Detecting Parasites Loads in Urine Diversion Toilets.

by

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Foreword

"… but worms are very unglamorous things, you talk of faeces and you talk of latrines, and there's no fashionable side to worms. You can always find a politician and policy makers who will love to come to open a brand new cardiac unit. Everybody's very willing to have a paediatric wing named after them or a special renal unit named after them. Just find someone who wants to have a toilet named after them, then you will control ascariasis" (Kan, 1989 in Crompton *et al*., 1989[a]).

[a]Crompton DWT, Nesheim MC and Pawloski ZS (1989). *Ascariasis and its prevention and control*. Taylor and Francis, London.

Abstract

In an attempt to supply sanitation to the growing communities in rural and peri-urban areas around Durban, the eThekweni Municipality has installed urine diversion (UD) toilets which have been modified to suit local conditions . These toilets are based on the ecological sanitation (EcoSan) system. The future aims are to reuse waste as a composting medium and minimize the use of water but the presence of microorganisms in the faecal waste poses a potential health risk to people in contact with it. Currently the Municipality has not deemed the waste safe for re-use but has suggested that after a one year standing period it should be free of all potential pathogens including *Ascaris lumbricoiodes* (human roundworm) ova. This study reports on the development of the AMBIC protocol for the recovery of *Ascaris* ova from the standing vaults of UD toilets. The protocol has been shown to consistently recover over 70% of *Ascaris* ova and has the added advantage of recovering the ova of other helminth species (*Trichuris trichiura* and *Taenia* sp.) present in a UD standing vault sample. Recoveries of *Ascaris* ova and ova of other parasite species, namely *Trichuris* and *Taenia* sp., are reported from waste which has been standing for one year. This is cause for concern as it shows one year is not a sufficient standing period to render the waste free of all microorganisms. Sampling from 124 UD toilet vaults that were in use, showed a high prevalence of both helminth (*Ascaris lumbricoiodes, Trichuris trichiura* and *Taenia* sp.) and protozoan (*Giardia* and *Cryptosporidium*) parasites.

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Preface

The experimental work described in this thesis was carried out in the School of Biological and Conservation Sciences, University of KwaZulu-Natal from January 2006 to January 2008, under the supervision of Professor M.T. Smith and Dr N. Rodda.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

Candidate: David James Hawksworth ……..

Supervisor: Professor Michael Smith …….

Supervisor: Dr Nicola Rodda ……..

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- Firstly, to my parents who supported me through this "project" from the beginning and continue to do so. As they would always remark that my brother and I were always in shit, it was just the depth that mattered. When I announced that I was going to make shit my career, they were not surprised. Go figure. . . . .

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- To Colleen (a.k.a. “Mrs A”, “**S**enior **S**hit **S**tirrer”, “Ouma” and the “Ou Tannie”), I could dedicate an entire chapter to you! You have taught me everything I know and I still know nothing? Where would I be without your cups of tea and treats – surely insane by now! You have not gotten rid of me yet and we still have loads more talking to do. . . . . I am forever grateful for your generosity!!

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Chapter 1: Background

1.1. General Introduction

Municipal authorities face challenges, both financial and logistical problems, in providing piped water and sewerage infrastructure to rapidly expanding, low-income peri-urban settlements. More affluent communities have waterborne sewerage, often termed the "*flush and forget*" option. It is widely perceived as the more prestigious system, but is more costly to implement. Other communities rely on either pit latrines or septic tanks for sanitation. In informal settlements and in rural communities, people may even defecate and urinate directly into their living environments. This potentially exposes the inhabitants to viral, bacterial and parasitic diseases.

There is, however, another means of waste disposal that has been developed with the specific aim of keeping faeces and urine separate (separated at source). This is based on the ecological sanitation (EcoSan) principle of *sanitise and reuse*. EcoSan is based on recirculation of nutrients rather than discharge with water by separating the urine and faeces at source. This promotes the recycling of human excreta and urine on-site, or as close to source as possible.

The urine diversion (UD) toilet is one such example of a dry sanitation system as it diverts the urine away from the faecal matter where the urine can be reused as a fertilizer and the faecal mater reused as a soil conditioner. The dry sanitation system becomes a practical solution in areas that are limited by water resources and inadequate sewage disposal systems, or a combination of both.

Urine diversion systems consist of a pedestal with a bowl divided into two sections. The front section collects the urine whereas the larger part of the bowl collects the faeces. The faecal matter is stored in an above ground vault and the urine is stored in collection tanks, or soaks away into the surrounding soil. The eThekwini Municipality has implemented the double vault system where the urine is not collected but is allowed to drain into the soil. As the first vault becomes full, the user moves the toilet pedestal to the adjacent empty vault and continues using the system after recording the last date of

use of the previous vault. Meanwhile, the faecal material in the first vault is allowed to biodegrade and the pathogen load is reduced over time. This is sometimes referred to as the standing period. Thereafter, the residue material is excavated and buried on site.

Since 2003, more than 73 000 UD toilets have been installed by the eThekwini Municipality. After the standing period of a year, thousands of UD toilets require emptying. This potentially poses a health risk to both the UD vault emptier and people in the immediate environment.

Ascaris lumbricoides is one of the pathogens found in UD waste, particularly in developing communities generally and, more specifically, along the KwaZulu-Natal coastline. It is a public health concern as the ova are extremely persistent in the environment outside the host. An important route of exposure to *Ascaris* ova exists in regions where human excreta are used as soil conditioners or fertilizers, so that both the person handling the waste and those consuming unprocessed crops grown in these soils are at risk.

1.2. Structure of Dissertation

This dissertation begins with a general introduction (Chapter 1) and a review of the relevant literature (Chapter 2). The study itself is presented as a number of stand alone chapters (Chapters 3 – 5). Each chapter deals with a specific objective (research question) investigated during the course of this study.

Chapter 3 investigates the development and refinement of the AMBIC protocol for detecting the presence of *Ascaris lumbricoides* ova in UD faecal waste incorporating faecal material and covering material (sand). Chapter 4 assesses the ability of the AMBIC protocol to recover *Ascaris* ova in both laboratory and UD field samples. This protocol needs to be reliable enough to be applied in field research and monitoring of *Ascaris* loads from household UD toilets. This forms the basis for Chapter 5, in which the AMBIC protocol is used as a laboratory tool to investigate the prevalence of helminths in the filling vaults of UD toilets.

Chapter 2: Literature Review

2.1. Setting the Scene

Throughout the developing world, the problems arising from the decreasing quality and quantity of water are becoming increasingly serious. This water crisis can be linked to the sanitation crisis, which, while much less often discussed, is responsible for major health and environmental problems around the world (Langergraber and Muellegger, 2005). Improvements in sanitation, access to water and hygiene education all contribute to improved health and overall wellbeing (Bartram *et al.*, 2005). It has been estimated that, worldwide, 2.5 billion people do not have access to adequate sanitation or wastewater treatment facilities (Werner *et al.*, 2003). In the South African context, it has been estimated that 18 million people lack adequate sanitation (Government White Paper, 2001), and adequate supplies of clean drinking water are still not widely available throughout the rural areas of South Africa (Kibel and Wagstaff, 1995; du Preez *et al*., 2008). Inadequate sanitation, lack of access to clean, potable water and poor domestic hygiene are the causes of approximately 80% of all infectious diseases *(e.g*. cholera, typhoid, hepatitis, polio, cryptosporidiosis, ascariasis and schistosomiasis) in the world and account for 10-25 million deaths each year, mostly in the under 5 years age group. These diseases are mainly transmitted via the faecal-oral route through faecally contaminated water, food or soil (WHO, 1989; WHO, 2007).

Included in the Millennium Development Goals, set by the United Nations in 2000, is a target to halve the number of people without adequate sanitation, by 2015 (UN, 2000). The current growth of South African cities due to the migration of people from rural areas has resulted in rapid population growth in peri-urban areas. This has resulted in areas that are typically, low-income, high-density, informal settlements without access to basic sanitation. The location and lack of planned structure in these communities makes installation and operation of conventional (reticulated) water and sanitation systems economically and logistically unfeasible.

2.2. Current Sanitation Systems Available in South Africa

2.2.1. *Waterborne*

Conventional waterborne sewerage has been the standard for sanitation in urban communities, but the costs of treating waste and the water requirements make it unsustainable and inappropriate for low-income communities (Paterson *et al.,* 2007). In South Africa, like other countries with water and sanitation backlogs, most of the population aspires to in-house, full-pressure water supply and flushing toilets linked to waterborne sewerage and wastewater treatment. The government recognises that the provision of these levels of services to all is neither technologically nor financially feasible, nor necessarily environmentally sustainable. Peri-urban and rural populations, particularly, are unsuited to the provision of such services. The government provides a subsidy for the installation of basic services, including water and sanitation, via the Municipal Infrastructure Grant. Subsidies for free basic sanitation cover the costs of hygiene promotion and the capital costs of providing a basic sanitation service to households (DWAF, 2003). This grant does not allow for the capital or maintenance costs of waterborne sewerage for such communities.

2.2.2. *On-site sanitation*

In a water-scarce country, like South Africa, sanitation options are needed that minimise the demand on water resources. On-site sanitation is one way of addressing this issue. The Department of Water Affairs and Forestry (DWAF) recognises this by specifying ventilated improved pit latrines (VIPs) or similar technology as the minimum standard for sanitation (DWAF, 2003).

Pit Latrines and Ventilated Improved Pit Latrines form part of the options for basic on-site sanitation but have potential problems. One of these is the removal of vault contents of the VIP when the vault is full. The use of heavy vehicles is often required. Mechanical desludging equipment is expensive and vulnerable to failure. It is often not always possible to access the site and the desludging equipment frequently cannot cope with the heavy sludge and solid matter found in the pit. The alternative is manual (by hand) emptying, where the excreta and degraded solid waste are dug out of the pit, using shovels, buckets and other implements. This work is unpleasant, and poses a

number of health risks if not managed carefully. For those pits that can be accessed by tanker, the average costs for emptying one pit ranges between R600 and R1 000. Also, topographical issues relating to hydro-geological conditions, as well as low cover on bedrock, clay and high water tables contribute to the difficulty in construction of these toilets (Brouckaert *et al*., 2004). Hence the eThekwini Municipality has sought an alternative to the ventilated improved pit latrine (VIP).

2.2.3. *On-site sanitation provision by urine diversion toilets*

Ecological sanitation (EcoSan), in the form of UD toilets, provides an alternative solution to the persistent problem of inadequate sanitation systems (Esrey *et al*., 2001). It saves water, does not pollute and returns the nutrients in human excreta to the soil (EcoSanRes, 2003). The eThekwini Municipality (Durban, South Africa) has selected double vault urine diversion (UD) toilets as the preferred sanitation option for rural and some peri-urban communities. This choice was driven by the logistical difficulties and excessive costs incurred by the municipality associated with the emptying of ventilated improved pit latrines (VIP) (Foxon *et al.,* 2005; Rodda *et al.,* 2006).

Urine diversion (UD) toilets (Figure 2.1) is a system based on the ecological sanitation (EcoSan) principal which aims to reuse urine as a fertiliser and faecal matter as a soil conditioner, thereby recycling nutrients back into the environment (Drangert, 1998; EcoSanRes, 2005; Lamichhane, 2007; Paterson *et al.,* 2007). Urine diversion is a sub-set of source separating systems, which in turn is a sub-set of EcoSan. Urine diversion toilets separate urine and faeces at source, thus eliminating odours and flies associated with mixing urine and faeces. The bowl of the toilet pedestal is divided into two parts. The front section collects the urine and is connected to a pipe, which diverts the urine into a soak-away. The back section directs the faeces into a storage vault beneath the toilet. The addition of sand or ash to the vault after each defecation aids in drying and neutralising the smell of the waste (Rodda *et al*., 2006). However, a disadvantage of on-site dry sanitation systems, like the UD toilet, is that they do not cater for greywater disposal.

The eThekwini Municipality has installed UD toilets purely as an on-site sanitation system at this stage. It does not promote the full ecological sanitation concept of reusing the stabilized waste, but instead encourages users to empty the vaults after a standing period of one year, and to bury the waste under a minimum soil covering of 250mm (Rodda *et al.*, 2006). This is because the fate of potential pathogens in the waste is still uncertain. eThekwini Municipality has modified the design of the UD toilets to suit local conditions by installing a dual vault system. Once the first vault is filled, the contents are left to stand whilst use of the second vault commences. Ideally, this allows the waste in the first vault to undergo drying and stabilizing processes, and also allows for harmful pathogens present in the waste to die off, thereby rendering it safe for potential re-use (Holmqvist and Stenström, 2006).

Figure 2.1: Operational urine diversion (UD) toilet in eThekwini Municipality, Durban (source: David Hawksworth).

2.3. Sanitary Risks Associated with UD Faecal Waste

2.3.1. *Emptying the UD vault*

Vault emptying and the burial of UD waste, as recommended by eThekwini Municipality, represents a potential risk to both the emptier and the environment. The potential risks associated with the use of UD faecal waste, as a soil conditioner must be addressed in order for this to be a viable disposable route. In Europe, the preferred

solution to the final use of sewage is recycling of sewerage sludge on agricultural soils due to its high organic content and fertilizers (Simonart *et al.*, 2003). However, microbiological risks exist and these are due to the presence of pathogens, microorganisms including enteroviruses, bacteria, protozoa as well as helminth parasites and fungi (Schönning *et al*., 2007).

2.3.2. *Pathogens associated with UD faecal waste*

The main risk associated with the reuse of excreta is that of the faeces and not of the urine, which is typically sterile when it leaves the bladder (Drangert, 1998; Schönning and Stenström, 2004). The average person, as reported in a study by Drangert (1998), excretes less than 500L of urine and 50-180 kg (wet weight) of faeces in a year, depending on food and water intake. Since most pathogenic microorganisms are found in the faeces, there is a potential health risk when the UD waste is handled.

There are relatively few pathogens that are traditionally associated with urine excretion. They include *Leptospira interrogans*, *Salmonella typhi*, *Salmonella paratyphi* and *Schistosoma haematobium* (Schönning and Stenström, 2004). On the other hand, although faeces typically have high moisture contents (70 – 85%), the rest is made up of organic material, including microorganisms (Drangert, 1998). The number of microorganisms range from 10^{11}-10^{12} /g of faecal material (Schönning and Stenström, 2004). These include bacteria (*e*.g. *Salmonella*), viruses (*e.g.* hepatitis A/E), parasitic protozoa (*e.g. Cryptosporidium parvum*) and the parasitic helminths (*e.g. Ascaris*). In developing countries it is the ova of helminths, such as *Ascaris* and *Taenia* sp.,that are of greatest concern because of their persistence in the environment (Meglitsch, 1967; Faust *et al*., 1975; Schönning and Stenström, 2004). *Ascaris* ova have been found to be resistant to fluctuating environmental conditions and this allows them to remain viable in the soil for years, provided that certain conditions are met in the external environment (Crompton and Pawlowski, 1985; Stephenson, 1987; O'Lorcain and Holland, 2000; Muller, 2002; Vincent, 2005). As a result they are frequently regarded as indicators of the hygiene quality of biosolids such as UD faecal waste (Schönning and Stenström, 2004; Capizzi-Banas *et al*., 2004). *Ascaris* ova can survive exposure to many chemicals and harsh environmental conditions and are able to remain viable in the soil for years, if

the ova shell remains undamaged (Yeager and O'Brien, 1983; Crompton, 1984). *Ascaris* is therefore typically used as an indicator organism from a health and safety point of view (Aladawi *et al*., 2006), and their disappearance suggests to that all other pathogens have also been inactivated (Feachem *et al*., 1983).

2.4. The geohelminth *Ascaris lumbricoides*

2.4.1. *Distribution*

Ascaris lumbricoides, a geohelminth parasitic worm, is one of the most common parasites of man. It has a world-wide distribution and is found in both temperate and tropical zones, but is more common in countries with warmer climates, where there is both adequate moisture and poor sanitation (Yamaguchi, 1981; Brown and Neva, 1983; Muller, 2002) and particularly where human faeces is used as fertilizer (Kibel and Wagstaff, 1995). According to O' Lorcain and Holland (2000), *Ascaris lumbricoides* is a highly infectious and persistent parasite that infects a quarter of the world's population, with global estimates ranging between 800 and 1000 million people.

2.4.2. *Morphology*

Ascaris is the largest of all the nematodes and the adult females reach lengths of 20-35 cm and 3-6 mm in diameter. The males are slightly smaller and attain a length of 12-31 cm and diameter of 2-4 mm. The female can lay up to 200 000 ova per day. These ova are broadly ovoid and measure 45-70 µm by 35-50 µm (Yamaguchi, 1981; Brown and Neva, 1983; Fripp, 2004). The ova have a thick shell which consists of four different parts, namely the ascaroside, chitinous, vitelline and uterine layers (Wharton, 1980; Fripp, 2004). The ascaroside layer consists of ascaroside lipids arranged in a double-layered membrane. It is composed of 75% ascaroside and 25% protein (Wharton, 1980). It is thought that the resistance of the ovum to chemicals is due to this ascaroside layer because if it is treated by liphophilic compounds such as ether or alcohol, the ova are inactivated. The chitinous layer is highly cross-linked and gives the ovum its structural rigidity (Wharton, 1980), while the vitelline layer is a thin proteinaceous layer derived from the membrane of the zygote (Wharton, 1980). The outer uterine layer consists of uneven deposits of mucopolysaccharides produced by the cells of the uterine wall of the female worm; however, this layer is not always present (Wharton, 1980). It is the

combined effect of these four layers which make the *Ascaris* ovum very resistant to fluctuating environmental conditions and allow it to remain viable in the soil for years, provided certain parameters are maintained (O'Lorcain and Holland, 2000; Muller, 2002). The ova are colourless in the uterus, though they are stained yellowish-brown by the bile pigment when passing down the alimentary tract (Yamaguchi, 1981; Fripp, 2004) and this makes them easier to identify under the microscope. If the external albuminoid layer is absent the ova become colourless and may be mistaken for the ova of hookworm (Yamaguchi, 1981).

2.4.3. *Life Cycle*

Ascaris ova are passed out in the faeces unsegmented and fertilized (Figure 2.2). After 10-15 days the embryo within the ovum moults into a first stage and thereafter into a second stage, the infective larva. The optimum temperature for development is between 21 and 35 ºC (Brown and Neva, 1983). These second stage larva can remain viable for up to seven years in the soil under optimal conditions (Russell-Hunter, 1979; Muller, 2002). The infective ovum, when ingested by a human, hatches in the duodenum and the larva then burrows through the gut wall, entering the lymphatic system. In the portal circulation the larva passes through the liver and heart to the lungs. It reaches the lungs between 1 and 7 days after infection, and then breaks through the alveoli to undergo a further two moults. The fourth, final stage is coughed up and swallowed, returning to the small intestine. Here it undergoes its final moult into an adult worm. The male and female worms pair up and mate and the female worms produce ova. The first ova produced by the female adult worm are passed 2 to 2½ months after infection (Otto, 1979; Brown and Neva, 1983; O'Lorcain and Holland, 2000; Muller, 2002; Fripp, 2004).

