Destruction of parasites in biosolids from wastewater treatment lagoons

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (PhD)

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Basma Ashour Khallaf

17/09/2018

Primary Supervisor: Professor Andy Ball

Secondary Supervisor: Dr. Esmaeil Shahsavari

Tertiary Supervisor: Adj. Professor Margaret Deighton
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Preface

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Chapter 3: 95%

Chapter 4: 96%

Chapter 5: 92%

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Data and techniques described in Chapter 3 & 4 have contributed to the following reports:

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The following publication is not described in this thesis:

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<tbody>
<tr>
<td>μm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>A</td>
<td>Ampere</td>
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<tr>
<td>AGRF</td>
<td>Australian genome research facility</td>
</tr>
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<td>ALS</td>
<td>Australian laboratory services</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>APS</td>
<td>Ammonium persulfate solution</td>
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<td>BH</td>
<td>Bushnell Hass mineral salts medium</td>
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<td>BOD</td>
<td>Biochemical oxygen demand</td>
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<td>ºC</td>
<td>Degrees Celsius</td>
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<td>Chemical grade</td>
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<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<td>CLSM</td>
<td>Confocal laser-scanning microscope</td>
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<td>CO₂</td>
<td>Carbon dioxide</td>
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<td>DAPI</td>
<td>4’,6’-diamidino-2-phenyl-indole</td>
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<td>Dd H₂O</td>
<td>Sterile ultra-pure water</td>
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<td>Pareto-Lorenz</td>
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<td>Waste stabilisation lagoons</td>
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<tr>
<td>WW</td>
<td>Wet weight</td>
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<tr>
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Abstract

Wastewater from domestic and industrial activities contains harmful compounds and pathogens which constitute significant health risks if released into the environment without adequate treatment. As well as the treated and discharged liquid sewage, wastewater treatment also results in the generation of biosolids which after post-treatment measures can be used as soil fertilizers, as road base or sent to landfills. However, biosolids, especially those from developing countries, may contain substantial levels of *Ascaris* spp. (helminths) eggs which survive most post-treatment measures. These can cause human infections when these biosolids are used in agriculture. Helminths such as *Ascaris* spp. infect about 1.2 billion people in the developing world causing morbidity and mortality and are of public health concern. Therefore, the aims of this study were to determine the prevalence of *Ascaris* spp. eggs in biosolids from waste treatment plants in Victoria and their decay rates following pan drying and stockpiling of biosolids. The microbial communities in biosolids were characterized using PCR-DGGE and metagenomics-based approaches, and their roles in the decay of *Ascaris* spp. were evaluated with MT2 Biolog-plate based assays.

Investigations of the prevalence of *Ascaris* spp. eggs were carried out on samples obtained from three wastewater treatment plants (WWTP) in Victoria (Heyfield, Rochester and Cobden WWTP). No eggs of *Ascaris* spp. were detected in these samples evaluated with the Tulane method indicating that helminths were either not present or that the Tulane method was not sensitive enough to detect them. Thereafter, biosolid samples were spiked with *Ascaris suum* eggs to assess the suitability of the Tulane method for egg detection. *Ascaris suum* eggs were detected (recovered) from spiked samples; the recovery efficiencies ranged from 64% in Cobden to 70% in Heyfield biosolid samples. The efficiency of egg recovery was inversely correlated with the concentration of spiked eggs. The Tulane method was found to be suitable
for egg recovery from biosolids confirming that helminths was largely absent in the wastewater samples examined.

The next experimental investigation was to determine the decay rates of *Ascaris suum* eggs in spiked biosolid samples (Cobden and Rochester) subject to pan-handling and stockpiling at a specific temperature (20 °C) in laboratory-based assays over 17 weeks. While the Tulane method was suitable for egg recovery, its use for determining the viability of eggs can be time-consuming. Therefore, a Live/Dead Baclight staining procedure was used for determining the viability of recovered eggs in this study. Results showed that the viability of *Ascaris suum* eggs was reduced by 22% in Cobden samples and 31% in Rochester samples. Consequently, if the die-off (decay rates) were constant, storing Cobden biosolids for 13 months and Rochester biosolids for 18 months would lead to complete elimination of viable *Ascaris suum* eggs and render the biosolid samples safe for use in agriculture-related applications. The Live/Dead Baclight staining procedure was also successfully used to discriminate between viable and non-viable eggs, making it an ideal additional technique to accompany the Tulane’s method.

Most research activities on the microbial composition of biosolids have assessed the prevalence of pathogens for public health safety. However, microorganisms naturally present in biosolids produce an array of enzymes, some of which may play important roles in the decay of *Ascaris suum* eggs alongside other factors. Therefore, two culture-independent approaches (PCR-DGGE and metagenomics) were used to characterize the microbial communities in selected biosolid samples.

Cluster analysis of the DGGE profiles of Cobden, Rochester and Heyfield samples indicated substantial differences (70-80%) between their microbial communities. Bacterial diversity (PCR-DGGE) assessed with Shannon diversity ($H'$) was highest in Rochester samples (3.5), followed by Cobden (2.3) and Heyfield (1.8) samples. The microbial community from these
samples was used to inoculate MT2 Biolog plates containing either chitin, lipid or protein substrates and incubated for 168 hours. Both Cobden and Rochester samples showed substantial utilization of protein substrates; Cobden samples also substantially degraded chitin and all the three samples showed some degree of lipid utilization. Key microbial groups from the bacterial community in the Biolog plates were identified and some putatively assigned to Flavobacteria, Cytophaga, Alpha-, Beta- and Gamma-proteobacteria groups. These groups produce enzymes such as lipases, proteinases and chitinases which can degrade the outer coating of *Ascaris suum* eggs rendering them non-viable.

Metagenomic analyses were used to evaluate the bacterial communities in the biosolid samples. The data indicated substantial differences in the biosolid communities. About 70% of the bacterial population belonged to the Gammaproteobacteria in Heyfield samples compared to ~25% in Rochester and ~12% in Cobden. In Rochester, both Gamma- and Beta-proteobacteria were dominant and Cytophagia, Betaproteobacteria and Epsilon bacteria were the key groups in the Cobden samples.

The final investigation involved the use of isolates biosolid samples to degrade the eggs of *Ascaris suum*. Pure bacterial isolates obtained from Heyfield, Rochester and Cobden samples, grown on chitin, lipid or protein substrates were screened based on growth and protein production characteristics in nutrient broth. Three isolates showing relatively high levels of activity, identified as *Pedobacter* sp., *Acidovorax* sp. and *Brevundimonas* sp were applied (as either cell-free, pellet-based, or uncentrifuged culture broth inocula) to *Ascaris* eggs to assess their effects on the rate of decay of *Ascaris* eggs compared to commercial enzymes. Individually, the egg decay efficiencies between the three isolates and the commercial enzymes were similar. However, when used as a mixture of the three isolates (uncentrifuged culture broth), there was a higher decay of *Ascaris* eggs (~23%) compared to the commercial enzyme
mixture (~19%) and individual isolates and cell-free samples (up to 19%). This indicated that microbial synergy was important in the decay of eggs in biosolids.

This study has successfully shown that the Tulane method was suitable for *Ascaris* eggs recovery while the Live/Dead Bclight staining procedure was excellent at discriminating between viable and non-viable eggs. Bacterial community composition was dependent on the biosolid source and specific bacterial species such as *Pedobacter* sp., *Acidovorax* sp. and *Brevundimonas* sp. working in synergy do play a role in the decay of *Ascaris* eggs in controlled conditions. This opens the possibility of a microbial (biological) approach to the decay of helminths eggs, used independently or as adjuncts to existing biosolid treatment processes. Future investigations should evaluate the impact of physicochemical and environmental factors on the synergistic decay of eggs in biosolid samples.
CHAPTER 1:

Literature Review
1.1 INTRODUCTION

Wastewater consists of water generated from household activities such as washing, cleaning, and use of showers as well as industrial activities. Wastewater generated by domestic and industrial activities can be toxic and constitute significant health risks as it may contain harmful compounds and pathogens. The increased volume of generated wastewater associated with increasing world population and urbanization exacerbates public health risks of wastewater (Lazarova and Bahri, 2005, Sato et al., 2013). Consequently, it is important that wastewater is properly treated before being discharged into the environment or re-used to reduce these public health risks (the spread of pathogens and incidence of infections) (Soller et al., 2003).

In most countries such as Australia, New Zealand and Singapore, wastewater is usually transferred via a network of pipes or sewer system to a centralised wastewater treatment plant for treatment purposes. There are currently a variety of methods for wastewater treatment such as those based on electrochemical technologies, use of adsorbent materials and aerobic and anaerobic biological resources (Chen, 2004, Burakov et al., 2018, Hargreaves et al., 2018). Wastewater treatment methods can be physical, chemical or biological in nature and many wastewater or sewage treatment procedures incorporate all these methods. Importantly, the source and component of the wastewater is a key determinant of the type of treatment applied.

A detailed review of the some of the various wastewater treatment methods has been carried out (ESCWA, 2010) with biological treatment approaches preferred for domestic or municipal wastewater treatment. Therefore, the focus of this introduction will be on the application of biological approaches for the treatment of municipal wastewater.

One widely used biological treatment approach in Australia is the use of lagoon-based wastewater treatment plants. Municipal and industrial wastewaters are treated by passing these wastes through a preliminary screening process, followed by primary treatment resulting in sedimentation of materials. From here, the effluent goes into aeration tanks (biosolids are
removed and independently treated) and then into a secondary sedimentation tank after which the water is treated with chlorination/filtration/UV prior to being re-used. However, lagoon-based wastewater treatment involves the use of a series of lagoons (anaerobic and or aerobic in nature) which utilise natural processes (sunlight, air and microbial activities) for wastewater treatment. Lagoon-based wastewater treatment systems are widely used because they are easier to construct and operate. However, they require greater space and footprint for operations. Lagoon-based treatment systems can cause significant reductions in contaminant levels, primarily because they include both physical and biological processes such as the settlement of solids, toxicity reducing biochemical reactions and bio-physical elimination of pathogens. However, lagoon-based systems require proper management to avoid or reduce odour generation and surface and groundwater contamination (Figure 1.1) (Bonomo and Patorelli, 1997, SWF, 2013a, Wilas et al., 2016).

The type of lagoon or treatment lagoons used is determined by the specific wastewater treatment application desired which in turn determines the system’s operating parameters. The different types of treatment lagoons or lagoons available include anaerobic lagoons, facultative lagoons, aerobic lagoons (wastes that settle at the base of the lagoon undergo anaerobic treatment) and maturation and oxidation lagoons. Detailed descriptions of these different lagoon types have been carried out (SWF, 2013a). While lagoon-based water treatment methods in wastewater treatment plants require significant footprints in terms of set-up and operational phases, the approach is considered as a sustainable approach to wastewater treatment. The potential advantages of the lagoon or lagoon-based systems include lower power consumption, natural oxygen supply to the systems, extensive pathogen removal and the potential to re-use the resulting biosolids as fertilizers.
A floating grid of duckweed is used at Harrington Sewage Treatment Works, New South Wales (Willet, 2005).

1.2 Wastewater Treatment

Wastewater generally undergoes four key processes or treatment phases before being discharged or re-used. These are termed preliminary, primary, secondary and tertiary treatment phases (Mittal 2004; SA Water 2010). The biosolid generated from the primary and secondary treatment processes is subject to additional treatment to produce biosolids which are used for multiple applications (Figure 1.2). Wastewater and the generated biosolids (biosolid) are subject to different treatments for public and environmental health reasons such as to reduce or eliminate pathogens and odour and limit the spread of enteric diseases (Australian Biosolids Partnership 2009).
1.2.1 Preliminary treatment

This is the first or initial treatment phase and is largely designed to remove large components of the waste materials. It usually involves the use of screens to remove large materials in the wastewater. These could be sticks, plastics, paper, rags and other types of large materials. This step is critical to protecting the equipment used in subsequent treatment phases as these materials, if not removed can clog and damage this equipment. Detritus and other heavy materials such as pebbles, rocks and soil settle at the bottom of the tank and are later removed from the tank (Mittal, 2006, Water, 2004) (Fig. 1.2).
Figure 1.2: Schematic representation of treatment processes in a wastewater plant (reproduced from ANZBP, 2018).
1.2.2 Primary treatment
Two processes occur in this treatment phase which is usually carried out in a tank called the primary sedimentation tank. Firstly, light, floating materials largely composed of grease and oil are removed. Secondly, any remaining heavy material settles to the bottom of the tank and are removed. The partly clarified wastewater with colloidal materials and biosolid debris are pumped to the next treatment facility for dewatering purposes (Mittal, 2006, Water, 2018, Water, 2004, Cheremisinoff, 2019)

1.2.3 Secondary treatment
Secondary or biological treatment leads to the removal of bacterial pathogens and organic compounds. Human waste and organic materials (biological pollutants) are degraded via microbially mediated reactions at this stage. Two types of secondary treatment processes occur; attached- and suspended- growth processes. With respect to attached growth processes (fixed film), micro-organisms are attached to multiple fixed media, usually plastic or ceramic in nature and the wastewater to be treated is allowed to flow over these microbial beds at a regular flow-rate. In contrast, in suspended growth processes, microbial groups are free floating and mixed with wastewater or sewage. In addition, secondary wastewater can be achieved using using reactors such as membrane bioreactors, biofilm beds and constructed wetlands After secondary treatment, the treated wastewater is pumped to another treatment facility (Mittal, 2006, Water, 2018, Krzeminski et al., 2019, Russell, 2019).

1.2.4 Tertiary treatment
Tertiary treatment is carried out by many treatment plants to improve the quality of the treated wastewater prior to its discharge or re-use for desired or prescribed activities. This involves the
use of mechanical and sand filters or microalgal biofilm for the removal of suspended organic matter. Sometimes, a disinfection step involving the application of ozone, UV radiation and chlorine is incorporated to eliminate microbial pathogens and other harmful organisms. The solid part of the treated waste (usually referred to biosolid or biosolids) can be subject to additional treatments (anaerobic digestion, pan-drying and stockpiling) before being re-used (Mittal 2004, SA Water 2010, Sukacova et al., 2015, Thompson et al., 2016).

1.3 Biosolid treatment systems

The different biosolid treatment systems are briefly described in this section and the advantages and disadvantages of these systems are discussed.

1.3.1 Anaerobic digestion

The resulting biosolid from the wastewater treatment can be treated by anaerobic digestion. Generally, anaerobic digesters are operated at 30–40°C or 50–60°C (mesophilic or thermophilic conditions respectively) for up to two weeks (Gavala et al., 2003). During this time-frame, anaerobic bacteria degrade or convert the organic component of the biosolid into multiple secondary and tertiary compounds and finally into water and organic acid. These processes occur below the surface of the biosolid (in the absence of oxygen) mediated by facultative and obligate anaerobic microorganisms. One common by-product of this process is the production of biogas (70% methane and 30% carbon dioxide) which can potentially be harnessed and used a source of energy (EPA Victoria, 2004, Cheremisinoff, 2001). The remaining pathogens in the biosolid are also inhibited or eliminated. The main factors thought to play important roles in pathogen inactivation during this process are the biosolid pH, temperature, antagonistic
microbial activities, the residence time of the biosolid and the design of the anaerobic reactor (Smith et al., 2005).

1.3.2 Drying pans

Following anaerobic digestion, the biosolid is air dried by pumping the biosolid into open lagoons (Figure 1.3). There are strict regulations guiding this process. For example, in Victoria, Australia, biosolid material must be retained in drying pans for close to a year (ten months). After drying, the biosolid must be stockpiled for an additional thirty-six months before it can be re-used (EPA Victoria, 2004). Before removal from the drying pans, between 10-30 % dry solids content of the biosolid or biosolid must be attained (Wang et al. 2007) (Fig. 1.2).

Figure 1.3: Process flowsheet of a treatment plant in Victoria. The example used is the Eastern Treatment Plant (reproduced from Melbourne Water, 2010).
1.4 Biosolids

Biosolids can be used as soil conditioners; in composting, as fertilizers in agriculture, as road base, for landscaping purposes, as topsoil, in landfilling, in phytocapping and potentially for power production (https://www.biosolids.com.au/info/what-are-biosolids) (Pritchard et al., 2010, Lamb et al., 2012). However, a significant proportion of the biosolids produced are used in agriculture for soil conditioning or as fertilizers in most countries including Australia (Martin and Kelso, 2009, Goh et al., 2018, McCabe et al., 2019). The agricultural sector is therefore a major end user of biosolids in Australia for soil fertilization and composting. Biosolids are usually stockpiled prior to being used. However as outlined earlier, the stockpiling of biosolids is challenging because it requires a large dedicated space and the costs of managing the process can be exorbitant (http://www.environment.gov.au/resource/biosolids-snapshot). The amount of biosolids produced in Australia vary in the different states and territory, ranging from 1,095 to 93,466 tonnes in 2013 (Figure 1.4). Biosolid stockpiles are usually maintained under anaerobic conditions and this process can generate biogas (methane), contributing to global warming.
1.4.1 Characteristics of biosolids

The physical characteristics of biosolids include their solid content, bulk characteristics, size of biosolid particles and moisture content while the chemical characteristics refer to the pH, organic matter content, metal concentrations, essential and non-essential elements content, micro- and macro-nutrient concentrations. The type and composition of wastewater, chemical treatment method applied (e.g. ferric chloride, polymers, etc.), stabilisation method utilized and the types of treatment (primary, secondary, tertiary), determine the chemical characteristics of the biosolids (Epstein, 2002).

The chemical properties of the biosolids are important in determining any beneficial effects on plant growth because they can influence the recipient soil’s chemical, biological and physical...
characteristics. Most biosolids (dry or semi-solid forms) are applied to topsoil before being mechanically ploughed into or mixed with soil, thereby introducing the organic and inorganic contents of the biosolids into the soil. The use of biosolids can increase the organic matter content of the receiving soil, although the extent of this increase is dependent on the application rate of the biosolids. Increases in the organic matter content of a soil are beneficial to the structure of the soil, soil water (moisture) content and cation exchange capacity (Epstein, 2002). However, these beneficial effects in soil structure require repeated biosolid applications.

Biosolids harbour a diverse microbial community derived largely from the wastewater itself and its various treatment phases. Some members of these communities can be harmful to humans, causing bacterial and viral infections such as gastroenteritis (enteric bacteria) and norovirus-related vomiting (Wei et al., 2010, Al-Gheethi et al., 2018). These microbial groups and their activities determine some of the characteristics of biosolids that are biological in nature. These characteristics cause changes in the microbial diversity and activities of the recipient soil and its environment, potentially affecting human health (Epstein, 2002).

1.4.2 Classification of biosolids

Biosolids are classified based on the type of contaminants they contain, the treatment they have been subject to and their microbial quality post-treatment. When classified based on contaminant type, they can be contaminant grade C1 or C2 and if by treatment, they can be treatment grade T1, T2 or T3. The type of microbiological treatment method used in wastewater treatment is also considered during classification in some instances. These microbiological methods function primarily to reduce or eliminate odour and reduce pathogen (microbial) growth (EPA Victoria, 2004). Table 1.1 presents a summary of the different types of classification of biosolids used in Victoria, Melbourne (Appendix A).
Table 1.1: Biosolids classification based on treatment and chemical grade and permissible end use.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Treatment</th>
<th>Chemical</th>
<th>Unrestricted Use</th>
<th>Restricted Agricultural Uses</th>
<th>Restricted Non-Agricultural Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>C1</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T2</td>
<td>C1</td>
<td></td>
<td>-</td>
<td>+^A</td>
<td>+</td>
</tr>
<tr>
<td>T3</td>
<td>C1</td>
<td></td>
<td>-</td>
<td>+^B</td>
<td>+^C</td>
</tr>
<tr>
<td>T1</td>
<td>C2</td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T2</td>
<td>C2</td>
<td></td>
<td>-</td>
<td>+^D</td>
<td>+</td>
</tr>
<tr>
<td>T3</td>
<td>C2</td>
<td></td>
<td>-</td>
<td>+^E</td>
<td>+^F</td>
</tr>
</tbody>
</table>

Note: Table modified from Victoria EPA (2004). + means biosolid with this grade can be used, +^A-C means biosolid with this grade can be used with some restrictions and – means biosolid with this grade cannot be used for prescribed activities.

Restricted agricultural uses: Biosolids used in direct or indirect contact with human food crops consumed raw, dairy and cattle grazing fodder, sheep grazing and fodder, food crops and woodlots.

Restricted non-agricultural uses: Landscaping (unrestricted public access) and landscaping (restricted public access), forestry, land rehabilitation.

+^A, +^B, +^D, +^F: Biosolid cannot be used in growing human food crop consumed raw if it allows for direct contact of biosolid with these crops.

+^B, +^E: Biosolid cannot be used in where dairy and cattle graze including poultry and in growing human food crops consumed raw

+^C, +^F: Biosolid cannot be used for landscaping (unrestricted public access)

T1-T3 and C1-C2 are biosolids generated from either conventional or lagoon-based wastewater treatment. T1-T3 classification based on treatment methods, microbiological quality, microbial inhibitors used, odour and vector attraction. T1 is the highest quality grade followed by T2 while T3 is the lowest grade. C1 and C2 are classified based on contaminant content/grade. C1 is of low contaminant content and of the highest grade. C2 is of lesser grade.
1.5 Lagoon treatment lagoons

Lagoons are a simple and cheap method of treating domestic, industrial and agricultural wastes (from piggeries, tanneries and abattoirs) leading to the generation of biosolids. Given the importance of lagoons in wastewater treatments and the fact that they influence the physical, chemical and biological properties of biosolids, an extensive review of lagoon treatment lagoons (types and mode of action) is carried out in subsequent paragraphs. “In simple terms, sewage lagoons are impoundments into which wastewater flows in and out after a defined retention period. Treatment relies solely on the natural processes of biological purification that would occur in any natural water body. No external energy, other than that derived from sunlight, is required for their operation. Waste treatment is optimised by using the most appropriate organic loadings, the best retention periods and lagoon depths to enhance the growth and activities of organisms involved in waste degradation (Mara et al., 1992, Dowson et al., 1996, Muga and Mihelcic, 2008).

Some of the advantages of lagoon treatment lagoons are related to their simplicity and low costs of construction. Lagoon lagoons have little or no machinery requirements to function and are therefore low maintenance sewage treatment systems. When properly operated, the final effluent is usually of high microbiological quality (although additional disinfection may be needed especially if effluent is to be used as recycled water in residential places). In addition, lagoon lagoons reduce the bioavailability of nutrients and organic compounds (reduced impact on receiving water), tolerate fluctuating hydraulic loads and cope with stormwater without any excessive loss of biomass. They are also efficient at treating many industrial wastewaters (removal of toxicants and heavy metals) (Mara et al., 1992). However, lagoon operations require more land area and land with a flat topography. In addition, the effluent from the lagoon lagoons may have higher soluble/dissolved solids and biochemical oxygen demand (BOD) and odour especially when overloaded, when compared to other treatment methods (e.g. trickling
filters or activated biosolid). Environmental factors associated with seasons and climatic changes can affect the efficiency of lagoon lagoons leading to lagoon lagoon operators having only partial control over the wastewater treatment process and efficiency (Mara et al., 1992).

1.5.1 Lagoon classification

Sewage treatment lagoons can be classified based on the type of lagoon, biological activities in the lagoon and whether aeration is supplied or not. The different types of lagoons are shown in Table 1.2.

Table 1.2: The classification of sewage lagoons.

<table>
<thead>
<tr>
<th>Lagoon Type</th>
<th>Biological Activity</th>
<th>Typical Depth (m)</th>
<th>Aeration Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobic</td>
<td>Does not require oxygen (anaerobic)</td>
<td>&gt;4</td>
<td>None</td>
</tr>
<tr>
<td>Aerated</td>
<td>Requires oxygen (aerobic)</td>
<td>3-4</td>
<td>Mechanical</td>
</tr>
<tr>
<td>Facultative (stabilisation)</td>
<td>Aerobic/anaerobic</td>
<td>1.2-2.5</td>
<td>Natural</td>
</tr>
<tr>
<td>Aerobic (oxidation and maturation)</td>
<td>Requires oxygen (aerobic)</td>
<td>0.9-1.5</td>
<td>Natural</td>
</tr>
</tbody>
</table>

Note: Table modified from Dowson et al. (1996).

A key differentiation is the oxygenation conditions in the lagoon as this affects the type and activities of bacteria in the lagoons. The strength of organic loadings determines the level of dissolved oxygen present in the system. For example, when wastewater with high organic content are pumped into the lagoons, anaerobic (or oxygen-deprived) conditions develop in sections of the lagoons where more oxygen molecules are consumed (greater microbial
activities), and less aeration is occurring. However, when wastewater with low organic loading is received, there are less microbial activities and aerobic conditions prevail in most parts of the lagoon system. Apart from organic loading, other physical and chemical factors may determine the level of dissolved oxygen present in the lagoon. Table 1.2 shows the general concept of lagoon design and classification based on the presence of oxygen in the lagoon system (Dowson et al., 1996).

Lagoons can also be classified based on treatment. The lagoon to which raw sewage is pumped into is called the primary lagoon; there can be more than one primary lagoon. Sewage from this lagoon is pumped into another lagoon called the secondary lagoon and this may be alone or in a series interlinked with connecting pumps. Secondary lagoons are also called maturation or polishing lagoon lagoons (Dowson et al., 1996).

The use of the various terms for lagoon classification is due to the different foci by lagoon operators. For example, when biochemical reactions are of interest, the terms that usually used are aerobic, maturation and facultative lagoons. When the interest is on the engineering nature of lagoon systems, the terms primary and secondary lagoons are used. However, these terms can be used interchangeably based on operators’ preferences or activities at that time.

1.5.1.1 Anaerobic lagoons

The processes that occur in these lagoons lagoon are anaerobic in nature resulting in the anaerobic digestion of components of the wastewater. The most important factor is anaerobiosis (lack of oxygen). Strong organic wastes are treated in anaerobic lagoons, especially those from manufacturing industries. They can also be used for treating wastewater of domestic origins. After initial treatment in the aerobic lagoon, these wastes are transferred to anaerobic lagoons for further biological treatment. There are two important microbial groups
that are critical to the degradation processes known to occur in anaerobic lagoons (Dowson et al., 1996).

One of these are the acid-forming bacterial groups. The microorganism found in this group mediate the initial series of degradative biochemical reactions. These reactions result in the formation of low molecular weight compounds such as alcohols and acids, volatile fatty acids, butyric (C4), propionic (C3) and acetic (C2) acids from the breakdown of complex organic molecules in the wastewater or sewage. These compounds are typically lipids, proteins and carbohydrates. These acid-forming groups produce little or no methane gas but only carbon dioxide and hydrogen. In addition, ammonia (NH₃) and hydrogen sulphide (H₂S) or metal sulphides from some complex organic wastes which are proteaceous in nature may be formed. The end product of these different processes is an acidic and odoriferous effluent (Spellman & Drinan, 2014).

This effluent is an excellent substrate for the activities of the second group of effluent microorganisms which is the methane-producing microbial group. Members of these groups are strict anaerobes, slow growing and very sensitive to oxygen levels and their activities; methane production is critical to the efficiency of anaerobic lagoon systems. Therefore, most lagoon operators are focussed on optimizing the conditions needed for effective activities of the methanogens. With the right conditions, methanogens convert the organic acids produced by the acid-forming groups into gases, largely composed of methane and CO₂. The production/evolution of gases results in the mixing of lagoon contents (Spellman & Drinan, 2014).

There are many strengths and weaknesses of anaerobic lagoons. These include the generation of low amounts of biosolid, bioenergy production in terms of methane generation and ability to handle wastewater with high BOD. However, microbial activities are slower in these lagoons
and are effluents are odoriferous in nature and these lagoons are vulnerable to shock, pH changes and sudden load changes (Dowson et al., 1996, United States Environmental Protection Agency, 2002).

Strong industrial and agricultural wastes are typically treated in anaerobic lagoons. In cases where industrial effluents are treated through the municipal system, anaerobic lagoons are used to substantially reduce the organic loadings of the wastewater prior to being passed through the municipal waste treatment scheme

### 1.5.1.2 Facultative lagoons

Facultative lagoons or lagoons are also referred to as waste stabilisation lagoons (WSP). These lagoons have a hybrid environment. There is a deeper (lower in the lagoon), anaerobic zone where microbial degradation of wastes (especially high strength waste) occurs. The surface of these lagoons is aerobic and oxidation of products from the anaerobic zones occur here (including odoriferous wastes), transforming these products into CO₂ and water. The dual nature of these lagoons results in them having a highly diverse microbial flora as they combine the activities of anaerobic and aerobic microorganisms (Mara et al., 1992, Dowson et al., 1996).

As a result of the need to create a hybrid environment, facultative lagoons present more design difficulties than any other lagoon type. The advantages these systems include (i) their ability to handle medium organic loads, (ii) promotion of medium biomass formation, (iii) conversion of pollutants to gaseous products, (iv) remaining odourless (provided aerobic zones are maintained) and (v) ability to handle shock loads (Dowson et al., 1996). However, these systems require a large land footprint, are temperature sensitive, may generate large undesirable algal biomass and can struggle with the maintenance of aerobic zone and limited
1.5.1.3 Aerobic lagoons

Aerobic lagoons are lagoons that have a high level of dissolved oxygen and are designed to be naturally aerated. Consequently, most of the microbial groups (activated biosolid) in these lagoons are aerobic organisms. These microorganisms break wastes, oxidize nutrients and by their antagonistic activities reduce or eliminate pathogenic micro-organisms. They are also sometimes referred to as oxidation lagoons or maturation lagoon (Goad, 2011). These lagoons are shallow to promote penetration by light and disinfection.

The factor that is very important in the process of aerobic lagoons is maintaining dissolved oxygen at a level sufficient enough to render the system aerobic. Bacterial degradation of wastes is dissolved oxygen intensive and if oxygen levels are substantially reduced, aerobic bacterial activities are inhibited. This is avoided in naturally aerated aerobic lagoons by two processes or mechanisms; firstly, photosynthetic microalgal activities replenish oxygen and secondly, oxygen can also diffuse from the atmosphere into the lagoons largely through wind action. Aeration is promoted by the fact that aerobic lagoons are shallow (1 – 1.5 m), and rectangular in shape. They are often aligned in such a way that there is wind access all the time. Oxygen consumption by lagoon bacteria is related to lagoon volume while lagoon aeration is correlated with the surface area (Dowson et al., 1996, Goad, 2011).

The aerobic microbial population is diverse. Bacteria and multicellular organisms degrade complex compounds into simpler compounds and through competition and antagonism reduce the population of or eliminate viruses and pathogenic micro-organisms (a key function of maturation lagoons). Microalga produces oxygen via photosynthesis during the daytime, using oxygen supply capacity (Dowson et al., 1996, Filipe & Leslie Grady, 1998, Sperling, 2007, United States Environmental Protection Agency, 2002).
CO₂ for anabolism. At night, they respire (no photosynthesis), consuming oxygen. The strengths of aerobic lagoons include its low cost of construction and operation and ability to cause substantial pathogen/virus reduction in the wastewater. However, aerobic lagoons require substantial land area for operation, can generate unwanted algal biomass while only being able to handle wastewater with low organic content and are temperature sensitive. The determined organic loading amount for most aerobic lagoons is dependent on the potential degradative power of the autochthonous microflora (Dowson et al., 1996, Lue-Hing, 1998).

1.6 Pathogenic health risks associated with lagoons

Given that human faeces and other human wastes are part of the domestic wastewater, a substantial number of human pathogens are often found in biosolids. Unfortunately, concentration of wastes occur during waste treatment through evaporation and de-watering steps and this leads to increased concentration of these pathogens in biosolids (Wéry et al., 2008). Consequently, substantial populations of human pathogens (enteric bacteria, protozoa, viruses, helminths etc.) are found in biosolids (Sidhu and Toze, 2009).
Table 1.3: Different types of pathogens of concern in municipal wastewater and sewage biosolid.

