Research into UD/VIDP (Urine Diversion Ventilated Improved Double Pit) Toilets:

Prevalence and Die-off of *Ascaris* Ova in Urine Diversion Waste

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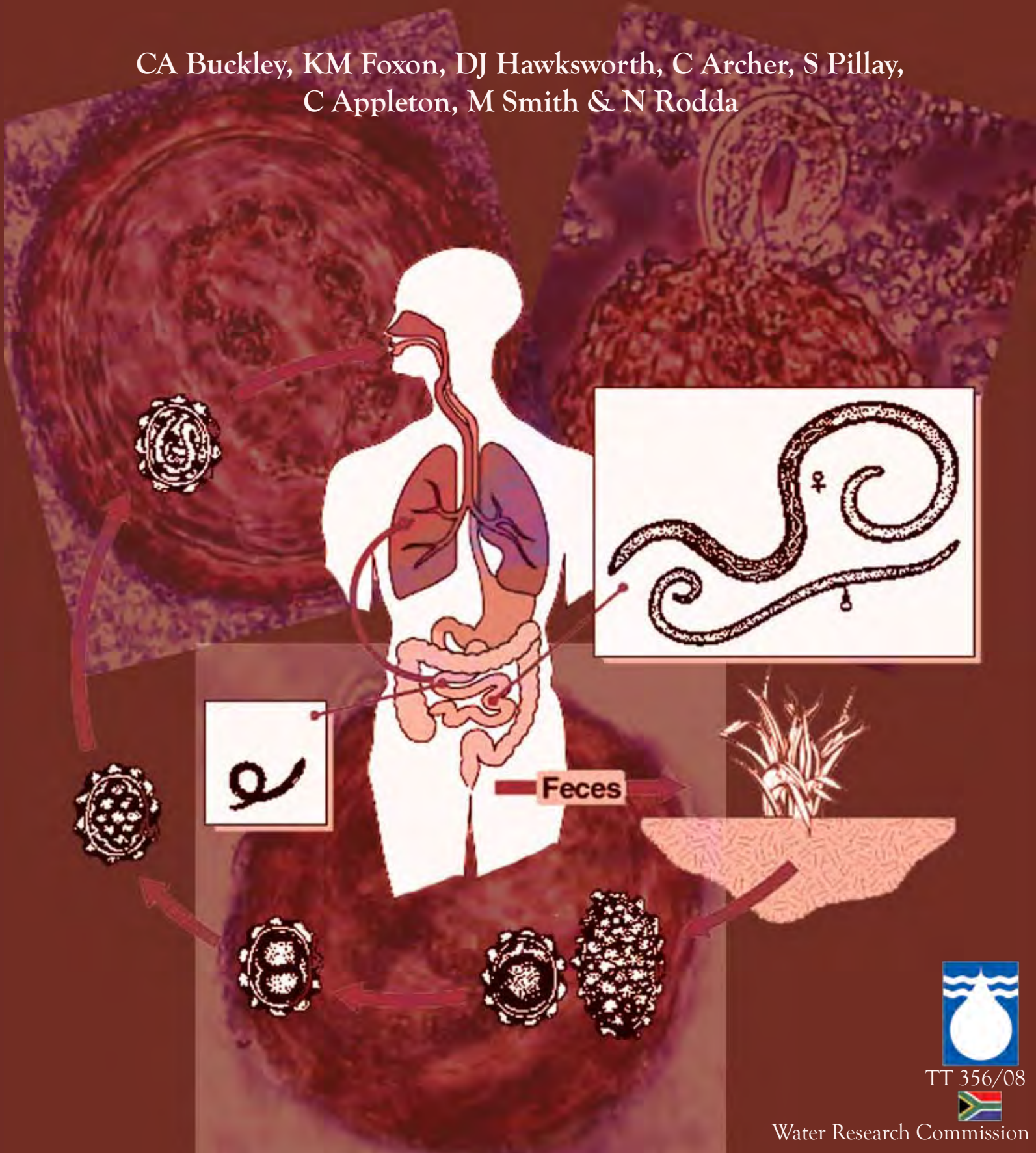
C Appleton, M Smith & N Rodda



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RESEARCH INTO UD/VIDP (URINE DIVERSION VENTILATED IMPROVED DOUBLE PIT) TOILETS: PREVALENCE AND DIE-OFF OF *ASCARIS* OVA IN URINE DIVERSION WASTE

CA Buckley, KM Foxon, DJ Hawksworth, C Archer, S Pillay, C Appleton, M Smith & N Rodda

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Water Research Commission

by

Pollution Research Group
University of KwaZulu-Natal

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The publication of this report emanates from a project entitled *Research into urine diversion toilets in eThekwini* (WRC Project No K5/1629).

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EXECUTIVE SUMMARY

eThekwini Municipality (EM) is one of the leading municipalities in South Africa with regard to the provision of basic sanitation services. The Municipality has selected a hybrid source-separating alternating twin pit ventilated pit toilets, also referred to as urine diversion toilets, as the preferred delivery mechanism for certain communities in their area of responsibility. Urine diversion VIP/VDVIP is the terminology used for the eThekwini implementation throughout this report. The first units were installed in communities outside the urban centre, built according to a constantly improving design that was initially based on a number of guiding principles identified by eThekwini Municipality. It is estimated that at the beginning of 2008 there are some 60 000 installed units in the eThekwini municipal area.

The eThekwini VDVIPs were installed because of the Municipality's experience in the logistical difficulties and excessive cost associated with the emptying of VIP toilets. The Municipality realised that they did not have a complete knowledge of urine diversion toilets, hence their request for research support. The risks associated with different disposal routes for the solids from vault also needed to be evaluated.

The eThekwini UDVIPs have a number of unique features which were developed to suit local conditions. The basic design is a double vault dry ventilated toilet with urine diverted to a soak-away located near the unit. A pedestal is located above one pit, into which faeces, anal cleansing material and bulking agent are dropped. Once the first vault is full, the pedestal is moved to the hole above the second vault and the first is sealed and allowed to stand. Once the second vault is full, the first vault is opened via a back plate and manually emptied by the householder or a contractor. The emptied contents are buried on the householder's property and, ideally, the burial site marked by planting a tree over it. The pedestal is then returned to its position above the first vault and the second vault is closed and left to stand while the first refills. It is expected that each vault will take between 6 months and one year to fill, resulting in a range of vault contents age of between 6 months and two years at the time at which they are removed from the vault.

The principle behind this design is twofold:

1. Biologically-mediated stabilisation and drying of organic material will occur in the pit, rendering it less unpleasant to handle; and

2. Sufficient pathogen deactivation will occur during the standing phase to reduce the risk of disease transmission during manual emptying of the pit

The motivation for a urine-diverting design is that the volume of material that requires handling is substantially less than in a pit latrine which receives urine and often grey water as well as faeces and cleansing material. Thus a far smaller vault needs to be constructed. The smaller volume of the vault contents and their relative dryness means that they can be removed relatively easily by the householder with an appropriately sized rake or spade.

Despite the obvious benefits of the design, there are a number of unresolved scientific, technological, social and health-related questions about how the design works from a biological and mass transfer perspective, and what the real health and environmental risks are to the

householder, community and any outsiders involved in the pit emptying process. In particular, the risk of infection by geohelminths such as *Ascaris* spp. has been identified as being an important factor in calculating the benefit of sanitation provision in terms of community health and quality of life. The infection cycle can be broken by a combination of hygiene education and practice, chemotherapy and improved sanitation. The fate of *Ascaris* spp. eggs in the urine diversion toilet is an important issue. Extended anaerobic conditions are expected to result in the ultimate death of the eggs, thus burying the contents of a urine diversion toilet may be effective in breaking the infection cycle.

Project aims

The project contract specifies the following aims for this project:

1. Provide a scientific basis for the design and operation of urine diversion toilets as used by eThekwini Municipality.

2. Evaluate the effectiveness of urine diversion toilets in improving the wellbeing of the user community.

3. Determine the fate of *Ascaris* spp. eggs from urine diversion toilets.

Products

The project contract specified the following products for this project:

1. An assessment of the health benefits of urine diversion toilets.

2. A qualitative model of the moisture balance and rate of stabilisation of the contents of a urine diversion toilet.

3. A risk assessment of the operation and maintenance of urine diversion toilets.

This report concentrates specifically on those aspects of the project associated with evaluation of the prevalence and die-off of *Ascaris* ova in the contents of urine diversion toilet vaults.

Methodology

This project involved a number of sub-projects, each of which followed its own particular methodology. The unifying principle was that each should contribute to the risk/benefit assessment of the Durban system as a whole, by providing scientific data to allow the evaluation of risks and benefits in the design and operation of the system. Some of the issues involved risks and benefits that were perceived at the start of the project, others were identified during the course of the investigations. Because of the wide scope of the investigations, where the risk involved in a topic appeared not to be critical, or the required data was available from elsewhere, it was not pursued.

In particular, the issue of the health benefits of providing UD toilets to communities was the subject of an extensive epidemiological investigation by the eThekwini Health Department and the University of KwaZulu-Natal Medical School, so that aspect was dropped from this project. A brief summary of the findings of that investigation appears in section 5.1 of the main report (not in this report).

The investigations undertaken in this project fell into three main groups:

1. physical and biological processes occurring in UD waste in the toilet vaults and after being removed from the vaults and buried;

2. the occurrence and survival of pathogens in UD waste;

3. the health risks associated with the process of vault emptying and burying the contents, as recommended by the eThekwini implementation of UD toilets.

The principal conclusion of the first and second groups of investigations was that greatest risk in the whole system was the spread of infection by geohelminths during the process of emptying the vaults and burying the contents, so the risk assessment focussed on a quantitative estimate of this risk, and the measures required to minimise it.

A substantial part of the experimental effort was devoted to developing and evaluating experimental techniques for characterising UD waste. Two techniques were chiefly used: firstly, the serum bottle test for tracking biodegradation processes, and secondly, an extraction process for helmith ova based on charge exchange between ova and the ammonium bicarbonate (AMBIC) extractant to remove helmith ova associated with soil. The AMBIC protocol represents a major advance over previous accepted methods, which were shown to seriously underestimate the incidence of ova in samples containing soil particles. This discovery brings into question the validity of previous studies on the survival of these pathogens in the soil-based systems or soil/faeces mixtures.

This report concerns itself exclusively with the development and application of the AMBIC protocol, within the framework provided by the objectives of the project. Results on tracking biodegradation using serum bottle tests are reported in the main report on this project.

Recovery and detection of *Ascaris* ova in UD waste

Ascaris is the largest of the common nematode parasites of man and has a relatively simple lifecycle. Each female worm has the potential to produce over 200 000 eggs per day. Eggs are passed in the faeces in the unembryonated state and humans contract ascariasis by ingestion of embryonated eggs through faecal contamination. It has been reported that adult worms can survive for 1 to 2 years and female worms can generate eggs for a period of one year while some may continue as long as 20 months. Diagnosis of helminth infection is normally through the detection of the eggs in the faeces. These eggs are highly infectious, and very robust.

It has been estimated that 10^{14} eggs pass daily into the global environment and an egg, once infective, is viable up to 15 years. In the context of agricultural wastewater reuse, it has been specified by the WHO that the recommended maximum permissible level of intestinal helminth egg load in sewage is 1 egg/L. Therefore it was imperative that an optimum detection method for *A. lumbricoides* ova in UD waste be established. This would aid in determining a realistic quantification of the *Ascaris* egg load in the UD waste. These results will provide guidelines for public and environmental health management.

The current methods for the isolation and detection of ova in faecal samples utilize the Kato-Katz or formal-ether methods. For the detection of *Ascaris* ova in wastewater samples, the modified Bailenger method, or the method currently implemented by the eThekwini municipality may be

used. There are currently no reported methods for the detection of *Ascaris* ova in a mixed faecal/soil sample.

The analysis of UD samples has proven to be a problem because of their composition. The eThekwini Municipality has advocated that users add a small amount of soil to the vault after defaecation. This is believed to assist in accelerating the drying process of the waste and to minimise odours from the filling vault. Preliminary studies had indicated that siliceous particles of different sizes were present in any unprocessed UD sample, and these rendered sample preparation and microscopic examination difficult. More significantly, no standard methods were available for recovery of ova from soil samples and general experience indicated very low recovery from samples that contain soil. An essential initial component of this study was therefore to develop a method with satisfactory recovery to avoid presenting an overly optimistic view resulting from a high proportion of false negative results. Initial results indicated that the presence of soil in a sample masks the presence of ova in some way, as all the standard flotation solutions tested did not recover any ova. It was hypothesised that electrostatic forces or extracellular polymers associated with the egg walls caused ova to bind to soil particles. This was tested by using an ammonium bicarbonate (AMBIC) extraction solution to disrupt electrostratic interactions between ova and soil particles. The recovery of ova using this AMBIC protocol represented a significant improvement on other methods tested.

The AMBIC protocol developed consists of three steps:

1. Sample preparation,
2. Mixing and extraction of ova with ammonium bicarbonate (AMBIC) solution at a SG of 1.3 - 1.4; and
3. Recovery with an adjusted zinc sulphate flotation procedure.

These steps essentially separate the ova from sand particles so that they can be counted microscopically.

The protocol was tested by inoculating UD waste samples with known numbers of *Ascaris* ova. Results yielded a mean recovery of 77% (SD 1.4%). This was significantly higher than recovery by any other method tested.

Inactivation of *Ascaris* ova in UD waste

The discovery that standard methods of detecting *Ascaris* ova in soil-based samples were relatively ineffective cast doubt on previous studies on the rate of *Ascaris* inactivation in UD waste.

The current minimum standing period of the UD toilet vault is one year. In order to ascertain whether this time period is safe for removal and handling of UD solid waste, reliable estimates of the egg load in the waste after a one-year standing period are needed. A study was undertaken to investigate the natural viability and die-off of *Ascaris* spp. ova in the UD solid waste, using the AMBIC protocol.

UD solid waste was sampled from five different UD toilets in the Zwelibomvu township near Durban in KwaZulu-Natal. The waste was cored out from the back opening of the toilet vaults. Those toilets which were situated such that the rear opening was difficult to reach, were sampled through the pedestal.

Samples (1 g) of well-mixed UD waste were placed into 15 mL conical test tubes, a total of 270 replicate tubes being set up for the duration of the experiment. Every fifth day, 15 tubes were analyzed. Of the 15 tubes, 5 were filled with waste from the top layer, 5 with middle layer waste and 5 with bottom layer waste.

Samples were processed and screened for *Ascaris* ova, using the AMBIC protocol. Of the five UD toilets sampled, two were found to contain *Ascaris*-infected waste. The waste from the toilet with the highest *Ascaris* egg load was used as a source of material for further experimentation and refinement of the AMBIC protocol.

One of the advantages of the AMBIC protocol is that it is non-destructive, thus viability of ova can be assessed on the basis of their appearance at the microscope examination stage. This was exploited in the study of *Ascaris* prevalence in samples of UD toilet waste. Counts of *Ascaris* ova were performed within 48 h, in order to allow for the developmental stage and condition of the ova, at a specific time, to be determined. Uniformity of timeframe was required for valid comparison of data sets. Ova were categorized as falling into one of 4 classes: (i) undeveloped, (ii) developed and motile, (iii) developed and immotile, and (iv) dead/damaged ova. In addition, ova were classified according to the condition of the viable ova, *i.e*. good or necrotic, as some of the ova that did become fully developed, began to die soon afterwards. Ova of other parasites were also observed.