Infective ova are transmitted via the faecal-oral route. Children, between the ages of 1 and 5 years, who come into contact with contaminated soil or food, are most at risk. Since ascariasis is essentially a household infection, it is closely associated with family hygiene and since there is no practical method by which soil in and around households can be rendered safe, prevention depends on the sanitary disposal of faeces and upon health education (Brown and Neva, 1983). Severe *Ascaris* infections can lead to

significant nutritional impairment, especially in children whose nutritional intake is low, thereby contributing to malnutrition of the host (Coovadia and Wittenberg, 1998). The survival time of the adult worm in the intestine is relatively short but in areas where soil contamination is high, due to unsatisfactory hygiene, daily exposure is common and people are continually reinfected (Brown and Neva, 1983).

2.4.4. *Pathology*

Helminth infections are widespread, especially in developing countries, and may include intestinal pain, fatty or watery stools, anaemia and weight loss. Although low to moderate worm loads are often asymptomatic, the indirect effects may contribute substantially to child morbidity when associated with malnutrition, pneumonia, other enteric diseases and vitamin A deficiency (Höglund, 2001).

2.4.5. *Link with Inadequate Sanitation*

It is estimated that 10^{14} *Ascaris* ova pass daily into the global environment and an ovum, once infective, is viable up to 15 years (O' Lorcain and Holland, 2000). In the context of agricultural wastewater reuse, it has been stated by the WHO that the recommended maximum permissible level of intestinal helminth ova load in sewage is 1 ovum/L (Gaspard and Schwartzbrod, 1995; Ayres *et al.*, 1996; WHO, 2007).

At the household level, the nature and concentrations of pathogens in human waste is dependent on the health and size of the family using the sanitation facility. The risk posed by a given type of sanitation facility is dependant on the technology, the health status of the family using the toilet, and the extent to which good hygiene practices are followed. The interaction amongst these factors is too seldom considered in studies aiming to establish the "safety" of a particular sanitation technology by demonstrating the absence of the parasite. Chale-Matsau (2005) points out that many communities in developing countries, such as South Africa, do not use antihelminthic prophylactics. Therefore contact with untreated or inadequately treated human waste, or waste residues such as UD waste, containing viable *Ascaris* ova could lead to re-infection.

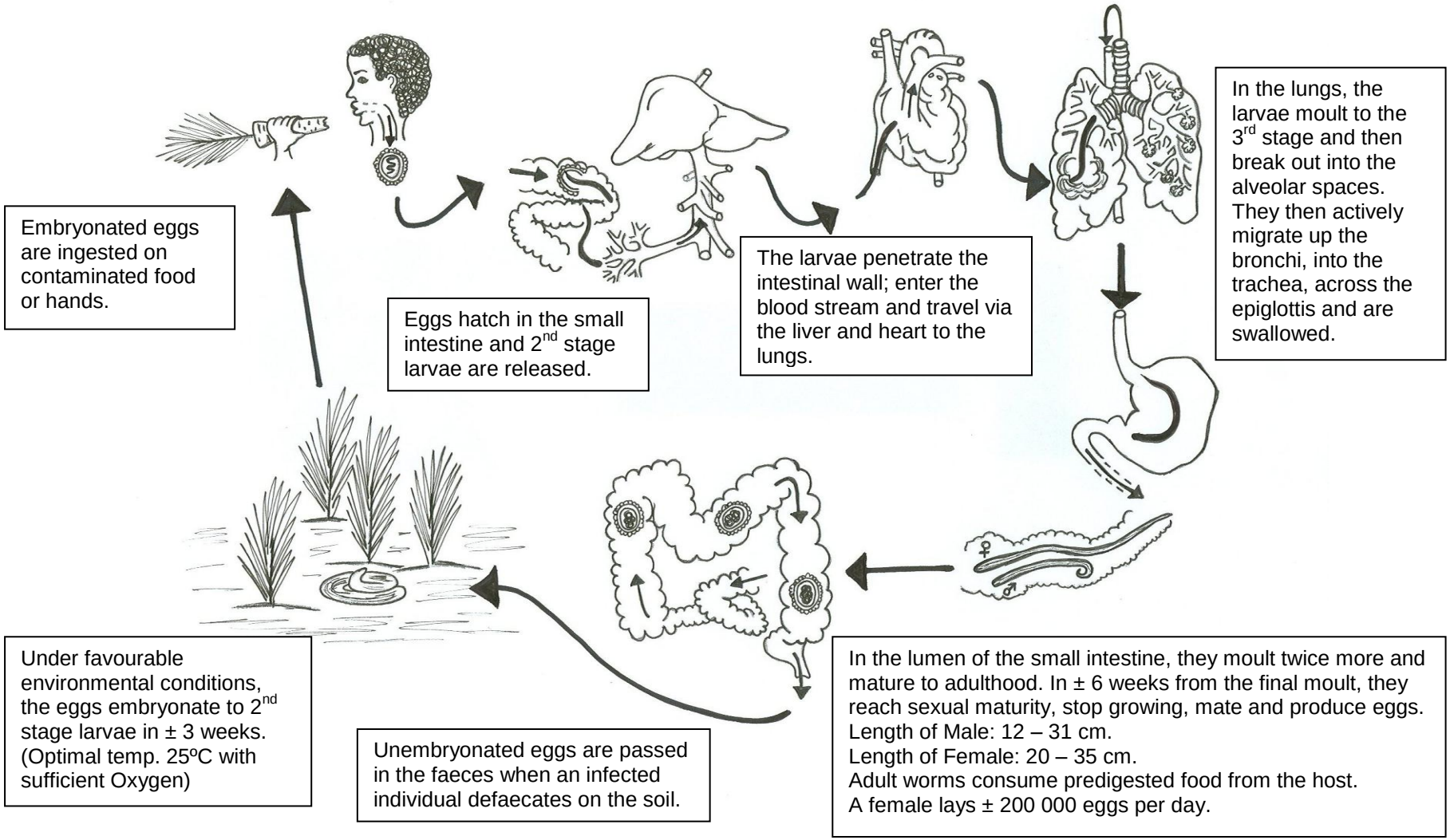

Figure 2.2: Life cycle of the roundworm *Ascaris lumbricoides* (Archer and Appleton, *in prep*. With permission)

Therefore it is imperative that an optimum detection method for *Ascaris* ova in UD waste is established. This would aid in determining a realistic quantification of the *Ascaris* ova load in UD faecal waste and provide indications of the infective potential of UD waste from a given vault. Furthermore, data on the *Ascaris* ova load in UD faecal waste will allow for the development of management guidelines for public and environmental health aspects in respect of UD toilets.

2.5. Recovery of *Ascaris lumbricoides*

2.5.1. *Recovery principles*

Methods used for recovery of *Ascaris* and other helminth ova from faecal samples can be broadly separated into two classes: those that concentrate ova by sedimentation (*e.g.* Kato-Katz and formal-ether methods) and those that concentrate ova by flotation (*e.g.* zinc sulphate flotation). In sedimentation approaches, the fatty matter is separated in an interphase solution (ether or ethyl acetate) while the parasites sediment into the aqueous, non-miscible buffer below. In flotation methods, parasite ova are floated away from the other debris in a solution of comparatively high relative density and recovered from the surface (Ayres and Mara, 1996). Both processes use centrifugation and the hydrophilic-lipophilic balance of the organism, and its relative density in relation to that of the separating solution to concentrate the helminth ova. The pH, presence of heavy metals, or alcohols in the reagents used to effect separation can interact with the surface properties of the parasite, as each species responds differently to these changes. Therefore no one single method concentrates all helminth parasite species with the same efficiency (Ayres and Mara, 1996).

Of the two approaches, sedimentation methods are more likely to recover ova and cysts of other helminthic and protozoan parasites in addition to *Ascaris* ova, thus providing greater information about total parasite loads (Allen and Ridley, 1970).

2.5.2. *Faecal samples*

The current conventional methods for isolation and detection of ova in faecal samples are either the Kato-Katz method (WHO, 1993) or formal ether (Allen and Ridley, 1970). According to Muller (2002), the Kato-Katz method can detect as few as 100 eggs per gram of faeces and for this reason has come to be regarded as the standard

protocol. Concentration methods, which are useful for prevalence surveys, are not especially useful for measuring the intensity of an infection because samples with large numbers of ova present make the counting process difficult (Muller, 2002). However, the formal-ether method can be used as a semi-quantitative estimate and has the advantage of being rapid and allows many samples to be processed simultaneously. Also, samples can be stored for a long period of time in formalin and transported to (better equiped) laboratories for more detailed analyses (Archer *et al*., 1997). In addition, it also isolates protozoan cysts and *Strongyloides* larvae (Muller, 2002). According to Allen and Ridley (1970), there are several disadvantages to the use of the formal-ether technique for the concentration of faecal ova, cysts and larvae: concentrations of *Taenia* and *Ascaris* (particularly the infertile stage) ova detected are usually unsatisfactory using this method; the technique makes use of hazardous chemicals, which are toxic to the environment (Archer *et al.*, 1997); expenses are incurred by this technique as it entails the use of a lot of equipment for the concentration method. Finally, adherence of the concentrated deposit to the microscope slide makes fixation and staining difficult and unsatisfactory results are obtained in the concentration of faecal specimens which are fixed in bulk and stored for days or weeks (Allen and Ridley, 1970).

Allen and Ridley (1970) described simple modifications to this method in order to overcome some of the problems experienced. The modified concentration procedure suggested by Allen and Ridley (1970) used formalin in water, instead of formal-saline, and centrifugation was done at 3000rpm instead of 2000rpm. This modified procedure was found to generate good results with all types of protozoan cysts as well as ova. The main advantage identified was in the concentration of *Ascaris*, *Taenia* and *Schistosoma* ova.

It was also mentioned that the use of a zinc sulfate flotation method failed to allow heavier ova to rise to the surface, whilst in the formal-ether sedimentation technique, all parasites accumulated at the bottom of the solution. The modified formal-ether method exhibited increased yields with all types of faecal parasites, produced relatively clean deposits, which enhanced visibility on examination by microscope, allowing structural details to be seen and therefore making it a viable routine diagnostic procedure. Further attempts to improve the efficiency of the method by

addition of wetting, or mucolytic agents, gave inconsistent results (Allen and Ridley, 1970).

According to Cheesbrough (1981), flotation methods utilize a solution that is heavier than the parasite ova and cysts, which then rise to the surface of the medium for recovery. One of the benefits of using this method is that if no centrifuge is available, the tubes can be left to stand in a rack for 45 minutes for the parasites to float to the surface. Furthermore, smaller parasites such as *Cryptosporidium* can be easily recovered. The disadvantages, however, are that many samples cannot be processed at once and the stool samples used should be fresh, or samples should be stored in a refrigerator for only a few days. In addition, not all parasites float consistently (ova of *Ascaris lumbricoides*, *Trichuris trichiura*, *Taenia* sp. and *Schistosoma* sp.), resulting in an incomplete recovery. Flotation methods cannot be used on fatty stools, as the fats will also float to the surface of the flotation medium with the recovered ova (Cheesbrough, 1981). According to Ayres and Mara (1996), of the wide range of flotation solutions tested by Bouhoum and Schwartzbrod (1989) for faecal analysis, iodomercurate concentrated the greatest range of species of parasitic helminth ova, but it was concluded that the reagent was too corrosive, and expensive, for routine use. It was also reported by Ayres and Mara (1996) that Arthur's method, in which saturated sucrose was used as a flotation solution, rapidly deformed ova, while zinc sulfate solution failed to concentrate *Trichuris* sp. or *Capillaria* sp. very well.

2.5.3. *Wastewater samples*

Gaspard and Schwartzbrod (1995) investigated three types of parasitological methods in order to establish an effective method for detection of *Ascaris* ova in wastewater. These included: physical methods (sedimentation or centrifugation); a combination of physical methods with various flotation liquids, and the diphasic methods combining hydrophilic and lipophilic reagents. A comparison of seven quantification techniques to recover helminth ova from wastewater suggested optimum results were obtained by using the diphasic technique which included a treatment with antiformine at 8 % and ethylacetate followed by a flotation with a 55% aqueous solution of zinc sulphate. Yields from this method (subsequently named Bailenger's method) were significantly independent of the ova concentration and produced good homogeneity of results. It was reported that the percentage of ova recovery by the Bailenger's method was 74 %

and this method was considered the best viable option at both a qualitative and quantitative level (Gaspard and Schwartzbrod, 1995; WHO, 1996).

2.5.4. *Towards an international standard method*

Various techniques for enumerating helminth ova in water and faecal samples have been published, however, according to Maya *et al.* (2006) there is still no widely accepted international method for evaluating these parasites in wastewater. According to Simonart *et al.* (2003), three detection methods have been described: the U.S. EPA modified method, the Triple Flotation (TF) method and the Norwegian method. It was also noted that up to that date no standard method existed as none of the methods demonstrated the capability of combining specificity, efficiency and viability in the detection of helminth ova. Simonart *et al.* (2003) reported that the Norwegian and EPA methods were relatively similar in their detection and enumeration principles. In the Norwegian method, after filtering, the diphasic step is followed by the flotation using sucrose whilst in the U.S. EPA method, after the straining, the flotation step is followed by the diphasic step using alcohol/ethanol. The use of a 38µm sieve in the Norwegian method could allow loss of smaller ova such as *Taenia* sp. and *Trichuris trichiura*. Also, sucrose was found to be an ineffective flotation solution as it allowed adhesion to surfaces. After comparison of the three methods, it was found that the most economical, simple and easiest to implement was the U.S. EPA method, followed by the Norwegian method whilst the TF method was found to be both time-consuming and expensive (Simonart *et al.*, 2003).

In order to determine the most appropriate method to implement in Mexico, four techniques were compared, including the U.S. EPA, Membrane Filter, Leeds I and Faust methods. All of the four techniques compared encompassed two general steps. The first was to separate, recover and concentrate the helminth ova from the sample sediment, and the second step involved the identification and enumeration of the ova with the aid of a microscope. After comparison of all the techniques, it was concluded that the EPA technique was the best method since it could be used for samples with both high and low solids content, allowed recovery of helminth ova with different specific gravities, and had the lowest total cost. The EPA technique was therefore recommended as the standard technique for quantification of helminth ova in wastewater and reclaimed water by the Mexican government (Maya *et al.*, 2006).

With the advent of medical parasitology came the development of several techniques for the enumeration of intestinal helminth ova and larvae in faeces, with the basic principles of these methods being adapted to the enumeration of helminth ova in sludge and compost (Ayres and Mara, 1996). According to Dumontet *et al.* (2001), technical limitations for detection and isolation procedures can be considered the main difficulties in monitoring sludge pathogens.

As mentioned earlier, the enumeration of intestinal helminth ova and larvae in wastewater is a much more complex process. This is because of the large variety of human and animal parasite species and free-living species, which may be present in wastewater, and to the varying degrees of size, specific gravity and surface properties of these species and their lower concentrations in wastewater than in faeces, sludge or compost. Ayres and Mara (1996) explained that many methods for the enumeration of helminth ova in wastewater have been described in the literature, with each method possessing its own advantages and disadvantages. Some techniques display high percentage recovery but are very time-consuming. Many techniques have not been reported in sufficient detail for replication to be possible or have unknown recovery rates. Some require expensive chemicals or are otherwise unsuitable for use in laboratories with limited equipment while others only recover a limited range of species.

Ayres and Mara (1996) and Bouhoum and Schwartzbrod (1989) concluded that Bailenger's method, which they adapted and modified for wastewater, was the best method overall as it required relatively inexpensive reagents and successfully concentrated the full range of species commonly found in wastewater. Although this modified Bailenger's method is generally useful, simple and cheap, it does possess recognized limitations, which need further evaluation. Nevertheless, it reliably recovers the ova of intestinal nematodes, is reproducible and has already been widely adopted in many laboratories globally (Ayres and Mara, 1996). Some of the many advantages it encompasses are that sample collection and preparation are uncomplicated and it utilizes only the basic of laboratory equipment for sample processing. Also, this method entails the use of McMaster slides, which are very efficient in counting of ova. In addition, operator error is reduced as compared to

alternative methods, which are long, tiring, laborious and are more prone to errors. However, weaknesses of this method also exist. Among these are the unknown percentage recovery of ova of this method and its unsuitability for many of the operculated or trematode ova, including those of *Clonorchis sinensis*, *Diphyllobothrium latum*, *Fasciola hepatica*, and *Schistosoma* spp. (Ayres and Mara, 1996). Some of these ova float in the zinc sulfate flotation solution but may sink quickly or become distorted, making accurate identification difficult. Also, ether is highly flammable and toxic but Ayres and Mara (1996) suggested that ether could be replaced by the safer solvent ethyl acetate for the extraction of parasite ova from faeces without any loss in efficiency.

2.5.5. *Recovery of Ascaris from UD waste*

The eThekwini Municipality has advocated that users, after defecation, add a small amount of soil to the vault. This is believed to assist in accelerating the drying and composting process of the waste. There are currently no reported methods for the detection of *Ascaris* ova in mixed faecal/soil samples. Preliminary studies had indicated that siliceous particles of different sizes were present in any unprocessed UD sample, and these rendered sample preparation and microscopic preparation difficult.

One possible solution to this problem would be the removal of the unwanted material (siliceous particles) from the sample by selective sieving (Visser and Pitchford, 1972) as pre-treatment preceding further processing by sedimentation or flotation-based methods. This possibility is further explored in Chapter 3.

2.6. References

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Chapter 3: The Development of a Method for the Recovery of *Ascaris* Ova from Faecal Waste in Urine Diversion Toilets.

3.1. Abstract

The greatest potential risks of urine diversion (UD) toilet operation and maintenance are associated with the removal and disposal of the faecal material from the toilet vault. Among these, exposure to viable ova of the nematode parasite, *Ascaris lumbricoides*, is thought to be the dominant hazard. It is not possible to characterise either the exposure or the associated health risks without reliable information on the occurrence of ova in UD waste. The investigation of the presence and viability of ova in UD systems has been hampered by the lack of an accepted and validated method for detection of *Ascaris* ova in soil-based samples (soil is used as the covering medium in UD toilets in the eThekwini municipal area). This study compares the application of current detection methods for ova in faecal and wastewater samples, to ova in soil-based samples. It reports an improved recovery protocol for separating eggs from soil using ammonium bicarbonate (AMBIC) to disrupt the association between ova and soil, thereby making them more readily available for enumeration.

3.2. Introduction

Inadequate or inappropriate sanitation exposes communities to viral, bacterial and parasitic diseases, both directly and through contamination of the surrounding environment, including water sources. It is estimated that in developing countries globally, 2.6 billion people lack access to basic sanitation. The provision of adequate sanitation has been recognised as an urgent environmental issue, both worldwide and nationally (Benatti *et al*., 2002), to the extent that Target 10 of the United Nations Millennium Development Goals is "to halve, by 2015, the proportion of people without sustainable access to safe drinking water and basic sanitation" (United Nations, 2000).