<table>
<thead>
<tr>
<th>Pathogen of Concern</th>
<th>Diseases or symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
</tr>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>Salmonellosis, typhoid fever</td>
</tr>
<tr>
<td><em>Shigella</em> spp.</td>
<td>Bacillary dysentery</td>
</tr>
<tr>
<td><em>Yersinia</em> spp.</td>
<td>Acute gastroenteritis (diarrhea, cramps)</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>Cholera</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td><strong>Viruses</strong></td>
<td></td>
</tr>
<tr>
<td>Polio virus</td>
<td>Poliomyelitis</td>
</tr>
<tr>
<td>Hepatitis A and E viruses</td>
<td>Hepatitis</td>
</tr>
<tr>
<td>Adenoviruses, Reoviruses</td>
<td>Respiratory tract infections, gastroenteritis</td>
</tr>
<tr>
<td>Astroviruses</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>Rotaviruses</td>
<td>Acute gastroenteritis, severe diarrhea</td>
</tr>
<tr>
<td><strong>Protozoa</strong></td>
<td></td>
</tr>
<tr>
<td><em>Cryptosporidium</em> spp.</td>
<td>Gastroenteritis, cryptosporidiosis</td>
</tr>
<tr>
<td><em>Entamoeba histolytica</em></td>
<td>Acute enteritis</td>
</tr>
<tr>
<td><em>Giardia lamblia</em></td>
<td>Giardiasis (diarrhea and abdominal cramps)</td>
</tr>
<tr>
<td><em>Balantidium coli</em></td>
<td>Diarrhea, dysentery</td>
</tr>
<tr>
<td><em>Toxoplasma gondii</em></td>
<td>Toxoplasmosis</td>
</tr>
<tr>
<td><strong>Helminth worms</strong></td>
<td></td>
</tr>
<tr>
<td><em>Ascaris lumbricoides</em></td>
<td>Digestive disturbances, abdominal cramps</td>
</tr>
<tr>
<td><em>Ascaris suum</em></td>
<td>Digestive, abdominal pains, coughing</td>
</tr>
<tr>
<td><em>Trichuris trichiura</em></td>
<td>Abdominal pain, diarrhoea, anaemia</td>
</tr>
<tr>
<td><em>Toxocara canis</em></td>
<td>Fever, abdominal discomfort, muscle aches</td>
</tr>
</tbody>
</table>

Table modified from Gerba and Smith (2005).
Therefore, the usage of biosolids for land application can create risks of contaminating food materials and water obtained from farmlands, ground and surface water. Human exposure tends to occur when these pathogens are ingested through food or water from environments where biosolids have been applied (Sidhu and Toze, 2009). Biosolids are therefore treated to reduce contamination by pathogens and toxicants that can adversely affect humans and the environmental health (Sidhu and Toze, 2009).

1.6.1 Enteric pathogens

Viruses and bacteria usually die-off after three months in stockpiled biosolids. However, helminths in form of eggs and protozoan (oocysts) survive longer, up to a year or two based on the kind of post-treatment process applied to the biosolids (Sidhu and Toze, 2009).

1.6.1.1 Enteric viruses

Enteric viruses are important human pathogens responsible for gastroenteritis and other infections of the gastrointestinal tract (GIT). These infections can be mild or severe and can be fatal in some instances. Pathogen transmission largely occurs through ingestion of contaminated materials or food, via faecal-oral routes (Romdhana et al., 2009). Different types of viruses are found in biosolids with hepatitis A and E viruses, Adenovirus, Astrovirus, Caliciviruses, Rotavirus and Enteroviruses being of major public health concern (Sidhu and Toze, 2009, Wong et al., 2010, Prado et al., 2013).

In addition to GIT related infections, a broad range of human illnesses such as myocarditis and meningitis in humans are caused by enteric viruses (Romdhana et al., 2009), some of which are found in all biosolids. However, the reported viral prevalence numbers and instances of disease are highly variable. This variability may be due to the different detection methods used
in studies, treatment methods or diverse geographical locations. For example, anaerobic
digestion which may lead to the elimination of bacterial pathogens tend to leave enteroviruses
unharmed in biosolids (Sidhu and Toze, 2009) leading to a higher number of viruses detected
in biosolids wholly treated by anaerobic methods.

Noroviruses, which cause acute human gastroenteritis can survive some wastewater treatment
process with limited information about the prevalence of noroviruses in biosolids being
available, primarily because these viruses cannot be cultured (Sidhu and Toze, 2009, Kittigul
et al., 2019). Another virus of public health concern in biosolids is the human rotaviruses), the
pathogen responsible for acute gastroenteritis in children (Schlindwein et al., 2010, Grant et
al., 2012). However, the population of rotaviruses in biosolids may be low compared to other
enteric viruses because, unlike common biosolid viruses, rotavirus are unable to adequately
adsorb to biosolid particles (Sidhu and Toze, 2009).

Human adenoviruses are also found in biosolids at a population higher than enteric viruses, up
to 10 times higher in wastewater and have been implicated in human gastroenteritis, respiratory
illness and eye infections (Romdhana et al., 2009, Wei et al., 2009, Pepper et al., 2010).
Adenovirus strains are slow growers, even when cultured using cell lines and are non-
cytopathogenic. This makes it difficult to detect them in biosolids and has contributed to the
lack of information on their prevalence in biosolids (Sidhu and Toze, 2009).

Hepatitis E virus (HEV) and Hepatitis A virus (HAV) are also found in biosolids (La Rosa et
al., 2010, Wei et al., 2010). These viruses induce similar disease symptoms in humans, with
HEV more fatal than HAV. In pregnant women, HEV fatality can be as high as 15–25%
(Romdhana et al., 2009). HAV are persistent in biosolids during wastewater treatment
processes and like other viruses, there is comparably limited information in the literature on
the prevalence of HAV in different biosolid samples.
1.6.1.2 Bacteria

Some bacterial species are major causes of human enteric diseases (Peng et al., 2003a). However, the total bacterial population in biosolids are not often reported during wastewater treatment (Sidhu and Toze, 2009), with the focus largely on pathogenic bacterial groups. The different types of pathogenic bacteria commonly found in biosolids include *Campylobacter jejuni*, *Salmonella* spp. and *Shigella* spp. *E. coli* O157, *Helicobacter pylori* and *Listeria monocytogenes* (Sidhu and Toze, 2009).

In sewage biosolid *Campylobacter* spp. are abundant (Jones, 2001, Sahlström et al., 2004, Sidhu and Toze, 2009). However, they are highly sensitive to oxygen and tend to be eliminated in biosolids generated via aerobic lagoon systems. Dissolved oxygen in wastewater and biosolid causes a rapid and significant impairment of this bacteria leading to cell death (Jones, 2001); in contrast *Campylobacter* spp. thrives under anaerobic conditions (Nicholson et al., 2005).

*Salmonella* spp. is another bacteria commonly found in raw biosolids but are thought to be substantially inactivated during wastewater treatment (Sahlström et al., 2004) although under certain (ideal) conditions, cellular growth may occur in stockpile biosolids (Sidhu and Toze, 2009). *Salmonella typhi* causes gastroenteritis in humans, a disease which can be fatal if left or not promptly treated (Romdhana et al., 2009). *Shigella* spp. on the other hand, is not as abundant as *Salmonella* spp. in wastewater (Techobanoglous et al., 2003, Sidhu and Toze, 2009).

1.6.1.3 Protozoan parasites

In biosolids, *Giardia lamblia*, *Cyclospora* and *Cryptosporidium parvum* are the protozoa of public health concern (Graczyk et al., 2008, Romdhana et al., 2009). This is because they can
cause disease in healthy and immune-deficient people. Unfortunately, compared to bacterial pathogens, there is limited research data available on the survival of these pathogens in biosolid stockpiles. In addition, protozoa-based research often involves the use of different methods for sampling and recovery of protozoa from samples, with the recovery rates of biosolid protozoa typically low (Quintero-Betancourt et al. 2003). This makes comparing data from different studies problematic.

Among the protozoa found in biosolids, *Giardia* spp. and *Cryptosporidium* spp. can survive wastewater treatment processes (type of wastewater treatment can influence their die-off rate) and have been found to be resistant to adverse environmental conditions. Although *Cryptosporidium* spp. oocysts have been less frequently isolated than those of *Giardia* spp. in some biosolids (Caccio et al., 2003), their frequency of isolation is dependent on the source, type and treatment of wastewater the biosolids were derived from. Therefore, treatment processes do have a significant effect on the population of protozoa. Whitmore and Robertson (1995) demonstrated that anaerobic digestion of wastewater/biosolid for about 2 weeks (18 days) was lethal to about 90% of oocysts of *Cryptosporidium* spp. Olson *et al.* (1987) showed that storing cattle manure for 7 days at 4°C or 20°C rendered *Giardia* cysts non-infective but *Cryptosporidium* cysts in the manure were unaffected and remained infective for up to 8 weeks.

1.6.1.4 Helminth parasites

Disease caused by helminth parasites occurs worldwide with some reports estimating up to 5 million infections (Clarke and Perry, 1988). Helminths are found in wastewater and often the eggs survive wastewater treatment. When the biosolid or biosolids derived from waste treatment are dewatered, the process also leads to concentration of the helminth eggs in the biosolid (Sidhu and Toze, 2009). The important helminth parasites in biosolids include *Ascaris*...
lumbricoides, Taenia spp., Trichuris trichiura, Toxocara canis, Necator americanus and Ancylostoma duodenale (Sidhu and Toze, 2009, Jimenez, 2007). Ascaris spp. are commonly encountered in biosolids in developing countries (Sidhu and Toze, 2009). Some reports indicate that high temperature and low water content or drying of biosolids over time inactivates some helminth eggs but anaerobic digestion has no adverse effect on the survival of the eggs (Collick et al., 2007).

1.7 Ascaris lumbricoides

1.7.1 Prevalence of Ascaris

The most predominant disease-causing helminth is Ascaris, infecting about 1.4 billion people global (Ascaris lumbricoides) with additional 4 billion people susceptible being infected by Ascaris (Rosypal et al., 2007, Brownell and Nelson, 2006). Infections by A. lumbricoides can be fatal with up to 60,000 annual deaths worldwide (WHO, 2001). Children between the ages of 5 and 15 are particularly susceptible (Bethony et al., 2006). Poor wastewater treatment and inadequate treatment of biosolids prior to being used in agriculture and for other purposes are thought to contribute to the high prevalence of Ascaris lumbricoides infections (Astudillo et al., 2008)

1.7.2 Symptoms and transmission

Ingestion of food or soil contaminated with A. lumbricoides eggs leads to human infections (ascariasis) with between 1–10 infectious larvae eggs sufficient to cause infections in humans. There are many symptoms of ascariasis and these include urticaria, fever and allergic reactions symptoms such as asthma. Once the larvae has grown into the adult worm and has stabilised in the intestine, it induces nausea, vomiting, diarrhoea and a host of other symptoms; children
become undernourished, lethargic and susceptible to other infections (Jimenez-Cisneros and Maya-Rendon, 2007).

1.7.3 The life cycle of Ascaris
The infective stage of Ascaris spp. is the larvae and not the eggs but viable eggs hatch into larvae when ingested (Fig. 1.5). In the soil, larvae are known to become fully developed in about 10 days under ideal soil moisture conditions and temperature of (Jimenez-Cisneros and Maya-Rendon, 2007). After eggs are ingested, the eggs survive stomach acidity and are transferred to the intestine where they adhere to the duodenum. The eggs hatch in the duodenum and move into the small intestine. At times, larvae enter the bloodstream leading to their transportation to different parts of the body. It is in the lung that the larva becomes established, developing into a juvenile worm (Astudillo et al., 2008). The juvenile worms eventually return to the small intestine via the pharynx, to become full adults which now lay eggs, eggs that are discharged outside the body alongside faecal materials (Brownell and Nelson, 2006).
1.7.4 Ascaris eggs

Ascaris spp. eggs are known to survive for a considerable time in the environment, with some eggs thought to survive for up to 15 years (Brownell and Nelson, 2006). This is because the eggs have a highly resistant wall (a 4 layered wall) (Brownell and Nelson 2005), resistant to chlorine, high pH etc. This characteristic also renders them more resistant to wastewater treatment and adverse environmental conditions compared to other helminths eggs.
Consequently, *Ascaris* spp. eggs can be used as markers or indicators of the rate of helminth die-off or decay during biosolids treatment and wastewater (Brownell and Nelson, 2006, Capizzi and Schwartzbrod, 2001, Collick et al., 2007, Pecson et al., 2007, Sidhu and Toze, 2009).

The female adult worm can produce eggs which are either fertilized or unfertilized (Table 1.7) (Peng et al., 2003a). The 4-layer shell of the fertilized egg is thick (3-4 µm thick) to protect the zygote from environmental stress and provide it with optimum survival chances. The four shell layers (uterine, vitelline, chitinous and lipid) are shown in Fig. 1.6 (Brownell and Nelson, 2006, Quilès et al., 2006).

The impermeable nature of the egg-shells of helminths is due to the lipid layer. This layer is composed of protein (25%) and lipids (75%) (Wharton, 1980). This layer is resistant to acids, bases, oxidants and a variety of chemical compounds (Brownell and Nelson, 2006). However, the lipid layer is not the thickest egg-shell layer, the chitinous layer is and this layer is the structural backbone of the egg. Detailed analysis of the chitinous layer showed that it is made up of chitinous microfibrils enveloped by protein molecules (Wharton, 1980). The fertilized egg has a vitelline membrane and part of this membrane which contributes to the formation of the vitelline layer (or lipoprotein vitelline layer). This layer is also resistant to some corrosive compounds and protects the egg from adverse environmental conditions (such as pH and UV radiation) and during wastewater treatment (Wharton, 1980).

There are distinct differences between fertilized and unfertilized eggs. For example, fertilized eggs are elliptical or rounded in shape, 35-50 um in width and 45-75 um in length, with a thin smooth irregularly mamillated layer and lack an outer wall and cause infections. In contrast, the unfertilized eggs are elongated, 35-44 um wide and 88-94 um in length, have thick shells with rough mamillated layer and are non-infective (Ash and Orihel, 1990; Peng et al., 2003a).
1.8 Physical factors and the survival of *Ascaris* spp. eggs

1.8.1 Temperature

The larvae of *Ascaris suum* is thought to require a temperature range between 16 ± 1 °C and 34 ± 1 °C for optimum development with a temperature of 27 °C to 29 °C required for embryonation (WHO, 2004). Some studies have shown that egg development is impaired at a temperature range of 8.9 °C to 15.6 °C, as the protoplasm is damaged at these low temperatures. However, there are other contrasting reports indicating the maintenance of egg viability (*Ascaris suum*) at extremely low temperatures of -18 °C to -27 °C for close to 6 weeks (WHO, 2004). The development of the egg is disrupted at 60 °C and above (for around 15 minutes) (WHO, 2004) as at this temperature all the egg’s physiological activities are inhibited, and this is why application of high temperature and dry heat destroys all infective eggs (Cram (1943) cited by WHO (2004)).
1.8.2 Sunlight and ultraviolet radiation

Sunlight and UV radiation exposure destroy the viability of helminth eggs over time. Sunlight exposure of water-submerged fresh *Ascaris suum* eggs for four to six hours rendered them non-viable, while dried fresh eggs lost their viability within 2 hours. Likewise, sunlight was found to be lethal to embryonated eggs within a 3 to 4-hour period (WHO, 2004). It is thought that sunlight exposure leads to heat absorption by the eggs, damaging the protoplasm.

UV radiation around 4000 Jm\(^2\) is required to render *Ascaris* spp. eggs non-viable, indicating high resistance to UV radiation. The UV dose required for *Ascaris* spp. egg inactivation is at least four times more than that required to inactivate the most resistant enteric virus (the adenoviruses) (Brownell and Nelson, 2006). The location of the eggs affects the potency of UV radiation, with eggs submerged in water more susceptible to UV radiation than those found in soils and biosolids (little or no UV penetration) (Mun et al., 2009).

1.8.3 Desiccation

*Ascaris* eggs are very sensitive to desiccation. It is believed that the drying out effects of sunlight contribute to its lethal effects on helminth eggs (WHO, 2004). The infective larva requires moisture too, dying off after desiccation for 37 days. *Ascaris* spp. eggs require at least 80% relative humidity at 22 °C for optimum development. The infective larvae usually die after 37 days of drying when kept at room temperature.

1.9 Chemical factors and the survival of *Ascaris* spp. eggs

1.9.1 pH

*Ascaris* eggs are resistant to acidic or basic environments. However, at most tested temperatures, acidic pH impaired the development of *Ascaris suum*, but the helminth was
unharmed in highly alkaline buffers (WHO, 2004). Another type of helminth, hookworms can survive in low and high pHs, developing into the infective larval stage in pH 4.6-9.4 environments (WHO, 2004).

1.9.2 Chemical compounds
Multiple investigations such as those by Seamster (1950), Arfaa (1968) and Cram (1924), have conclusively demonstrated *Ascaris* spp. eggs resistance to a variety of chemical compounds. *Ascaris* eggs were unharmed and developed into their infective stage in different toxic solutions (14% hydrochloric acid, 9% sulphuric acid, 8% acetic acid, 4% formaldehyde, 0.4% nitric acid, 1% mercuric chloride, 0.3% carbonic acid and 0.5% sodium hydroxide) (Fairbairn, 1957, Morishita, 1972). However, fumes of concentrated ammonium hydroxide fumes were lethal to *Ascaris* spp. eggs after 36 hours (WHO, 2004). *Ascaris* eggs are inactivated or destroyed by compounds such as chlorine (Bandala et al., 2012), ozone (Velasquez et al., 2004), ammonia (Pecson and Nelson, 2005) and lime treatment (Eriksen et al., 1996).

1.10 Biological factors and the survival of *Ascaris* spp. eggs

1.10.1 Fungi and invertebrates
Fungi and invertebrates can feed on *Ascaris* eggs with ovicidal fungi known to degrade *A. lumbricoides* eggs under experimental conditions. However, the rate of egg degradation is determined by the fungal type. For example, the fungal species, *Cylindrocarpon radicola* can grow on *A. lumbricoides* eggs destroying them in the process and proteases produced by *Pochonia chlamydosporia* have been shown to destroy A. suum eggs (WHO, 2004, de Freitas Soares et al., 2015). Gastropods and insects are also known to feed on *Ascaris* spp. eggs through direct ingestion. In addition, some fungi such as *Aspergillus terreus* and *Fusarium oxysporum*
can inhibit the development of *A. suum* eggs causing up to 60% mortality of the larvae in some studies (Jaborowska et al., 2006, Blaszkowska et al., 2013). However, not all the ingested eggs are digested, with about 10-20% of the eggs ingested by organisms such as *Planorbis corneus*, *Galba palustris*, *Planorbis planorbis*, *Bithynia tentaculate*, *Physa fontinalis* and *Succinea purtis* found intact in their faecal materials (WHO, 2004).

1.10.2 The processes of biosolid treatment

Table 1.4 showed different processes in wastewater and biosolid treatment and their effects on helminths eggs. Survival is dependent on the type of process. For example, anaerobic digestion is generally less effective on a short-term basis as *Ascaris* spp. eggs are known to remain infective after 3 months under anaerobic conditions. In contrast, when these eggs stay longer under anaerobic conditions (i.e. for longer than 6 months), 90% of the eggs were destroyed. Aerobic digestion also leads to the destruction of *Ascaris* spp. eggs, particularly at elevated temperatures (Bitton, 2011, Jimenez, 2007) with the drying pans being an effective method for the destruction *Ascaris* spp. eggs (100% egg deaths at moisture levels <5% (WHO, 2004)).
Table 1.4: Effect of biosolid treatment processes on helminth eggs.

<table>
<thead>
<tr>
<th>Unit of operation</th>
<th>Stabilization processes</th>
<th>Decontamination processes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic digesters</td>
<td>Promotes egg development (increases destruction with increased temperature)</td>
<td>None</td>
</tr>
<tr>
<td>Anaerobic digesters</td>
<td>Retards egg development (increases destruction with increased temperature)</td>
<td>None</td>
</tr>
<tr>
<td>Incineration</td>
<td>-</td>
<td>100% destruction</td>
</tr>
<tr>
<td>Drying beds</td>
<td>-</td>
<td>100% kill at 5% moisture content (moisture content may vary with temperature)</td>
</tr>
<tr>
<td>Composting</td>
<td>-</td>
<td>100% effective if temperature is maintained at 60°C for at least 2 hours</td>
</tr>
<tr>
<td>Routine chlorination</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>Sonication</td>
<td>-</td>
<td>80% effective at 30-50 KHZ and 600 watts</td>
</tr>
<tr>
<td>Gamma radiation</td>
<td>-</td>
<td>100% effective at 200 KRADs</td>
</tr>
<tr>
<td>Heat</td>
<td>-</td>
<td>100% effective at 70°C for 30 minutes, or lower at higher temperature. Effectiveness dependent on temperature and exposure time</td>
</tr>
<tr>
<td>Lagoon storage</td>
<td>-</td>
<td>50-100% (depends on time and temperature) 80-100%</td>
</tr>
<tr>
<td>Lime stabilization</td>
<td>-</td>
<td>Depends on dosage and pH</td>
</tr>
<tr>
<td>Ammonification</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from (WHO, 2004). Stabilisation and decontamination processes are carried out to reduce/eliminate odour by preventing putrefaction, reduce/eliminate pathogens and allow it to be stored for longer periods without significant loss of biosolid properties/nutrients (as the biosolid is dry) and microbial (anaerobic) activity is inhibited.
1.11 Assessment of the Viability of *Ascaris* eggs

1.11.1 Morphological Methods

The morphological microscope-based method for assessing *Ascaris* egg viability is a direct counting method (WHO, 2004). The use of this method can be challenging as it requires some experience and skill to recognize viable and non-viable eggs (observations of non-motile larvae are not a sign of dead eggs). This approach also lacks an objective standard. Identification of the differences between live and dead eggs is often difficult. Non-viable eggs are confirmed only once degeneration of egg membranes are observable which may require an extended period (weeks and months (WHO, 2004). Ayres (1992), cited by WHO (2004), observed that culture-based methods were better than direct morphological methods for assessing egg viability as there are distinct structural changes between a living (viable) and non-viable (dead) eggs. Dead eggs will have shrinkages in their membranes, large granules and vacuolation in the cytoplasm and exhibit degeneration of key structures and egg shell membranes (Ayres, 1992 cited by WHO, 2004).

1.11.2 Conventional Incubation Methods

While the conventional incubation method is widely used for assessing helminth egg viability due to its reliability, the processes involved are labour and time-consuming. Recovery of helminth eggs from wastewater, waste materials (faeces), biosolid and soil, faeces and discrimination between viable and non-viable eggs typically involved the application of flotation and sedimentation techniques (Bowman et al., 2003).

A commonly used method, the Tulane method, incorporates both flotation and sedimentation processes (Bowman et al., 2003). Sedimentation results in separation based on particle size, with the large particles removed with sieves of different mesh-sizes. Flotation procedures on
the other hand involve the use of solutions; these solutions have greater specific gravity than the eggs and so the eggs float and are separated from other heavier particles (Bowman et al., 2003, Ravindra et al., 2019). The floating eggs are subsequently harvested from the supernatant using sieves of appropriate mesh size. Finally, the collected *Ascaris* eggs are incubated at 25 °C for up to 28 days, optimum conditions for larval development (Bowman et al., 2003). The application of the Tulane method has led to egg recovery rates of 60-76% from biosolid samples (Bowman et al., 2003) and this method can be used to recover and discriminate between viable and non-viable eggs in biosolid samples (Bowman et al., 2003, Ravindra et al., 2019).

1.11.3 Polymerase Chain Reaction (PCR)

Different PCR primers and protocols have been developed for the detection and identification of parasites and helminths in wastes, wastewater and soil (Aboobaker and Blaxter, 2003, Nejsum et al., 2008, Zhu et al., 1999). Unfortunately, no currently published PCR method is able to detect *Ascaris* spp. eggs embedded in biosolids. This limitation hampers the development of a PCR-based molecular method for *Ascaris* spp. eggs detection (Zarlenga and Trout, 2004). Detection of the adult worms is however possible with PCR (Sidhu and Toze, 2009).

1.11.4 Denaturing Gradient Gel Electrophoresis (DGGE)

This method is a fingerprinting to the study of microbial ecology of different environments such as soils (Cheung and Kinkle, 2001, Mao et al., 2012), aquatic environments (Beier et al., 2008, Kaartokallio et al., 2008, Hale et al., 2010), wastewater (Svobodová et al., 2018, Turki et al., 2017) and the human gut (Kennedy et al., 2014). DGGE generates a profile of the
microbial community diversity based on the separation PCR amplicons and allows for the comparison of microbial communities from different environments. Purified nucleic acids (DNA and cDNA) from a desired source can be amplified using desired primers and the amplicons subject to DGGE analysis to create a banding pattern (or fingerprint) based on the GC components and changes on the electrophoretic mobility of different DNA sequences (amplicon) in a polyacrylamide gel. Each band is assumed to represent a microbial species whose putative identity can be determined. A number of excellent reviews of this method, (principle, the advantages and disadvantages) are available (Muyzer, 1999, Muyzer and Smalla, 1998, Nocker et al., 2007, Alvarez and Illman, 2005).

DGGE as a tool has been applied to the study of microbial communities associated with waste treatment and biosolids (Yan et al., 2015, Mohan et al., 2016). However, they have not been applied to the study of the decay of *Ascaris* eggs. Some members of the biosolid microbial communities may potentially affect the decay rates of *Ascaris* eggs, if they are able to produce hydrolytic enzymes that adversely affect the membranes of these eggs. DGGE therefore, offers an opportunity to study the microbial community associated with egg decay provided suitable primers are used.

### 1.11.5 Vital Staining Methods

Changes in the permeability of *Ascaris* spp. eggs can be exploited to differentiate between non-viable and viable eggs through the use of staining reagents (Clarke and Perry, 1988). Viable eggs are not stained as certain dyes will only stain the damaged cell membranes of non-viable *Ascaris* spp. eggs (de Victorica and Galván, 2003). Viable staining is a simple and fairly rapid method which can be carried out or completed within 10 minutes. Evaluation of multiple dyes for use in the staining of eggs has been carried out with mixed results (WHO, 2004).
Dyes such as potassium iodide solution and iodine were observed not to stain dead eggs (Boyd, 1941 cited in WHO, 2004). The application of another stain, Trypan blue did not allow for precise detection of dead eggs (Hudson and Hay 1980, cited by WHO, 2004). Other stains such as Trypan blue, Sudan III, Eosin malachite green, Thionine blue, Methyl green, Congo red, Neutral red or Kresofuchin were also unable to selectively stain living or dead eggs (Keller 1951, cited by WHO, 2004). However, 0.05% methylene blue applied for 5 minutes stained dead eggs (larvae) but not Ascaris spp. eggs with larvae (Arene, 1986). The use of crystal violet gave mixed results and was found to be unreliable when eggs were in media with extreme pH values (Hindiyeh 1995, cited by WHO 2004).

The use of fluorescent dyes in determining the viability of oocysts of parasites and eggs of helminths has been explored in multiple studies. Immunofluorescence can be used to stain some microbes (e.g. Cryptosporidium and Giardia) and antibodies conjugated to fluorescein diacetate (FDA) was found to stain only viable cysts, resulting in green fluorescence as protozoal enzymes are degraded in dead oocysts (Jarmey-Swan et al., 2000, Zarlenga and Trout, 2004). 6’-diamidino-2-phenyl-indole (DAPI) fluorescence stains and Propidium iodide (PI) and 4’ colour viable Ascaris suum eggs blue (fluorescence (DAPI)) and non-viable eggs red (fluorescence (PI)) (Sidhu and Toze, 2009).

The Live/Dead® BacLight™ bacterial viability kit is a nucleic acid fluorescence staining kit developed by Molecular Probes and is usually used to differentiate between living and dead bacteria. The kit has two types of fluorescence dyes. The first one, Syto 9 dye penetrates live and dead bacteria and stains their membrane green. The second dye, propidium iodide (PI) only stains dead bacteria resulting in a red colouration (Manual, 2004). The kit has been successfully used in multiple bacterial-based studies (Boulos et al., 1999, Biggerstaff et al., 2006, Pascaud et al., 2009). Given its ease of use and reliability compared to other staining methods, it should be possible to use this stain to distinguish between non-viable and viable Ascaris eggs.
1.12 Current regulations

There is currently scope for studying the microbiological safety wastewater biosolids in detail using recently developed high-resolution microbiological methods. The microbiological safety has not been studied in detail of some biosolids produced in Australia, which becomes the main limiting main factor restricting the application of biosolids as fertilizers.

Victorian EPA Biosolids Land Application Guidelines as stated in Smart Water report (2013) are reproduced _verbatim_ below;

I. “The treatment grade T1 require verification for prescribed treatment systems for <1 *Salmonella*/50 g dry weight, <100 *E. coli* MPN/g dry weight and ≤1 enteric virus PFU/100 g” (EPA Victoria, 2004).

II. “Victorian EPA Biosolids Land Application Guidelines prescribed the T1 treatment grade biosolids which are obtained by any alternative processes require <1 *Salmonella*/50 g dry weight, <100 *E. coli* MPN/g dry weight, >3 log10 reduction of enteric viruses and >2 log10 reduction of *Ascaris* eggs”.

Given that lagoon-based wastewater treatment generates biosolids, it is crucial that their safety is validated. Process verification should be carried out to demonstrate log10 reductions of enteric viruses, parasites, or designated indicators for every batch of biosolids produced.

III. “Victorian EPA Biosolids Land Application Guidelines prescribe the biosolids from alternative systems for T2 certification require that the biosolids contain <10 *Salmonella*/50 g dry weight, <1000 *E. coli* MPN/g dry weight, <1 *Taenia* egg/10 g dry weight and <2 enteric virus PFU/10 g dry weight” (SWF, 2013a).

Enteric viruses and helminth concentrations are very low level making demonstrating their log reductions problematic in Victorian biosolids. For lagoon based biosolids under the treatment grade T3 biosolids, they must be subject to aerobic digestion for ≥40 days at ≥ 20°C, and ≥60 days at ≥15°C.
A key limitation in ensuring the safety of biosolids is the lack of or insufficient validated laboratory-based methods for identifying pathogens in biosolids. Some of these methods are time-consuming, expensive and require advanced laboratory and expertise. Therefore, it can be challenging and costly to certify lagoon-based wastewater treatment processes and biosolids. Consequently, there is a need to optimize existing methods for pathogen detection and develop or apply newer more efficient and cost-effective techniques for testing biosolids from different sources. In this study, the focus has been on the helminth, *Ascaris* spp. given its ability to survive in biosolids longer than most other pathogens. Biosolid samples from lagoon-based systems were selected for use in this study because of the ease of sample access and project support.

1.13 Overview of Project

The aims of this project are:

(a) To assess initial concentrations of *Ascaris* spp. eggs in biosolids from three regional lagoon treatment systems;

(b) To assess the decay of *Ascaris* spp. eggs in simulation studies in pan drying and stockpiling treatment of selected biosolid samples.

(c) To assess the role of microorganisms and enzymes in the decay or die off *Ascaris* spp. eggs in selected biosolids.

This was carried out by assessing the suitability and efficacy of the Tulane assay for detecting *Ascaris* eggs in lagoon samples (biosolids) from three different wastewater treatment plants in Victoria, Australia. An existing method, the Tulane method, was optimized and used to assess the presence and recovery efficiency of *Ascaris* spp. eggs in biosolid samples (Chapter 3). Using the optimized method, the decay rates of *Ascaris* spp. eggs were determined in simulated
pan drying experiments under laboratory conditions. Live and dead staining kits were successfully used in determining the viability of *Ascaris* spp. eggs in different biosolid samples (Chapter 4). Using a PCR-Denaturing Gradient Gel Electrophoresis (DGGE) approach, the microbial community found in biosolid samples were examined and their potential roles in the decay of *Ascaris* spp. eggs assessed via enzymatic analyses. Additionally, the effects of enzymes produced by bacteria in lagoon on the viability of *Ascaris* eggs were assessed (Chapter 5). Overall, the long term aim of this research investigation is to provide useful information and advice to wastewater treatment plants on better management of solid waste and the removal of helminth eggs.
CHAPTER 2:

Materials and Methods
2.1 Introduction
This chapter describes the materials and methods used in this project. It describes the source of samples, sampling procedures, analytical techniques and methods used for data analysis. Some of the details on operating systems and treatment procedures were supplied by the different wastewater treatment plants.