After 90 days, there was a relatively higher number of non-viable *Ascaris* ova (63% dead, 17% necrotic) than viable ova (6% undeveloped, 12% viable immotile and 2% viable motile). The percentage viable *Ascaris* ova was found to decrease sharply initially, particularly between day 10 and day 20. Thereafter, viability decreased more slowly and appeared to plateau towards the end of the 90-day period. A very small percentage (~1%) of infertile *Ascaris* ova and hatched ova was detected at the end of the experiment. Total ova counts for *Ascaris*, *Trichuris* and *Taenia spp.* were found to increase initially, and then to decrease with time. A far greater number of *Ascaris* ova than *Trichuris and Taenia spp.* ova were detected. In addition, fungal spores, larval worms and plant tissue were observed in the samples. It should be noted that adequate training by experienced personnel is essential to avoid false positive scoring of some of these substances as *Ascaris* ova, and of false negative scoring of *Ascaris* ova with atypical appearance.

One of the problems encountered in this study was the wide variation in the number of helminth eggs detected in each replicate tube. Although the UD waste was mixed thoroughly, the different replicate tubes exhibited very different *Ascaris* ova counts. This could be attributed to heterogeneity of the UD waste, which has also been reported for other characteristics of UD waste (see main report).

The comparison between samples taking from the different levels in the vaults indicated that was the prevalence of viable ova was similar at all levels, and that their response to the experimental conditions also did not differ significantly.

Since the level in the vault is assumed to be correlated with the age of the material, this suggests that the die-off that was observed must have been initiated by the test conditions, and did not represent what had been taking place in the vaults before the samples were taken. In Feachem *et al*. (1983) it is stated that “climatic factors influence egg development in the soil and, therefore, transmission. Temperatures of 20-32°C are ideal, with little development taking place below 18°C. A moist, shady environment also encourages egg development”. Later, the following is also mentioned: “in anaerobic conditions development is arrested but recommences when air is introduced.” Thus it is possible that development of ova and subsequent decay occurs predominantly once the waste pile is disturbed in such a way that aerobic conditions are re-established, *e.g.* during excavation and burial of the waste at the end of the standing period.

Future studies on the viability and die-off of *Ascaris* ova in UD waste should aim at comparing different conditions (*e.g.* temperature, pH and humidity) in the waste that could potentially affect the longevity of the ova in the waste.

Discussion

The AMBIC protocol represents a very significant contribution, since it has demonstrated that previous methods were unreliable in detecting *Ascaris* ova in soil-based samples, which brings into question previous ideas about the survival of these pathogens in UD waste and other soil-based systems. It is possible that previous studies underestimated helminth persistence and hence overestimated the safety of UD waste and similar waste residues.

Recommendations

The AMBIC protocol for detection of helminth eggs should be disseminated to laboratories engaged in this kind of analysis. Training of technicians in the recognition of the appearance of *Ascaris* and other helminth ova at various developmental stages is imperative to the successful implementation of the AMBIC protocol.

A comprehensive and systematic study should be undertaken to investigate the die-off of Ascaris eggs under a range of relevant environmental conditions using the AMBIC protocol.

ACKNOWLEDGMENTS

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Research Into Urine Diversion Toilets In eThekwini

The Steering Committee responsible for this project consisted of the following persons:

Mr JN Bhagwan	:	Water Research Commission (Chairman)
Prof GA Ekama	:	University of Cape Town
Mr B Pfaff	:	eThekwini Water Services
Mr JL Harrison	:	eThekwini Water Services
Mrs SAF Jackson	:	eThekwini Water Services
Mr MVG White	:	Development Corporation Ireland
Prof F Bux	:	Durban Institute of Technology
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Mr N Walker	:	Partners in Development cc
Mr LM Austin	:	CSIR
Ms S Büning	:	Sol Plaatje Housing Corporation
Mr H Sussens	:	Department of Water Affairs

The financing of the project by the Water Research Commission and eThekwini Water Services, and the contribution of the members of the Steering Committee is gratefully acknowledged.

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1 INTRODUCTION

eThekwini Municipality is one of the leading municipalities in South Africa with regard to the provision of basic sanitation services. The Municipality has selected source-separating toilets, also referred to as urine diversion toilets, as the preferred delivery mechanism for certain communities in their area of responsibility. Urine diversion (UD) is the terminology used for the eThekwini implementation throughout this report. The first units were installed in communities outside the urban centre, built according to a constantly improving design that was initially based on a number of guiding principles. It is estimated that at present (beginning 2008) there are some 60 000 installed units in the eThekwini municipal area.

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1.2 Project aims

The project contract specifies the following aims for this project:

1. Provide a scientific basis for the design and operation of urine diversion toilets as used by eThekwini Municipality.

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3. Determine the fate of *Ascaris* spp. eggs from urine diversion toilets.

1.3 Products

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3. A risk assessment of the operation and maintenance of urine diversion toilets.

This report concentrates specifically on those aspects of the project associated with evaluation of the prevalence and die-off of *Ascaris* ova in the contents of urine diversion toilet vaults.

1.4 Methodology

The project methodology can be summarised as comprising two parts:

1. A reliable experimental protocol for the recovery of ova of *Ascaris* and other helminths was developed and characterised.

2. The protocol developed in 1. above was applied to the investigation of prevalence and survival of *Ascaris* ova in UD waste excavated from toilets in the field and incubated in the laboratory for 90 days under what were considered to be typical summer conditions in a UD vault (29 to 30˚C and 100% relative humidity).

3. Recommendations were developed on the basis of the results of the experimental studies.

1.5 Report Outline

This report, entitled “Prevalence and Die-Off of *Ascaris* Ova in Urine Diversion Waste”, represents Part B of the Final Project Report entitled “Research into Urine Diversion Toilets in eThekwini”.

The report itself consists of 4 main sections:

1. Introduction,
2. Literature review,
3. Development of AMBIC protocol for detection and quantification of *Ascaris* ova in UD waste, and its application to evaluate prevalence and die-off of *Ascaris* ova in UD waste in eThekwini,
4. Discussion and recommendations.

2 LITERATURE REVIEW

2.1 Potential Health Impacts Associated with UD Toilets

South Africa, along with many other countries is faced with the dilemma of inadequate disposal of excreta-related human wastes into the environment (Butare and Kimaro, 2002). The World Health Organisation (WHO) estimates that 80% of all deaths in developing countries are related to water- and excreta-related diseases (Petterson and Ashbolt, 2002). Untreated excreta and wastewater contains organic matter, plant nutrients, trace elements and micronutrients as well as pathogenic bacteria, viruses and helminths, endocrine substances and medical residues .

The inadequate and unsanitary disposal of infected human faeces leads to contamination of the ground and of sources of water. Often it provides the sites and the opportunity for certain species of flies and mosquitoes to lay their eggs, to breed, or to feed on the exposed material and to carry infection. It also attracts domestic animals, rodents and other vermin which spread the faeces and with them the potential for disease. There are a number of diseases related to excreta and wastewater which commonly affect people in the developing countries, the incidence of which can be reduced by the introduction of safe excreta disposal. Major examples are intestinal infections and helminth infestations, including cholera, typhoid and paratyphoid fevers, dysentery, hookworm, schistosomiasis and filariasis. Those most at risk of these diseases are children under five years of age, as their immune systems are not fully developed and may be further impaired by malnutrition. The diarrhoeal diseases are by far the major underlying cause of mortality in this age group, (WHO, 1992)

Among the pathogenic organisms which may be associated with faecal wastes are as follows (Butare and Kimaro, 2002):

1. *Viruses*: poliomyelitis, hepatitis (A/E), enteroviruses, rotavirus, enteric adenoviruses.

2. Of growing concern is Hepatitis A, which has been recognized as a pathogenic virus of major concern when applying wastes to land and is considered a risk for water- and food-borne outbreaks. Hepatitis E is a viral disease of emerging importance in water- and food-borne infections (Schonning and Stenstrom, 2004).

3. *Bacteria*: *Salmonella* spp. (causing typhoid paratyphoid fevers), *Shigella* (causing bacterial dysentery), *Vibrio cholerae* (causing cholera), *Mycobacterium* spp. (causing tuberculosis – relative importance of water as a transmission route??), *Escherichia coli* 0157:H7 (gastroenteritis with complications including haemolytic uremic syndrome)

4. *Protozoa*: *Entamoeba histolytica* (causing amoebic dysentery)

5. *Cryptosporidium parvum* and *Giardia lamblia/intestinalis* have been studied intensively due to the high environmental resistance and low infection doses. *Entamoeba histolytica* has recently been recognized as an infection of concern in developing countries(Schonning and Stenstrom, 2004).

6. *Helminths*: roundworms, pinworms, sheep liver flukes and shistosomes (causing bilharzias).

7. Because of their persistence in the environment, *Ascaris* and *Taenia* ova are regarded as hygienic quality indicators. Nearly one billion people world wide are infected by hookworm, particularly in the moist tropics and subtropics. In developing nations, these infections are exaggerated by malnutrition. The infective eggs from *Ascaris* and hookworms are excreted in faeces and requires a latency period and favourable conditions in soil or a deposit of faeces to develop into larvae and become infectious (Schonning and Stenstrom, 2004).

   Inadequate sanitation, lack of access to clean potable water and poor domestic hygiene are the causes of ~ 80% of all infectious diseases *(e.g*. cholera, typhoid, hepatitis, polio, cryptosporidiosis, ascariasis, and schistosomiasis) in the world and responsible for 10-25 million deaths each year, most of them 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).

   Community studies show that the number of pathogens present in excreta varies as a function of the health of the host and the local environment (Schonning and Stenstrom, 2004). Communities characterised by poor hygiene and a large proportion of children will generate excreta rich in enteric pathogens. The chances of these pathogens resulting in new infections in other susceptible individuals is a function of contact and exposure, which are governed by factors such as the excreted quantity and the infective dose. The probabilities of contact and exposure are further governed by the ability of different species and strains of pathogens to withstand environmental conditions outside the host's body and to persist in a stage where they can infect a new individual upon exposure. Healthy individuals do not normally excrete pathogens, therefore the pathogen load in the environment is linked to the general health status of the community. There are five possible exposure routes for pathogens spread by the faecal-oral route: 1) direct contact with untreated excreta, 2) direct contact with inadequately treated excreta, 3) consumption of crops watered or fertilised with untreated or inadequately treated excreta (with or without a withholding period since the last application of waste), 4) inhalation of pathogens, 5) transmission from animals to humans by contact with animal excreta, where the animal host may also amplify the pathogen in the environment.

   Helminth infections are of particular concern in developing countries and many of these parasitic worms have human hosts. 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. *Ascaris lumbricoides* is one of the most significant human pathogens in the UD waste, particularly in developing communities generally and, more specifically, along the KwaZulu-Natal coastline (*i.e.* including many areas served by eThekwini Municipality). Its importance derives from the fact that it has ova which are extremely persistent in the environment outside the host. An important source of exposure for humans to *Ascaris* ova exists in regions where 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 of infection (Faust, 1955).

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 (Carrington, 2001). 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 eggs (Peasey, 2000). Chale-Matsau (2005) pointed out that many communities in developing countries such as South Africa do not de-worm themselves (*i.e.* practice chemotherapeutic prophylaxis), therefore contact with untreated or inadequately treated sewage sludge, or other waste residues such as UD waste, containing viable *Ascaris* ova could lead to heavy worm infestations. Symptoms of helminth infestation are widespread, especially in developing countries, and may include gut 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).These ailments affect humans and animals and are directly linked to faecal contamination (Simonart *et al*., 2003). They are also linked to poor socio-economic conditions, so it is not surprising that poor communities in developing areas are characterised by a high prevalence of ascariasis. Especial care must therefore be taken when introducing a sanitation technology that potentially increases contact between householders and excreta which may contain viable *Ascaris* ova. The risks of handling waste must be clearly identified and methods of reducing risk must be instituted wherever possible.

The application of health risk assessment methodology is helpful in identifying the extent of risk and the potential benefit of various barriers to infection, allowing treatment and hygiene interventions to be targeted specifically at the most vulnerable among the exposed population and at the interventions most likely to yield the highest benefit (WHO, 2006).

2.2 Health hazards associated with *Ascaris*

Ascaris is the largest of the common nematode parasites of man and has a relatively simple lifecycle. Each female worm has the potential to produce over 200 000 eggs per day. Eggs are passed in the faeces in the unembryonated state and mature to an embryonated stated in the environment. Humans contract ascariasis by ingestion of embryonated eggs through faecal contamination of their environment. It has been reported that adult worms can survive for 1 to 2 y in the human host, and female worms can generate eggs for a period of one year while some may continue as long as 20 months (O'Lorcain and Holland, 2000). Diagnosis of helminth infection is normally through the detection of the eggs in the stool (Simonart *et al.*, 2003). These eggs are highly infectious, and very robust.

According to O'Lorcain and Holland (2000), it is estimated that 10^{14} eggs pass daily into the global environment and an egg, once infective, is viable up to 15 years. In the context of agricultural wastewater reuse, it has been specified by the WHO that the recommended maximum permissible level of intestinal helminth egg load in sewage is 1 egg/L (Gaspard and Schwartzbrod, 1995; Ayres *et al.,* 1996). 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* egg load in the UD waste and provide indications of the infective potential of UD waste from a given vault (bearing in mind that vault contents will contain

Ascaris eggs only if members of the household are infected with *Ascaris* worms). Data on the *Ascaris* ova load in UD waste will provide input for development of guidelines for public and environmental health management with respect to UD toilets.

2.3 Current detection methods for *Ascaris* ova

General 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) (Allen and Ridley, 1970). In sedimentation approaches, the fatty matter is separated in an interphase solution (ether or ethyl acetate) while the parasites sediment into a non-miscible buffer below. In flotation methods, parasites eggs 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 rely on centrifugal force. The hydrophilic-lipophilic balance of the organism and its relative density in relation to that of the separating reagent, are the main factors that govern the success of the concentration of specific parasite species detected. This suggests that pH or the presence of heavy metals or alcohols in the reagents used can have an effect on the surface properties of the parasite, as each species responds differently to these changes. Therefore no one single method concentrates all species with the same efficiency (Ayres and Mara, 1996).

Comparing 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).

Faecal samples

The current conventional methods for isolation and detection of ova in faecal samples are sedimentation techniques, *viz.* the Kato-Katz method (WHO, 1993) or formal-ether method (Allen and Ridley, 1970). According to Muller (2002), the Kato-Katz method can detect low numbers of ova in faeces and was long regarded as the standard for faecal samples. Concentration methods, which are useful for prevalence surveys, are not useful for measuring the intensity of an infection because samples with large numbers of eggs 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 quick because many samples can be processed simultaneously. Furthermore, samples can be stored for a long period in formalin and preserved samples can be transported to more sophisticated laboratories for more detailed analyses (Archer *et al*., 1997). The technique also separates protozoal cysts and *Strongyloides* larvae (Muller, 2002), so enables presence of parasites besides *Ascaris* to be evaluated.