South African policy on water and sanitation similarly addresses the water and sanitation backlog, proclaiming free basic water supply and basic, dignified and sustainable sanitation as a right for all people (DWAF, 2003). However, municipal

authorities face many challenges in implementing this policy. Established, more affluent communities have waterborne sewerage, which is widely perceived as the most prestigious system, and that to which most people who lack basic services aspire (Redlinger *et al*., 2001). However, flush toilets with waterborne sewerage are extremely costly to implement and have many drawbacks (Drangert, 1998). Large volumes of potable water are used as the transport medium for human waste (sewerage), making this high quality water unavailable for other more beneficial uses. In developing countries, few wastewater treatment plants are operated effectively and discharge effluents high in microbial and nutrient load into surface watercourses. Domestic waste is often mixed with industrial wastewater *en route* to the treatment plant. Traces of heavy metals from other industrial sources make sewage difficult to treat and if not removed during aerobic and anaerobic digestion of waste, make recycled biosolids (sludge) unacceptable for reuse in agriculture (Nacheva *et al*., 2002).

It is therefore recognised at national level that providing waterborne sewerage to low-income, often densely populated, rural and peri-urban communities is simply not feasible (Redlinger *et al*., 2001; DWAF, 2003). Other options include on-site sanitation, utilising technologies in which human waste is handled and treated within the boundaries of the property in pit latrines or septic tanks. The minimal acceptable standard of on-site sanitation recognised by the South African government is the ventilated improved pit latrine (VIP) (DWAF, 2003). However, VIPs are not suitable in all locations. Factors that influence this include local geohydrology, a high water table, elevation and road access to the pits for possible emptying. A further problem with waste originating from on-site sanitation is the unpleasant odours that arise from the mixing of urine and faeces (Drangert, 1998; Schönning and Stenström, 2004), due to the abundance of bacteria, such as *Micrococcus urea*, in the faeces that decompose urea into ammonia gas, which then dissipates into the atmosphere (Drangert, 1998).

There is, however, another means of human waste disposal that has been developed with the specific aims of keeping faeces and urine separate, and of promoting the recycling of human excreta. Ecological Sanitation (EcoSan) is based on the principle of local reuse of nutrients arising from human waste rather than their discharge with water.

EcoSan can be achieved through the use of a dry sanitation system, which requires neither water nor sewerage infrastructure (Drangert, 1998; Redlinger *et al*., 2001; Schönning and Stenström, 2004). Dry sanitation systems become a practical solution in areas that have limited water resources and inadequate sewerage disposal systems. Urine diversion toilets are an application of dry sanitation. These urine diversion (UD) systems divert the urine away from the faecal matter and do not use any water, greatly reducing the chance of either surface or groundwater becoming contaminated with sewerage.

The urine diversion toilet is a dry toilet system engineered and built to handle urine and faeces, which are separated at source. The first component diverts the urine with a specially designed pedestal and carries it through a pipe to a storage tank or below-ground urine soak-away pit. In eThekwini Municipality, urine is diverted away to a soakaway. The second component consists of an above-ground vented faecal receptacle or vault where faeces dehydrate, desiccate and decompose, a process which assists in killing faecal pathogens and renders the faecal contents safer for handling. Ash, dry soil or sawdust sprinkled over the faeces after defecation reduces the visual impact, moisture, odours and flies in the toilet.

A noteworthy feature of the UD toilet used in eThekwini Municipality is that the prefabricated concrete faeces vault is divided into two. As soon as one vault is full, the second can be used whilst the contents of the inactive vault are left to stand. The faecal waste is cleared from the storage receptacle and buried or bagged for disposal once the second active vault is filled.

The storage time of the faecal waste allows for the reduction of the pathogen load in faeces, through processes such as desiccation and die-off. The time period is referred to as the standing period. The first vault is evacuated once the second vault is full (a period of approximately one year). The waste is then buried. This differs from the full EcoSan principle which involves reuse of urine as a liquid fertiliser and the dry faecal matter as soil conditioner (Winblad and Simpson- Hébert, 2004), which is not practised in eThekwini.

Urine diversion toilets have been implemented by the eThekwini Municipality (Durban) as a sanitation option in areas situated outside access to the waterborne sewerage network or where pit latrines are unhygienic or full.

The main risk associated with any handling of human waste originates from the faeces and not from the urine, which is typically sterile when it leaves the bladder (Drangert, 1998; Schönning and Stenström, 2004). Faecal matter contains moisture (70 – 85%) and organic material, including microorganisms (Drangert, 1998). The number of microorganisms range from 10^{11}-10^{12} /g of faecal material (Schönning and Stenström, 2004). These include bacteria (such as *Salmonella*), viruses (such as hepatitis or rota virus), parasitic protozoa (including *Cryptosporidium parvum*) and the parasitic geo-helminths (such as *Ascaris lumbricoides*). Helminths that persist in the environment, such as the ova of both *Ascaris* and *Taenia* species, are of greatest public health concern. (Faust *et al*., 1975; Schönning and Stenström, 2004). *Ascaris* ova are resistant even to harsh and fluctuating environmental conditions and can remain viable in the soil for years (Crompton and Pawlowski, 1985; Stephenson, 1987; O'Lorcain and Holland, 2000; Muller, 2002; Vincent, 2005). The presence of *Ascaris* ova is regarded as an indicator of the safety of biosolids (Capizzi-Banas *et al*., 2004; Schönning and Stenström, 2004).

In order to evaluate the safety of biosolids associated with the use and maintenance of UD toilet vaults, a reliable, sensitive and accurate method to detect and enumerate *Ascaris* in mixed soil-faecal waste samples is necessary. The current methods for the isolation and detection of ova in faecal samples are the Kato-Katz (WHO, 1993) or formal-ether (Allen and Ridley, 1970) methods. For the detection of *Ascaris* ova in wastewater, the modified Bailenger method (WHO, 1996), or the method currently implemented by the eThekwini municipality (Gaspard and Schwartzbord, 1995) may be used. There are currently however, no reliable methods for the detection of *Ascaris* ova in mixed faecal-soil samples.

Preliminary studies of UD faecal waste obtained from the toilet storage vaults have indicated that siliceous particles (sand) of different sizes are always present and these

make sample preparation and microscopy using the standard detection methods difficult. A number of possible solutions can be employed to separate the ova from the organic material and unwanted siliceous particles. One method is by selective sieving of the sample using a series of filters of decreasing mesh size, known as a Visser Filter®. The Visser Filter® method was developed as a rapid and simple method for the recovery of helminth eggs from organic faecal matter (Visser and Pitchford, 1972).

The objective of the present study was to compare available methods for the isolation of *Ascaris* ova, and developed a new protocol, specifically for mixed faecal-soil samples, which proved more effective than methods intended for recovery of ova from either wastewater or faeces.

3.3. Materials and Methods

3.3.1. *Quantifying Ascaris ova in a sample*

The composition of a UD waste sample is a mixture of both faecal and soil material. It was decided to investigate if either the faecal or soil component was affecting the overall recovery of ova. To determine this, a series of test samples (A – C, as previously described) were seeded using ova recovered from the adult *Ascaris lumbricoides*.

Samples (n = 6) of faecal waste from six UD vaults, provided by eThekwini Municipality, were tested using a standard density flotation method using $ZnSO_4$ with a specific gravity (SG) of 1.3. A standard sample of 1 g in 15 mL conical polypropylene (Bibby-Sterilyn®) test tubes (ct tube) was used throughout all experiments reported here. The $ZnSO_4$ flotation method can be summarised as follows. Deionised water was added to the sample, and the sample vortexed, sieved through a mesh strainer and centrifuged. The supernatant was removed, $ZnSO_4$ solution of SG 1.3 was added, the ct tube vortexed and centrifuged again. The top 1.5 mL of the supernatant was removed and examined using a microscope (x 100 magnification). This method failed to yield any *Ascaris* ova from the samples provided.

Since it had been expected that *Ascaris* ova would be present, it was not clear how these negative results should be interpreted. It was therefore decided to *seed* the sample with a

known number of *Ascaris* ova and check the sensitivity and specificity from such a standardised test mixture. Ova were dissected from the proximal 3cm of the uterus of an adult female *Ascaris lumbricoides* (24.2 cm long) worm. The extracted eggs from the worm were diluted in 8.5% saline solution. Three protocols of 10 replicates each were chosen to accurately establish the number of eggs contained in a 60 µL drop from the *stock solution*.

Methods of enumeration were as follows: (1) The ova suspension (*stock solution*) was well mixed, by vortexing, and a full pipette (3 mL) was removed. Ten slides were then prepared using successive drops of 60 µL each, and the ova enumerated using a light microscope (x 100). (2) The suspension was well mixed, and a full pipette (3 mL) removed. A single slide was then prepared using a 60 µL drop. The remaining fluid in the pipette was then expelled back into the original suspension. This process was repeated a further nine times to produce ten slides in total. Each slide was examined using a light microscope and individual ova counted. (3) After mixing the suspension well, 3 mL was withdrawn and 60 µL drops were added to each of five slides. The remaining fluid was returned to the original suspension. This process was repeated. Each slide was then examined using a light microscope and individual ova counted.

3.3.2. *Flotation test protocols using seeded samples*

Four specific gravity (SG) flotation protocols were tested using the following solutions, NaCl (SG 1.2), a saturated sucrose solution (SG 1.2) and two $ZnSO_4$ solutions (SG 1.2 and 1.3). Three test samples (n = 5 each) were chosen, as follows: (A) 60 µL drop from the saline ova *stock solution*, (B) uncontaminated human faecal (1g) material seeded with ova, and (C) local soil mixture of Berea Red (1g) seeded with ova. Samples A - C were each seeded with 60 µL drop of ova suspension. The procedure is outlined below.

Deionised water was added to the 15 mL ct tubes containing the test samples (A – C). These were vortexed, sieved through a mesh strainer, centrifuged for 3 min, and the supernatant poured off. Flotation solution (12 mL) was added to each ct tube, which was vortexed and centrifuged again for 3 min. The top 1.5 mL of the supernatant was removed and placed into a clean 15 mL ct tube. Deionised water was added up to the

14 mL mark. The ct tube was then vortexed for a minute and centrifuged for a further 3 min. The supernatant was poured off and retained. The pellet was examined microscopically (x 100) for ova. The middle supernatant (±8 mL) was removed with a pipette and placed into a clean 15 mL ct tube. Deionised water was added up to the 14 mL mark. The ct tube was then vortexed for a minute and further centrifuged for 3 min. The supernatant was poured off and the pellet examined microscopically (x 100) for ova. The remaining deposit (the pellet in the original 15 mL ct tube) was made up to 3 mL by diluting with distilled water and the ova counted. This was to determine the distribution of ova among the top, middle and bottom fractions of the flotation solution. This process was repeated for all four flotation solutions for test samples A – C.

Since the concentration of ova in a 60 µL aliquot of the *stock solution* had been quantified, this procedure allowed for a quantitative estimate of total percent recovery and proportion recovered in each fraction (top, middle and bottom) of the ct to be made.

3.3.3. *Separating Ascaris ova from organic and siliceous residue – the AMBIC protocol*

An ammonium bicarbonate (AMBIC) solution was used to separate ova from organic and siliceous residue by disrupting the electrostatic interactions between ova and these soil components.

The AMBIC protocol developed in this study consisted of three steps:

1) Sample preparation.
2) Mixing with ammonium bicarbonate (AMBIC) solution.
3) Recovery of ova by $ZnSO_4$ flotation.

The three steps are outlined below.

Step one: A 1g sample of UD faecal waste was placed into a 15 mL ct tube.

Step two: The sample was mixed with a saturated AMBIC solution (pH 8.6 at 22 ºC), made up to the 14 mL mark on the ct tube and vortexed for 3 min. The screw cap was placed on the ct tube and it was left to stand for an hour. Thirty minutes into the

standing period, the ct tube was again vortexed for 3 min and manually shaken for a further 2 min. After an hour had elapsed, the ct tube was centrifuged (± 940 x g for 3 min) and the entire supernatant discarded. Deionised water (±14 mL) was added, the contents mixed and vortexed for 2 min. The ct tube was then centrifuged (± 940 x g for 3 min) and the supernatant discarded. This constituted a wash step to remove excess AMBIC solution.

Step three: $ZnSO_4$ with specific gravity adjusted to 1.3 was added to the pellet from the previous step (±12 mL). The mixture was vortexed for 2 min, and then centrifuged (± 600 x g) for 3 min. The entire supernatant was divided equally among four 15 mL ct tubes. The pellet was retained for a further flotation trial. The four ct tubes were topped up (± 12 mL) with deionised water and centrifuged (± 1850 x g) for a further 3 min. The supernatant was discarded, the four pellets viewed using a light microscope and the ova counted. The retained pellet was subjected to another flotation using the same methodology outlined above and any ova that may have been retained at this step were counted.

3.3.4. *Preparation of comparable environmental samples*

The recovery efficiency of the AMBIC protocol was tested in two stages. The first was a semi-quantitative assessment based on the mean innoculum in 60 µL ova stock suspension. The second stage was the determination of the number of ova innoculated into each 1 g sample to allow more accurate calculation of recovery.

Ten individual slides were prepared, each with 60 µl of ova suspension. The ova on each slide were counted and recorded. The contents were then carefully washed into five 15 mL ct tubes, each containing a laboratory prepared 1g UD faecal sample. Berea Red, a local used as covering medium in UD vaults, was mixed with faecal material on a 1:1 basis. The slide and cover slip were re-examined for ova that may have been retained. None were found on any of the five slides or cover slips. The ct tubes were then vortexed for 3 min to randomly distribute the ova in the waste before being subjected to the AMBIC protocol described above. The recovered ova were counted and the percent recovery calculated.

3.3.5. *Comparison of AMBIC protocol with Visser Filter® method*

Counts were made of 60 µL aliquots of the ova suspension, using 10 separate slides for the process, as described above. The ova samples from each slide were washed off into each of ten 15 mL ct tubes, each containing 1g of laboratory prepared UD waste. The ct tubes were vortexed for 3 min, and then subjected to selective size filtration using two meshes of a three mesh Visser Filter® (100 µm and 35 µm). The middle filter (80 µm) was not incorporated as it might have retained some of the ova, thus introducing another variable to the experiment.

After vigorous filtration using a strong jet of water, the accumulated fluid was tapped off into 50 mL ct tubes and centrifuged (± 1358 x g) for 4 min. The supernatant was poured off and the 50 mL ct tube filled with $ZnSO_4$ of SG 1.3. The 50 mL ct tube was vortexed for 2 min and then centrifuged for 3 min (± 940 x g). The entire supernatant was then poured into four 15 mL ct tubes and filled with deionised water. The pellet was retained for further processing. The four ct tubes were vortexed and centrifuged (± 940 x g) for 3 min. The supernatant was poured off and the pellet examined for ova using a light microscope. The original pellet in the 50 mL ct tube was subjected to a second $ZnSO_4$ flotation (as outlined above) to account for any ova left after the first flotation.

3.3.6. *Statistical methods*

Statistical Package for Social Sciences (SPSS) version 15.0 was used for the statistical analysis of the data. The Kolmogorov-Smirnov test was used to check the normality of distribution of the data, analysis of variance (ANOVA) with post-hoc testing (Tukey) was used to compare more than two treatments and the Student's *t*-test to compare differences between two treatments. Significance was taken at the 0.05 level.

3.4. Results and Discussion

3.4.1. *Quantifying Ascaris ova in a sample using standardised zinc flotation method*

No ova were recovered in preliminary studies when $ZnSO_4$ of SG 1.3 was used as the flotation medium on an unseeded UD waste samples (n = 6), supplied by the eThekwini

Municipality. The decision was therefore taken to inoculate a laboratory prepared UD waste sample with ova from the uterus of an adult female worm. Further investigations were conducted to determine whether the choice of flotation solution or pretreatment steps had an effect on the recovery of *Ascaris* ova.

To estimate the number of ova per 60µL drop of *stock solution* (8.5 % saline), three enumeration methods were conducted (previously described). There was no significant difference among the three enumeration methods ($p = 0.281$) and the mean innoculum number established was 1208 (±SE 58) per 60µL drop of ova suspension. Throughout the counting process, ova were consistently observed to aggregate. This was attributed to the presence of residual uterine wall tissue from the dissection of the female worm.

3.4.2. *Flotation test protocols*

Protocols performed using test sample A were conducted to determine which of the four flotation solutions gave the best ova recovery from the inoculated (1208±SE 58) 15 mL ct tube (Figure 3.1). When the contents of the 15 mL ct tubes were passed through a mesh strainer (570 µm – 760 µm x 443 µm – 538 µm), as part of the pre-treatment step, it was noted that ova were retained in the mesh strainer. This was taken as further evidence of the inherent adhesiveness of the dissected ova.

There was no significant difference ($p = 0.228$) among the ova recoveries from protocols using the four flotation solutions (Figure 3.1). $ZnSO_4$ of SG 1.2 gave the highest recovery (68.2% ±SE 7.2) and $ZnSO_4$ solution of SG 1.3 the lowest (33.9% ±SE 0.79).

Documented parasitological methods based on flotation techniques advocate that the top 1.5 mL is removed from the flotation solution, distilled water added, and washed down by centrifugation (Faust *et al*., 1975). The residual deposit or pellet can then be examined microscopically. The flotation solution that recovers the most ova from the top 1.5 mL would be optimal.

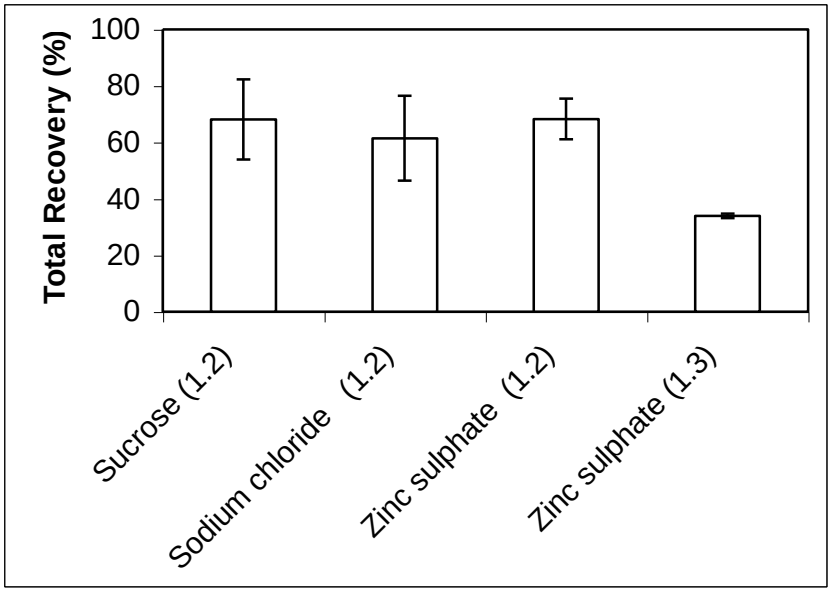

Figure 3.1: Total recovery (top 1.5 mL, middle supernatant and pellet) of inoculated ova (1208 ±SE 58) from pure ova test sample (A) for the four flotation solutions tested (SG in brackets). Error bars show standard error of mean.

When the top 1.5 mL, middle layer (± 8 mL) and residual pellet ('deposit' in Figure 3.2) were examined individually for ova and their percentage contribution to the overall recovery calculated, it was seen that of the 33.9% (±SE 0.79) recovery achieved with $ZnSO_4$ of SG 1.3 (Figure 3.1), 94.1% ova were located in the top 1.5 mL (Figure 3.2). This represented the highest recovery in the top section of the four solutions tested. No ova were detected in the pellet, and 5.7% recovered from the middle. Although $ZnSO_4$ of SG 1.2 yielded a higher total recovery (68.2% ±SE 7.2; Figure 3.1), only 1.8% of the recovered ova were detected in the top 1.5 mL of the ct tube (Figure 3.2). Similarly, recovery from the top 1.5 mL of NaCl of SG 1.2 was only 2.5% of the overall recovery (Figure 3.2).