2.2 Source of material
The wastewater treatment (WWTP) plants used in this study were based in regional Victoria. These were Heyfield WWTP (Gippsland Water, Fig. 2.1 A), Cobden Wastewater Reclamation Plant (WRP) (Wannon Water, Fig. 2.1 B) and Rochester WWTP (Coliban Water, Fig. 2.1 C). These plants use a two-lagoon lagoon-based treatment for wastewater treatment. Biosolids or biosolids were collected from the primary lagoons at each of these waste-treatment plant.
Figure 2.1: The Study area and location in Australia.
(A) Gippsland Water (Heyfield biosolids) (B) Wannon Water (Cobden biosolids) (C) Coliban Water (Rochester biosolids).
2.3 General description of the study area

2.3.1 Heyfield Waste Water Treatment Plant (WWTP)

Heyfield WWTP is used to treat only domestic sewage. After treatment, biosolids are transported to the Gippsland Water’s Soil and Organic Recycling Facility (SORF) at Dutson Downs (Fig 2.2). At this facility, the biosolids are stockpiled for composting and after maturity used as fertilizers in agriculture. For this study, samples (60 kg of biosolid) were taken from Heyfield WWTP and transported to the RMIT University, Melbourne and used to set-up laboratory based drying pan and stockpile simulation experiments.

Figure 2.2: Wastewater treatment system at Heyfield WWTP.

Note: Samples (15% DS) obtained at the shaded area. Reproduced from SWF, (2013a).

2.3.1.1 Harvesting of biosolid

A polymer (cationic polyacrylamide (ZETAG®8165, BASF)) was used for harvesting the biosolid or biosolids. It was prepared by mixing the powder with water to get a polymer of 0.25% (W/W) and applied a rate of 5 kg per tonne of biosolid (dry weight). The batch solution was then dosed into the incoming feed at a rate ranging between 2,000 to 4,000 L per h. The polymer was mixed with incoming feed (97.5% water and 2.5% solids; flow rate of 120 m³ per
hour). The polymer and feed mixture form a floc before they are mechanical separated (SWF, 2013a).

### 2.3.2 Cobden (WWTP)

After the removal of large items, the wastewater was allowed to flow into a primary lagoon and aerated twice in 24 hours, with each aeration phase lasting for 60 minutes. Aeration stimulates microbial growth and the digestion of organic compounds (Fig 2.3). From here, the partially digested sewage was transferred into a maturation lagoon and retained in this lagoon for 30 days. In the maturation lagoon or lagoon, microbial activities continued, and heavy sewage particles settled at the bottom of the lagoon. After maturation, the supernatant was pumped to another facility for storage purposes.

Biosolid/biosolid samples were collected with a positive displacement pump (10 L/ min) from the primary lagoon and mixed using a shovel in a 300 L container. Aliquots of 15 L were collected and transported to RMIT University for further analysis.
2.3.3 Rochester WWTP

Sewage was initially pumped into an Imhoff Tank where large particles settle to the bottom and organic matter degradation/stabilization via microbial activities occurs (Fig 2.4). Biosolid/biosolid samples were obtained from this lagoon (primary lagoon B) using a displacement pump, as described in the Cobden WWTP sampling regime. Rochester WWTP is unique in that alum wastes are treated. It was estimated that the WWTP handled approximately 200 kg every 24 hours with a typical alum concentration of 300 mg/L sewage; sufficient to induce major flocculation (Malhotra et al., 1964). Dewatering of the biosolid is presumed to lead to a higher alum concentration in the biosolid or biosolid samples.
Figure 2.4: Schematic representation of the wastewater treatment system at Rochester WWTP.

Reproduced from SWF (2013a). Biosolid samples were obtained at point B (orange arrow). The blue arrow represents raw biosolid entry point into the Imhoff tank. Coarse screen (bars about 2 cm apart) are used to remove large objects in sewage. Green arrows indicate sewage entry points into the 2 lagoons. Wastewater eventually gets into the secondary lagoons for polishing and finally into the storage tank.

2.4 Sampling Protocol

Heyfield (April-winter) and Cobden (June-winter) and Rochester (February-summer) biosolids were examined for the presence of *Ascaris* (all in 2012). Samples were stored at 4°C for four weeks until used for further investigations. These samples were used to assess the prevalence of *Ascaris* spp. in biosolid samples, die-off rates of *Ascaris* spp. eggs in laboratory-based pan-drying and stockpiling simulations.
2.5 Chemicals and Media

All the media and chemicals of used in this study were of analytical grade. They were obtained from Oxoid Ltd (Basingstoke, Hampshire, U.K.) and Sigma–Aldrich Chemical Company (NSW, Australia) except otherwise mentioned in the text.

2.5.1 Bushnell and Haas (BH) medium for bacterial isolation

Bushnell Haas (BH) mineral salts medium containing 0.02 g L\(^{-1}\) CaCl\(_2\)-2H\(_2\)O, 0.2 g L\(^{-1}\) MgSO\(_4\)-7H\(_2\)O, 1.0 g L\(^{-1}\) KH\(_2\)PO\(_4\), 0.05 g L\(^{-1}\) FeCl\(_3\)-6H\(_2\)O, 1.0 g L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), 1.0 g L\(^{-1}\) (NH\(_4\))\(_2\)HPO\(_4\); pH 7.0. No carbon sources added and sterilised at 121 °C for 15 min (Bushnell and Haas, 1941).

2.5.2 Nutrient broth and nutrient agar (bacterial growth)

Nutrient Broth (NB) and Nutrient Agar (NA) (13 g L\(^{-1}\) and 24 g L\(^{-1}\)) were dissolved in 1 L MilliQ-water respectively and autoclaved at 121 °C for 15 min.

2.5.3 1% “7X” solution (1%) (MP Biomedicals, Australia).

Thirty-four grams of potassium dihydrogen phosphate (KH\(_2\)PO\(_4\)) (Merck Pty, Australia) was added to 1 L of RO water to make 1 L of phosphate buffer. The pH was adjusted to 7.2 ± 0.5 with NaOH (1 M). Phosphate buffered water (99 mL) was added to 1 mL of “7X” solution (MP Biomedicals, Australia) to make a 1% aqueous solution.
2.5.4 Magnesium sulphate (MgSO$_4$) floatation solution.
To 1 L of RO water 215.2 g of MgSO$_4$ (Sigma-Aldrich, Australia) was added. For ease of preparation, 40 g was added to 500 mL RO water and mixed in batches using a magnetic stirrer. The specific gravity was measured using a hydrometer.

2.6 Biosolid analysis
The biosolid/biosolid samples were dewatered prior to being used for any investigation. Approximately 10 kg of each biosolid sample was added to designate steel tanks to form stockpiles (biosolid dry solids (DS) was at ~15%, based on protocols from previous simulations performed in an earlier project, (SWF 611-001) carried out at RMIT (project details are confidential).

Briefly, 250 L of lagoon biosolid from Cobden, Heyfield and Rochester WWTP were pumped into the collection tubs and mixed properly. Biosolid samples (15 L) were aliquoted into 20 L plastic containers and allowed to settle for 4 days at RMIT University. About 10 L of biosolid supernatant was discarded from each can (after four days) leaving 5 L of biosolid which was used for further investigation.

All the biosolid/biosolid samples were subject to scientific investigations designed to detect the presence of helminth eggs (specifically *Ascaris* spp. eggs). In addition, biosolid samples from Heyfield WWTP and Cobden WRP were also used to evaluate the egg recovery efficiencies and detection threshold for *Ascaris* spp. eggs of a selected method. The details of the methods used are described in Section 2.7.11. In addition, 4.5 L of biosolid from each of the sites were added to three stainless steel tanks and used for laboratory-based simulation experiments to assess *Ascaris* spp. die-off rates. The details of the methods used are described in Chapter 4 of this thesis. Aliquots of biosolid samples were also analysed for their nutrient content.
2.6.1 Dry weight of biosolid

The dry weight of biosolid samples was determined as previously described (SWF, 2013a) in triplicate samples. Firstly, porcelain crucibles were labelled and weighed, after which 10 g of fresh biosolid sample was added to each crucible and the weight of biosolid-crucible determined. The crucibles were placed in a preheated oven to 105°C for 24 h. The weight of the biosolid-crucible was determined after 24 h. The samples were allowed to cool down for at least 1 h at room temperature prior to being weighed. The biosolid dry weight was calculated using the formula described below:

- Crucible weight
- Fresh weight of sample (FW) = fresh biosolid weight
- Dry weight of sample (DW) = total dry weight (weight of crucible + dry biosolid) - weight of crucible

Sample dry solids content (DS) is calculated from: \( \text{DS\%} = \frac{(\text{DW}/\text{FW}) \times 100\%} \)

2.6.2 Nutrient analysis

Samples from the three representative lagoon treatment systems were sent to Australian Laboratory Services (ALS) for analysis of total Kjeldahl nitrogen, total nitrogen, Olsen extracted phosphate and nitrate and nitrite (SWF, 2013a). Triplicate samples were used for each system.

2.6.3 Volatile solids content

Dissolved and volatile solids (VS) analyses were carried out for all samples using standard methods (Eaton et al., 1995). Triplicate samples (1 g wet weight) were used throughout.
2.7 *Ascaris* spp.

2.7.1 Source of eggs

*Ascaris lumbricoides* is a human pathogen with similar characteristics and life cycle to *Ascaris suum* found in pigs (Aitken et al., 2005, Johnson et al., 1998); Therefore, *Ascaris suum* was selected for use in this study due to ease of availability and to reduce the risk of personnel infection. The *Ascaris suum* eggs used in this project were gifts from Mr Patrick Daniel of the Department of Primary industries (DPI). The presence of the eggs in pig faeces was validated by carrying out a 1:2 dilution of 0.5 g of faecal material with saline solution. Aliquot of this dilution were examined with a light microscope. Unused faecal samples were stored at 4 °C before being discarded.

2.7.2 Collection and quality control of eggs

Faeces from pigs infected with *Ascaris suum* were also collected from a pig farm in Victoria, Australia whose location is confidential. Faecal materials were collected from pig pens by washing the floors with water and allowing the faecal material-water mixture to sediment for up to 24 hours. The supernatant was subsequently discarded, the faecal material (sediment) drained, centrifuged to get rid of excess water and transported to the laboratory at 4°C.

2.7.3 Coating glassware

All glassware used in this study were coated with SIGMACOTE®. SIGMACOTE® is a chlorinated organopoly siloxane in heptane that combines with surface silanol groups found on glass surfaces resulting in the formation of a neutral, hydrophobic thin film. This thin film prevents eggs from adhering to the glassware. All glassware were initially cleaned and oven-
dried before the application of undiluted SIGMACOTE®, using plastic 5 L containers to hold the excess SIGMACOTE®. All treated glassware were air-dried and prior to use, briefly rinsed in water to remove undesired by-products (Sigma-Aldrich, 2010).

2.7.4 Recovery of eggs via Tulane method

The Tulane method was used in this study with regards to investigations pertaining to *Ascaris suum* eggs (Bowman et al., 2003) (Appendix B up to step 20). It was used in a modified form to recover eggs from pig faeces, assess the presence of helminths eggs in the three biosolid samples and in recovery efficiency assays (Chapters 3 and 4). In brief, the dry solids (DS) content of the faeces was measured, and 5-8 g of total solids were washed in water and blended for 1 min at high speed (to ensure proper mixing) and poured into a 1 L beaker. One percent ‘7X’ solution (v/v) was added to this mixture and incubated overnight at room temperature. The supernatant was discarded, water was added to the sediment and blended for 1 min at high speed. This homogenous sample was transferred to a clean 1 L beaker and 1% ‘7X’ solution added, mixed and incubated for up to 3 hours at room temperature. This incubation period allowed the faeces to settle, permitting the separation of small particles from large particles. After 3 h, the supernatant was discarded and 300 mL of 1% ‘7X’ solution added, stirred with a magnetic stirrer for 5 min before being sieved with clean stainless steel sieves of 38 µm pore sizes. The sieved samples were allowed to settle with solution ‘7X’ for 2 h after which the supernatant was discarded. The sediment was briefly mixed before being aliquoted into 50 mL tubes. Aliquots were centrifuged for 10 min at 800 x g. Magnesium sulphate (MgSO₄, 1.8 M) solution was added to the supernatant and briefly vortexed. The eggs were observed to float (specific gravity of 1.20) and the mixture was centrifuged for 5 min at 800 x g to ensure proper egg separation. The eggs were recovered from the supernatant using a 38 µL sieve.
The sediment was collected, transferred into clean tubes, centrifuged at 800 x g for 3 min and the supernatant discarded. The pellet (*Ascaris suum* eggs) were stored in 0.5% formalin (in culture fluid) at 4°C (Bowman et al., 2003). The egg recovery procedure (Tulane method) was carried out in replicate in order to obtain sufficient egg concentration for pan-drying and simulation experiments (Appendix C).

2.7.5 Viability and total egg count

2.7.5.1 Conventional incubation method to determine percentage of viable eggs

Aliquots of eggs were centrifuged at 800 x g for 3 min. The supernatants were discarded and a few drops of 0.5% formalin was added to the pellet, then gently mixed before being transferred to sterile Petri dishes. These Petri dishes were covered with Parafilm (for moisture retention) and aluminium foil (to prevent illumination) at 25 °C for 28 days. The eggs were gently shaken on a weekly basis to aid hatching. After incubation, the samples were transferred to a 15 mL tube, centrifuged at 800 x g for 3 min and the supernatant discarded. The eggs were bleached for 10 min with household bleach (10% concentration) to remove the outer eggshell layer. This makes microscopic examination of the eggs easier. After bleaching, the samples were centrifuged for 3 min at 800 x g to remove the 10% household bleach (supernatant). The bleached eggs were rinsed with water and examined under a light microscope at 40 x and 100 x magnification for the counting of viable and non-viable eggs. The accuracy of counting was improved by dividing the coverslip into nine equal squares. Each of these squares was in turn divided into another nine squares. Eggs were recorded as viable eggs when motile larvae or hatched larvae were seen. However, if no larvae were observed, the eggs were recorded as being non-viable. Each sample (n=4) was counted thrice (n=12). The total number of eggs present was the sum of viable and non-viable eggs.
2.7.5.2 Photographs of eggs
Digital records of the recovered and the 28 day-incubated eggs were taken using a Leica DM2500 compound microscope with a Nikon digital camera attached.

2.7.6 Preparation of biosolid concentrations for the examination of eggs of A. suum
The Tulane method (Bowman et al., 2003) was used for the examination of helminth eggs. Biosolid samples from three WWTPs were added in different volumes based on the dry solids content (DS%). Examination of the biosolid samples was carried out with 50 mL of biosolid from Heyfield WWTP (15% DS, 7.5 g total solids), 90 mL of biosolid from Rochester WWTP (9% DS, 8.1 g total solids) and 200 mL of biosolid from Cobden WWTP (3% DS, 6 g total solids). Biosolid samples were blended at high speed for 1 min after which 1% “7X” (an anionic detergent) (MP Bio, Seven Hills, Australia) was added to the selected biosolid samples and allowed to settle for 18 hours at room temperature. The supernatant was discarded, the samples blended again at high speed after the addition of ~ 300 mL of RO water. Detergent “7X” was added again at a ratio of 1:10 of biosolid and the mixture allowed to settle for up to 3 hours. The supernatant was discarded, 300 mL of “7X” added to the biosolid sediment, stirred for 5 min, sieved using 850 and 350 µm mesh size sieves and the rest of the protocol in the Tulane method was followed leading to the generation of a final volume of 1.5 mL of sediments (Bowman et al., 2003, Karkashan et al., 2015). Slides were prepared and examined under a light microscope at 100 x magnification (20 µL per slide and covered by a 22 x 22 mm coverslip) to count the eggs in the biosolid as described by Karkashan et al. (2015). Each slide was counted twice to ensure the accuracy of the counting process (Figure 2.5).
Figure 2.5: Outline of the Tulane protocol.

(A) Sedimentation of the blended sample in 1 L beakers (B) 38 µm sieves used for the removal of large particles (C) sample concentrated in magnesium sulphate flotation solution (D) microscopic examination of samples for *Ascaris* eggs.

### 2.7.7 Recovery efficiency of *A. suum* eggs in sewage biosolid.

To assess the recovery rate of *Ascaris suum* ova, 10 mL of the egg suspension was added to the selected biosolid. This was carried out on 50 mL of biosolid from Heyfield WWTP (15% DS, 7.5 g total solids), 90 mL of biosolid from Rochester WWTP (9% DS, 8.1 g total solids) and 200 mL of biosolid from Cobden WWTP (3% DS, 6 g total solids). The different concentrations of biosolid samples used based on the total solids have been measured and can
be seen in Appendix D. Biosolid and eggs mixture were placed in a blender and mixed together as prescribed in the Tulane method (Bowman et al., 2003).

Recovery efficiencies were calculated by \( \% \text{ Recovery} = \frac{\text{Total No. of eggs counted per deposit}}{\text{the density of eggs initially added to the biosolid}} \times 100. \)

**2.7.8 Determination of the limits of detection**

After the recovery rate was determined, further testing was required to investigate the minimum number of eggs able to be detected by this method. Using the same method as described in Section 2.7.4, smaller numbers of eggs were added to the biosolid each time. First, serial 1:2 dilutions were prepared (12 mL egg suspension + 12 mL RO water) from the original suspension used for recovery rate testing. From this stock, subsequent dilutions were carried out (1:4, 1:8, 1:16, 1:32, and 1:40). After that, 10 mL of the desired dilution was added to an aliquot of biosolid (volume determined by DS%) and the modified Tulane method for egg counting described in Bowman et al. (2003) was followed. Approximately 1.5 mL of a final solution was examined, using 20 µL volume per slide covered by a 22 x 22 mm coverslip under a light microscope at 100 x magnification. The number of eggs detected were counted per volume of biosolid examined. The number of eggs recovered was compared with the number of eggs initially added and the percentage recovery calculated. The same method was repeated for all the other dilutions of egg suspensions carried out until the dilutions with no eggs detected was reached. Duplicate counting was performed per slide to confirm the detection of eggs and to ensure the accuracy of the counting procedure. The limit of detection was determined as the lowest dilution with a number of eggs detected after processing by the modified Tulane method (the dilution before no eggs were detected).
2.7.9 Statistical analysis
GraphPad Prism 7 (GraphPad Software) was used to conduct the statistical analysis. A linear regression relationship between the egg number and recovery efficiency was established by pooling the data for all samples.

2.7.10 Sample collection and laboratory simulation
Laboratory-based simulation procedures were developed to examine the rate of decay of *Ascaris* eggs during pan drying and stockpiling. Three steel containers (4.5 L each) were used in the simulation experiment with each of these containers filled with biosolid and placed in a Biological Safety Cabinet Class II system (SWF, 2013a). To provide the infrared component of the solar radiation, which is critical for pan drying process, a ceramic infrared heating system was placed in the cabinet. The temperature was maintained at ~20 °C for 17 weeks because it is the temperature in Melbourne during summer. The simulation set-up is described in Figure 2.6. Two simulation experiments were carried out; one for Cobden and another one for Rochester biosolids. Sampling was carried out specific time intervals; 0, 4, 7, 10, 13 and 17 weeks.
Figure 2.6: System for drying-pan and stockpiling simulation.

Three tanks were set up with dewatered lagoon biosolid, to allow for triplicate sampling. One biological safety cabinet was required for each simulation (Cobden and Rochester) (SWF, 2013a).

2.7.11 Determining egg viability within biosolids samples

2.7.11.1 Parasite assay chamber

Assay chambers for holding egg-biosolids mixture were prepared within a large container (4.5 L) because it was impossible to collect sufficient eggs for the direct seeding of 4.5 L of biosolids. The following method was developed by Dr Duncan Rouch and Chahak Kakar (SWF, 2013b) in-house at RMIT University and used for this phase of the work. The protocol is reproduced verbatim in the paragraph below.

The centrifuge filter’s inner tube (0.45 µm) (ODGHPC34, pk 100, Pall; UFC30HV00, Millipore) was taken out of the outer tube and by using a sterile scalpel held in the Bunsen burner for 10 seconds, the end of the outer tube was cut off. A hole was made in both the lid of the outer tube and the supplementary centrifuge tube. This was done by heating a spatula in a Bunsen burner for around 10 seconds. To roughen the caps on the outer tube and supplementary tube, a metal file was employed. The purpose of this was to encourage effective binding of the
glue. Subsequently, a membrane filter was fixed between the caps of the outer tube and supplementary tube. A mixture of a small amount of epoxy glue made using a toothpick, which was then used to apply a thin circle of glue on the two caps and surrounding the holes. Tweezers were then employed to add a filter on top of one tube and covering the hole, and this was done by firmly pressing down on the filter with the tweezers. After this, the cap of the second tube was placed over the filter and to enable effective and strong bonding, was left for 24 hours to set. Then, physical and leak tests were carried out, with a total of 18 assay chambers being prepared, 15 of which were for the simulation experiment for each mixture. Across the tops of the chambers, paired notches were cut by applying a heated scalpel knife. The same method was used to cut the bottom of the assay chamber. In order to provide support for the wire circle, the notches were cut in line. Weights (nuts) were then joined to assay chambers, in order to sink the chamber. Coloured electrical tape (yellow) was stuck around the middle of the chamber for identification purposes (Figure 2.7).

2.7.11.1 Physical tests I

The end of the tubes was examined for presence of residual glue. Some of the glue had set properly while others did not, leaving the caps open. For these tubes, the glue was scraped off and the process repeated until the cap was fully attached to the filter.

2.7.11.2 Leak tests I

Leak test 1 was performed by placing the inner tube inside the outer tube and adding 400 μL of saline solution. The cap was closed and inverted on a rack. The absence of any leakage (visual observation) validated that the tube was properly closed again.
2.7.11.2 Seeding eggs into lagoon samples

2.7.11.2.1 Cobden simulation

Recovered egg samples were centrifuged at 800 x g for 10 min. The pellets containing eggs were combined from multiple tubes and made up to a final volume of 2.5 mL. This volume was estimated to contain 610,725 eggs in 10 mL of Cobden biosolid. Approximately 500 µL of the eggs and biosolid mixture was added to sterile centrifuge tubes (~24,429 eggs per chamber).

2.7.11.2.2 Rochester simulation

Recovered egg samples were centrifuged at 800 x g for 10 minutes. The pellets containing eggs were combined from multiple tubes and made up to a final volume of 2 mL. This volume was estimated to contain 502,270 eggs in 8 mL of Rochester biosolid. Approximately 500 µL from the mixture of eggs and lagoon sample was added to each centrifuge tube (~25,113.5 eggs per chamber).
2.7.11.3 Suspending assay chambers in a simulated drying pan
A picture wire was tied in a circle around the length of the chamber and used to suspend assay chambers in the artificial lagoon. The chamber was submerged and anchored with weight nuts (Nut Hex MTRC HDG M8, pk 50, 4.6 g each, Bunnings), attached to the picture wire. The chambers were tethered with paper clips to grids located on the top of the 4.5 L biosolid containers. After attachment, the chambers were pushed down into the biosolid using a spoon previously sterilised with 70% ethanol (Figure 2.8 A and B).

(a) 

(b) 

Figure 2. 8: Positioning the assay chambers in a container.
(a) shows the container in the chamber and (b) submerging assay chambers into the biosolid using a sterilised spoon

2.7.11.4 Recovering *Ascaris* samples from assay chambers
Three assay chambers were extracted from three steel containers at each sampling point (4, 7, 10, 13 and 17 weeks) and the concentration of *Ascaris* eggs determined. Retrieved chambers were washed with RO water before being opened. A sterile scalpel heated for 15 s was used to cut the end of a 250 µL pipette tip. Using this cut tip made it easier to remove the biosolid
content of the assay chambers. RO water (250 µL) was added to the chamber and mixed with the biosolid. This mixture was transferred into a 15 mL tube and the process repeated until all the content of the assay chamber was transferred into the tube.

A 100 µL pipette was used to remove any remaining material at the bottom of the inner tube and made up to 5 mL with RO water. Then, aliquots (1 mL) were transferred into three 15 mL tubes, each containing 10 mL of 10% household bleach. The tubes were allowed to stand for up to 15 min after which the samples were washed with RO water and centrifugation at 10,000 × g for 5 min, and the final volumes made to 1 mL by adding RO water. Approximately 10 µL of this solution was examined under the light microscope to validate the bleaching process and subsequently stored at 4 °C.

2.7.12 Viability staining

2.7.12.1 Staining and recovering of Ascaris eggs from assay chambers and determining egg viability during the drying pan simulation

As suggested in the Molecular Probes Manual, a BacLight staining kit (Live/Dead® BacLight™ Bacterial Viability Kit Staining) was employed. For a short time, suspensions of the relevant biosolid samples were prepared and mixed using the vortexing technique. Following the mixing, 250 µL of three replicate samples were collected at relevant time periods and put into a 1.5 mL Eppendorf tube. Furthermore, 0.35 µL from every BacLight kit component (A and B) was applied to the samples. Aluminium foil was used to cover the tubes to keep the light out and then incubated at room temperature for 15 minutes. Following incubation, vortexing was carried out on the samples and 10 µL from each sample was placed into the wells of a 24-tissue culture plate. A Pasteur pipette (150 mm) was employed to spread the sample. Aluminium foil was then used to cover the plate before the samples were then exposed to microscopic testing through a confocal laser-scanning microscope (CLSM) (Nikon...
A1R equipped with Nikon Eclipse Ti Fluorescence Microscope) in order to see the stained eggs. From the three chambers, samples were gathered at the relevant times and the eggs were counted in triplicates (total of nine counts), as outlined earlier. (Karkashan et al., 2015) (Appendix E).

2.7.12.2 Confocal Laser Scanning Microscope (CLSM).
The stained eggs were examined with a Nikon A1R confocal microscope with using a 20 x objective (200 x total magnification). A 488 nm excitation filter and a 570-620 nm emission filter (red fluorescence) and a 488 nm excitation filter and 500-550 nm emission filter (green fluorescence (Syto 9)) were used. The channels used in this phase of work included Ch2 Alexa 488 antibody and Ch3 Texas Red. Four image types, merged, Syto9, PI and transmitted light were recorded using a Nikon CLSM (Karkashan et al., 2015).

2.7.12.3 Data Analysis
GraphPad Prism 7 (GraphPad Software) was used to perform different statistical analyses. These included T-tests and one-way ANOVA to determine the differences between each week during simulation and to compare the viable numbers of *A. suum* eggs between samples.

2.8 Determination of the bacterial community structures

2.8.1 DNA extraction from biosolid samples
DNA extractions from replicate Heyfield, Cobden and Rochester biosolid samples (0.25g) were carried out with ZR Soil Microbe DNA Kits™ (ZYMO Research Corp, CA, USA). These extracts contained DNA from bacteria and other biological entities in the selected samples. Total DNA concentrations and purity were measured with Nanodrop. An aliquot of the DNA
extracts (1 µL) was introduced unto the nanodrop. DNA concentration was determined by reading double stranded DNA absorbance of 260 nm. DNA samples were stored at -20°C until subject to downstream analyses for total bacterial community analysis using PCR-DGGE and metagenomics-based approaches.

2.8.2 Assessment of the abilities of bacterial isolates to utilize selected substrates

Biolog MT2 is a special type microplate, containing 96 wells (Fig. 2.9). Each well contains a wide variety of bacterial-buffered nutrient medium and tetrazolium redox dye. Biolog MT2 plate wells were either inoculated with biosolid samples or bacterial isolates.

Biolog MT2 microplates (tetrazolium redox dye incorporated into the wells) (Bochner, 1989) were used for assessing the abilities of bacterial isolates from the three WWTP to utilize different substrates. The substrates tested were commercially purchased chitin, protein and lipid (concentrations 0.5%) and added to desired wells. Re-suspended bacterial pellets (150 µL) were aseptically added to the desired wells (containing desired substrates) on the Biolog microplates in a Bio-cabinet. Bacterial cultures (150 µL) were also added to selected wells which did not contain any substrate and these wells were designated as control wells. The lids of inoculated plates were closed and incubated at 20 °C without any illumination. Replicated samples of selected bacterial isolates (n=3) were assessed on the three different substrates (chitin, protein and lipid). The rate of colour development in the plates was measured over time using a multiscan microplate reader (Labsystems, Finland, Multiscan EX Version 1.0) at 595 nm. Measurements were taken at each hour over time 0-6 h, every three hours from 6-18 h and every twelve hours from 18-96 h (up to 4 days) The measured absorbance values of inoculated wells with substrates and those without substrates (control wells) were deducted from the initial zero hour measured absorbance values. The final absorbance values were subsequently calculated (the OD<sub>600</sub> of control wells deducted from OD<sub>600</sub> of substrate wells).
The average absorbance values of each inoculated well were determined over the experimental time-frame.

![Biolog MT2 plates](image)

**Figure 2.9: An example of the Biolog MT2 plates used in this study.**

Blue colour indicates oxidation of the substrate occurring as a result of degradation.

### 2.8.3 Carbon substrates

#### 2.8.3.1 Chitin

Forty grams of chitin was added to 400 mL of concentrated 1 M HCl and shaken at 300 rpm for 50 minutes. Cold water (2 L, 5 to 10°C) was added to filtered chitin; the wash step was repeated until the pH reached 3.5. After that, the sample was dried at 100°C for 24 hours (Hsu and Lockwood, 1975).
2.8.3.2 Casein
Casein (10 g) was autoclaved and added into PPF and heated at 80°C for 10 minutes according to the manufacturer's protocol (Sigma-Aldrich, Australia).

2.8.3.3 Lipid
Egg yolk emulsion was purchased as a sterile liquid formula and used directly in MT2 plate (Thermo Scientific, Oxoid Microbiology Products, Australia) (Willis and Hobbs, 1959, Willis, 1977).

2.8.4 Identifying putative *Ascaris*-degrading bacteria in the biosolid samples from different lagoon systems
To identify the component of the total bacterial community that could potentially degrade *Ascaris* eggs, a microtitre plate assay approach was used (Biolog MT2 microplates). Replicate biosolid samples (1 g) from Heyfield, Cobden and Rochester were added to sterile water to obtain a 1:10 dilution. This dilution was agitated for 20 min at a speed of 200 rpm (Floch et al., 2011) cause bacteria adhering to biosolid or biosolid samples to go into solution. The resulting mixtures were diluted in phosphate buffered saline solution until the 10⁻³ diluent was achieved. Centrifugation at 1500 × g, 10 min was applied to sediment large biosolid particles. Testing of individual biosolid suspensions (n=3) was performed on the desired carbon substrates (0.5% of chitin, protein and lipid) (Sigma Aldrich, Australia) which were added to the MT2 plates based on the description by Mansur et al. (2014). Aliquots of biosolids suspension (150 μL) from the 10⁻³ diluent were added to the Biolog MT2 plates wells. Control wells (without any substrates added) were inoculated with desired biosolid sample (150 μL). Plates’ incubation, measurement of colour formation and calculations were performed as described in the previous.
Measurements were taken at each hour over time 0-6 h, every three hours from 6-18 h and every twelve hours from 18-168 h (up to 7 days)

2.8.5 DNA extraction from Biolog MT2 plates

DNA was extraction from the Biolog plates was accomplished by the use of phenol-chloroform-bead beating method (Steffan et al., 1988) after 7 days of incubation. The content of each inoculated well was aseptically removed and added to sterile 2 mL Eppendorf tubes containing sterile sodium phosphate buffer (0.5 mL, 100 mM, pH 7.5) and sterile beads (0.5 g glass beads, 300 µm, Sigma/Aldrich). The mixture was bead-beaten twice for 30 s with a Bead-Beater (Biospec Product-USA). A 10 mM Tris pH 8.0, 1 mM EDTA (0.5 mL, Sigma) saturated phenol-chloroform-isoamyl alcohol (25:24:1) solution was added to the DNA solution. Centrifugation was at 12,000 × g at 4°C for 10 min. The aqueous phase was aseptically transferred into a new sterile Eppendorf tube. Phenol-chloroform-isoamyl alcohol was added to this tube at a volume equivalent to recovered aqueous phase. Repeat centrifugation was performed at 4°C 12,000 × g for 10 min. This described phenol-chloroform-isoamyl alcohol wash repeated thrice. The resultant crude DNA extract was purified using a GENECLEAN TURBO Clean up kit (MP Biomedicals LLC). This DNA was then used in generating DGGE profiles for the microbial communities in samples subject to substrate assays.