The formal-ether method for the concentration of faecal ova, cysts and larvae suffers from three major drawbacks: (i) concentrations of *Taenia* and *Ascaris* (particularly the infertile stage) ova detected are usually unsatisfactory using this method; (ii) the technique makes use of hazardous chemicals, which are toxic to the environment; and (iii) it is expensive as a

considerable amount of equipment is used for concentration (Allen and Ridley, 1970; Archer *et al.*, 1997). In addition, concentrated deposits may prove difficult to fix and stain on microscope slides and results obtained from the concentration of faecal specimens which are fixed in bulk and stored for days or weeks can be unsatisfactory (Allen and Ridley, 1970). However, Allen and Ridley (1970) described simple modifications to this method in order to overcome some of these problems. The concentration procedure adopted by Allen and Ridley (1970) comprised the use of formalin in water instead of formal saline and centrifugation was done at 3 000 rpm instead of 2 000 rpm. This modified procedure was found to yield good results with all types of protozoal cysts and ova. The main advantage of the modified method was in the concentrations of *Ascaris*, *Taenia* and *Schistosoma* ova detected. Other benefits included a relatively clean deposit and enhanced visibility of the structural detail of ova or cysts obtained, therefore making it a viable routine diagnostic procedure. Further attempts to improve the efficiency of the method were suggested, by addition of wetting or mucolytic agents, but none was found to give consistent success (Allen and Ridley, 1970).

Flotation methods utilize a solution that is denser 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 min for the parasites to float to the surface. Also, small, light parasite cysts, *e.g*. *Cryptosporidium,* can be easily recovered. The disadvantages are that many samples cannot be processed at once and that stool samples used should be fresh, therefore samples can be stored in a refrigerator for only a few days. In addition, not all parasite eggs float, therefore an incomplete parasite picture is obtained. Eggs of *Ascaris lumbricoides*, *Trichuris trichiura*, *Taenia* spp. and *Schistosoma* spp. can be recovered by this method. Flotation methods cannot be used on fatty stools, as the fats, being the least dense constituent of the stool, will float on the surface (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 eggs, but the reagent was too corrosive and expensive for routine use. Saturated saccharose was found to deform eggs rapidly, while zinc sulphate solution did not concentrate *Trichuris* spp. or *Capillaria* spp. very well. Zinc sulphate remains the most common flotation solution for *Ascaris* ova.

The basic principles of methods for detection and enumeration of helmith ova in faeces were adapted to the enumeration of helminth eggs in sludge and compost (Ayres and Mara, 1996). One adapted method for detecting, monitoring and enumerating *Ascaris* ova from wastewater, sludge and compost provides for processing of samples by blending with buffered water containing a surfactant. This test method was considered useful for providing a quantitative indication of the level of *Ascaris* ova contamination of wastewater, sludge and compost. It has also been used in evaluating the effectiveness of treatment (Geenen *et al*., 1999). Other methods have been described in the literature, but technical limitations continue to complicate detection and isolation of helminth ova and other pathogens in sludge and other mixed-media solid samples, *e.g.* sludge, (Dumonetet *et al*., 2001).

The Visser Filter® method relies on separating ova on the basis of size. The apparatus comprises a series of either two or three interlocking sieves of decreasing mesh size. *Ascaris* ova are theoretically retained by the smallest mesh and are counted using light microscopy. This method is not used widely outside South Africa, but within South Africa it is used by a

number of municipal laboratories for solid samples such as sludge, and in the analysis of veterinary samples which are predominantly animal faeces.

Wastewater

The enumeration of intestinal helminth eggs and larvae in wastewater is more complex than in faeces (Ayres and Mara, 1996). This is due to the large variety of human and animal parasite species and free-living species which may be present in wastewater, to the varying size, specific gravity and surface properties of these species, and to their relatively lower concentrations in wastewater than in faeces, sludge or compost. Many methods for the enumeration of helminth eggs 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, while 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 recover only a limited range of species.

For the detection of *Ascaris* ova in wastewater samples, the modified Bailenger method (WHO, 1996), or the method currently implemented by the eThekwini Municipality (Gaspard and Schwartzbrod, 1995) are typically used. These are flotation methods, which depend on the specific gravity of the flotation solution, typically zinc sulphate, to float *Ascaris* ova to the surface, from which they can be recovered and enumerated using standard light microscopy.

The method ultimately adopted by the WHO for recovery of *Ascaris* and other helminth ova from wastewater was a modified Bailenger method (WHO, 1996), which was judged to be the best method overall as it required relatively inexpensive reagents and successfully concentrated the full range of species commonly found in wastewater (Ayres and Mara, 1996). The modified Bailenger method is generally useful, simple and cheap. It reliably recovers the eggs of a range of intestinal nematodes, is reproducible, and has already been widely adopted in many laboratories globally (Ayres and Mara, 1996). One of its advantages is that sample collection and preparation are uncomplicated and utilize only the most basic of laboratory equipment for sample processing. Also, this method entails the use of McMaster slides, which are very efficient in counting of ova. Operator error is reduced compared to other methods, which are long, tiring, and laborious and are more prone to errors. However, weaknesses of this method also exist. Among these are the unknown percentage recovery of eggs of this method and its unsuitability for many of the percolated or treated eggs, including those of *Clonorchis sinensis*, *Diphyllobothrium latum*, *Fasciola hepatica*, and *Schistosoma* spp. (Ayres and Mara, 1996). Some of these eggs float in the zinc sulphate flotation solution but may sink quickly or become distorted, making accurate identification difficult. Also, ether (the original organic extractant recommended for the biphasic extraction) is highly flammable and toxic, but this can be replaced by ethyl acetate for the extraction of parasite eggs from faeces without any loss in efficiency. Ethyl acetate is much safer than ether, less toxic and has a lower boiling point (Ruse *et al.,* 1987; Ayres and Mara, 1996).

Gaspard and Schwartzbrod (1995) investigated, three types of parasitological methods to define an effective method for detection of *Ascaris* eggs in wastewater. These included: the physical methods employing sedimentation or centrifugation; the physical methods using various flotation liquids and the biphasic methods combining hydrophilic and lipophilic

reagents. A comparison of seven quantification techniques relating to helminth eggs in wastewater was undertaken. The best results were displayed by the biphasic technique which included a treatment with antiformine at 8% and ethyl acetate followed by a flotation with zinc sulphate at 55%. The corroboration of performance achieved showed that the yield of the method was significantly independent of the egg concentration and results showed good homogeneity of results. Mean recovery was found to be of the order 74%, thus this method was identified by the authors as the most viable option on both a qualitative and quantitative level for wastewater samples (Gaspard and Schwartzbrod, 1995).

2.4 Efforts to standardise methods across sample types

Various techniques for enumerating helminth ova in water and faecal samples have been published, yet, according to Maya *et al.* (2004) there is still no widely accepted international method for evaluating these parasites in wastewater. At the European level, three detection methods have been described. These include: the US EPA modified method, the Triple Flotation (TF) method and the Norwegian method (Simonart *et al.*, 2003). To date, no standard method exists as none of the methods has demonstrated the capability of combining specificity, efficiency and viability in the detection of helminth eggs. The Norwegian and EPA methods are relatively similar in their detection and enumeration principles. In the Norwegian method, after filtering, the biphasic organic extraction step is followed by flotation using sucrose, whilst in the US EPA method, after the straining, the flotation step is followed by the biphasic step using alcohol/ethanol. The use of a 38 µm sieve in the Norwegian method, however, could allow the loss of eggs such as *Taenia* and *Trichuridae*. 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 method which was most economical, simple and easiest to implement was the US EPA method, followed by the Norwegian method whilst the TF method was found to be very time consuming and expensive (Simonart *et al.*, 2003).

For the determination of the most adequate method for Mexico, four techniques were compared These included the US EPA, Membrane Filter, Leeds I and Faust methods (described fully by Maya *et al.*, 2004). All four techniques encompassed two general steps. The first was to separate, recover and concentrate the helminth ova from the sample sediment. 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 by Maya *et al.* (2004) that the EPA technique was the best since it can be used for samples with both high and low solids content, allows recovery of helminth ova with different specific gravities, and has the lowest total cost. The EPA technique was therefore selected and recommended as the standard technique for quantification of helminth ova in wastewater and reclaimed water by the Mexican government.

In work conducted by Maya, Archer and Hawksworth (publication in preparation) in South Africa since then, the Maya/EPA method was compared to the AMBIC protocol (described below) developed by Hawksworth and Archer as part of this project, and as input to another WRC project concerned with the development of standard methods for analysing sludge. A set of consensus methods was developed, for application to a number of different waste media, including sludge, UD waste, compost and wastewater. South African municipal

laboratory technicians are presently being trained in these consensus methods and they will be published as part of the development of a set of national sludge guidelines.

3 DEVELOPMENT OF AMBIC PROTOCOL FOR DETECTION AND QUANTIFICATION OF *ASCARIS* OVA IN UD WASTE, AND ITS APPLICATION TO EVALUATE PREVALENCE AND DIE-OFF OF *ASCARIS* OVA IN ETHEKWINI

3.1 Development of the AMBIC protocol for detecting *Ascaris* ova in UD waste

The aim of this study was to optimise the protocol for detecting the presence of *Ascaris* ova in UD solid waste, after the one-year standing period. Such a protocol would help indicate whether a one-year standing period is sufficient to render the waste safe for handling during excavation and burial, or potentially for use as a soil conditioner as per EcoSan principles, without contributing to the *Ascaris* load already in the environment.

As described above, the current methods for the isolation and detection of ova in faecal samples utilize the Kato-Katz (WHO, 1993) or formal-ether (Allen and Ridley, 1970) methods. For the detection of *Ascaris* ova in wastewater samples, the modified Bailenger method (WHO, 1996), or the method currently implemented by the eThekwini municipality (Gaspard and Schwartzbrod, 1995) are the most widely used. There are currently no reported methods for the detection of *Ascaris* ova in a mixed faecal/soil sample.

The analysis of UD samples for *Ascaris* ova has proven to be a problem because of their composition. eThekwini Municipality has advocated that users add a small amount of soil to the vault after defecation. This is believed to assist in accelerating the drying of the waste and the amelioration of unpleasant odours. Preliminary studies had indicated that siliceous particles of different sizes were present in any unprocessed UD sample, and these rendered sample preparation and microscopic examination 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 method had been reputed to be rapid and simple for the recovery of helminth egg from excreta.

3.1.1 Materials and methods

Trial phase

Initial UD samples provided by the eThekwini Municipality were tested using a standard zinc sulphate flotation method, with zinc sulphate at a specific gravity (SG) of 1.3 (Faust *et al*., 1975). Samples of UD waste (1 g) were weighed into 15 mL conical polypropylene (Bibby-Sterilyn®) centrifuge tubes. The use of these 15 mL conical test tubes was kept constant throughout the course of the experiment. Deionised water (5 mL) was added to each tube and the conical test tube vortexed. The contents of the conical test tube were pre-treated by sieving through a mesh strainer (570 µm – 760 µm x 443 µm – 538 µm) and collected in a 100 mL polystyrene cup. The contents of the polystyrene cup were then washed back into a clean 15 mL conical test tube, centrifuged for 3 min at 940 x g and the supernatant decanted. Zinc sulphate solution of SG 1.3 was added (12 mL) to the pellet and the conical test tube vortexed for 3 min. The tube was then centrifuged (940 x g) for 3 min. The top 1.5 mL of the

supernatant was removed and examined using a microscope at 100x magnification. This method failed to detect any *Ascaris* ova.

Since it was not clear whether the UD waste sample provided by eThekwini contained *Ascaris* ova, or at what levels, it was decided to seed the sample with a known number of *Ascaris* ova and to evaluate 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 (24.2 cm long), which has been preserved and stored in formalin. The extracted eggs from the worm were diluted in 8.5% saline solution.

Three treatments of 10 replicates each were chosen to establish accurately the number of eggs contained in 60 µL from the stock solution. These were:

1. The ova suspension (stock solution) was mixed well by vortexing, and a 3 mL aliquot was removed. Ten slides were then prepared using successive drops of 60 µL each, and the ova enumerated using a light microscope (100x magnification).

2. The suspension was mixed well and a 3 mL aliquot was 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 then repeated a further nine times to produce ten slides in total. Each slide was then examined using a light microscope and individual ova counted.

3. After mixing the suspension well, a 3 mL aliquot was withdrawn and 60 µL drops were added to five slides. The remaining fluid was returned to the original suspension. This process was repeated. Each slide was then examined under a light microscope and individual ova counted.

Flotation protocols tested:

The four flotation solutions tested were: sodium chloride (SG 1.2), a saturated sucrose solution (SG 1.2) and two zinc sulphate solutions (SG 1.2 and 1.3). The four test samples chosen were as follows: 60 µL drop from the saline ova stock solution; (A), uncontaminated stool (1 g) sample seeded with ova (B); soil mixture (1 g) seeded with ova (C); and unseeded UD waste (1 g) from a low prevalence area (D).

Test samples A - C (as above) were seeded with 60 µL drop of ova suspension.

Test sample D was not seeded. It was necessary to determine if there were any ova in the UD waste sample from the low prevalence area, supplied by the eThekwini Municipality, as the waste was to be seeded at a later stage with ova.

Deionised water (5 mL) was added to a series of 15 mL conical centrifuge tubes (containing test samples A – D) and vortexed for 3 min. Pre-treatment consisted of straining the contents of the 15 mL conical test tubes through a sieve (mesh size 570 µm – 760 µm x 443 µm – 538 µm) into a 100 mL polystyrene cup. The contents of the polystyrene cup were poured back into a clean 15 mL conical test tube, centrifuged for 3 min at 940 x g and the supernatant decanted. Flotation solution (12 mL) was added to the 15 mL conical test tube, vortexed and then centrifuged at 940 x g for 3 min. The top 1.5 mL of the supernatant was removed with a pipette and placed into a clean 15 mL conical test tube. Deionised water was added up to the

14 mL mark. The tube was then vortexed for a minute and further centrifuged (940 x g) for 3 min. The supernatant was decanted and the pellet examined microscopically (magnification 100x) for ova.

The 15 mL conical test tube containing the original flotation solution, after the top 1.5 mL had been removed, was not discarded. The middle supernatant (±8 mL) was removed with a pipette and placed into a clean 15 mL conical test tube. Deionised water was added up to the 14 mL mark. This tube was then vortexed for a minute and further centrifuged (940 x g) for 3 min. The supernatant was poured off and the pellet examined microscopically (magnification 100x) for ova. The original deposit (pellet in the original 15 mL conical test tube) was made up to 3 mL by diluting with distilled water and the ova counted as before. This was to determine the distribution of ova throughout the flotation solution and pellet. This process was repeated for all four flotation solutions for test samples A – D.

Ammonium bicarbonate (AMBIC) protocol:

The AMBIC protocol developed in the PRG laboratory consists of three steps:

1. Sample preparation;
2. Mixing of sample with ammonium bicarbonate (AMBIC) solution; and
3. Recovery with an adjusted zinc sulphate flotation solution.

The steps are outlined in greater detail below.

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

Step two: The sample was mixed with a saturated AMBIC solution (pH 8.6, 22°C). The AMBIC solution was added up to the 14 mL mark on the conical test tube and the tube vortexed for 3 min. An applicator stick was used to dislodge any larger particles from the bottom of the conical test tube during vortexing. A screw-top lid was placed on the conical test tube and it was left to stand for an hour. Thirty minutes into the standing period, the tube was again vortexed for 3 min and manually shaken for a further 2 min. After the full hour had elapsed, the conical test tube was centrifuged (940 x g for 3 min) and the entire supernatant discarded. Deionised water (±14 mL) was added to the pellet, the contents mixed and the tube vortexed for 2 min. An applicator stick was again used to dislodge any larger particles. The tube was then centrifuged (940 x g for 3 min) and the supernatant discarded. This constituted a wash step to remove excess AMBIC solution which would otherwise damage the ova.