When the same protocols were repeated using an inoculated stool sample (test sample B), there was again no significant difference ($p = 0.776$) among the recovery rates of the four tested flotation solutions (Figure 3.3). NaCl with a SG of 1.2 showed the highest total recovery of ova of 47.4% (±SE 0.25) and $ZnSO_4$, of the same SG, returned the lowest recovery of 28.1% (±SE 17.6). The total recovery for all four stool test samples

was below 50%, and there was more variation among trials than observed with Sample A. This suggests that stool composition had an effect on the overall recovery.

This decreased recovery from all the flotation methods tested may have been the result of the pre-treatment step, during which samples were sieved through a mesh before the flotation protocols. It was observed that a large proportion of the stool remained behind after sieving, and this retained material may have contained ova that were not counted. Enumerating ova in the deposit was time-consuming and supported the principle embodied in the original method, *viz*. that any protocol should rely only on the top 1.5 mL. The use of saturated sucrose of SG 1.2 was unsuitable due to the sticky nature of this flotation medium and the discolouration of samples which hampered microscopic examinations.

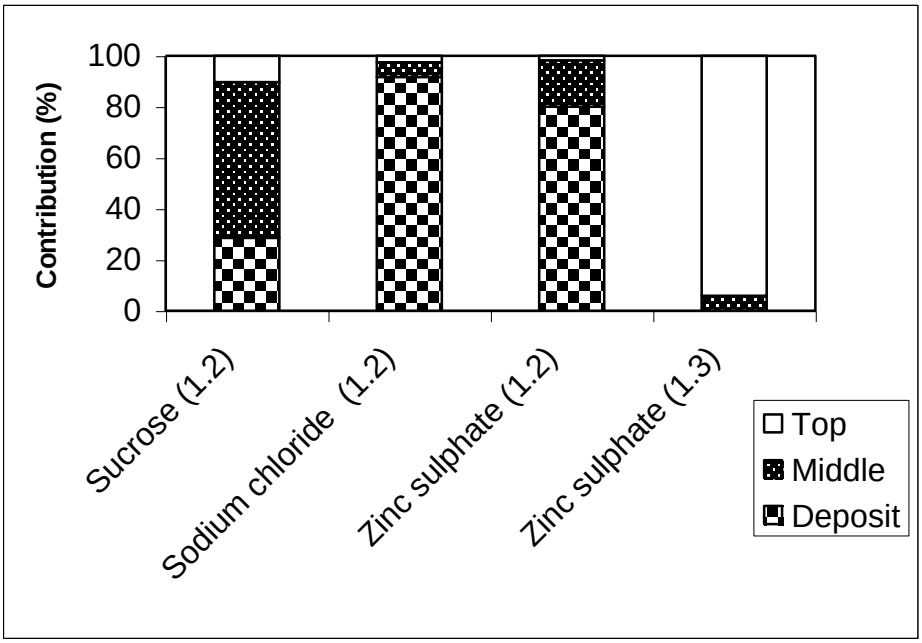

Figure 3.2: Relative contributions of the ova recovered from the three levels (top 1.5 mL, middle supernatant and retained pellet/deposit) in the pure ova test sample (A) to total recovery, using the four flotation solutions tested (shown in Figure 3.1) (SG in brackets).

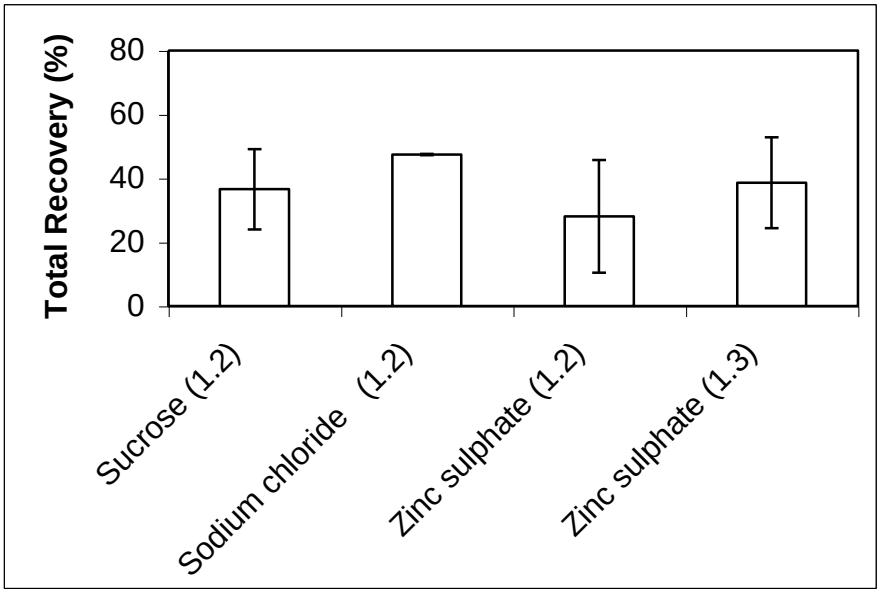

Figure 3.3: Total recovery (all three levels) of inoculated ova (1208 ±SE 58) from the inoculated stool test sample (B) for the four flotation solutions tested. Bars show standard error of mean (SG in brackets).

Although NaCl yielded the highest recovery (47.4% ±SE 0.25), both this and the low variability were found to be misleading. When all three layers were examined, all of the 47.4% ova recovered were located in the pellet (Figure 3.4). These results are in contrast with the recoveries observed for the pure egg test treatments (Figure 3.2), where ova were detected in all layers. Of the total ova recovery of 38.6% (±SE 14.2) achieved with the $ZnSO_4$ solution of SG 1.3 (Figure 3.3), 27.3% were in the top 1.5 mL (Figure 3.4). This was the highest recovery in the top 1.5 mL of the four flotation solutions tested, and contrasts with $ZnSO_4$ of SG 1.2, where all the ova detected were in the deposit (Figure 3.4). The top 1.5 mL of the saturated sucrose solution of SG 1.2 yielded 2.2% (Figure 3.4) of the total recovery of 36.6% (±SE 12.6) (Figure 3.3), the second best recovery for the top 1.5 mL. It was thought that the recovery of ova from both the top 1.5 mL and the middle portion of the sucrose flotation were due to a combination of the "stickiness" of the solution and some degree of binding of floating organic material rather than purely a density phenomenon.

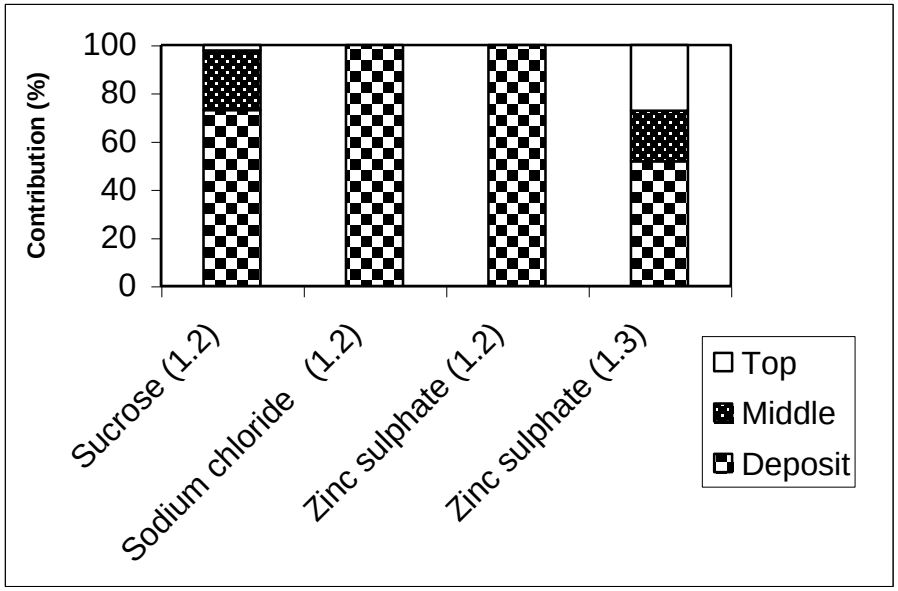

Figure 3.4: Relative contributions of ova recovered from the three levels (top 1.5 mL, middle supernatant and retained pellet/deposit) in the stool test sample (B) to total recovery, using the four flotation solutions (shown in Figure 3.3) (SG in brackets).

Figure 3.5 represents the percentage recovery of ova from seeded soil sample C, using the four flotation protocols. These recoveries varied significantly ($p = 0.013$). NaCl of SG 1.2 recovered 88.9% (±SE 14.9) of the inoculated ova from sample C, whereas $ZnSO_4$ of SG 1.3 recovered 27.3% (±SE 0.5), which was very similar to $ZnSO_4$ of SG 1.2. Sucrose of SG 1.2 yielded the lowest total recovery, 20.4% (±SE 8.1). Multiple comparison testing showed a significant difference ($p = 0.023$) between NaCl of SG 1.2 and the other three flotation test solutions. There was no significant difference ($p = 0.764$) between sucrose and either of the two $ZnSO_4$ test solutions. Recoveries by the two $ZnSO_4$ test solutions were nearly identical ($p = 0.999$).

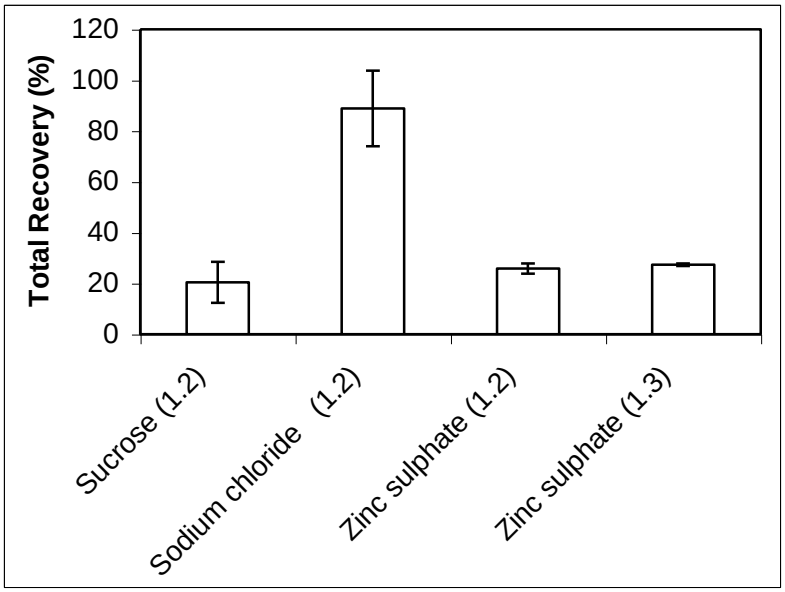

Figure 3.5: Results of combined total recovery (top 1.5 mL, middle supernatant and pellet/deposit) of inoculated ova (1208 ± 58) from the soil test samples (C) for the four flotation solutions tested. Values in brackets represent the SG of the solution. Error bars show standard error of mean.

It is clear from Figure 3.6 that soil masks the presence of ova in some way because none of the flotation solutions tested yielded ova in either the top 1.5 mL or middle section of the supernatant. On this basis, it was hypothesised that perhaps some electrostatic forces or extracellular polymers associated with the egg walls caused ova to bind to soil particles. Examination of the pellet remaining after flotation became prohibitively time-consuming, precluding accurate estimates of ova not suspended by the flotation solutions. UD samples are a mixture of faecal and soil material, both of which returned poor recoveries for all four solutions tested (Figures 3.4 and 3.6). Because of the poor recoveries, an attempt was made to modify the extraction and flotation protocol using ammonium bicarbonate (AMBIC) and a $ZnSO_4$ solution with a SG of 1.3.

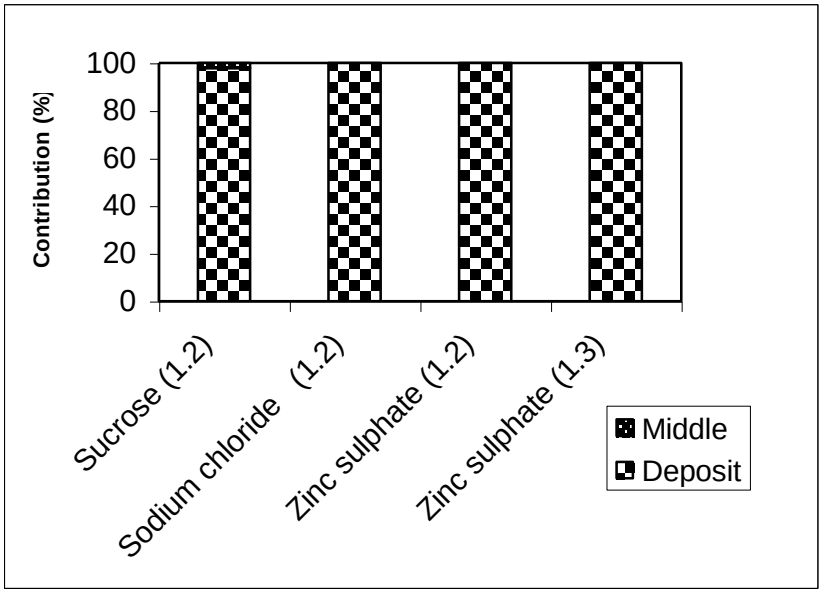

Figure 3.6: Relative contributions of ova recovered from the three levels (top 1.5 mL, middle supernatant and retained pellet/deposit) in the soil test sample (C) to total recovery, using the four flotation solutions (shown in Figure 3.5) (SG in brackets).

3.4.3. *Separating Ascaris ova from organic and siliceous residue: the AMBIC protocol*

Preliminary investigations with a saturated ammonium bicarbonate (AMBIC) solution showed that it was effective in a model system, where a pure soil test sample was seeded with ova from the *stock solution*. This is probably due to hydroxyl and bicarbonate anions, originating from dissociation of AMBIC, binding to the cation exchange sites located on the surface of soil particles. These anions may displace the ova from the surface of soil particles, and/or prevent trivalent phosphate anions located on the surface of the *Ascaris* ova from binding to the cation exchange sites located on the surface of soil particles. Trivalent phosphate anions are found in the uterine (outermost) layer of the egg wall, which consists of uneven deposits of mucopolysaccharides (Crompton and Pawlowski, 1985). The action of the bicarbonate is not perfectly understood, but may either affect electrostatic forces, which release ova from soil, or modify extracellular polymers that bind the soil to the ova.

In most flotation protocols documented in the diagnostic parasitology literature, samples are typically pre-sieved to remove large particles and debris (such as sieving through a

mesh sieve in the flotation protocols). Pre-sieving was not included in the AMBIC protocol reported here, as it was thought that ova bound to soil or any faecal debris could possibly be retained and therefore not be enumerated. This resulted in large amounts of brown organic debris in the upper layer (top 1.5 mL) along with the ova, which complicated the preparation of slides for the enumeration step.

The results obtained during quantitative evaluation of the AMBIC protocol are shown in Table 3.1. The recoveries were not significantly different ($p = 0.831$) among ten replicates. The range in recovery in Table 3.1 was 11.3%. Quantitative assessment of this proposed new method shows that it is capable of consistently achieving recoveries above 70%. Trials with different flotation protocols, reported above, yielded on average much lower recoveries overall. Furthermore, for seeded faecal and sand samples, most of the ova were not in the fraction of the flotation supernatant, which is typically examined for ova enumeration. Thus the AMBIC protocol reported here represents an improvement over standard flotation methods for the analysis of UD waste samples.

Table 3.1: Quantitative recovery of *Ascaris* ova from seeded UD waste samples (1g), using the top 1.5 mL and middle supernatant from the final flotation step of the AMBIC protocol.

Sample Number	Inoculated Ova	Recovered Ova	Recovered Ova (%)
1	**1064**	**868**	**81.6**
2	**1239**	**987**	**79.7**
4	**837**	**603**	**72.0**
5	**1176**	**889**	**75.6**
6	**1271**	**947**	**74.5**
7	**968**	**715**	**73.9**
8	**1143**	**865**	**75.7**
9	**1205**	**847**	**70.3**
10	**1076**	**872**	**81.0**
		Mean Recovery (%)	**76.0**
		Standard Error	**1.2**

A further advantage of the AMBIC protocol over other described methods is that it floats (and recovers) not just *Ascaris* ova, but also many other structures of interest from a health-related perspective, such as fungal spores and eggs of other parasites, *e.g. Trichuris trichiura* and *Taenia* sp. (results not shown).

3.4.4. *Comparison of AMBIC protocol with Visser Filter® method*

Finally, the AMBIC protocol was compared quantitatively with another standard protocol using a pre-treatment step aimed at dissociating ova from sand, using the Visser Filter® method. The highest recovery recorded with this method was 72.8%, and the lowest 30.3% (Table 3.2). Despite the wide range, there was no significant difference ($p = 0.192$) among the recoveries. However, there was a significant difference between the recovery rates of the Visser Filter® and the newly developed AMBIC protocol ($p = 0.010$).

Table 3.2: Quantitative recovery of *Ascaris* ova from seeded UD waste samples (1g), using the Visser Filter®.

Sample Number	Inoculated Ova	Recovered Ova	Recovery (%)
1	**1083**	**788**	**72.8**
2	**1278**	**608**	**47.6**
3	**1084**	**850**	**78.4**
4	**1092**	**567**	**51.9**
5	**1077**	**326**	**30.3**
6	**897**	**589**	**65.7**
7	**1314**	**808**	**61.5**
8	**1188**	**547**	**46.0**
9	**1141**	**758**	**66.4**
10	**1488**	**757**	**50.9**
		Mean recovery (%)	**57.1**
		Standard Error	**4.6**

Although the Visser Filter® had a lower overall mean recovery compared to the AMBIC protocol, the samples were easier to prepare and examine microscopically. This was due to the absence of both organic material and soil particles in the final preparation. Also minimal clumping of ova was observed using the Visser Filter®, whereas in the AMBIC protocol clumping was observed. The presence or absence of clumping makes a significant difference to the overall recovery of ova because clumps can contain anywhere between 20 and 150 ova. *Taenia sp.* and *Trichuris* ova were not encountered as frequently using the Visser Filter® method as with the AMBIC protocol (results not shown)

3.5. Conclusion and Recommendations

In conclusion, the AMBIC protocol described here shows considerable improvements in recovering *Ascaris* ova from soil-based UD waste samples, when compared with standard flotation protocols and, to a lesser extent, the Visser® filter method. With future work, it is hoped that further development and refinement of the AMBIC protocol will occur, possibly involving pre-filtration steps and using a $ZnSO_4$ flotation only once in the procedure.

Future research is planned to determine the efficiency of the AMBIC protocol on field samples collected from UD toilets and to compare results to samples seeded with *Ascaris lumbricoides* ova.

3.6. Acknowledgements

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Chapter 4: Assessment of the Recovery Efficiency of the AMBIC Protocol.

