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Water Sanitation & Hygiene Research & Development Centre

A COMPREHENSIVE INVESTIGATION OF EXISTING SANITATION HELMINTH ENUMERATION METHODS WITH THE AIM OF PRODUCING AN INTERNATIONAL STANDARD

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Date of submission: 02 September 2022

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Publication 1 - Submitted to Journal of Parasitology

Naidoo, D. & Archer, C.E., 2022. The effects of all reagents and chemicals used in existing helminth test methods on the viability of *Ascaris suum* eggs.

Author contributions: DN conducted all experimentation and both completed the microscopy. DN wrote up the manuscript, and CEA thoroughly proofread and edited it, and provided guidance and supervision.

Publication 2 - Submitted to Journal of Parasitology

Naidoo, D. & Archer, C.E., 2022. The effects of all technical steps used in helminth test methods on *Ascaris suum* egg recovery from pig faeces.

Author contributions: DN conducted all experimentation, completed the microscopy and wrote up the manuscript, and CEA thoroughly proofread and edited it, and provided guidance and supervision.

Publication 3 - Submitted to Journal of Parasitology

Naidoo, D. & Archer, C.E., 2022. *Ascaris suum* egg recovery from fecal sludge samples after phase extraction. Journal of Parasitology.

Author contributions: DN conducted all experimentation, completed the microscopy and wrote up the manuscript, and CEA thoroughly proofread and edited it, and provided guidance and supervision.

Publication 4 - in preparation, aimed at a methods journal

The WRDC Helminth Method: an optimised and highly adaptable procedure for helminth egg recovery that is suitable for sanitation and environmental samples

Author contributions: DN wrote up the manuscript, and CEA thoroughly proofread and edited it, and provided guidance and supervision.

Signed: Date: 06/07/2022

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PREFACE

This thesis has been formatted in "publication-style" and comprises a general introduction, publications 1, 2, 3 and 4 (the last of which is still in manuscript form and has not been submitted to a journal as yet), followed by conclusions and recommendations for future work. The main reference list at the end of the thesis includes those cited in the general introduction and conclusions, and not those in the 4 publications, as each of these has a reference list of its own. The main reference list and in text citations are in APA style. Layout options, such as abbreviations and spacing, have been kept consistent throughout the entire thesis, to facilitate better understanding and ease of reading.

The format of the first 3 publications is in accordance with the Journal of Parasitology, including both in text citations and the final reference list. This is an American journal; therefore, the language has been adjusted to American English (U.S. English). These manuscripts have, however, been changed slightly for the purpose of this thesis, to make it easier for the examiners to read through.

Publication 4 is written in South African English and is still to be submitted to a journal as the first 3 need to be submitted and published as evidence and support in terms of efficacy and egg recovery, before a new method will be considered for publication. We are aiming for a methods journal; thus, the manuscript has been formatted accordingly, and not as a regular research article. We have used the layout and guidelines from Nature Protocols for writing up the manuscript.

Appendix F (Paper 0 - Naidoo et al., 2020) is the first article published towards the PhD degree. It was a continuation of my Master's research, focusing on heat treatment and drying of *Ascaris suum* eggs in water and composite faecal sludge samples. Whilst the PRG Helminth Method was employed for all samples processed in this study, it did not fit the scope of method development for the purposes of this thesis, and was therefore included as an appendix and not part of the body.

Lastly, all *Ascaris suum* eggs used for experimentation were sourced from experimental pigs that were kept for a separate research project (ethical clearance attached as Appendix D). A moderate infection was maintained in these pigs as a source of helminth eggs for experimentation and prototype toilet testing.

FOREWORD

Dedicated to my dad, Roland Naidoo (07 June 1965 – 16 June 2022)

This one's for you dad – the final feather in your cap for me, as you would have said. My heart is so sad that you aren't here to witness this, but I know you'd be proud. Thank you for raising me to be who I am, and teaching me to shoot for the moon and beyond.

I can do all things through Christ who strengthens me

Philippians 4:13

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To God be all the glory, for the strength, courage and fortitude to have persevered through this year and degree, despite all adversities and setbacks.

To my incredible family – thank you for the constant love and support. To dad, thank you for your countless sacrifices and always pushing me to the best of my ability, so that I could climb this final mountain. Mum, thank you for believing in me, my ability and career, and for always nurturing me and being the force in my corner. Damian, you have always been my protector. Thank you for always showing up for me (sampling trips included). Denali, particularly, for always being "my person", I appreciate you and your willingness to always listen and defend me. Lastly, to Gladys Qwabe, for always taking such good care of me.

Dr Colleen Archer, for mentoring, training and guiding me for the last 10 years, and supporting me throughout the duration of this degree and my career in general. You have taught me so much, and I know that was just the tip of the iceberg. I look forward to the many years ahead of working with you.

Merissa Naidoo – thank you for your laboratory and academic support, but also for being an incredible friend and cheerleader throughout. Your faith in me has always been boundless and I appreciate you for it. Also, thank you for stomaching my complaints!

Phumzile Afrika, you have been my confidant throughout. Thank you for always being willing to proofread my work and allowing me to bounce ideas of you. Most importantly, thank you for praying for and believing in me. Megan Watson – my other go-to person for advice and a shoulder to cry on– you encouraged me through the worst and cheered me on through the best! Your heart is everything.

To my girls, Amy Naidoo, Jade Brimiah & Desiree Naidoo – thank you guys for always having my back, for reassuring me when I felt like giving up. To my extended friends and family, I am grateful for the unconditional love and encouragement.

To the entire WASH R&D Centre, particularly the laboratory team, I am grateful for the technical and academic assistance. The University of KwaZulu-Natal for use of the facilities to complete this degree. To the Water Research Commission (WRC) for funding this project, and the National Research Foundation (NRF) for funding my doctoral studies.

ABSTRACT

Helminth testing in faecal sludge should be consistent so data are comparable. New faecal sludge treatments for on-site toilet technologies are constantly being developed in order for municipalities in developing countries to supply dignified alternatives to sewered systems that waste large amounts of potable water and require pumping to wastewater treatment works for centralised treatment. In order to ensure that these new, onsite toilet technologies adequately sanitise the faecal matter, helminth eggs are spiked into these systems to test inactivation according to the ISO-30500 standard for non-sewered sanitation systems (NSSS). A sensitive, standard helminth isolation and enumeration method, accredited to the ISO-17025 international standard for testing and calibration laboratories, is therefore required for application in laboratories globally. Internationally, laboratories and groups have used variations of the standard United States Environmental Protection Agency (USEPA) Method (2003), the Mexican Standard for Wastewater Analysis (2012), the Bailenger Method (1996) and the Pollution Research Group (PRG) Helminth Method (2017) previously used by the Water, Sanitation and Hygiene Research and Development Centre (WRDC) for helminth testing, and formed the foundation of this study. Conventional helminth methods can be broken down into five steps: washing and sedimentation of samples to separate eggs from larger particles, flotation using density gradients to separate eggs from heavier particles, centrifugation after both washing and flotation, extraction, that involves the use of a buffer and solvent combination to further separate organic material from eggs, and microscopic analysis. Some methods also include incubation that allows for egg-viability assessment. Every reagent used in these helminth methods was tested on *Ascaris suum* eggs for varying time intervals; ammonium bicarbonate and 7X® (a brand of ionic surfactant) performed best in terms of egg development and viability. Washing samples under pressure and no pressure were compared and the former produced the best egg recovery. Different flotation solutions were tested at different specific gravities, and zinc sulphate at specific gravity of 1.3 recovered the most eggs. Centrifugation speeds and times were tested after the washing and flotation steps, and 3000 rpm for 10 minutes and 2000 rpm for 15 minutes produced optimal egg recovery, respectively. Different extraction combinations were tested, and it was discovered that eggs were lost in this step. It was therefore recommended that extraction be removed from the method. Different wash solutions were then tested against various sample types to determine which resulted in the highest percentage egg recovery and which solutions facilitated easier microscopic analysis. Based on data from each experiment, a final SOP was produced for the new WRDC Helminth Method, that accommodates different sample types and egg viability assessment post incubation.

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LIST OF ABBREVIATIONS

A. suum	*Ascaris suum*
g x force	gravitational force
g	gram(s)
ISO	International Organisation for Standardisation
JMP	Joint Monitoring Programme
L	litre(s)
min	minute(s)
ml	millilitre
NSSS	non-sewered sanitation systems
PRG	Pollution Research Group
rpm	rotations per minute
SDG(s)	Sustainable Development Goal(s)
sp. gr.	specific gravity
SOP	standard operating procedure
STH(s)	soil-transmitted helminth(s)
UDDT	urine diversion dry toilet
UKZN	University of KwaZulu-Natal
UN	United Nations
USEPA	United States Environmental Protection Agency
VIP	ventilated improved pit latrine
WASH R&D CENTRE	Water Sanitation & Hygiene Research & Development Centre
wk	week
WHO	World Health Organisation
WRC	Water Research Commission
WRDC	WASH R&D Centre
WWTW	wastewater treatment works
yr	year
µm	micrometre

CHAPTER 1: INTRODUCTION

Background

Approximately 2.3 billion people globally lack access to basic sanitation facilities and one third of the world's population (1.4 billion people) are infected with soil transmitted helminths (STHs), which can cause diarrhoeal disease (JMP, 2017; Cooper & Hollingsworth, 2018). One in eight children under the age of 5 years (yr) die due to diarrhoeal diseases, with 1.3 million global deaths per yr (Kotloff, 2017). *Ascaris lumbricoides*, also known as the human roundworm, is the most common STH of human health importance (Brownell & Nelson, 2006). An estimated 804 million people worldwide are infected with *A. lumbricoides*, particularly in areas that lack a source of potable water, improved sanitation, and proper hygiene practices (Jourdan et al., 2018).

The World Health Organisation (WHO) has developed an agenda of goals that aim to eradicate poverty, protect the planet, and ensure prosperity for all (Joshi et al., 2015). These are known as the Sustainable Development Goals (SDGs), and are targeted for accomplishment by 2030 (WHO, 2015). The South African government's Department of Water and Sanitation considers the provision of potable water and improved sanitation to the previously disadvantaged a priority development goal (Friedrich et al., 2015) that will contribute towards alleviating the transmission of diarrhoeal diseases. The combination of an effective sanitation regime, educating people on good hygiene practices, and the implementation of proper faecal sludge disposal programs or onsite sanitation systems is imperative in reducing diarrhoeal disease transmission. Faecal sludge is an extremely rich source of nutrients (especially phosphorus) for growing of crops, and is much cheaper than chemical fertilizers (Semiyaga et al., 2015) for use as soil conditioner. However, it must be treated and decontaminated prior to reuse (land application), to reduce the number of viable helminth eggs and other pathogens entering the environment (Fewtrell & Bartram, 2001; Clasen et al., 2014).

Pathogens found in faecal sludge

Pathogens found in faecal sludge can be divided into four groups: bacteria, viruses, protozoans, and helminths (Sidhu & Toze, 2009). It is impossible to isolate every microorganism that is found in sludge, due to a lack of specificity of detection methods, as well as the fact that it is extremely costly and time consuming (Sidhu and Toze, 2009). Faecal coliform bacteria are now used as indicators of water and biosolid safety (Horan, 2003). Issues and limitations associated with bacteria include short survival periods and a ubiquitous nature, indicating an origin from sources other than human faecal matter and regrowth outside the host (Sidhu and Toze, 2009). Somatic coliphages and male-specific coliphages can also be tested for, such as human adenovirus, however methods are complex and

expensive (Horan, 2003). *Giardia* sp. and *Cryptosporidium* sp. cysts are used as protozoan model organisms, as there is no seasonal aspect to the life cycle, and they are found in large numbers in faecal sludge. These methods however, are also expensive and complex. *Clostridium* sp. spores are used as a surrogate for the aforementioned protozoan parasites, however, it is very difficult to successfully culture the spores (Horan, 2003; Sidhu & Toze, 2009).

This thesis therefore focused on optimising a simple, cheap method for detecting helminth eggs. Diarrhoeal diseases can be symptomatic of infection by parasitic worms (helminths), where *Ascaris lumbricoides* (Linnaeus, 1758) is of importance to human health (Brownell & Nelson, 2006). Common helminth eggs that are recovered from sanitation samples include *Ascaris lumbricoides*, *Trichuris* spp. (Roederer, 1761), *Taenia* spp. (Linnaeus, 1758), hookworm species, *Toxocara* spp. (Filleborn, 1921), *Hymenolepis diminuta* (Rodolphi, 1819), *Hymenolepis nana* (Bilharz, 1857), *Fasciola* spp. (Linnaeus, 1758), and *Schistosoma* spp. (Bilharz & Ernst, 1851). Figure 1(A) shows the life cycle of *Ascaris lumbricoides*, which is a geohelminth, meaning that part of the life cycle requires eggs to develop in soil (after open defaecation) to an infective state, and Figure 1(B) shows these developmental stages of the eggs. The life cycles of *Taenia saginata* and *T. solium* are shown in Figure 2, as an example of helminths that require an animal as an intermediate host and food source for transmission of the disease (undercooked beef and pork, respectively). Figure 3 shows an example of two helminths, commonly known as blood flukes, *Schistosoma mansoni* (found in human faeces) and *S. haematobium* (found in human urine), that have a phase of the lifecycle that requires a water body, plus specific snail species as the intermediate hosts, for development of the organism and successful transmission. All of the above species produce eggs that can be found in water and sanitation samples (Sidhu & Toze, 2009).

Ascaris sp. eggs are used as an indicator of inactivation and treatment efficacy due to their hardy nature. It is assumed that if a process can kill off these eggs, then all other pathogens would be inactivated as well (Horan, 2003). *Ascaris suum* (Goeze, 1782) eggs are used as a surrogate for the human roundworm, *Ascaris lumbricoides*, as the latter can be difficult to source due to ethical and logistical constraints. Both species are morphologically identical in all developmental stages, but differ genetically. Furthermore, the human is the definitive host for *A. lumbricoides* and the pig for *A. suum*, thus being, mostly, non-pathogenic to humans (Daugschies et al. 2013). *Ascaris suum* infection in pigs can be ethically and inexpensively set up and maintained for harvesting of this roundworms' eggs to use as a test organism source for research purposes. A standard, internationally accepte, helminth method is therefore required for pathogen testing of water, sanitation, and other environmental samples.