2.8.6 PCR reactions

DNA extracts from replicate samples of the three lagoon biosolid samples (Section 2.8) and from Biolog MT2 (section 5.2.3) plates were subject to Polymerase Chain Reaction (PCR) in a 50 µL PCR master mix. The 314F-907R primer set (Muyzer and Smalla, 1998) amplifying bacterial V3 and V5 regions were used in this study. The 314F primer had a GC clamp
(5’CGCCCGCCGCGCCCGCCGGCCGGGGGGACGGGGGGCCCTACGGGAGGCAGCAG-3’) and the reverse primer was 907R (5’- ATTACCGCGGCTGCTGG-3’). The reactions were prepared using the primers 341 FGC (2 µL, 10 µM), 2 µL of 907R (10 µM) reverse primers, 25 µL of Promega GoTaq® Green Mastermix, 18 µL nuclease-free molecular grade water and 3 µL of DNA sample on a T100 Thermal cycler (Bio-Rad® Laboratories, Vic, Australia). The initial DNA denaturation was performed for 5 min at 94°C, followed by a 30-cycle amplification process, denaturing at 94°C for 5 min, annealing at 52°C for 30 s and extension at 72°C for 30 s. Amplifications were completed with a 7 min extension step at 72°C. When needed, a Touchdown PCR procedure was also applied for the amplification of DNA from biosolid samples (Table 2.1).
Table 2.1: PCR conditions for amplification of DNA from biosolids

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (minutes)</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94</td>
<td>10:00</td>
<td>Denaturation</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>1:00</td>
<td>Denaturation</td>
</tr>
<tr>
<td>3</td>
<td>52</td>
<td>1:00</td>
<td>Annealing</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>1:00</td>
<td>Extension</td>
</tr>
<tr>
<td>5</td>
<td>Repeat Step 2-4, 29 times.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>72</td>
<td>5:00</td>
<td>Extension</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>∞</td>
<td>Holding</td>
</tr>
<tr>
<td>1</td>
<td>94</td>
<td>5:00</td>
<td>Denaturation</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>1:00</td>
<td>Denaturation</td>
</tr>
<tr>
<td>3</td>
<td>65, -0.5 per cycle</td>
<td>1:00</td>
<td>Annealing</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>3:00</td>
<td>Extension</td>
</tr>
<tr>
<td>5</td>
<td>Repeat Step 2-4, 20 times</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>94</td>
<td>1:00</td>
<td>Denaturation</td>
</tr>
<tr>
<td>7</td>
<td>55</td>
<td>1:00</td>
<td>Annealing</td>
</tr>
<tr>
<td>8</td>
<td>72</td>
<td>1:00</td>
<td>Extension</td>
</tr>
<tr>
<td>9</td>
<td>Repeat Step 6-8, 15 times</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>72</td>
<td>7:00</td>
<td>Extension</td>
</tr>
<tr>
<td>11</td>
<td>12</td>
<td>∞</td>
<td>Holding</td>
</tr>
</tbody>
</table>

2.8.7 Agarose gel electrophoresis

Agarose gels (1.5%) were used for analysing DNA amplicons. The 16S rDNA amplicons (5 µL) were mixed with a loading dye (3 µL) and ran on agarose gel at 80 V and 400 A for 30 min. Gels were stained with ethidium bromide (Fluka) for 10 min then de-stained for 15-20 min with a stream of water. Finally, DNA was visualised using a Gel Doc™ (Bio-Rad
Laboratories, Gladesville, Australia) and imaging computer running Quantity One software. Gel Doc 2000 UV transilluminator (Bio-Rad, Vic, Australia).

2.9 Molecular techniques

2.9.1 Identification of bacterial isolates

Bacterial isolates of interest were cultured in nutrient broth (10 mL) for 48 h, harvested by centrifugation at 4 °C at 16,000 × g for 2 min and washed twice with sterile distilled water. DNA extraction of the pellet was carried out using ZR Soil Microbe DNA Kit™ (Zymo Research, USA) according to the manufacturer’s protocol. The purified DNA samples were subject to PCR using a 50 μL PCR master mix (Go Tag® green master mix). Each PCR reaction was prepared using bacterial primers 63f (10 μM) (5′CAGG CCTAACACATGCAAGTC-3′) as the forward primer and 1389r (10 μM) (5′-ACGGGCGGTGTACAAG-3′) as the reverse primer, together with 3 μL of purified DNA. The PCR thermocycling conditions were an initial denaturing step of 95 °C for 5 min and then 95 °C for 1 min 65 °C for 1 min and 72 °C for 2 min (10 cycles in touchdown mode from 65 to 55 °C (1 °C decrease per cycle) and 20 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min followed by a final extension step at 72 °C for 10 min. Amplified DNA were cleaned up, sequenced and their putative identities determined as described in Section 2.9.3.3.

2.9.2 Denaturing gradient gel electrophoresis (DGGE)

2.9.2.1 Assembly of DGGE plates

Two clean glass plates were used (200 mm x 180 mm and 200 mm x 160 mm) (Bio-Rad, U.S) for DGGE. A backing film, PAG bond paper (Cambrex Bioscience Rockland Inc, Rockland, U.S.A) (required for silver staining) was carefully attached to the smaller plate (avoiding the
formation of air bubbles) by adding a thin film of water to the hydrophobic side of this paper. This plate with the film (on the inside) was placed onto the big plate (200 mm x 180 mm) with the hydrophilic side facing the big plate. Two thin spacers were carefully inserted in between the plates on either side and the plates clamped with Bio-Rad clamps.

2.9.2.2 Setting of gels

The assembled plate was placed upright in the casting stand, the bottom blocked with parafilm and plugged with 1-2 mL of cross-linked 0% denaturant to prevent the leaking of denaturing gel. Polymerization of the denaturing gel was carried out through the addition of 1% (v/v) of a 10% APS solution and 0.1% (v/v) of Temed (N, N, N, N'-tetra-methyl-ethylene diamine) to the denaturant. The cross-linked 0% denaturant was allowed to set for up to an hour.

Two different concentrations of denaturing solutions (low and higher concentrated denaturants prepared based on experimental requirements) were used to form a gradient. The two cross-linked denaturants were loaded into tubes (30 mL) at a volume of 12.5 mL each (125 µL of 10% APS, 12.5 µL of Temed) and discharged via a T junction into the plates by means of a gradient discharger Model 475 (Bio-Rad, Inc., CA, USA). This led to the higher concentrated denaturant being discharged before the lower concentrated denaturant. A gel with an increasing parallel denaturant concentration was therefore formed which was set after a 60 min waiting period. A well comb (Bio-Rad, USA) was inserted into the set gel and filled with 4 mL cross-linked 0% denaturant, and allowed to set for up to 30 min. In this way, wells into which samples would be loaded were created. After setting, the comb was removed, and the formed wells flushed with 1X TAE. PCR products from desired samples or cultures, mixed with loading buffer (containing equal volumes of fast orange G, bromophenol blue, xylene CFF) were loaded into the DGGE
well. DGGE markers made up of a mixture of PCR products generated from purified DNA fragments of pure microbial cultures were loaded in appropriate lanes as controls.

2.9.2.3 Denaturing gradient gel electrophoresis

PCR products were analysed using a Universal Mutation Detection System (Bio-Rad Inc., CA, USA) using 6% acrylamide: bis gel for the lower denaturant and 12% acrylamide: bis gel for the higher denaturant. For the analysis of 16S rDNA, a 40 to 60% urea-formamide denaturant gradient was used. The gel was run for 18-20 h at 60°C and 60 V for bacterial analysis. This was fixed overnight in fixing solution I which was poured off before a 0.2 % silver nitrate solution was added. The gel was then gently shaken on a A600 Rocker (Denley, UK) before being placed in developing solution for at least 20 min or until the desired exposure was attained. The developed gel was then placed in fixing solution II for 10 min and preserved in preservative solution for 10-20 min.

The components of the solutions used to develop the DGGE gels are presented below:

<table>
<thead>
<tr>
<th>Denaturant Type</th>
<th>Volume (mL)</th>
<th>Solution Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% denaturant</td>
<td>15</td>
<td>40% Acrylamide: bis solution (37:1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>50 X TAE</td>
</tr>
<tr>
<td></td>
<td>83</td>
<td>dd H$_2$O</td>
</tr>
<tr>
<td>40% denaturant (pH 8.0)</td>
<td>15</td>
<td>40% Acrylamide: bis solution (37:1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>50 X TAE</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>Formamide</td>
</tr>
<tr>
<td></td>
<td>16.8 g</td>
<td>Urea</td>
</tr>
<tr>
<td>Denaturant made up to</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>60% denaturant (pH 8.0)</td>
<td>30</td>
<td>40% Acrylamide: bis solution (37:1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>50 X TAE</td>
</tr>
<tr>
<td></td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>Component</td>
<td>Quantity</td>
<td>Description</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>----------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>28 mL Formamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25.2 g Urea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturant made up to 100 mL with dd H_2O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium persulphate solution*</td>
<td>0.1 g</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td></td>
<td>1 mL</td>
<td>dd H_2O</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.1%</td>
<td>TEMED solution</td>
</tr>
<tr>
<td>Fixing solution I</td>
<td>50 mL</td>
<td>Ethanol (100%)</td>
</tr>
<tr>
<td></td>
<td>2.5 mL</td>
<td>Glacial acetic acid</td>
</tr>
<tr>
<td></td>
<td>447.5 mL</td>
<td>dd H_2O</td>
</tr>
<tr>
<td>Fixing solution II</td>
<td>3.75 g</td>
<td>Sodium carbonate</td>
</tr>
<tr>
<td></td>
<td>500 mL</td>
<td>dd H_2O</td>
</tr>
<tr>
<td>Silver nitrate solution*</td>
<td>0.2 g</td>
<td>Silver nitrate</td>
</tr>
<tr>
<td></td>
<td>200 mL</td>
<td>dd H_2O</td>
</tr>
<tr>
<td>Developing solution*</td>
<td>0.02</td>
<td>Sodium borohydride</td>
</tr>
<tr>
<td></td>
<td>0.80 mL</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td></td>
<td>3 g</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td></td>
<td>200 mL</td>
<td>dd H_2O</td>
</tr>
<tr>
<td>* Solutions were prepared just before use.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preservative solution</td>
<td>125 mL</td>
<td>Ethanol (100%)</td>
</tr>
<tr>
<td></td>
<td>50 mL</td>
<td>Glycerol</td>
</tr>
<tr>
<td></td>
<td>325 mL</td>
<td>dd H_2O</td>
</tr>
</tbody>
</table>
2.9.3 Denaturing gradient gel analysis

2.9.3.1 Similarity relationships

DGGE gels were scanned with Epson Expression 1600 Pro as tiff. files before being analysed with Phoretix 1D advanced analysis package (Phoretix Ltd, UK). The relatedness of the microbial communities was expressed as similarity coefficients. UPGMA dendrograms were also generated using the Phoretix 1D advanced analysis package. The software uses a sequential clustering algorithm to generate hierarchical similarity relationships between samples based on the degree of similarities between the bands in different samples on a DGGE gel (Krebs, 1999). The algorithm used is expressed in the equation below:

\[
\text{Similarity between a sample and an existing cluster} = \frac{1}{t_J t_K} \left( \sum S_{J(K)} \right)
\]

Where;

- \( S_{J(K)} \); similarity between two clusters J and K
- \( t_J \); number of samples in cluster J (\( \geq 1 \))
- \( t_K \); number of samples in cluster K (\( \geq 2 \))

2.9.3.2 Diversity and equitability index

The intensities of bands on DGGE community profiles were calculated using Phoretix 1D advanced analysis package and the Shannon Weaver diversity (\( H' \)) and equitability index (J) determined. The Shannon Weaver diversity index is a general diversity value which reflect the number of species (bands). The equitability index is a measure of the relative abundance of the different species (bands) in the sample (Dilly et al., 2004, Krebs, 1999). Gaussian volumes
generated from profile deconvolution analysis were used for analysis. The noise levels and minimum peak thresholds of the software were set at optimum values to eliminate background noise peaks allowing the detection of only genuine peaks. The diversity and the equitability index were then calculated using the formula below;

\[ H' = -\sum p_i \ln p_i \]

Where

\( H' \); is the Shannon Weaver diversity index.

\( p_i \); is the proportion of the community that is made of species \( i \) (intensity of the band \( i \) / total intensity of all bands in the lane).

\( \ln p_i \); is the natural log of \( p_i \).

\( J = H' / (\ln n_i) \)

Where;

\( J \); is the Shannon Weaver equitability index.

\( \ln n_i \); is the natural log of the total number of species in a lane.

2.9.3.3 Recovery of bands from DGGE gels and sequence analysis.

Bands selected for further investigations (sequence analysis) were retrieved from the DGGE profiles of samples obtained from Biolog MT2 plates. Bands were aseptically excised and placed into a 2 mL Eppendorf tube containing 50 µL of sterile water. This tube was incubated overnight at ambient temperature. DNA re-amplification (PCR) was carried out in a 50 µL PCR master mix. with 2 µL each of Universal bacterial Muyzer primers 341 FGC (10 µM) and 907R (10 µM), 25 µL Promega GoTaq® Green Mastermix, 18 µL nuclease-free molecular grade water and 3 µL of DNA from incubated bands. Molecular grade nuclease-free water was used.
as inoculant for the negative controls of all performed PCR assays. The PCR program used for re-amplification was as followed; the first denaturation cycle was performed for 5 min at 94°C; followed by a 35-cycle amplification process; denaturing at 94°C for 5 min, annealing at 52°C for 30 s and extension at 72°C for 30 s. Amplifications were completed with a final extension step at 72°C for 7 min (Mansur et al., 2014). Amplicon purification was carried out with PCR clean-up kit (Wizard SV PCR clean-up system, Promega, USA). Nanodrop (Thermo Scientific, USA) was used for DNA quantification and DNA sequencing performed at the Australian Genome Research Facility (AGRF), Melbourne (www.agrf.org.au). DNA sequence data were analysed with Sequencher (version 5) (Gene Codes Corporation, USA) and the aligned sequences submitted to BLASTN to determine their putative identities.

2.9.3.4 Metagenomics analysis

Purified DNA extracts (Section 2.8) from replicate Heyfield, Cobden and Rochester biosolid samples were subject to Next Generation Sequencing via the Illumina platform. Metagenomic analyses were carried out as previously described (Koshlaf et al., 2016). DNA libraries were prepared with Nextera® XT Index Kit (Illumina, San Diego, CA) as described in Illumina’s 16S Metagenomic Sequencing Library Preparation Protocol for replicate biosolid samples. Library quantifications of these samples were carried out on a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Samples were pooled together, and sequencing performed on a MiSeq platform (Illumina, San Diego, CA) at RMIT University (Koshlaf et al., 2016). Despite repeated attempts, the DNA extracted from the MT2 Biolog plates Section 2.10) were not of sufficiently good quality and so metagenomic analyses were not carried out on these samples.
2.9.3.5 Statistical Analyses

Bacterial community fingerprint analysis was undertaken using the computer software Phoretix 1D as described in previous sections (Muyzer et al., 1993; Patil et al., 2010). The Shannon Diversity index ($H'$) (Dice-Sorensen index) was calculated to evaluate the diversity of the bacterial community of each biosolid sample based on their DGGE profiles (Girvan et al., 2003). Community evenness and functional organization in the biosolid samples were analysed using the Pareto–Lorenz (PL) distribution curve (Marzorati et al., 2008; Erklens et al., 2012). DGGE gels were analysed using Phoretix TotalLab 1D. Band intensities (Gaussian values) were ranked from high to low and the normalized cumulative intensities of these bands were plotted on the y-axis. The cumulative normalized band numbers were plotted on the x-axis. The community was analysed at 20% (0.2 × axis intercept) of the population to determine the proportional cumulative intensities with a line of perfect evenness drawn at 45% (Erkelens et al., 2012, Marzorati et al., 2008). Principal-component analysis (PCA) was performed by XLSTAT. Data were subjected to ANOVA (SPSS 25) to determine whether there were significant differences between the biosolid bacterial communities.

2.10 Bacterial Isolations

Microbial (bacteria) isolations using culture media were performed on biosolid samples from three sources: (i) WWTP Cobden, (ii) WWTP Rochester, and (iii) WWTP Hayfield. From each source, 1 g of biosolid sample was aseptically added to sterile distilled water (9 mL) and subject to serial dilution with phosphate-buffered saline (PBS) solution until the $10^5$ dilution ratio was attained. These dilutions were carried out in triplicate for each sample. Aliquots of selected diluents were plated on Bushnell Hass (BH) mineral salts medium (Bushnell and Haas, 1941). This medium does not contain a carbon, protein or lipid source allowing it to be supplemented with any of these compounds. BH agar plates, containing 0.5 % (v/v) of either
chitin, protein or lipid (Sigma Aldrich, Australia) (as substrates) were inoculated using a spread plate method. The inoculated plates were at 20 °C for up to 36 hours or 3 days. Detected colonies were purified by repeated sub-culturing on BH agar and nutrient agar plates. Purified isolates were aseptically added to nutrient broth (10 mL; Acumedia, Lansing, MI, USA). Inoculated tubes were incubated at 20°C for 48 h at 120 rpm for the production of bacterial cells for long-term storage. Desired cultures were preserved at −80 °C in 20 % glycerol until needed for other downstream processes such as using MT2 plate-based assays to screen them for their abilities to utilize Ascaris sp. eggs components.

2.11 Preparation of bacteria isolates for Biolog MT2 plates-based assay
Bacterial isolates from stock cultures were reactivated by adding 0.5 mL of stock bacterial culture to 15 mL of nutrient broth (15 mL). The inoculated broth was at 120 rpm for 48 hours shaken at 20 °C. This incubated broth was centrifugation for 2 min at 16,000 × g. The centrifuged culture was with MilliQ grade water (twice) and standardized (Kadali et al., 2012). The supernatant was discarded and the culture pellets re-suspended in sterile water (1 mL) prior to use.

2.12 Effect of bacterial growth on the die-off rates Ascaris spp. eggs
The effects of selected bacterial isolates from the different WWTP samples on the die-off rates of Ascaris spp. eggs were assessed using a culture-based approach.

2.13 Effect of bacterial growth on the decay of Ascaris spp. eggs
The three selected bacterial isolates, #16, #42 and #26 were grown in broth culture for 24 h and aliquots of the culture centrifuged. The supernatant (cell-free), washed bacterial pellets and the
uncentrifuged cultures were applied to *Ascaris* eggs to assess their effects on the decay of the eggs using replicate samples. Sterile tubes containing 300 µL of PBS buffer and 3,000 eggs of *Ascaris* were used for the different listed assays;

(i) For cell free-based assays, 300 µL of culture supernatant was added to the PBS-eggs buffer at 1X.

(ii) For the uncentrifuged bacterial broth, 300 µL of the broth at 1 X concentration was added to replicate PBS-eggs buffers.

(iii) The bacterial pellets were dissolved in 300 µL of PBS buffer (pH 7.2) and added to designated tubes at 1X concentration.

(iv) An equal volume of the uncentrifuged broth of the three bacterial species (100 µL each) was mixed together and added to the PBS-eggs buffer.

(v) For control samples, 300 µL of sterile nutrient broth was added the PBS-eggs buffer

All the tubes were incubated at 20°C and at 120 rpm and the decay of *Ascaris* eggs was observed by microscopy every 5 days over a 30 day-experimental period.

### 2.14 Commercial enzyme-based assays

#### 2.14.1 Effects of commercial enzymes on the decay of *Ascaris* sp. eggs

The effects of commercially available enzymes on the decay of *Ascaris* eggs were also assessed. Commercial variants of chitinase, lipase and proteinase (purchased from Sigma, Australia, Table 1) were used to assess their effect on the decay of *Ascaris* eggs. Five U of each enzyme was taken and dissolved in 300 µL PBS buffer pH 7.2 and added to 300 µL PBS buffer containing 3,000 eggs and incubated as earlier described. These experiments were carried out in triplicate. In addition, assays were carried out to assess the combined effects of these enzymes on egg decay. This involved adding 300 µL of enzyme mixture (100 µL of each enzyme) to PBS buffer containing 3,000 eggs and incubating the mixture as earlier described.
The decay of the eggs was observed every 5 days for 30 days. The decay of eggs was observed by microscopy.
CHAPTER 3:

Assessing the suitability and efficacy of the Tulane assay for detecting Ascaris eggs in lagoon samples from Victorian Wastewater treatment plants.
3.1 Introduction

There is an extensive history of wastewater use in the agricultural industry, with treated or untreated wastewater being used in irrigating up to 20 million hectares of agricultural land worldwide (Sengupta et al., 2011, Jiménez and Asano, 2008, Contreras et al., 2017). Crop irrigation with untreated wastewater is dangerous as it poses considerable risks to human health via consumption of contaminated or poorly washed fresh produce such as vegetables and crops which have accumulated pathogens or toxic chemical compounds from the wastewater (Sengupta et al., 2011). Pathogens include bacterial, viral and parasitic pathogens, of which one or more are generally found in untreated, occasionally treated and partially treated wastewaters (Sengupta et al., 2011, Karkashan et al., 2015, Sidhu and Toze, 2009).

Among parasites, helminth parasite eggs which are often found in untreated wastewater (Sengupta et al., 2011). As a result of common inefficient treatment, an excessive number of cases of helminthic diseases has been reported in lower-middle-income and low-income nations. These include infections caused by roundworms (*Ascaris lumbricoides*), hookworms (*Ancylostoma duodenale*) and whipworms (*Trichuris trichiura*) (Amoah et al., 2017). Helminth parasites are thought to survive in wastewater for a long time, up to 15 years (Sengupta et al., 2011, Tudor, 2015, Mitrea, 2011, Bowman et al., 2003, Stott, 2003, Yaya-Beas et al., 2016, Gaspard and Schwartzbrod, 2003, Zdybel et al., 2016, Rocha et al., 2016b, Black et al., 1982, Brownell and Nelson, 2006). Therefore, the safe use of wastewater in irrigated agriculture should include wastewater treatment processes which lead to the elimination of helminth eggs (Stott, 2003, Sengupta et al., 2011). The World Health Organization (WHO) in its latest updates has recommended that less than one helminth egg per litre of wastewater should be present in wastewater being used in agriculture, in the absence of the availability of any other risk reduction options (WHO, 2006). In addition, less than 1 helminth egg in 4 g dry soil (DS) is
required by the USEPA part 503 biosolids rule (USEPA, 2003, Bowman et al., 2003, Karkashan et al., 2015, Berendes et al., 2015).

*Ascaris lumbricoides* is one of the most important intestinal worms (the human roundworm) in human beings. It is estimated that about 1.2 billion people are affected by ascariasis worldwide (Sengupta et al., 2011, Crompton, 2001, Bethony et al., 2006, Karkashan et al., 2015, Dold and Holland, 2011, Rai et al., 2000, Lamberton and Jourdan, 2015, Steinbaum et al., 2017). For ease of availability, researchers have chosen an alternate model for *A. lumbricoides* (the human pathogen), which is *Ascaris suum* (pig roundworm) because they are similar in the morphology, life cycle and *A. suum* may cross-inflect humans (Jeandron et al., 2014, Sá et al., 2017).

It is often challenging to recover the eggs of *Ascaris* sp. from biosolids material because the eggs are very small in size (45-75 µm in length and 35-50 µm in width) and can be trapped and effectively hidden within the particles of biosolids material (Charitha et al., 2013, Amoah et al., 2017, Ash and Orihel, 2007). At present, researchers have no technique to recover 100% of the eggs from biosolids (Hawksworth et al., 2012, Steinbaum et al., 2017). However, in the last few years, different culture-based methods (USEPA, 2003) involving centrifugation have been developed to detect, recover and identify helminth eggs in wastewater (Bowman et al., 2003, Sá et al., 2017, Charitha et al., 2013, Maya et al., 2006). A brief review of these methods (termed conventional methods) compared to newer molecular techniques (PCR, qPCR, LAMP) and emerging techniques (digital PCR, BacLight assay) has been carried out (Amoah et al., 2017). This review concluded that the conventional methods remain the easiest and cheapest group of methods to use.

The Tulane method in particular (Bowman et al., 2003) is thought to be the best technique for egg recovery because it can be used for samples with both low and high solids content, allows the recovery of *Ascaris* eggs with varying specific gravities, and has the lowest cost (USEPA,
2003). However, it is a time-consuming process and may not be suitable when samples results are urgently required (Traub et al., 2004, Traub et al., 2007, McCarthy et al., 2012). Another method is the sedimentation and flotation method. It is basically a combination of two methods and is now widely used to recover helminth eggs from soils, faeces and biosolids (Bowman et al., 2003, Sá et al., 2017, Dryden et al., 2005, Karkashan et al., 2015, Amoah et al., 2017).

The main objective of this chapter was to (i) evaluate the suitability of the Tulane method for the detection *Ascaris* spp. eggs in different lagoon samples in Victoria and (ii) determine the efficiency of this recovery method for each lagoon sample from different waste treatment plants.
3.2 Results

3.2.1 Dry weight determination of biosolid

The average dry weight results of replicate samples (n=3) of the three different WWTPs are presented in Table 3.1. The dry weight is important when using the Tulane method because at least 5 g dry weight (Total Solids (TS)) of biosolid sample is needed to recover the eggs. From the results presented, the amount of wet biosolid containing 5 g total solids was calculated to be between 50 to 200 g wet weight.

Table 3.1: Biosolid requirements (in terms of Total Solids) for the Tulane method

<table>
<thead>
<tr>
<th>% Dry Solids Value</th>
<th>Total Solids g/100 mL</th>
<th>Volume Biosolid Required (mL) for Tulane method (min 5 g TS)</th>
<th>Total Solids being Tested (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3</td>
<td>200</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>90</td>
<td>8.1</td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>50</td>
<td>7.5</td>
</tr>
</tbody>
</table>

3.2.2 Nutrient analysis

The results (averages) of the nutrient analyses of replicate samples (n=3) of the three different types of biosolids are shown in Table 3.2. The concentration of total nitrogen was only similar in Heyfield and Rochester samples ($2.9 \times 10^4$ mg kg$^{-1}$ and $2.6 \times 10^4$ mg kg$^{-1}$ respectively). Levels of soluble phosphate (Olsen P) were lowest in Rochester biosolid samples at $1.3 \times 10^4$ mg kg$^{-1}$. 
Table 3.2: Physiochemical characteristics of biosolid from WWTP

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Units</th>
<th>Lagoon Biosolid from Heyfield WWTP, ~15% DS</th>
<th>Lagoon Biosolid from Cobden WWTP, ~3% DS</th>
<th>Biosolid from Rochester WWTP, ~9% DS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate + Nitrite, as N</td>
<td>mg N / kg</td>
<td>1.3</td>
<td>5.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Total Kjeldahl Nitrogen</td>
<td>mg/kg</td>
<td>2.9 x 10^4</td>
<td>4.7 x 10^4</td>
<td>2.6 x 10^4</td>
</tr>
<tr>
<td>Total Nitrogen</td>
<td>mg N / kg</td>
<td>2.9 x 10^4</td>
<td>4.7 x 10^4</td>
<td>2.6 x 10^4</td>
</tr>
<tr>
<td>Olsen P</td>
<td>mg/kg</td>
<td>4.4 x 10^2</td>
<td>5.3 x 10^3</td>
<td>1.3 x 10^3</td>
</tr>
</tbody>
</table>

3.2.3 Eggs number and viability in quality control sample

The number of recovered eggs was counted in triplicate using universal worm eggs counting chambers. The total number of eggs recovered from the pig faecal sample was 55,490 eggs in a total volume of 10 mL. By using the conventional incubation method, an average of 83 eggs was classified as viable and 3 eggs non-viable for every ~15 µL of egg suspension (96% viable eggs and 4% as non-viable eggs) (Table 3.3). Incubating the eggs at 28 °C caused the larvae to become mobile with some worms being observed to move out of the weakened the egg shell (hatching). Eggs in which fully developed larvae were observed were deemed to be viable. Unfertilized eggs were characterized by longer oval shapes (Figure 3.1).
Table 3.3: Percentage of viable and non-viable eggs *Ascaris suum* determined after incubation for 28 days.

<table>
<thead>
<tr>
<th>Incubated</th>
<th>Replicate</th>
<th>Viable eggs</th>
<th>Non-viable eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st incubation</td>
<td>1</td>
<td>80</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>82</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>82</td>
<td>4</td>
</tr>
<tr>
<td>2nd incubation</td>
<td>1</td>
<td>82</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>83</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>84</td>
<td>4</td>
</tr>
<tr>
<td>3rd incubation</td>
<td>1</td>
<td>85</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>81</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>83</td>
<td>3</td>
</tr>
<tr>
<td>Mean</td>
<td>9</td>
<td>82</td>
<td>3</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>1.51</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>(%)</td>
<td>96</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1: *Ascaris suum* eggs after incubation for 28 days at 400 x magnification.

The image was captured using a Leica DM2500 microscope. The outer shell was removed by 10% household bleach (A) Viable *Ascaris suum* with fully developed larva is exiting the eggs shell (B) Viable *Ascaris suum* eggs (C) Non-Viable (unembryonated) *Ascaris suum* eggs.

3.2.4 Egg suspension

Ascaris eggs were serially diluted a number that is easily counted prior to assessing their recovery rates in biosolids. Suspension A was determined to contain approximately 744 ova of *Ascaris suum*/mL. All serial dilutions were counted and the 1:2, 1:8 and 1:32 dilutions were used in the recovery rate experiment. (Table 3.4). Note that the original suspension contained 14,880 eggs and dilutions were prepares in the appropriate %DS.

<table>
<thead>
<tr>
<th>Dilution Factors (DF)</th>
<th>A (1:2)*</th>
<th>B (1:4)</th>
<th>C (1:8)*</th>
<th>D (1:16)</th>
<th>E (1:32)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Approx. no. of eggs/10 mL</td>
<td>7440</td>
<td>3720</td>
<td>1860</td>
<td>930</td>
<td>465</td>
</tr>
</tbody>
</table>

*: the dilution that has been used in this experiment.
3.2.5 Parasites level in biosolid

No eggs of *Ascaris* spp. were observed or detected in any of three biosolid types (SWF, 2013a) (Table 3.5) prior to being inoculated with eggs from the pig's faecal materials.

<table>
<thead>
<tr>
<th>Biosolid origin</th>
<th><em>Ascaris lumbricodes</em> (eggs/g DS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heyfield 15% DS (centrifuged)</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Cobden 3% DS (settled)</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Rodchester 9% DS (settled)</td>
<td>&lt;0.2</td>
</tr>
</tbody>
</table>

3.2.6 Recovery rate of *Ascaris* eggs in biosolid and limit of detection

Table 3.6 showed that the recovery efficiencies of *A. suum* eggs from biosolid samples via the Tulane method was between 33.3 % to 73.3 %. The recovery efficiency and egg number had an inverse relationship, with the highest recovery efficiency observed when the lowest numbers of eggs were used to inoculate the biosolid (Figure 3.2). The relationship between egg number and recovery efficiency was calculated by pooling the data for all samples, and determined with this equation:

\[
\text{Recovery efficiency (\%)} = -0.004484 \times X + 71.15, \quad \text{where } X \text{ is the number of eggs in the sample}
\]

\[
R^2 = 0.9146, \quad P = <0.0001
\] (Figure 3.3).

Since the recovery efficiency increased with the decline in the egg concentration, determining the detection limit from the biosolid samples with the highest dilution rate of eggs (1:32) was logical. Using the standard recovery method for biosolid aliquots from Heyfield, the theoretical limit of detection of total eggs (viable and non-viable) corresponded to 1 egg/7.5 g⁻¹ DS or 0.13 eggs g⁻¹ DS. The efficiency at the 1:32 dilution equates to 73.3%. With regards to the
biosolid from Rochester, the theoretical limit of detection was 0.12 eggs g$^{-1}$ DS, or 1 egg/8.1 g$^{-1}$ DS, which represented the maximum recovery efficiency (66%); the biosolid from Cobden had a theoretical limit of detection of 0.16 eggs g$^{-1}$ DS or 1 egg/6 g$^{-1}$ DS, and the maximum recovery efficiency (63.6%). The average recovery efficiency percentage determined at the highest egg dilution rate for the standard solids value (5 g DS biosolid samples) was found to be 67.6% (Karkashan et al., 2015).