4.1. Abstract

While pathogenic bacterial die-off is considered rapid, exposure to viable and persistent ova of the helminth parasite, *Ascaris lumbricoides*, is considered the greatest single hazard associated with operation and use of Urine Diversion toilets. Thus an accurate and reproducible method for the enumeration of *Ascaris* ova in urine diversion (UD) faecal waste, which is easy to implement, would be a necessary prerequisite to asses risk associated with the removal, possible reuse and burial of UD contents. This study was conducted in two stages. The first part of this study examined the recovery efficiency of the AMBIC protocol and showed a recovery of over 70% of all *Ascaris lumbricoides* ova present with the added advantage that the protocol detected the ova of other helminth species (*Trichuris trichiura* and *Taenia* sp.). Having established these high recovery values, the protocol was applied, and also shown to be reliable, in field trials. The ova of *Ascaris lumbricoides*, as well as other helminth parasites, were recovered from the standing vaults of sampled UD toilets, including a toilet that reportedly had stood unused for a period of one year. It was concluded that none of the faecal material sampled from the toilets could be considered as safe for communities for potential re-use as a soil conditioner.

4.2. Introduction

The provision of adequate sanitation is one of the leading concerns of municipalities worldwide, especially those of urban and periurban areas in developing countries (Redlinger *et al*, 2001; Nakagawa *et al*., 2006). Despite progress towards improved water supplies, almost half of the people living in South Africa do not have adequate sanitation facilities. The growth of cities due to the migration of people from rural areas has resulted in rapid population growth in peri-urban areas, resulting in areas which are typically, low-income, high density, informal, and often illegal settlements (Langergraber ad Muellegger, 2005). The location and structure, or lack thereof, of these communities makes installation

and operation of conventional water and treatment systems complex (Paterson *et al*., 2007). According to Redlinger *et al*. (2001) and Von Münch and Mayumbelo (2007), this has a number of contributing factors, such as the lack of finance and space to set up the infrastructure required for proper sanitation and poor water supplies.

Poor sanitation is responsible for the spread of diseases such as epidemic cholera and dysentery and endemic infections such as diarrhoea, scabies and intestinal parasites amongst populations (Prüss *et al*., 2002). In areas where there are no sanitation facilities, residents have to make use of pit latrines, buckets or even dispose of their faecal waste in and around their living environments. This directly exposes them to diseases, bacteria and parasites that are carried in faecal material (Vinnerås, 2007; Schönning *et al*., 2007). People can become infected with these pathogens and parasites by coming into contact with faecal material in their environments. In order to combat the spread of viral, bacterial and parasitic diseases that are aggravated by poor sanitation, municipalities in South Africa and other areas of the world have introduced Urine Diversion (UD) toilets. This provides populations of low income communities with a safe way of disposing of their waste. These toilets have been introduced in preference to Ventilated Improved Pit (VIP) latrines because of the difficulty in and high cost of emptying the VIP toilets (von Münch *et al*., 2006; Bhagwan *et al*., 2008).

In the implementation of UD toilets as used by eThekwini, the full vault is sealed and left to stand for approximately 1 year. After this standing period has elapsed, the material needs to be removed from the vault. This poses potential health risks during the emptying process (Schönning *et al*., 2007). This is particularly so for the ova of *Ascaris lumbricoides* (Schönning and Stenström, 2004; Jiménez *et al*., 2006; Jensen *et al*., 2008). *Ascaris* ova are resistant environmental conditions and can remain viable in the environment for years (Muller, 2002; Vincent, 2005). It is for this reason that they are regarded as indicators of the safety of biosolids such as faecal waste in UD toilets (Capizzi-Banas *et al*., 2004; Jiménez, 2007).

Householders apply sand/ash after defeacation and it appeared that siliceous particles (sand) of different sizes present in unprocessed UD samples, was the principal reason for unreliable results. In order to evaluate the health risks associated with faecal waste from UD toilets, a reliable, sensitive and accurate method to detect and enumerate *Ascaris* ova in mixed soil-faecal samples is necessary.

Published methods for the isolation and detection of *Ascaris* ova in faecal samples utilize the Kato-Katz (WHO, 1993) or formal-ether (Allen and Ridley, 1970) was deemed unsatisfactory for UD waste samples (Hawksworth *et al*., *submitted*). Similarly, methods for the detection of *Ascaris* ova in wastewater samples, currently implemented by the eThekwini Municipality (Gaspard and Schwartzbord, 1995), gave variable results..

One possible solution to this problem would be the removal of the unwanted material (siliceous particles) from the sample by selective sieving using a series of filters of decreasing mesh size, known as a Visser® filter method. This technique is reputed to be rapid and simple for the recovery of helminth ova from excreta (Visser and Pitchford, 1972). A disadvantage of this method is that samples can only be prepared individually and large amounts of water are used for the sieving process.

The objectives of the present investigation was to assess the recovery efficiency of the developed AMBIC protocol (described in Chapter 3) and to compare it to the Visser filter® method (Visser and Pitchford, 1972), with or without pre-treatment with ammonium bicarbonate (AMBIC), and a standard Zinc Sulphate ($ZnSO_4$) flotation method using a mixed faecal-soil sample inoculated with known numbers of *Ascaris lumbricoides* ova. The assessment of the protocol would allow for the accurate quantification of the recovery of *Ascaris* ova. The final objective was to assess the suitability of the protocol for field faecal samples collected from UD toilet vaults.

4.3. Materials and Methods

4.3.1. *Experimental outline*

The experimental procedure was conducted in three stages:

1. The first stage involved assessing and comparing both the recovery efficiency and the effect, if any, of ammonium bicarbonate (AMBIC) on the four selected methods. A 1 g laboratory prepared UD faecal waste sample was prepared and inoculated with *Ascaris* ova from the uterus of a mature female worm (Table 4.1).
2. Secondly, the AMBIC protocol was tested on four randomly selected, well mixed UD samples taken from UD filling vaults in the field (Zwelibomvu community). Recovery efficiency was compared with that of the inoculated samples.
3. The last stage involved the screening of four randomly selected UD toilet waste samples (from standing vaults) to test the applicability of the developed AMBIC protocol under field conditions.

Table 4.1: Four methods assessed for recovery of *Ascaris lumbricoides* from laboratory prepared UD sample.

Method	Method Name	Replicates (n)	Ammonium Bicarbonate Added
1	**Visser Filter®**	**20**	**No**
2	**Visser Filter®**	**20**	**Yes**
3	**Standard $ZnSO_4$ Flotation**	**20**	**No**
4	**AMBIC Protocol**	**20**	**Yes**

4.3.2. *Recovery of Ascaris lumbricoides ova for inoculation*

Since the exact number of *Ascaris lumbricoides* ova in any UD waste sample is unknown, it was decided to seed a laboratory-prepared sample with a known number of *Ascaris lumbricoides* ova and to calculate the recovery levels from such a standardised test mixture. Ova were dissected from the proximal 3 cm of the uterus of an adult female *Ascaris lumbricoides* worm and placed in a Petri dish containing a 10% formalin solution. The extracted ova from the worm were diluted in 8.5% saline solution. Ova were enumerated (~200 – 300 ova) using a light microscope, taking care to ensure no clumping of ova occurred, and carefully washed back into eighty 1 g samples that had been pre-weighed into

15 mL conical polypropylene (Bibby-Sterilyn®) centrifuge tubes. This was used as the initial assessment step for the four recovery methods (Table 4.1).

4.3.3. *Collection, preparation and inoculation of waste material*

Two types of UD waste material were utilised for this experiment. The first was a laboratory-prepared sample intended to simulate UD waste typically recovered from UD vaults in eThekwini Municipality. *Ascaris* free faecal material was mixed with soil (local Berea Red soil) in a 2:1 ratio to mimic faecal waste in UD toilet vaults. The faecal material was first examined for the presence of any *Ascaris* ova, using the formal-ether method (Allen and Ridley, 1970), before being mixed with soil. This was then inoculated with isolated (~200 – 300 ova) *Ascaris* ova removed from a mature female worm.

The second set of samples comprised actual UD toilet faecal waste samples collected from four randomly selected UD toilets in the Zwelibomvo community of eThekwini Municipality (Table 4.2). The vaults had been standing for varying periods of time since the last known date of use. The standing time was established after consultation with the homeowner. This UD waste was utilised in two different ways.

1. Firstly, the UD faecal waste from all four vaults was mixed, to ensure homogeneity, and twenty (n = 20) 1 g samples were removed and analysed using the AMBIC protocol. The AMBIC protocol was repeated until no *Ascaris* ova were recovered.

2. Secondly, each toilet (1- 4) was analysed on an individual basis for *Ascaris* ova load. This was then expressed as ova/g Total Solids (TS) and then corrected to ova/g UD waste for the particular waste material used.

Table 4.2: Standing period of four household UD vaults sampled from the Zwelibomvo community (standing period as reported by householder).

Sample number	House number	Standing period (months)
Toilet 1	**421166**	**12**
Toilet 2	**401840**	**7**
Toilet 3	**426301**	**Unknown**
Toilet 4	**426302**	**1**

4.3.4. *Standard $ZnSO_4$ flotation and AMBIC protocol* (Chapter 3)

The AMBIC protocol applied in this study consisted of three steps:

1. Sample preparation.

2. Mixing with ammonium bicarbonate (AMBIC) solution.

3. Recovery of ova by $ZnSO_4$ flotation.

The standard $ZnSO_4$ flotation (Table 4.1; Method 3), used in this experiment, and the AMBIC protocol (Table 4.1; Method 4) differ in the addition of ammonium bicarbonate (AMBIC), step 2, as a pre-treatment. This was done to determine if the treatment of the UD waste with ammonium bicarbonate affected the recovery of ova.

Approximately 1 g of sample was weighed out and placed into 15 mL conical polypropylene (Bibby-Sterilyn®) centrifuge tubes. Twenty replicates (n = 20) were performed for both the laboratory-prepared sample (Table 4.1; Methods 3 and 4) and the mixed UD waste samples collected from Zwelibomvu community (Table 4.2) to asses the recovery efficiency. Where the four toilets were analysed on an individual basis, six replicates (n= 6) of 1 g each were taken.

For the AMBIC protocol, ammonium bicarbonate was added gradually to each test tube, vortexing between additions and stirring with an applicator stick to ensure that the entire sample was exposed to the ammonium bicarbonate. The addition of ammonium bicarbonate was omitted for the standard $ZnSO_4$ protocol. For the AMBIC protocol, the tubes were left to stand for 60 minutes and were mixed by hand at 5 minute intervals. The tubes were then

centrifuged at ±950 x *g* for 2.5 minutes and the supernatant was discarded. Deionised water was added gradually to the tubes; again the tubes were vortexed in between additions to ensure adequate washing of the sample. The tubes were centrifuged at ±950 x *g* for 2.5 minutes and the supernatant was discarded.

For both the AMBIC and the $ZnSO_4$ protocols, $ZnSO_4$ with a specific gravity (SG) of 1.3 was gradually added to the tubes, which were thoroughly vortexed and stirred with an applicator stick. The tubes were centrifuged again at ±950 x *g* for 2 minutes. The top 3 mL of supernatant was removed with a pipette and placed in a clean test tube containing approximately 3 mL of deionised water. The next 3 mL was removed and placed in a second clean test tube containing approximately 3 mL of deionised water and the remaining supernatant was equally distributed between a further two clean test tubes. The tubes were vortexed to ensure adequate mixing and deionised water was added to the 14 mL mark to reduce the SG. The tubes centrifuged at ±950 x *g* again and the supernatant was discarded. The pellet was prepared on a slide and examined using a light microscope (magnification x 100). The number of *Ascaris* ova on each slide was counted and recorded.

4.3.5. *Visser Filter® Method (Visser and Pitchford , 1972)*

This method relies on separation of particles on the basis of size classes. This is accomplished by passing the sample through two or more filters, with different mesh sizes, under a pressurized jet of water. The water forcefully removes the ova from the soil particles and washes them through the pores of the larger filter, whilst the larger soil particles are retained by the coarser filter. The ova are trapped in the second, finer meshed, filter and are then collected in a test tube through a tap at the base of the apparatus.

Approximately 1 g of sample was weighed out and placed into 15 mL conical polypropylene (Bibby-Sterilyn®) centrifuge tubes. Replicates (n = 20) were prepared for the laboratory-prepared samples (Table 4.1; Methods 1 and 2). Samples analysed by Method 2 were pre-treated with ammonium bicarbonate (AMBIC) for 60 minutes as described

previously. This, again, was done to determine if prolonged exposure to AMBIC had any affect on the recovery of ova.

Only the 100 µm and 35 µm filters of the Visser Filter® were used for study (the 85 µm filter was not used). The 1 g sample was added to the 100 µm inner filter and washed with a strong jet of water for 2-3 minutes. The inner filter was then removed, allowed to drain completely into the outer filter, and the sediment collected in the 35 µm outer filter. This was then washed for 2-3 minutes. The excess water was allowed to drain away and the remaining sample was collected in a 50 mL test tube through the tap at the base of the apparatus. The 35µm filter was rinsed with small amounts of water, which were collected in the test tube, to ensure that any remaining ova were washed out via the tap into the tube.

These tubes were centrifuged at ±950 x*g* for 6 minutes and the supernatant was carefully poured off and discarded. The pellet was transferred into a 15 mL test tube. $ZnSO_4$ (SG = 1.3) was gradually added to the tubes, which were then thoroughly vortexed and stirred with an applicator stick. The tubes were centrifuged again at ±950 x *g* for 2 minutes. The top 3 mL of supernatant was removed with a pipette and placed in a clean test tube containing approximately 3 mL of deionised water. The next 3 mL was removed and placed in a second clean test tube containing approximately 3 mL of deionised water and the remaining supernatant was equally distributed between a further two clean test tubes. The tubes were vortexed to ensure adequate mixing and deionised water was added to the 14 mL mark to reduce the SG. They were centrifuged at ±950 x *g* again and the supernatant was discarded, 3 mL of saline was added to each sample to keep them in a suspended state until they were viewed using a light microscope. The pellet was prepared on a slide and examined at magnification x 100. The number of *Ascaris* ova on each slide was counted and recorded.

4.3.6. *Total solids and ova/g UD waste calculations*

Total solids (TS) were determined by drying ten (n = 10) 10 g UD waste samples in an oven at 105 ^{O}C for 24 h, after which they were cooled and weighed (Standard Methods,

1995). This was then used to express the recovery as *Ascaris* ova / g TS. This was done in an attempt to standardise the reporting of *Ascaris* ova load in UD toilet waste.

4.3.7. *Statistical analysis*

Statistical Package for Social Sciences (SPSS) version 15.0 was used for the statistical analysis of the data. The Kolmogorov-Smirnov test was used to check the normality of the data distribution. Levene's test was used to check the homogeneity of variances. Analysis of Variance (ANOVA) was used to determine whether there was a significant difference among samples. Post Hoc (Tukey) testing was used to determine where the difference lay between the samples.

4.4. Results and Discussion

4.4.1. *Inoculation and recovery of seeded samples*

Laboratory-prepared UD samples, TS content 86.6 % ± SE 0.05, were inoculated (seeded) with *Ascaris* ova dissected from the uterus of a female *Ascaris lumbricoides* worm and subjected to different recovery methods (Table 4.1; Methods 1 – 4). There were no significant ($p = 0.875$) difference among the ova inoculation numbers for the four methods (Table 4.3). There was no significant ($p = 0.641$) difference in total solid (TS) values among the four methods. This then facilitated recovery comparisons directly across methods.

Table 4.3: Summary of mean ova inoculum per 1 g total solids for laboratory-prepared UD samples for the four methods compared. Twenty replicates (n = 20) were performed for each method.

	Method Name			
	Visser Filter®	Visser Filter®	Standard $ZnSO_4$	AMBIC Protocol
Method Number	**1**	**2**	**3**	**4**
AMBIC Solution Added	**No**	**Yes**	**No**	**Yes**
Mean Inoculation	**235.7**	**228.3**	**236.2**	**229.7**
Standard Deviation	**32.5**	**28.1**	**35.9**	**36.0**
Standard Error Mean	**7.3**	**6.3**	**8.0**	**8.0**

There were significant differences ($p = 0.0023$) among ova recovery efficiencies of the four tested methods (Figure 4.1). The AMBIC protocol (Method 4) recovered the most *Ascaris* ova from the laboratory-prepared UD sample (75.7%). The standard $ZnSO_4$ flotation (Method 3) yielded the lowest recovery (6.2%). The AMBIC protocol was shown to be the most effective method for recovering ova from 1 g laboratory-prepared UD samples. Post-Hoc (Tukey) testing showed a significant difference ($p = 0.0037$) between Method 4 (AMBIC Protocol) and the other three methods.

The pre-treatment of UD samples with ammonium bicarbonate (AMBIC) solution proved to have a significant effect ($p = 0.0397$) (Figure 4.1).This was shown in two ways. Firstly, a significant difference ($p = 0.0027$) in recoveries was seen between the standard $ZnSO_4$ flotation (no pre-treatment, Method 3) and the AMBIC protocol ($ZnSO_4$ flotation with AMBIC pre-treatment; Method 4). Secondly, there was a significant difference ($p = 0.0412$) between the Visser Filter® method after pre-treatment with AMBIC (Method 2, 47.0%) and the standard Visser Filter® method (Method 1, 34.1%) as outlined by Visser and Pitchford (1972). The overall recovery of *Ascaris* ova with the AMBIC protocol was found to be significant and therefore indicates that the AMBIC protocol had better recovery efficiency. These observations supported earlier findings reported in Chapter 3 (Hawksworth *et al*. *submitted*).

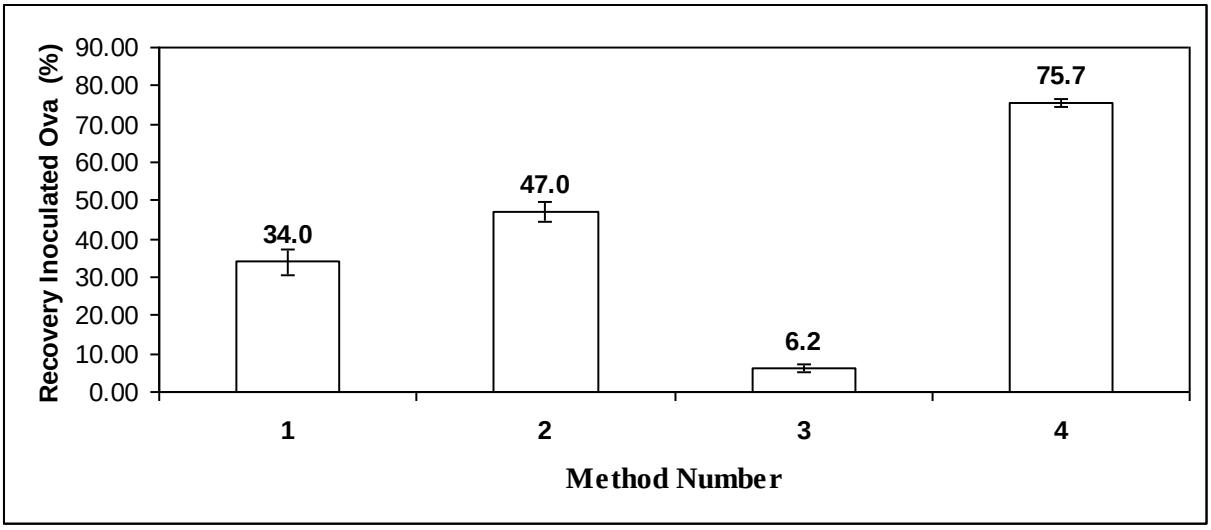

Figure 4.1: Total recovery of inoculated *Ascaris* ova from laboratory-prepared UD waste using the four selected methods (n = 20). Error bars represent standard error of mean.