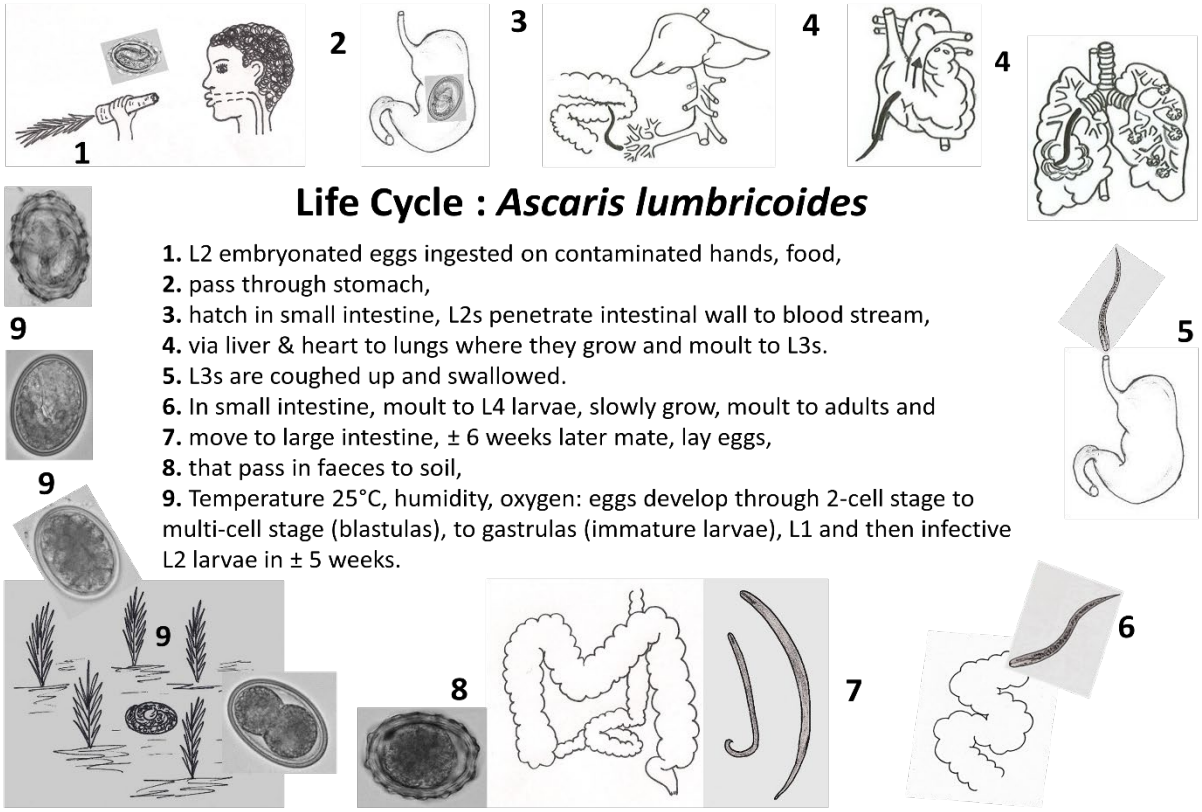

Figure 1(A): Life cycle of *Ascaris lumbricoides* (C.E. Archer, 2022)

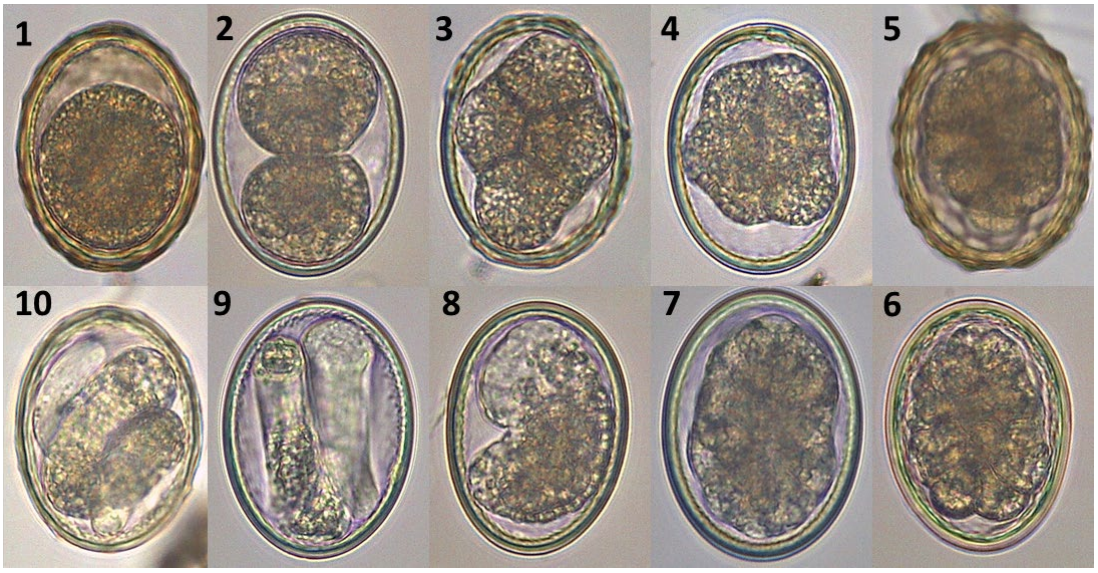

Figure 1(B): The developmental stages of *Ascaris* eggs that occurs in the soil phase of the life cycle (steps 8 and 9 in Figure 1A). (1) Undeveloped (one-celled); (2) Two-celled; (3) Four-celled; (4) Eight-celled; (5) Sixteen-celled; (6) Morula; (7) Blastula; (8) Gastrula; (9) and (10) Larval eggs.

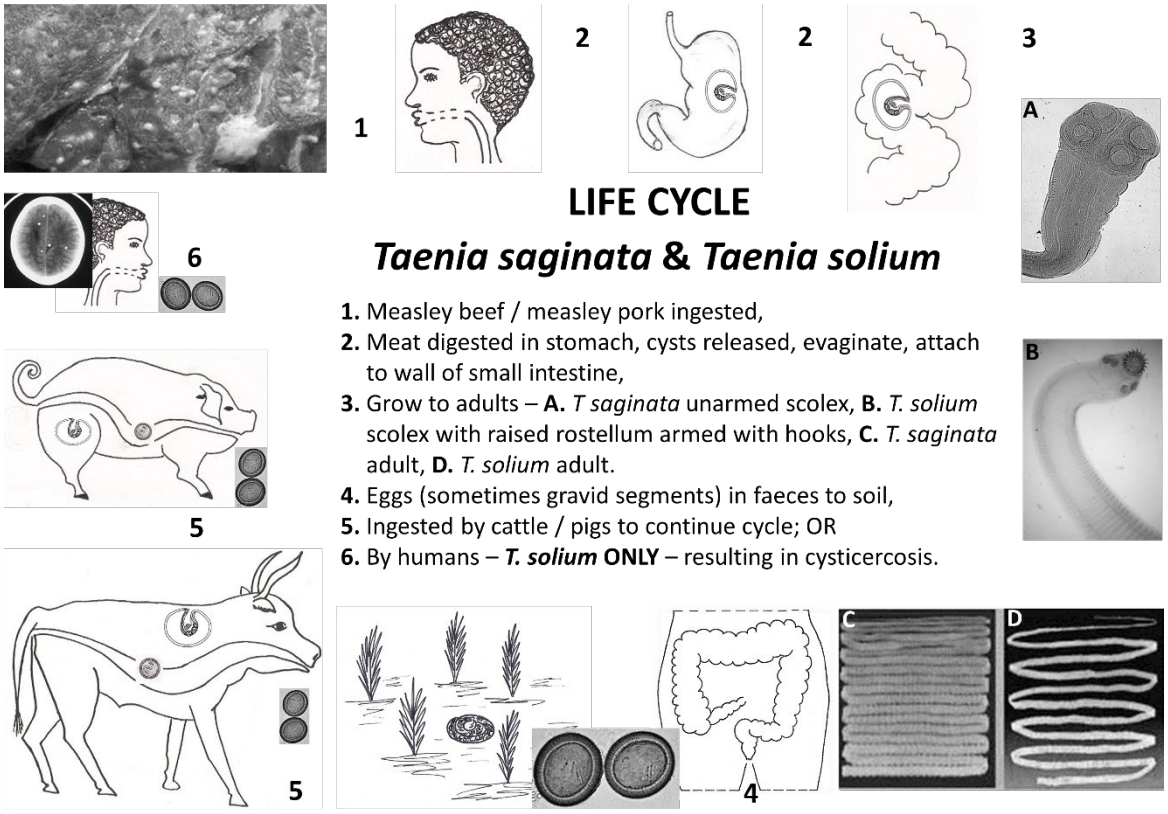

Figure 2: Life cycle of *Taenia saginata* and *Taenia solium* (C.E. Archer, 2022).

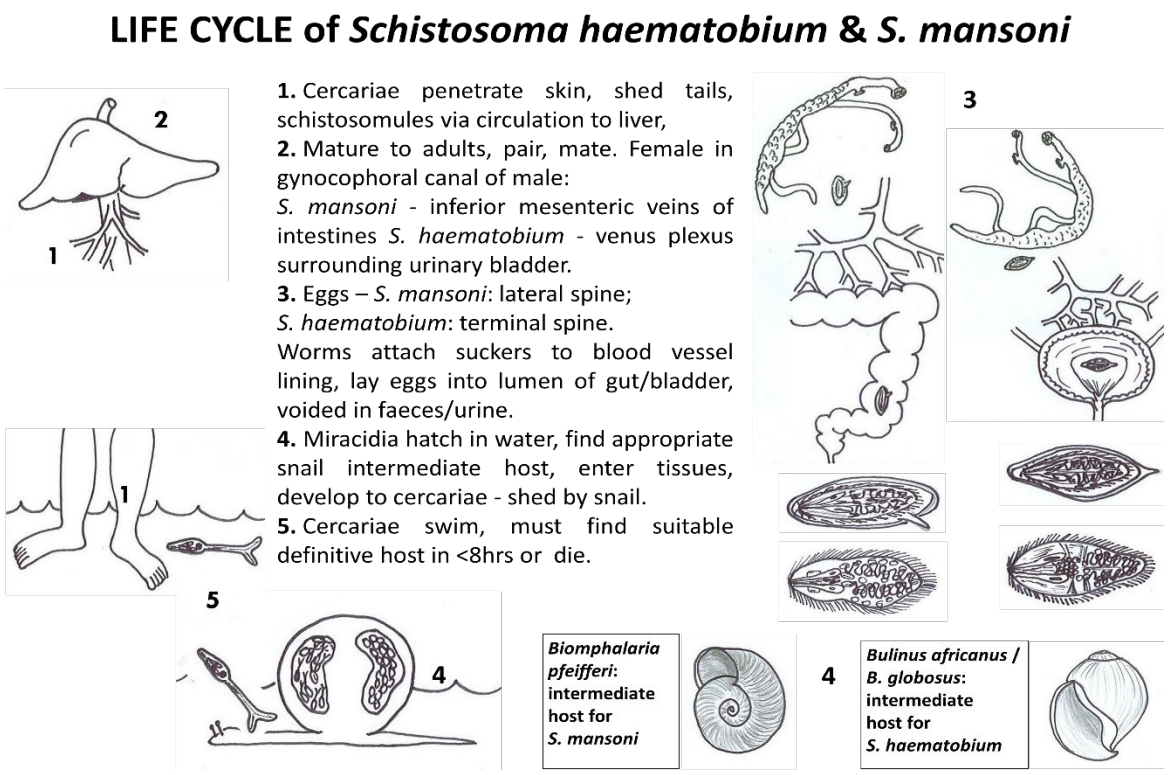

Figure 3: Life cycle of *Schistosoma haematobium* and *Schistosoma mansoni* (C.E. Archer, 2022).

Basic principles of helminth testing

Helminth test methods comprise of the same basic principles and steps: washing and sedimenting or washing of the sample over a set of pan sieves to facilitate breaking up of the sample matrix and separation of eggs from larger particles, flotation, that uses the principle of density gradients to allow eggs to float up the supernatant column, phase extraction, that uses hydrophilic and lipophilic reagents to create an interphase that can separate lipids and protein from eggs to facilitate easy sample analysis, incubation of eggs to determine egg viability and microscopy to assess, quantify and categorise eggs (Collender et al. 2015; Rocha et al. 2016).

The first step of processing a sanitation sample for egg recovery is washing and involves the use of one of a variety of solutions. Different wash solutions are used for different sample types to maximise egg recovery from a specific matrix (Rocha et al., 2016). Most wash solutions are anionic detergents or surfactants that can break bonds between particles and eggs. Some are also able to dislodge eggs from samples that are granular and where eggs are trapped between particles (Amoah et al., 2017).

After washing a sample to remove larger particles, flotation is performed. The density gradient created during flotation allows for eggs to float up the column to the surface whilst particulate matter sediments down and gets packed into a pellet when centrifuged (Rocha et al. 2016). The flotation solution is made up to a certain density. According to David & Lindquist (1982), the relative density of soil-transmitted helminth eggs ranges from 1.05 to 1.27, thus a specific gravity (sp. gr.) of 1.3 would allow for eggs to float up efficiently without exerting too much pressure on the egg wall.

The third step is extraction, that traps sample debris, including proteins and lipids, between an acidic aqueous and a lipophilic phase of the mixture, thus reducing the size of the final pellet for microscopy. Both extraction reagents are thus added to the pellet in the test tube, and the tube is shaken vigorously to allow for phase separation to occur. Some studies have indicated that extraction solutions can have detrimental effects on the eggs (Nelson & Darby, 2001; Rocha et al., 2016), thus recommending the extraction step should not be included in sample processing. If it is however included, then exposure time should be minimal (Nelson & Darby, 2001).

Incubation solutions are used to suspend and contain the eggs and allow growth and development over a 28-day incubation period. An ideal incubation solution should therefore possess antimicrobial and antifungal properties to prevent the growth of contaminants, but it should also be mild enough to not affect the development, survivability, and viability of the eggs (Amoah et al., 2017). Many methods do not include the incubation step, but it is imperative when determining egg viability, especially when testing sewage treatment technologies.

The final step is microscopy, though certain methods perform this prior to incubation to determine the initial state of the eggs. Different types of slides can be prepared, such as a wet mount preparation, a Sedgewick-Rafter counting chamber, a Doncaster disc or McMaster slide, and a compound microscope is used at 100 X and 400 X magnification. Depending on the specified method, eggs are simply assessed for presence or absence, or counted only, or counted and also categorised based on the developmental stage and condition of the egg and recorded according to the different helminth species (Ayres & Mara 1996; USEPA 2003; Secretaría de Economía 2012; Velkushanova *et al.* 2021).

Overview of Existing Helminth Methods

Most helminth test methods were designed for wastewater samples, and are thus unable to accommodate semi-solid or solid faecal sludge samples. This led to the development of the PRG Helminth Method by Archer and Hawksworth in 2005, later published in Hawksworth et al. (2010), targeting urine diversion dry toilet (UDDT) sludge samples that generally contain soil. This method was modified by Moodley et al. (2008) for a Water Research Commission report, then by Archer, who used it in a study focusing on isolating eggs from soil samples in baboon foraging sites (Pebsworth et al., 2012). Figure 4 details the evolution of the method over time, from initial development to date. The standard operating procedure (SOP) for the method was continuously modified over the years and employed at the Pollution Research Group (PRG) laboratory at the University of KwaZulu-Natal (we are now the Water Sanitation and Hygiene Research and Development Centre (WASH R&D Centre, abbreviated even further to WRDC)) for helminth testing, and it formed the foundation for this project.

