figure 4.10. The relationship

between these two measures was quantified by calculating the Spearman's Rho correlation coefficient (ρ) for the data set, according to:

$$\rho = 1 - \frac{6\sum d_i^2}{n(n^2-1)} \quad [4\text{-}2]$$

where $d_i^2$ = difference between the ranks of corresponding variables, and $n$ = the number of observations. In this case n = 15. Because ρ = 0.76071, the qPCR-based measurements of viable *Ascaris* ova are strongly correlated to those made via microscopy. Similarly, the linear regression of the data set can be described with an $R^2$ value of 0.6405. Finally, the linear regression has a slope of 0.89795 and y-intercept equal to 10.653. If the qPCR- and microscopy-based measures of ova are in 100% agreement, then the linear regression would have a slope equal to one and a y-intercept equal to 0. Therefore, the slope and y-intercept of the linear regression in Figure 4 also indicate that the two measures are in good agreement. Previous studies derived similar relationships between qPCR and microscopy-based *Ascaris* ova counts, e.g., a linear relationship of qPCR-based counts =1.1265*microscopy-based counts + 8.812 was found by Soto et al. (2017) and Raynal et al. found that qPCR-based counts were 0.8646 times the microscopy-based counts (2012).

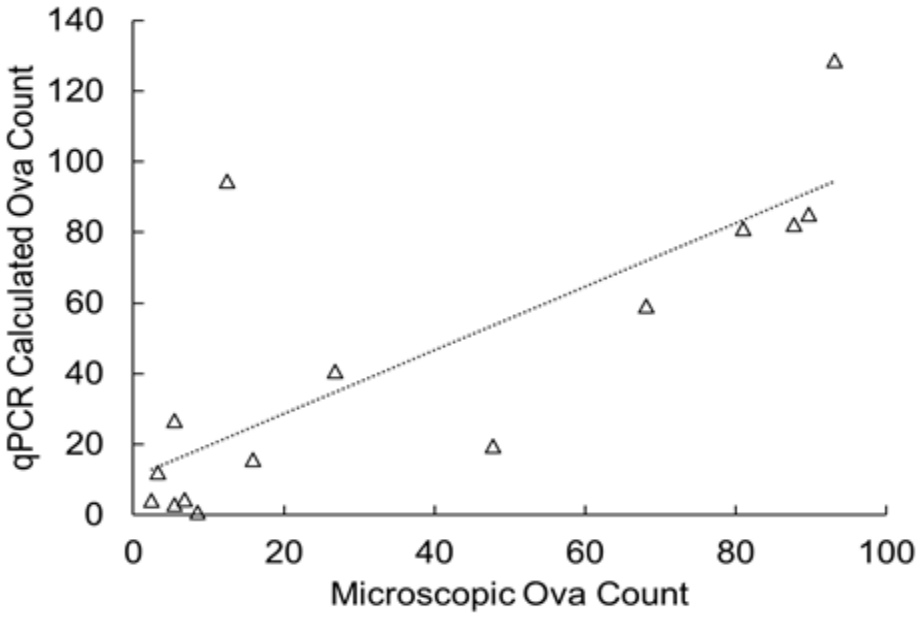



Figure 4.10: Linear regression of *Ascaris* ova numbers of ova calculated based on ITS-1 copy number versus microscopic ova counts .

### 4.3.3 Determination of the Optimal PMA Concentration

PMA binds to and precipitates double-stranded DNA, which enhances the removal of DNA derived from non-viable cells during DNA extraction and purification and prevents its amplification (Nocker et al., 2006). At the same time, elevated PMA concentrations are known to be cytotoxic (Taylor et al., 2014). As a result, amplification of DNA derived from viable cells could potentially be inhibited by using elevated concentrations of PMA. Therefore, steps were taken to determine a PMA concentration that would: (1) not significantly reduce the qPCR signal associated with DNA derived from viable *Ascaris* ova, and (2) maximize the reduction of the qPCR signal associated with DNA derived from non-viable *Ascaris* ova, To determine the PMA dose that meets the first criterion, viable second-stage *Ascaris* ova were treated with different concentrations of PMA and the resulting qPCR signals were compared to the signal derived from untreated ova. To compare each data set, a t-test was performed according to:

$$t = \frac{(x_1 - x_2)}{\sqrt{\frac{(s_1)^2}{n_1} + \frac{(s_2)^2}{n_2}}} \quad [4\text{-}3]$$

where $x_1$ = mean of 1st set of values; $x_2$ = mean of 2nd set of values; $s_1$ = standard deviation of 1st set of values ; $s_2$ = standard deviation of sample 2nd set of values; $n_1$ = number of samples in 1st set of values; and $n_2$ = number of samples in 2nd set of values.

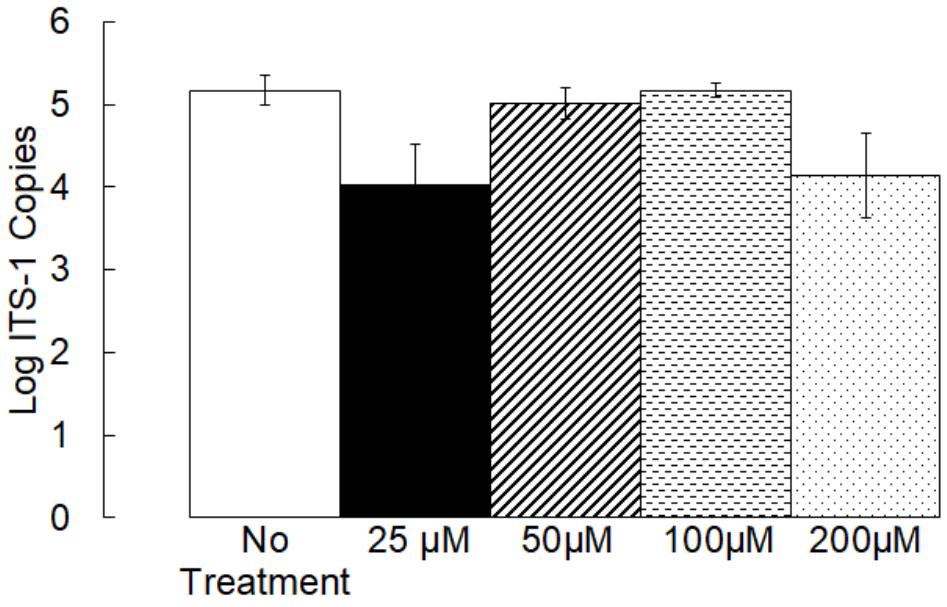



Figure 4.11: Control ova treated with different concentrations of propidium monoazide. Error bars represent 95% confidence.

As summarized in Figure 4.11 (and Appendix D), treatment of *Ascaris* ova with 50 µM or 100 µM treated samples did not have a significant impact ($p < 0.05$) on the number of ITS-1 sequences amplified relative to the number of ITS-1 sequence copies ($1.5 \times 10^5$) in the control samples that were not treated with PMA. In contrast, treatment of *Ascaris* ova with either 25 µM or 200 µM of PMA significantly reduced the qPCR yield relative to the untreated controls ($p < 0.05$).

In practice, a wide range of PMA concentrations have been used to prevent amplification of target DNA from a variety of nonviable microorganisms. For example, Yuan et al. (2018) found that a minimum of 5 µM PMA was needed to bind the DNA from $10^5$ colony forming units (CFU)/mL of dead *Escherichia coli* cells, whereas 50 µM PMA was cytotoxic to, and reduced the qPCR signal from, viable *E. coli* cells. However, a PMA concentration of 50 µM is frequently used for treatment of bacterial cells (e.g., Nocker et al., 2006); and 100 µM PMA was used to distinguish DNA derived from viable versus nonviable helminth (hookworm) ova (Gyawali et al., 2006; Gyawali et al., 2007). Presumably, in the current study, 200 µM PMA exerted a cytotoxic effect on viable *Ascaris* ova and inhibited the amplification of the ITS-1 DNA sequence target. It is not clear why treatment of viable *Ascaris* ova with 25 µM PMA reduced the number of ITS-1 sequences amplified from ova relative to the untreated controls. The most likely explanation is that during the photoactivation of the PMA, the 25 µM sample became overheated. This could compromise the integrity of ova structures and allow PMA to permeate cells.