4.4.2. *Recovery of Ascaris from mixed standing UD vault waste*

A well-mixed UD sample, TS content 86.3% ± SE 0.03, was subjected to the AMBIC protocol. The previously weighed out 1 g sample was then repeatedly subjected to gradient flotation separation, as outlined in the AMBIC protocol, until no *Ascaris* ova were recovered in the supernatant using flotation with $ZnSO_4$ (SG = 1.3). The values for recovery per flotation were added to give a total recovery value. The remaining deposit (pellet) in the centrifuge tube was examined and found to free of any remaining *Ascaris* ova. A total of nine successive flotations (Table 4.4) were required to recover all *Ascaris* ova from the mixed sample obtained from the standing vaults of UD toilets. There was a progressive decrease in recovery of *Ascaris* ova with each successive flotation on the 1 g UD waste sample.

Table 4.4: *Ascaris lumbricoides* ova recovery per gram UD waste (TS) for nine successive flotations (n = 20).

	Recovery *Ascaris* per Flotation (ova/g TS)									
Flotation Number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Total**
Mean Recovery	**265.6**	**58.5**	**21.8**	**7.4**	**2.2**	**2.6**	**1.1**	**0.2**	**0.00**	**355.4**
Standard Deviation	**58.7**	**19.1**	**9.1**	**4.0**	**2.2**	**2.7**	**1.3**	**0.4**	**0.00**	**68.5**
Standard Error Mean	**13.1**	**4.3**	**2.0**	**0.9**	**0.5**	**0.6**	**0.3**	**0.1**	**0.00**	**15.3**

From the total recovery (355.4 ± SE 15.3 ova/g TS) in Table 4.4, it is possible to determine the percentage recovery of *Ascaris* ova per individual flotation (Figure 4.2). This indicates the percentage recovery of ova from a single flotation from a 1 g UD waste sample subjected to the AMBIC protocol. The AMBIC protocol recovered 74.6% (± SE 1.36) of the total enumerated ova after being subjected to just a single flotation (Figure 4.2). This was found to be significantly ($p = 0.0027$) different from successive flotation recoveries. There was a slight increase in recovery between flotation 5 (0.6% SE ± 0.2) and flotation 6 (0.8% ± SE 0.2). This was not significant ($p = 0.795$).

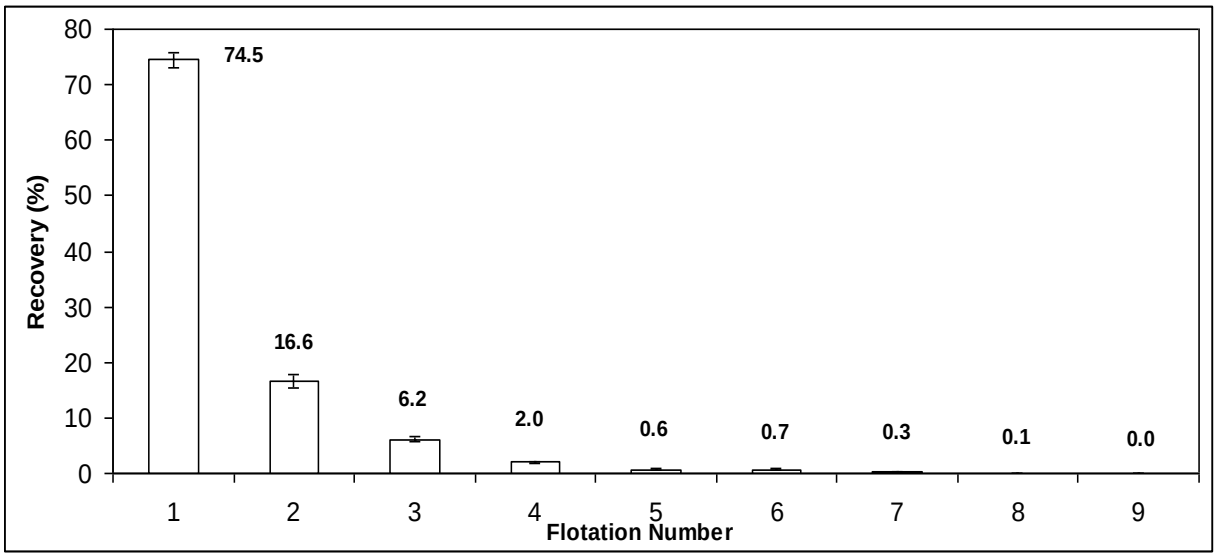

Figure 4.2: Percentage recovery of *Ascaris* ova from a 1 g UD waste sample subjected to the AMBIC protocol (n = 20) until no further ova were recovered. Error bars represent standard error of mean.

4.4.3. *Comparison of recoveries among seeded samples and mixed field samples*

A comparison in recovery efficiencies could be drawn between the laboratory seeded (Figure 4.1) sample and the mixed field samples from four standing vaults (Figure 4.2) in the Zwelibomvo community. Often laboratory-prepared tests cannot be applied directly to actual field samples as the initial load in a field sample is unknown. The results of laboratory tests can therefore only be regarded as a qualitative guideline. This may lead to uncertainty in the application of laboratory methods to field-based studies. Thus the importance of this assessment lay in simulating testing of these two sample types (inoculated versus field samples) as the load (ova/g TS) was known and could therefore be used to compare recoveries and possibly predict, within a margin of error, actual ova loads in field samples.

The inoculated sample and field (standing vault) sample showed no significant difference ($p = 0.891$) in TS content ($n = 10$), but did show a significant difference ($p = 0.0382$) in total ova numbers in a sample ($n = 20$) (Figure 4.3). There was no significant difference ($p = 0.671$) between the recoveries (Figure 4.3). The inoculated laboratory sample recovery was 75.7% (± SE 1.03) recovery of *Ascaris* ova from the first flotation compared to 74.6% (± SE 1.36) from the field (mixed standing vault) sample (Figure 4.3). This is important as it illustrates that although the ova numbers may be different, it is still possible to recover more then 70% of any *Ascaris* ova present in a sample. Results in Chapter 3 showed a 76.0% (± SE 1.18) recovery of ova inoculated into laboratory prepared samples ($n = 10$). This was achieved with inoculation numbers greater then 950 ova/g UD waste. Unfortunately the TS content was not measured in that particular study and therefore the effect that TS content may have had on recovery is unclear.

Given the approximate recovery efficiency of a method, it is possible to estimate the *Ascaris* ova load in a field sample without having to undertake successive flotations to ensure complete recovery.

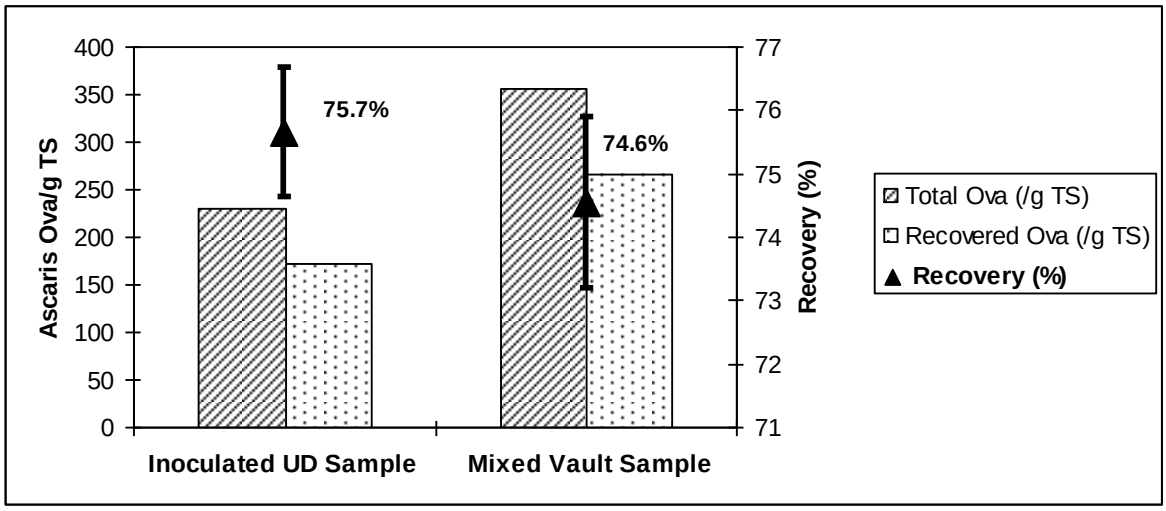

Figure 4.3: Overall comparison of recoveries between inoculated UD sample and the four mixed standing vault samples (n = 20). Values illustrated are *Ascaris* ova/g and mean overall percentage recovery (%); the error bars represent standard error of mean.

4.4.4. *Recovery of other species of helminth ova*

An advantage of the AMBIC protocol is its ability to concentrate ova of other helminth species, in this case the ova of *Trichuris trichiura* and *Taenia* sp. Although the development of the AMBIC protocol focused primarily on recovery of *Ascaris* ova, it is seen as a major advantage that other helminth ova can be enumerated simultaneously.

During the experimental process of enumerating *Ascaris* ova from UD waste material collected from standing vaults in the Zwelibomvo community, it was noted that ova from both tapeworm (*Taenia* sp.) and whipworm (*Trichuris trichiura*) were present. It was decided that these ova were to be incorporated into the recovery efficiency of the AMBIC protocol. Any ova detected from these two species were enumerated until no further ova were recovered by flotation. The remaining deposit (pellet) was examined, as in for the case of *Ascaris*, and found to be negative for *Trichuris trichiura* and *Taenia* sp.

Trichuris trichiura ova were present in all twenty of the 1 g samples examined. All *Trichuris* ova were recovered after 5 flotations, which gave a recovery number of 6.7 ± SE 0.7 ova/g TS. Processing of the field UD faecal sample resulted in an 83.3% (± SE 3.6) recovery of all *Trichuris* ova present in the first flotation (Figure 4.4). This was significantly different for successive flotations ($p < 0.0021$). The initial recovery number was low (6.7 ± SE 0.7 ova/g TS) and this indicates that the AMBIC protocol may allow quantitative extraction of initial low numbers of *Trichuris* in a UD faecal sample. The validity of this statement has not been tested or compared. This needs further testing using laboratory-seeded samples as a model.

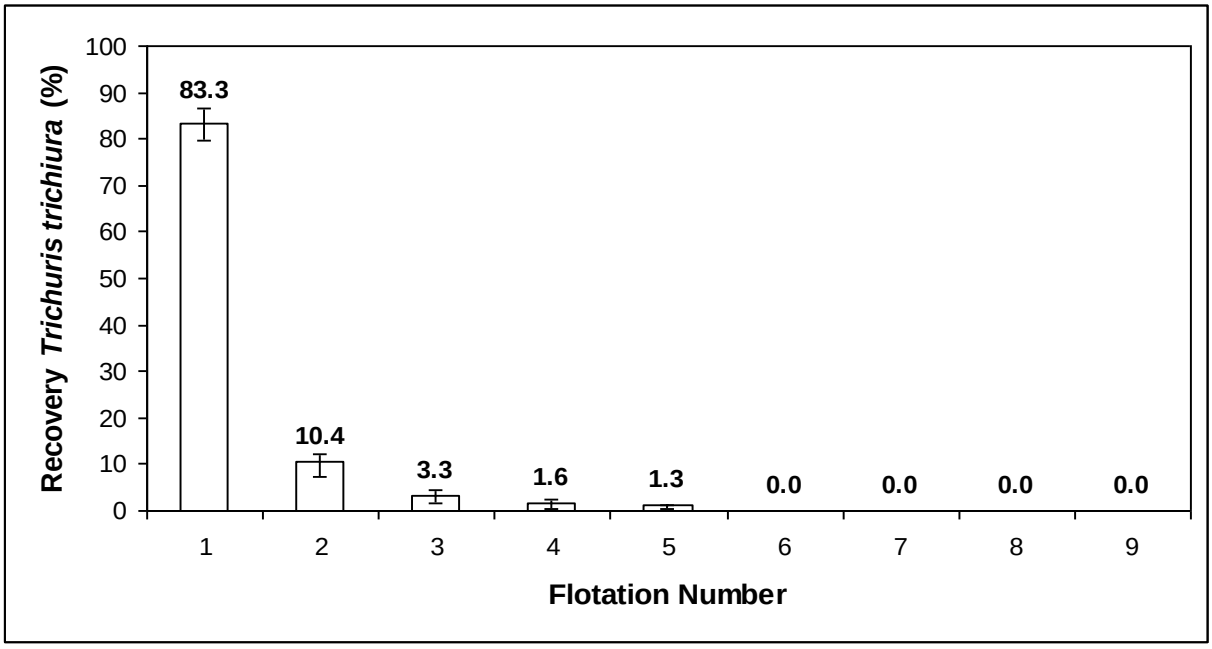

Figure 4.4: Percentage recovery of *Trichuris trichiura* ova from a 1 g UD waste sample subjected to the AMBIC protocol (n = 20) until no further ova were recovered. Error bars represent standard error of mean.

Taenia sp. were detected in eighteen of the twenty 1 g samples, to give a recovery of 3.0 ± SE 0.4 ova/g TS. All ova were recovered in the first 5 flotations. Processing of the UD sample resulted in a 71.2% (± SE 7.6) recovery of all present *Taenia* sp. ova in the first flotation (Figure 4.5). This was significantly different from successive flotations

(p = 0.0032). As was the case with *Trichuris* levels in the UD waste, the overall ova load was low, and the AMBIC protocol enabled a high recovery (> 70%) for this particular study. However, it must be emphasised that in the case of *Taenia* sp. ova, this method can only be used as a qualitative and not quantitative tool. The ova of *Taenia* sp. are excreted in proglottids that may contain thousands of ova.

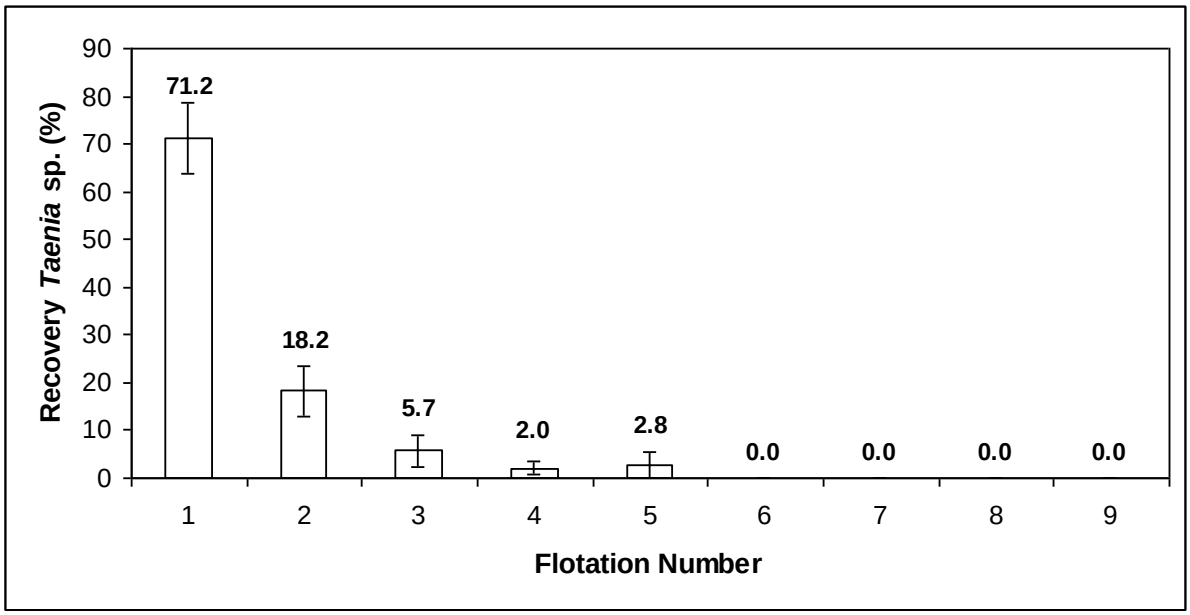

Figure 4.5: Percentage recovery of *Taenia* sp. ova from a 1 g UD waste sample subjected to the AMBIC protocol (n = 20) until no further ova were recovered. Error bars represent standard error of mean.

4.4.5. *Small – scale field trial using the AMBIC protocol*

Having established the recovery efficiency of the AMBIC protocol for *Ascaris* ova under laboratory conditions (inoculated versus collected and mixed waste), the identical four UD vaults used for the mixed waste were revisited and samples taken from their standing vaults to determine the future suitability of the protocol for possible large scale investigations into the helminth load in UD toilets.

Figure 4.6 shows recovery of *Ascaris* ova using the AMBIC method. Counts showed that toilet 3 (unknown standing period) had an average ovum count of 159.3 (± 49.6) ova/g TS and toilet 4 (1 month standing period) had an average ovum count of 56.0 (± 10.3) ova/g TS. These are much higher ova loads than toilet 1 (12 months standing period) and toilet 2 (7 months standing period) which had average ova counts of 1.5 (± 0.7) ova/g TS and 1.5 (± 0.7) ova/g TS respectively. The ANOVA test showed a significant difference ($p = 0.03$) in the overall recoveries among the different toilets. Post Hoc (Tukey) test showed no significant differences in ova loads ($p = 0.694$) between the recoveries of toilets 1 and 2.

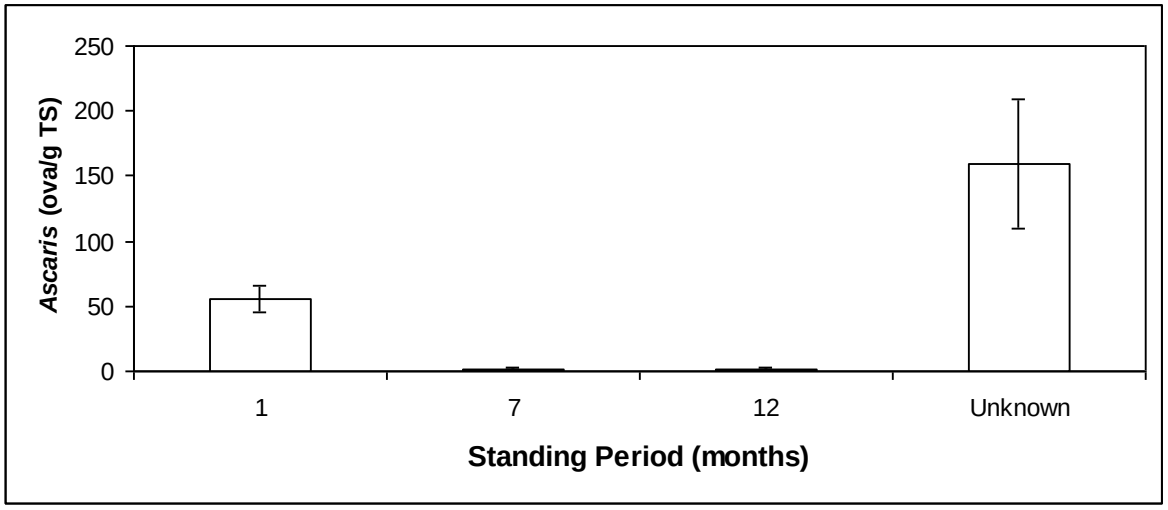

Figure 4.6: Results showing the total recovery (n= 6) of *Ascaris* (ova/g TS) from UD standing vault waste in four randomly selected toilets using the AMBIC protocol. Error bars represent standard error of mean.