Step three: Zinc sulphate (±12 mL) with an adjusted (modified) specific gravity of 1.4, was added to the conical test tube and vortexed for 2 min. An applicator stick was used to dislodge any larger particles from the bottom of the conical test tube. The tube was centrifuged (± 600 x g) for 3 min. The entire supernatant was then poured equally into four clean 15 mL conical test tubes. The pellet (deposit) was retained for a further flotation trial. The four conical test tubes were topped up with deionised water (±12 mL) and centrifuged (± 1850 x g) for a further 3 min. The supernatant discarded from each of the four tubes and the pellet from each was viewed using a light microscope and the ova counted. The original

deposit (pellet) was then subjected to another flotation using the same methodology outlined above and any ova that may have been retained at this step were also counted.

Recovery efficiency of AMBIC protocol for *Ascaris* ova:

The recovery efficiency of the developed AMBIC protocol was tested. This testing was conducted in two stages:

Stage one: Aliquots of 1 g of UD waste were weighed into fifteen 15 mL conical test tubes. Thereafter, 3 mL of distilled water and a 60 µl drop of ova suspension were added to each tube. Tubes were vortexed for 3 min to randomly distribute the ova throughout each sample. This yielded a total of 15 replicates from the original ova suspension together with UD waste. AMBIC solution (as above) was then added to each tube, the modified zinc sulphate flotation performed, the ova enumerated and results recorded (as described above).

Stage two: Five 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 off into five 15 mL conical test tubes, each containing 1 g of UD waste. 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 tubes were then vortexed for 3 min to distribute the ova randomly throughout the UD waste sample. Each of the five 15 mL conical test tubes was subjected to the AMBIC protocol (as described above). The recovered ova were counted and the results recorded. This represented 5 replicate assessments of the ova suspension alone and 5 replicates of ova plus UD waste.

These two procedures represent a quantitative assessment of the AMBIC protocol.

Comparison of the AMBIC protocol with the Visser Filter® method:

Counts were made of 60 µL aliquots of the ova suspension, using 10 separate slides for the process. The individual ova aliquots were then washed off into each of ten 15 mL conical test tubes, each containing 1 g of UD waste. Slides and cover slips were individually inspected to ensure full recovery of ova had taken place. The tubes were vortexed for 3 min to distribute the ova randomly throughout the sample. Tube contents were subjected to selective size filtration using a Visser Filter®. The 100 µm and 35 µm mesh sizes were used. The middle filter (80 µm) was not incorporated as it might have retained some of the ova thus giving an additional, uncontrolled variable in the experiment.

After vigorous filtration using a strong jet of water, the accumulated fluid from each sample was collected in 50 mL conical centrifuge tubes from the tap at the base of the 35 µm filter and centrifuged (± 1358 x g) for 4 min. The supernatant was poured off and the 50 mL conical centrifuge tube was filled with zinc sulphate of SG 1.4. The 50 mL conical centrifuge tube was vortexed for 2 min and an applicator stick was used to dislodge any large particles from the bottom of the tube. The tube was then centrifuged for 3 min (940 x g). The entire supernatant (not just the top 1.5 mL, as before) was then poured into four 15 mL conical test tubes and these were filled with deionised water. The four tubes per sample were vortexed and centrifuged (940 x g) for 3 min. The supernatant was then poured off and the pellet examined for ova using a light microscope. The original deposit (pellet) in the 50 mL conical centrifuge test tube was not discarded. The 50 mL conical centrifuge tube was subjected to a second

zinc sulphate flotation (as outlined above). This was done to attempt to account for any ova which may not have been recovered during the first flotation.

Statistical analysis:

Statistical Package for Social Sciences (SPSS) version 11.0 was used for the statistical analysis of the data. The Kolmogorov-Smirnov test was used to check for normality of the data distribution. Analysis of variance (ANOVA) was used to determine if there were significant differences among treatments. Post Hoc (Tukey) testing was conducted to determine where the differences lay between treatments. Student's t-test was used to determine if there was significant difference between paired treatments. All tests were conducted at the p=0.0.5 level of significance.

3.1.2 Results and Discussion

Trial phase:

Preliminary studies showed that no ova were recovered when zinc sulphate of SG 1.3 was used as a flotation medium on an unseeded UD waste sample (D), supplied by the eThekwini Municipality from an area considered to ha low prevalence of *Ascaris* in the population and hence in UD waste. The decision was therefore taken to seed a UD waste sample from a low *Ascaris* prevalence area, (Mzinyathi), with ova from the uterus of a dissected adult female *Ascaris lumbricoides* worm. Further investigations were conducted to determine whether the choice of flotation solution or pre-treatment steps had an effect on the recovery of *Ascaris* ova. 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, described in materials and methods) were seeded using ova recovered from the female *Ascaris lumbricoides* worm.

To estimate the number of ova per 6 0µL drop of stock solution (ova in 8.5% saline), three sampling protocols were conducted (as described in materials and methods). The inoculation number was established as 1208 (± 58) ova per 60 µL drop of stock suspension. There was no significant difference among the three treatments, A – C (p=0.281). 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.

Flotation test protocols:

The first trial (test sample A) was conducted to determine which of the four flotation solutions would return the best ova recovery of from the inoculated (1208 ± 58) 15 mL conical centrifuge tube.

When the contents of the 1 mL conical test 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 (but not counted) in the mesh strainer. This was taken as further evidence of the inherent adhesiveness of the isolated ova or the result of possible extraneous ovarian tissue causing clumping.

There was no significant difference (p=0.228) among the ova recoveries for the four flotation solutions tested (Figure 3.1), using the protocol outlined in the materials and methods. Zinc

sulphate of SG 1.2 returned the highest recovery rate (68% ± 7.2) and zinc sulphate solution of SG 1.3 the lowest recovery rate of 34% (± 0.79).

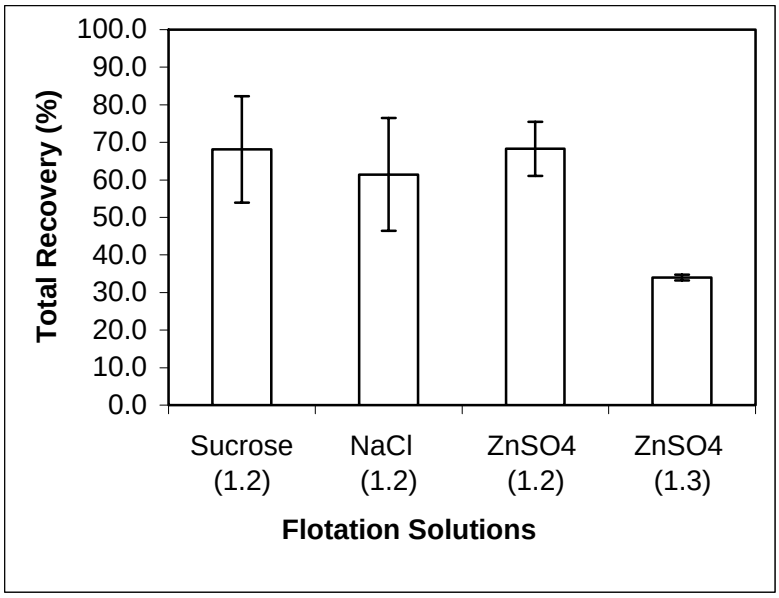

Figure 3.1: Results of combined total recovery (top 1.5 mL, middle supernatant and pellet) of inoculated ova (1208 ± 58) from pure ova test sample (A) for the four flotation solutions tested. Values in brackets represent the SG of the solution and the standard error bars for the four flotation solutions are shown.

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). As a result, a pellet is formed that can be examined microscopically. Therefore, the flotation solution that recovers the most ova from the top 1.5 mL would be optimum.

However, when the top 1.5 mL, middle (± 8 mL) layer and pellet were examined for recovered ova and their percentage contribution to the overall recovery calculated, it was seen that of the 34% (± 0.79) recovery achieved with zinc sulphate of SG 1.3 (lowest recovery), 94% of the recovered ova were located in the top 1.5 mL. No ova were detected in the pellet of this preparation, and 6% recovery was observed for the middle region of the 15 mL conical centrifuge tube. The highest recovery (68% ± 7.2) of the four solutions tested was achieved with zinc sulphate at SG 1.2. However, in this case only 2% of the recovered ova were detected in the top 1.5 mL of the 15 mL conical centrifuge tube. This represents a poor recovery for the upper layer, and similarly with that achieved with sodium chloride of SG 1.2 where only 2.5% of the overall recovery was detected in the top 1.5 mL (Figure 3.2).

When the same protocol (as outlined in materials and methods) was repeated using a stool sample (test sample B) inoculated with ova (1208 ± 58), there was no significant difference ($p=0.776$) among the recovery rates of the four tested flotation solutions (Figure 3.3). Sodium chloride with a SG of 1.2 showed the highest total recovery for ova with 47% (± 0.25), and zinc sulphate of the same SG, returned the lowest recovery with 28% (± 17.6). The total recoveries for all four stool test samples were all lower than 50% (Figure 3.3), although there

was considerable variation between trials. Therefore the stool content appeared to be having a negative effect on the overall recovery.

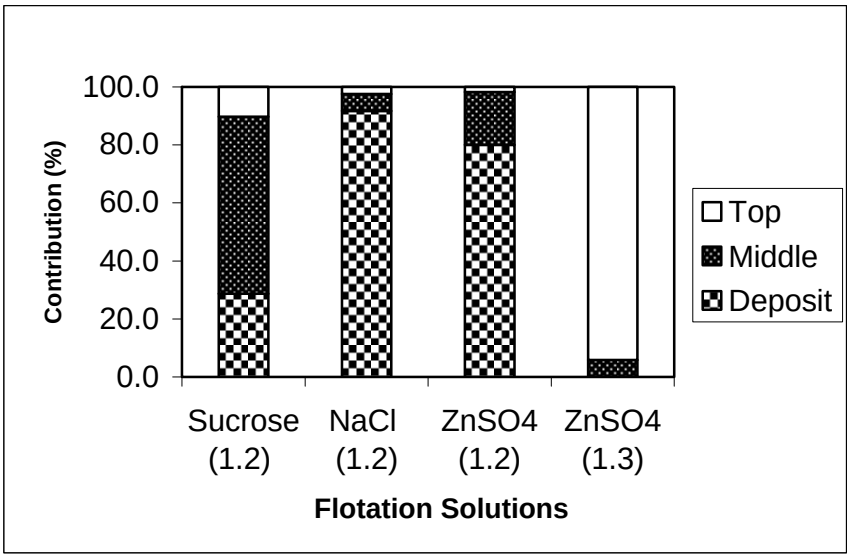

Figure 3.2: Relative contribution of recovered ova from the three regions (top 1.5 mL, middle supernatant and pellet/deposit) in the pure ova test sample (A) from the original recovery for the four flotation solutions tested. Values in brackets represent the SG of the solution.

This decreased recovery may have been the result of the pre-treatment step of sieving through a mesh before the flotation protocol (outlined in materials and methods). It was observed that a large proportion of the stool remained behind after sieving. As the sample was vortexed before sieving, the ova would nonetheless have been distributed randomly throughout the sample. Therefore any material retained by the sieve may have contained ova that were not counted. Enumerating ova in the pellet was very time consuming and supported the original statement that any protocol developed should rely on recovery in the top 1.5 mL of the supernatant of the flotation solution after centrifugation. It was concluded that the saturated sucrose of SG 1.2 was difficult to work with. The use of sucrose led to discoloured samples, which made microscopic examinations difficult. The excessively sticky nature of the flotation medium also made processing samples difficult.

Sodium chloride yielded the highest recovery rate at 47% (± 0.25), but the high recovery rate, and low variability observed, was found to be misleading. When all three layers (top 1.5 mL, middle supernatant and deposit) were examined, 100% of the 47% (± 0.25) ova recovered were located in the pellet (Figure 3.3). These results are in striking contrast to the recoveries observed for the pure egg test treatments (Figure 3.2), where ova were detected in all the zones (top 1.5 mL, middle supernatant and deposit) of the 15 mL conical centrifuge test tube (Figure 3.2). Examining the pellets for all the stool controls was time consuming.

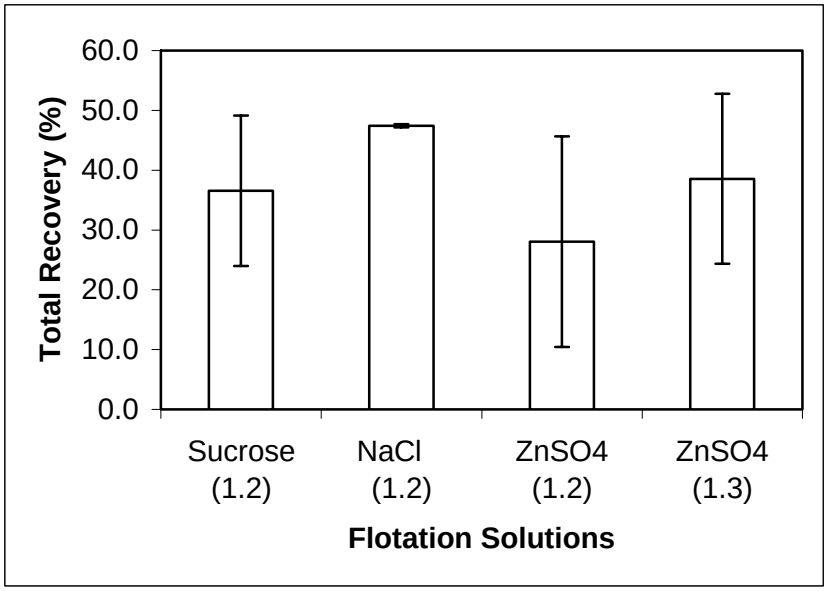

Figure 3.3: Results of combined total recovery (top 1.5 mL, middle supernatant and pellet) of inoculated ova (1208 ± 58) from the stool test samples (B) for the four flotation solutions tested. Values in brackets represent the SG of the solution and error bars represent the standard error for the four flotation solutions are shown.

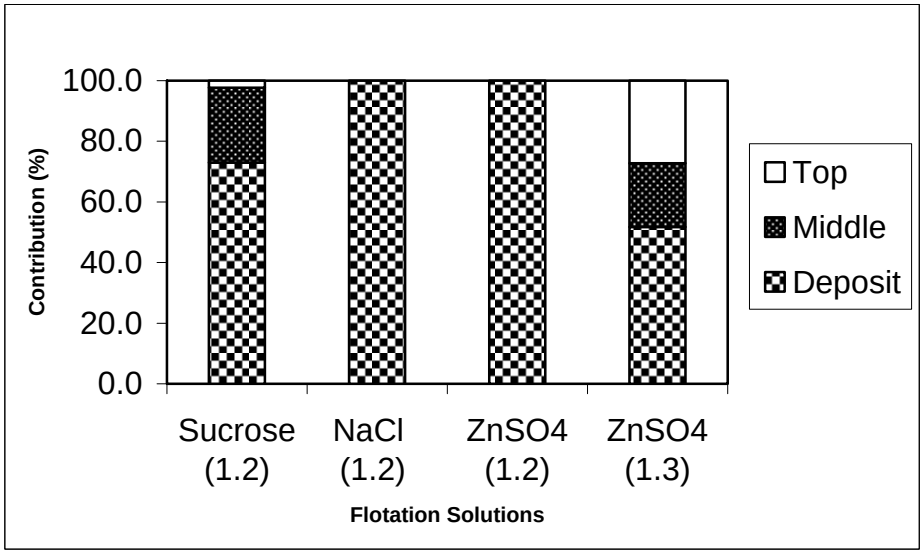

Figure 3.4: Relative contribution of recovered ova from the three regions (top 1.5 mL, middle supernatant and deposit/pellet) in the stool test sample (B) from the original recovery rate for the four flotation solutions tested. Values in brackets represent the SG of the solution.