Based on the results obtained with viable *Ascaris* ova, the effect of PMA on the amplification of DNA derived from non-viable ova was examined using a concentration of 100 µM PMA. To confirm that the treatment methods (incubation in 10% bleach for 15 minutes at room temperature, and incubation at 70 ˚C for 5 min or 15 min) effectively inactivated *Ascaris* ova, the treated ova were inspected microscopically. If no viable ova were detected, i.e., no ova exhibited movement in the microscope field, the treatment was considered effective. In addition, *Ascaris* ova that had a bubbly appearance were considered inactivated (Figure 4.12).

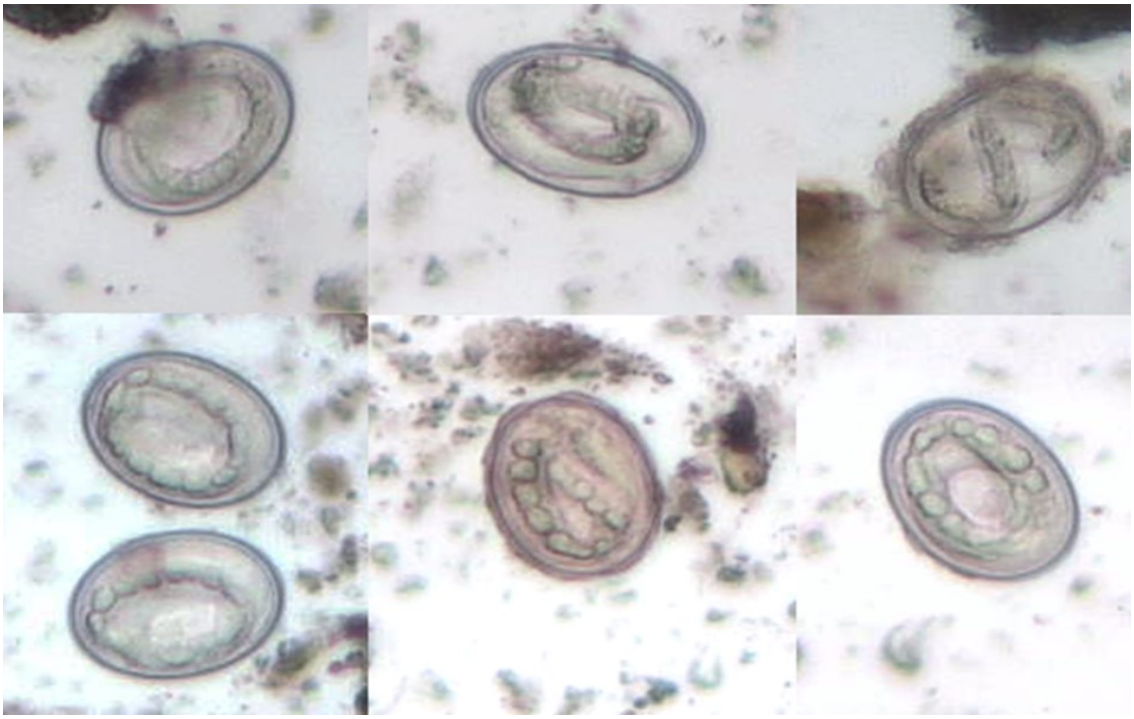


Figure 4.12: Viable *Ascaris ova* taken from control samples (top row) and ova ,with formation of bubbles, that have been inactivated using high heat treatment

The appearance of bubbles on inactivated *Ascaris* ova also was also noted by Schmitz (2016) and is not observed on viable ova. It should be noted that the bleach treatment was chosen to test the hypothesis that fully developed worms contributed to the high qPCR-based counts of viable second-stage ova relative to the microscopy-based counts on days 15 and 20 in the direct air drying pile, as described above. Specifically, it was thought that bleach would inactivate the worms, make them permeable to PMA, and thus reduce the qPCR-based signal associated with the worms. The combination of bleach and PMA treatments was used successfully to prevent amplification of DNA derived from juvenile potato cyst nematodes (Christoforou et al., 2014). In contrast, bleach was not expected to inactivate viable *Ascaris* ova. In fact, *Ascaris* ova are routinely treated with bleach to remove coloration and the corticated layers well as to induce them to move during microscopic inspection, as described above.

As expected, the number of viable second-stage ova in the controls and samples treated with 10% bleach estimated using qPCR alone were not significantly different ($p=0.05$; Figure 4.13). However, the PMA + qPCR-based measure of viable second-stage ova in the bleached sample were also not significantly different relative to the control sample. This indicates that treatment with bleach and PMA cannot be used to prevent amplification of DNA from emerged *Ascaris* worms via qPCR.

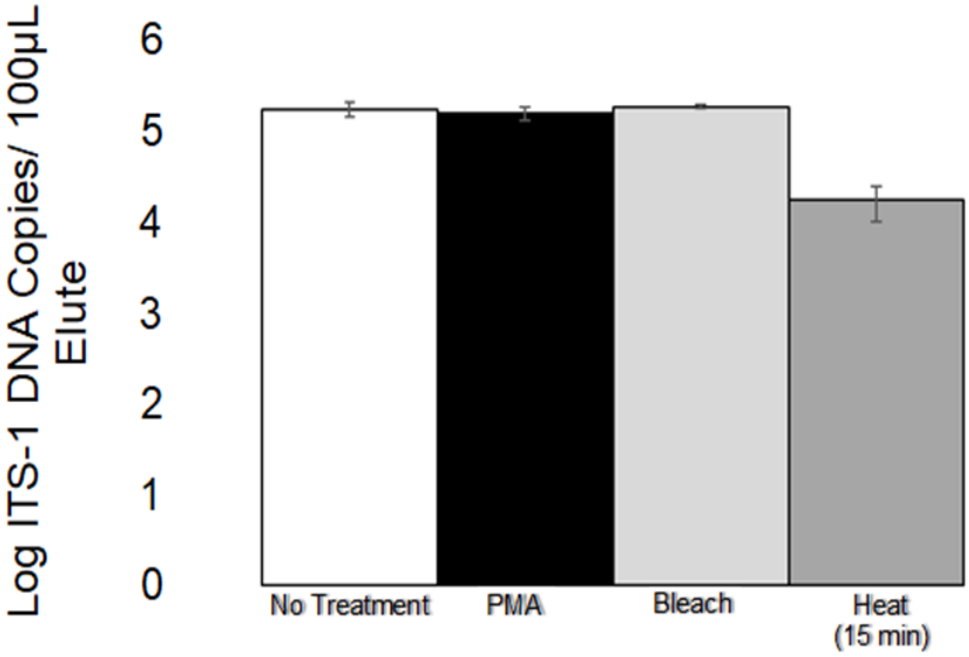



Figure 4.13: Ova Treated with either no treatment, PMA only, Bleach and PMA, or Heat and PMA

Thus, if worms are present, an overestimation of viable second stage *Ascaris* ova will be obtained using qPCR (with or without PMA).