Although this is a small sample size (4 toilets), it has implications when considering the emptying of UD toilets. If there is no significant difference between the *Ascaris* ova loads at 7 (toilet 2) and 12 (toilet 1) months, then presumably the emptying can be done at an earlier stage. However, although the ova counts in these toilets were low, under the light microscope some ova appeared to be viable (Figure 4.7) as there were fully formed, and in some cases motile, larva. Further in-depth viability studies would have to be conducted to confirm this finding. More importantly these findings give cause to question eThekwini's

statement that ‘a one year standing period is sufficient time to render the waste safe to use as composting material’ as there are still viable *Ascaris* ova remaining in the waste, based on the limited study presented here.

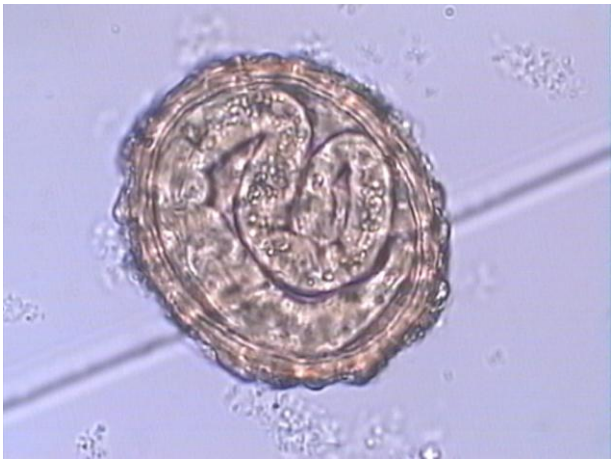

Figure 4.7: Viable *Ascaris lumbricoides* ovum recovered from a UD toilet vault that had been standing for a year (12 months), recovered using the AMBIC method. Note the second stage infective larva (Phase Contrast 400x).

It is acknowledged that there is no previously recorded data from any of these toilets, so therefore the initial load of *Ascaris* ova of the toilets is unknown. The ova recoveries recorded in this experiment cannot be compared to any initial amount so it cannot be stated that after a one year standing period the number of ova in the waste decreased. Furthermore, the ova may have entered the vault in the soil that was added to the vault after each time the toilet was used rather than by being introduced in the faeces of an infected user. This would mean that the ova may have already been exposed to environmental conditions and viability may have been affected by these conditions and not necessarily by the conditions present in the vault.

Households utilising toilets 3 (unknown standing period) and 4 (1 month standing period) both had high *Ascaris* ova counts and subsequently large numbers of viable ova. This shows a high *Ascaris* ova load in the UD waste. As the standing vaults were sampled and

not individuals, the comparison can only be drawn at a household level and not at an individual level.

The standing UD vault of Toilet 3 (Figure 4.8; unknown standing period) also contained ova of *Trichuris trichiura* (0.8 ± SE 0.3 ova/g TS) and *Taenia* sp. (0.8 ± SE 0.5 ova/g TS). Unfortunately the users (Toilet 3) did not know when last this toilet had been used. There was confusion amongst the users as to the time frame the toilet had stood (ranging from 9 – 12 months). Toilet 4, which had the shortest standing period of a month, showed the highest ova load of both *Trichuris trichiura* (6.7 ± SE 1.3 ova/g TS) and the presence of *Taenia* sp. (1.7 ± SE 1.0 ova/g TS). In Toilet 1 it was noted that even after 12 months, *Trichuris* ova (0.5 ± SE 0.3 ova/g TS) were present (Figure 4.7). This is cause for concern as, theoretically, toilet 1 had passed the minimum one year standing period and the presence of a motile *Trichuris* larva indicated that, even without considering *Ascaris*, the waste was not yet safe to handle.

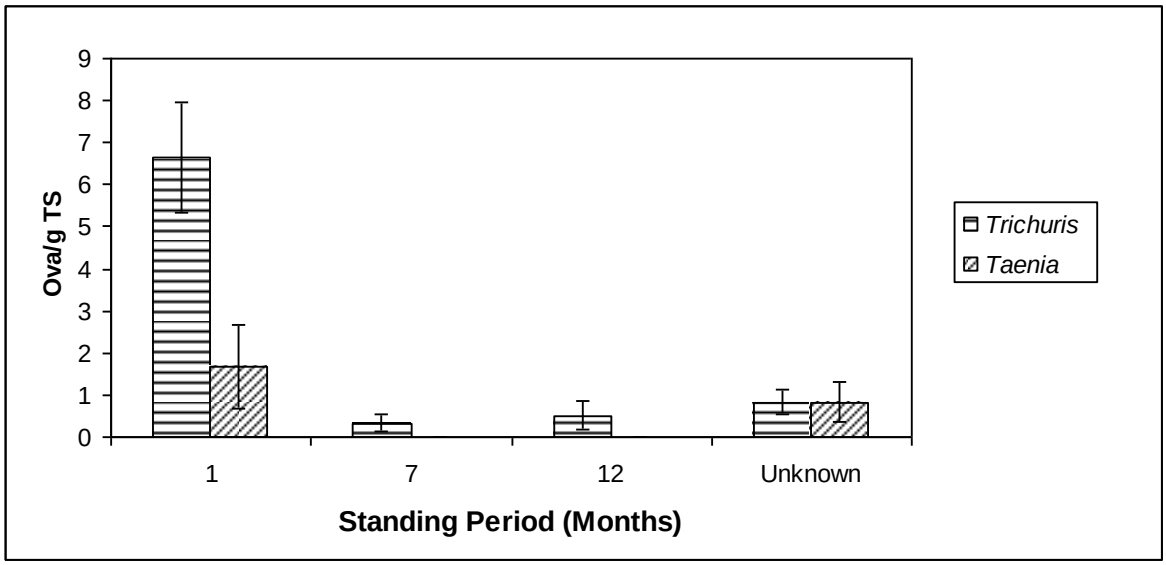

Figure 4.8: Results showing the total recovery (n= 6) of both *Trichuris trichiura* and *Taenia* sp. (ova/g TS) from UD standing vault waste in four randomly selected toilets using the AMBIC protocol. Error bars represent standard error of mean.

4.4.6. *Significance of brief field trial and future research*

The limited number of toilets sampled and the lack of previous data meant that the initial ova loads of the toilets were not known. Thus this study was essentially a *blind* assessment of the applicability of the AMBIC protocol in a field assessment. However, the field trial has indicated that the waste from all four UD toilets, irrespective of standing period, was unsuitable for re-use in any way. It is possible to state tentatively that the suggested standing period of one year is insufficient to deem the waste free of all *Ascaris* ova, although these initial studies also indicated that there was a low ova load in the older (7 and 12 month) UD vaults. It is therefore necessary to do follow-up studies which are more extensive and extend over a longer period of time. This would allow a more accurate assessment of die-off in a field-based study. These studies should include ovum counts of other parasite species *e.g. Trichuris* and *Taenia* sp. (Figure 4.9) These helminth species are often not considered a priority and are often not even included in studies either because of a lack in capacity or because the focus of a study is primarily on the ova of *Ascaris lumbricoides*. If present, these ova could potentially constitute a health risk to people reusing the waste. Further die-off studies are envisaged to determine how long the waste needs to stand before no viable *Ascaris* ova can be detected

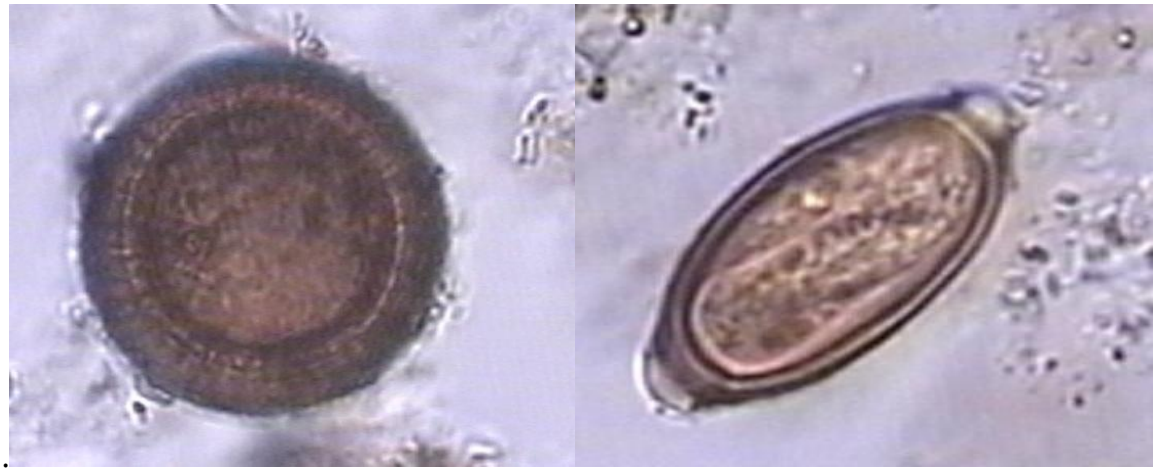

Figure 4.9: *Taenia* sp. (left) and *Trichuris trichiura* (right) ova recovered from UD toilet standing vaults (x400).

4.5. Conclusions

The AMBIC protocol proved to be very efficient (74.6%) in recovering *Ascaris lumbricoides* ova from the mixed soil/faecal waste of UD toilets. The method was also able to isolate ova from other helminth species. This can be very useful when incorporated into risk assessments of emptying UD standing vaults. However, a worrying aspect was that, although only low ova numbers were found, there were still viable *Ascaris* ova recovered in the vault of a UD toilet that had stood for 12 months.

4.6. Acknowledgements

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Chapter 5: Prevalence of Helminth and Protozoan Parasites in the Filling Vaults of Urine Diversion (UD) Toilets.

5.1. Abstract

Diarrhoeal disease is still very common in eThekwini municipality. A combined programme of improved water supply, sanitation supplied by a urine diversion (UD) toilet, health and hygiene education resulted in significant reduction in diarrhoea in people living in the homesteads exposed to the combined intervention. Although the prevalence of reported helminth infections was relatively low, there was no significant reduction following the water, sanitation and hygiene intervention. The present study measured the prevalence of protozoa and helminths in faecal residue obtained from a sample of the faecal storage vaults in household urine diversion toilets in the water, sanitation and hygiene project.

A total of 124 UD faecal waste samples from two geographical areas were screened for the protozoans *Giardia* and *Cryptosporidium,* and helminths *Ascaris lumbricoides, Trichuris trichiura* and *Taenia* sp. A staggering 89.5% of the UD toilets contained protozoan and helminth parasites. The overall prevalence of *Ascaris* was 61% *Trichuris* 51% and *Taenia* 12%, *Giardia* 55% and *Cryptosporidium* 21%.

5.2. Introduction

In South Africa since 1994, an estimated 2.8 million households have been provided with improved sanitation facilities, thereby improving the lives of over 9 million people. Improved sanitation comprises a flush toilet, ventilated improved pit latrine or dry (composting) toilet. Nationally, 91% of households have basic sanitation (88% in KwaZulu-Natal province and 97% in eThekwini). However, 24% only have access to unimproved sanitation (an unimproved pit latrine or a bucket system), which reflects as a severe burden on the health system**.**

The greatest burden of diarrhoeal diseases falls on children under the age of five, mostly in poor communities with insufficient water supply, inadequate sanitation and

little health awareness (Gasana *et al*., 2002; Naish *et al*., 2004). It is therefore necessary for the government or local municipalities to provide improved sanitation services in order to control diarrhoeal disease (Palamuleni, 2002; Mara, 2003; Lamichhane, 2007).

A recent study completed in eThekwini showed that the diarrhoea episode incidence rate was 1.9 per 1000 person days (0.71 episodes per person per year) in areas where householders had access to the multiple water, sanitation and hygiene (WSH) interventions and 3.3 per 1000 person days (1.23 episodes per person per year) in areas where these interventions were not in place (Lutchminarayan *et al*., 2008a). Occupants of households exposed to WSH interventions exhibited a 41% reduction in episodes of diarrhoea. Relatively few household members in the study reported having geo-helminths and the WSH intervention did not significantly reduce the burden of these parasitic infestations in the short term (Lutchminarayan *et al*., 2008b). In contrast, a study conducted in Brazil by Carneiro *et al*. (2002) reported that *Ascaris* infections were 4.6 times higher in children from overcrowded households with no water in their homes when compared to those with water in their homes. The risk of infection was 2.5 times higher in children from lower socioeconomic groups with lower sanitation and hygiene levels. Asaolu and Ofoezie (2003) concluded, after reviewing various health education and sanitation interventions around the world, that access to sanitation and water reduced the prevalence of helminth infections as well as the risk of re-infection, within a community.

In a water-scarce country like South Africa, sanitation options are needed that minimise the demand on water resources. There are currently three options. The first two, 'flush and forget', which is the normal reticulated sanitation option, and 'drop and store', which are represented by pit latrines or Ventilated improved pit latrines (VIP), have been used extensively. The third, more recent option is the urine diversion (UD) toilet based on the concept of separating urine and faeces at source. Separation is accomplished by dividing the toilet pedestal into a front section that collects the urine whilst the back portion collects the faecal material. The urine bowl is connected to a pipe that drains into a soak-away. The faeces are collected in a collecting vault. In the application of UD toilets adopted by eThekwini Municipality, two vaults are used in

sequence. After the first vault has filled, the contents are allowed to stand for about a year during which time the second vault is allowed to fill. When the second vault is full, the faecal waste in the first vault is removed and buried. The benefits of this sanitation system are multiple: no water is used for flushing; it is more affordable than conventional sewage systems; it decreases the pressure on municipal resources; and it has the potential of utilising the urine and faecal waste as a resource (fertilizer) although this is not currently practised in eThekwini.

The present study focused on UD toilets as implemented by eThekwini Municipality. Since 2003, more than 73 000 households in per-urban and rural areas within eThekwini Municipality have been provided with free on-site UD toilets, safe water and hygiene education. The combined WSH package has been implemented as part of a Municipal strategy to address the infrastructure backlog.

The objective of this study was to determine the prevalence of helminth (*Ascaris lumbrocoides, Trichuris trichiura* and *Taenia* sp.) and protozoan parasites (*Giardia* and *Cryptosporidium*) in a sub-sample of the faecal residue from UD toilets included in the eThekwini EcoSan (Ecological Sanitation) study by Lutchminarayan (2006). Attention was focused on these organisms because of the relative resistance of their ova/(oo)cysts and the possibility of surviving the one-year standing phase which is part of a management strategy for UD toilets.

The survey of protozoan parasites was conducted by a Masters student from the Swedish Institute for Infectious Disease Control, in collaboration with the experimenter. The survey of geohelminths was conducted by the experimenter.

5.3. Materials and Methods

5.3.1. *Study sites*

Samples were collected from UD toilet vaults from two peri-urban communities outside Durban (Sawpitts and Mtamuntengayo), approximately 3.2 km apart. In each community, households were selected to participate in the study based on the previously completed epidemiological EcoSan study (Lutchminarayan, 2006). Half of the

households chosen in each area had reported an elevated incidence of diarrhoea; the other half reported low diarrhoea incidence.

The study was performed as a double blind study so that the information on whether a household belonged to the high or low risk group was not known in advance to anyone participating in sampling or screening. Of the selected households, faecal samples collected were obtained from 124 UD toilets (67 in Mtamuntengayo and 57 from Sawpitts).

5.3.2. *Sampling procedure*

A miniature pickaxe was used to loosen vault doors and a spade weas used to take the samples from the filling UD vaults. The samples were immediately put in wide mouthed plastic honey jars with sealing lids. Water was added to the jars in order to prevent the possible drying of the protozoan (oo)cysts. All jars were stored in a cool room at $4^{o}C$ until further processing. All samples were screened for the presence of helminth and protozoan parasites.

5.3.3. *Screening for Giardia and Cryptosporidium*

Approximately 1 g of faeces was added to a 50 mL conical test tube and 15 mL of water was added. The samples were mixed and filtered through a single layer of wet gauze. Concentration and detection of (oo)cysts was conducted, for 1 mL of each sample, by the United States Environmental Protection Agency (USEPA) Method 1623 consisting of immunomagnetic separation (IMS) and immuno-fluorescence (IF) microscopy with fluorescein isothiocyasnate (FITC) labelled antibodies (USEPA, 2001). IF-microscopy was conducted on a 5mL sub-sample of the IMS concentrated samples.

Sample preparation was conducted in South Africa at the University of KwaZulu – Natal. Concentration and microscopy were performed at the Swedish Institute for Infectious Disease Control by a Masters Student of that Institution.

5.3.4. *Screening for helminths*

The AMBIC protocol developed by the Pollution Research Group was used to screen for *Ascaris, Trichuris* and *Taenia* ova (Chapter 3).

5.3.5. *Statistical analysis*

Statistical Package for Social Sciences (SPSS) version 15.0 was used for the statistical analysis. Results were analysed with respect to the frequency of parasite occurrence and correlation of the different parasites with each other, and with the overall occurrence of diarrhoeal disease as recorded in the epidemiological study.

5.4. Results and Discussion

5.4.1. *Parasite prevalence*

Most (111) of the 124 faecal samples household urine diversion toilets (89.5%) sampled were found to contain one or more protozoan or helminth parasites (Table 1). The overall prevalence of *Ascaris* was 61%, *Trichuris* 51%, *Taenia* 12%, *Giardia* 55% and *Cryptosporidium* 21%. The prevalence of *Ascaris* in Mtamuntengayo was 60.7%, followed by *Giardia* (54.8%), *Trichuris* (50.5%), *Cryptosporidium* (21.0%) and *Taenia* (11.6%).

Table 5.1: Prevalence of helminth and protozoan parasites in UD vaults sampled in Sawpitts and Mtamuntengayo communities (n=124 households).

Parasite	Positive Vaults (n=124)	Prevalence (%)
Ascaris	**75**	**60.7**
Trichuris	**62**	**50.5**
Taenia	**14**	**11.6**
Giardia	**68**	**54.8**
Cryptosporidium	**26**	**20.7**

Sawpitts (n = 57 households) had higher prevalences of *Ascaris* (73.7%), *Trichuris* (56.1%) and *Taenia* (15.8%) than Mtamuntengayo (47.8%, 44.8% and 7.5% respectively; n = 67 households) (Figure 5.1). A possible explanation for this could be the greater population density in Sawpitts. It was also visually noted during sample

collection, that although some households in Sawpitts were supplied with UD toilets, they were not being used, suggesting that the householder might in fact be practicing (random) open defaecation. This would have increased the possibility of transmission via unsanitary conditions. Prevalence of common intestinal nematodes in the KwaZulu-Natal province has a direct relationship with the altitude up to approximately 1 700 m. The climatic change experienced with an increase in altitude influences the prevalence of soil-transmitted nematodes (Appleton and Gouws, 1996). Although Sawpitts and Mtamuntengayo were only approximately 3.2 km away from each other at their nearest points, there is a marked difference in altitude between the two communities. Mtamuntengayo is located at an altitude of 310 to 400 m above sea level and Sawpitts at just above sea level (50 to 100 m).