Of the total ova recovery of 39% (± 14.2) achieved with the zinc sulphate solution of SG 1.3 (Figure 3.3), 27% of the ova were detected in the top 1.5 mL (Figure 3.4). This represented the highest recovery in the top 1.5 mL for the four flotation solutions tested, and contrasted sharply with results for zinc sulphate of SG 1.2, where all the ova detected were located in the pellet (Figure 3.4). The top 1.5 mL of the saturated sucrose solution of SG 1.2 detected 2% of the total ova recovery of 37% (± 12.6) (Figure 3.3). This was the second best recovery for the top 1.5 mL of the flotation medium. It was observed that the recovery of ova in both the top

1.5 mL, and the middle portion of the sucrose flotation were due to an apparent combination of the stickiness of the solution and some degree of binding of floating organic material rather than purely a density phenomenon.

Figure 3.5 represents the recovery efficiencies of ova from seeded soil samples (C, as outlined in materials and methods), using the four flotation solutions. The recoveries were significantly different (p=0.013) among the flotation solutions. Sodium chloride of SG 1.2 recovered 89% (± 14.9) of the inoculated ova from the soil sample, whereas zinc sulphate of SG 1.3 solution achieved a recovery rate of 27% (± 0.5). Sucrose of SG 1.2 recorded the lowest total recovery with 20% (± 8.1).

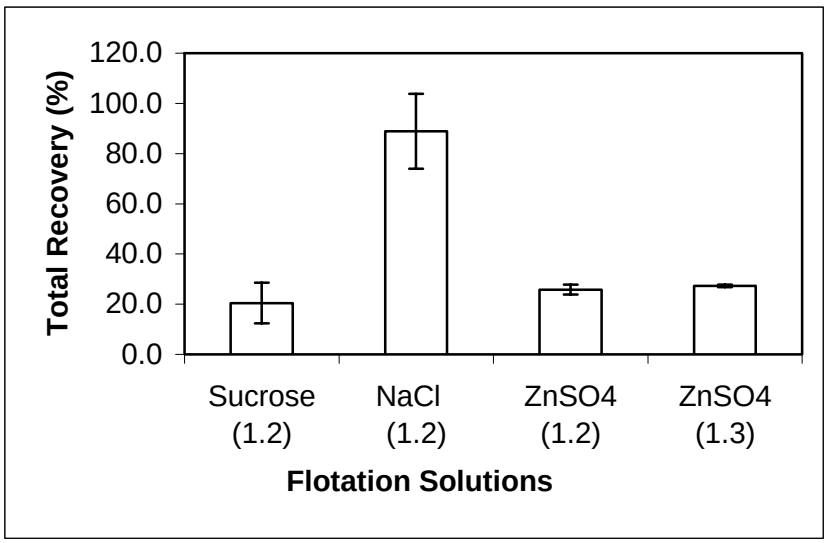

Figure 3.5: Results of combined total recovery (top 1. 5 mL, middle supernatant and pellet) of inoculated ova (1208 ± 58) from the soil test samples (C) for the four flotation solutions tested. Values in brackets represent the SG of the solution and the standard error bars for the four flotation solutions are shown.

Multiple comparison testing (Table 3.1) showed a significant difference ($p<0.05$) between sodium chloride of SG 1.2 and the other three flotation test solutions. There was no significant difference ($p>0.05$) between sucrose and either of the two zinc sulphate test solutions. Recoveries of the two zinc sulphate test solutions were nearly identical with $p=0.999$.

It is clear from Figure 3.6 that the presence of soil masks the presence of ova in some way, as all four of the flotation solutions tested did not recover any ova in either the top 1.5 mL or middle supernatant of the 15 mL conical centrifuge tubes when examined. From this it was concluded that perhaps some electrostatic forces or extracellular polymers associated with the egg walls resulted in the binding of the ova to the soil. When examining the deposit, preparation of microscope slides to count the ova became prohibitively time consuming. UD samples are a mixture of both faecal and soil material, both of which returned poor recoveries for all four solutions tested (Figures 3.4 and 3.6) using flotation protocols. Because of the poor recoveries, an attempt was made to modify the extraction and flotation protocol using ammonium bicarbonate (AMBIC) and a zinc sulphate solution with a SG of 1.4. AMBIC was chosen because it is used in soil extraction procedures to displace negatively charged ions such as phosphates from soil particles.

Table 3.1: Results of multiple comparisons testing for the four flotation solutions tested with soil test samples (C). Bold p values show significance at the 0.05 level.

Flotation Solution	Comparison Solution	p Value
Sucrose (1.2)	NaCl	**0.016**
	ZnSO4 (1.2)	0.967
	ZnSO4 (1.3)	0.936
NaCl (1.2)	Sucrose	**0.016**
	ZnSO4 (1.2)	**0.022**
	ZnSO4 (1.3)	**0.024**
ZnSO4 (1.2)	Sucrose	0.967
	NaCl	**0.022**
	ZnSO4 (1.3)	0.999
ZnSO4 (1.3)	Sucrose	0.936
	NaCl	**0.024**
	ZnSO4 (1.2)	0.999

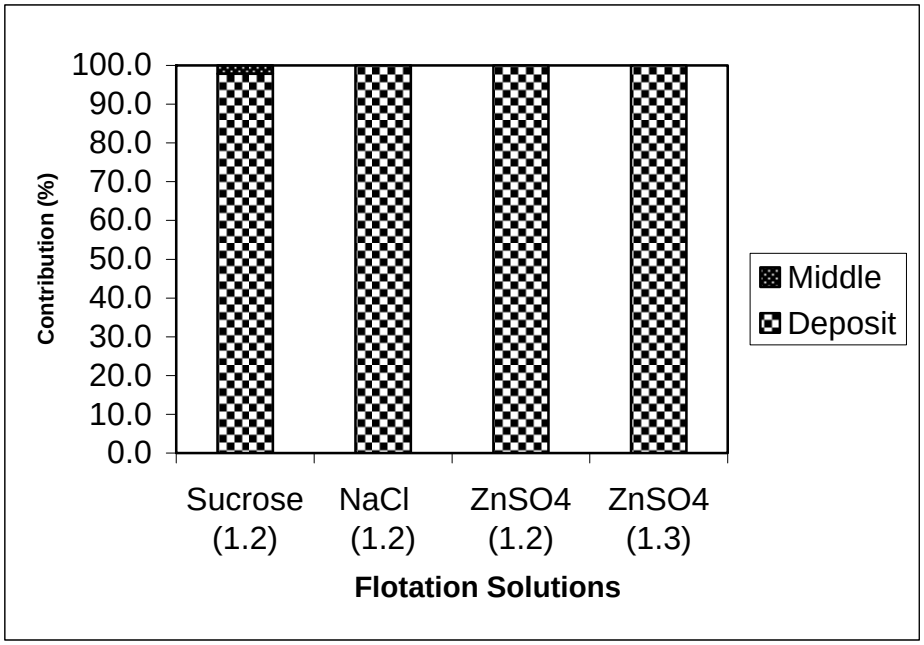

Figure 3.6: Relative contribution of recovered ova from the three regions (top 1.5 mL, middle supernatant and pellet/deposit) in the soil test sample (C) from the total recovery rate for the four flotation test solutions. Values in brackets represent the SG of the solution.

Extraction technique using AMBIC protocol:

Preliminary investigations with a saturated ammonium bicarbonate (AMBIC) solution showed that it was effective in a model system, where a soil test sample was seeded with ova from the stock solution. This is thought to be due to the hydroxyl and bicarbonate anions binding to cation exchange sites located on the surface of soil particles (Hughes, pers. comm., 2005). These anions may displace the ova from the surface of soil particles and prevent the 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. 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. Trivalent phosphate anions are repeatedly found in the uterine (outermost) layer of the egg wall, which consists of uneven deposits of mucopolysaccharides (Crompton and Pawlowski, 1985). Since AMBIC is used to extract phosphates from soil, it is unsurprising that it would displace ova containing phosphate groups in their outer layers from soil.

In most flotation protocols documented in diagnostic parasitology, samples are typically pre-sieved to remove large particles and debris. Pre-sieving was not included in the protocol developed here, as it was thought that ova bound to soil or any faecal debris could possibly be retained and therefore not enumerated. This resulted in large amounts of brown organic debris in the upper layer (top 1.5 mL) along with the ova. This complicated the preparation of slides for the enumeration step.

Ova were recovered from seeded samples in stage one (outlined materials and methods) of the AMBIC protocol (Figure 3.7). Although the lowest recorded recovery was 53% (± 12.6) and the highest 89% (± 5.8), there were no significant differences among recoveries from the five samples (p=0.366).

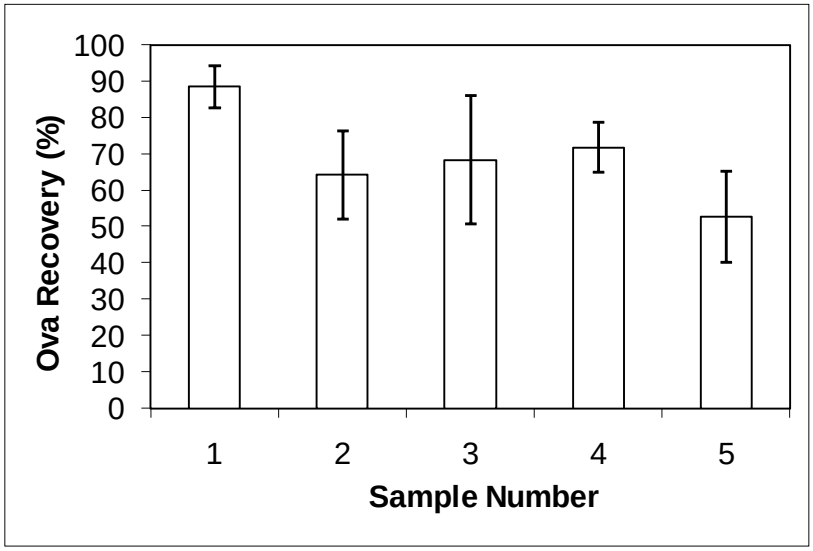

Figure 3.7: Mean recovery of ova, using the AMBIC protocol (top 1.5 mL and middle supernatant), using zinc sulphate of SG 1.4 as the flotation solution. The 1g samples of UD waste were each inoculated with 1208 (±58) Ascaris ova. Standard error bars are shown.

The results obtained in stage two (previously described materials and methods) of the AMBIC protocol testing are shown in Table 3.8. The recoveries were not significantly different

(p>0.05). Comparison of recoveries between stage one and two of the AMBIC protocol (using the Student's t-test) showed no significant difference (p=0.168). The range in recoveries in Table 3.8 was 7%, compared to the 36% in Figure 3.7. Therefore stage two (Table 3.8) represents a quantitative assessment of the new AMBIC protocol. The AMBIC protocol is capable of yielding recoveries above 70%. Unreported work done earlier in the study achieved, at best, only 5% recovery rates with other methods from similar samples.

Table 3.2: Quantitative recovery from stage two (top 1.5 mL and middle supernatant) of the AMBIC protocol, using 1 g samples of UD waste inoculated with a known number of *Ascaris* ova. The mean and standard error are shown.

Sample Number	Inoculated Ova	Recovered Ova	Recovery %
1	1064	868	82
2	1239	987	80
3	837	623	74
4	1176	878	75
5	1271	967	76
		Mean (%)	77
		Standard error	1

An advantage of the new AMBIC protocol over other described methods is that it floats and recovers not just *Ascaris* ova, but also many other organisms of interest from a health-related perspective, such as fungal spores and parasitic eggs of *Trichuris trichuria* and *Taenia* spp. (Figure 3.8).

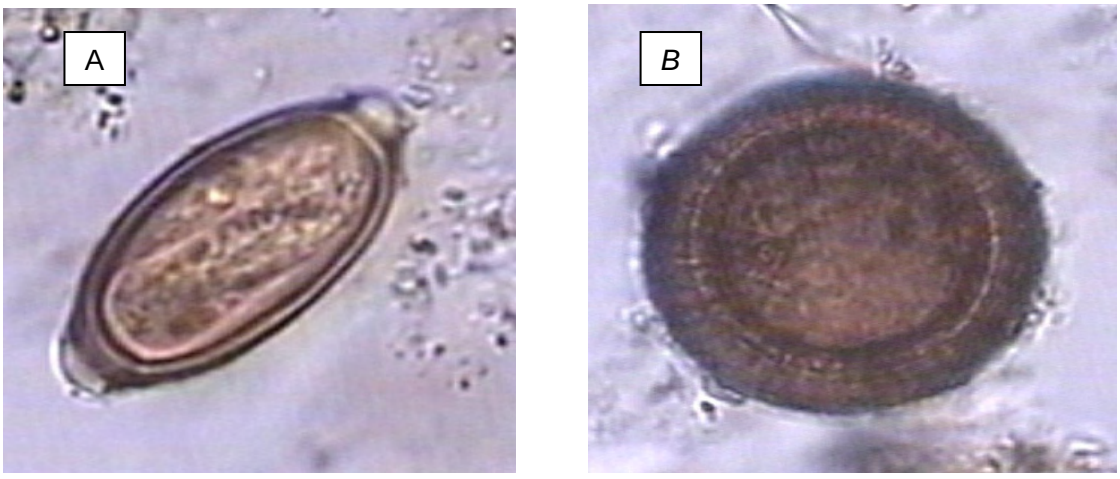

Figure 3.8: *Trichuris trichuria* (A) and *Taenia sp.* (B) ova recovered using the AMBIC protocol.

Comparison with Visser Filter® method:

Finally, the AMBIC protocol was compared with the Visser Filter® method (outlined in materials and methods). Table 3.3 gives the recovery of ova using the Visser Filter® method. The highest recovery recorded was 73%, and the lowest was 30%.

There was no significant difference ($p>0.05$) between the recoveries among samples using the Visser Filter® method. However, there was a significant difference between the recoveries using the Visser Filter® method and the newly developed AMBIC protocol ($p=0.010$).

Table 3.3: Ova recovery, using the Visser Filter® method, from 1 g samples of UD waste inoculated with a known number of Ascaris ova. The mean and standard error have been included.

Sample Number	Inoculated Ova	Recovered Ova	Recovery %
1	1083	788	73
2	1278	608	48
3	1084	850	78
4	1092	567	52
5	1077	326	30
6	897	589	66
7	1314	808	62
8	1188	547	46
9	1141	758	66
10	1488	757	51
		Mean (%)	57
		Standard error	5

Although the Visser Filter® yielded a lower overall recovery compared to the AMBIC protocol, the samples were nonetheless easier to prepare and examine microscopically. This was attributed to the absence of both organic material and soil particles. In addition, minimal clumping of ova was observed using the Visser Filter®, whereas clumping was observed using the AMBIC protocol. The presence or absence of clumping makes a significant difference to the overall recovery of ova. This is because clumps can contain anywhere from 20 to 150 ova. *Taenia sp.* and *Trichuris* ova were not enumerated as frequently using the Visser Filter® method as with the AMBIC protocol.

Future studies will incorporate pre-treating the UD samples with a saturated AMBIC solution before sieving in the Visser Filter®. This may facilitate releasing ova bound to soil particles. At present, it is assumed that ova not recovered are being retained by soil particles. Future studies will be done to validate this statement.