In contrast, the number of viable second-stage ova in the untreated controls and the samples incubated for 15 min at 70 ˚C were significantly different based on qPCR alone ($p < 0.05$; Figure 4.14). These results indicate that heat is effective at inactivating viable second-stage *Ascaris* ova. Moreover, they show that most

of the DNA derived from heat-inactivated *Ascaris* ova is not amplified using qPCR. Presumably, this is the result of heat induced unwinding of AT-base pair rich regions of the DNA from heat treated ova, as discussed above, and/or the removal of this DNA from samples through DNA purification steps. Treatment of heat-inactivated *Ascaris* ova with PMA did not further decrease the yield of ITS-1 sequences amplified via qPCR ($p < 0.05$). Due to the thick shell of Ascaris ova and its resistance to different chemicals, PMA may not have been able to permeate any of the the *Ascaris* ova, or, DNA derived from heat-inactivated *Ascaris* ova was removed during DNA purification (Hill, 2013).

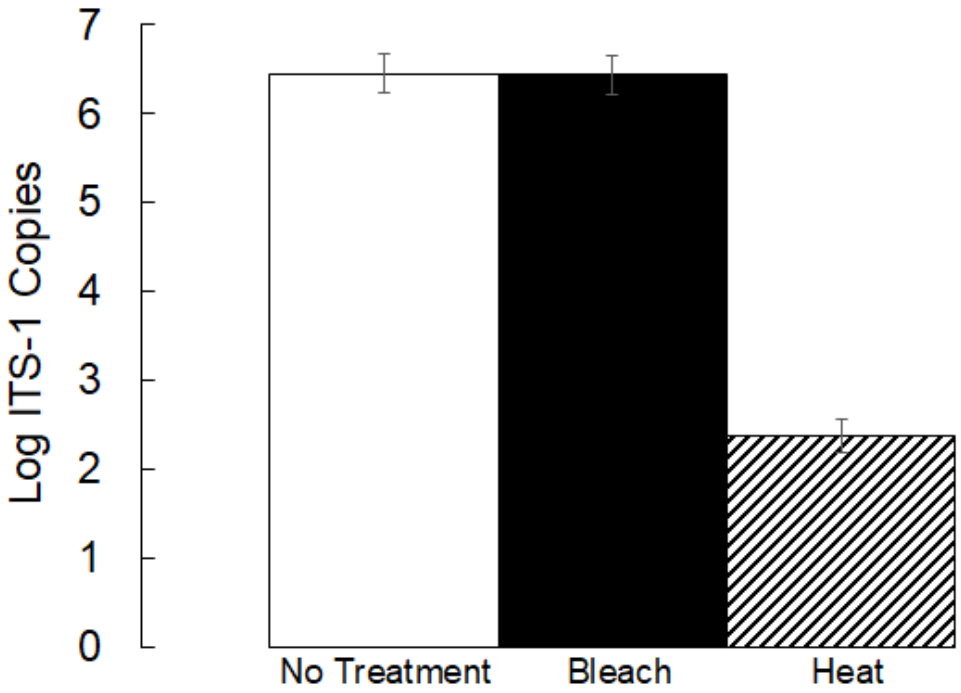



Figure 4.14: Ova Treated with either no treatment, Bleach, or Heat all without PMA

In contrast, the number of ITS-1 copies derived from *Ascaris* ova heated to 70 ˚C for 5 min and amplified via qPCR was not significantly different ($p < 0.05$) compared to those obtained from the untreated control (Figure 4.15). When ova

that were heated for 5 min were inspected microscopically, they did not exhibit movement and their surfaces were bubbled, indicated that they were inactive. However, the 5 minute heating period, unlike the 15 minute heating period, did not melt or damage DNA enough to prevent qPCR amplification. The addition of PMA to the *Ascaris* ova maintained at 70 ˚C for 5 min did significantly reduce the ITS-1 sequence qPCR signal ($p < 0.05$); however, a substantial amount of DNA from the heat inactivated ova was still amplified. It is possible that the PMA treatment method could be optimized to improve PMA binding of DNA derived from inactivated ova, but it seems unlikely that amplification of this DNA cannot be completely eliminated using only PMA unless the conditions that result in ova inactivation are extreme enough to cause irrervisble damage to, or degradation of, the DNA. In that case, there really is no benefit of adding PMA, as observed the analyses of samples taken from the biosolids long-term storage beds and air drying beds, as well as the samples that were heated to 70 ˚C for 15 min. In those scenarios, DNA extraction and qPCR appear to results that are consistent with microscopic ova measurements, unless fully developed *Ascaris* worms are present.

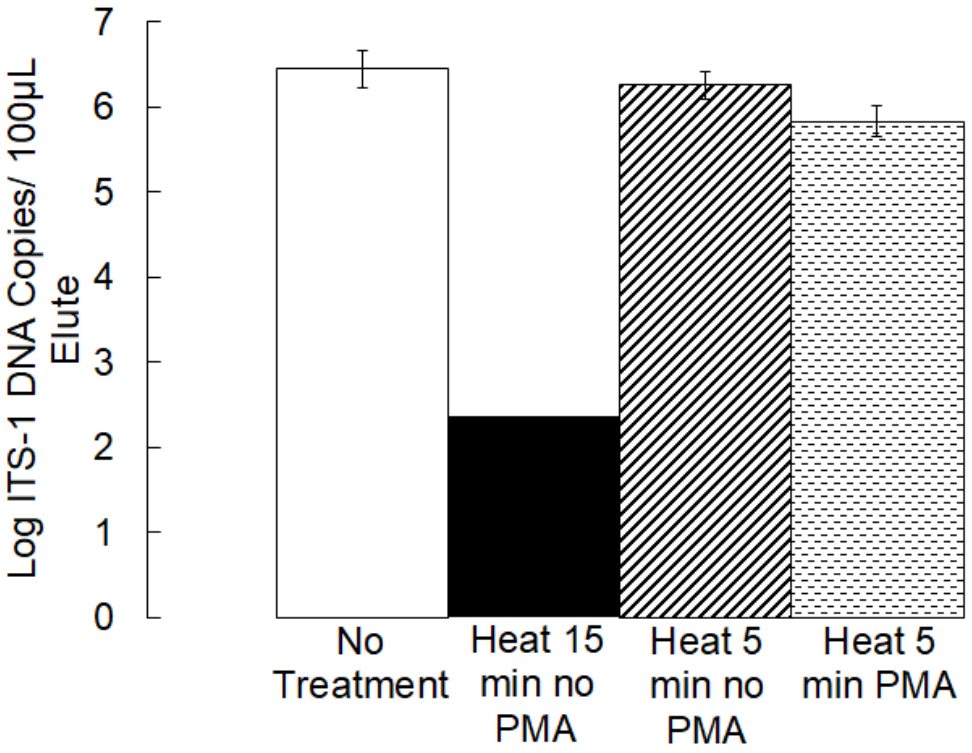



Figure 4.15 : Log ITS-1 DNA copies per 100 µL of eluent in no treatment controls and in samples treated with heat (for 15 min) and no PMA, heat (for 5 min) no PMA, or heat (for 5 min) plus PMA.

## 4.4 Conclusions

PMA concentration and treatment methods that have previously been applied to a wide range of organisms, including hookworms, were used to treat *Ascaris* ova in the current study. Treatment of *Ascaris* ova with 100 µM PMA did not have significant negative impacts on the qPCR amplification of ITS-1 sequence DNA derived from viable ova. However, treatment with PMA did not completely suppress the qPCR amplification of DNA from *Ascaris* ova that were inactivated via heat treatment, unless the heat treatment itself was sufficiently severe to damage the ova and DNA enough that the qPCR signal was reduced by four orders of magnitude, even in the absence of PMA. Thus, there appears to be

little benefit to treating *Ascaris* ova with PMA to distinguish between ITS-1 sequence copy numbers derived from viable and non-viable ova. This was illustrated by comparing the number of viable second-stage *Ascaris* ova enumerated via traditional microscopy and qPCR in biosolids that were subjected to long-term storage and air drying. qPCR captured the qualitative trends and accurately predicted the quantity of viable *Ascaris* ova in these systems, unless fully developed (juvenile) *Ascaris* worms were present. Combined treatment with bleach and PMA did not significantly eliminate the large qPCR signal derived from the juvenile worms. Therefore, future research should focus on identifying methods that can be used to account for the potential impacts of juvenile *Ascaris* worms on qPCR-based estimate of viable ova.