Table 3.6: Recovery efficiencies of *Ascaris suum* eggs seeded into lagoon-treated sewage biosolid samples (Karkashan et al., 2015).

<table>
<thead>
<tr>
<th>Biosolid origin</th>
<th>DS content</th>
<th>Dilution Factor (DF)</th>
<th>Theoretical total no. of eggs added</th>
<th>Total no. of eggs recovered</th>
<th>Mean and standard deviation of recovery rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WWTP 3%</td>
<td>1:2</td>
<td>7,440</td>
<td>3,109</td>
<td></td>
<td>41.8±12.1</td>
</tr>
<tr>
<td>Cobden</td>
<td>1:8</td>
<td>1,860</td>
<td>1,111</td>
<td></td>
<td>59.7±7.0</td>
</tr>
<tr>
<td></td>
<td>1:32</td>
<td>465</td>
<td>295</td>
<td></td>
<td>63.6±2.4</td>
</tr>
<tr>
<td>WWTP 9%</td>
<td>1:2</td>
<td>7,440</td>
<td>2,765</td>
<td></td>
<td>37.2±9.5</td>
</tr>
<tr>
<td>Rochester</td>
<td>1:8</td>
<td>1,860</td>
<td>1,197</td>
<td></td>
<td>64.4±5.4</td>
</tr>
<tr>
<td></td>
<td>1:32</td>
<td>465</td>
<td>307</td>
<td></td>
<td>66±1.8</td>
</tr>
<tr>
<td>WWTP 15%</td>
<td>1:2</td>
<td>7,440</td>
<td>2,480</td>
<td></td>
<td>33.3±8.9</td>
</tr>
<tr>
<td>Heyfield</td>
<td>1:8</td>
<td>1,860</td>
<td>1,297</td>
<td></td>
<td>69.7±5.0</td>
</tr>
<tr>
<td></td>
<td>1:32</td>
<td>465</td>
<td>341</td>
<td></td>
<td>73.3±2.4</td>
</tr>
</tbody>
</table>

WWTP: Wastewater treatment plants. Dry solids (DS) contents from three wastewater treatment plants in Victoria, Australia determined using the conventional Tulane technique.
Figure 3.2: *Ascaris suum* eggs (200×) in biosolid samples.

Some debris is present in the eggs. The image was captured by using Leica DM2500.

Figure 3.3: The Relationship between *A. suum* eggs number and recovery efficiency.

Linear regression was calculated using GraphPad software. Data presented with 95% upper and lower confidence interval.
3.3 Discussion

The standard method of detecting viable and non-viable helminth eggs is the Tulane method (Bowman et al., 2003). This study assessed the suitability of this method to count helminth eggs in control samples (pig faeces), and biosolid samples from three different wastewater treatment plants. The findings from this study suggest that this method was suitable for helminth egg recovery in all the samples tested.

Ninety-six percent of the recovered eggs were calculated as viable from pig faeces (Table 3.3). This indicated a high percentage of active worm infection in donor pigs. *Ascaris* eggs have two forms; fertilized and unfertilized (Peng et al., 2003b). During helminth egg incubation, only fertilized eggs will develop into larval forms. That 3% of the eggs were evaluated as non-viable might be related to the fact that they were unfertilized and less likely to the 4°C storage conditions used in this study. Other storage conditions such as eggs storage in 0.5% formalin at 28°C have been shown to have negligible effects on egg viability (Ash and Orihel, 2007).

When the magnesium sulfate solution’s specific gravity was higher than 1.20, the heavier particles present in the sediment floated alongside the eggs of *Ascaris*. However, when the specific gravity was below 1.20, no floatation of *Ascaris* eggs occurred. Therefore, the magnesium sulfate solution’s specific gravity was kept between 1.05 and 1.20 allowing eggs to float and be easily collected while the debris sank (Bowman et al., 2003, Dryden et al., 2005).

No *Ascaris* eggs were detected in any of the three biosolid samples evaluated (Heyfield, Rochester and Cobden biosolids). The *Ascaris* spp. level in the three WWTPs was less than 0.2% /g DS, and suggested little or low-level incidence of *Ascaris* in wastewater biosolids from Victoria. This is not unusual as several other studies have confirmed that the rates of helminth infections are extremely low in Victoria (Irwin et al., 2017). It was therefore highly unlikely
that this method was not suitable for detecting *Ascaris* spp. but rather eggs were not present in the samples used for this study (Steinbaum et al., 2017).

Steinbaum et al. (2017) reported that *Ascaris* larvae can hatch from eggs in soil. In seeded soil samples, other studies have used a similar protocol as the one used in this study (Steinbaum et al., 2017, Charitha et al., 2013, Baker and Ensink, 2012), with larvae being detected in some samples from one of these studies with a viability of 58%. This shows that there may be a high health-risk of using untreated wastewater for agricultural purposes.

Several research workers have developed different techniques to recover helminths eggs from biosolids (Sá et al., 2017). Bean and Brabant showed 12.02% recovery for *Ascaris* eggs in 2001 (Bean and Brabants, 2001) while Huyard et al. reported a rate of recovery of 50% (Huyard et al., 2000). Bowman et al. reported a 60% recovery of the eggs (Bowman et al., 2003). The rate of recovery reported by Charitha et al. (2013) was 31%. Other reports state recovery efficiencies of 25% and 72.7% respectively (Collender et al., 2015, Steinbaum et al., 2017). Overall, compared to previously published studies, this study showed a higher egg recovery efficiency 73.3% (Karkashan et al., 2015) (Table 3.5). This value was obtained with Heyfield biosolid while the lowest recovery percentage, 63.6% was obtained in Cobden biosolid. The source of the wastewater biosolid therefore affected the egg recovery efficiency. While the reason for this was not investigated in this study, it could be related to the different sources of wastewater and processing conditions used at these different wastewater treatment plants. It was also observed in this study that the recovery rate improved as the total number of eggs originally seeded into the biosolid decreased. The egg recovery efficiency was inversely related to the number of eggs used for biosolid inoculation.
Sample weight may play a critical role in the number of eggs recovered (Amoah et al., 2017). For example, it was observed that the recovery of *Ascaris* eggs from 50 g biosolid sample was higher than from 200 g of biosolid (Bean and Brabants, 2001). Some authors have observed that the recovery of eggs can be affected by centrifugal flotation. In addition, sample size, soil texture, pretreatment, the degree of soil contamination, flotation time and solution (Zenner et al., 2002) can affect egg recovery rates. Other factors such as the use of Falcon tubes and pipettes can affect egg recovery rates (Jeandron et al., 2014). Generally, eggs showed a greater adherence to plastic material than glass slides; this might be because of the lower electrostatic attraction (Kleine et al., 2016).

### 3.4 Conclusions

In conclusion, this study showed that the recovery rates of *A. suum* eggs from wastewater samples was very variable. The results showed that the Tulane method was suitable and led to the recovery of higher numbers of *A. suum* and can be used for biosolid in Victoria. Egg recovery efficiency was inversely related to the concentration of egg inoculants with Heyfield biosolid having the highest recovery efficiency of 70% and Cobden biosolid samples with the lowest at ~64%.
CHAPTER 4:

Evaluating the die-off rates of *Ascaris suum* eggs in laboratory-based simulation assays.
4.1 Introduction

*Ascaris lumbricoides* is a nematode found in soil which is known to cause human infections. These infections occur through the oral route when food contaminated with embryonated nematode eggs are consumed (Bethony et al., 2006, Kim et al., 2012). *Ascaris lumbricoides* infections are common in countries with moist and warm soils such as those found in developing subtropical and tropical countries in Asia and Africa (Bangladesh, Pakistan, India, Ghana and Nigeria). *Ascaris lumbricoides* is of significant public health concern in human population especially in children, as they are the most commonly infected group. These worm infections, alongside endemic malnutrition and infection by other helminths, have been associated with impaired cognitive development and increased risks of asthma development in children (Bethony et al., 2006, Palmer et al., 2002, Ezeamama et al., 2005).

This parasite infects human beings, but its eggs are commonly found in soils, especially those exposed to sewage and animal wastes. Its prevalence in soil is affected by different factors such as the viability of parasite eggs, temperature, pH, moisture content, soil ammonia content and humidity. A common source of *Ascaris* eggs is sewage biosolid. Sewage biosolid is a product of physical, biological and chemical (precipitation with lime, ferric chloride, or alum) treatment of wastewater with treatment methods determining the prevalence of pathogens in biosolid (Straub et al., 1993). The biosolid or biosolid produced is rich in nutrients and can be applied to agricultural land reducing or eliminating the need for fertilizers (Ghiglietti et al., 1997). The different methods of biosolid (biosolid) treatment in Australia and guidelines on the use of biosolids based on Environmental Protection Authority and National Water Quality Management Strategy (NWQMS) documentations have been described (O’ Connor et al., 2017).
Reuse of wastewater/biosolids/biosolids that have been inadequately treated for helminths removal in agriculture can cause increased infections by *Ascaris lumbricoides*. *Ascaris* eggs can survive in conditions typical for biosolids (low moisture conditions) and are resistant to different treatment conditions, remaining viable and infectious post-treatment (Yaya-Beas et al., 2016, WHO, 2006, Jiménez, 2006, Schwartzbrod et al., 1998, Pecson et al., 2007, O'Donnell et al., 1984, Crompton, 2001). Appropriate biosolid treatment should reduce the prevalence of parasites in the biosolid (Kim et al., 2012, Pecson et al., 2007, O'Donnell et al., 1984). Long-term storage post-treatment (up to 3 years) which allows the parasites to die off naturally is required to reduce risks of *Ascaris* infections (O'Connor et al., 2017, Rouch et al., 2011). However, long-term storage impairs the agronomic quality of the biosolid and so a balance must be struck if the biosolid is to be used for agricultural purposes.

*Ascaris lumbricoides* eggs are thought to be susceptible to the direct effects of high temperatures, with high temperature inactivating *Ascaris* eggs in biosolid and the eggs rapidly dying off at temperatures above 50°C (Barnard et al., 1987, Katakam et al., 2014, Nordin et al., 2009). However, this approach must be balanced by the high energy costs and adverse effects on valuable biosolid microbial resources if biosolid heating was performed artificially. Conversely, at a lower temperature of 38°C, 77% of *Ascaris* eggs have been shown to be viable for 25 days in biosolid (Black et al., 1982, Fitzgerald and Ashley, 1977).

Also, some *Ascaris* eggs are still infective even after severe treatment conditions (Nelson and Darby, 2002). For instance and in contrast to most reports, Maya et al. (2012) investigated the inactivation rate of eggs of *Ascaris lumbricoides* when submitted to 80°C; less than 25% of these parasites were inactivated under these conditions (Rocha et al., 2016a). Nevertheless, using treated sewage biosolid in agriculture in most developed countries has been carried out with substantially reduced risks to public health and environment. However, given that potential adaptation of parasites can occur in the environment, it is important to periodically
evaluate the efficacy of biosolid treatment conditions on the viability of *Ascaris* for public health reasons.

In multiple studies, *Ascaris suum*, a helminth that infects pigs is often used as a model for *A. lumbricoides*, a human parasite (Boes et al., 1998) (Jeandron et al., 2014, Sá et al., 2017, Decrey et al., 2011). This is because *A. suum* is widely distributed worldwide and available in developed and developing countries and thus easily studied in developed countries unlike *A. lumbricoides* (prevalent in developing countries). In addition, *A. suum* and *A. lumbricoides* are closely related *Ascaris* species with similar life cycles with *A. suum* especially easily enumerated and processed in the laboratory. Indeed, *in vitro* tests carried out on embryonation of eggs confirmed that *A. suum* eggs was an excellent substitute model for *A. lumbricoides* (Johnson et al., 1998, Cruz et al., 2012, Holmqvist and Stenström, 2001). Therefore, in this study, *A. suum* has been used as the surrogate *Ascaris* for assessing the die-off rates of *Ascaris* under laboratory conditions.

This chapter is focused on the assessment of the fate of *Ascaris* eggs in biosolid samples. The aim was to determine the effect of temperature on the decay of *Ascaris* eggs in a laboratory simulation under conditions of pan drying and stockpiling. The storage time required to reduce or completely eliminate the levels of *Ascaris* eggs would be determined.
4.2. Results

4.2.1. Determining the utility of Live/Dead BacLight system for assessing eggs viability

A BacLight staining kit (Live/Dead® BacLight™ Bacterial Viability Kit Staining) was used in this investigation with selected suspensions biosolid samples being stained with kit components as described in section 2.7.12.1. The eggs were stained with two fluorescence dyes; the red PI and green Syto 9 from the BacLight staining kit. The lipid layer of viable eggs was stained while in non-viable eggs, the inner egg contents were stained (Figure 4.1). Yellow colourations were formed when the two dyes were merged into a single image (all Figures part A). After 2 months, the larvae were observed to have developed with some motile larvae detected inside the eggs. However, the dead or non-viable larvae stained differently; red and green fluorescence were observed in these samples (Figure 4.2).
Figure 4.1: Viable and non-viable *Ascaris suum* eggs in different biosolids samples, stained with Live/Dead® Baclight™ bacterial viability kit. Magnification was 200x

Note: Outer shell removed using household bleach (10%). Viable eggs (light arrow) inner membrane stained by green and red fluorescence dyes. Non-viable egg (heavy arrow) with the dead zygote (middle of egg) completely stained with both dyes. The green fluorescence was observed with a 488 nm excitation filter and 500-550 nm emission filter. Red fluorescence was observed with a 488 nm excitation filter and 570-620 nm emission filter. A: merged (syto9, PI, transmitted light), B: Syto9, C: PI and D: transmitted light.
Figure 4.2: Non-viable *Ascaris suum* larvae in biosolids sample after 10 weeks in simulated drying pan, stained with Live/Dead®Baclight™bacterial viability kit at 200x magnification.

Note: Outer shell removed using household bleach (10%). Both dyes, the green and red fluorescence dyes stained the non-viable larvae. The green fluorescence was observed with a 488 nm excitation filter and 500-550 nm emission filter. Red fluorescence was observed with a 488 nm excitation filter and 570-620nm emission filter. A: merged (syto9, PI, transmitted light), B: Syto9, C:PI and D: transmitted light.
4.2.2 Determining viability of the eggs during the dry pan simulation

The percentage of viable eggs from Rochester and Cobden simulation experiments was observed to decrease during incubation. Figure 4.3 shows that the viability of *Ascaris suum* eggs for Rochester and Cobden decreased steadily from 100% to 69% and 78% respectively over the 17 weeks’ incubation period. Statistically significance differences were observed in the *P*-value of *T*<sub>0</sub> and *T*<sub>17</sub> in both simulations with *P*-value <0.0001.

![Figure 4.3: Percentage of viable *Ascaris suum* eggs during pan drying and stockpiling simulation with Rochester and Cobden biosolids.](image)

Results are means of three replicate samples with standard error bars displayed.
4.2.3 The relationship between viable eggs and Dry solids percentage.

Figure 4.4 shows an inverse relationship between viability and DS percentage. As the percentage of egg viability decreased during simulation the DS percentage increased over the experimental period. In Rochester simulation the eggs viability decreased from 100% to 69% while the DS content increased from 9% to 34%; for the Cobden simulation egg viability decreased from 100% to 78% whereas the DS increased from 3% to 59%.
Figure 4.4: The relationship between viability and DS content. (A) Cobden simulation (B) Rochester simulation. Values presented are means of three replicates with standard error bars.

4.2.4. Data analysis using the 95% confidence interval test

The viability of *Ascaris suum* eggs decreased steadily by 22% for Cobden and 31% for Rochester during 17 weeks of simulation period (Table 4.1). This data was plotted to show the
decay trends for *Ascaris suum*, with the 95% confidence limits (Figure 4.5). The decay rate of *Ascaris suum* in Cobden biosolid was about 2-fold lower than for Rochester. The confidence limits slightly increased with increasing treatment time but remained relatively narrow compared to the decreasing trends in survival of *Ascaris suum*. That is, we can be confident that significant decay of *Ascaris suum* had occurred during treatment in both cases.

**Table 4.1: The percentage of live eggs, upper and lower confidence level (95%) for live eggs seeded in biosolids after incubation in pan drying**

(A)

<table>
<thead>
<tr>
<th>Replicates</th>
<th>T0</th>
<th>T4</th>
<th>T7</th>
<th>T10</th>
<th>T13</th>
<th>T17</th>
</tr>
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<tbody>
<tr>
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<td>98</td>
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<td>87</td>
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<tr>
<td>SD</td>
<td>3</td>
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<td>2.872</td>
<td>2.759</td>
<td>3.346</td>
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<td>94.51</td>
<td>90.46</td>
<td>84.77</td>
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</tr>
<tr>
<td>UCL</td>
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<td>98.58</td>
<td>96.82</td>
<td>94.87</td>
<td>89.01</td>
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(B)

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<th>Replicates</th>
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<th>T7</th>
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<td>91.7</td>
<td>87.49</td>
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</tr>
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</table>

(A) Cobden simulation (B) Rochester simulation. Tc= treatment time in weeks, SD=standard deviation, LCL=lower confidence level, UCL= upper confidence level. Values presented are averages of three replicate samples.
Figure 4.5: The mean, upper and lower 95% confidence interval for the proportion of live eggs in seeded lagoon simulation after staining with Live/Dead® BacLight™ Bacterial Viability Kit.

(A) Cobden simulation (B) Rochester simulation. N=3 and standard error bars are presented.
4.2.5 The values of log reduction during the simulation.

In the simulation from Cobden and Rochester ($R^2$ values for decay curves were 0.918 and 0.9656, respectively). The log reduction in the viability of *Ascaris* eggs per g DS during Cobden simulation was $\sim 0.1 \log_{10}$, and $\sim 0.2 \log_{10}$ in Rochester simulations (Figure 4.6). To provide a $\sim 1 \log_{10}$ reduction in egg viability in both simulations, approximately 85 weeks for Rochester biosolid, and 187 weeks for Cobden biosolid, would be required.

Figure 4.6: Decay of *Ascaris suum* in simulation, over 17 weeks.

(A) Cobden simulation (B) Rochester simulation. Linear regression was calculated as shown. Means of three replicate samples with standard error bars are presented.
4.3. Discussion

The Live/Dead BacLight (Invitrogen, San Diego, California, United States) fluorescence-based assay is a nucleic acid staining kit that binds to the nucleic materials (DNA and RNA) of biological structures such as *Ascaris suum* eggs. There are two stains which have different abilities of penetrating healthy cells allowing for discrimination between the living and dead cells. Syto 9 stains penetrate live and dead cells and stain them with a green colouration. In contrast, the (PI) stains damaged cell membranes with a red colour. In this study, this kit was applied to the study of the viability of *Ascaris* eggs.

For complete staining to occur in *Ascaris suum* eggs, the outer egg shell (layer) has to be detached, allowing the stain to penetrate the outer lining before staining the inner lipid layer. Detachment of this outer layer renders the egg non-viable. The Syto® 9 (Invitrogen) and red fluorescent propidium iodide (PI) was observed to stain the nucleic acid materials of some eggs after penetrating the lipid layer and these eggs were therefore non-viable. With viable eggs, staining was only at the peripheral layers and the inner contents of live *Ascaris* eggs were not affected. After fertilization, the outer shell is formed (this layer is lacking in unfertilized egg) and so simple dyes such as the ones used in this study can enter readily (Karkashan et al., 2015, Bleve-zacheo et al., 1993, Wharton, 1983). None of the dyes used in this kit adversely affected the viability or mobility of viable larvae after 180 minutes. In contrast, acridine orange and eosin stains which have demonstrated some toxic effects on larvae, according to Tennant (1964), as cited by WHO (2004). Therefore, the Syto 9 and propidium iodide dyes are better than other simple dyes and are recommended for use in future studies designed to evaluate the viability of helminths’ eggs.

The stained eggs were viewed under confocal laser scanning microscopes using specific excitation and emission filters. Recommended excitation and emission filters; Syto 9 480 nm and 500 nm were and for propidium iodide stains, they were 490 nm and 635 nm respectively.
In this experiment, the excitation filter used was at 488 nm (Karkashan et al., 2015, Berney et al., 2007, Biggerstaff et al., 2006), while the emission filters used were as described in a recent study (Karkashan et al., 2015) for green (500-550 nm) and red (570-620 nm) fluorescent stains. These were similar to the filters used by Bernay et al. (2007) in their flow cytometry measurements (red and green fluorescent stains viewed at 630 nm and 520 nm respectively).

One observed problem with examining eggs using CLSM was the need for continually refocussing of the microscope for efficient counting of eggs in different areas of view. As Ascaris eggs were larger than the biosolid materials (biosolid sample diluted for better staining effects), they were easily distinguished from inert biosolid materials. Typically after a few weeks of drying, the larva develops and emerges from the eggs (Ash and Orihel, 2007). Time, temperature and dryness of the biosolids may affect the die-off or decay rates of Ascaris eggs. Samples were in drying pans used in this study for over a 4-month period resulting in the decay rates of 22% and 31% at 20°C as shown in Table 4.1; this die-off rate decreased over the experimental time-frame. This is in agreement with the finding that some Ascaris eggs are resistant to adverse environmental conditions with some eggs remaining infective from 6 to 20 years (Ligocka and Paluszak, 2009).

In the present study, the eggs were exposed to 20°C temperatures for 120 days in drying pans. We noted a 22% and 31% reduction in the viability of Ascaris eggs; Holmqvist and Stenström (2002) showed a decrease in viability of Ascaris suum eggs of ~21% at 30°C after 31 days. Another study reported that the viability of Ascaris eggs decreased by 15% at 20°C after 150 days (Katakam et al., 2014). According to Pecson et al. (2007) 99% of the eggs cannot survive more than 450 days at 20°C. Temperatures greater than 25°C over 16 months reportedly caused the inactivation of more than 90% of Ascaris eggs (O’Donnell et al., 1984). From these studies, high temperature plays a significant role in the die-off of helminth eggs (Feachem et al., 1983).
Temperature and stockpile time-frame play important roles in pathogen decay and loss of viability (Lang and Smith, 2008, Smith et al., 2005).

The viability of *Ascaris* can potentially be used an indicator of the viability of the eggs of other helminths in biosolids. The results indicate that the viability of *Ascaris* eggs reduced by 22% and 31% after 17 weeks drying pan simulation and if the die-offs continued at the rate observed in this study, by 13 months and 18 months the viability of *A. suum* reduction would be 100% in Cobden and Rochester biosolids, respectively. The die-offs probably occurred as result of the combination of different factors; the adverse effect of the temperature of study and the accompanying reduction in moisture content over the incubation period.

### 4.4 Conclusion

This study has shown that substantial decreases in the viability of *Ascaris suum* eggs occurred over 17 weeks in Cobden and Rochester biosolids. The Live/Dead® BacLight™ staining kit was successfully applied and allowed for clear differentiation between viable and non-viable eggs. The results suggest that this approach may be more accurate than the popular Tulane method, and so could be useful in future studies on the viability of helminth eggs. The data obtained in this study suggests that safety from Ascarid infections (total removal of *Ascaris* eggs) in Cobden and Rochester biosolids can be achieved within 18 months of stockpiling. From this work we can suggest that that temperature and time are in part responsible for the *Ascaris* dies off. Further studies detailed in the next chapter further examine the possible reasons for higher decay rate of *Ascaris suum* during the Rochester biosolid simulation when compared with the Cobden simulation.
CHAPTER 5:

Culture-dependent and independent assessments of the biosolids’ bacterial community diversity and their ability to degrade *Ascaris* sp. eggs.
5.1 Introduction

Biological treatment of municipal and industrial wastewaters is widely used because it is effective in wastewater detoxification with low economic costs. The application of additional post-treatment measures for waste biosolid allows for further minimization of public health risks associated with the treatment processes and re-use of waste treatment by-products (biosolid or biosolids) (Kumari et al., 2009, Hesham et al., 2011). This risk minimization is achieved through the reduction or elimination of pathogens in the biosolids prior to their use on farmland as agricultural fertilizers or for other purposes. The treatment and post-treatment processes usually applied include mesophilic anaerobic digestion (MAD), thermophilic aerobic digestion, use of lagoons, air drying and composting (Manser et al., 2015, Brisolara and Qi, 2015).

Although sewage treatment can significantly reduce pathogen numbers in biosolids, the extent of this reduction is dependent upon the specific processes employed (Watanabe et al., 1997, Gantzer et al., 2001, Amahmid et al., 2002, George et al., 2002, Yasunori et al., 2002, Muhammad et al., 2007, Rouch et al., 2011). Given the public health risks associated with their use, the prevalence of pathogens in biosolids or biosolid should be determined prior to their re-use. The term biosolids is commonly used for biosolids treated to acceptable microbiological standard for use on land (Pepper et al., 2006). The sewage or biosolids treatment method employed is determined by cost, its efficiency, sewage components and source of wastewater.

A detailed study of the microbial composition of wastewater treatment and the derived biosolid is important for understanding the mechanisms underpinning the biological treatment processes. This knowledge is especially important for process improvement; preventing biosolid bulking or foaming (Kumari et al., 2009, Hesham et al., 2011) and for effective removal of pathogens. Improved understanding of microbial composition and activities in biosolids has been possible in recent times because of the application of molecular tools.
Traditional molecular biological techniques include methods such as 16S rDNA clone library analyses (McGarvey et al., 2004, Otawa et al., 2006), ribosomal intergenic spacer analysis (RISA) (Yu and Mohn, 2001, Baker et al., 2003), 16S-restriction fragment length polymorphism (16S-RFLP) (Baker et al., 2003, Gilbride and Fulthorpe, 2004) and PCR-denaturing gradient gel electrophoresis (DGGE) (Liu et al., 2002). These methods allow for culture-independent studies of the microbial ecology of wastewater and biosolids’ treatment systems. PCR-DGGE, in particular is a widely used traditional molecular method which, compared to other methods, is cheap, fast, and can be used to generate microbial community fingerprints of different systems (Liu et al., 2002, Casserly and Erijman, 2003, Kaksonen et al., 2004, Rowan et al., 2003).

Studies on bacterial composition and dynamics during wastewater and biosolid treatment using DGGE based approaches have been successfully performed (Boon et al., 2002, Ibekwe et al., 2003, Gilbride et al., 2006, Liu et al., 2007a, Liu et al., 2007b, Xu et al., 2018, Bassin et al., 2018, Zou and Wang, 2017). These DGGE-based analyses have provided useful information on the diversity, structure, potential activities and identities of microbial communities present in biosolid and wastewater samples (Heuer et al., 1999, Riemann and Winding, 2001). However, DGGE profiles are affected by variable DNA and PCR-related biases, imprecise taxonomic information and species estimation and a poor detection limit (Theron and Cloete, 2000), (v Wintzingerode et al., 1997), (Duarte et al., 2012), (Michaelsen et al., 2006, Nakatsu et al., 2000).

Sewage bacterial groups such as those belonging to the *Proteobacteria* and *Firmicutes* and eukaryotic and cyanobacterial groups such as *Chlorella* sp., *Arthropsira* sp. and *Planktophrix* sp. have been identified via DGGE (Ding et al., 2011), (Eland et al., 2018). Sewage processes (denitrification, phosphorus-accumulation, sulfate- and phosphate reduction and complex organic compounds degradation) resulting in wastewater detoxification have also been studied
These microbial groups continue to be active in the degradation processes in composted biosolids with age and time influencing the dominant taxa (Novinscak et al., 2009, Novinscak et al., 2008, Belyaeva et al., 2012).

In recent times, next-generation sequencing (NGS) approaches such as metagenomics have provided greater details on microbial communities in different environmental systems. These approaches require more expertise and are more expensive than PCR-DGGE systems but generate detailed and more accurate data on microbial taxonomy and function (Uhlik et al., 2013). The different types of next-generation sequencing platforms and principles of operation have been reviewed (Mardis, 2013). Consequently, NGS based approaches are becoming the preferred method for studying microbial communities. Metagenomics in particular, has been successfully applied to the study of viral pathogens in biosolids (Bibby and Peccia, 2013), bacterial diversity in activated biosolid (Zhang et al., 2012), antibiotic resistance genes in biosolids (McCall et al., 2016) providing excellent data on microbial diversity and putative functions.

Waste (biosolid) detoxification and pathogen reduction are affected not only by the activities of indigenous flora (Ward et al., 1981, Sidhu et al., 2001) but by other factors such as temperature (Smith et al., 2005), retention time (Lang and Smith, 2008), pH (Feng et al., 2003) and moisture content (Ward et al., 1981, Yeager and Ward, 1981). The application of PCR-DGGE and metagenomic-based approaches should provide a better understanding of how these factors influence the survival of pathogenic microorganisms and viruses in biosolids; a knowledge critical to their safe use in agriculture.

Biosolids may also contain other non-microbial pathogens such as the helminths which are of significant health risks and can cause human fatalities (Palmer et al., 2002, WHO, 2001). Scientific focus on reductions in microbial pathogen levels in biosolids has led to the
identification of critical gaps in the knowledge of possible microbial roles in the decay of helminth pathogens (eggs in biosolid). These gaps in knowledge can be addressed through molecular, NGS and culture-dependent approaches. As the decay of helminths eggs (Ascaris sp) is the focus of this study, a review of past work was carried out by the author, with no publication specifically dealing with microbial interactions with helminth eggs subsequently identified.

Biosolid and biosolids teem with different microbial groups which mediate the bulk of biosolid biological activities (organic matter degradation) (Kallistova et al., 2014, Shchegolkova et al., 2016) through the use of a wide array of enzymes including proteases, lipases and chitinases. It is possible that these catabolic enzymes may affect the survival of other non-microbial pathogens such as the helminths (Ascaris sp.) degrading their cellular and reproductive structures. For example, proteases, lipases and chitinases produced by microbial groups in biosolids can potentially affect the protein, chitin and lipid components of helminths (Ascaris sp.) eggs, rendering them non-infective (decay).

Therefore, the aim of this chapter is to assess bacterial diversity in biosolid samples and evaluate their microbial functionality with respect to the decay of Ascaris sp. eggs using culture-dependent and independent approaches. Firstly, PCR-DGGE and metagenomic-based approaches will be applied to study the diversity and identities of key microbial groups in biosolid samples. Secondly, a culture-dependent MT2 plates-based approach will be used to assess the potential effects of the biosolid’s microbial enzyme activities and bacterial isolates (cultures) on the decay of Ascaris eggs. Bacterial isolates showing substantial egg decaying potential would then be applied directly to Ascaris sp. eggs and compared with commercial enzymes to determine their egg decay efficiencies.
5.2 Results

5.2.1 Bacterial community in biosolid samples

Figure 5.1 shows the bacterial community profiles generated from PCR-DGGE analysis of the different biosolid samples used in this study. The communities in each of the biosolid samples were substantially different from one another. For example, the bacterial community in Cobden samples was 70% and ~80% dissimilar to the ones in Heyfield and Rochester samples respectively (Fig. 5.1).

![UPGMA dendrogram of bacterial community generated from PCR-DGGE analyses of biosolid samples from Heyfield, Cobden and Rochester Waste Treatment Plants. N=3 and scale represents similarity percentages.]

5.2.2 Bacterial diversity in biosolid samples

The highest diversity value $H'$ of 3.5 was obtained in Rochester samples followed by 2.3 in Cobden and the lowest value of 1.8 in Heyfield. The community in Rochester was therefore significantly (P<0.05) more diverse than those in Cobden and Heyfield. Evaluation of the equitability values indicated a high degree of similarities between all the samples analysed.
Pareto-Lorenz values ranged from ~32% in Cobden to 47% in Heyfield suggesting that the communities in these samples were highly even (Figure 5.3).

Figure 5.2: Shannon Weaver diversity index for the biosolid’s microbial community, derived from DGGE gels of samples from Heyfield, Cobden and Rochester Waste Treatment Plants. N = 3 and standard error bars are presented. SDI refers to Shannon Diversity index while E refers to the equitability index.
Figure 5.3: Pareto-Lorenz curves of bacterial population in Heyfield, Cobden and Rochester biosolid samples. The black straight line at 45 degrees represents the perfect evenness of a community. Coloured arrows represent the average Pareto Lorenz values of the biosolid samples.