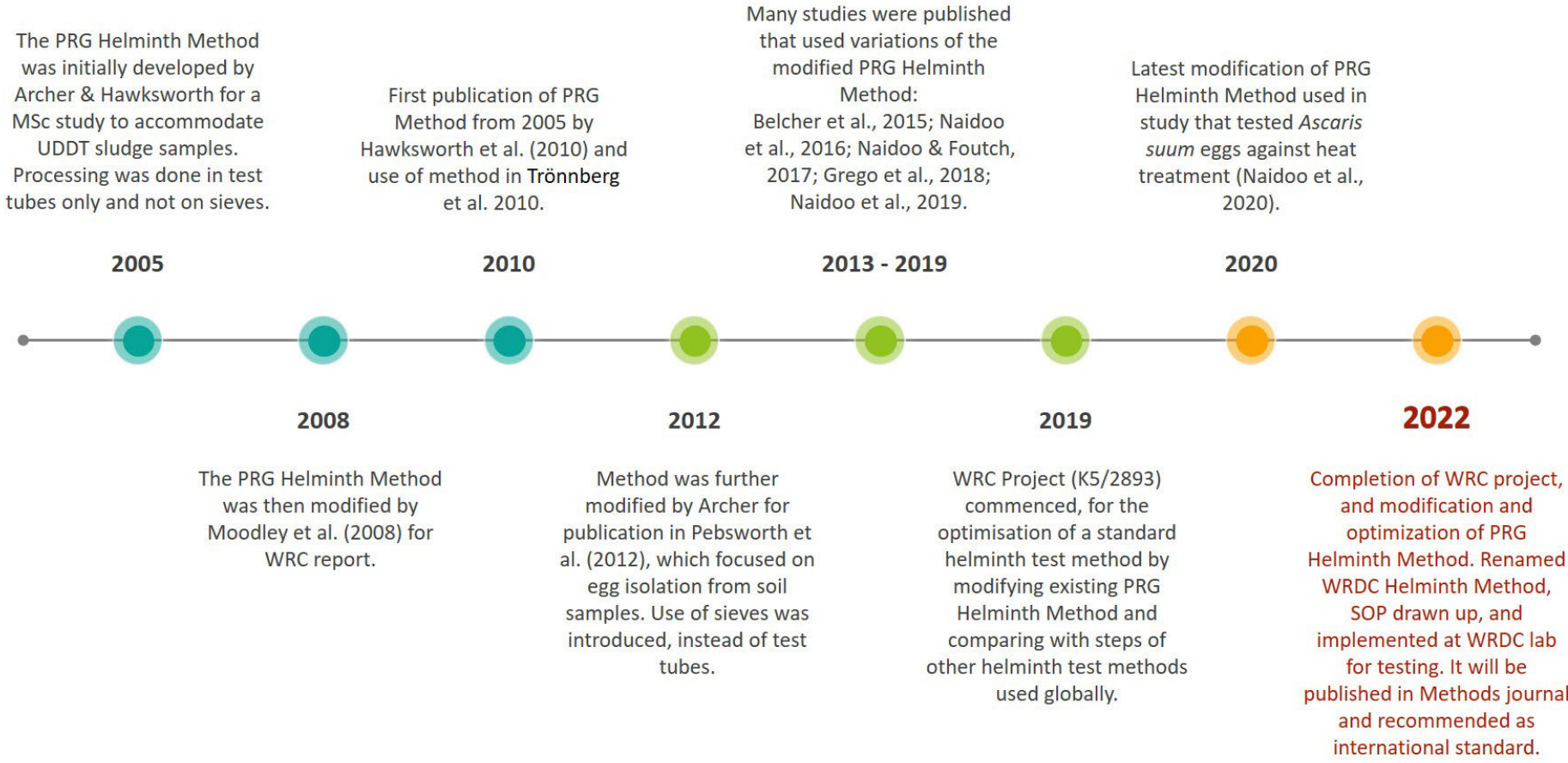

Figure 4: Evolution of the PRG Helminth Method, from initial conception in 2005, to the modification and renaming in 2022 to the WRDC Helminth Method. The timeline includes improvements over the years, and subsequent publications that used these variations, showing how it changed from the PRG Helminth Method to the WRDC Helminth Method.

The PRG Helminth Method

The PRG helminth method can be broken down into five steps: washing, flotation, centrifugation, microscopic analysis, and incubation. Depending on the type of sludge sample, a given amount (either 10 – 20 g of solid sludge, or 200 ml – 1 L of liquid sample) is soaked in ammonium bicarbonate. A magnetic stirrer bar is added, and the sample is placed on a magnetic stirrer plate to break down the sample matrix and allow for dislodgement of helminth eggs from sludge particles. The sample is then poured through a set of pan sieves (a 100 µm sieve over a 20 µm sieve, both 200 mm in diameter) (Belcher et al., 2015; Naidoo et al. 2020). It is washed thoroughly on the sieves, using tap water under pressure and by breaking any clumps using the back of a gloved hand. The 100 µm sieve is then removed and the retentate discarded. The retentate (containing the *Ascaris* eggs) on the 20 µm sieve is then washed thoroughly and collected into 4 x 15 ml graduated plastic test tubes (Falcon tubes). These tubes are centrifuged at 1512 x g (3000 rpm) for 10 minutes (min) and the supernatant discarded (Velkushanova et al., 2021).

Zinc sulphate is added to each tube in ± 3 ml aliquots to a total of 14 ml while mixing on a vortex to break up the pellet and homogenise the suspension. The test tubes are centrifuged at 672 x g (2000 rpm) for 10 min, to allow eggs to separate out of the sediment and float up into the supernatant (Belcher et al. 2015; Naidoo et al. 2020). The supernatant is then poured onto a smaller 20 µm sieve (100 mm diameter), washed with water, and the retentate collected into a single 15 ml Falcon tube. The final sample is centrifuged at 1512 x g (3000 rpm) for 10 min, after which the supernatant is discarded, and the final pellet microscopically analysed. Sometimes, if the sample is too thick or the pellet too large, an extraction step is performed before microscopy using 10% formalin and ethyl acetate or diethyl ether (Velkushanova et al., 2021).

The sample is then washed back into the test tube, incubated for 28 days at 25°C and re-analysed microscopically (Belcher et al. 2015; Grego et al. 2018; Naidoo et al. 2019). Categorisation of eggs by microscopy is done as follows: a) Potentially viable eggs – motile (eggs with a fully developed, plump, motile larva at the point of examination), immotile (eggs with a fully developed, plump, immotile larva, inactive at the point of examination), undeveloped (eggs with a one-celled embryo) and developing (eggs with a developing embryo, from a 2-celled stage to a late gastrula stage); and b) Non-viable eggs – dead (eggs with a globular, or ruptured embryo or collapsed or broken wall), necrotic (eggs with a shrivelled larva that has pulled away from the walls of the egg) and infertile (eggs that have not been fertilised) (Naidoo et al. 2016; Naidoo et al. 2020).

Comparisons between existing methods

Other commonly employed helminth methods include the United States Environmental Protection Agency (USEPA) Method (2003), the Mexican Standard for Wastewater Analysis (2012) and the Bailenger Method (1996). Different laboratories and groups have used variations of these protocols for helminth testing, therefore the steps from each were used for comparison against the steps of the PRG Helminth Method, for this project. Table I below highlights the differences between the four mentioned methods at each step.

Table II (immediately below Table I) shows the versatility of the four test methods compared in this project, and how each can be adapted or modified to suit the sample type and helminth prevalence of a given region. A common trend across these studies is, however, a lack of consistency in terms of processing steps, microscopy counting methods and how data should be reported for publication purposes. Furthermore, some methods do not take egg viability into account, and only report on presence and absence of different helminth species eggs in samples. A gold standard would therefore allow for better cross-comparisons of what does and does not work for faecal sludge treatment technologies, and the consequent effect on disease prevalence worldwide.

Table I: Differences between the PRG Helminth Method, the standard USEPA Method, the Mexican Standard for Wastewater Analysis, and the Bailenger Method.

Step	PRG Method	USEPA Method	Mexican Standard	Bailenger Method
Sample preparation	Solid/liquid samples are covered in ammonium bicarbonate and stirred using a magnetic stirrer bar and magnetic plate. Fatty/dirty samples are soaked in 1% Tween®80, stirred, then washed over pan sieves.	Sample is soaked overnight in water, then blended in water prior to washing over a set of pan sieves. 1% *7X® is added, and sample is left overnight to sediment.	Method was designed for wastewater samples. Samples are collected in 5 L lots in plastic jugs.	Method was designed for wastewater samples. Depending on the wastewater type, 1 – 10 L if sample is collected and allowed to sediment for 1-2 hr.
Apparatus	Only plasticware is used, except for microscopy, where glass slides and coverslips are used. Plastic beakers, test tubes and Pasteur pipettes are the main pieces of apparatus used.	Glassware is used, all of which must be coated in organosilane. Additional glassware is used, such as Erlenmeyer flasks and large beakers.	Plasticware is mostly used, evenin the case of microscopy, where a Sedgewick-Rafter or Doncaster counting chamber is used.	Specialised sampling containers are not required and minimal laboratory equipment is needed. Plasticware is mostly used, including a McMaster slide used for microscopy.
	A 100 µm sieve is used for large particle separation and a 20 µm sieve for small particle separation and collection of helminth eggs	A 297 or 841 µm sieve is used for large particle separation and a 38 µm sieve for small particle separation and collection of helminth eggs.	A single 150-170 µm sieve is used for separation of particulate matter from eggs that pass through the sieve and are collected in a container beneath by sedimentation.	Sieves are not used.
Washing & Sedimentation	Ammonium bicarbonate was found to be successful for dissociation of eggs from sludge and silica particles and is therefore used here. A 1% Tween®80 solution is also an option, depending on sludge type. Only one wash, and one centrifugation step is performed.	1% 7X® is the only wash solution used. Gravitational sedimentation is performed on the sample multiple times and a vacuum pump is used to suction off the supernatant.	The sample is allowed to settle (gravitational sedimentation) for 3 hours or centrifuged at 400 x g (1543 rpm) for 5 min.	There is no washing step performed, thus no wash solution is used.
	Soaked sample is washed straight onto a set of drum sieves – 100 µm placed over a 20 µm sieve.	Sample is then washed on a 297 or 841 µm sieve placed into a funnel over a beaker, resulting in slow drainage. A second blend, wash, sedimentation (for 2 hr) and vacuum separation of the permeate is performed.	The supernatant is either vacuum aspirated or poured off slowly.	Gravitational sedimentation is performed, where a liquid sample is allowed to settle prior to processing, rather than washing over sieves.

Step	PRG Method	USEPA Method	Mexican Standard	Bailenger Method
	Washing is done under pressure (using a hose attached to the tap). A spray bottle is used only for retentate collection.	A wash bottle (only) is used for washing of the samples through the set of sieves.	The sample is passed through a 150-170 µm sieve. This is then washed with 5 L of water and the wash water is recovered with the filtered sediment.	Sample is not washed, thus separation of possible particulate matter from eggs under pressure is not performed.
	Sample is pipetted into 15 ml Falcon tubes.	Sample is washed into 50 ml Falcon tubes.	The sample is transferred back to the original jug or test tubes.	After settling, supernatant is suctioned off using vacuum pump.
Centrifugation	First and third centrifugation (before microscopy) after washing is conducted at 1512 x g (3000 rpm for 10 min). Second centrifugation after flotation is conducted at much slower speed to allow for eggs to float up into the supernatant column (672 x g or 2000 rpm for 10 min).	First and third (before microscopy) centrifugation after washing is conducted at 1000 x g (2500 rpm for 10 minutes). Second centrifugation after flotation is at a similar speed (800 – 1000 x g or 2200 - 2500 rpm for 5-10 min).	The jug allowed to settle for 3 hr and is then transferred back to original test tubes and centrifuged at 1543 rpm for 5 min. If not by gravitational sedimentation, the sediment can immediately be transferred to test tubes and centrifuged. The supernatant is then discarded or vacuum aspirated.	The sediment is then transferred to test tubes, which are then centrifuged at 1000 x g (2500 rpm) for 15 min. The supernatant is discarded and all sediment is transferred to one tube. The initial beaker and additional test tubes are rinsed with 0.1% Triton®X-100 or Tween®80 and 'rinsings' are transferred to the sediment. Test tube is centrifuged at 2500 rpm for 15 min again.
Flotation	Eggs in the washed sample are floated using zinc sulphate (sp. gr. 1.3)	Eggs in the washed sample are floated using magnesium sulphate (sp. gr. 1.2) and poured over a 37 µm sieve.	Eggs in the washed sample are floated using zinc sulphate at sp. gr. 1.3. The sample is then centrifuged at 1000 x g (2500 rpm) for 5 min. The supernatant is then poured into a plastic container, diluted up to 1 L and then allowed to settle for 3 hr or centrifuged at 2500 rpm for 5 min. The supernatant is then discarded.	Eggs in the washed sample are floated (after extraction) in zinc sulphate at sp. gr. 1.18. An aliquot is quickly removed using a Pasteur pipette and transferred to a McMaster slide.
Extraction	Phase extraction is not performed or included in the SOP, however, was performed IF necessary, when the final pellet was too large for microscopy. Chemicals used included 10% formalin and ethyl acetate or diethyl ether.	Phase extraction is not performed. The old USEPA method (1999) employed an extraction step using acid-alcohol and ethyl acetate, but was excluded from the 2003 version.	Extraction is performed after flotation, using acid-alcohol and ethyl acetate. The sample is then centrifuged at 660 x g (1982 rpm) for 3 min. The supernatant is aspirated.	Extraction is performed before flotation, using acetoacetic buffer and ethyl acetate. Sample is then centrifuged at 2500 rpm for 15 min.

Step	PRG Method	USEPA Method	Mexican Standard	Bailenger Method
Incubation	Sample is suspended in a drop/few drops of water and analysed immediately after processing. Eggs are categorised based on morphology. Sample is washed back into the test tube, centrifuged, water removed, pellet suspended in 0.1N sulphuric acid, incubated for 3 - 4 weeks (wk) at 25°C.	Sample is suspended in 0.1N sulphuric acid, then incubated first for 3 - 4weeks at 26°C, then analysed via light microscopy. A control culture is incubated to determine embryonation time and the next point of microscopic analysis in test samples.	Incubation is not performed; thus, viability assessment is not entirely possible.	Incubation is not performed; thus, viability assessment is not entirely possible.
Microscopy	Wet mounts are prepared by pipetting one or more drops of sample onto a glass microscope slide, covered with a coverslip, and analysed under the microscope. The entire sample is analysed on one or more slides	Sedgwick-Rafter counting chamber is used to examine the concentrated sample after incubation, and the entire concentrate is analysed. Only *Ascaris* sp. eggs are classified.	The entire sample is analysed in aliquots to facilitate easier microscopy. Each aliquot is distributed in either a Sedgewick-Rafter or Doncaster counting chamber. The sample is counted for all helminth eggs, but viability assessment is not necessarily carried out.	The McMaster slide, with the eggs suspended in the zinc sulphate is left to stand for 5 min to allow eggs to float to the surface. An aliquot of the sample is counted and all helminth egg species are then counted under 10X and 40X magnification.
Overall Processing Time	Total processing and initial microscopic analysis time take 2 – 2.5 hr. Viability is assessed immediately, and incubation is done only if necessary when assessing viability is difficult or when eggs are in the undeveloped stage.	Total processing time can take 2.5 days for liquid samples and almost 5 days for solid samples, including overnight soaking and sedimentation steps. Actual results are obtained 3 - 4 weeks after the incubation step has been completed.	Total processing time if gravitational sedimentation is performed is 12 hours, and 3 hours if samples are centrifuged instead.	Total processing time is 3.5 hours, including gravitational sedimentation and centrifugation steps. It is claimed that microscopy can be completed in 1 - 2 min per slide.
Overall Chemical Exposure	Chemical exposure is significantly reduced to prevent egg viability being affected.	Eggs in test samples are exposed to more chemicals for prolonged periods of time.	Eggs are exposed to more chemicals than the first two methods, due to the extraction step.	Eggs are exposed to more chemicals than the first two methods, due to the extraction step.