## 4.5 References


Ahmed, A. U., & Sorensen, D. L. (1997). Autoheating and Pathogen Destruction During Storage of Dewatered Biosolids with Minimal Mixing. *Water Environment Research, 69*(1), 81-94.

Alonso, J. L., Amorós, I., & Guy, R. A. (2014). Quantification of viable Giardia cysts and Cryptosporidium oocysts in wastewater using propidium monoazide quantitative real-time PCR. *Parasitology Research, 113*(7), 2671-2678.

Alum, A., Rock, C., & Abbaszadegan, M. (2014). A unified method to process biosolids samples for the recovery of bacterial, viral, and helminths pathogens. *Journal of Environmental Science and Health, 49*, 679-684.

Amoah, I. D., Reddy, P., Seidu, R., & Stenström, T. A. (2018). Concentration of soil-transmitted helminth eggs in sludge from South Africa and Senegal: A probabilistic estimation of infection risks associated with agricultural application. *Journal of Environmental Management, 206*, 1020-1027.

Amoah, I. D., Singh, G., Stenström, T. A., & Reddy, P. (2017). Detection and quantification of soil-transmitted helminths inenvironmental samples: A review of current state-of-the-art and future perspectives. *Acta Tropica, 169*, 187-201.

Bae, S., & Wuertz, S. (2009). Discrimination of Viable and Dead Fecal Bacteroidales Bacteria by Quantitative PCR with Propidium Monoazide. *Applied and Environmental Microbiology, 75*(9), 2940-2944.

Bärenbold, O., Raso, G., Coulibaly, J. T., N'Goran, E., Utzinger, J., & Vounatsou, P. (2017). Estimating sensitivity of the Kato-Katz technique for the diagnosis of *Schistosoma mansoni* and hookworm in relation to infection intensity. *PLOS Neglected Tropical Diseases*.

Becker, J. G., & Seagren, E. A. (2018). *High-Tech Analysis of Low-Tech Methods for Sustainable Class A Biosolids Production: Phase 2, Long-Term Storage and Air Drying Pilot-Scale Studies at PLWSA*. Retrieved from Water Environment & Reuse Foundation

Berhe, N., Medhim, G., Erko, B., Smith, T., Gedamu, S., Bereded, D., . . . Gundersen, S. G. (2004). Variations in helminth faecal egg counts in Kato–Katz thick smears and their implications in assessing infection status with Schistosoma mansoni. *Acta Tropica, 92*(3), 205-212.

Bethony, J., Brooker, S., Albonica, M., Geiger, S. M., Loukas, A., Diemert, D., & Hotez, P. J. (2006). Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *The Lancet, 367*, 1521-1532.

Bowman, D. D., Little, M. D., & Reimers, R. S. (2003). Precision and Accuracy of an Assay for Detecting Ascaris eggs in Various Biosolids Matrices. *Water Research, 37*, 2063-2072.

Burgos, J. S., Ramírez, C., Tenorio, R., Sastre, I., & Bullido, M. J. (2002). Influence of reagents formulation on real-time PCR parameters. *Molecular and Cellular Probes, 16*, 257-260.

Burnham, J. C. (1988). *The alkaline stabilization and disinfection of municipal wasteater sludge.* Paper presented at the Residuals Management Specialty Conference, Atlanta, GA.

CDC. (2018). Ascariasis Prevention & Control. Retrieved from https://www.cdc.gov/parasites/ascariasis/prevent.html

Chaiyanan, S., Chaiyanan, S., Huq, A., Maugel, T., & Colwell, R. R. (2001). Viability of the Nonculturable Vibrio cholerae O1 and O139. *Systematic and Applied Microbiology, 24*, 331-341.

Council, N. R. (2002). *Biosolids Applied to Land: Advancing Standards and Practices*. Washington D.C.: The National Academies Press.

Cruz, L. M., Allanson, M., Kwa, B., Azizan, A., & Izurleta, R. (2012). Morphological Changes of Ascaris Spp. Eggs During Their Dvelopment Outside the Host. *Journal of Parasitology, 98*(1), 63-68.

Direito, S. O. L., Marees, A., & Röling, W. F. M. (2012). Sensitive life detection strategies for low-biomassenvironments: optimizing extraction of nucleic acids adsorbingto terrestrial and Mars analogue minerals. *FEMS Microbiology Ecology, 81*, 111-123.

Dold, C., & Holland, C. V. (2011). Ascaris and ascariasis. *Microbes and Infection, 13*, 632-637.

Drake, L., Jukes, M., Stemberg, R., & DAP, B. (2000). Geohelminth infections (ascariasis, trichuriasis, and hookworm): Cognitive and developmental impacts. *Seminars in Pediatric Infectious Diseases, 11*(4), 245-251.

Easton, A. V., Oliveira, R. G., O'Connell, E. M., Kepha, S., Mwandawiro, C. S., Njenga, S. M., . . . Nutman, T. B. (2016). Multi-parallel qPCR provides increased sensitivity and diagnostic breadth for gastrointestinal parasites of humans: field-based inferences on the impact of mass deworming. *Parasites & Vectors, 9*(38).

Standards for the Use or Disposal of Sewage Sludge, (1993).

EPA. (2003). EPA/625/R-92/013 Appendix I Test Method for Detecting, Enumerating, and Determining the Viability of Ascaris Ova in Sludge".

Everett, K. R., Rees-George, J., Pushparajah, I. P. S., Janssen, B. J., & Luo, Z. (2010). Advantages and disadvantages of microarrays to study microbial population dynamics- a minireview. *New Zealand Plant Protection, 63*, 1-6.

Fairbairn, D. (1961). The In Vitro Hatching of Ascaris Lumbricoides Eggs. *Canadian Journal of Zoology, 39*, 153-162.

Fittipaldi, M., Nocker, A., & Codony, F. (2012). Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. *Journal of Microbiological Methods, 91*(2), 276-289.

Girones, R., Ferrús, M. A., Alonso, J. L., Rodriques-Manzano, J., Calgua, B., de Abreu Corrêa, A., . . . Bofill-Mas, S. (2010). Molecular detection of pathogens in water- The pros and cons of molecular techniques. *Water Research, 44*, 4325-4339.

Green, H. C., & Field, K. G. (2012). Sensitive detection of sample interference in environmental qPCR. *Water Research, 46*, 1251-3260.

Guzmán, C., Jofre, J., Montemayor, M., & Lucena, F. (2007). Occurrence and levels of indicators and selected pathogesn in different sludges and biosolids. *Journal of Applied Microbiology, 103*, 2420-2429.

Gyawali, P., Ahmed, W., Sidhu, J. P. S., Very, S. V., Clements, A. C., Traub, R., . . . Toze, S. (2016). Quantitative detection of viable helminth ova from raw wastewater, human feces, and environmental soil samples using novel PMA-qPCR methods. *Environmental Science and Pollution Research, 23*(18), 18639-18648.

Gyawali, P., Sidhu, J., Ahmed, W., Jagals, P., & Toze, S. (2017). Comparison of Culture-based, vital stain and PMA-qPCR methods for the quantitative detection of viable hookworm. *Water Science & Technology, 75*, 2615-1621.