In conclusion, the AMBIC protocol developed in this project shows considerable improvement in recovering *Ascaris* ova from UD waste samples, relative to other documented

methods for *Ascaris* recovery from different media. With future work, it is hoped that further development and refinement of the AMBIC protocol will occur, possibly involving filtration steps and using a zinc sulphate flotation only once in the protocol.

3.2 Prevalence and Die-off of *Ascaris* ova in UD waste in eThekwini

The current minimum standing period of the filled UD vault in the eThekwini implementation of UD toilets is expected to be one year, although shorter standing periods have been reported by users. In order to ascertain whether this one year period renders UD waste safe for handling during removal from the vault, reliable estimates of the egg load in the waste are needed after the one-year standing period.

This section of the report addresses viability and die-off of *Ascaris* ova from UD solid waste. The results of these experiments will support the development of guidelines for management of UD toilets by eThekwini Municipality.

3.2.1 Materials and Methods

Study area:

UD solid waste was sampled from five different UD toilets situated in the Zwelibomvu township near Durban in KwaZulu-Natal.

Sampling procedure:

Appropriate safety and containment measures were taken during the handling of UD waste. The waste was cored out from the back opening of the vaults, unless toilet vaults which were situated such that the rear opening was difficult to reach, in which case vaults were sampled through the toilet pedestal. Samples were taken from the bottom, middle and top of the waste heap in the vault, representing differing ages of waste (from oldest at the bottom to most recent at the top).

Samples were screened for *Ascaris* ova using the AMBIC protocol developed by Hawksworth *et al*. (2005) and described in detail in the preceding section. Of the five different UD toilets sampled, only two were found to contain *Ascaris*-infected waste. The waste from the toilet with the highest *Ascaris* egg load was used as the source of material for further experimentation and refinement of the AMBIC protocol.

The AMBIC protocol encompasses three steps: 1) sample preparation, 2) mixing with ammonium bicarbonate (AMBIC) solution (pH 8.6 and 22°C) and 3) recovery with an adjusted zinc sulphate flotation solution (as described above).

Sample preparation:

A 1 g sample of well-mixed UD waste was placed into each of 15 mL conical test tube. A total of 270 replicates were set up for the duration of the experiment, allowing for 15 tubes to be analyzed every fifth day. Each batch of 15 tubes was labelled accordingly. Of the 15 tubes, 5 contained waste from the top layer, 5 from the middle layer and 5 from bottom layer. In addition, 1 mL distilled water was added to each tube to provide moisture in the tube. The 270 tubes were then placed into an incubator at 29.5 °C for a period of 90 days. A glass beaker with distilled water was placed inside the incubator, as a further source of moisture. Thus this

scenario represented a “worst case” scenario in terms of drying (100% relative humidity in the vault).

Every fifth day, 15 replicate tubes were removed and processed according to the AMBIC protocol for enumeration of *Ascaris* ova. Ova were also assessed for viability

Sample extraction using AMBIC:

The prepared samples were made up to 14 mL with saturated[1] AMBIC solution and vortexed for 3 min. This was done by adding AMBIC a little at a time to the tube and vortexing each time, thereby allowing the AMBIC to mix thoroughly with the waste sample. An applicator stick was used to help dislodge larger particles from the bottom of the test tube during vortexing. The tubes were then left to stand for an hour. After each 5 min into the standing period, the tubes were again vortexed for 1 minute and manually shaken for a further 1 min. After an hour had elapsed, the tubes were centrifuged (±650 x g for 3 min) and the entire supernatant discarded. Deionised water (± 14 mL) was added to each tube and the contents mixed and vortexed for 2 min, as before. The tubes were then centrifuged again (±650 x g for 3 min) and the supernatant discarded. This was done as a wash step for removal of excess AMBIC solution.

Zinc sulphate flotation:

Approximately 12 mL zinc sulphate solution with an adjusted specific gravity of exactly 1.3 was added to the tubes and the tubes vortexed for 2 min. The tubes were then centrifuged (±416 x g) for 3 min and the supernatant from each tube was decanted equally into four clean 15 mL conical test tubes.

These four test tubes (four tubes originating from each of the original 15 tubes) were then topped up (±12 mL) with deionised water and centrifuged (±936 x g) for 3 min. This centrifugation step was repeated to ensure thorough washing out of the zinc sulphate. Thereafter the supernatant layers of the four tubes were combined into one tube. This was done for all 15 replicates. The tubes were then centrifuged (±936 x g) for 2 min.

The pellet from each tube was viewed under a light microscope and the ova counted. Counts of *Ascaris* ova present were done within 48 h, in order to allow for standardisation of the developmental stage and condition of the ova, as these are liable to change with time. Counts were categorized[2] according to the numbers of undeveloped, developed and motile, developed and immotile and dead/damaged ova. In addition, ova were classified according to the condition of the viable ova, *i.e*. good or necrotic, as some of the ova that did become fully developed, soon after began to die. The presence of ova of other parasites was also noted.

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[1] Hawksworth *et al*. (2005) did not stipulating the exact quantity of AMBIC required to yield a saturated solution. In this study, the amount of ammonium bicarbonate required was calculated from the solubility factor. This gave the saturation quantity of ammonium bicarbonate as 11.9 g. This amount added to 100 mL distilled water at pH 7 87 was the concentration of AMBIC used for the experiment.

[2] The ova in 1, 2 and 3 were classified as viable whilst only those in 2 and 3 were classified as potentially infective. All the ova in 4 were classified as non-viable.

Light microscopy was used to count ova (100 x magnification for counts and 400 x magnification for determining viability) whereas phase contrast microscopy (500 x) was used to obtain detailed images of the ova at different developmental stages as categorized in Table 3.4.

Statistical Analysis:

All data were initially entered in Microsoft Excel where graphs were produced and standard deviations and standard errors of the mean were determined. Statistical analysis was performed the Statistical Package for Social Sciences (SPSS) version 11.0. Data were tested for normality of distribution using the Kolmogorov-Smirnov test. Significance of differences was assessed using the Student's t-test (two samples compared) or one-way ANOVA (more than two samples compared).

3.2.2 Results

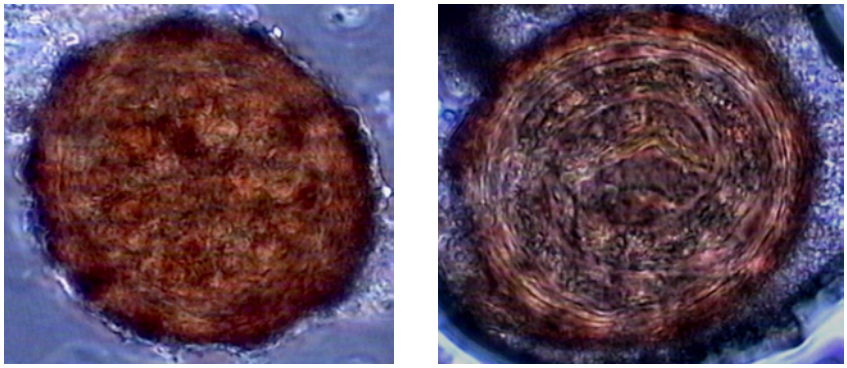
Observations of ova viability by light microscopy:

Over the 90 day period, it was found that there were a relatively higher numbers of non-viable *Ascaris* ova (63% dead, 17% necrotic at 90 days) than viable ova (6% undeveloped, 12% viable immotile and 2% viable motile at 90 days) (Table 3.4). A very small percentage (~1%) of infertile *Ascaris* ova and hatched ova was detected. Ova counts for *Ascaris*, *Trichuris* and *Taenia spp.* were found to increase initially and then decrease with time. A far greater number of *Ascaris* ova were detected than those of *Trichuris and Taenia spp.*. Typical appearances of viable and non-viable *Ascaris* ova, and of ova of other helminth species are shown in Fig. 3.9 to 3.11. In addition, spores, larval worms, plant tissue and one hookworm egg were observed during counts.

Table 3.4: Counts of *Ascaris* ova and ova of other helminths, and assessment of viability of *Ascaris* ova, over the 90 day experimental period

Time	Under developed Ascaris ova	N	Developed *Ascaris* ova (non-motile lava) good	N	Developed *Ascaris* ova (non-motile lava) necrotic	N	Developed *Ascaris* ova (motile lava)	N	Developed *Ascaris* ova	N	*Taenia* ova	N	*Trichuris* ova	N	Comments
5	27-40	15	27-38	15	0	15	8-16	15	20-26	15	0-1	15	1-2	15	3% Infertile *Ascaris* Ova 50% undeveloped *Ascaris* Ova but maturing 7% viable Ova hatched
10	51-69	15	58-73	15	0	15	14-25	15	49-64	15	0-1	15	1-3	15	4% Infertile *Ascaris* Ova
15	15-27	15	51-75	15	0	15	5-9	15	27-40	15	0	15	0-1	15	3% Infertile *Ascaris* Ova
20	13-24	15	34-59	15	31-70	15	2-6	15	102-173	15	0-1	15	2-2	15	-
25	10-12	15	50-66	15	67-104	15	10-16	15	103-129	15	1-3	15	3-4	15	2% Infertile *Ascaris* Ova 1% viable Ova hatched
30	6-8	15	22-31	15	47-64	15	7-8	15	80-101	15	1-2	15	3-4	15	1% Infertile *Ascaris* Ova 5% viable Ova hatched
35	4-8	15	13-15	15	37-48	15	5-7	15	115-159	15	2-4	15	2-3	15	1% Infertile *Ascaris* Ova 6% viable Ova hatched
40	8-10	15	18-23	15	37-59	15	5-6	15	128-188	15	0-1	15	2-3	15	2% Infertile *Ascaris* Ova 4% viable Ova hatched
45	6-9	15	17-23	15	40-70	15	1-2	15	159-208	15	1-1	15	2-3	15	2% Infertile *Ascaris* Ova 2% viable Ova hatched
50	7-12	15	17-21	15	33-47	15	2-3	15	146-209	15	0-1	15	3-4	15	1% Infertile *Ascaris* Ova 6% viable Ova hatched
55	7-12	15	15-22	15	26-40	15	3-5	15	196-247	15	1-2	15	2-3	15	1% Infertile *Ascaris* Ova 2% viable Ova hatched
60	3-6	15	15-28	15	25-40	15	3-7	15	105-352	15	1-1	15	2-3	15	1% Infertile *Ascaris* Ova 2% viable Ova hatched
65	6-10	15	20-29	15	46-70	15	2-3	15	188-264	15	0-1	15	3-4	15	1% viable Ova hatched
70	6-10	15	22-31	15	45-67	15	3-5	15	169-248	15	0	15	2-3	15	1% Infertile *Ascaris* Ova 2% viable Ova hatched
75	4-8	15	15-21	15	42-66	15	3-4	15	161-215	15	0-1	15	2-3	15	2% Infertile *Ascaris* Ova 4% viable Ova hatched, 1 hookworm egg
80	5-8	15	18-25	15	43-52	15	2-3	15	199-238	15	0-1	15	2-2	15	2% Infertile *Ascaris* Ova 4% viable Ova hatched
85	2-3	15	11-16	15	33-49	15	1-2	15	148-178	15	0	15	1-2	15	1% viable Ova hatched
90	0	15	1	15	2-4	15	0	15	14-19	15	0	15	0	15	10% Infertile *Ascaris* Ova 10% viable ova hatched

Values were reported as ova counts per 1 g UD waste. Numbers are reported as Average ± Standard Error of Mean, N = number of replicates tubes.



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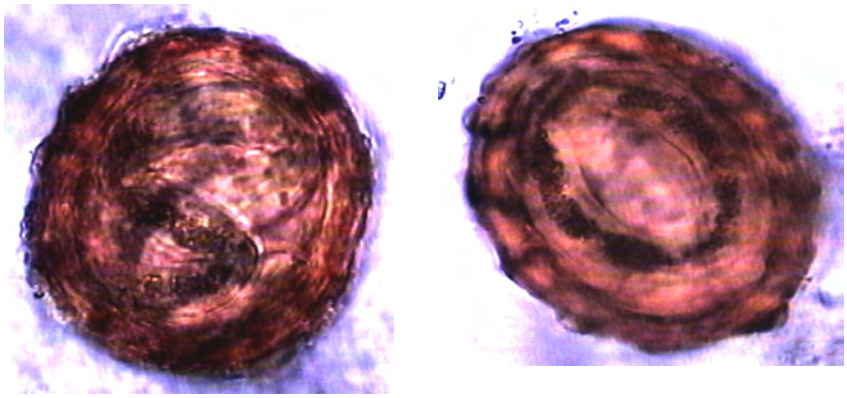

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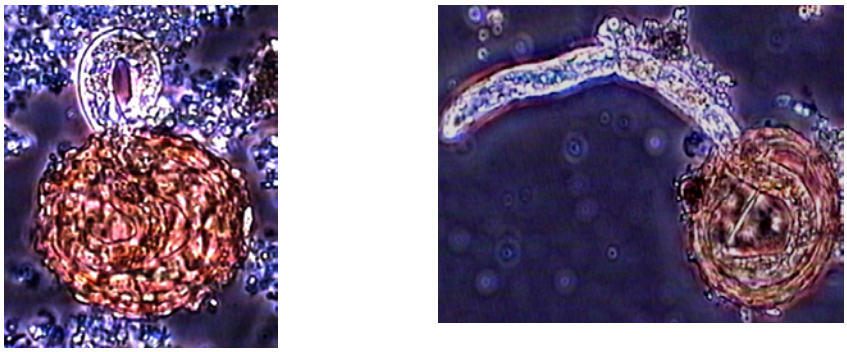
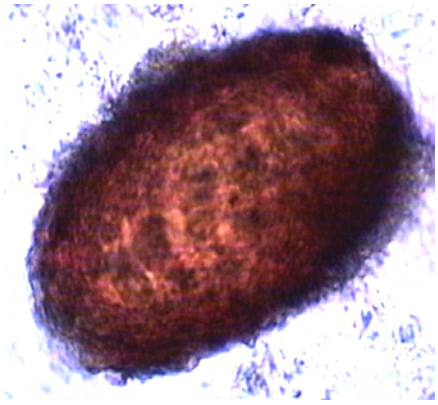


(d) (e)

Figure 3.9: **Viable *Ascaris* ova in UD solid waste: (a) Undeveloped egg (100 x, magnification, phase contrast microscopy, PCM); (b) Developed egg, immotile larvae – good condition (100 x magnification, PCM); (c) and (d) Developed eggs, motile larvae (100 x magnification, PCM) and (e) and (f) Developed eggs (motile larvae – hatching) (50 x magnification, PCM)**



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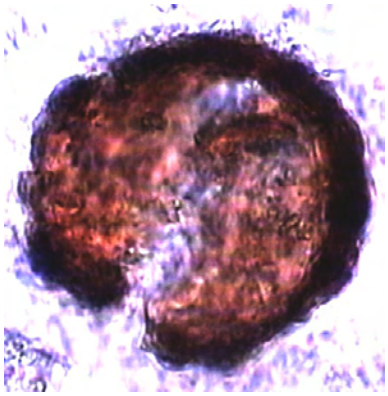