5.2.3 Metagenomics analyses

Metagenomic analysis of the biosolids from the three wastewater treatment plants was carried out to further assess the microbial community at each site; metagenomic analysis allows the detection of much more of the microbial community than achieved with PCR-DGGE. Figure 5.6 confirms that the bacterial communities in the biosolid samples were different, as determined by PCR-DGGE. In Heyfield samples, the community was dominated (~75% of total community) by bacterial groups belonging to Gammaproteobacteria. Other groups such as those belonging to the Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Actinobacteria and Clostridia were also detected. In Rochester samples, the dominant bacterial groups belonged to either the Gammaproteobacteria or Betaproteobacteria (~45% of total bacterial community). However, in Cobden samples, both Epsilonbacteria and
Betaproteobacteria had equal dominance with other bacterial groups such as those belonging to Deltaproteobacteridia, Bacteroides and Clostridii also detected (Figure 5.4). Shannon ($H'$) diversity showed that Heyfield samples had the highest bacterial diversity of 5.3, followed by Rochester, 5.0 and Cobden 3.7 (data not shown). PCoA of the taxonomic data showed distinct differences between Rochester, Heyfield and Cobden bacterial communities (Figure 5.5).

Figure 5.4: Bacterial groups in biosolid samples from Heyfield, Rochester and Cobden wastewater treatment plants. Data derived from metagenomic analysis of DNA extracts from these biosolid samples. For each sample, DNA extracts from three replicate samples were pooled together and subject to next-generation sequencing on the Illumina platform (MiSeq).
Figure 5.5: Principal component analysis of bacterial communities in biosolid samples from Heyfield, Rochester and Cobden wastewater treatment plants. Figure generated from the analysis of metagenomic dataset of these biosolid samples with MEGAN6. For each sample, DNA extracts from three replicate samples were pooled together before being sequenced on the Illumina platform (MiSeq).

5.2.4 Assessment of biosolids microbial community substrate utilization potential

Analysis of the potential of the biosolid microbial community to degrade the outer layer of the *Ascaris* sp. eggs was assessed using Biolog MT2 plates using protein, chitin and lipid as substrates as they represent the components of the outer layer; substrate utilization revealed distinct trends. For example, only samples from Cobden showed substantial utilization of chitin from 96 hours to the end of the incubation period at 168 hours or 7 days (0.2 to 0.3 OD$_{595}$) (Fig. 5.4a). In Biolog plates supplied with protein substrates, both Cobden and Rochester samples showed substantial utilization of the supplied substrate after 48 hours or Day 2. The increase in substrate utilization stopped at 120 hours (0 to 2.5 OD$_{595}$) with utilization levels remaining the same until 168 hours in Cobden samples while continuing to increase in Rochester samples (0 to 2.1 OD$_{595}$). Protein substrate utilization was not detected in Heyfield and the Cobden samples had higher rates of protein utilization compared to Rochester samples (Fig. 5.4b). All
the samples showed some utilization of the supplied lipid substrates but the extent of utilization was substantially lower than observed with the protein substrates. Nevertheless, Cobden had the highest lipid utilization levels (0.3 OD$_{595}$), followed by Heyfield (0.18 OD$_{595}$) and Rochester (0.12 OD$_{595}$) (Fig. 5.4c). PCR-DGGE analysis of the composite samples from Heyfield, Cobden and Rochester samples from MT2 plates with chitin, lipid and protein substrates is presented in Fig. 5.5. The results confirm that the microbial communities from the three wastewater treatment plants were capable of degrading each of the three substrates. The presence of a significant number of bands confirm that a number of microorganisms were present in each biosolids sample that were capable of degrading each of the main components of the outer layer of *Ascaris* ova (Fig. 5.6).
Figure 5.6: Substrate utilization patterns of the bacterial communities in Cobden, Heyfield (HF) and Rochester biosolid samples in MT2 Biolog plates containing (a), protein (b) chitin and lipids (c) over 168 hours (7 days). Values presented are averages of three replicate samples with standard error bars.
5.2.6 Putative identities of bacterial species from the DGGE of the community in MT2 plates.

DNA was extracted from selected MT2 plate samples (after the completion of BIOLOG analysis), amplified with universal primers 314F and 907R and subject to DGGE after which bands of interest were excised from the generated DGGE profiles. The identities of the excised and sequenced selected bands from Figure 5.7 are shown in Table 5.1. The community was dominated by Proteobacterial species with other detected groups belonging to the *Flavobacteria, Sphingobacteria* and *Cytophaga* (Table 5.1).
Figure 5.7: The DGGE gel of bacterial community from MT2 plates with chitin, protein and lipid substrates. Numbered boxes represent bands of interest which were excised and sequenced to determine their putative identities.
Table 5.1: The putative identities of selected bacterial species from MT2 plates DGGE.

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<td>Alphaproteobacteria; Rhizobiales</td>
<td>proteinase</td>
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</table>


5.2.7 Bacteria isolated from biosolid samples using culture media

Using chitin, protein and lipid (0.5 % (v/v)) as the main substrates in BH medium, a cumulative total of 51 pure isolates or phenotypes were obtained from the biosolids from Cobden, Heyfield and Rochester (data not shown). The highest number of bacterial isolates capable of growing on Bushnell Hass (BH) mineral salts medium was 38 (protein, lipid and chitin supplemented BH medium plates). Thirteen (13) isolates were obtained from chitin supplemented BH medium. Representative plates showing bacterial growth are shown Figure 5.8.
5.2.8 Bacterial isolates utilization of substrates in MT2 plates

Most of the microorganisms isolated from the different biosolid samples were able to utilize the substrate sources on the Biolog plates after a 4-day (96 h) incubation. Amongst the three tested substrates, the highest microbial substrate utilization was associated with lipids and yet most were isolated on chitin. Overall, the ability to utilize all the supplied substrates was observed in 30 out of the 51 isolates (Figure 5.9). Figure 5.9 also showed that the highest bacterial utilizers were different in each of the tested substrates. The only exception was isolate 26, which was in the top 5 utilizers of all the three substrates. The five highest substrate degraders were isolates 17, 26, 33, 10 and 32 for chitin, 42, 26, 43, 13 and 56 for protein and 16, 26, 15, 50 and 41 for lipids.

Figure 5.8: Bacterial growth on BH agar supplemented with lipid substrate (A) and purified isolates grown on nutrient agar (B).
![Graphs showing the change in average absorbance (595nm) over incubation time (hours) for Chitin and Protein](image-url)

**Chitin**

- The graph shows the absorbance over time for different samples labeled 17, 44, 26, 33, and 10.

**Protein**

- The graph shows the absorbance over time for different samples labeled 56, 43, 13, 26, 42, and 6.
Figure 5.9: Substrate utilization patterns of selected bacterial isolates from Heyfield, Cobden and Rochester biosolid samples in MT2 plates supplemented with chitin (a), protein (b) and lipids (c) substrates at selected time points over 98 hours (4 days). N=3 and standard error bars are presented. The numbers, 16, 50, 15, 26, 38 and 41 refer to denotations of bacterial isolates.

5.2.9 The effects of bacterial culture, pellets and cell-free extracts on the decay rate of *Ascaris* sp. eggs

Initially, the effects of each bacterial species pellets at 1X (B100) and 0.5X (B50), uncentrifuged bacterial at 1X (NB + B100) and 0.5X (NB + B50), the cell free extracts at 1X (NB100) and 0.5X (NB50), the mixture of uncentrifuged cultures of the three selected bacterial cultures (All B) and control (no bacteria) were compared. For ease of understanding, the data was presented over two graphs for each isolate; Ai and Aii for bacterial isolate no 16, Bi and Bii for bacterial isolate no 42 and Ci and Cii for bacterial isolate no 26 (Fig 5.10). In all cases, the lowest percentage of live eggs at ~ 77% was observed in samples with the three isolates.
mixed together. Compared to the mixed culture, a higher percentage of live eggs was observed when individual pellets of each test isolate, at different dilutions and their cell free extracts were used. The rates of *Ascaris* egg decay were also largely not substantially different when the 3 isolates were compared (Figure 5.10A, 5.10B and 5.10C).

The effects three different commercial enzymes (chitinase, protease and lipase) and the mixture of these enzymes were also assessed on the decay of *Ascaris* eggs with the lowest percentage of live eggs (~81%) observed in samples with the commercial enzyme mixtures (Figure 5.10D). Individually, the commercially enzymes egg decay effects were largely not substantially different from the decay effects of the cell-free extracts, bacterial pellets and the uncentrifuged bacterial cultures (Figure 5.10).

However, Fig. 5.10E showed that the bacterial cultures from the biosolids samples demonstrated greater abilities to degrade *Ascaris* eggs, when used as a mixture rather than as monocultures. The mixed cultures caused ~23% decay in the eggs compared to 19% decay observed when the three commercial enzymes were used as a mixture. In the control samples, there was little or no decay of eggs over the 30-day experimental period (Figures 5.10 and 5.11 (i) and (ii)) Therefore, the bacterial community working in synergy caused a higher percentage of decay in *Ascaris* eggs than when applied individually. This is validated by Figure 5.11 (ii and iii) which showed the damage caused to *Ascaris* eggs by a mixed culture on days 15 and 30 respectively compared to the intact eggs (Figure 5.11 (i)) at day 0. The identities of the three bacterial isolates used in this study are shown in Table 5.2.
Figure 5.10: The percentage of live *Ascaris* eggs detected during incubation with selected and mixed bacterial species and commercially available enzymes over 30 days

Note that control refers to the sterile nutrient broth, NB refers to the cell-free supernatant, NB+B refers to uncentrifuged bacterial culture, B refers to the bacterial pellet, 100 and 50 refers to 1X and 0.5X sample concentrations respectively. All B refers to the three bacterial mixed together and All C refers to all the commercial enzymes mixed together.

<table>
<thead>
<tr>
<th>Band no.</th>
<th>Nearest taxon</th>
<th>Accession no.</th>
<th>Accession no. Similarity (%)</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td><em>Pedobacter</em> sp</td>
<td>KP899202</td>
<td>99</td>
<td>Sphingobacteria</td>
</tr>
<tr>
<td>42</td>
<td><em>Brevundimonas</em> sp</td>
<td>MH142375</td>
<td>99</td>
<td>Alpha-proteobacteria</td>
</tr>
<tr>
<td>26</td>
<td><em>Acidovorax</em> sp</td>
<td>KT321698</td>
<td>99</td>
<td>Betaproteobacteria</td>
</tr>
</tbody>
</table>
Figure 5.11: The effects of mixed bacterial culture on the layers of *Ascaris* eggs.

Note: (i) refers to eggs in control samples at day 0, (ii) refers to eggs in control samples at day 30, (iii) refers to eggs in mixed cultures at day 0, (iv) refers to eggs in mixed cultures and the effect of enzyme in eggs layers (v) refers to eggs in mixed cultures and the break layers.
5.3 Discussion

To date, most research has focussed on how the chemical and biological components of biosolids affect environmental and human health when they are used as fertilizers or for landfill purposes. For example, the tracking of aerosolized microorganisms from land-applied sewage biosolid have been carried out with a view to assessing their implications on human health (Baertsch et al., 2007). Extensive research of the prevalence of human pathogens and their indicators in biosolids have also been performed (Sidhu and Toze, 2009). The efficacy of naturally occurring and specific biosolid disinfection procedures has also been assessed (Fall and Silva-Hernández, 2017), along with the human and environmental health risks associated with biosolid use (Khuder et al., 2007, Dowd et al., 2000). In addition the effects of disinfection on microbial components and activities have also been evaluated (Chen et al., 2017, Lau et al., 2017, Kao et al., 2006, Kraas et al., 2017, Barbarick et al., 2004). These investigations have been carried out using a range of conventional and molecular tools.

However, there have been only limited studies focussed on characterizing the microbial components of biosolids and determining how these components differ from one treatment batch or waste treatment plants to another and their effects on specific groups of pathogens. This knowledge is critical in determining how the microbial components of biosolids can adversely affect pathogenic helminths, which is the focus of this study. In studies where the microbial components of biosolids have been investigated, the focus has been on the prevalence of microbial pathogens. For example, a pyrosequencing-based characterization of pathogens in biosolids detected predominantly opportunistic bacteria belonging to *Clostridium* and *Mycobacterium* (Bibby et al., 2010). This study is therefore novel because the biosolids’
bacterial communities have been studied with a view to assessing their potential effects on the removal of helminths pathogens.

A comparison of the microbial community profiles of biosolids from the three different wastewater treatment plants confirmed that the bacterial communities were highly diverse. The dissimilarity percentages ranged from 70 to 80% indicating the bacterial groups in Heyfield, Cobden and Rochester biosolids were substantially different (based on DGGE analysis). Metagenomic analyses also showed a similar trend with ~70% of bacterial population belonging to *Gammaproteobacteria* in Heyfield compared to ~25% in Rochester and less than 5% in Cobden. In Rochester, both *Gamma-* and *Beta- Proteobacteria* were dominant and *Cytophagia, Betaproteobacteria* and *Epsilonbacteria* being key groups in the Cobden samples. PCoA analysis of the taxonomic data also indicated differences between the biosolid samples. This is not unusual as previous investigations have shown this. A comparison of four composting processes including those based on sewage biosolids showed distinct differences in DGGE community profiles of these processes/biosolids. These differences were thought to be due to the different concentrations of dissolved organic materials (Ishii and Takii, 2003).

Wastewater components can differ from one batch to another and these differences can determine the residual content of biosolids. Differences in composting procedures and types of anaerobic biosolid have been correlated to differences in the rates of organic matter decomposition and the dominant bacterial communities observed via DGGE (Nakasaki et al., 2009) and metagenomic profiles. A comparison of bacterial communities from biosolids from 14 different wastewater treatment systems and plants showed substantial differences in the bacterial community as assessed by pyrosequencing, based on temperature, pH and geographical locations (Wang et al., 2012, Zhang et al., 2012). In addition to biosolid content and location, other factors, such as biosolid water content and temperature can vary and affect the biosolid microbial community activities (Liang et al., 2003). Therefore, the observed
differences in the UPGMA dendrograms, metagenomic profiles and community diversity of
Heyfield, Cobden and Rochester biosolids could be due to differences in biosolid content,
location, processing conditions, temperature and moisture content. These communities may
change over different seasons and the extent of these changes requires further investigation.
Therefore, future research work should include the use biosolid samples from other seasons to
determine the extent of seasonal changes in the microbial community of biosolid samples.

This study showed that Rochester biosolid samples had the highest bacterial community
diversity index followed by Cobden and Heyfield samples based on PCR-DGGE analysis. In
contrast, the metagenomic data showed that Heyfield had the highest diversity closely followed
by Rochester and Cobden samples. Critically, both methods showed that the bacterial diversity
in the biosolid samples were different from one another. The reason for this difference is related
to the differences in the basic principles of the two approaches. PCR-DGGE-based diversity
results are generated from the top 1-10% dominant microbial groups, excluding a large
proportion of non-dominant groups. Therefore, the diversity values from DGGE are reflective
of the key, dominant and important microbial groups in the biosolids. In contrast, a
metagenomics-based approach generates its diversity indices using both dominant and non-
dominant groups and is therefore a representation of actual total bacterial community diversity.

In order to assess whether the high diversity observed using the PCR-DGGE analysis could be
correlated with functionality, Pareto-Lorenz (PL) analyses were carried out. These analyses
showed that this significantly higher diversity in Rochester biosolids samples did not translate
to increased community functionality. The PL value for the Rochester community was ~ 44%
compared to 47% in Heyfield and ~32% in Cobden. The interpretation of Pareto-Lorenz values
is based on the assumption that the distribution of bacterial species in a microbial community
is related to the community’s functionality (Marzorati et al., 2008). The closeness of the PL
values of Rochester and Heyfield indicated that the community had similar potential
functionality despite the fact that Heyfield had significantly lower diversity. These communities had medium evenness (PL 45%) or functionality and probably have well defined internal microbial community structure.

The functional capacity of the microbial community was also assessed with MT2 plates. Helminths (Ascaris sp.) egg consists of different layers. For example, the fertilized eggs shell found in biosolids is composed of four layers 3-4 μm thick; uterine, vitelline, chitinous and lipid layers (Figures 1.8 and 1.9) (Brownell and Nelson, 2006, Quilès et al., 2006, Wharton, 1980). Therefore, assessing the abilities of the community to degrade chitin and, lipid substrates could give an indication whether the microbial community in these biosolid samples can render Ascaris eggs non-infective or non-viable.

Analysis of the MT2 plate data showed that the bacterial communities in some of the biosolid samples were able to degrade the supplied substrates and could potentially render Ascaris eggs non-infective. Both Cobden and Rochester showed significant degradation of supplied protein substrates; the Cobden community (and to some extent, Rochester community) degraded the chitin substrates and Cobden, Heyfield and Rochester samples showed some degree of lipid degradation. Therefore, further investigations were carried out to determine the identities of the key members of the community. In general, most of the key isolates in the community such as Flavobacterium, Pedobacter and Janthinobacteria sp. are known lipase enzyme producers. Others isolates such as Bosea and Pseudomonas sp. are known proteinase producers while chitinase producers such as Brevundimonas, Xanthomonas and Flavobacterium sp. were also identified from the DGGE profiles (Männistö and Häggblom, 2006, Shivaji, 2017, Kobayashi et al., 1995, Sakka et al., 1998, Yamaoka et al., 1999). It is, therefore, possible that the observed degradation of the test substrates in this study was due to the enzymes produced by these members of the microbial community.
The results obtained from using culture-dependent and independent approaches demonstrated that the potential to degrade *Ascaris* eggs via chitinase, proteinase and lipase activities was available in the microbial community of the tested biosolid samples. However, it is also important to validate this potential using bacterial isolates, especially if this potential is to be exploited in future biosolid treatment (for helminths decay). The isolation of 51 bacterial phenotypes in this study from the different biosolid samples that could grow on chitin, protein and lipid supplemented media suggested that this potential is intrinsic in the biosolid samples.

As previously stated, helminth (*Ascaris* sp.) eggs are composed of uterine, vitelline, chitinous and lipid layers (Brownell and Nelson, 2006, Quilès et al., 2006, Wharton, 1980). Therefore, the 51 bacterial isolates that degraded these substrates in Biolog MT2 plates could potentially be capable of degrading the *Ascaris* sp. eggs during storage. This finding is important given that extensive research has been carried out demonstrating the effectiveness of physical and chemical processes in the decay of helminth eggs (Koné et al., 2007, Nordin et al., 2009, de Souza et al., 2011) but little or no research on microbial roles in egg decay. It might be possible that a microbial (biological) approach to the decay of helminth eggs could be used independently or as adjuncts to existing processes in the future.

Using their growth characteristics, three bacterial species identified as *Pedobacter* sp., *Acidovorax* sp., and *Brevundimonas* sp. were selected for further investigations. Their efficiencies in the decay of *Ascaris* eggs were evaluated individually and as a mixture and compared with the efficiency of commercial enzymes. Individually, there was not much difference in the egg decay efficiencies between the three isolates and when compared with individual commercially sourced enzymes. This suggests that the bacterial groups were efficient producers of chitinase, lipase and protease enzymes. However, when used as a mixture, there was a higher decay of *Ascaris* eggs (~23%) compared to the commercial enzyme
mixture (~19%) and individual isolates and cell-free samples. This indicated that microbial synergy contributed to the efficient decay of eggs in biosolid samples.

Microbial activities in biosolid samples are affected by storage time, temperature and moisture content (Liang et al., 2003, Lang and Smith, 2008) and interactions with other microbial groups. Microbial community interactions can be antagonistic or synergistic. Antagonistic interactions probably play some roles in the elimination of enteric bacteria (alongside other physicochemical factors) while synergistic activities are known to improve soil fertility in soils to which biosolids have been added (Cele and Maboeta, 2016). This study now demonstrates that synergy between microbial groups could be playing vital roles in the decay (breakdown) of Ascaris eggs in biosolid samples. Also, it is important to analyse the genes responsible for the secretion of such enzymes involved in the degradation of Ascaris eggs. Additionally, it would be useful to combine different bacterial genera synthesizing enzymes and determine the minimum inhibitory concentration that can degrade a certain amount of helminth eggs within a certain time. These additional experimental investigations can be carried out in future studies.

Bacterial decay or breakdown of other components of biosolids is well known. For example, coliphages (bacterial viruses that infect coliform bacteria) are usually used as indicators of enteric viruses in wastewater systems (Harwood et al., 2005, Costán-Longares et al., 2008). While indigenous microflora, process pH, temperature and retention time have been associated with the loss of viability of sewage coliphage (Feng et al., 2003, Nappier et al., 2006), the specific mechanisms of viral decay have not been adequately clarified. Bacterial proteases are thought to play some roles in the inactivation of sewage coliphage and enteric viruses by breaking down their protective protein coats (Nasser et al., 2002). Biological degradation does occur in biosolids, a process mediated by multiple bacterial enzymes, including proteases, (Dueholm et al., 2001, Gessesse et al., 2003, Gerardi, 2006, Nasser et al., 2002). These arrays
of bacterial enzymes involved in other degradative activities could also be acting on helminth eggs.

By conclusively showing that bacterial species in biosolids can degrade *Ascaris* eggs, this study has provided valuable insight into the potential role of biodegradation in the die off of *Ascaris* eggs in biosolids. However, the extent of bacterial degradation of *Ascaris* eggs in biosolid samples is unknown. Also, while this study has demonstrated the degradation of *Ascaris* eggs by bacteria, the role of other factors such as pH, temperature, water content and other microbial groups (protozoa and fungi) on the process was not investigated. These factors may enhance or impair the bacterial mediated decay of eggs and should be investigated in future studies. The mechanisms by which synergy enhances egg decay are unknown. In addition, it is unclear whether these bacterial mediated processes are applicable to other helminths found in biosolids.

### 5.4 Conclusions

In conclusion, the bacterial communities in biosolid samples from Heyfield, Cobden and Rochester were substantially different, as assessed by both PCR-DGGE and metagenomic analyses. MT2 Biolog plate assays conclusively demonstrated high protein utilization, especially in Cobden and Rochester samples. All the biosolids samples also demonstrated some utilization of chitin or lipid indicating that the potential to degrade these components in *Ascaris* eggs exists in the microbial communities in biosolids. Bacterial isolates that could degrade components of *Ascaris* sp. eggs were successfully isolated using modified BH medium. These isolates also successfully degraded the key egg components (chitin, lipids and protein) on Biolog MT2 plates. Selected bacterial isolates applied directly to *Ascaris* sp. eggs also degraded the eggs leading to up 23% reduction in egg viability. Bacterial-mediated egg decay was
enhanced by the synergistic effect of the bacterial isolates and was more efficient than observed in commercial enzymes.
CHAPTER 6:

General Discussion
6.1 General Discussion

Biosolids or sewage biosolid that are derived from wastewater treatment processes are widely used for many purposes in the agricultural and construction industries. These include the use of biosolids for soil conditioning, as fertilizers in agriculture, as road base in road construction, for landscaping purposes, as topsoil and for landfilling purposes (https://www.biosolids.com.au/info/what-are-biosolids). However, the most common uses of biosolids are for soil conditioning and as fertilizers for growing crops (Rouch et al., 2011, Correa and Silva, 2016, Pritchard et al., 2010, O'Connor et al., 2017).

Treatment processes are generally designed to remove chemical toxicants and pathogens from wastewater and biosolids with remaining pathogens in waste treatment solid by-products (biosolids) eliminated during the ageing process (up to 2 years). However, this stockpiling process can reduce their efficacy as fertilizers because desirable plant nutrients can be lost during this process. In addition, it is possible that the eggs of some of the parasitic worms or helminths such as *Ascaris lumbricoides*, *Necator americanus*, *Ancylostoma duodenale*, *Taenia spp.*, *Trichuris trichiura* and *Toxocara canis* found in biosolids (Sidhu and Toze, 2009, Jimenez, 2007) can survive the ageing process. These remain infective even when the biosolids with these parasitic forms are applied to soils as fertilizers and may constitute a significant health risk to farmers and to consumers of crops such as vegetables cultivated on such farms.

Helminthiasis are one of the most common diseases worldwide infecting a significant number of people in the developing world (Clarke and Perry, 1988). One helminth, *Ascaris lumbricoides* is an important human intestinal worm with up to 1.2 billion people infected worldwide (Karkashan et al., 2015, Lamberton and Jourdan, 2015, Steinbaum et al., 2017). It causes weight loss, delayed growth, learning difficulties, childhood asthma (Palmer et al., 2002) and can result in severe ill-health (WHO, 2001). It is highly probable that the soil contamination by biosolid in most developing countries is a contributor to the high rates of
Ascaris sp. infections in these countries. Therefore, one way of achieving the sixth UN sustainable development goal of sanitation and health for the human population could involve adequate treatment of biosolids (to eliminate pathogens) before the biosolids are used on farms. Reductions in the incidence of helminthiases will require the optimization of post-treatment processes and development of new and novel ways of rapidly eliminating them in biosolids. Reductions in helminth infection rates lessen the public health burden of helminthiases in developing countries, freeing up resources that can be used for other beneficial purposes.

However, there are a number of challenges associated with efforts to reduce helminthiases especially from a scientific research-based point of view. These include, lack of or sub-optimal resources for research on helminthiases reduction, outdated and less effective detection methods and the extended time required for ageing processes critical to the decay of helminth eggs. For example, the challenge of limited resources for research activities on the optimization of biosolid treatments for helminths removal in developing countries can be addressed by provision of research grants and/or carrying out the required research activities in developed countries such as Australia. Although helminths such as Ascaris lumbricoides have been largely eliminated in Australia, an alternative model parasitic worm, found in pigs, Ascaris suum (pig roundworm) can be used. This is possible because of its similarities to Ascaris lumbricoides in terms of its morphology and life cycle (Jeandron et al., 2014, Sá et al., 2017). Therefore, in this study Ascaris suum was been used for all the scientific investigations.

Another challenge of studying helminths such as Ascaris suum in biosolids is their detection. Their eggs are small and can be hidden in the biosolids. There are legitimate questions regarding the suitability of existing detection methods such as modified formol ethyl acetate sedimentation, modified Wisconsin floatation, simple gravity sedimentation and Tulane method (Goodman et al., 2007, Bowman et al., 2003) for detection of Ascaris eggs in biosolid from different sources. Therefore, the first result chapter of this study was focussed on
assessing the suitability of the most commonly used method (Tulane’s method) for the
detection of *Ascaris suum*’s eggs in different biosolids from different wastewater treatment
plants.

Biosolid samples obtained from three different WWTP (Heyfield, Cobden and Rochester) in
Melbourne, Victoria, Australia were used in this phase of work. Preliminary analyses indicated
the absence of Ascarid eggs in these samples confirming that *Ascaris* was non-existent or of
extremely low-level incidence in wastewater biosolids in Victoria. This result was not
unexpected as stringent health and environmental regulations have significantly reduced
infections by helminths in most developed countries. Indeed, several studies have confirmed
that the rate of helminth infections was extremely low in Victoria (Irwin et al., 2017).

The Tulane method is a widely used method for helminth egg detection in soils and biosolids
(Butarewicz, 2006, Verbyla et al., 2016). However, there are questions on its suitability for use
on the different types of biosolid and its limit of detection. Therefore, in addition to using this
method to assess the prevalence of *Ascaris* sp. eggs in different biosolid samples, this study
also used the Tulane method to for egg detection in biosolid samples deliberately contaminated
with *Ascaris* eggs. This was done by seeding the biosolid samples with *Ascaris suum* eggs
which had been previously collected from infected pigs in Victoria.

The results obtained confirmed that the Tulane method was a suitable method for detecting
*Ascaris* sp. eggs in biosolids, as a substantial percentage of the seeded eggs were recovered
from the biosolid samples. The highest egg recovery efficiency of 73.3% was obtained in
Heyfield biosolid while the lowest recovery percentage of 63.6% was obtained in Cobden
biosolid. The egg recovery efficiency appeared to be affected by the source of the biosolids.
Apart from the source, egg recovery efficiency is known to be affected by type of helminth
eggs, sample matrix and the expertise of the researcher (Amoah et al., 2017). However, in this
study, *Ascaris* sp. eggs were enumerated by the same researcher but using biosolids sourced from different WWTP’s. Differences in egg recovery percentages in different biosolids observed in this study were likely to be related to the biosolids components. Given the focus of this part of study on the detection and prevalence of *Ascaris*, analysis of the components of these biosolids was not carried out.

This study also observed that the egg recovery efficiency was inversely related to the number of eggs used for biosolid inoculation; the recovery rate improved as the total number of eggs originally seeded into the biosolid decreased. The reason for the decrease in egg recovery efficiency as the number of seeded eggs increased is unknown but the result suggests that the egg recovery threshold of the Tulane’s method might have been exceeded in this study.

The Tulane method is a widely used method for helminth egg detection in soils and biosolids (Butarewicz, 2006, Verbyla et al., 2016) and despite its known limitations, remains a preferred method. In this study, the Tulane method was excellent in egg recovery studies, but it was difficult to detect the viability of recovered eggs using this approach. A review of methods did show that the Tulane method was laborious, time consuming and prone to user-related errors (Amoah et al., 2017). Apart from this, a pertinent research question was, “what percentage of the recovered eggs was viable, that is, able to infect the secondary host?” In addition, the impact of the ageing process on the viability of the eggs in biosolids used in this study was unknown.

Therefore, Chapter 4 focussed on the fate of *Ascaris* eggs in selected biosolid samples (Rochester and Cobden samples) and the effects of temperature on the decay (viability) of *Ascaris* eggs in laboratory simulations (pan drying and stockpiling). This was assessed with a view to determining the time-frame needed to reduce or completely eliminate the viability of *Ascaris* eggs in these samples. The viability of eggs was determined using a BacLight staining
kit (Live/Dead® BacLight™ Bacterial Viability Kit Staining). This is a fluorescent dye and digital colorimetric approach that should be an improvement in the conventional Tulane incubation method (Karkashan et al., 2015, Dabrowska et al., 2014, Włodarczyk et al., 2017).

The use of this approach was found to be more efficient and less labour intensive than the Tulane method. A steady decline in the viability of eggs over 17 weeks at 20°C was observed with Ascarid egg viability decreasing by 31% and 22% in Rochester and Cobden samples respectively. An inverse relationship between the viability of eggs and the percentage of dissolved solids was observed in this study. The Live/Dead® BacLight™ staining kit was successfully applied in this study and adequately differentiated viable eggs from non-viable eggs. This method was therefore more suitable for determining the viability of helminth eggs than the popular Tulane method and should be used in future studies on the viability of helminth eggs. Modelling the data obtained in this study indicated that total removal of *Ascaris* eggs in Cobden and Rochester biosolids can be achieved within 18 months of stockpiling. However, care must be taken in the extrapolation of these results as environmental factors such as temperature and pH and variable wastewater content (affects biosolid content) which were not adequately accounted for in the modelling may affect the egg removal rates.

Rather than focus on the role of physical and chemical factors on helminth egg viability and recovery, this study focused on potential microbial roles for the reduction of helminth egg viability which represents a novel approach. Temperature, pH, ammonia content and exposure time are known to affect the viability of helminth eggs in biosolids and manure (Pecson et al., 2007, Pecson and Nelson, 2005, Katakam et al., 2014). As an addendum to the natural disinfection processes, some waste treatment plants will carry out further physical and chemical treatment of the biosolids using a variety of methods which have been reviewed by Acquisto et al. (2006). These may include heat drying, irradiation with gamma rays, alkaline treatment
and acid liming. However, there is little to no information available on natural, microbial-mediated helminth egg inactivation processes.

Although biosolids do contain diverse groups of microorganisms, the focus of most research activities have been on the prevalence, identification and removal of microbial pathogens from biosolids using different treatment methods (Yergeau et al., 2016, Fatunla et al., 2017, Flemming et al., 2017). This study is novel and different from most studies in that the focus was on investigating the potential role of biosolid microbial communities on the inactivation of Ascarid eggs. This was initially carried out with a culture-independent approach.

Using a PCR-DGGE approach and UPGMA dendrograms, this study evaluated the bacterial community diversity and potential role in the loss of viability of Ascaris eggs in the different biosolids samples. The microbial communities in Heyfield, Cobden and Rochester biosolid samples were substantially different (70-80% dissimilarity). Rochester biosolid samples had the highest bacterial community diversity and were significantly different from other samples. These differences may be related to the different wastewater components of the WWTP.