*7X is a brand of ionic surfactant commonly used for washing of samples

Table II: Summary of studies that have made use of the PRG Method, the USEPA Method, the Mexican Standard for Wastewater Analysis and the Bailenger Method for helminth egg detection and enumeration.

Method	Sample Type/ Matrix	Wash Solution Used	Flotation Solution Used	Observations/Findings	Reference(s)
PRG Helminth Method	Water	None	None	*Ascaris* eggs that are spiked into water are easily recoverable and do not require washing and flotation.	Naidoo et al., 2016; Naidoo et al., 2017; Naidoo et al., 2019.
	VIP and UDDT sludge samples	Ammonium bicarbonate (119 g/L)	Zinc sulphate (sp. gr. 1.3)	*Ascaris suum* eggs were spiked into sludge samples and heat treated. Eggs were successfully recovered.	Naidoo et al., 2020
	Composite VIP sludge sample – liquid VIP sludge mixed with potato flakes.	Ammonium bicarbonate (119 g/L)	Zinc sulphate (sp. gr. 1.3)	No reports of steps of the processing method affecting egg viability and recovery. *Ascaris* eggs were spiked, and equal numbers were recovered after treatment and processing.	Belcher et al., 2015
	Blackwater	Ammonium bicarbonate (119 g/L)	Zinc sulphate (sp. gr. 1.3)	*Ascaris* spp., hookworm spp., *Trichuris* spp., *Hymenolepis nana, Hymenolepis diminuta* and other helminth species were recovered.	Grego et al., 2018
USEPA Method	Faecal sludge	0.1% 7X®	Magnesium sulphate (sp gr. 1.3 rather than 1.2)	There was no report of the method affecting egg viability. *Ascaris* spp., *Trichuris* spp., & *Toxocara* spp. were recovered.	Gantzer et al., 2001; Kone et al., 2007
	Faecal sludge	0.1% 7X®	Magnesium sulphate sp. gr. 1.2)	No report of reagents affecting egg viability. *Ascaris* sp. eggs were recovered.	Capizzi-Banas et al., 2004
	Pig Faeces	None	Zinc sulphate (sp. gr. 1.2)	*Ascaris suum* eggs were recovered.	Katakam et al., 2014
	Faecal sludge	0.1% 7X®	Zinc sulphate (sp. gr. 1.2)	*Ascaris* sp., *Trichuris* spp., *Hymenolepis* spp. & *Toxocara* spp. were recovered. Extraction step with ethyl acetate and acid-alcohol also performed.	Maya et al., 2010; Maya et al., 2012
	Faecal sludge	0.1% Tween®80	Zinc sulphate (sp. gr. 1.2)	*Ascaris* sp. eggs were recovered. Extraction step with ethyl acetate and acid-alcohol also performed.	Nelson & Darby, 2001
Mexican Standard for Wastewater Analysis	Faecal sludge	0.1% 7X®	Zinc sulphate (sp. gr. 1.2)	*Ascaris* sp., was recovered. Extraction step with ethyl acetate and sulphuric acid-ethyl alcohol also performed. Care was taken to minimize exposure of eggs to extraction reagents.	Pecson & Nelson, 2003; Pecson & Nelson, 2005; Pecson et al., 2007
	Wastewater	0.1% 7X®	Zinc sulphate (sp. gr. 1.3)	*Ascaris* sp., hookworm sp., *Trichuris* sp., *Taenia* sp. & *Toxocara* sp. eggs were recovered.	de Victorica & Galvan, 2003

Method	Sample Type/ Matrix	Wash Solution Used	Flotation Solution Used	Observations/Findings	Reference(s)
Bailenger Method	Faecal sludge	0.1% Tween®80	Zinc sulphate (sp. gr. 1.2)	*Ascaris* sp. eggs were recovered. Extraction step with ethyl acetate and acid-alcohol also performed.	Nelson & Darby, 2001
	Wastewater	Tween®80 or Triton®X-100	Zinc sulphate (sp. gr. 1.18)	*Ascaris* sp., hookworm sp., *Trichuris* sp., *Taenia* sp. & *Toxocara* sp. eggs were recovered.	Garcia et al., 2013
	Irrigation water and potatoes	None	Saturated sodium chloride-glucose solution	Helminth eggs were successfully recovered from both sample types. Exact species, however, were not mentioned.	Forslund et al., 2010
	Vegetables	Physiological saline for vegetables and no wash solution for other samples	Sucrose (sp. gr. 1.2)	*Toxocara* sp., *Strongyloides* sp., *Taenia* sp., hookworm, *Trichuris* sp. and *Enterobius* sp. eggs were recovered from samples.	Maikai et al., 2012

Circular Economy

The faecal sludge from the off- and on-site sanitation systems (such as VIP latrines, UDDTs, septic tanks and experimental toilets) should be safely managed to convert it to resource-recoverable by-products. From the perspective of a circular economy, management of faecal sludge should benefit the environment by generating less waste, as well as promoting innovation and developing markets for reuse of the by-products (Rayavellore Suryakumar & Pavithra, 2020). Waste reuse and land application of faecal sludge plays an important role in resource recovery by reintroducing nutrients back into the soil (Amoah et al., 2017). It is also a viable business option that may propagate the sanitation management service chain as well as the local farming trade in rural areas. (Verbyla et al., 2013). Waste must be tested for pathogens prior to reuse to avoid reintroduction of disease-causing organisms into the environment. The WHO recommends 1 egg per litre of wastewater and 1 egg per gram of total dried solids (faecal sludge) as the acceptable limits for the presence of helminth eggs (WHO, 2006). The processing and recovery of helminth eggs in faecal sludge must be consistent if those data are to be comparable.

The provision of potable water and proper sanitation, education in terms of proper hygiene practices, and treatment regimens act as a combined barrier that can disrupt the cycle of poverty (Guigas and Molofsky, 2015). New on-site faecal sludge treatment and toilet technologies are constantly being developed for more effective waste management, partially in response to the 'Reinvent the Toilet Challenge' launched by the Bill & Melinda Gates Foundation (Sutherland et al., 2021). Helminth eggs

are spiked into these system to test treatment efficacy according to the ISO-30500 standard for non-sewered sanitation systems (NSSS). A highly sensitive, standard helminth test method, accredited to the ISO-17025 international standard for testing and calibration laboratories, is therefore required for application in laboratories globally.

Problem Statement

Most pathogen testing in the water and sanitation sector is expensive, resource-intensive and requires specialised skills, whereas helminth testing can offer the opposite. Due to the lack of consistency in helminth testing, however, there is currently an urgent need for the new WRDC Helminth Method to be accredited for acceptance as an internationally recognised standard. The method should be adaptable to sample type, rather than the sample being manipulated to suit the method, and it should accommodate all sample types. It needs to be cost and time efficient so that it is applicable to laboratories in both developed and developing nations. It should also not compromise egg viability or recovery in any way.

Aims and Objectives

The essence of this study was thus the thorough interrogation of the existing PRG Helminth Method against steps of the USEPA Method, the Mexican Standard for Wastewater Analysis and the Bailenger Method, to determine the best chemicals to use and optimal exposure times, the optimal washing mode, centrifugation times and speeds, and the minimum steps that should be used based on sample type. A final optimised method will then be derived and renamed the WRDC Helminth Method for publication in a methods journal.

It should be noted that our laboratory was not specifically set up for the other three methods against which the steps of the PRG Helminth Method were tested and were thus not run in parallel. Such a set up would have been time-consuming and expensive, and with the COVID-19 pandemic and resultant national shutdowns, laboratory access was limited at many stages during sampling and experimentation. We therefore opted to separate the individual steps of the PRG Helminth Method into various experiments, whereby data from one could feed into the design of the next, and then compare the same steps from other methods, to determine the best choices for egg viability and recovery towards recommendations for the final method.

This will be achieved after completion of the following, that all form part of publications 1, 2 and 3, to culminate in publication 4, a standard method:

1) Every reagent used in all common international helminth methods will be tested on fresh, viable *Ascaris suum* eggs for varying time intervals, to determine whether viability of these eggs was

affected or not. The reagents will include wash solutions, flotation solutions, individual extraction solutions, combination extraction solutions, and incubation solutions (included in Publication 1).

2) The best mode of washing, and subsequent centrifugation speeds and times will be tested against egg recovery (included in Publication 2).
3) Based on the data from 1 above, the most efficient flotation solution and optimum specific gravity (sp. gr.) to use will be tested in combination with centrifugation speeds and times to determine which combinations recover the most eggs (included in Publication 2).
4) Based on the best wash solutions from 1, different sample types, including water, WWTW effluent, VIP sludge, UDDT sludge, septic tank sludge, dried sludge, fatty sludge, and soil, will be washed and processed to determine the best wash solution for maximum egg recovery (included in Publication 2).
5) Phase extraction will be done on different sample types to determine the effects on egg recoverability. The necessity for doing this step was also assessed, as the chemicals used here are the least environmentally friendly of those used in all environmental helminth methods (included in Publication 3).
6) The data from all experiments will then be collated to compile one robust method, the WRDC Helminth Method, that is adaptable to various sample types (included in Publication 4).
7) All the above were conducted in statistically significant enough replicates to provide strong evidence for the best procedure to follow to create an accredited method that will stand up to peer scrutiny. The final step after an optimum method is decided upon, will be testing the method for accuracy and reproducibility between technicians of varying capabilities.

After all experimentation in this project is complete, the newly improved WRDC Helminth Method will be implemented at our laboratory henceforth and used when training other water and sanitation laboratories on helminth testing.

CHAPTER 2: PUBLICATION 1

THE EFFECTS OF ALL REAGENTS AND CHEMICALS USED IN EXISTING HELMINTH TEST METHODS ON THE VIABILITY OF *ASCARIS SUUM* EGGS.

Submitted to Journal of Parasitology

THE EFFECTS OF ALL REAGENTS AND CHEMICALS USED IN EXISTING HELMINTH TEST METHODS ON THE VIABILITY OF *ASCARIS SUUM* EGGS

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ABSTRACT

Various helminth test methods are currently in use; however, no single standard exists. Development of such a method firstly involves testing the effects of all reagents used in current methods on helminth egg viability. This study investigated the effects on viability and development of *Ascaris suum* eggs when exposed to: wash solutions (water, ammonium bicarbonate, Tween® 20, Tween® 80, Triton® X-100, Sunlight® Liquid, bentonite and 7X®), flotation solutions (zinc sulphate, magnesium sulphate, sodium nitrate, brine, and sucrose), extraction solutions (10% formalin, acetoacetic buffer, acid-alcohol, ethyl acetate and diethyl ether), extraction combinations (10% formalin + ethyl acetate, 10% formalin + diethyl ether, acetoacetic buffer + ethyl acetate, acetoacetic buffer + diethyl ether and acid-alcohol + ethyl acetate) and incubation solutions (water, 0.1N sulfuric acid, physiological saline and 0.5%, 2% and 5% formalin), for various time periods. Ammonium bicarbonate and 7X® performed best as wash solutions (including for overnight soaking), and zinc sulphate is recommended for flotation up to 30 minutes (min) exposure time. Individually, all extraction solutions had minimal effects on egg viability, and in combination, acid-alcohol and ethyl acetate was successful, for up to 15 min exposure time. Sulfuric acid allowed optimal egg development and clear samples for microscopy, post-incubation.

Keywords: Extraction; flotation; helminth; incubation; viability; wash solution

INTRODUCTION

Globally, approximately 2 billion people lack access to potable water, 3.6 billion to safely managed sanitation, 2.3 billion lack basic hygiene practices, and 1.5 billion people are infected with soil-transmitted helminths (STHs) (United nations, 2021). The provision of improved sanitation can reduce

STH prevalence by 36%, thus many non-sewered sanitation systems have been developed for implementation in underprivileged communities. These systems are not water dependent and can treat waste on-site, consequently supporting resource recovery (Sutherland et al., 2021). The South African government considers the provision of potable water and improved sanitation a priority development goal, thus working towards alleviating diarrheal disease transmission (Friedrich et al., 2015).

The four categories of pathogens found in fecal waste are bacteria, viruses, protozoans, and helminths. Adult helminths (worms) live in the host's gut, but eggs passed in feces are either directly infective or require time in the soil to develop to the infective stage (geohelminths) (Sidhu and Toze, 2009). *Ascaris* spp. are the most resilient of all helminths and are therefore used as an indicator organism when testing for pathogen inactivation. Due to ethical and logistical issues, it is often difficult to source *Ascaris lumbricoides* (human roundworm) eggs, thus eggs of the pig roundworm, *Ascaris suum*, are used as a surrogate. Both species are morphologically identical in all developmental stages (Daugschies et al., 2013).

One of the Millennium Development Goals (MDGs) aimed at halving the number of people living without access to sustainable sanitation and potable water (Friedrich et al., 2015; Moe and Rheignans, 2006). Sustainable Development Goal (SDG) 6 is a continuation of this MDG but includes the improvement of water quality by reducing water pollution, improved water usage efficiency, ecosystem protection, community involvement, and water and sanitation management, and refers to all countries worldwide rather than just to developing nations. SDG 6 also focuses on the development of reuse technologies (Hoekstra et al., 2017), and reuse depends on the complete hygienization of waste material by pathogen removal or destruction (Verbyla et al., 2013).

New fecal sludge treatments and toilet technologies are constantly being developed, where helminth eggs must be spiked into the system to test treatment/ inactivation efficacy. The processing and recovery of helminth eggs in fecal sludge should be consistent so that data are comparable. Different laboratories and groups have used variations of the standard United States Environmental Protection Agency (USEPA) Method (2003), the Mexican Standard Method for Wastewater Analysis (2012), the Bailenger Method (1996) and the Pollution Research Group (PRG) Helminth Method (2017) for helminth testing (Ayres and Mara, 1996; USEPA, 2003; Secretaría de Economía, 2012; Velkushanova et al., 2021). According to the WHO (2006), the recommended limit for helminth eggs should be: 1 egg per liter of wastewater and 1 egg per gram of total dried solids (fecal sludge), however, counts also vary from country to country (Maya et al., 2012). This variation in egg load, and the fact that egg viability is not taken into consideration calls for these limits to be re-evaluated. There is also no single

"gold standard" in existence for helminth egg recovery and analysis from sanitation and environmental samples. A highly sensitive, standard isolation and enumeration method is therefore required for application in laboratories globally.

The basic principles of sample processing and egg recovery are standard across the various methods currently in existence: washing and sedimentation (using a surfactant or wash solution), flotation (utilizing density gradients to allow eggs to separate from particulate matter), extraction (phase separation using lipophilic and hydrophilic solutions to trap organic matter between two phases), incubation (to determine egg viability) and microscopic analysis (before and after incubation using a light microscope) (Amoah et al., 2017a; Rocha et al., 2016). Table I summarizes all reagents and chemicals used in existing methods.

Table I: Chemicals and reagents tested in this study, and corresponding methods and literature. Additional references were only included from studies that modified an existing method or included a single processing step from a method.