Hill, G. B., Baldwin, S. A., & Lalander, C. (2013). The Effectiveness and Safety of Vermi-Versus Conventional Composting of Human Feces with Ascaris Suum Ova as Model Helminthic Parasites. *Journal of Sustainable Development, 6*(4).

Jaskoski, B., & Colucci, A. V. (1964). In Vitro Hatching of Ascaris suum Eggs. *Transactions of the American Microscopical Society, 83*(3), 294-300.

Jiménez-Cisneros, B. E., Maya-Rendon, C., & Salgado-Velazquez, G. (2001). The elimination of helminth ova, faecal coliforms, Salmonella and protozoan cysts by various physicochemical processes in wastewater and sludge. *Water Science & Technology, 43*(12), 179-182.

Karim, M. R., Fout, G. S., Johnson, C. H., White, K. M., & Parshionikar, S. (2015). Propidium monoazide reverse transcriptase PCR and RT-qPCR fordetecting infectious enterovirus and norovirus. *Journal of Virological Methods, 219*, 52-61.

Karkashan, A., Khallaf, B., Morris, J., Thurbon, N., Rouch, D. A., Smith, S. R., & Deighton, M. (2015). Comparison of methodologies for enumerating and detecting the viability of Ascaris eggs in sewage sludge by standard incubation-microscopy, the BacLight Live/Dead viability assay and other vital dyes. *Water Research, 68*(1), 533-544.

Kongs, A., Marks, G., Verfe, P., & Van der Stuyft, P. (2001). The unreliability of the Kato-Katz technique limits its usefulnessfor evaluatingS. mansoni infections. *Tropical Medicine and International Health, 6*(3), 163-169.

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

Li, D., Tong, T., Zeng, S., Lin, Y., Wu, S., & He, M. (2014). Quantification of viable bacteria in wastewater treatment plants by using propidium monoazide combined with quantitative PCR (PMA-qPCR). *Journal of Environmental Sciences, 26*, 299-306.

Long, S. C., D'Amato, V., & Bahe, A. (2009). *An Investigation into Biosolids Sampling and Handling Methods for U.S. EPA-Approved Microbial Detection Techniques*. Retrieved from

Manser, N. D., Wald, I., Ergas, S. J., Izurieta, R., & Mihelcic, J. R. (2015). Assessing the Fate of Ascaris suum Ova during Mesophilic Anaerobic Digestion. *Environment Science & Technology, 49*, 3128-3135.

Maya, C., Jiménez, B., & Schwartzbrod, J. (2006). Comparison of Techniques for the Detection of Helminth Ova in Drinking Water and Wastewater. *Water Environment Research, 78*(I), 118-124.

Maya, C., Torner-Morales, F. J., Lucario, E. S., Hernández, E., & Jiménez, 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.

Nocker, A., Cheung, C.-Y., & Camper, A. K. (2006). Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *Journal of Microbiological Methods, 67*, 310-320.

Nocker, A., Sossa, K. E., & Camper, A. K. (2007). Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. *Journal of Microbiological Methods, 70*, 252-260.

Nocker, A., Sossa-Fernandez, P., Burr, M. D., & Camper, A. K. (2007). Use of Propidium Monoazide for Live/Dead Distinction in Microbial Ecology. *Applied and Environmental Microbiology, 73*(16), 5111-5117.

Oberhelman, R. A., Guerrero, E. S., Fernandez, M. L., Silio, M., Mercado, D., Comiskey, N., . . . Mera, R. (1998). CORRELATIONS BETWEEN INTESTINAL PARASITOSIS, PHYSICAL GROWTH, AND PSYCHOMOTOR DEVELOPMENT AMONG INFANTS AND CHILDREN FROM RURAL NICARAGUA. *The American Journal of Tropical Medicine and Hygiene, 58*(4), 470-475.

O'Connell, E. M., & Nutman, T. B. (2016). Molecular Diagnostics for Soil-Transmitted Helminths. *American Journal of Tropical Medicine and Hygiene, 95*(3), 508-513.

O'Connor, N. A., Surapaneni, A., Smith, D., & Stevens, D. (2017). Occurrence and fate of *Ascaris lumbricoides* Ova in Biosolids in Victoria, Australia: a Human Health Risk Assessment of Biosolids Storage Periods. *Water Science & Technology, 76*(6), 1332-1346.

Palmer, P. E. S., & Reeder, M. M. (2001). *The Imaging of Tropical Diseases* (Vol. 2). Germany: Springer-Verlag Berlin Heidelberg.

Parshionikar, S., Laseke, I., & Fout, G. S. Use of Propidium Monoazide in Reverse Transcriptase PCR To Distinguish between Infectious and Noninfectious Enteric Viruses in Water Samples. *Applied and Environmental Microbiology, 76*(13), 4318-4326.

Pecson, B. M., Barrios, J. A., Johnson, D. R., & Nelson, K. L. (2006). A Real-Time PCR Method for Quantifying Viable *Ascaris* Eggs Using the First Internally Transcribed Spacer Region of Ribosomal DNA. *Applied and Environmental Microbiology, 72*(12), 7864-7872.

Postollec, F., Falentin, H., Pavan, S., Jérõme, C., & Sohier, D. (2011). Recent Advances in Quantitative PCR (qPCR) Applications in Food Microbiology. *Food Microbiology, 28*(5), 848-861.

Rafael Lozano, M. N., Kyle Foreman, Stephen Lim, Kenji Shibuya, Victor Aboyans*, Jerry Abraham*, Timothy Adair*, Rakesh Aggarwal*, Stephanie Y Ahn*,, Mohammad A AlMazroa*, M. A., H Ross Anderson*, Laurie M Anderson*, Kathryn G Andrews*, Charles Atkinson*, Larry M Baddour*, Suzanne Barker-Collo*,, David H Bartels*, M. L. B., Emelia J Benjamin*, Derrick Bennett*, Kavi Bhalla*, Boris Bikbov*, Aref Bin Abdulhak*, Gretchen Birbeck*, Fiona Blyth*, Ian Bolliger*,, Soufi ane Boufous*, C. B., Michael Burch*, Peter Burney*, Jonathan Carapetis*, Honglei Chen*, David Chou*, Sumeet S Chugh*, Luc E Coff eng*, Steven D Colan*,, Samantha Colquhoun*, K. E. C., John Condon*, Myles D Connor*, Leslie T Cooper*, Matthew Corriere*, Monica Cortinovis*, Karen Courville de Vaccaro*, William Couser*,, Benjamin C Cowie*, M. H. C., Marita Cross*, Kaustubh C Dabhadkar*, Nabila Dahodwala*, Diego De Leo*, Louisa Degenhardt*, Allyne Delossantos*, Julie Denenberg*,, . . . Anthony D Woolf*, S. W., Pon-Hsiu Yeh*, Paul Yip*, Azadeh Zabetian*, Zhi-Jie Zheng*, Alan D Lopez†, Christopher J L Murray. (2012). Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *The Lancet, 380*, 2095-2128.

Ravindran, V. B., Surapaneni, A., Crosbie, N. D., Schmit, J., Shahsavari, E., Haleyur, N., . . . Ball, A. S. (2019). A modified approach to recover and enumerate Ascaris ova in wastewater and sludge. *PLOS Neglected Tropical Diseases, 13*(2).

Raynal, M., Villegas, E. N., & Nelson, K. L. (2012). Enumeration of Viable and Non-viable Larvated Ascaris Eggs with Quantitative PCR. *Journal of Water and Health, 10*(4), 594-604.