Wastewater composition have been shown to cause shifts (differences) in the microbial community of biosolids. For example, the distribution and dominance of Archaea were observed to change as the wastewater component changed from a predominantly glucose containing waste to that containing pharmaceutical wastes. While Archaea were still present, changes in the relative contribution of different Archaeal species were detected on DGGE profiles (Akarsubasi et al., 2005). Pharmaceutical residues (Kraigher et al., 2008), heavy metals (Tsai et al., 2005) and nanoparticles (Yang et al., 2014) in wastewater have been shown to determine the bacterial structure in wastewater treatment systems. In a study of the bacterial community in 14 different wastewater treatment plants, wastewater characteristics were
reported to be the major factors responsible for variation in the microbial community (Wang et al., 2012).

Evaluation of the expressed functionality (assessed with MT2 Biolog plate assays) indicated that the bacterial communities in the tested biosolids samples produced enzymes that were able to digest the protein coat of helminth egg cell walls, especially in biosolids samples from Cobden and Rochester. All the samples demonstrated some utilization of chitin or lipid substrate indicating that the potential to degrade these components in Ascaris eggs exist in the biosolid’s microbial communities. Putative identities of key members of the bacterial community in these biosolid samples indicated that they belonged to bacterial genera known to produce chitinases, lipases and proteinase. It is highly likely that these microbial groups including Pedobacter sp., Pseudomonas fluorescens and Brevundimonas bullata strain would be involved in the degradation of Ascaris eggs resulting in the steady decline in the viability of eggs over 17 weeks at 20°C observed in this study.

PCR-DGGE-diversity analysis of the dominant bacterial community did show that Rochester samples had the highest Shannon diversity values. Samples from Rochester also had the highest observed egg decay (31%) over the experimental period. It is therefore possible that some of the comparatively high rates of decay in Ascaris egg viability in Rochester samples are due to microbial mediated egg decay activities. Adjunct research on the decay of Salmonella Birkenhead and coliphages in biosolids have demonstrated bacterial roles in their decay. Indigenous biosolid microorganisms produced enzymes such as proteases which were shown to digest bacterial and viral proteins, inactivating coliphages, contributing to the loss of viability observed in Salmonella (Mondal et al., 2015). These indigenous microorganisms potentially would be digesting Ascaris sp. eggs in the biosolid or biosolids samples. However, this decay processes had not yet been investigated; a knowledge gap that this study has now been filled.
The potential for bacterial mediated *Ascaris* decay was further investigated using a culture-based approach. Culture-based assays with BH media supplemented with either chitin, protein and lipid substrates and seeded with biosolid samples from Rochester, Heyfield and Cobden yielded distinct isolates. These isolates were further screened on Biolog MT 2 plates supplemented with the desired substrates and based on their enzyme activities and growth characteristics three high performing isolates were selected. These isolates were identified as *Pedobacter* sp., *Acidovorax* sp. and *Brevundimonas* sp.

The *Ascaris* eggs decay efficiencies of these three isolates were evaluated individually and as a mixture and compared with decay percentages obtained with commercial enzymes. Individually, the egg decay efficiencies of the three isolates (pellets, cell-free and uncentrifuged cultures) were similar and when compared with individual commercially obtained enzymes were not substantially different. This indicated that the bacterial groups were highly efficient producers of chitinase, lipase and protease enzymes as their activities were similar to those of purified commercially obtained enzymes. However, when used as a mixture, there was a higher decay of *Ascaris* eggs (~23%) compared to the commercial enzyme mixture (~19%), individual isolates and cell-free samples. This suggested that microbial synergy was essential for the efficient decay of eggs in biosolids samples.

In conclusion, this study has demonstrated the suitability of the Tulane method for egg detection and recovery in biosolids from different WWTP. The BacLight kit was also applied to different biosolids, successfully discriminating viable and non-viable *Ascaris* eggs, and is a suitable replacement for the Tulane method. Using traditional (PCR-DGGE) and metagenomics microbiological tools and MT2 plates, this study evaluated the microbial community in biosolids from different WWTP highlighting differences in bacterial community structure (diversity and dominance) and functionality. Focusing on the microbial factors responsible for egg decay, this study has also conclusively demonstrated that the bacterial
communities in biosolids from Rochester, Heyfield and Cobden can cause the decay of *Ascaris* eggs. Some bacterial species (*Pedobacter* sp., *Acidovorax* sp. and *Brevundimonas* sp.) responsible for this decay were also identified. The author is unaware of any similar published research on the effects of bacteria on the decay of *Ascaris* eggs in biosolids. This report, therefore, represents one of first report of the effects of these bacterial species on egg decay.

### 6.2 Future direction

This study confirmed that the Tulane method was a suitable method for the detection of *Ascaris* sp. eggs in biosolid samples but using a BacLight Staining kit offered a faster and more reliable approach for differentiating viable and non-viable eggs. Investigation of the role microbial community in egg decay in biosolids using Biolog Assays indicated that members of these community can potentially utilize chitin, lipids and protein components of *Ascaris* sp. eggs. Culture based assays using isolates from biosolid samples showed that *Pedobacter, Acidovorax* and *Brevundimonas* species can degraded *Ascaris* sp. eggs. Mixture of cell free aliquots of the three isolates caused a greater decay of eggs than all other tested samples.

Given the focus and nature of this study (one-time sampling carried out), detailed analyses of biosolid samples were not carried out. The effects of seasons on the wastewater treatment process and microbial community was also not evaluated. However, these could be carried out in future studies and should allow for the assessment of *Ascaris* eggs recovery and decay over different seasons. The limit of the detection/recovery of eggs of the Tulane methods in different samples were also not evaluated in this study due to time constraints. Further investigations can be conducted to determine the range of this detection/recovery threshold using a higher number of biosolids from more diverse sources and varying egg seeding concentrations.
The efficiency of the microbial mediated decay of *Ascaris* eggs observed in this study should be evaluated and optimized in future studies. The optimized decay efficiencies can be compared with other physical or chemical processes designed to eliminate *Ascaris* eggs. It is possible that this approach could be used solely for *Ascaris* eggs decay or as part of the physio-chemical biological approach to biosolid treatment. The feasibility of both approaches can be tested in future studies. The extent of bacterial degradation of *Ascaris* eggs in biosolid samples is presently unknown and the influence factors such as pH, temperature, water content and other microbial groups (protozoa and fungi) on the process was not investigated. The mechanisms by which bacterial synergy enhances egg decay and whether these decay processes are applicable to other helminths found in biosolids are not known. These should be investigated in future studies.
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Appendix
Appendix A: The various treatment grades of biosolids (EPA, 2004)

<table>
<thead>
<tr>
<th>Treatment process</th>
<th>Associated controls</th>
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<tr>
<td><strong>Treatment Grade T2</strong> Microbiological criteria</td>
<td></td>
</tr>
<tr>
<td><strong>Verification</strong> (of prescribed processes listed below)</td>
<td>≤ 1 Salmonella/50g (dw), &lt;100 E.coli MPN/g (dw) and ≤1 enteric virus PFU/200g with analysis detailed in 4.2.1. Verification of inhibition of pathogen regrowth is also required (4.2.2).</td>
</tr>
<tr>
<td><strong>Routine monitoring</strong> (of prescribed processes listed below) is based on ≤ 1000 E.coli MPN/g (dw) (4.2.2).</td>
<td></td>
</tr>
<tr>
<td><strong>Alternative process</strong> microbiological described on case-by-case basis (detailed 4.2.2) Vector attraction reduction controls also required (see Table 4).</td>
<td></td>
</tr>
<tr>
<td>Composting processes that simultaneously heat all material (e.g. in-vessel) Temperature of all compost material to be maintained at 25°C for 21 days, including 5 turnings of the windrow. Process control as per AS-4454.</td>
<td>Relevant vector attraction reduction controls (refer Table 4) and production of product that does not generate offensive odours. Weed seed controls may be needed in landscaping or agricultural applications.</td>
</tr>
<tr>
<td>Composting window method Temperature of compost material maintained at 35°C for 21 days, including 5 turnings of the windrow. Process control as per AS-4454.</td>
<td>Relevant vector attraction reduction controls (refer Table 4) and production of product that does not generate offensive odours. Weed seed controls may be needed in landscaping or agricultural applications.</td>
</tr>
<tr>
<td>High pH and high temperatures Biosolids pH raised to 2.02 for 372 continuous hours and during this period, maintained at 25°C for 212 continuous hours. Final biosolids product to be air-dried to a solids content of 25%.</td>
<td>Relevant vector attraction reduction controls (refer Table 4) and production of product that does not generate offensive odours.</td>
</tr>
<tr>
<td>Heating and drying Biosolids dried by heating particles to ≥80°C to a total solids content of 25%.</td>
<td>Relevant vector attraction reduction controls (refer Table 4) and production of product that does not generate offensive odours.</td>
</tr>
<tr>
<td>Long-term storage Sludge is digested, dewatered to ≤10% w/w solids and stored for ≥3 years.</td>
<td>Product must be stored in a manner that ensures no recontamination and not generate offensive odours.</td>
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<td>Thermophilic digestion processes EPA endorsement of processes operating at greater than 55°C will be considered on a case-by-case basis depending on retention time, process stages and batch versus continuous feed/draw.</td>
<td>Relevant vector attraction reduction controls (refer Table 4) and production of product that does not generate offensive odours.</td>
</tr>
</tbody>
</table>

### Suggested Treatment Process

| Treatment Grade T2 Microbiological criteria |
| **Routine monitoring** (of prescribed processes listed below) | ≤ 1 Salmonella/50g (dw), <1000 E.coli MPN/g (dw) (described in 4.2.3). |
| **Alternative process** Based on achieving Salmonella and E.coli criteria and demonstration of a log Taenia saginata and enteric virus removal or batch testing to demonstrate ≤ 1 Taenia ova per 50g and ≤1 enteric virus PFU per 50g. Vector attraction reduction controls also required (see Table 4). |
| Composting method The temperature of all compost material to be 25°C for 21 continuous days at 25°C for 21 continuous days. (NB. Although this criteria is comparable to T1, it is also included as a T2 process in reflection that achieving the stringent T1 E.coli limits may require specialist techniques. | Relevant vector attraction reduction controls (see Table 4) and production of product that, coupled with management controls, does not generate offensive odours. Weed seed controls may be needed in landscaping or agricultural applications. |
| Heating and drying Biosolids are heated to 200°C and dried to a solids content of at least 25% w/w. | Relevant vector attraction reduction controls (see Table 4) and production of product that, coupled with management controls, does not generate offensive odours. |
| Aerobic thermophilic digestion Aerobic conditions at 55-60°C for ≥10 continuous days. Final product dried to 25% solids. (NB. Could also achieve T1 process). | Relevant vector attraction reduction controls (see Table 4) and production of product that, coupled with management controls, does not generate offensive odours. |

### Treatment Grade T3

| **Routine monitoring** (of prescribed processes listed below) | ≤ 2,000,000 E.coli MPN/g (dw). |
| **Alternative process** Based on E.coli criteria and 1 log reductions in Salmonella and enteric viruses. Vector attraction reduction controls also required (Table 4). |
| Anaerobic digestion 255 days at 35°C or ≥60 days at 25°C. | For all Grade T3 treatment processes: Relevant vector attraction reduction controls (see Table 4) and product that, coupled with management controls, does not generate offensive odours. Weed seed controls may be needed in landscaping or agricultural applications. |
| Aerobic digestion 240 days at ≥20°C or ≥60 days at 25°C. | |
| Composting Aerobic conditions maintained 25 days at 26°C including 24 hours at 25°C. | |
### Contaminant upper limits for classifying biosolids as grade C1 or C2

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Grade C1 &amp; RSCL (mg/kg)</th>
<th>Grade C2 (mg/kg)</th>
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Appendix B: Tulane Method

The recovery and incubation of *Ascaris* eggs from faecal and biosolid sample. Source Bowman et al., 2006.

**PROCEDURE FOR THE EXAMINATION OF WASTEWATER SLUDGE OR SLUDGE PRODUCTS FOR HELMINTH EGGS**

**SEAGENTS, MATERIALS, EQUIPMENT**

**Reagents**
1. 1% aqueous solution of 7X (Lindeco) (v/v), an anionic detergent.
2. MgSO4 (USP) solution, specific gravity 1.20, adjusted with hydrochloric acid.
3. Culture fluid, 0.5% formalin (5 mL formalin (40% formaldehyde) + 950 mL H2O).
4. Bleach solution, 0.05% solution of sodium hypochlorite (10% vol.) of household bleach.
5. Pross 28" (organosilane for coating glassware). All glassware should be treated according to instructions.

**Materials**
1. Beakers, 100 mL, bail form, Duran glass.
2. Beakers, 100 mL, low form.
3. Funnel, glass, short stem, 50 mm diameter.
4. Funnel, powder filter, 150 mm diameter, Naigene.
5. Centrifuge tubes, conical, 50 mL, polypropylene.
6. Centrifuge tubes, conical, 15 mL.
7. Petri plates, Petri dish, disposable, 5.94 inches.
8. Cover glasses, 24 x 40, 24 x 50 mm.
9. Microscope slides, glass, 3 x 2 inches.
10. Sieve, 20 mesh, 5 in diameter, brass frame.
11. Sieve, 50 mesh, 5 in diameter, brass frame.
12. Sieve, 400 mesh stainless-steel screen attached to bottom of centrifuge stainless-steel funnel (5 in diameter, 400 mesh screen can be obtained from Small Parts Inc., P.O. Box 31699, Miami, FL 33233-6990). Stainless steel wire cloth, Twilled square weave, Part No. D-6X4-400, Tel: 305-751-0050.
13. Hydrometer, 1.0 - 1.3 sp gr.
14. Culture dishes (5 X 15 mm glass Petri dishes).
15. Wash bottles, plastic, 500 mL.
16. Spray bottles, 15 mL.
17. Paraffin, 4 in wide roll.
18. Carrot hair brushes, small.
19. Agitators, wooden, 6 in long.
20. Tongue depressors, wooden, 5 in long.

**Equipment**
1. Centrifuge, with head and cups (swinging) for 50 mL tubes.
2. Centrifuge, clinical model, with head and cups (swinging) for 15 mL tubes.
4. Magnetic stirrers.
5. Microscope, compound, binocular.

**PROCEDURE**
1. For liquid sludges, take an amount that will contain at least 5 g total solids (dry weight basis) or more, place in blender container, add about 250 mL of water, blend for 1 min at high speed.
2. For dewatered sludges, take an amount that will contain at least 1 g, while dried or more (usually 30 - 50 g), place in blender container, add about 200 mL of water, blend for 1 min at high speed.
3. Pour blended sample into a 1000 mL ball form beaker and using a wash bottle, thoroughly rinse blended sample into beaker. Add 7X to 750 mL.
4. Let settle for 3-4 hrs (or overnight). While settling, the solution may need to be stirred occasionally to ensure that any material floating on the surface will settle.
5. After settling, pour off the supernatant, transfer to blender container, add water to 300 mL, and blend again for 1 min at high speed.
6. Transfer to ball form beaker, add 7X to 900 mL, and allow to settle for 2 hr. Pour off supernatant.
7. Add 300 mL of 7X, stir for 5 min on a magnetic stirrer.
8. Pour homogenized sample through a 20-mesh sieve into another ball beaker, carefully rinsing all material from beaker. The sieve is placed on a powder flaring funnel sitting on a ball beaker and the sample is washed through the sieve with the aid of spray water from a spray bottle.
9. Pour this mixture into the original tall beaker through a 50-mesh sieve as described above. If there is difficulty in rinsing the material through the sieve, a caron hair brush can be used to stir the material on sieve. This stirring is then followed with spray water from a spray bottle.
10. Add 7X to 900 mL, settle for 2 hr. or longer.
11. Decant and discard supernatant; mix sediment and divide to 50 mL centrifuge tubes. Thoroughly wash any sediment in beaker into tubes with the aid of a wash bottle. Bring volume in each tube to 50 mL with water. The number of tubes required will depend upon the amount of sediment. Usually at least 2 tubes are required.
12. Centrifuge for 5 - 10 min at 800g. Decant and discard the supernatant. The packed sediment in each tube should not exceed 5 mL. If so, add water, mix and distribute evenly among additional tubes. Repeat centrifugation and then discard supernatant.
13. Add about 15 mL of MgSO4 solution to each tube, leave sediment from tube with applicator stick, cap and mix for 15 - 25 sec on a vortex mixer.
14. Add additional MgSO4 solution to each tube to bring to 50 mL, (one applicator stick into one tube; centrifuge for 5 - 10 min at 800g).
15. After centrifuging has come to a complete stop (without use of break), while rotating the tube, pour most of the supernatant from each tube through the 400 mesh sieve (sieve may be placed over a tall beaker).
16. If using the spray bottle, wash the excess collection fluid and fine particles through the sieve. If there is difficulty in passing the supernatant through the sieve, a caron hair brush may be used to stir the sediment on the surface of the sieve while using the spray.
17. Rinse the sediment collected on the sieve into a 100 mL beaker by directing a stream of water from a wash bottle onto the upper surface of the sieve and washing the material into the beaker while the sieve is partially inverted.
18. Transfer the suspension in the beaker to one or more 15 mL centrifuge tubes, taking care to remove the beaker thoroughly.
19. Centrifuge the tubes for 3 min at 800g, then decant the supernatant and discard.
20. Add 0.5 mL of culture fluid (0.5% formaldehyde) to the sediment in each used tube, mix, and add culture fluid to fill tube.
21. Centrifuge for 5 min at 800g, then decant supernatant.
22. Add a few drops of culture fluid to sediment, transfer mixture into a culture dish. Thoroughly rinse tube with culture fluid from a wash bottle, transfer to culture dish. Add culture fluid, if needed, to have the fluid completely cover bottom of dish (to a depth of 3-4 mm). Place a sheet of Parafilm over Petri dish bottom to seal, then cover with Petri dish top.
23. Store culture dish in dark or subdued light at 22-28°C. Beakers should be agitation every few days to mix sediment and to aerate fluid.
24. After a minimum of 28 days, the cultured sediment is transferred to one or more 15 mL centrifuge tubes, depending on the volume of the sample, by placing a small glass funnel into the tube and mixing the suspension into the tube.
25. Centrifuge at 800g for 3 min. then decant supernatant.
26. Add 10 mL of 10% household bleach (1 part to 9 parts water) to sediment and mix. Allow to stand for 10 min (this desiccates the outer shell of the eggs of *Ascaris* and *Trichuris* and permits the control of the eggs to be obtained more easily when examined microscopically).
27. Centrifuge at 800g for 3 min; decant supernatant.
28. Add a few mL of water, mix, fill tube with water, then centrifuge as in step 40.
29. With pipette, mix sediment with the small amount of water remaining in the tube (it may be necessary to add more water), transfer an appropriate amount of the sediment to a microscope slide, and cover with an appropriate size of coverslip. (fluid should not extend beyond edges of coverslip) [in order to induce any larvae in eggs to become actively mobile, submerge bottom of tube in a beaker containing tap water at 38-40°C for a few minutes.]
30. Systematically examine the preparation under at least 10X magnification of the microscope. If necessary identify objects under 40X magnification. 31. Count all of each type of parasitic helminth egg present (*Ascaris*, *Toxocara*, *Trichuris*, etc.) and record. 32. Repeat the number of viable eggs of each type present in the amount of the sample examined (e.g., 5 viable *Ascaris* eggs present in 5.1 g TSS sample). If no eggs found, record: less than one helminth egg found in the sample size examined (e.g., <1 viable helminth egg found in 5.1 g TSS sample).
Appendix C: The number of recovered eggs was counted in three cells of the Universal worm eggs counting chamber.

A: first sample

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### Appendix D: Biosolid requirements for Tulane Method

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<th>%DS Value</th>
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<th>Volume Biosolid Required (mL) for Tulane method (min 5g TS)</th>
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Appendix E: Manufacturer’s instruction LIVE/DEAD® BacLight™ Bacterial Viability Kits (Molecular Probes manual 2004)

Staining Bacteria in Suspension with either Kit L7007 or L7012

2.1 Combine equal volumes of Component A and Component B in a microfuge tube, mix thoroughly.

2.2 Add 3 μL of the dye mixture for each mL of the bacterial suspension. When used at the recommended dilutions, the reagent mixture will contribute 0.3% DMSO to the staining solution. Higher DMSO concentrations may adversely affect staining.

2.3 Mix thoroughly and incubate at room temperature in the dark for 15 minutes.

2.4 Trap 5 μL of the stained bacterial suspension between a slide and an 18 mm square coverslip.

2.5 Observe in a fluorescence microscope equipped with any of the filter sets listed in Table 1.

<table>
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<tr>
<th>Omega Filters*</th>
<th>Chroma Filters*</th>
<th>Notes</th>
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<tr>
<td>XF25, XF26, XF115</td>
<td>11001, 41001, 71010</td>
<td>Longpass and dual emission filters useful for simultaneous viewing of SYTO 9 and propidium iodide stains</td>
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<td>Bandpass filters for viewing SYTO 9 alone</td>
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* Catalog numbers for recommended bandpass filter sets for fluorescence microscopy. Omega® filters are supplied by Omega Optical Inc. (www.omegafilters.com). Chroma filters are supplied by Chroma Technology Corp. (www.chroma.com).
Publications
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

Alaa Karkanash, Basma Khalil, Jacqueline Morris, Nerida Thurbon, Duncan Rouch, Stephen R. Smith, Margaret Deighton

School of Applied Sciences, RMIT University, Plenty Road, Bundoora 3083, Victoria, Australia
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**Abstract**

The aim of this study was to evaluate the Live/Dead BacLight viability kit as a method for enumerating viable eggs of *Ascaris suum* in sewage sludge as a surrogate for the human roundworm. The number and viability status of eggs of *A. suum* were accurately measured directly in sewage sludge samples by the BacLight method, compared to the conventional incubation-microscopy procedure. BacLight stains were not toxic to *A. suum* eggs, in contrast to some conventional vital dyes which disrupted viable eggs. The method was effective for the direct examination of eggs in heavily contaminated samples or seeded sludge containing 300 eggs/g DS in sludge with 5% DS content. However, a recovery method would be necessary to examine samples with small numbers of eggs, for instance in sludge from regions where the prevalence of infection with *Ascaris lumbricoides* is low. The BacLight technique may therefore be an alternative to conventional incubation-microscopy for enumerating *Ascaris* eggs in contaminated field samples or to validate sludge treatment processes by examining decay rates of inoculated *A. suum* eggs in laboratory simulations. Most field samples would require recovery from an appropriate number of composite samples prior to vital staining.

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1. Introduction

Treated sewage sludge (biosolids) application to land provides benefits to soil from the addition of plant nutrients, such as nitrogen and phosphorus, and by improving soil structural properties and water holding capacity (NRC, 1996; Rouch et al., 2011a,b). However, biosolids may contain an array of pathogenic microorganisms, including bacteria, viruses, protozoa and helminths, excreted by infected individuals resident in
the wastewater collection catchment. These microbiological agents represent a significant potential risk to human health from the agricultural use of sludge through the faecal-oral transmission pathway of infection, mainly from contaminated food crops (Sidhu and Toos, 2003). Biocides use on agricultural land is therefore carefully regulated and controlled to protect human health by providing barriers to pathogen transmission. This is achieved by the treatment of sludge using effective processes to reduce or eliminate the pathogen content, supported by, but not replacing, on-farm storage and application. Therefore, a rapid and reliable method is required to distinguish between infective and non-infective eggs and to enumerate infective eggs in environmental samples. The published methods for assessing the viability of Ascaris spp. eggs include: (i) conventional microscopic examination, (ii) use of various vital dyes, (iii) incubation under conditions to promote larval development, and (iv) polymerase chain reaction (PCR) techniques. Conventional microscopic examination for determining viability lacks objective standards (WHO, 2004). The qPCR method shows promise as a method for viability assessment of Ascaris spp. but requires further evaluation for use in sewage sludge eggs (Peason et al., 2009; Raynal et al., 2012).

The Live/Dead BacLight bacterial viability kit (Molecular Probes Inc., Eugene, USA) was primarily designed for determining the viability of bacterial cells (Boullon et al., 1999; Qu et al., 2010). The fundamental principle of the BacLight test is based on detecting differences in the membrane integrity of viable and non-viable cells. This is determined by their differential uptake of two specialized membrane-permeable DNA labelling dyes: Syto 9, which fluoresces green (maximum emission 498 nm), and propidium iodide (PI), which fluoresces red (maximum emission 617 nm). Syto 9 is taken up by viable cells, but PI is excluded, and live cells emit green fluorescence; however, damaged cells and quenchers Syto 9, therefore non-viable cells exhibit red fluorescence (Lisle et al., 1999). A recent publication showed that BacLight bacterial viability kit could be used to distinguish viable eggs from non-viable eggs of Ascaris suum (as a surrogate for the human roundworm) when inoculated into water or dewatered sewage sludge (Dabrowska et al., 2014). However, the relative efficiency of this method compared to conventional staining methods is unknown.

The aim of this study was to further evaluate the Live/Dead BacLight viability kit as a method for enumerating viable eggs of A. suum in sewage sludge. The objectives were to: (i) compare Ascaris egg viability using the BacLight kit with conventional incubation and staining with simple vital dyes, (ii) further investigate the ability of the BacLight viability kit to enumerate viable A. suum eggs in sewage sludge and, (iii) observe the effect of the different dyes on embryonated eggs and exiting larvae.

2. Materials and methods

2.1. A. suum as a surrogate for A. lumbricoides

Eggs of A. suum are morphologically indistinguishable from eggs of A. lumbricoides and the two species exhibit similar
using a Leica DM2500 compound light microscope with 40×, 100× and 200× magnification and equipped with a Nikon digital camera. Eggs were recorded as viable if they did not take up dye or showed leaking internal components and non-viable if the internal parts of the egg were stained. Eggs that could not be classified were recorded as uncertain. Three replicates were examined for each dye.

2.5. Direct determination of egg viability in sewage sludge

2.5.1. Use of BacLight method to determine viability in seeded sludge

The BacLight method was applied to eggs suspended in a sample of digested sewage sludge (2% DS) obtained from the output of a mesophilic anaerobic digester. An aliquot of 1 ml of heat-treated or untreated eggs was added to 2 ml of a diluted sludge mixture (20 g/100 ml in RO water) and mixed to ensure the eggs were thoroughly distributed in the material. After bleaching, the final volume was made up to 2 ml an aliquot of 0.5 ml of the seeded, bleached sludge was stained with the BacLight vital dyes and 20 µl volumes of the reaction mixture were examined by CLSM (following the procedure described above). The total numbers of viable and non-viable eggs were counted. Staining and microscopy procedure was repeated five times, the total volume of stained material examined was 100 µl.

2.5.2. Recovery efficiency of A. suum eggs in sewage sludge by the standard methodology

The application of BacLight staining to sewage sludge samples containing small numbers of eggs would require a recovery step so that sufficient eggs may be collected for counting and viability testing. Therefore, we determined the recovery efficiency of the BacLight technique for A. suum eggs seeded into sludge samples collected from two lagoon treatment systems at operational wastewater treatment plant (WWTP) in regional Victoria, Australia. Sludge from WWTP A had a DS content of 19% (dewatered), and the DS content of sludge from WWTP B was 3.0% (fresh). Since the Tulane method requires sludge samples of ≤ 5% total solids (Bowman et al., 2003), 50 ml aliquots of WWTP A sludge and 200 ml aliquots of WWTP B sludge were used in this experiment, giving 7.5 g and 6.0 g of total solids per aliquot, respectively. Suspensions of recovered A. suum eggs (undiluted) containing >744 eggs/ml were diluted in RO water in the ratios of 1:8 and 1:32. These dilutions were chosen to provide a suitable number of eggs for counting following extraction and concentration. Two 10 ml aliquots of the undiluted suspension, and the two diluted egg suspensions (containing 7440, 1860 and 465 eggs/10 mL, respectively), were used to seed duplicate sludge samples collected from each WWTP 50 ml of sludge was used for WWTP A and 200 mL of sludge for WWTP B to account for the contrasting DS contents. Eggs were recovered from the seeded sludge samples using the Tulane method, as described previously, the deposit, containing the seeded eggs was collected and made up to 1.5 ml. For each sample of recovered eggs, the entire deposit (1.5 ml) was examined by light microscopy, and the total number of eggs was counted and recorded. Recovery efficiencies were calculated by dividing the total number of eggs counted per deposit by the density of eggs initially added to the sludge.

2.6. Statistical methods

Since the total number of eggs obtained from the stock suspension for staining varied slightly from sample to sample, percentage values rather than absolute numbers of eggs were used to compare the different techniques of viability determination. Data obtained from staining eggs suspended in RO water or sludge with vital stains were analysed using the 95% confidence interval test of the proportion of viable and non-viable samples determined by the conventional methodology. This test measures the reliability of the results by calculating the upper and lower confidence limit for the population mean using the following formula:

\[ \hat{p} \pm 1.96 \sqrt{\frac{\hat{p}(1-\hat{p})}{n}} \]

where \( \hat{p} \) is the proportion mean of the samples, 1.96 is the confidence level which is equivalent to 95%, \( \hat{p} \) is \((1 - \hat{p})\) and \( n \) is the number of replicates.

3. Results

3.1. Egg recovery and assessment of viability after conventional incubation by light microscopy and by CLSM

The total egg count (viable and non-viable) recovered from pig faeces, determined by light microscopy using a universal egg counting chamber was 2970 eggs/mL. However, the total number of eggs counted in a total volume of 30 µl by the conventional incubation and light microscopy procedure was 127 eggs (40, 44, and 43 were counted respectively in three replicate 10 µl aliquots). Assuming the uniform distribution of eggs in the sample, the estimated number of eggs in this volume, based on conventional microscopy of the sample of pig faeces, was 89. The larger number of eggs measured by incubation-microscopy compared to the direct counting procedure was probably explained by sampling errors associated with the pipetting of dense suspensions of A. suum eggs, for instance: (i) during transfer of 15 ml of the stored eggs suspension to a Petri dish for incubation, or (ii) while collecting 10 µl samples of the incubated egg suspension for examination by light microscopy. Viable and non-viable eggs were distinguished by the incubation-microscopy procedure from the presence of actively motile larvae within or exiting the egg; of the total number of eggs counted (127), 109 (85.8%) were indicated as viable and 18 (14.2%) were non-viable (Table 1, Fig 1).

3.2. Staining of incubated eggs using the Live/Dead BacLight procedure

Staining of incubated eggs with BacLight vital dyes and examination by CLSM showed that larvae did not take up either Syto 9 or PI (Fig 2 row A) and remained actively motile inside and outside of the eggs following exposure to the stains. Only the inner lipoprotein layer of eggs containing active larvae took up the stains and eggs demonstrating this behaviour
were therefore classified as viable. By contrast, the internal structures of incubated eggs lacking recognizable larvae, or that had been previously heat treated, were comprehensively stained with both dyes (Fig. 2 row b). Ascaris eggs exhibiting this response to the BacLight vital stain treatment were classified as non-viable.

3.3. Direct staining of recovered eggs using the Live/Dead BacLight procedure

Direct viability staining of *A. suum* eggs recovered from pig faeces (without incubation) showed that the inner membrane (lipoprotein layer) of the majority of eggs stained with both Syto 9 and PI, but internal structures remained unstained. Based on the behaviour observed in the experiments with incubated eggs, eggs responding in an identical manner to direct staining were therefore recorded as viable (Fig. 2 row C). Unfertilized eggs, recognizable by their characteristic elongated shape, also showed a similar response to non-viable, incubated eggs and were saturated by both Syto 9 and PI stains. The inner membranes, as well as the internal contents, of all heat-treated eggs were also stained with both dyes (Fig. 2 row D). In both cases, these eggs were classified as non-viable. A small proportion of eggs showed partial staining of their internal components and their classification was “uncertain”.

The total number of eggs counted after recovery from pig faeces was 389 and exceeded the estimated number of 323 based on the inoculation density by 20%. The mean percentage of eggs assessed as viable (internal structures unstained) was equivalent to 70–88% (mean 78%), which was marginally smaller compared to the mean result (86%) from conventional incubation and light microscopy; however, the difference was not statistically significant (p > 0.05) (Table 1, Fig. 3). Both the BacLight (internal contents stained) and the conventional Tulane method estimated the number of non-viable eggs as 14%. A further 8% of eggs showed partial internal staining by the BacLight method and their viability could not be classified (uncertain category) (Table 1). Recovered heat-treated eggs were assessed as non-viable by both conventional incubation and vital staining: 313 heat-treated eggs were examined and the viability status of one egg was indicated as uncertain by the BacLight method (Table 1).