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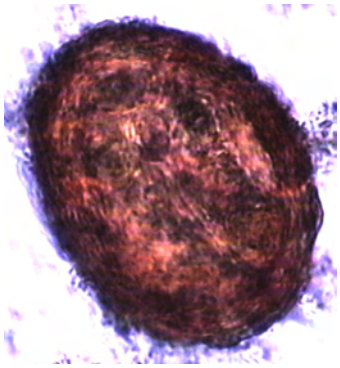
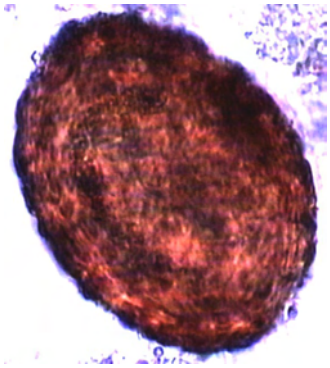


(c)



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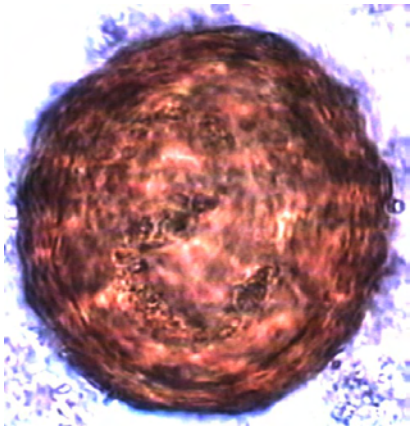

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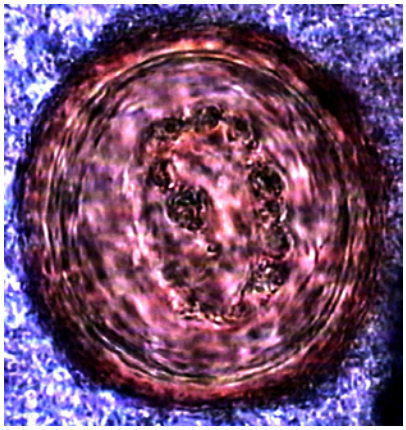
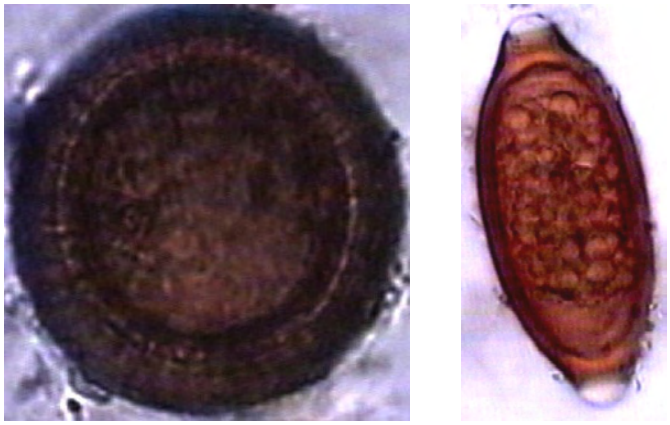


(f)

Figure 3.10: Non-viable *Ascaris* ova in UD solid waste: (a) Infertile egg (100 x magnification, phase contrast microscopy, PCM) (b) Damaged egg (100 x magnification, PCM); (c, d) Dead eggs (100 x magnification, PCM); (e, f) Developed egg, immotile larvae – necrotic (100 x magnification, PCM)



(a) (b)

Figure 3.11: (a) *Taenia spp.* ova (100 x) and (b) *Trichuris spp.* ova (100 x) detected in UD solid waste.

Recovery of *Ascaris* ova:

The total number of *Ascaris* eggs recovered varied widely with time (Fig. 3.12). The lowest recoveries were recorded in the first three experimental runs and probably represent the time required for the experimenter to become fully acquainted with the technique. The wide scatter in results thereafter is consistent with other reports (see above) of the inherent heterogeneity in the composition of UD waste, even from the same vault and the same sub-sample from that vault.

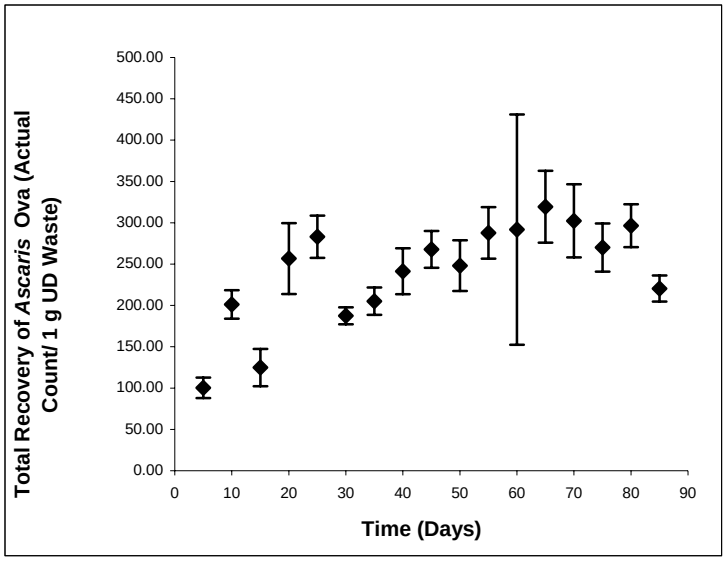


Figure 3.12: Average total percentage recovery of *Ascaris* ova over time from UD waste samples incubated at 29.5˚C and 100% relative humidity

The heterogeneity of individual samples is further supported by the variability observed across replicate tubes. Results for each tube were averaged across sampling times. Had *Ascaris* counts had been relatively homogeneous within each sample, the means across

replicate tubes would have been similar, at the least within each set of 5 tubes from each UD layer. Instead, statistically different ($p<0.05$) mean counts were recorded for replicate tubes (Fig. 3.13) The middle five tubes, corresponding to the middle layer of the UD heap, showed the greatest variability.

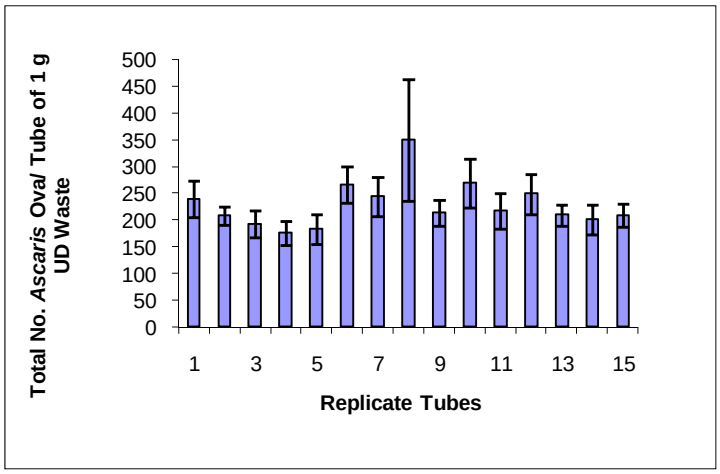


Figure 3.13: Average number of *Ascaris* ova detected in each replicate tube in UD waste samples incubated at 29.5˚C and 100% relative humidity.

Despite inherent variability in the data. incidence of viable *Ascaris* was found to decrease significantly with time over the experimental period. A sharp decrease in viability was seen at day 20. Thereafter, the decline slowed, but continued throughout the 90-day experimental period (Fig. 3.14). The decline in percent viable ova over the experimental period was statistically significant ($p<0.05$).

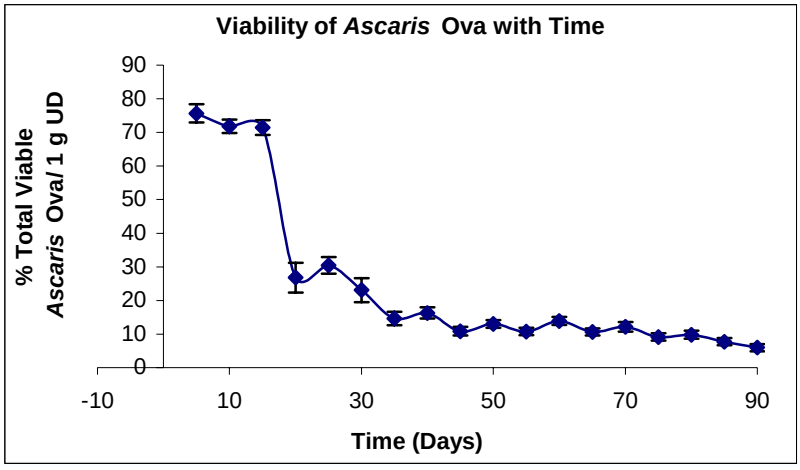


Figure 3.14: Total percentage recovery of viable *Ascaris* ova with time in UD waste samples incubated at 29.5˚C and 100% relative humidity.

Viable immotile *Ascaris* ova were found to make up the highest proportion of viable ova, while viable undeveloped ova and viable motile ova followed respectively. The percentage of viable motile *Ascaris* ova detected was found to be quite low. The percentage of potentially infective *Ascaris* ova (viable immotile and viable motile ova) was found to remain significantly higher

(p<0.05) than the proportion unlikely to be infective, although the total percentage of viable ova showed a marked decrease overall, with the sharpest decline occurring in the first 30 days (Fig. 3.15)

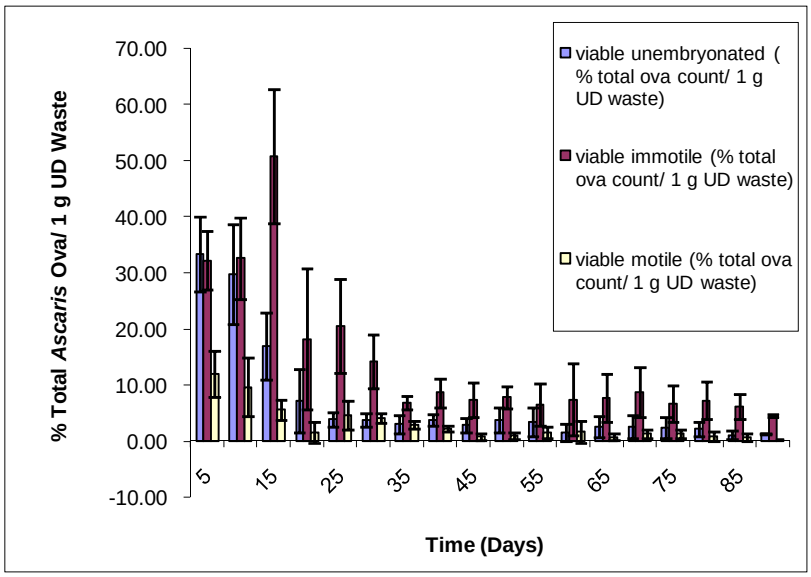


Figure 3.15: Comparison of the different categories of the total viable *Ascaris* ova with time in UD waste samples incubated at 29.5˚C and 100% relative humidity

At the initial stages of the experiment (days 5–15), a very high percentage of viable immotile *Ascaris* ova in good condition was seen, whilst no viable necrotic ova were detected. However, from day 20 onwards an approximately constant percentage of necrotic *Ascaris* ova was noted, while the number of ova appearing to be in good condition decreased markedly after the first 30 days (Fig. 3.16).

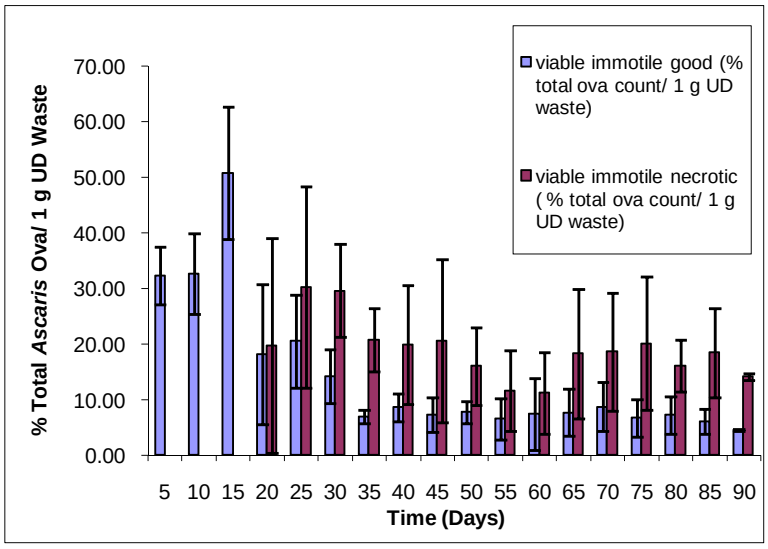


Figure 3.16: Total percentage of viable immotile *Ascaris* ova, characterised by good condition *vs* necrotic condition, with time in UD waste samples incubated at 29.5˚C and 100% relative humidity

The evidence presented in Fig. 3.15 and 3.16, taken together, suggest a declining trend in infective potential of the already decreasing number of viable *Ascaris* ova remaining after 20-30 days (as suggested by Fig 3.14).

The total contribution of ova with low to no infective potential to the number of *Ascaris* ova recovered can be seen in Fig 3.17. Up to day 15 of the experiment, infective *Ascaris* ova of various developmental stages made up the greatest proportion of recovered ova (around 70%). From day 20 onwards, however, the sum of non-viable and necrotic viable ova rapidly approached 100% of total ova recovered. This supports the data presented in Fig. 3.14.

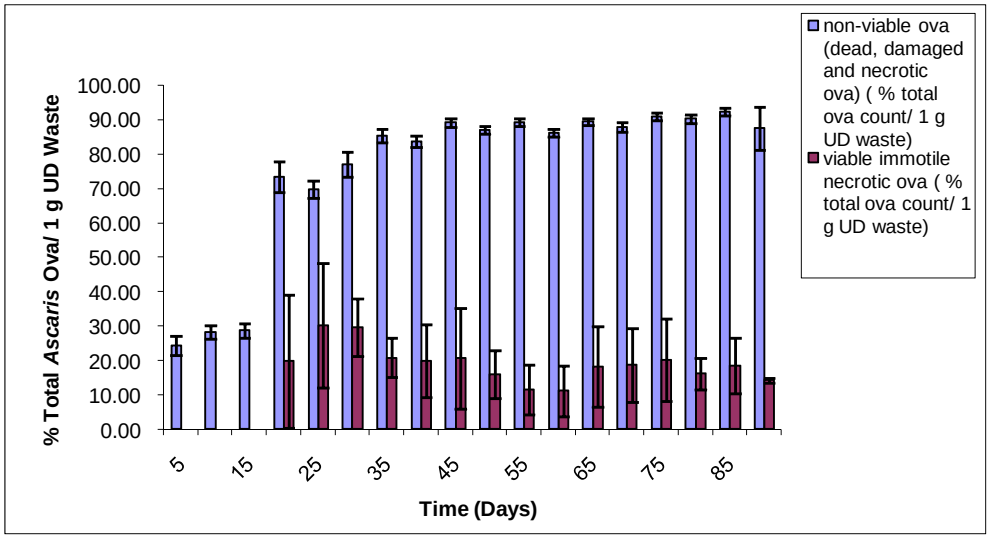


Figure 3.17: Total percentage of non-viable *Ascaris* ova and viable immotile (necrotic) ova over time in UD waste samples incubated at 29.5˚C and 100% relative humidity

The total number of *Ascaris* ova (viable and non-viable), recovered from each of the bottom, middle and top layers of the UD waste heap in the vault is show and averaged over the experimental period, is shown in Fig. 3.18. There was little difference among the layers, but counts were slightly higher in the middle layer.