Chemical/ Reagent	Step of method used in	Related method or reason for testing	Reference(s) (in alphabetical order)
Water	Washing/ Sedimentation	PRG Helminth Method	Velkushanova et al., 2021
Ammonium bicarbonate	Washing/ Sedimentation	PRG Helminth Method	Velkushanova et al., 2021
Tween® 20	Washing/ Sedimentation	Used as an alternative wash solution	Paquet-Durand et al., 2007
Tween® 80	Washing/ Sedimentation	PRG Helminth Method; Mexican Standard for Wastewater Analysis; Bailenger Method	Ayres and Mara, 1996; Secretaría de Economía, 2012; Velkushanova et al., 2021
Triton® X-100	Washing/ Sedimentation	Bailenger Method	Ayres and Mara, 1996
Bentonite	Washing/ Sedimentation	Used to help separate eggs from pig faeces	Burden and Hammet, 1976
7X®	Washing/ Sedimentation	PRG Helminth Method; USEPA Method	USEPA, 2003; Velkushanova et al., 2021
Zinc sulphate ($ZnSO_4$)	Flotation	PRG Helminth Method, Mexican Standard for Wastewater Analysis; Bailenger Method	Ayres and Mara, 1996; Secretaría de Economía, 2012; Velkushanova et al., 2021
Magnesium sulphate ($MgSO_4$)	Flotation	USEPA Method	USEPA, 2003
Sodium nitrate ($NaNO_3$)	Flotation	Used as a cheaper alternative flotation solution (saturates easily)	Mizgajska, 1997; Dubná et al., 2007; Rosa Xavier et al., 2010

Chemical/ Reagent	Step of method used in	Related method or reason for testing	Reference(s) (in alphabetical order)
Sodium chloride solution (NaCl)	Flotation	Used as a cheaper alternative flotation solution (saturates easily).	Faust et al., 1939; Gaspard et al., 1996; Forslund et al., 2010; Sengupta et al., 2011; Yaya-Beas et al., 2016
Sucrose solution	Flotation	A possible cheaper alternative flotation solution	Maikai et al., 2012; Horiuchi et al., 2013; Fallah et al., 2016
10% Formalin + Ethyl acetate	Extraction	PRG Helminth Method	Velkushanova et al., 2021
10% Formalin + Diethyl ether	Extraction	PRG Helminth Method	Velkushanova et al., 2021
Aceto-acetic buffer + Ethyl acetate	Extraction	Bailenger Method	Ayres and Mara, 1996
Acid-alcohol + ethyl acetate	Extraction	Old USEPA Method; Mexican Standard for Wastewater Analysis	USEPA, 1999; Secretaría de Economía, 2012
0.1N Sulfuric acid (H_2SO_4)	Incubation	PRG Helminth Method; USEPA Method; Mexican Standard for Wastewater Analysis	USEPA, 2003; Secretaría de Economía, 2012; Velkushanova et al., 2021
0.5 - 5% Formalin	Incubation	PRG Helminth Method	Bowman et al., 2003; Velkushanova et al., 2021

To develop such a standard method, the first step would be to evaluate the effects of the listed chemicals and reagents on helminth egg viability, as the chemicals in a method should have no impact on the outcome of the test. The choice of wash solution plays a critical role in the number of eggs recovered from a given sample type (Rocha et al., 2016), however, the effects of wash solutions alone on egg viability have not been widely explored. Some studies have shown that long term exposure to some of these chemicals compromises the viability of eggs. Gaspard et al. (1996) and Smith (1991) state respectively that exposure to zinc sulphate and magnesium sulphate, used as flotation solutions, can be toxic to eggs. Nelson and Darby (2001) state that extraction solutions, such as ethyl acetate and diethyl ether, reduce egg viability. The present study was therefore aimed at determining these effects and which chemicals and reagents perform best and can thus be recommended for inclusion in an international standard processing and enumeration method.

MATERIALS AND METHODS

Chemical exposure

Testing was separated into 5 different experiments and the viability of *Ascaris suum* eggs was quantified. Over and above the chemicals and reagents highlighted in Table I, others were included in experimentation to ensure a well-rounded dataset. Exposure times were chosen based on existing

methods, as well as logical time frames needed to complete the intended steps when performing the method (including centrifugation times), and all combinations were done in replicates of 5. *Ascaris suum* eggs were isolated from faeces of research pigs.

Wash solutions: Experiment 1 involved the exposure of eggs to various wash solutions for different periods of time, including overnight soaking that is necessary for very dry sample types. Solutions that were tested included ammonium bicarbonate (at a concentration of 119 g/L), 0.1% Tween® 20, 0.1% Tween® 80, 0.1% 7X®, 1% Triton® X-100, 0.1% Sunlight® Liquid (a commonly used South African brand of dishwashing liquid), 1% bentonite suspension, and tap water as a control. Exposure times included 10 minutes (min), 30 min, 2 hours (hr), 6 hr, and 24 hr.

*Flotation solutions***:** Experiment 2 involved the exposure of eggs to commonly used flotation solutions, including zinc sulphate (specific gravity (sp. gr.) 1.3), magnesium sulphate (the solution saturated at sp. gr. 1.25, and formed crystals at the bottom of the bottle when stored), sodium nitrate (sp. gr. 1.3), sodium chloride (saturated at sp. gr. 1.18 even when the solution was heated), and sucrose (saturated at sp. gr. 1.2), for 30 min, 1 hr and 2 hr.

Extraction solutions: These are usually used in combinations of a preservative-type solution plus a solvent, thus Experiment 3 involved the exposure of eggs to all extraction solutions separately first, to determine the individual effect of each solution on egg viability, then Experiment 4 explored these solutions in combinations, to simulate those used in actual extraction processes. Solutions included 10% formalin, aceto-acetic buffer, acid-alcohol, ethyl acetate and diethyl ether, individually, and in combinations of 10% formalin and aceto-acetic buffer each with ethyl acetate and diethyl ether, and acid-alcohol with ethyl acetate, for 15 min, 30 min and 1 hr.

Incubation solutions: The final experiment (number 5) involved the exposure of eggs to various incubation solutions: tap water, physiological saline, 0.1 N sulfuric acid, and 0.5%, 2%, and 5% formalin for 28 days.

Egg stock solutions were made up in deionized water, with an approximate egg count of 300 eggs per milliliter (ml) of suspension. Approximately 13 ml of each test chemical was pipetted into 15 ml conical, graduated, plastic test tubes (Falcon tubes), and 1ml of well mixed egg stock was then spiked into each sample, and exposure times were monitored.

Sample processing and microscopic analysis

Test tubes containing samples and the respective test chemicals were then poured over a 20 µm sieve (100 mm diameter) after each sample's exposure times elapsed. The test tube was rinsed several times and poured over the sieve. The sample was then thoroughly washed with tap water to ensure

that all residue from the wash solution was removed, before collecting the retentate back into the same (well rinsed) test tube and centrifuging at 3000 rpm (1512 x g) for 5 min. The supernatant was subsequently discarded, and the pellet was analyzed using light microscopy, after which the sample was washed back into the test tube, incubated for 28 days at 25 - 27°C in water, then re-examined microscopically. Eggs were categorized as potentially viable: undeveloped (one-celled), developing (two-celled to gastrula), motile (plump, developed larva that moved) and immotile (plump, developed larva that did not move); and non-viable: necrotic (thin, shriveled larva), dead (globular, ruptured, or with irregular contents), and infertile (unfertilized).

For the incubation solution samples, an initial check of the egg stock was done using light microscopy (as detailed above) in triplicate, to produce an average initial egg count and reference point for post-incubation samples (i.e., initial viability as a percentage of the total egg count). Eggs (1 ml aliquots of the stock solution) were pipetted into 15 ml Falcon tubes. The tubes were centrifuged at 3000 rpm (1512 x g) for 5 min, the supernatant was discarded, and 2 ml of each incubation solution was added to the respective tubes. Wooden applicator sticks were used to dislodge the pellets to allow for eggs to be fully exposed to the incubation solution. The tubes were then incubated for 28 days at 25 - 27°C and subsequently analyzed to determine egg development and viability according to the categories described above.

Statistical analyses

Data from all experiments were statistically analyzed using the Kolmogorov-Smirnov test for normality of data, followed by two-way ANOVAs together with the Shapiro-Wilk test for normality of residuals and Levene's test for homogeneity of variance of residuals from the ANOVA. The analyses were run on IBM SPSS Statistics (version 25, IBM Corp., Armonk, NY, USA) and R (version 3.5.2. R Core Team 2018). Percentage viability was calculated as follows:

*Potential viability (PV) = (Total viable eggs recovered/Total number of eggs recovered) *100 (equation 1)*

*Actual viability (AV) = (Total larval eggs recovered/Total number of eggs recovered) *100 (equation 2)*

Potential viability refers to the percentage of viable eggs before incubation, and thus includes undeveloped and developing eggs, whilst actual viability refers to the percentage of eggs that reached the larval stage after incubation. The criterion set for successful inactivation for this study was < 10% viable eggs recovered after treatment (Naidoo et al., 2016).

RESULTS

Samples were exposed to the respective test chemicals and then thoroughly rinsed. The pH measurements indicated in the tables below are therefore of the chemicals that eggs were exposed to for each time period, however after the time elapsed, all eggs were returned to water (neutral pH) for microscopy and subsequent incubation. Biological development, referred to below as contamination, was noted and differentiated from debris based on what the sample looked like before and after incubation, as debris would have appeared in both instances, but growth only after incubation. After incubation, eggs from some of the exposure times sometimes appeared “webby”, “matted” or clumped. Furthermore, egg development was halted in these cases and eggs looked damaged. Inconsistent fluctuations in actual viability after incubation can be seen across exposure times for some of the experiments below, and this was attributed to contamination and resultant egg damage.

Wash solutions

Data indicated that the wash solution alone had no significant effect on potential egg viability (P = 0.790), but a significant effect was observed on actual viability ($P < 0.001$). Exposure time alone had a significant effect on both potential and actual viability (P = 0.04 and $P < 0.001$ respectively). The interactive effects of wash solutions combined with exposure times were also significant for both potential and actual viability (P = 0.027 and $P < 0.001$ respectively). Eggs looked healthy (mostly at the one-celled, undeveloped stage) when analyzed directly after reagent exposure, accounting for the high potential viability figures for all reagents and exposure times, however, not all these eggs developed to the larval stage after incubation. Table II shows that across exposure times, ammonium bicarbonate and 7X® allowed for the highest larval development of eggs. The samples looked the healthiest, with minimal contamination and excellent development, where larvae were plump and motile. The water samples resulted in web-like contamination after incubation, causing severe clumping of eggs. The same ‘webiness’ was seen in Tween® 20 and Tween® 80, as well as eggs appearing dark and in an unhealthy state. Sunlight® Liquid and Triton® X-100 samples were clear and easy to analyze, displayed good egg development, and performed second best. Bentonite samples were very particulate and therefore messy to analyze, with severe contamination (hyphae visible), and eggs were dark.

Table II: Effects of wash solutions on the potential and actual viability (%) of *Ascaris suum* eggs.

Wash solution	pH	Exposure time									
		10 min		30 min		2 hr		6 hr		24 hr	
		PV ± SD	AV ± SD	PV ± SD	AV ± SD	PV ± SD	AV ± SD	PV ± SD	AV ± SD	PV ± SD	AV ± SD
Water	7.45	86.4 ± 3.4	37.6 ± 17.2	83.2 ± 1.4	58.4 ± 30.9	88.9 ± 1.8	39.7 ± 26.5	84.8 ± 7.6	39.6 ± 32.6	81.8 ± 2.1	35.6 ± 15.6
Ammonium bicarbonate	8.51	85.4 ± 4.0	54.3 ± 20.3	84.5 ± 2.4	77.5 ± 5.7	86.9 ± 1.0	75.8 ± 4.6	81.3 ± 3.2	79.7 ± 1.4	85.3 ± 1.5	72.7 ± 6.9
Tween® 20	3.36	88.4 ± 2.9	40.2 ± 14.9	89.6 ± 2.0	53.6 ± 19.3	83.3 ± 1.4	34.7 ± 21.0	84.3 ± 2.5	67.8 ± 18.5	84.3 ± 1.2	32.0 ± 16.0
Tween® 80	4.05	84.4 ± 4.1	27.9 ± 27.7	88.1 ± 4.6	65.8 ± 16.3	83.4 ± 3.0	35 ± 28.1	83.6 ± 2.5	65.1 ± 30.3	87.6 ± 3.4	11.5 ± 9.5
Sunlight® Liquid	7.87	87.1 ± 2.0	18.4 ± 7.3	88.8 ± 2.1	74.2 ± 7.2	87.4 ± 3.9	46.4 ± 24.5	87.8 ± 3.3	74.3 ± 9.2	84.8 ± 2.2	42.9 ± 19.4
Triton® X-100	6.33	85.7 ± 1.9	8.7 ± 9.3	84.8 ± 2.6	77.4 ± 3.7	87.3 ± 1.2	38.0 ± 33.3	86.1 ± 2.6	59.9 ± 20.6	88.1 ± 4.0	33.6 ± 19.6
Bentonite	8.00	86.9 ± 1.6	2.6 ± 4.5	89.9 ± 2.7	71.6 ± 8.2	89.7 ± 1.4	24.5 ± 17.0	66.8 ± 37.4	8.4 ± 6.3	85.6 ± 2.6	8.2 ± 6.9
7X®	4.76	87.2 ± 1.9	79.6 ± 5.0	82.1 ± 7.1	82.0 ± 2.9	86.4 ± 4.1	80.8 ± 3.8	86.2 ± 5.1	82.2 ± 1.5	83.8 ± 2.0	79.7 ± 5.9

PV – Potential viability; AV – Actual viability; n = 5

Flotation solutions

Flotation solution alone had a significant effect on both potential viability and actual viability of eggs ($P < 0.001$). Exposure time alone had a significant effect on both potential and actual viability ($P < 0.001$). The interactive effects of flotation solutions combined with exposure times were also significant for both potential and actual viability ($P < 0.001$). As mentioned above, eggs that appeared healthy directly after reagent exposure accounted for the high potential viability figures, however percentage larval development was not as high after incubation. Table III indicates that at 15 min, zinc sulphate, magnesium sulphate and sodium nitrate resulted in the highest percentage larval development.

Table III: Effects of flotation solutions on the potential and actual viability of *Ascaris suum* eggs.

Flotation solution	pH	Exposure time					
		15 min		1 hr		2 hr	
		PV ± SD	AV ± SD	PV ± SD	AV ± SD	PV ± SD	AV ± SD
Zinc sulphate	4.49	88.9 ± 1.5	66.0 ± 10.4	82.9 ± 2.3	18.0 ± 5.5	81.6 ± 3.0	36.8 ± 12.1
Magnesium sulphate	6.04	87.6 ± 1.3	69.0 ± 9.7	85.0 ± 3.7	16.1 ± 14.3	82.3 ± 0.9	32.1 14.1
Sodium nitrate	7.35	87.5 ± 1.3	67.4 ± 5.4	69.8 ± 5.5	15.0 ± 2.7	80.4 ± 1.9	28.9 ± 9.0
Sodium chloride	6.63	78.6 ± 2.9	34.2 ± 9.4	80.6 ± 1.9	9.9 ± 7.6	75.0 ± 2.1	14.5 ± 2.6
Sucrose	6.85	82.8 ± 2.6	23.6 ± 3.8	85.9 ± 1.2	6.1 ± 1.9	83.3 ± 3.3	18.3 ± 14.3

PV – Potential viability; AV – Actual viability; n = 5

Exposure times greater than 15 min resulted in poor larval development, and sucrose and sodium chloride resulted in severe contamination of the samples post-incubation and very low larval development (Table III). Sodium chloride samples resulted in halted/ terminated development of eggs, whilst sucrose caused samples to appear murky and slimy.