3.4. Direct staining of recovered and incubated eggs with other vital dyes

Direct staining of untreated recovered eggs with simple dyes generally caused leaking of the internal contents of viable eggs after 2–3 min of exposure (Fig. 4 row A). Viable incubated eggs, showing actively motile larvae that were usually already hatched (Fig. 4 row C), did not stain initially with any of the four dyes tested. However, larvae took up the stains within 5 min and stopped moving. Safranin caused the larvae to lyse completely after several minutes of exposure to the stain. The total numbers of untreated eggs counted after staining by the vital dyes were in the range 82 (trypan blue) to 150 (crystal violet), compared to the number determined by the standard method (127) (Table 1). The percentage of eggs indicated as viable by methylene blue (87%) was similar to and not statistically significantly different (p > 0.05) from the standard procedure (86%), although 24% fewer eggs were detected overall by this stain compared to the Tulane method. Safranin and crystal violet overestimated the proportion of viable eggs.

![Viable and non-viable Ascaris suum eggs (200x). The egg in the top right is non-viable (unembryonated) (red arrow), and the two eggs on the left have fully developed larvae (green arrow). Some debris is present between the eggs.](image-url)
Fig. 2 – Ascaris suum eggs stained using Live/Dead BacLight viability kit. Column a: merged images from both channels viewed with transmitted light (Syto 9 and PI), transmitted light; Column b: Syto 9; Column c: PI; Column d: transmitted light. Row A: Incubated A. suum eggs; viable larva; the larva (blue arrow) appears only in images Column a and Column d; some debris is present around the larva; The larva has exited the egg following the 28 day incubation period; the larva was actively motile and did not stain with either dye. Row B: Incubated heat-treated A. suum eggs containing fully developed larvae (red arrow); five non-viable eggs are visible; all the larvae inside the eggs stained with both dyes, suggesting membrane damage; some debris was present around the eggs. Row C: Direct staining of recovered eggs; the inner membrane (lipid layer) stained with both dyes (green arrow); some debris was present around the eggs. Row D: Direct staining of heat-treated (non-viable) eggs; the centre of the eggs (non-viable syncyte) and the inner membrane (lipid layer) stained with both dyes (red arrow). Row E: Enlargement of eggs shown in images Ca and Da. The lipid membrane of viable eggs showed staining with both dyes, while the internal structures of non-viable eggs showed variable amounts of staining with PI.
Fig. 3 – Mean values and confidence limits for the fraction of viable eggs assessed by seven different methods. Conventional incubation (CI), BacLight (BL), methylene blue (MB) safranin (S), trypan blue (TB), crystal violet (CV), and BacLight in sludge (BLS). UCL lower confidence limit, UCL higher confidence limit. Bars indicate 95% confidence limits; midpoints of bars indicate mean values.

compared to conventional incubation-microscopy, by 11% and 6%, respectively. The viability status of a large proportion of eggs, equivalent to approximately 60% of the total number of eggs counted, could not be determined with trypan blue (Table 1). Thus, trypan blue significantly underestimated egg viability by almost 50% relative to the conventional method (Table 1, Fig. 4).

Heat-treated eggs were consistently indicated as non-viable and generally stained strongly with all dyes, except for trypan blue (Fig. 4, row B). However, all dyes generally underestimated the number of non-viable eggs in heat-treated samples due to the higher proportion of uncertainties compared to conventional incubation-microscopy. Only safranin provided results for non-viable egg counts (97%) that were comparable to and not significantly different (P > 0.05) from the standard incubation (Table 1). Thus, in order of increasing uncertainty at establishing the viability status of heat-treated A. suum eggs, the dyes were arranged as: safranin (9%) < methylene blue (11%) < crystal violet (16%) < trypan blue (100%) (Table 1).

3.5. Recovery efficiency and limit of detection by the conventional Tulane procedure for A. suum eggs in sewage sludge

Recovery efficiencies of A. suum eggs from sludge samples by the Tulane procedure ranged from 33.3% to 73.3% (Table 2). For particular dilution mixtures, the results for sludge samples from WWTP A and WWTP B were approximately similar.

Fig. 4 – Ascaris suum eggs stained with vital stains. Column a: crystal violet; Column b: safranin; Column c: trypan blue; Column d: methylene blue. Row A: Non-incubated viable eggs; all the viable eggs showed leaking of their internal components after the staining. Row B: non-viable (heat-treated) eggs (non-incubated); the entire egg stained strongly with the dye. Row C: viable eggs after incubation for 28 days. Row D: non-viable (heat-treated) eggs after incubation for 28 days; all the non-viable larvae inside the eggs were stained; the egg in the safranin-stained sample is inside an air bubble; some debris was present in trypan blue and safranin-stained samples.
and within a 10% margin. An inverse relationship was observed between egg number and recovery efficiency, with the largest overall recovery efficiency values occurring with the smallest numbers of eggs. The data for all samples was pooled to calculate a linear regression relationship between the recovery efficiency and egg number, and this was represented by the following equation:

\[
\text{Recovery efficiency}(\%) = -0.0096 \times N_e + 71.7
\]

where \(N_e\) is the number of eggs in the sample (\(R^2 = 0.89, P = 0.005\)).

Given that the recovery efficiency increased as the egg concentration declined, it is reasonable to estimate the detection limit from the sludge samples containing the highest dilution rate of eggs (1:32). The theoretical limit of detection of total eggs (viable and non-viable) by the conventional recovery method for sludge aliquots from WWTP A was equivalent to 1 egg/7.5 g DS or 0.13 eggs/g DS. Adjusting for the recovery efficiency at the 1:32 dilution (73.3%), the limit of detection was 0.18 eggs/g DS. For sludge from WWTP B, the theoretical limit of detection was 1 egg/6 g DS, or 0.16 eggs/g DS, and the adjusted detection limit, accounting for the maximum recovery efficiency (63.5%), was 0.25 eggs/g DS. Using the average recovery efficiency value measured at the highest dilution rate of eggs for both sludge samples (68.5%) and a standard solids value of 5 g DS (theoretical limit of detection 0.2 eggs/g DS), the overall limit of detection was 0.29 eggs/g DS.

3.6. Staining sludge samples seeded with non-incubated eggs using Live/Dead BacLight

The total number of eggs enumerated in 100 mL of seeded and bleached anaerobically digested sewage sludge was 215. This value was larger than the estimated number of eggs (180) contained in the sludge based on the inoculation density and assuming homogeneous distribution of eggs in the sample.

As may be expected, the seeded sludge samples contained a considerable amount of debris when examined microscopically. Nevertheless, viable and non-viable eggs of A. suum were readily identified due to their large size and characteristic appearance (Fig. 5). Viable eggs are shown in Fig. 5 row A, heat-treated eggs are illustrated in Fig. 5 row B, and viable and unfertilized eggs are presented in Fig. 5 row C.

The viability results (a total of 219 eggs were assessed in a total volume of 100 mL of sludge, based on the examination of 5 replicate aliquots, each of 20 µL) determined by BacLight staining indicated that 60% of eggs were viable, 13% were non-viable and the remainder were indeterminate. This was entirely consistent with, and within the 95% confidence interval for, the proportion of viable and non-viable eggs determined by conventional incubation methods of the seeded sludge sample (85.8% viable, 14.2% non-viable) (Fig. 5). Heat-treated eggs seeded into sludge samples were all identified as non-viable by both detection methods.

4. Discussion

Direct staining with vital dyes to enumerate and determine the viability of Ascaris eggs potentially offers major advantages compared to the Tulane method of detection; in particular, they are simple and inexpensive, and avoid the extended period of incubation necessary for egg enumeration by the standard enumeration and viability assessment procedure. Here we present an assessment of the use of vital dye components of the Dead/Live BacLight kit: Syto 9 and PI for the enumeration and viability assessment of eggs of Ascaris spp. Our results are in general agreement with those of Dabrowska et al. (2014), who were also able to distinguish viable eggs from non-viable eggs of A. suum on the basis of BacLight staining.

The BacLight kit contains two nucleic acid stains (Syto 9 and PI), which bind to both DNA and RNA. According to the manufacturer (Molecular Probes Inc. Manual 2004), PI selectively stains non-viable bacterial cells, whereas Syto 9 penetrates both viable and non-viable bacterial cells. In tests with Ascaris eggs, only the lipoprotein layer of viable eggs was saturated with Syto 9 and PI, whereas the internal components of non-viable eggs stained with both dyes. In our experiments, Syto 9 did not enter intact A. suum eggs. This may be explained because the eggs of Ascaris spp. have a tough outer shell and an extremely impermeable lipoprotein layer and both provide barriers to the diffusion of molecules into the viable egg (Fairbairn, 1957; Wharton, 1983). Removing the external layers of the egg by sodium hypochlorite treatment allowed staining of the lipoprotein layer, but not penetration of the lipoprotein layer of viable eggs by either dye. The images presented here, exhibiting only surface staining of viable eggs, differ from those of Dabrowska et al. (2014), which appear to show staining of internal structures, which however, is likely due to background fluorescence from upper and lower surfaces of eggs. As well as exhibiting reduced background fluorescence, confocal microscopes can display thin layers of a sample and therefore show internal structures. Other differences in methodology are less likely to be the cause of the differences between images. Eggs used in the simulation studies by Dabrowska et al. (2014) were collected from the uterine content of worms obtained from the intestines of infected pigs, and thus would have had immature outer costs and possibly more permeable lipid layers. In contrast, when eggs are collected from pig feces, these will generally have fully developed outer costs (Fairbairn, 1957).
Fig. 5 – Ascaris suum stained directly in sludge with Live/Dead BacLight bacterial viability kit. Column a: merged images from both channels viewed with transmitted light (Syto 9 and PI), transmitted light; Column b: Syto 9; Column c: PI and Column d: transmitted light. Row A: viable eggs; two viable eggs are visible (green arrows). Row B: heat-treated eggs; three eggs are visible (red arrows), the egg in the top right is dechorinated. Row C: viable and unfertilized eggs in sludge sample; one unfertilized egg (brown arrow) is visible in the top right corner and one viable egg is visible below it (green arrows).

and are therefore, more representative of eggs present in wastewater. However, the source of eggs only appeared to have an only minor effect on egg staining as eggs present in naturally contaminated sewage sludge differed only slightly from those in experimentally enriched sludge and in both cases the entire egg appeared to be stained (Dabrowska et al., 2014). Furthermore, it is unlikely that the difference in staining eggs was due to the different proportions of dye used in the two studies, as Dabrowska et al., showed that 1 μL of dye mixture per mL of sample was sufficient for full staining, while in the present study, a higher proportion was used, 2.8 μL per mL of sample, as recommended by the manufacturer.

The lipoprotein layer of Ascaris spp. consists of an unusual class of lipids called ascarosides in association with protein. Ascarosides form after egg fertilization and are stored as acetate or propionate esters in the oocyte; they are secreted by evisceration of granules to the surface of the egg cytoplasm and are further strengthened after fertilization and formation of the egg shell (Wharton, 1983). Fairbairn (1977) reported that fertilized eggs also have a supply of cytoplasmic DNA. Therefore, the staining reaction with Syto 9 and PI could be explained by the possible secretion and incorporation of DNA into the lipid lipoprotein layer during its formation. Alternatively, staining with the nucleic acid dyes could result from the non-specific binding to the lipid lipoprotein layer.

The non-visible eggs observed in this study included heat-killed eggs, unembryonated eggs and unfertilized eggs. Both Syto 9 and PI penetrated the lipoprotein layer and stained the internal nuclear material of all these types of non-visible eggs. The ability of the dyes to enter heat-killed eggs was probably linked to damage and increased permeability of the lipid layer surrounding the eggs caused by the heat treatment. The lipoprotein layer becomes more impermeable following egg fertilization and shell formation (Wharton, 1983), which may explain why both vital dyes were able to saturate the internal structures of unfertilized eggs and unembryonated eggs.

Several authors (de Victoria and Galván, 2002; WHO, 2004; Jensen et al., 2009) reported the use of vital stains to differentiate viable and non-visible Ascaris spp. eggs. Despite their potential advantages, however, some stains are toxic to embryos and require sample examination within a few minutes of application (WHO, 2004). The Integrated Guide to Sanitary Parasitology (WHO, 2004) lists crystal violet staining followed by microscopy as a suitable method for enumerating and assessing the viability of Ascaris spp. eggs recovered from sewage sludge. de Victoria and Galván (2002) were able to reliably identify viable and non-visible eggs of A. suum with methylene blue, safranin and eosin Y, but not with trypan blue. More recently, Jensen et al. (2009) found methylene blue discriminated between viable and non-visible eggs of A. suum
seeded into human excreta. In the present study, viabilities estimated with BacLight and methylene blue were within the 95% confidence limits of the viable egg counts by the standard enumeration method; these viable stains also gave similar numbers of eggs recorded as “uncertain,” equivalent to approximately 8% of the total eggs counted by both methods. By contrast, viability was significantly overestimated by crystal violet and safranin, and underestimated by trypan blue. Most, but not all, heat-treated eggs were identified as non-viable and by staining with cotton blue, safranin and methylene blue; the most accurate estimate of non-viable eggs, compared to standard incubation-microscopy, was obtained with safranin.

A potential disadvantage of simple stains for Ascaris enumeration is their toxicity to viable eggs and larvae causing leaching of internal components, which can interfere with the microscopic observation of sample material. Therefore, rapid counting within a few minutes of administering the stain is advisable. In contrast, Syto 9 and PI had no apparent toxic effects on viable eggs or mature larvae; therefore, time constraints on performing egg counts were less critical after staining with these vital dyes compared to the simple stain types examined here. Both BacLight dyes penetrated non-viable Ascaris eggs, but neither entered viable structures. Therefore, the vital staining procedure could be adapted and simplified to use either Syto 9 or PI.

In the second part of this investigation, we inoculated eggs of A. suum into sewage sludge in order to discover whether viable eggs in a sludge matrix could be distinguished from non-viable eggs using the BacLight staining method followed by CSLM examination. Incubation of sludge with eggs was necessary because, if eggs were present in the sludge, their numbers were below the limit of detection. The BacLight method provided an accurate assessment of the numbers of viable and non-viable eggs present in sewage sludge, compared to standard incubation-microscopy. Using the BacLight method we counted a total of 186 viable eggs of A. suum in a 100 μL volume of sewage sludge, however, given the ease with which viable eggs were identified in the sludge, it would have been possible to detect as few as 1–2 viable eggs in the same volume. This corresponds to a limit of detection of ~10 eggs/mL (or 200 eggs/g DS in sludge with a DS content of 5%). Furthermore, by starting with a conventional recovery step and following by a bleaching and centrifugation step, the limit of detection can be substantially decreased, to ~0.29 A. suum eggs/g DS. This addition to the method may be useful, as field samples of sewage sludge in temperate regions of more economically-developed countries may contain very small numbers of eggs of Ascaris spp. We have used the BacLight staining procedure successfully to measure decay coefficients of inoculated A. suum eggs in laboratory simulations of sludge treatment by both air drying of anaerobically digested sludge and lagoon based treatment systems (data not shown). This suggests that the method would also be appropriate for assessing the efficiency of decay of Ascaris spp. eggs in field treatment processes in which raw sewage influents are heavily contaminated with eggs.

While viability assessment of A. suum eggs suspended in MAD sludge using BacLight staining showed good agreement with the conventional incubation method, it cannot be assumed that BacLight staining would be equally reliable for eggs suspended in sewage sludge or recycled water that has undergone some other form of treatment, since some types of treatment can damage the internal components of the egg causing loss of viability without altering the lipoprotein layer (no morphological changes). For example, UV radiation, often used to treat recycled water, mainly damages the internal components of the eggs with only minimal effect on the protein of the outer layers (Brownell and Nelson, 2006). Likewise, failure of eggs of A. lumbricoides to develop fully when suspended in wastewater effluent and exposed to gamma irradiation was attributed to damage to cellular organelles or enzymes (de Souza et al., 2011). In addition, eggs of A. suum exposed to high hydrostatic pressure as used in the food industry, showed no structural changes on examination by light microscopy, but did not undergo cellular division and were unable to embryonate, probably due to damage to proteins required for full development (Roxypal et al., 2007, 2011). It is unknown whether similar affects occur in sewage sludge treated by hydrothermal processes, which involve high temperature and pressure conditions (Hili et al., 2013). Ultracentrifugation also damages the internal components of A. suum eggs without causing damage to the lipoprotein layer (Beams and King, 1937). In these instances, non-viable eggs would appear viable if examined by the procedures described in the present study.

Inaccuracies in viability determination of Ascaris spp. eggs using BacLight could also occur in eggs that ceased development at different stages, resulting in variable degradation of proteins in the lipoprotein layer, leading to incorrect interpretation as viable (minimal lipoprotein damage, late cessation of development) or non-viable (substantial lipoprotein depletion damage, early cessation of development). Variable degradation of proteins could also explain the “uncertain” classifications recorded in Table 1. Discrepancies in viability classification between dye penetrance microscopy and the gold standard (mouse infectivity) have also been reported for oocysts of the protozoan pathogen Cryptosporidium parvum (Lorenzo–Lorenzo et al., 1993; Kato et al., 2001).

Nevertheless, the BacLight method shows promise as an alternative to conventional incubation for assessing the viability of Ascaris spp. eggs in air drying of sewage sludge following anaerobic digestion or lagoon pond treatment. Given that the current standard method requires 28 days of egg incubation before results are available, this method would be an extremely valuable alternative to prolonged incubation for assessment of egg viability in anaerothically digested sludge or lagoon pond sludge. Whether the BacLight method could be used for counting viable Ascaris spp. eggs following other types of sludge treatment is uncertain and requires further research.

5. Conclusions

- The number and viability status of eggs of A. suum, which is an effective surrogate for the important human roundworm parasite, were accurately measured directly in sewage sludge samples by the BacLight, vital dye staining method, compared to the conventional incubation-microscopy (Tulane) procedure.
• The BacLight methodology also reduced the significant laboratory resource and time constraints required for enumeration by the standard technique by avoiding the extended incubation period necessary for embryonation of Ascaris eggs.

• BacLight stains were not toxic to A. suum eggs, in contrast to other simple dyes which disrupted viable eggs possibly interfering with the enumeration or viability assessment of Ascaris.

• Methylene blue was equally as effective as BacLight as a direct method of enumerating the viability of Ascaris eggs.

• The BacLight technique may be an effective alternative to conventional incubation-microscopy for enumerating Ascaris eggs in contaminated field samples or to validate sludge treatment processes by examining decay rates of inoculated A. suum eggs in laboratory simulations.

• Most field samples (containing < 200 eggs/g DS) would require recovery from an appropriate number of composite samples prior to vital staining.

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References


Method Article

A modified assay for the enumeration of ascaris eggs in fresh raw sewage

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GRAPHICAL ABSTRACT

ABSTRACT

Soil-transmitted helminths (STHs) pose a significant public health problem, infecting approximately 2 billion people globally. Despite relatively low prevalence in developed countries, the removal of STHs from wastewater remains crucial to allow the safe use of biosolids or recycled water for agriculture. Wastewater helminth egg count data can contribute to an assessment of the need for, or success of, a parasite management program. Although the World Health Organisation (WHO) has recommended a standard method for counting helminth eggs in raw sewage based on the method of Bailenger (Ayres et al., 1996), the method generally results in low percentage egg recoveries. Given the importance of determining the presence of STHs, it is essential to develop novel techniques that optimise the recovery rate of eggs from raw sewage. In the present study:

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Method details

The new method (Modified Bowman Method)

The main outlines of the protocol are presented in Fig. 1.

1. Allow the raw sewage (1 L) sample to sediment in the sample bottle overnight at 4 °C.
2. Aspirate and discard the supernatant to just above the sediment layer (~100 mL volume remaining) and resuspend the sediment by vortexing and transfer to a blender.
3. Rinse the sample bottle twice with ~100 mL of 1% 7× detergent each rinse (MP Biomedicals) and transfer and combine each rinse to the blender in step 2. Add sterile reagent water to a final volume of 300 mL if necessary.
4. Add 1 mL antifoam-B (Sigma-Aldrich) to the sample and blend on high speed for 1 min.
5. Transfer the homogenized sample into a tall 1000 mL graduated cylinder. Rinse the blender twice with ~100 mL of 1% 7× detergent each rinse and transfer each rinse into the graduated cylinder. Add additional 1% 7× detergent to a final volume of ~900 mL.
6. Allow sample to sediment overnight or a minimum of 4 h at 4 °C.
7. Aspirate and discard the supernatant to just above the sediment layer (~100 mL volume) and resuspend the sediment by vortexing and transfer to a blender.
8. Rinse the graduated cylinder twice with ~100 mL of 1% 7× detergent each rinse and transfer each rinse to the blender in step 7. Add sterile reagent water to a final volume of 300 mL if necessary.
9. Add 1 mL antifoam-B to the sample and blend on high speed for 1 min.
10. Repeat steps 6 through 9 allowing the sample to sediment in the blender (three blending cycles in total).
11. Allow sample to sediment in the blender overnight or a minimum of 4 h at 4 °C.
12. Aspirate and discard the supernatant to just above the sediment layer (~100 mL volume) and transfer the sediment to a 250 mL conical centrifuge tube.
13. Rinse the blender with ~50 mL of 1% 7× detergent each rinse and transfer each rinse to the centrifuge tube in step 12. Add 1% 7× detergent to a final volume of 250 mL if necessary.
14. Centrifuge the sample in a swinging bucket rotor at 800 × g for 10 min at ambient temperature.
15. Aspirate and discard the supernatant to just above the sample pellet.
16. Transfer the sample pellet to a 50 mL centrifuge tube. If the pellet is greater than 5 mL then evenly distribute the pellet into multiple 50 mL centrifuge tubes containing no more than 5 mL of pellet in each tube.
17. Add 50 mL MgSO₄ (specific gravity 1.25) and resuspend the sample pellet by vortexing.
18. Centrifuge the sample in a swinging bucket rotor at 800 × g for 10 min at ambient temperature.
19. Transfer the supernatant containing the helminth eggs to a second 250 mL conical centrifuge tube.
20. Repeat steps 16 and 17.
21. Transfer and combine the supernatant containing the helminth eggs to the centrifuge tube in step 18.
22. Add sterile reagent water to a final volume of 250 mL and centrifuge the sample in a swinging bucket rotor at 800 × g for 10 min at ambient temperature.
23. Aspirate and discard the supernatant.
24. Resuspend the pellet in at least five volumes of MgSO₄ (specific gravity 1.25).
25. Mix the final concentrate by vortexing and transfer ~0.5 mL aliquots into each chamber of a Whitlock Universal 4 chamber worm egg counting slide.
26. Leave the prepared slide to stand on a flat surface for 5 min before examination to allow the eggs to float to the surface.
27. Examine the entire sample at 50× or greater magnification.
Table 1
Percentage recoveries of spiked A. suum eggs from different raw sewage samples using the modified Bowman method.

<table>
<thead>
<tr>
<th>Source of raw sewage samples</th>
<th>Recovery rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borneo Treatment Plant, Borneo</td>
<td>87</td>
</tr>
<tr>
<td>Pakenham Treatment Plant, Pakenham</td>
<td>50</td>
</tr>
<tr>
<td>Mt Martha Treatment Plant, Mt Martha</td>
<td>84</td>
</tr>
<tr>
<td>Somers Treatment Plant, Somers</td>
<td>66</td>
</tr>
<tr>
<td>Eastern Treatment Plant, Carrum</td>
<td>34</td>
</tr>
<tr>
<td>Eastern Treatment Plant, Carrum</td>
<td>64</td>
</tr>
<tr>
<td>Western Treatment Plant, Werribee</td>
<td>40</td>
</tr>
<tr>
<td>Western Treatment Plant, Werribee</td>
<td>52</td>
</tr>
<tr>
<td>Western Treatment Plant, Werribee</td>
<td>63</td>
</tr>
<tr>
<td>Western Treatment Plant, Werribee</td>
<td>74</td>
</tr>
<tr>
<td>Mean</td>
<td>57</td>
</tr>
<tr>
<td>Range</td>
<td>14-87</td>
</tr>
</tbody>
</table>

Note: Original Bowman method and WHO protocol details can be found in Refs. [1] and [2] (http://www.who.int/water_sanitation_health/wastewater/labmanual.pdf) respectively.

Method validation

Ascaris suum eggs were prepared from infected pig faeces by Excelsior Scientific (USA). An aliquot of the eggs was diluted in phosphate buffered saline to a final mean concentration of 100 eggs per 100 µL (based on triplicate counts). Raw sewage samples were collected from different sewage treatment plants in Victoria, Australia and 1 L homogenous samples were prepared for processing. Samples were processed in parallel, 1) following the modified Bowman method with the addition of 100 µL of the stock A. suum egg preparation, 2) following the modified Bowman method without the addition of 100 µL of the stock A. suum egg preparation, and 3) following the WHO method [2] with the addition of 100 µL of the stock A. suum egg preparation. The percentage recovery of the spiked A. suum eggs following the modified Bowman and the WHO methods were calculated as the number of eggs counted in the corresponding spiked sample less the number of indigenous eggs counted in the sample that was not spiked. No indigenous Ascaris spp. eggs were detected for those samples processed without the addition of the spiked A. suum eggs.

The percentage recoveries of A. suum eggs following the modified Bowman method are presented in Table 1. The results showed that the recoveries varied between 14 and 87% with a mean recovery of 57%. For the comparison of the WHO and modified Bowman methods the mean recoveries were 11 and 42%, respectively (Table 2). The greater mean percentage recovery of helminth eggs following the

Table 2
Comparison of percentage recoveries of spiked A. suum eggs from different raw sewage samples processed following the WHO and modified Bowman methods.

<table>
<thead>
<tr>
<th>Source of raw sewage samples</th>
<th>Recovery rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WHO method</td>
</tr>
<tr>
<td>Borneo Treatment Plant, Borneo</td>
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<td>Pakenham Treatment Plant, Pakenham</td>
<td>10</td>
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<td>Mt Martha Treatment Plant, Mt Martha</td>
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<td>Longwarry Treatment Plant, Longwarry</td>
<td>9</td>
</tr>
<tr>
<td>Koo Wee Rup Treatment Plant, Koo Wee Rup</td>
<td>11</td>
</tr>
<tr>
<td>Blind Bight Treatment Plant, Blind Bight</td>
<td>11</td>
</tr>
<tr>
<td>Mean</td>
<td>11</td>
</tr>
</tbody>
</table>
How rapidly do pathogens decay in sewage sludge treatment?

Can sewage sludge be treated for shorter times than those currently required by national regulations? Key plant nutrients are lost by long-term storage of biosolids. Shorter treatment times are desirable, provided microbiological safety is assured. We have, therefore, investigated decay times of key pathogens present in raw sewage.

Introduction

Raw sludge can potentially contain pathogenic microorganisms, including viruses, bacteria, protozoa and helminths. Enteric viruses found in biosolids can be divided into two major groups: enteroviruses such as poliovirus, coxsackievirus, hepatitis A virus and echovirus, and a heterogeneous group including adenovirus, human rotavirus and astroviruses. Bacterial organisms found in sewage sludge include Salmonella spp. and Campylobacter spp., which cause gastroenteritis in humans. The most common protozoan parasites of concern in sludge are Cryptosporidium spp. and Giardia lamblia, which cause diarrheal illness. Helminths such as Ascaris lumbricoides may also be present in Australian sewage, although in very low numbers.

Results

Field studies

We have evaluated the decay of indicator microorganisms during the sludge air drying and stockpiling processes at two wastewater treatment plants (WWTPs) in the Melbourne area. These plants use anaerobic digestion, followed by pan drying and storage in stockpiles for at least three years. Escherichia coli and Salmonella spp. were indicators for pathogenic enteric bacteria and coliphages represented enteric viruses.

In drying-pans from both WWTPs, E. coli showed substantial decay during pan drying. Levels of E. coli dropped from 1 x 10^3 cfu/g D5 (T3 treatment grade, restricted use) on entry into the drying pan to <100 cfu/g by 8 to 10 months (T1 treatment grade, unrestricted use). There were only minor differences between the two WWTPs plants in the length of time required to reach each treatment grade. The decay rate of Enterococcus spp. was slightly less than for E. coli, while coliphages decayed at the slowest rate, Figure 1. Salmonella spp. was not detected in a selected range of drying-pans and stockpiles.

Laboratory simulation

We chose seven pathogens and indicators, to assess pathogen decay in sewage sludge treatment by experimental simulation. Three of these were present in the field sewage; E. coli, Coliphage and Enterococcus spp. (Figure 1). Also included was...
Salmonella Typhimurium, Ascaris suum, enteric adenoviruses (porcine adenovirus and human adenovirus type 40/41), and the protozoan pathogen Cryptosporidium parvum. The data enabled us to compare log reductions and derive decay coefficients for indicators in the field and in the laboratory, and pathogens in laboratory simulations.

Large tanks (25 l) or containers (5 l) were used to follow the decay of indicators and bacterial pathogens, while assay chambers (0.5 ml volume) were used for seeding biosolids with human adenovirus (HAV type 41), porcine adenovirus (PAV-6), oocysts of Cryptosporidium parvum and eggs of Ascaris suum, due to restricted availability of pathogens or to improve their recovery.

**Estimating the decay of indicators and pathogens**

The falling numbers of indicators and pathogens over treatment time in biosolids treatment can be estimated using the following equation (1):

\[ N_t = N_0 e^{-kt} \]

![Figure 1](image1.png)

**Figure 1.** Indicator decay in WWTP 1 pan 20 (full year drying). Drying time (by days). Symbols: ●, E. coli; ●, K-12 Coliphage; ●, Enterococcus spp. At time zero the sludge from an anaerobic digester was used to start filling a drying pan. Error bars show +/-1 STD. Limit of detection is 20 cfu/g dry solids (DS) for bacteria and 20 pfu/g DS for bacteriophages.

![Figure 2](image2.png)

**Figure 2.** Decay of bacteria in drying-pan simulation 3. Symbols: ●, E. coli; ●, Enterococcus spp. ●, Salmonella Typhimurium. Error bars show 1 sd (most too small to be visible).
Smart Water Fund

Final Report

Pathogen Die Off In Air Dried and Stockpiled Biosolids Harvested from Wastewater Lagoon Processes

Project: 9TR4-001
June 2013
Pathogen Die Off In Air dried and Stockpiled Biosolids Harvested from Wastewater Lagoon Processes

Project: 9TR4-001 (2012-2013)
Smart Water Fund
Round 9

Final Report

Environmental and Industrial Microbiology Group

Harvesting lagoon pond sludge at Heyfield WWTP, Gippsland, Victoria.
Smart Water Fund

Final Report

Verifying Microbial Safety in Pan-Dried and Stockpiled Biosolids Treatment

Project: 611 – 001
July 2012
Verifying microbial safety in pan-dried and stockpiled biosolids treatment

Project: 611-001 (2009-2011)
Smart Water Fund
Round 6

Final Report
Environmental and Industrial Microbiology Group

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Parasitologist
Associate Professor Peter Smooker

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Research Assistants
Venessa Fleming and Nerida Thurbon

Casual Research Assistance
Yue Qu

Daniella Petrovska

Sasikumar Vesuvanathan

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Tanja Mondal (bacterial decay)
Basma Khallaf (Ascaris) (funded by Saudi Government)

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Tim Casey (did not complete) (Viruses)
Sasikumar Vesuvanathan (Viruses)
Frederick Kong (Cryptosporidium)
David Garrick (bacteriophage)

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Vishal Mistry (Cryptosporidium)
Sneha Pai (Salmonella methods)
Alaa Karkashan (Ascaris) (funded by Saudi Government)
Ronak Hojatpanah (Ascaris)
Basma Khallaf (Ascaris) (funded by Saudi Government)
Nadin Al Mosnid (DNA decay) (funded by Saudi Government)
Shengxi Zeng (Ascaris)

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Naveen Rayala
Sauzinha Fernandes
Wei Gan
Kel Win Low
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Sasikumar Vesuvanathan
Melissa Turner
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Jessica Kidd
John Leong
Josh Fraser
Lana Rengey
Tom Kivin
Will Howden