As the prevalences of both *Giardia* and *Cryptosporidium* were similar between Sawpitts and Mtamuntengayo (Figure 5.2), this result suggested that the protozoan parasites may be endemic at different altitudes.

The prevalence of protozoan parasites was higher than expected. The prevalence of *Giardia* and *Cryptosporidium* in individuals with diarrhoea attending a health facility in KwaZulu-Natal was between 2.9 and 3.7% for *Cryptosporidium* and 2.9 and 3.0% for *Giardia* respectively (Jarmey-Swan *et al*., 2001). It is likely that people in remote areas do not seek care at health facilities for minimally symptomatic or asymptomatic infections.

In contrast, prevalence of these parasites in filling UD toilet vaults was an order of magnitude higher for *Giardia* and *Cryptosporidium*.

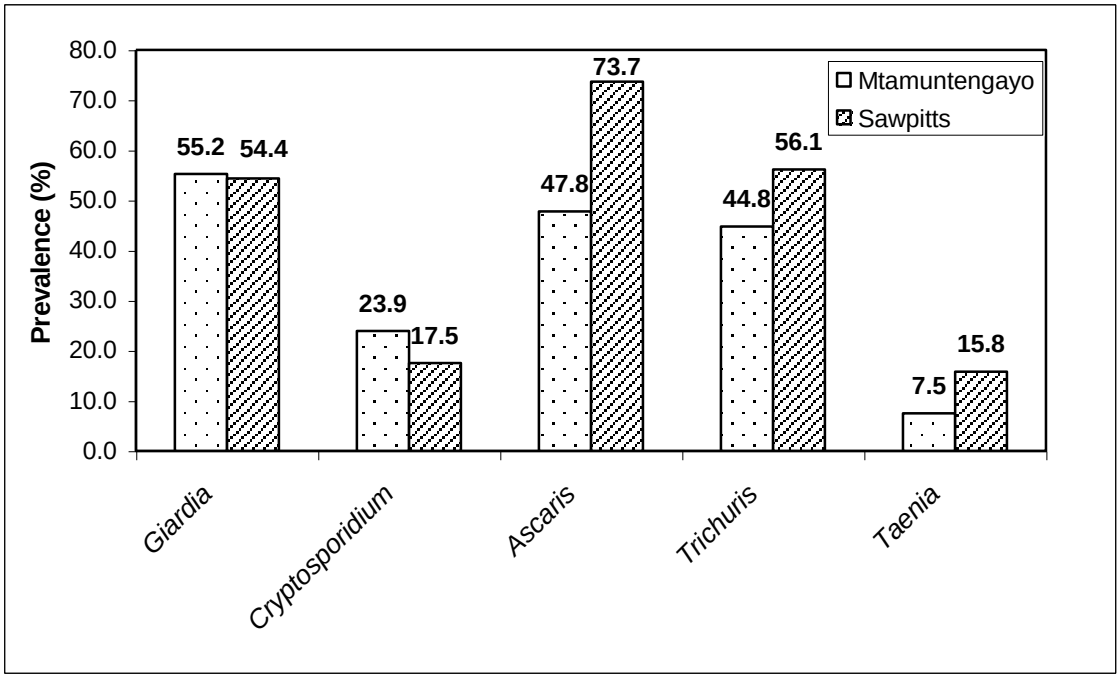

Figure 5.1: Comparative prevalences in parasites in filling vaults of UD toilets between the two peri -urban communities, Mtamuntengayo and Sawpitts.

Almost half of the household UD vaults sampled (41.9 %) were contaminated by a single species of helminth ova while a further 29.0% of the UD vaults harbored dual infections. When considering both the sampled communities, only 21.8% of the households were negative for helminth infections (Figure 5.2). When separating the two communities on the basis of number of positive samples per vault, a different prevalence pattern emerged (Figure 5.3): Sawpitts had elevated double infections and fewer negative samples compared to Mtamuntengayo. This may be associated with the population density in this particular peri-urban settlement, the non-use of the provided sanitation intervention (UD toilet) and the fact that Sawpitts is located within the coastal belt where prevalence can be greater then 70% (Appleton and Gouws, 1996; Appleton *et al.*, 1999).

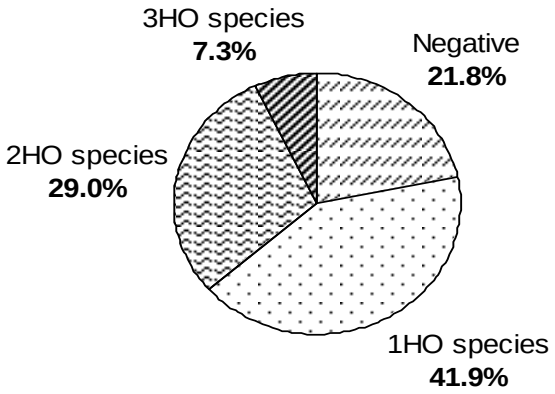

Figure 5.2: Prevalences of multiple helminth ova (HO) contamination in UD vault samples from Sawpitts and Mtamuntengayo, ranging from negative prevalence to three helminth species (3HO).

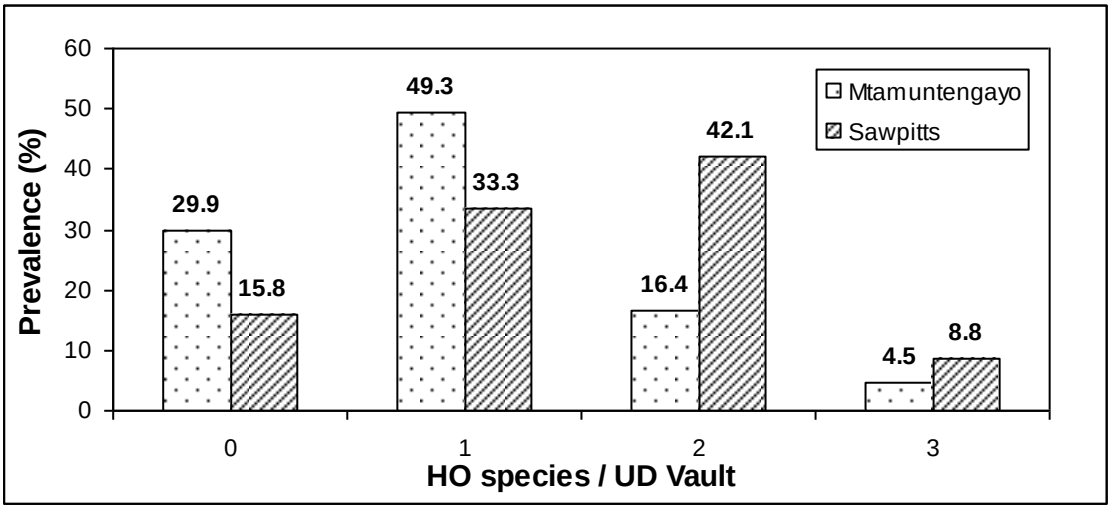

Figure 5.3: Distribution of multiple helminth species (HO) contamination per UD vault for Sawpitts and Mtamuntengayo.

Urine diversion vaults in Mtamuntengayo (n = 67 households) were positive for either *Cryptosporidium* or *Giardia* in 62.7% of the samples (Table 5.2). In 55.2% of the samples only *Giardia* was found and 23.9% of the samples only *Cryptosporidium* was present. Both *Cryptosporidium* and *Giardia* were found in 16.4% of the samples. Urine

diversion samples in Sawpitts (n = 57 households) were positive for either *Cryptosporidium* or *Giardia* in 57.9% of the samples. In 54.4% of the samples, only *Giardia* was found and 17.5% of the samples were positive for *Cryptosporidium* only. Both *Cryptosporidium* and *Giardia* were found in 14.0% of the samples (Table 5.2). However, 60.3% of all (n = 124 households) UD vault samples contained either *Cryptosporidium* or *Giardia* (oo)cysts.

Table 5.2: Occurrence of protozoan parasites in UD vault samples from the two peri - urban communities, Sawpitts and Mtamuntengayo.

	Location of UD Vault		
Protozoan Parasite	**Sawpitts (%)**	**Mtamuntengayo (%)**	**Overall (%)**
Giardia	**54.4**	**55.2**	**54.8**
Cryptosporidium	**17.5**	**23.9**	**20.7**
Giardia* or *Cryptosporidium	**57.9**	**62.7**	**60.3**
Giardia* and *Cryptosporidium	**14.0**	**16.4**	**15.2**

The results for the prevalence of protozoan parasites indicate a high level of contaminated UD vaults in the peri-urban areas chosen for investigation. These results are consistent with another study with a similar approach, in which 82% of the investigated households tested positive for *Giardia*, 70% for *Cryptosporidium* and 65% for both pathogens (Redlinger *et al*., 2002). Both studies re-inforce suggestions that intestinal pathogenic protozoa are likely to be endemic in areas with limited hygiene resources and very low socio-economic standards.

5.4.2. *Link of parasite prevalence to possible infection*

Before drawing any conclusions regarding prevalence of pathogens based on sampling from UD toilets, the number of people using any one UD toilet should be known. It is therefore not possible to link the results from a study such as this, which is based on sampling from family toilets with a varying number of users, to prevalence on an individual level. However, the results give an indication of the potential number of infected individuals. The current study shows that when considering protozoan prevalence rates, of the 124 samples taken, 54.8% contained *Giardia* and 20.7% contained *Cryptosporidium* (Table 5.1). Assuming an average family structure of five

people and extrapolating these results, this translates to a possible 340 individuals infected with *Giardia* and, possibly, 128 people with *Cryptosporidium*. Using the same line of reasoning, 373 individuals (60.3%) in the selected households may be infected with one or both of these (Table 5.2).

Further extrapolation of the results, to include all of the 1 337 households in the original EcoSan epidemiological study (Lutchminarayan, 2006), indicates that it is possible that 806 households may have had one or more members infected with one or both protozoan parasites or 45 990 of the 73 000 households currently being serviced by UD toilets.

5.4.3. *Limitations and statistical results*

No correlation was found between the prevalence of both protozoan and helminth parasites and reported diarrhoeal frequencies in the studied communities were found (Spearman's Rank: $p > 0.05$). Detection of a possible correlation could have been limited by the small sample size ($n = 124$ households, 10.5% of total households) or because diarrhoea is a symptom of mainly bacterial and viral pathogens, so this suggests parasites do not necessarily co-occur with bacterial and viral pathogens.

Initially, it was hypothesized that there should be a higher occurrence of protozoan and helminth parasites in the Sawpitts area when compared with Mtamuntengayo. Sawpitts was a more densely populated community with houses in visually worse condition than in Mtamuntengayo, which was more remote and had, in some cases, comparatively large stretches of land separating households. However, the occurrence of protozoans was lower in Sawpitts than in Mtamuntengayo (Table 5.2), although this difference was not significant (Mann-Whitney: $p > 0.05$). The difference was significant (Mann-Whitney: $p < 0.05$) when considering helminth occurrence between the two study areas. Helminths were higher in Sawpitts, in accordance with the hypothesis (Figure 5.3).

Some households that were visited, especially in Sawpitts, did not use their UD toilet and since samples could not be taken from those particular toilets, some households were excluded from the sampling in this study. In addition, at times the householders

practiced open defecation instead of using their UD toilet. This was observed on more then one occasion by the research team. This was more the case in Sawpitts than in Mtamuntengayo could contribute to problems with estimating the true prevalence. An investigation is being planned, by Lutchminarayan and co-workers, to investigate the non-use of the UD toilets in this particular community (Sawpitts).

The statistical results did show a positive correlation between helminth ova in the toilets of households and incidences of *Giardia* (Fishers exact: $p < 0.05$), but not between helminth ova and *Cryptosporidium* (Fishers exact: $p > 0.05$). Therefore, if a household UD vault contained *Giardia* then it was likely that there would be helminth ova present in the UD vault and vice versa. This was not necessarily the case with *Cryptosporidium*. If a UD vault contained *Cryptosporidium* there was likely to be helminth ova present but if a UD vault waste was contaminated with helminth ova, the vault material would not necessarily contain *Cryptosporidium*. This further supports the possibility that parasites do not co-occur with bacterial and viral pathogens which cause diarrhoea.

5.5. Conclusions

A high prevalence of both protozoan and helminth parasite infections was found in the two communities, suggesting that although the two communities have received an intervention strategy (UD toilet, water and hygiene education); there has been a breakdown in proper hygiene practices. Asaolu *et al*. (2002) reported that there is a 'threshold investment' level below which providing clean water and sanitation may not significantly improve human health but improvement in health status in terms of diarrhoea was demonstrated for these communities.

It is recommended that further studies be conducted to investigate the importance of hygiene as a possible 'missing link' between the successes of providing sanitation, clean water and health education to peri-urban communities. A comprehensive and regular hygiene education programme has been shown to improve knowledge about the control of intestinal parasites (Gungoren *et al*., 2007) and be cost effective (Mascie-Taylor *et al*., 2003). Since diarrhoea was reduced, it is more likely that either parasite transmission is a general environmental phenomenon and not as easily addressed at

household level, or parasites are more resistant to these interventions then previously thought.

Sampling directly from the deposited faecal material is useful as a screening assessment of family-based health, and as a further base for environmental risk assessment of the material in UD toilets. The parasites ova/(oo)cysts are located in the UD vault and this greatly reduces the risk of environmental contamination. The possible risk of exposure to these parasites will occur when, after the one-year standing period has elapsed, the UD vault requires emptying.

5.6. Acknowledgements

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Chapter 6: Concluding Remarks.

6.1. AMBIC Protocol

The AMBIC protocol, developed in the studies reported here, proved to be an effective laboratory tool to recover the ova of *Ascaris lumbricoides* from urine diversion (UD) waste, and an improved method over other reported methods. This was due mainly to the effect that the AMBIC solution, in conjunction with both a standing and mixing phase, had on the ova bound in the faecal-soil UD waste samples. The zinc sulphate flotation ($ZnSO_4$) with a specific gravity (SG) of 1.3 was found to be the most reliable density flotation medium for the recovery of *Ascaris* ova. An added advantage was the ability of the protocol to recover the ova from other helminth species, namely those of *Trichuris trichiura* and *Taenia* sp. (Chapter 3) The AMBIC protocol proved successful and robust enough to be implemented in a field trial on 124 households which built on data from an existing epidemiological study (Lutchminarayan, 2006) (Chapter 5).

The Visser Filter®, surprisingly, recovered nearly 30 % fewer ova than the AMBIC protocol (Chapter4). This is a worrying aspect, as this apparatus and methodology are currently used in many South African laboratories that routinely conduct laboratory work on biosolids (including UD faecal waste and sewage sludge). Future studies are envisaged to investigate whether this is a regular occurrence or could be associated with poor quality control after manufacturing and assembly of the filters. This could potentially be investigated by scanning electron microscopy (SEM) on new filters to determine the actual size of mesh pores or if there are any potential tears. It was noted that the tap arrangement, located on the 35 μm filter, for draining of the retained material into a centrifuge tube, was not robust and retained UD waste material. This had to be washed several times to remove all retained material and therefore increased the work load and may decrease detection efficiency if lab technicians do not routinely take such precautions in analyses.

6.2. Sampling from filling vaults of UD toilets

Sampling from the filling vaults of UD toilets (Chapter 5) indicated a high prevalence of both protozoan (*Giardia* and *Cryptosporidium*) and helminth parasites (*Ascaris lumbricoides, Trichuris trichiura* and *Taenia* sp.) within the communities (Sawpitts and Mtamuntengayo) studied in eThekwini Municipality. This further emphasises that the presence of all helminth ova should be recorded in laboratory analysis of UD waste. It is not uncommon in routine laboratory analysis of biosolids, for emphasis to be placed on the ova from *Ascaris* and to neglect other helminth species present. It needs to be considered that the prevalence of *Ascaris* throughout South Africa is not evenly distributed. For example, in the Eastern Cape the ova from *Taenia solium* are more prevalent (Mafojane *et al*., 2003) due to the common practice of informal free-range pig farming (Krecek *et al*., 2008) usually coupled with the provision of poor sanitation facilities in these areas. However in the context of the present study, the faecal material was recovered from the filling UD vaults and therefore had not been directly deposited into the surrounding environment.

The potential risks are now associated with the emptying of these UD vaults after the one-year standing period has elapsed. It was shown that there were high prevalence rates in the two communities studied (Mtamuntengayo and Sawpitts) and this is cause for concern with regards to emptying. It is possible that these two communities could represent isolated incidents and with this in mind, it is recommended that a similar study is widened to include other areas where UD toilets have been installed. This would give the eThekwini Munincipality an indication of the extent of potential risk areas and affords the possibility to formulate a pre-emptive pit emptying management plan to safeguard those communities or contractors involved with emptying. A mass prophylactic chemotherapy treatment plan should be administered to those areas identified as having high prevalence in the survey, starting with children under the age of 5 years.

6.3. Future research

The AMBIC protocol has provided future researchers with a reliable method to investigate the helminth load in UD faecal waste. The information gathered during the course of this research has indicated that the UD faecal waste sampled should not be

regarded as safe for handling after a one-year standing period has elapsed. This may prove to be a problem as after a year the waste needs to be removed as the UD vault system is designed to be rotated on a yearly basis. It is therefore recommended that a comprehensive risk assessment of UD vault emptying be conducted to determine possible exposure routes and hazards to both the emptier and the receiving environment, and to recommend suitable exposure barriers.

It is also envisaged that an extensive die-off study be conducted to determine the die-off of *Ascaris* ova under various combinations of temperature and relative humidity; and to determine *Ascaris* die-off under field conditions. This will then provide a comprehensive analysis of *Ascaris* die-off under various conditions of temperature, relative humidity and time likely to be encountered in the field. This can then be used to determine the conditions necessary for *Ascaris* die-off, either in UD vaults or in secondary treatment, such as composting, which could possibly be achieved either on site or as a community-based project. It would be beneficial to conduct experiments on samples from a number of different vaults in different communities, in order to establish die-off over a range of possible waste compositions.

6.4. Closing remarks

The provision of adequate sanitation facilities has been shown, in numerous studies, to improve both the livelihoods and health of communities. Yet, the provision of adequate sanitation is often regarded as the *ugly sister* to the provision of clean water and is often allocated a smaller budget. The provision of on-site sanitation such as UD toilets is starting to address the sanitation backlog. UD toilets represent a dry system, reducing the need for water to flush on an already stressed water supply.

There are, however, still problems associated with the removal and reuse of the faecal material before the UD toilet can be regarded as the *complete sanitation solution*. There is a need to establish adequate protocols for the safe handling of waste from UD vaults to ensure that potential risk factors are correctly managed. This study makes a valuable contribution to laying foundations for future management policies in eThekwini with regards to the operation and maintenance of UD toilets.

6.5. References

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