Reimers, R. S., Little, M. D., Akers, T. G., Henriques, W. D., McDonell, D. B., & Mbela, K. K. (1990). *Persistance of pathogens in lagoon-stored sludge*. Retrieved from

Reimers, R. S., Little, M. D., Englande, A. J., McDonell, D. B., Bowman, D. B., & Hughes, J. M. (1986). *Investigation of parasites in sludges and disinfection techniques*. Retrieved from EPA:

Reimers, R. S., Little, M. D., Lopez, A., & Mbela, K. K. (1991). *Final Testing of the Synox Municipal Sludge Treatment for PFRP Approval—Phase II*. Retrieved from Bethesda, MD:

Rodgers, W. P. (1958). Physiology of the Hatching of Eggs of Ascaris lumbricoides *Nature, 181*, 1410-1411.

Rosa, B. A., Supali, T., Gankpala, L., Djuardi, Y., Sartono, E., Zhou, Y., . . . Mitreva, M. (2018). Differential human gut microbiome assemblages during soil-transmitted helminth infections in Indonesia and Liberia. *Microbiome, 6*(1).

Santos, S. S., Nielsen, T. K., Hansen, L. H., & Winding, A. (2015). Comparison of three DNA extraction methods for recovery of soil protists DNA. *Journal of Microbiological Methods, 115*, 13-19.

Schmitz, B., Pearce-Walker, J., Gerba, C. P., & Pepper, I. L. (2016). A Method for Determining *Ascaris* Viability Based on Early-to-Late Stage In-Vitro Ova Devolopment. *Journal of Residuals Science and Technology, 13*(4), 275-286.

Schriewer, A., Wehlmann, A., & Wuertz, S. (2011). Improving qPCR efficiency in environmental samples by selective removal of humic acids with DAX-8. *Journal of Microbiological Methods, 85*, 16-21.

Simeon, D. T., Grantham-McGregor, S. M., Callender, J. E., & Wong, M. S. (1995). Treatment of Trichuris trichiura Infections Improves Growth, Spelling Scores and School Attendance in some Children. *The Journal of Nutrition, 125*(7), 1875-1883.

Soto, L. A., Santisima-Trinidad, A. B., Bornay-Llinares, F. J., Gonzalez, M. M., Valero, J. A. P., & Munoz, M. R. (2017). Quantitative PCR and Digital PCR for Detection of *Ascaris lumbricoides* Eggs in Reclaimed Water. *BioMed Research International*.

Starr, M. C., & Montgomery, S. P. (2011). Soil-Transmitted Helminthiasis in the United States: A Systematic Review. *American Journal of Tropical Medicine and Hygiene, 85*(4), 680-684.

Stephenson, L., Latham, M., & Ottesen, E. (2000). Malnutrition and parasitic helminth infections. *Parasitology, 121*(S1), S23-S38.

Stephenson, L. S., Latham, M. C., Adams, E. J., Kinoti, S. N., & Pertet, A. (1993). Physical Fitness, Growth and Appetite of Kenyan School Boys with Hookworm, *Trichuris trichiura* and *Ascaris lumbricoides* Infections are Improved Four Months after a Single Dose of Albendazole. *The Journal of Nutrition, 123*(6), 1036-1046.

Tarafder, M. R., Carabin, H., Joseph, L., Balolong Jr, E., Olveda, R., & McGarvey, S. T. (2010). Estimating the sensitivity and specificity of Kato-Katz stool examination technique for detection of hookworms, Ascaris lumbricoides and Trichuris trichiura infections in humans in the absence of a 'gold standard'. *International Journal for Parasitology, 40*, 399-404.

Taskin, B., Gozen, A. G., & Duran, M. (2011). Selective Quantification of Viable Escherichia coli Bacteria in Biosolids by Quantitative PCR with Propidium Monoazide Modification. *Applied and Environmental Microbiology, 77*(13), 4329-4335.

Tata, P., Lue-Hing, C., Bertucci, J. J., Sedita, S. J., & Knafl, G. T. (2000). Class A Biosolids Production by a Low-Cost Conventional Technology. *Water Environment Research, 72*(4), 413-422.

van Fankenhuyzen, J. K., Trevors, J. T., Lee, H., Flemming, C. A., & Habash, M. B. (2011). Molecular pathogen detection in biosolids with a focus on quantitative PCR using propidium monoazide for viable cell enumeration. *Journal of Microbiological Methods, 87*(3), 263-272.

Varma, M., Field, R., Stinson, M., Rukovets, B., Wymer, L., & Haugland, R. (2009). Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. *Water Research, 43*, 4790-4801.

Vasileski, G. (2007). *Beneficial Uses of Municipal Wasteater Residuals-Biosolids*. Retrieved from Ottawa, ON:

Venglovsky, J., Martinez, J., & Placha, I. (2006). Hygienic and ecological risks connected with utilization of animal manures and biosolids in agriculture. *Livestock Science, 102*(3), 197-203.

Viau, E., & Peccia, J. (2009). Survey of Wastewater Indicators and Human Pathogen Genomes in Biosolids Produced by Class A and Class B Stabilization Treatments. *Applied and Environmental Microbiology, 75*(1), 164-174.

Vishnivetskaya, T. A., Layton, A. C., Lau, M. C. Y., Chauhan, A., Cheng, K. R., Meyers, A. J., . . . Onstott, T. C. (2014). Commercial DNA extraction kits impact

observed microbial community composition in permafrost samples. *FEMS Immunology and Medical Microbiology, 87*, 217-230.

Vondrakova, L., Turonova, H., Scholtz, V., Pazlarova, J., & Demnerova, K. (2018). Impact of various killing methods on EMA/PMA-qPCR efficacy. *Food Control, 85*, 23-28.

Wang, S., & Levin, R. E. (2006). Discrimination of viable Vibrio vulnificus cells from dead cells in real-time PCR. *Journal of Microbiological Methods, 64*, 1-8.

WHO. (2008). Action Against Worms. *WHO Newsletter*(11).

Zeibig, E. A. (2013). *Clinical Parasitology: A Practical Approach* (2nd Edition ed.). St. Louis, MO: Elsevier.

# 5 Conclusions and Future Directions

## 5.1 A Comparison of Methods for Inducing Viable Larvated *Ascaris* suum Ova Motility Using Heating and Bleach Treatments

### 5.1.1 Inducing Movement via heat and/or bleach treatment

The goal of the 3rd chapter of this chapter was to improve the methods used for determining viability of *Ascaris* ova that have developed over the course of a treatment process, i.e. the long term storage of biosolids. When compared with samples that had not been treated, using 1% bleach, heating up to 40 $^{o}$C, as well as using a combination of both increased the number of ova seen to move over the course of 1 minute.

### 5.1.2 Future Directions

Other methods to determine viability such as dyes, other viability testing, or molecular methods such as qPCR could be tested to provide other methods for determining viability where just incubation would not be useful. Other chemical/heat treatments could also improve the viability counts or reduce the time needed to observe each ova.

## 5.2 Use of Molecular Methods for the detection and quantification of *Ascaris* Ova During Long-term Biosolids Storage

### 5.2.1 Enumeration of *Ascaris* ova using Microscopy vs qPCR

Several studies have shown the ability to use qPCR to test for the presence of *Ascaris* ova in wastewater samples, however few have attempted to correlate qPCR results with microscopic ova counts. In this study, we attempted to correlate 15 ova-spiked biosolids samples that had been subjected to long-term storage treatment. Using control larvated ova, a ITS-1 copies/ova was

determined for our DNA extraction method and used to determine the number of viable ova. Using this, the number of ova was able to be determined via qPCR for a majority of the samples. However, two samples had qPCR results suggesting an ova concentration several times higher than that which was counted microscopically.