Extraction solutions (individual exposure)

Data indicated that the extraction solution alone and exposure time alone had a significant effect on potential egg viability ($P = 0.044$ and $P = 0.001$, respectively) and on actual viability ($P < 0.001$ for both). The interactive effects of extraction solutions combined with exposure times had no significant effect on potential viability ($P = 0.095$), but a significant effect was seen on actual viability ($P = 0.007$). Potential viability figures across all extraction solutions and exposure times were ± 80%, however actual viability decreased for samples exposed to the solvents for time periods > 15 min. The 10% formalin samples were clear to look at with minimal development of contamination. Aceto-acetic buffer resulted in blackened eggs that appeared damaged with some fungal contamination, and samples were not as clear as those exposed to formalin. Ethyl acetate also caused blackening of the eggs with extensive contamination, but samples were clear of debris and easy to analyze. Diethyl ether caused some eggs to rupture and displayed extensive contamination, with eggs looking the worst when compared with all 5 flotation solution exposures. Acid-alcohol caused hatching of eggs after incubation.

Table IV: Effects of extraction solutions (individually) on the potential and actual viability of *Ascaris suum* eggs.

Extraction solution	pH	Exposure time					
		15 min		30 min		1 hr	
		PV ± SD	AV ± SD	PV ± SD	AV ± SD	PV ± SD	AV ± SD
10% Formalin	4.46	79.8 ± 4.1	71.3 ± 5.8	85.3 ± 2.3	44.1 ± 22.8	83.3 ± 4.5	62.3 ± 4.1
Aceto-acetic buffer	5.12	79.7 ± 2.5	72.9 ± 11.4	86.9 ± 2.1	14.6 ± 5.9	84.6 ± 4.4	13.8 ± 25.5
Acid-alcohol	1.74	85.5 ± 3.2	82.1 ± 4.0	88.0 ± 2.3	80.2 ± 1.0	84.9 ± 2.8	81.1 ± 2.9
Ethyl acetate	5.15	81.7 ± 1.8	59.1 ± 32.9	83.4 ± 3.2	39.3 ± 22.7	84.1 ± 1.5	40.0 ± 28.3
Diethyl ether	4.89	82.7 ± 1.8	47.5 ± 19.6	84.4 ± 1.4	25.8 ± 8.3	83.4 ± 2.6	14.9 ± 12.9

PV – Potential viability; AV – Actual viability; n = 5

Extraction solutions (combinations)

The effect of extraction combination alone was significant for potential egg viability but not for actual viability ($P < 0.001$ and $P = 0.279$, respectively). Exposure time alone had a significant effect on both potential and actual viability ($P < 0.001$ and $P = 0.002$, respectively). The interactive effect of extraction combination with exposure times was also significant for both potential and actual viability ($P = 0.002$

and $P = 0.021$). As seen with the individual exposures, 15 min resulted in the best development of eggs. Actual viability then fluctuated for longer exposures, and although figures may appear similar, the condition of the eggs deteriorated. The formalin and ethyl acetate samples looked clear, were easy to analyze and the developed eggs looked healthy. The formalin and diethyl ether samples resulted in blackened eggs and some contamination was evident. Aceto-acetic buffer and ethyl acetate samples were clear, but some contamination was evident. Aceto-acetic buffer and diethyl ether samples were also easy to analyze but eggs again appeared blackened and damaged, post-incubation. Acid-alcohol and ethyl acetate resulted in extensive hatching of eggs.

Table V: Effects of extraction solutions (in combination) on the potential and actual viability of *Ascaris suum* eggs.

Extraction solution (combinations)	**Exposure time**					
	15 min		30 min		1 hr	
	PV ± SD	AV ± SD	PV ± SD	AV ± SD	PV ± SD	AV ± SD
10% Formalin + ethyl acetate	78.8 ± 1.1	74.8 ± 4.2	81.9 ± 2.5	61.5 ± 15.6	82.5 ± 2.0	74.9 ± 11.4
10% Formalin + diethyl ether	78.5 ± 2.4	68.1 ± 20.8	80.0 ± 3.3	75.3 ± 6.6	80.9 ± 2.5	65.0 ± 27.1
Acid-alcohol + ethyl acetate	89.6 ± 3.8	79.4 ± 4.7	91.7 ± 1.2	81.3 ± 4.1	87.0 ± 2.6	74.0 ± 5.8
Aceto-acetic buffer + ethyl acetate	78.6 ± 1.2	76.9 ± 6.6	85.6 ± 2.8	61.8 ± 11.6	86.9 ± 3.5	78.2 ± 5.0
Aceto-acetic buffer + diethyl ether	76.3 ± 3.8	75.3 ± 5.0	78.0 ± 3.2	46.7 ± 30.1	82.4 ± 3.9	81.2 ± 5.9

PV – Potential viability; AV – Actual viability; n = 5

Incubation solutions

Table VI: Effects of incubation solutions on the potential and actual viability (%) of *Ascaris suum* eggs.

Incubation solution	**pH**	**Incubation solution**	
		PV ± SD	AV ± SD
Initial egg stock assessment	N/A	88.9	NA
Water	7.45	85.0 ± 4.3	82.6 ± 4.5
Sulfuric acid	1.47	81.5 ± 4.2	79.6 ± 4.3
Physiological saline	5.94	82.1 ± 3.5	78.5 ± 4.1
0.5% Formalin	5.34	84.8 ± 2.6	81.3 ± 2.5
2% Formalin	4.16	78.5 ± 3.7	74.6 ± 4.9
5% Formalin	4.57	65.5 ± 3.7	57.9 ± 4.5

PV – Potential viability; AV – Actual viability; n = 5

A significant effect was seen on both potential and actual viability when looking at the incubation solution used ($P < 0.001$). Samples incubated in water and physiological saline were contaminated and eggs were caught in webby clumps that made microscopic analysis difficult. Sulfuric acid samples were very clean and easy to analyze, and eggs were well developed with plump, motile larvae. The formalin

samples displayed some contamination, the most being at a concentration of 0.5% and the least at 5%, but eggs looked increasingly damaged as the formalin concentration increased.

DISCUSSION

A variety of methods exist for the isolation and enumeration of helminth eggs from environmental and sanitation samples, but some were designed for wastewater samples and are time-consuming and consumables-heavy (USEPA Method and Mexican Method for Wastewater Analysis), whilst some are not robust enough to isolate all helminth eggs, e.g., the Bailenger Method (Ayres and Mara, 1996; USEPA, 2003; Secretaría de Economía, 2012). The chemicals and reagents in these methods may have inhibitory or detrimental effects on helminth eggs. The development of a single, cost efficient and inclusive method is therefore essential.

Ammonium bicarbonate and 7X® performed the best across all exposure times when looking at the effects of the 8 wash solutions on egg viability, indicating that even prolonged soaking of samples in a wash solution is possible. Furthermore, these solutions resulted in clean deposits post-incubation with minimal development of biological contaminants, indicating that these could have sufficient antimicrobial properties to thoroughly clean samples prior to incubation, for viability assessment. Tween®80 and Tween®20 both affected egg viability and samples developed contamination that could have further impacted viability figures. Whilst many studies focus on the effects of wash solutions to recover helminth eggs from different sample types, few have focused on the isolated effects of the solutions on egg viability. Ravindran et al., (2019) stated that 7X® does not form a precipitate when reacting with a flotation solution, thus making it a good dissociation solution. Amoah et al., (2017b) also stated that ammonium bicarbonate and Tween®80 did not affect egg viability (71.1% and 87.9%) and were successful for egg recovery, but prolonged exposure to the latter did damage eggs, which was in line with the findings of this study. Data also suggest that Triton®X-100 and Sunlight® Liquid may be used as alternatives to ammonium bicarbonate and 7X®, but for shorter exposure times, not exceeding 6 hr. Naidoo et al., (2016) reported that sodium hypochlorite-based detergents damage eggs, reduce egg viability, thin out the eggshell and initiate hatching of larval eggs, therefore any surfactant with this as the active ingredient would be ineffective as a wash solution.

When looking at the ideal flotation solution(s), egg viability was similar for zinc sulphate, magnesium sulphate and sodium nitrate when exposed for 15 min, with ± 67% forming viable larvae. The difference between potential and actual viability could be attributed to the contamination that formed after incubation, as samples were not processed with a wash solution, which would generally reduce microbial activity. After 15 min of exposure, actual viability dropped quite rapidly, indicating that exposure time played a role in egg development and should be kept to a minimum. Gaspard et al.

(1996) stated that prolonged exposure to flotation solutions may damage the eggs due to the inhibitory nature of some salts on egg development, thus reduced exposure times were advised.

Nelson and Darby (2001) and Amoah et al., (2017b) reported similar findings, that zinc sulphate, magnesium sulphate and sodium chloride did not affect egg viability at sp. gr. 1.2 (81.2%, 85.0% and 83.9%) and sp. gr. 1.3 (88.5%, 88.5%, and not-applicable), respectively. We favored zinc sulphate over magnesium sulphate and sodium nitrate. Magnesium sulphate precipitated into salt crystals over time but also proved difficult in terms of reaching a sp. gr. of 1.3 and precipitated at sp. gr. 1.25. Smith (1991) reported that exposure to magnesium sulphate was toxic to eggs, whilst Amoah et al., (2017b) further elaborated that increased exposure time exacerbated the toxic effects. Sodium nitrate samples were more particulate, indicating a reduced ability to separate eggs from finer debris and resulted in extensive contamination post incubation. Gaspard et al. (1996) recommended a double flotation with sodium chloride as the solution was found to have no toxic effects on eggs, however, it was found that the solution precipitated out before reaching sp. gr. 1.3 even when heated. Karkashan et al., (2015) reported 85.8% viability when eggs were processed with 7X® as the wash solution for at least 1 hr and $MgSO_4$ as the flotation solution for at least 5 min, however, the individual effects on egg viability were not mentioned.

The extraction step aims to remove any protein and lipid contaminants from the remaining sample containing helminth eggs (Rocha et al., 2016), with some methods recommending it before flotation (Bailenger Method), and some after (old USEPA Method (1999) and Mexican Standard for Wastewater Analysis). Data from this study indicated that whilst the hydrophilic solutions had little to no effect on egg viability (both potential and actual), with acid-alcohol affecting egg development the least, viability was reduced when eggs were exposed to ethyl acetate and diethyl ether (lipophilic solvents). In combination, acid-alcohol and ethyl acetate performed the best after 15 min of exposure, with a potential viability of 89.6% and actual viability of 79.4%. Acetoacetic buffer and ethyl acetate also produced promising results, but all combinations were unable to yield an actual viability figure > 80%, possibly due to the synergistic effects of the hydrophilic and lipophilic solutions on the eggshell. Nelson and Darby (2001) reported that acid-alcohol alone resulted in the inactivation of eggs (52.2% viability), whilst diethyl ether alone did not have any effect on the viability of eggs (85.3%), contradicting the findings of our study. Acid-alcohol and diethyl ether, acid-alcohol and ethyl acetate and acetoacetic buffer and diethyl ether combination extractions resulted in 85.7% and 26.5%, 82.4% and 4%, and 84.5% and 87.2% viability after 2 and 30 min of exposure, respectively (Nelson and Darby, 2001). Amoah et al., (2017b) reported 74.4% and 3% egg viability for ethyl acetate and acetoacetic acid alone respectively, and 13.2% and 59.1% for acetoacetic acid and ethyl acetate and acetoacetic acid and formalin combinations, respectively. Although the latter is not a combination of a hydrophilic

and a lipohilic reagent, but rather 2 hydrophilic reagents, this study did explore the effects of the same chemicals that we had tested, and individual findings aligned with the findings of this study, as acetoacetic buffer combinations were found to be less toxic to eggs than those combined with formalin.

Nelson and Darby (2001) therefore recommended that exposure time to the extraction combination (acetoacetic buffer and diethyl ether in this case) should be limited to 30 min and samples should be rinsed before incubation. After 15 min exposure, the viability figures fluctuated across the extraction combinations in this study. Although potential viability was > 80% and actual viability was > 65% after an hour of exposure to the combinations, the prolonged effects on the eggshell must be considered. Nelson and Darby (2001) reported that acid-alcohol, both alone and in combination with diethyl ether, reduced egg development as a result of increased eggshell permeability. It is possible that solvents also increase permeability by interfering with the lipid layer of the eggshell (Nelson and Darby, 2001). We therefore recommend that exposure time is a maximum of 15 min, and samples should be rinsed prior to incubating. Nelson and Darby (2001) also reported 30% egg viability after extraction of sludge samples with acid-alcohol and diethyl ether, whilst acetoacetic-buffer and diethyl ether extractions did not reduce egg viability. We found that acid-alcohol was the least toxic to egg development, both alone and in combination with ethyl acetate even up to an hour of exposure. It is therefore recommended as a preferred hydrophilic solution for extractions, with acetoacetic buffer being the second-best option.

Satchwell (1986) reported a reduction in contaminants and debris in samples when extracted using a formaldehyde and diethyl ether combination, however the result was a 95% reduction in egg viability. Diethyl ether is hazardous to human health and more flammable. Ethyl acetate is thus recommended as a suitable alternative, as it has a lower flash point and is more effective in recovering eggs (Rude et al., 1987). Data from this study indicated that diethyl ether was more toxic to eggs and should therefore be replaced with ethyl acetate for extractions. Nelson and Darby (2001) stated that egg recovery was 48% lower in extracted sludge samples, and therefore recommended that the extraction step be removed from sample processing where possible. The present study focused solely on the effects of reagents on egg viability, thus eggs were not spiked into sludge samples and recovery was not reported. So, based on our data, extractions performed using acid-alcohol and ethyl acetate, at the minimum exposure time caused the least amount of damage to *Ascaris* eggs.

An ideal incubation solution should have antimicrobial properties thereby inhibiting bacterial and fungal growth that could interfere with egg development as incubation success is dependent upon the composition of the medium in which eggs are allowed to develop (Cruz et al., 2012). We found that

sulfuric acid resulted in the best egg development (81.5% potential and 79.6% actual viability), where eggs looked healthy, larvae within were motile, minimal contamination had developed and samples were easy to analyze. Oksanen et al., (1990) found 90-97% viability for eggs incubated in tap water and sulfuric acid, and 88% viability in 1% formalin, with slower egg development. Nelson and Darby (2001) reported 72.8%, 82.4% and 82.7% viability in water, sulfuric acid and 0.5% formalin respectively, and Pecson and Nelson (2005) reported 95% viability in sulfuric acid. Amoah et al., (2017b) reported 89.7% viability in 0.5% formalin, all of which support the findings of this study. The highest actual viability seen in the present study was in samples incubated in tap water (85% potential and 82.6% actual viability), however, the eggs clumped together and made sample analysis very difficult. The same can be said for 0.5% formalin, and eggs were not as clear and healthy with plump larvae. Studies have also indicated that fungal development could be toxic to eggs, as some strains possess ovicidal properties that could halt egg development (Ciarmela et al., 2002; Ferreira et al., 2011; Blaszkowska et al., 2014). Furthermore, microbial development results in oxygen competition that may further damage eggs (Gaspard et al., 1996).