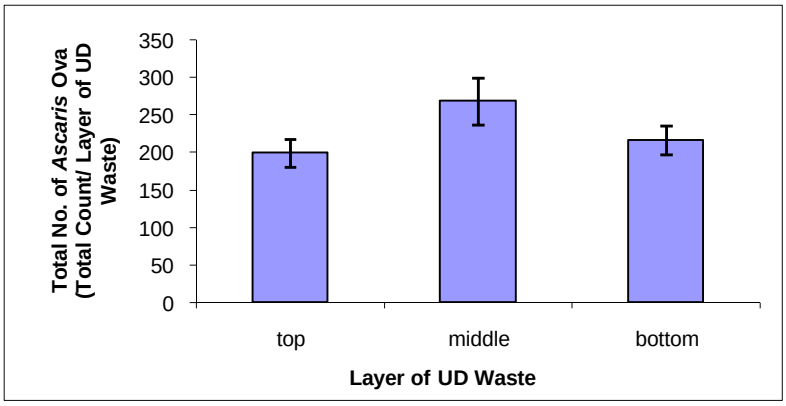


Figure 3.18: Total number of *Ascaris* ova recovered from different layers of the UD waste heap in UD waste samples incubated at 29.5˚C and 100% relative humidity for 90 days.

When viable ova were differentiated from non-viable ova, the mean counts of each were similar across the three layers, but the percentage of non-viable ova was significantly higher than the percentage of viable ova in each layer (Fig. 3.19, $p<0.05$).

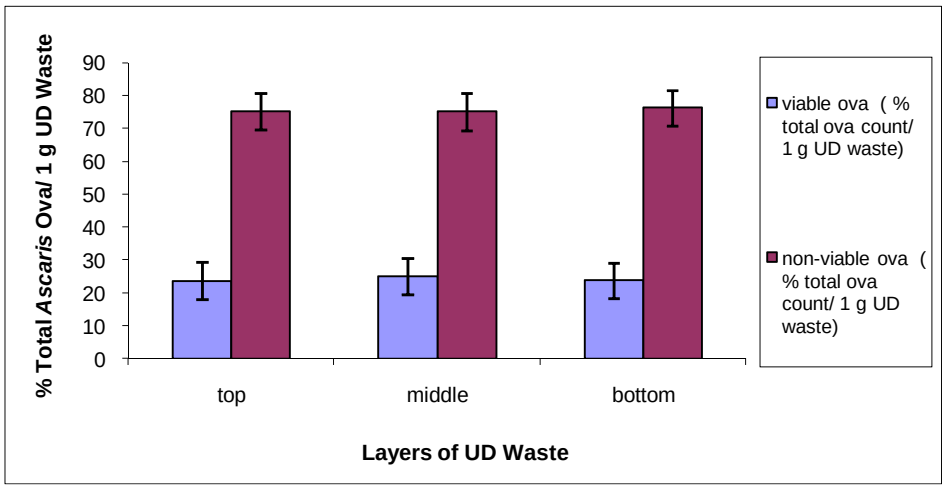


Figure 3.19: Comparison of percentage viable and percentage non-viable *Ascaris* ova recovered from each of the UD waste heap layers in UD waste samples incubated at 29.5°C and 100% relative humidity for 90 days.

The decline in percentage viability of *Ascaris* ova in each of the three layers (top, middle and bottom) of the UD waste heap over the experimental period is shown in Fig. 3.20. Several points are worth noting. Firstly, although UD vaults which had completed the standing period were sampled, the initial viability of recovered *Ascaris* ova is between 70% and 80%. This suggests that, contrary to general belief and published data, there is considerable survival of *Ascaris* in UD vaults. This is a cause for potential concern and an indicator for urgent further investigation, with regard to the health implication to UD waste handlers who empty the vaults.

Secondly, the die-off pattern of ova, separated by layer, is essentially the same as that obtained when data are averaged over all three layers (Fig. 3.14). Die-off is initially slow, shows a marked drop around days 20-30, and then continues at a slower rate for the rest of the experimental period. The decrease in percentage viable ova is significant for each layer, when taking the entire experimental period into account.

Lastly, differences in percentage viable ova among the three layers at each time point are slight and do not show any consistent trend. Therefore the fate of *Ascaris* ova does not appear to depend on the position in the waste layer. Since the level in the vault must be correlated with the age of the material, this suggests that the die-off that was observed must have been initiated by the test conditions, and did not represent what had been taking place in the vaults before the samples were taken.

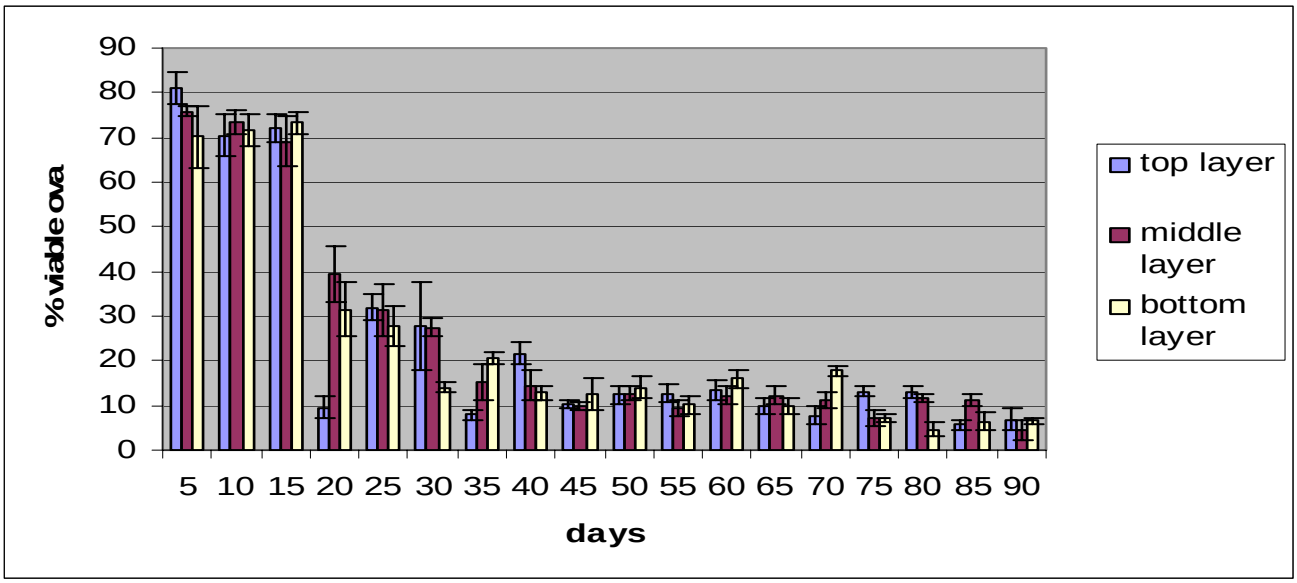


Figure 3.20: Change in viability of *Ascaris* ova in each of three layers of the UD waste heap over time, in UD waste samples incubated at 29.5°C and 100% relative humidity .

3.2.3 Discussion and Recommendations:

After the 90 day period of the experiment, more non-viable *Ascaris* ova (63% dead, 17% necrotic) than viable ova (6% undeveloped, 12% viable immotile and 2% viable motile) were detected. A very small percentage (~1%) of infertile *Ascaris* ova and hatched ova found in the waste. Such infertile eggs are typically narrower and more elliptical (88 to 93.5 µm by 38.5 to 44 µm) than fertilised eggs (Faust, 1955). Internally, infertile eggs contain a mass of disorganized granules and globules which completely fill the shell. Fertilized eggs of *Ascaris lumbricoides* measure ~ 45 to 75 µm by 35 to 50 µm and are broadly ovoidal (Faust, 1955). This description was borne out by the observations made in this study.

One of the prominent features in this study was the large variation in the number of helminth eggs detected among replicate tubes for the same ample. Although the UD waste was mixed thoroughly to render an apparently homogeneous mixture, replicate tubes exhibited very different *Ascaris* ova counts. This indicated that the waste was not homogeneous. Such variation cannot be accounted for by multiplication since helminths cannot multiply outside the host's body (Schwartzbrod, 2003). Hence, the only possible explanation is inherent heterogeneity of the material being tested (Gallizzi, 2003).

Hawksworth *et al*. (2005) noted that a zinc sulfate flotation solution at a S.G. of 1.3 floated not only *Ascaris* ova, but also many other spores and parasites of medical importance. This is borne out by the results reported above. Apart from *Ascaris* eggs, *Trichuris* and *Taenia* spp. eggs were also detected and enumerated. In addition, spores, larval worms, plant tissue and 1 hookworm egg were observed.

UD waste analysed in this study yielded a far greater number of *Ascaris* ova than *Trichuris* and *Taenia* spp. ova, therefore the household from which the waste was sampled had members with high worm loads of *Ascaris* (roundworm infection).

Ascaris eggs are highly infectious and very robust. They are resistant to desiccation and putrefaction, and can withstand considerable desiccation and cold (Faust, 1995). They can survive in the environment for a very long time even without a suitable host. *Ascaris* infections are therefore common in communities where the soil acts as a reservoir of eggs . Adult *Ascaris* worms can survive for 1-2 years in the host and female worms typically generate eggs for a period of one year, while some may continue as long as 20 months. Eggs are passed in the faeces in the undeveloped state and humans contract ascariasis by ingestion of developed eggs through faecal contamination of food or water, or direct contact with contaminated faeces (O' Lorcain and Holland, 2000). The fertile eggs of are passed in the one-celled stage (Faust, 1995). This explains the large number of undeveloped ova seen in the UD waste during the early stages of the incubation period. Feachem *et al*. (1983, quoted by Gallizi, 2003), argue that high temperatures are the main factor responsible for the die-off of helminth eggs. At temperatures of 40°C and above, the embryos are killed, whereas at lower temperatures (22°C to 33°C) they complete development to the first stage (rhabditoid larvae) in 9 to 13 days (Faust, 1995). This explains the observation that from day 10 of the experimental period onwards more developed than undeveloped ova were detected.

In this study, the viability of *Ascaris* ova was found to decrease with time, with a very steep decrease seen 20-30 days after initiation of the experimental period. Thereafter, a slower but steady decrease in the percentage of viable ova was noted. Strauch (1998, quoted in Gallizi, 2003) described studies that found a combination of pH 12 and temperatures in the range of 60-70°C destroyed *Ascaris* ova and a range of viruses within 24 hours. As the incubator temperature used in this experiment was 29.5°C, continued exposure to the temperature over the 90 day period caused the eggs to become more susceptible to dying and damage (larvae disintegrating), but this occurred at a much slower rate because the temperature was much lower. The temperature used in this experiment was considered to be representative of the temperature of UD vaults in the field in the eThekwini municipal area.

At the initial stages of the experiment (days 5 – 15), a very high percentage of viable immotile (good condition) *Ascaris* ova was seen but from day 20 onwards, more viable immotile (necrotic) *Ascaris* ova were seen. As a result of continued exposure to moderately elevated temperature, the larvae within the ova began to disintegrate and become necrotic. From day 25 onwards, there was a substantially higher percentage of viable immotile (necrotic) ova seen in comparison to the viable immotile (good) ova. The percentage of non-viable *Ascaris* ova found in the different layers of waste was found to be similar. The total percentage of non-viable *Ascaris* ova was seen to be much higher than that of viable ova by the end of the experimental period.

From this study, which portrayed the fate of *Ascaris* ova in UD waste under 100% relative humidity and 29-30°C (typical of the upper temperature range in summer in eThekwini municipal area), it was clear that *Ascaris* ova viability decreased, rapidly in the first 25-30 days and then more gradually. However, the material taken from different depths in the waste heap showed no significant differences in either the prevalence of viable ova or their die-off curve with time. Since the level in the vault must be correlated with the age of the material,

this suggests that the die-off that was observed must have been initiated by the test conditions, and did not represent what had been taking place in the vaults before the samples were taken. In Feachem *et al*. (1983) it is stated that “climatic factors influence egg development in the soil and, therefore, transmission. Temperatures of 20-32°C are ideal, with little development taking place below 18°C. A moist, shady environment also encourages egg development”. Later, the following is also mentioned: “in anaerobic conditions development is arrested but recommences when air is introduced.” All this strongly suggests that the ova survived in a dormant state in the UD vault, but were induced to resume development by the experimental conditions, which subsequently lead to their death.

The present study is a preliminary study only and much further work is needed to characterise the fate of helminth ova over a wider range of moisture content, temperature and time combinations. A worrying observation was the significant levels of *Ascaris* ova and their high viability in waste on excavation from the UD vault in the community, considering that the vault in question had already undergone a standing period.

The recently revised World Health Organisation Guidelines for Safe use of Wastewater, Excreta and Wastewater in Agriculture (WHO, 2006) specify a limit of ≤1 helminth ova per litre for unrestricted use in agriculture, based on health risks to consumers and handlers.

Since the UD waste in this study was still found to contain a high number of ova in the UD waste ***and*** at the end of the experimental period, it is possible that, in the eThekwini application, the assumed one year standing period for the waste in the toilet vault may be too short to render the waste safe for handling without instituting additional barriers to exposure (see next section) or subjecting the waste to further treatment, *e.g.* composting.

Recommendations for future work

Further studies on viability and die-off of *Ascaris* and other helminth ova in UD waste should aim at comparing different conditions that could potentially affect the longevity of the ova in the waste.

This would involve testing various combinations of the factors thought to be most directly related to pathogen die-off in UD toilet vaults, *viz.* standing time, temperature and moisture content. An additional possibility is to test the effect of secondary treatments (*e.g.* composting, vermicomposting, pH adjustment) on die-off of helminth ova remaining at the end of the standing period in the toilet vault.

4 DISCUSSION AND RECOMMENDATIONS

This was an exploratory project in several respects: firstly, since the team of researchers had no previous direct experience in onsite sanitation, and secondly, since there has been very little previous research into the processes occurring in UD wastes. As a result, much of the effort was directed towards developing and proving the techniques that could be used to characterise various aspects of UD waste.

4.1 Measurement and testing protocols

Two measurement techniques received the most attention: the anaerobic serum bottle test for characterising various aspects of biodegradation processes, chiefly biodegradability and anaerobic inhibition (reported in the main report); and the AMBIC protocol for recovering helminth eggs from UD solids in order to assess their prevalence and viability (reported in this report).

The AMBIC protocol represents a very significant contribution to the analysis of UD wastes (and other soil-based samples) for *Ascaris* ova, since it has demonstrated that previous methods were unreliable in detecting the eggs. This brings into question previous ideas about the survival of these pathogens, since they may have been derived from unreliable measurements.

4.2 Recommendations

The results of the project support the assumption, which was the basis of the decision to adopt the UD sanitation system in eThekwini, that it would lead to a safer and healthier environment for the communities to which the service is provided. The continued roll-out of the system is therefore supported.

The risk of *Ascaris* infection is significant for anyone who has to handle the UD waste, but it can be readily managed. The issue is most critical where emptying is to be carried out by contractors from outside the household, since they will be more frequently exposed, and they will be more likely to spread the infection. There is a strong case for combining the provision of UD toilets with a chemo-therapeutic campaign to reduce the prevalence of ascariasis in school children and hence the load of *Ascaris* ova in the environment. This would enable the cycle of re-infection to be broken.

The AMBIC protocol for detection of helminth eggs should be disseminated to analytical laboratories engaged in this kind of analysis.

A comprehensive and systematic study should be undertaken to investigate the die-off of *Ascaris* eggs under a range of relevant environmental conditions using the AMBIC protocol.

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