### 5.2.2 PMA Treatment to Prevent False-Positives

PMA has been used as a way to prevent false-positives during molecular methods as it binds to DNA in non-viable cells. PMA was first tested in several concentrations to determine the maximum that would still amplify viable ova, and was found that  ova treated with 100 µM had similar results to none treated ova, while 200 µM treated ova were significantly lower. Next, qPCR and PMA-qPCR was tested on several samples using bleach, short-heat, long heat, and no treatment methods. Generally, qPCR found no difference between the use of PMA or not for the no, bleach, or long-heat treatment methods. However, when ova samples were treated with a higher temperature for shorter duration, PMA-qPCR resulted in lower ITS-1 copy concentrations than that of normal qPCR. While PMA-qPCR was more accurate than regular qPCR for short heat ova, it was it did not accurately amplify DNA from only inactivated ova, as there were no viable of in the sample after heat treatment.

### 5.2.3 Future Direction

The use of qPCR is a promising approach that could be used to determine the number of viable ova in a biosolids sample. Further research is needed to improve the correlation, as well as increase the number of samples run. Different matrices such as feces, wastewater, differently treated biosolids need to be tested to determine if further correlation could be found. Different extraction methods such as freeze-thaw, different bead-beading/mixing, as well as different extraction kits should be tested to determine which one can get the best recover of DNA ova from different matrices. The number of ITS-1 copies/ ova that we

determined was based on the DNA extraction method we used. More studies could determine a more accurate number of ITS-1 copies/ ova that could be used to accurately predict the number of ova that would be quantified using the accepted microscopy method.

PMA-qPCR may be a promising approach to determine a more accurate number of ITS-1 copies from only viable ova in a sample. Further research is needed to test if concentrations between 100 and 200 μM can consistently reduce the number of ITS-1 copies amplified from inactivated ova. Other PMA protocols such as longer retention time, longer light exposure times, or other deviations may cause greater binding of PMA to inactivated DNA and should be looked into. Different treatment methods such as UV, different chemical treatments (pH, lime, etc), or different heat treatments should be tested to determine if PMA would be more effective for samples treated in different ways.

# A Copyright documentation

The image used in this thesis is from the Center for Disease Control webpage on *Ascaris lumbricoides*. CDC materials displayed on the website are public domain and are free of copyright restrictions. Please see below for additional citation details and attribution information.

Figure 1.1: "*Ascaris* lumbricoides Life Cycle" by DPDx at the US Center for Disease Control. Licensed under Public Domain - https://www.cdc.gov/parasites/ascariasis/biology.html Accessed August 2017.

# B Purification Kit Protocols

## B.1 Powersoil DNeasy Extraction Kit

1. To the **PowerBead Tubes** provided, add 0.25 grams of sample

2. Gently vortex to mix

3. **Check Solution C1**. If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.

4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.

5. Secure **PowerBead Tubes** horizontally with tape on flat-bed vortex pad. Vortex at a maximum speed for 10 minutes.

6. Make sure the **PowerBead Tubes** rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x g for 30 sec at room temperature. **CAUTION**: Be sure not to exceed 10,000 x g or tubes may break

7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).

8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.

9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g

10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).

11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.

12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.

13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided)

14. Shake to mix **Solution C4** before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.

15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant into the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room

16. temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature.

17. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x g.

18. Discard the flow through.

19. Centrifuge again at room temperature for 1 minute at 10,000 x g.

20. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**

21. Add 100 µl of **Solution C6** to the center of the white filter membrane.

22. Centrifuge at room temperature for 30 seconds at 10,000 x g.

23. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application.

24. Storage at -20°C to -80°C

## B.2 Test Method for Detecting, Enumerating, and Determining the Viability of *Ascaris* Ova in Sludge

### 1.0 Procedure

**1.1** The percentage moisture of the sample is determined by analyzing a separate portion of the sample, so the final calculation of ova per gram dry weight can be determined. The concentration of ova in liquid sludge samples may be expressed as ova per unit volume.

**1.2** Initial preparation:

**1.2.1** Dry or thick samples: Weigh about 300 g (estimated dry weight) and place in about 500 ml water in a beaker and let soak overnight at 4 - 10EC. Transfer to blender and blend at high for one minute. Divide sample into four beakers.

**1.2.2** Liquid samples: Measure 1,000 ml or more (estimated to contain at least 50 g dry solids) of liquid sample. Place one half of sample in blender. Add about 200 mL water. Blend at high speed for one minute transfer to a beaker. Repeat for other half of sample.

**1.3** Pour the homogenized sample into a 1000 mL tall form beaker and using a wash bottle, thoroughly rinse blender container into beaker. Add 1% 7X to reach 900 ml final volume.

**12.4** Allow sample to settle four hours or overnight at 4 - 10EC. Stir occasionally with a wooden applicator, as needed to ensure that material floating on the surface settles. Additional 1% 7X may be added, and the mixture stirred if necessary.

**1.5** After settling, vacuum aspirate supernatant to just above the layer of solids. Transfer sediment to blender and add water to 500 ml, blend again for one minute at high speed.

**1.6** Transfer to beaker, rinsing blender and add 1% 7X to reach 900 ml. Allow to settle for two hours at 4 - 10EC, vacuum aspirate supernatant to just above the layer of solids. 168

**1.7** Add 300 ml 1% 7X and stir for five minutes
on a magnetic stirrer.

**1.8** Strain homogenized sample through a 20 or 50 mesh sieve placed in a funnel over a tall beaker. Wash sample through sieve with a spray of 1% 7X from a spray bottle.

**1.9** Add 1% 7X to 900 mL final volume and allow to settle for two hours at 4 - 10EC.

**1.10** Vacuum aspirate supernatant to just above layer of solids. Mix sediment and distribute equally to 50 mL graduated conical centrifuge tubes. Thoroughly wash any sediment from beaker into tubes using water from a wash bottle. Bring volume in tubes up to 50 ml with water.

**1.11** Centrifuge for 10 minutes at 1000 X G. Vacuum aspirate supernatant from each tube down to just above the level of sediment. (The packed sediment in each tube should not exceed 5 mL. If it exceeds this volume, add water and

distribute the sediment evenly among additional tubes, repeat centrifugation, and vacuum aspirate supernatant.)

**1.12** Add 10 to 15 mL of MgSO4 solution (specific gravity 1.20) to each tube and mix for 15 to 20 seconds on a vortex mixer. (Use capped tubes to avoid splashing of mixture from the tube.)

**1.13** Add additional MgSO4 solution (specific gravity 1.20) to each tube to bring volume to 50 mL. Centrifuge for five to ten minutes at 800 to 1000 X g. DO NOT USE BRAKE.

**1.14** Allow the centrifuge to coast to a stop without the brake. Pour the top 25 to 35 mL of supernatant from each tube through a 400 mesh sieve supported in a funnel over a tall beaker.

**1.15** Using a water spray bottle, wash excessive flotation fluid and fine particles through sieve.

**1.16** Rinse sediment collected on the sieve into a 100 mL beaker by directing the stream of water from the wash bottle onto the upper surface of the sieve.

**1.17** After thoroughly washing the sediment from the sieve, transfer the suspension to the required number of 15 mL centrifuge tubes, taking care to rinse the beaker into the tubes. Usually one beaker makes one tube.

**1.18** Centrifuge the tubes for three minutes at 800 X G, then discard the supernatant.

**1.19** If more than one tube has been used for the sample, transfer the sediment to a single tube, fill with water, and repeat centrifugation.

**1.20** Aspirate the supernatant above the solids.

**1.21** Resuspend the solids in 4 mL 0.1 N H2SO4 and pour into a 20-mL polyethylene scintillation vial or equivalent with loose caps.

**1.22** Before incubating the vials, mark the liquid level in each vial with a felt tip pen. Incubate the vials, along with control vials containing *Ascaris* ova mixed with 4 mL 0.1 N H2SO4, at 26EC for three to four weeks. Every day or so, check the liquid level in each vial. Add reagent grade water up to the initial liquid level line as needed to compensate for evaporation. After 18 days, suspend, by inversion and sample small aliquots of the control cultures once every 2 - 3 days. When the majority of the control *Ascaris* ova are fully embryonated, samples are ready to be examined.