Gaspard et al. (1996) looked at incubating eggs in bacteriological culture media that resulted in the development of bacteria around eggs. The culture media were treated with organic compounds that had inhibitory effects on egg development. Similarly, organic material that promotes bacterial development is present in sanitation samples and could therefore explain contamination development post-incubation, subsequent inhibition of development, and toxicity to eggs.

Many studies have looked at the effects of elevated pH on the inactivation of helminth eggs (Ghiglietti et al., 1997; Bina et al., 2004; Pecson and Nelson, 2005) but few have focused on lower pH. Ghiglietti at al. (1997) and Pecson and Nelson (2005) showed that a 99% inactivation rate was achieved at pH levels > 11 when exposed for 90 days. Bina et al., (2004), however, showed that lime treatment at pH 11-12 for 5 days, did not successfully inactivate eggs. Naidoo et al., (2016) reported that certain detergents with a pH > 11.5 were also successful in killing off eggs when exposed for relatively short periods of time. Inactivation required high pH levels, mostly prolonged exposure times (at least a month) or both for successful inactivation. The reagents tested in this study went up to a maximum pH of 8.51, and eggs were exposed for up to a day, thus high pH levels were most likely not what caused egg damage in the present study.

Butkus et al., (2011) looked at the ability of short-chain fatty acids to inactivate *Ascaris suum* eggs at a pH range of 2-7. It was found that most of the acids did not inactivate eggs, however, pentanoic, butanoic, and propanoic acid, as well as some acid mixtures thereof at very high concentrations and low pH levels were successful after 20 hr of exposure. When looking at the acidic scale, the pH values

of the tested reagents and chemicals in this study varied between 4 and 7, with the exception of acid alcohol and sulfuric acid (1.74 and 1.47), both of which performed the best in their respective experiments with regards to egg viability. This, and the fact that the highest exposure time was 24 hr in the wash solution experiment, indicates that low pH (and prolonged exposure) would most likely not be the driving factor behind egg inactivation, when looking at the correlation between pH and percentage viability in each experiment. Furthermore, pH of the stomach is strongly acidic (between 1 and 2), but pH of the gastrointestinal tract is 6.5–7.5 (Fallingborg, 1999). *Ascaris* eggs would therefore be adapted to very low pH values, as well as to moderate increases in pH, further supporting the reasoning that pH did not cause the reduction in egg viability.

CONCLUSION

Recent studies have aimed at developing more modern enumeration techniques, such as qPCR (real-time PCR) for the molecular detection of the presence of helminth eggs (DNA) in fecal sludge (Gyawali et al., 2015), and the BacLight staining technique that requires a specialized confocal microscope for helminth egg quantification (Karkashan et al., 2015). These more sophisticated methods are not always applicable to small laboratories in developing countries, where a constant supply of electricity, financial support, or specialized equipment and reagents may be lacking for the successful implementation of such techniques. Furthermore, these techniques might have shortfalls that have not yet been solved, e.g., PCR can only give an indication of the presence or absence of eggs, and not their viability status (an indicator of risk to human health) (Amoah et al., 2017a). A simple, cost- and labor-effective method is therefore needed.

The present study served as the foundation for the development of such a method, and the following is recommended for each step thereof: ammonium bicarbonate and 7X® were found to be the best wash solutions, with Triton® X-100 and Sunlight® Liquid being acceptable alternatives. Zinc sulphate is recommended for flotation, and magnesium sulphate and sodium nitrate may be used as replacements. Precipitation of the salts and contamination development are however problems that were encountered for these two flotation solutions. With regards to extractions, the hydrophilic solutions have a less toxic effect than solvents in the phase extraction step. Acid-alcohol or acetoacetic buffer proved to be ideal, whilst ethyl acetate is recommended as the solvent, as it is less hazardous and inhibitory to egg development. Similarly, in combination, acid-alcohol and ethyl acetate was the best combination, followed by acetoacetic buffer and ethyl acetate as an alternative. Sulfuric acid produced the clearest samples after incubation, with healthy eggs containing plump, motile larvae. Water may be used as a replacement incubation solution, but contamination development resulted in eggs being clumped together.

The next steps in our development of a standardized helminth method were to test the physical aspects, such as how to wash samples, centrifugation speeds and times, various possible densities of flotation solutions, egg recovery experiments after washing, flotation and extractions and how to handle all sample types or matrices. These experiments needed to be conducted such that modifications could be made at each step to optimize and develop a final method adaptable to different sample types while optimizing recovery and maintaining egg viability. This work will be presented in subsequent publications.

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CHAPTER 3: PUBLICATION 2

THE EFFECTS OF ALL TECHNICAL STEPS USED IN HELMINTH TEST METHODS ON *ASCARIS SUUM* EGG RECOVERY FROM PIG FAECES.

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THE EFFECTS OF ALL TECHNICAL STEPS USED IN HELMINTH TEST METHODS ON *ASCARIS SUUM* EGG RECOVERY FROM PIG FAECES

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ABSTRACT

There are many technical aspects associated with helminth egg isolation and enumeration that affect how efficiently eggs are recovered from samples. This study investigated egg recoverability when samples were washed under pressure and using no pressure, and from different sample types (water, effluent from wastewater treatment works (WWTW), VIP, UDDT, dried, fatty, and septic tank sludges, and soil) when processed with water, ammonium bicarbonate, and 7X®. We also looked at egg recovery after flotation with zinc sulphate, magnesium sulphate and sodium nitrate at specific gravities of 1.18, 1.2 and 1.3, at respective centrifugation speeds and times after washing (2500 and 3000 rpm for 5, 10 and 15 minutes) and after flotation (2000 and 2500 rpm for 5, 10 and 15 minutes). We found that samples should be washed under pressure to ensure full dissociation and separation of eggs from the sample matrix and then centrifuged at 3000 rpm for 10 minutes. For sludge samples (or samples with high fat content), 7X® produced the best egg recovery and clearest samples for microscopic analysis, whilst soil and soil-containing samples (UDDT sludge) were best processed with ammonium bicarbonate. Flotation was optimal with zinc sulphate at specific gravity 1.3 after centrifugation at 2000 rpm for 15 minutes.

Keywords: Centrifugation; egg recovery; helminth eggs; flotation; specific gravity; wash solution

INTRODUCTION

There is no universally accepted helminth recovery method for processing sanitation and soil samples (Gyawali, 2017). Existing methods entail following these basic principles: washing of a sample (using a solution that can dissociate bonds between helminth eggs and the sample matrix) and subsequent filtration over sieves, flotation (using density gradients to separate eggs from other particles), phase

extraction to remove protein and lipids, microscopic analysis of the resulting pellet to quantify eggs, and incubation for viability assessment (Rocha et al., 2016; Ravindran et al., 2019). Furthermore, there are many technical aspects related to these steps that can impact on the efficacy of the method in terms of how well it recovers eggs. These include centrifugation speeds and times, choice of wash and flotation solutions, and specific gravity (sp. gr.) of the latter (Collender et al., 2015; Amoah et al., 2017).

The first attempts to isolate helminth eggs from fecal sludge samples were based on stool techniques, but the difference in volume between fecal sludge and stool samples, and the fact that eggs would be more concentrated in the latter, required that these methods be adapted (Faust et al., 1939; Allen and Ridley, 1970; Rocha et al., 2016). Common sanitation helminth methods currently used globally include the United States Environmental Protection Agency (USEPA) Method, the Mexican Standard for Wastewater Analysis, the Bailenger Method, and the PRG Helminth Method. The latter was established by our group and implemented in our laboratory for helminth testing (Ayres and Mara, 1996; USEPA, 2003; Secretaría de Economía, 2012; Velkushanova et al., 2021). Other than the PRG Method, that separates the protocol according to sample type, none of the other methods are as specific. Table I outlines the technical steps for each of these methods and forms the basis upon which all experiments in this study were designed.

Collender et al. (2015) noted that samples of high particle concentration could trap helminth eggs. Homogenization of a sample enhances consistent egg recovery prior to processing, as well as after soaking in the wash solution, the latter of which ensures dissociation of eggs from the matrix (Bowman et al., 2003). Few studies have looked at egg recovery from water samples, but Maya et al. (2006) reported that the use of Tween®80 or no wash solution at all were both efficient for egg recovery from well water. For sludge samples, it is recommended that a surfactant is used, such as 7X®, Triton®X-100, Tween®20, or Tween®80 (Collender et al., 2015; Velkushanova et al., 2021), and 7X® was reportedly the most efficient for egg recovery (Bowman et al., 2003). Furthermore, some surfactants possess antimicrobial properties, thus preventing excessive biological contamination (Amoah et al., 2017). Soil type and texture of samples can affect the recovery of eggs but soaking and homogenization of soil samples in ammonium bicarbonate allows for dissociation of bonds that form between the surface layer of the eggshell and silica particles in soil (Nunes et al., 1994; Hawksworth et al., 2010). Gaspard et al. (1996) stated that the wash solution should be made up to as low a concentration as possible to avoid egg damage.

Often, the use of sieves and respective mesh or pore sizes are looked at to determine the efficacy of egg recovery relative to size of eggs of various helminth species (Amoah et al., 2017; Ravindran et al., 2019). No studies, however, investigated how the mode of washing over these sieves affects

separation of eggs from the sample matrix. The USEPA Method recommends that samples be washed over the sieve using a spray bottle, thus no pressure is applied to push eggs released from the sample through the top sieve (USEPA, 2003). The Mexican Standard for Wastewater Analysis uses an exact, measured amount of water to wash the sample over the sieve, most likely from a beaker, thus without pressure (Secretaría de Economía, 2012). Furthermore, both methods recommend that the first washing be done on a sieve placed over a beaker such that the wash water can be collected. This could allow egg loss over the sides of the sieve if it does not fit exactly over the beaker. The PRG Helminth Method uses a 100 µm sieve over a 20 µm sieve for all helminth eggs and samples are washed under pressure using a hose or shower head attached to the tap such that the retentate on the 20 µm sieve contains the eggs (Velkushanova et al., 2021). Use of pressure to break up a sample and push eggs through a mesh was only practiced when sieves were introduced for washing (Pebsworth et al., 2012), rather than as previously done, by adding a wash solution to the sample in test tubes and mixing them on a vortex mixer (Moodley et al., 2008; Trönnberg et al., 2010).

There is a lack of comparative data in terms of the best wash solution for specific sample types and optimal centrifugation speeds and times for egg retrieval (Amoah et al., 2017; Ravindran et al., 2019). Many methods recommend gravitational sedimentation of the sample after washing, followed by vacuum aspiration of the supernatant (Ayres and Mara, 1996; USEPA, 2003), after which the sediment is transferred to test tubes and centrifuged for up to 1000 x g (Bowman et al., 2003). Centrifugation after washing is essential for effective sedimentation of eggs in the filtrate, such that eggs are not lost in the supernatant (Ravindran et al., 2019).

Flotation solutions are used to create a difference in density between eggs, other particles in the sample, and the suspension medium, such that eggs can be separated from residual matter that was not removed during the washing and sieving step (Dryden et al., 2005; Amoah et al., 2017). The flotation solution needs to be denser than the eggs with a minimum sp. gr. of 1.25 and is made up (using a hydrometer) to a sp. gr. that allows all eggs to float (Dryden et al., 2005), thus eggs that are lighter than the other particulate matter and the flotation solution, are buoyant (David and Lindquist, 1982; Ravindran et al., 2019). Once centrifuged, the particulate matter packs tightly into a pellet at the bottom of the tube, eggs float up towards the surface of the flotation medium, and the supernatant is collected. According to David and Lindquist (1982), the relative density of soil-transmitted helminth eggs ranges from 1.05 to 1.23, with *Ascaris suum* eggs having a relative density of 1.13.

Commonly used flotation solutions include zinc sulphate and magnesium sulphate, at sp. gr. of 1.18, 1.2 and 1.3 (Ayres and Mara, 1996; USEPA, 2003; Secretaría de Economía, 2012; Velkushanova et al.,

2021). A solution that is too dense will result in some eggs imploding due to a drastic shift in pressure as eggs move up the column. A sp. gr. of 1.30 would allow for eggs to float up efficiently without exerting too much pressure on the egg wall and is therefore used in the PRG Helminth Method (Velkushanova et al., 2021). Dryden et al., (2005) stated that centrifugation, rather than passive flotation, was essential to allow eggs to float, and thus improved egg recovery. Some method modifications place a coverslip at the top of the test tube (after either centrifugation or passive flotation) to allow for adhesion of eggs to the coverslip that then gets placed onto a microscope slide and examined. Others pipette the supernatant out and dispense it, or decant it, onto a 20 µm sieve and wash off the flotation solution (Dryden et al., 2005), to return the eggs to a neutral sp. gr.

Table I: Comparison of all technical steps across the 4 test methods

Method	Washing Type	Sieve Size(s)	Centrifuge Speed & Time	Flotation Solution	Specific Gravity (sp. gr.)	Centrifuge Speed & Time	Reference
PRG Method	Under pressure	100 µm over 20 µm	3000 rpm (1512 x g) for 10 min	Zinc sulphate	1.3	2000 rpm (672 x g) for 10 min	Pebsworth et al., 2012; Velkushanova et al., 2021
USEPA Method	No pressure (using spray bottle)	297 µm or 841 µm	± 2500 rpm (1000 x g) for 10 min	Magnesium sulphate	1.2	± 2200 - 2500 rpm (800 - 1000 x g) for 5 - 10 min	USEPA, 2003
Mexican Standard	No pressure (pouring from beaker)	150 – 170 µm	± 1543 rpm (400 x g) for 5 min	Zinc sulphate	1.3	± 2500 rpm (1000 x g) for 5 min	Secretaría de Economía, 2012
Bailenger Method	No washing step – just a series of sedimentation and aspiration steps	N/A	± 2500 rpm (1000 x g) for 15 min	Zinc sulphate	1.18	Gravitational sedimentation and subsequent flotation for 5 min	Ayres and Mara, 1996

The Bailenger Method uses a McMaster counting chamber for quantification of eggs after the sample is processed and floated in zinc sulphate at sp. gr. 1.18 by standing for 5 min to allow for gravitational sedimentation of heavier particles and passive flotation of eggs (Ayres and Mara 1996). An aliquot (1.5

milliliter (ml)) of the solution is then pipetted into the two chambers of the McMaster slide and counted using a microscope. The grid etched on the top side of the chamber is focused on, and eggs are identified and counted at 100X magnification. This makes morphological identification and categorization to assess viability difficult. It also performs poorly when helminth infections are low (Cringoli et al., 2010). Furthermore, egg recovery might not be optimum as there is no centrifugation step, which facilitates more efficient flotation (Collender et al., 2015).

The present study is the second in a series of 3 and was designed around recommendations made by Naidoo & Archer (the first study in the series), that looked at the effects of all reagents and chemicals used in existing helminth test methods on the viability of *A. suum* eggs. The optimum chemicals were therefore selected for testing in the technical steps explored in this study: selection of the best flotation solution relative to sp. gr. and testing different sample types against wash solutions. Other technical aspects investigated included mode of washing, and optimal centrifugation speeds and times after washing and flotation, all for the highest egg recovery. Data from this study will be used for the design of a final helminth test method to use on a variety of sanitation and environmental samples.