**1.23** Examine the concentrates microscopically using a Sedgwick-Rafter cell to enumerate the detected ova. Classify the ova as either unembryonated, embryonated to the first, second, or third larval stage. In some embryonated *Ascaris* ova the larva may be observed
to move. See Figure 1 for examples of various *Ascaris* egg categories.

**2.0 Calculation**

**2.1** Calculate % total solids using the % moisture
result:
% Total solids = 100% - % moisture
**13.2** Calculate catagories of ova/g dry weight in
the following manner:
Ova/g dry wt = (NO) x (CV) x (FV)

(SP) x (TS)

## B.3 Zymo Plasmid Miniprep

Perform the following procedure at room temperature.

1. Transfer 600lowing procedure at roomgrown in LB medium to a 1.5ml microcentrifuge tube.

**Note:** If you wish to process larger volumes of bacterial culture (up to 3.0ml), use the protocol provided in

Section 4.C.

2. Add 100C.to process larger voer, and mix by inverting the tube 6 times.

The solution should change from opaque to clear blue, indicating complete lysis.

**Note: Proceed to Step 3 within 2 minutes.** Excessive lysis can result in denatured plasmid DNA. If

processing a large number of samples, process samples in groups of ten or less. Continue with the next set of

ten samples after the fi rst set has been neutralized and mixed thoroughly.

3. Add 350een neutralized and mixed thoroughly.ly.zed and mixed thoroughly.ess. Continue with t

The sample will turn yellow when neutralization is complete, and a yellow precipitate will form. Invert the

sample an additional 3 times to ensure complete neutralization.

4. Centrifuge at maximum speed in a microcentrifuge for 3 minutes.

5. Transfer the supernatant (~900 a microcPureYield supernatant.

Do not disturb the cell debris pellet. For maximum yield, transfer the supernatant with a pipette.

6. Place the minicolumn into a PureYieldureYield PureYiel PureYieldo a PureYieldor maximum yield, transfer the

for 15 seconds.

7. Discard the fl owthrough, and place the minicolumn into the same PureYield same Pur same Pur

8. Add 200same PureYield PureYieldlace dor maxminicolumn. Centrifuge at maximum speed in a microcentrifuge

for 15 seconds. It is not necessary to empty the PureYieldconds. It is n. It

9. Add 400onds. Column Wash Solution to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for

30 seconds.

10. Transfer the minicolumn to a clean 1.5ml microcentrifuge tube, then add 30hen add 30tube, theer directly to the

minicolumn matrix. Let stand for 1 minute at room temperature.

**Notes:**

1. Nuclease-free water at neutral pH can also be used to elute DNA.

2. For large plasmids (>10kb), warm the Elution Buff er to 50oC prior to elution, and increase elution volume

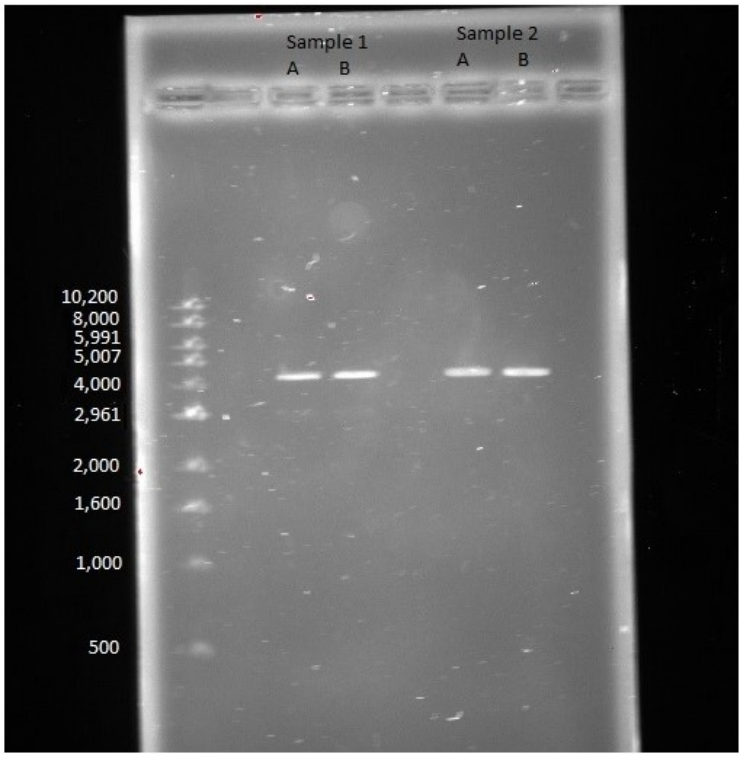
to 500oC prior to elution, and increase eltemperature (22o 50oC prior to elution, and increase elutio

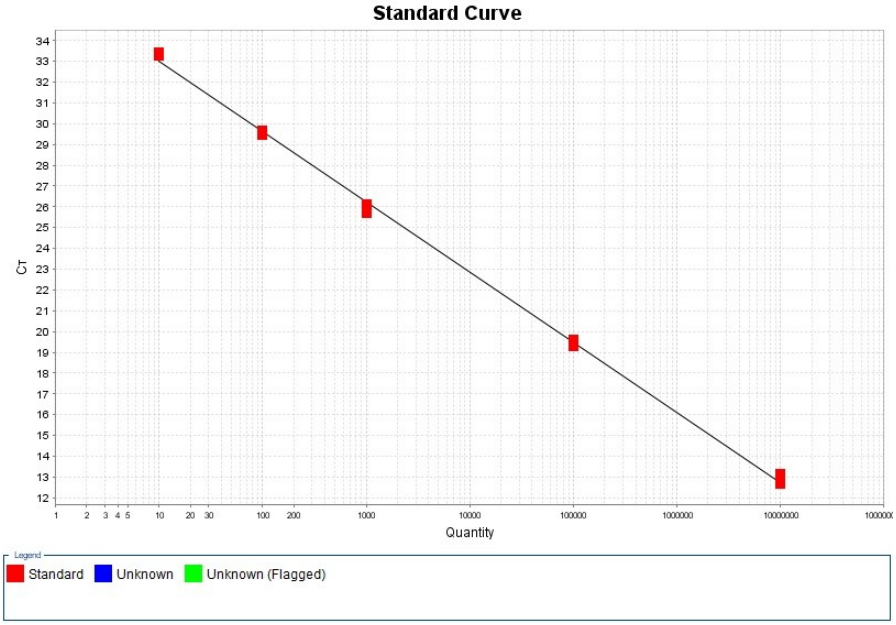
Step 11.

11. Centrifuge at maximum speed in a microcentrifuge for 15 seconds to elute the plasmid DNA. Cap the

microcentrifuge tube, and store eluted plasmid DNA at or 15

# C Plasmid Standard Preparation



# D PMA Concentration Optimization

| PMA Concentration (μM) | Geometric mean ± 95% confidence interval of ITS-1 copy number in samples treated with PMA |
|---|---|
| 0 | $1.5 \times 10^5 \pm 5.7 \times 10^4$ |
| 25 | $1.5 \times 10^4 \pm 1.8 \times 10^4$ |
| 50 | $1.0 \times 10^5 \pm 4.1 \times 10^4$ |
| 100 | $1.5 \times 10^5 \pm 2.8 \times 10^4$ |
| 200 | $1.8 \times 10^4 \pm 1.4 \times 10^4$ |

Table D-1. ITS-1 sequence copies in 100 μL of eluent derived from ova treated with varying PMA concentrations.