MATERIALS AND METHODS

Ascaris suum eggs were isolated from feces of research pigs. Egg stocks were made up in 0.5% formalin at approximately 200 eggs per ml. A slurry of negative pig feces was made up by blending the feces with water to facilitate efficient spiking and mixing of eggs, as pig feces alone is dry. Spiking was then performed with 1 ml egg stock per 10 g slurry samples for each experiment below. Every comparison factor in each experiment was performed using 5 replicates.

Washing mode: washing samples using pressure and without pressure

The mode of washing used, i.e., using pressure from a tap with a hose or shower nozzle fitted, and without pressure using a wash bottle, was tested against egg recovery. Spiked samples were either washed with pressure or without, onto a set of sieves (200 mm diameter and 50 mm deep, always with the 100 µm sieve on top of the 20 µm). Retentate on the 20 µm sieve was collected into 15 ml graduated, conical, plastic test tubes (Falcon tubes) and centrifuged at 2500 and 3000 rpm (1050 and 1512 x g) for 5, 10 and 15 min.

Since we were testing the efficacy of the mode of washing and centrifugation speeds and times only, to avoid confounding results, we could not perform the flotation step on the samples. After centrifugation, all supernatants were discarded. Pellets were topped up with water to 5 ml and dislodged using a vortex to homogenize them, and 1 ml was removed for immediate analysis. Egg recovery was then extrapolated to 5 ml. This was to establish an estimated egg recovery without any

possible egg loss during the normal subsequent flotation step. The remaining 4 ml was stored at 4°C for full processing later once the flotation step had been optimized.

After determining the optimal flotation solution, sp. gr., centrifugation speeds and times, pellets from the 4 ml samples were floated using zinc sulphate as per flotation experiment below and analyzed for egg recovery. This figure was added to the recovery from the 1 ml sample to establish total egg recovery.

Flotation solutions, specific gravities and centrifugation speeds and times

Zinc sulphate, magnesium sulphate, and sodium nitrate were tested for optimal egg recovery, each at sp. gr. of 1.18, 1.2 and 1.3. Centrifugation speeds and times for flotation were tested against egg recovery – 2000 (672 x g) and 2500 rpm for 5, 10, and 15 min. Spiked samples were washed under pressurized tap water over the set of sieves. Retentate on the 20 µm sieve was collected into 15 ml Falcon tubes and centrifuged at 3000 rpm for 10 min (the optimum mode of washing, and centrifugation speed and time, were selected based on the previous experiment). The supernatant was discarded, and the pellet dislodged with an applicator stick whilst simultaneously pipetting 3 ml at a time of flotation solution into each tube and using a vortex mixer to ensure homogenization. The sample was topped up to 14 ml with flotation solution and centrifuged. The supernatant was then poured onto a 20 µm sieve (100 mm diameter, 50 mm deep) and rinsed with tap water. The retentate was pipetted into 15 ml Falcon tubes and centrifuged at 3000 rpm for 10 min. The supernatant fluid was discarded, and the pellet analyzed for egg recovery using light microscopy.

Determining the most suitable wash solution for different sample types

Different wash solutions were used on various sample types to determine which solution resulted in the best egg recovery. Sample types included water, WWTW effluent, VIP, UDDT, dried (soaked for both 4 and 24 hr), fatty, and septic tank sludges, and soil. Selection of wash solutions was based on data from the first study in the series by Naidoo and Archer. We therefore tested ammonium bicarbonate (119 g/L), 0.1% 7X® and water as a control, in quintuplicate (Table II).

Table II: Sample types and respective volumes treated with the tested wash solutions

Sample Type	Volume (ml)/ Weight (g)	Treatment with wash solutions
Water (control)	1000 ml	Samples were spiked with approximately 200 eggs. Wash solution was made up directly into the sample. 1 ml of 7X® or 119 g of ammonium bicarbonate were added directly and mixed well.
WWTW Effluent	1000 ml	

Sample Type	Volume (ml)/ Weight (g)	Treatment with wash solutions
Ventilated improved pit latrine (VIP) sludge	10 g	Samples were spiked with approximately 200 eggs. Water, ammonium bicarbonate (119 g/L) or 0.1% 7X® was added to each sample – sufficient to cover the surface. The sample was homogenized and left to stand for 10 min.
Urine diversion dry toilet (UDDT) sludge	10 g	
Dried sludge (4 and 24 hr)	10 g	
Fatty sludge	10 g	
Septic tank sludge	250 ml	
Soil	50 g	

Samples were then washed over a set of sieves under pressurized tap water. Retentate on the 20 µm sieve was collected into as many 15 ml Falcon tubes as was required, depending on sample type. The tubes were centrifuged at 3000 rpm for 10 min, supernatants were discarded, and pellets floated with zinc sulphate (sp. gr. 1.3), processed, and analyzed using light microscopy. The final pellets of the fatty sludge samples were large, and required an extraction step, but eggs were being lost in this process (third study in this series by Naidoo & Archer). Fatty sludge samples were re-run a few times until we found an effective way of counting eggs in the final pellet. Eventually, the resulting pellets were resuspended to 1 ml with water in the test tubes and homogenized. The drops were counted, and half the number were analyzed. The number of eggs counted was doubled to calculate total egg recovery.

Statistical Analyses

Data from all experiments were statistically analyzed using the Kolmogorov-Smirnov test for normality of data, followed by nested ANOVAs and multiway ANOVAs, together with the Shapiro-Wilk test for normality of residuals and Levene's test for homogeneity of variance of residuals from the ANOVAs. Analyses were run on IBM SPSS Statistics (version 25, IBM Corp., Armonk, NY, USA) and R (version 3.5.2. R Core Team 2018). Egg recovery was calculated as follows:

*Egg recovery in 1 ml (%) = (Total number of eggs recovered in 1 ml*5/Total number of eggs spiked) *100* *(equation 1)*

Total number of eggs recovered in 5ml = Total number of eggs recovered in 1 ml + Total number of eggs recovered in 4 ml *(equation 2)*

*Total egg recovery in 5 ml (%) = (Total number of eggs recovered in 5 ml/ Total number of eggs spiked) *100* *(equation 3)*

*Total egg recovery (%) = (Total number of eggs recovered/ Total number of eggs spiked) *100*

(equation 4)

RESULTS

Washing mode: Washing samples using pressure and without pressure

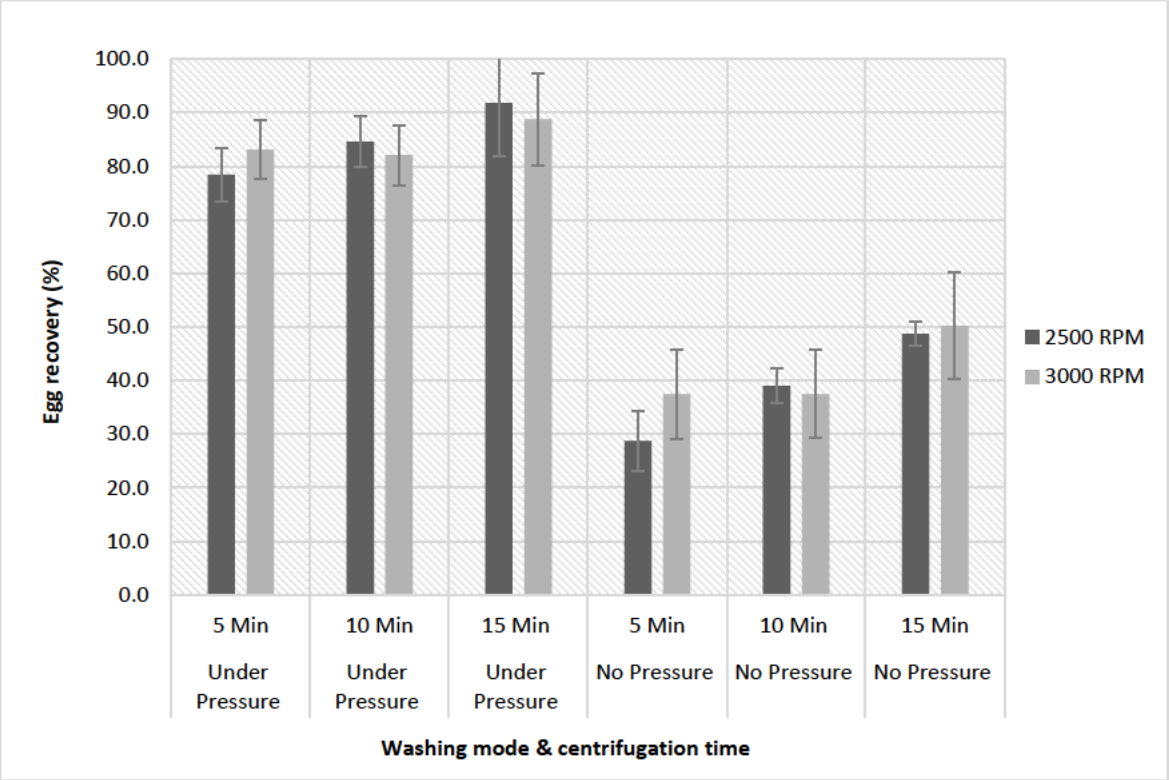

Figure 1: Egg recovery (%) of samples that were washed under pressure and using no pressure, and then centrifuged at 2500 and 3000 rpm for 5, 10 and 15 min (for each mode of washing and each centrifugation speed and time, n = 5). These are extrapolated figures that excluded a flotation step.

When looking at the effect of washing mode and centrifugation time alone, significant effects on egg recovery were noted ($P < 0.001$), but centrifugation speed alone was insignificant ($P = 0.540$). The combination of washing mode and centrifugation speed did not impact on egg recovery ($P = 0.630$), but when nested with centrifugation time, egg recovery was significantly affected ($P < 0.001$). Figures 1 and 2 clearly indicate that samples need to be washed under pressure. This physically separates larger particles on the 100 µm sieve from eggs, whilst also pushing eggs through the 100 µm mesh of the top sieve onto the 20 µm mesh bottom sieve. Furthermore, the back of a gloved hand can be used to mechanically break up clumps and gently push eggs and smaller particulate matter through the top sieve. Using a wash bottle to wash the sample on the 100 µm sieve made it difficult to break up larger

particles and push eggs through the sieve pores with the finer debris. The sample never quite looked adequately washed, whereas with pressure, the water eventually ran clear from the 100 µm sieve, indicating that washing was complete.

The extrapolated figures, when the flotation step was not included, (Figure 1) indicated that both 10 and 15 min resulted in > 90% egg recovery. It also indicated that 2500 rpm resulted in better recovery than 3000 rpm at 10 and 15 min. The actual recovery after including the flotation step (Figure 2), however, indicated that 3000 rpm produced higher egg recovery than 2500 rpm, for both 10 and 15 min (> 90% egg recovery for both times). Furthermore, it was noted that the pellet was better compacted at the bottom of the test tube at 3000 rpm, making it easier to discard the supernatant without losing eggs prior to performing the flotation step.

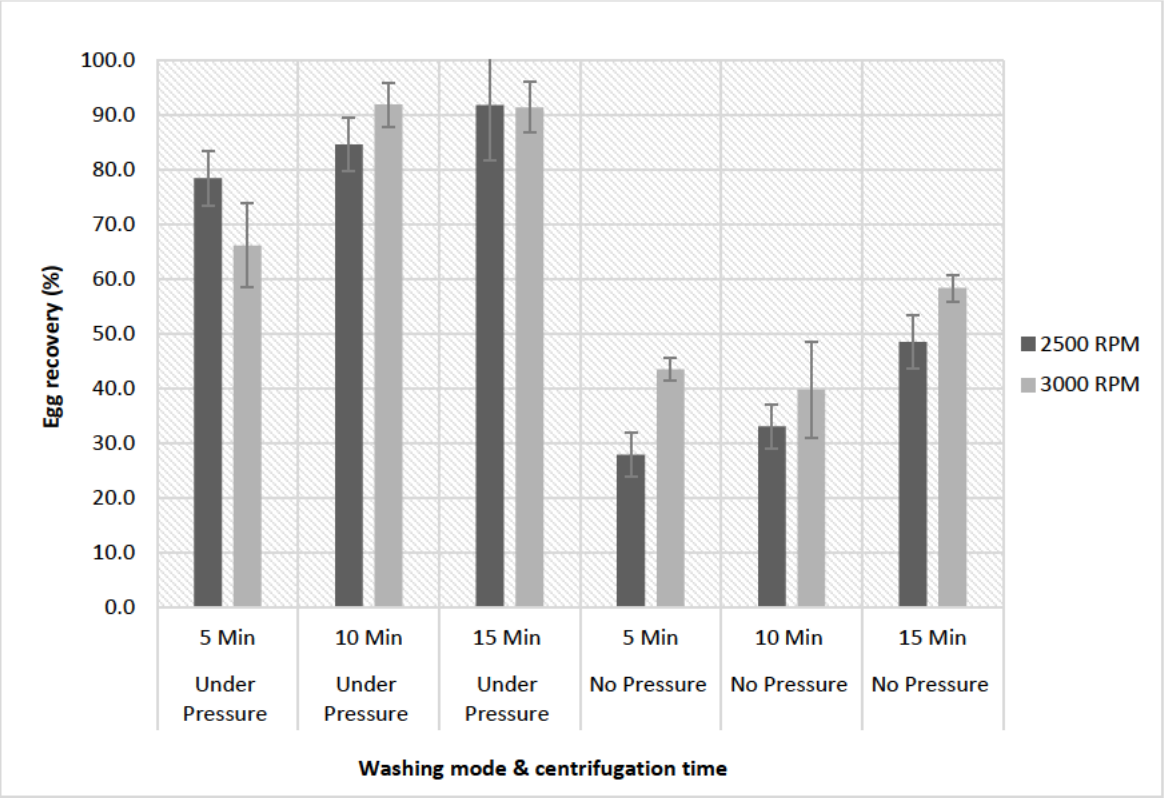

Figure 2: Egg recovery (%) of samples that were washed under pressure and no pressure, and then centrifuged at 2500 and 3000 rpm for 5, 10 and 15 min (n = 5). These are actual recovery values after samples were floated (inclusive of the eggs recovered in the initial 1 ml).

Flotation solutions, specific gravities and centrifugation speeds and times

Flotation solution alone played a significant role in egg retrieval ($P < 0.001$), and across all 3, egg recovery increased as the sp. gr. of solutions increased (Figures 2 - 4; $P < 0.001$). Furthermore, a

significant effect was seen when recovery was nested with centrifugation speed alone ($p < 0.001$) and speed and time together ($P < 0.001$). Egg recovery at sp. gr. 1.18 was extremely low for all 3 flotation solutions, indicating that *A. suum* eggs require a denser solution for better separation from particles (sp. gr. alone on egg recovery: $P < 0.001$).

The nested effect of specific gravity, centrifugation speed, and centrifugation time was also significant in terms of how well eggs were recovered ($P < 0.001$). At sp. gr. 1.2, egg recovery was still very low, with all 3 solutions recovering < 50% of the spiked eggs. This indicated that the density of solutions plays a more important role in egg recovery than centrifugation speeds and times. Even when samples are spun down for a longer period (Figures 2 - 4), separation from particles was not completely successful at lower specific gravities.

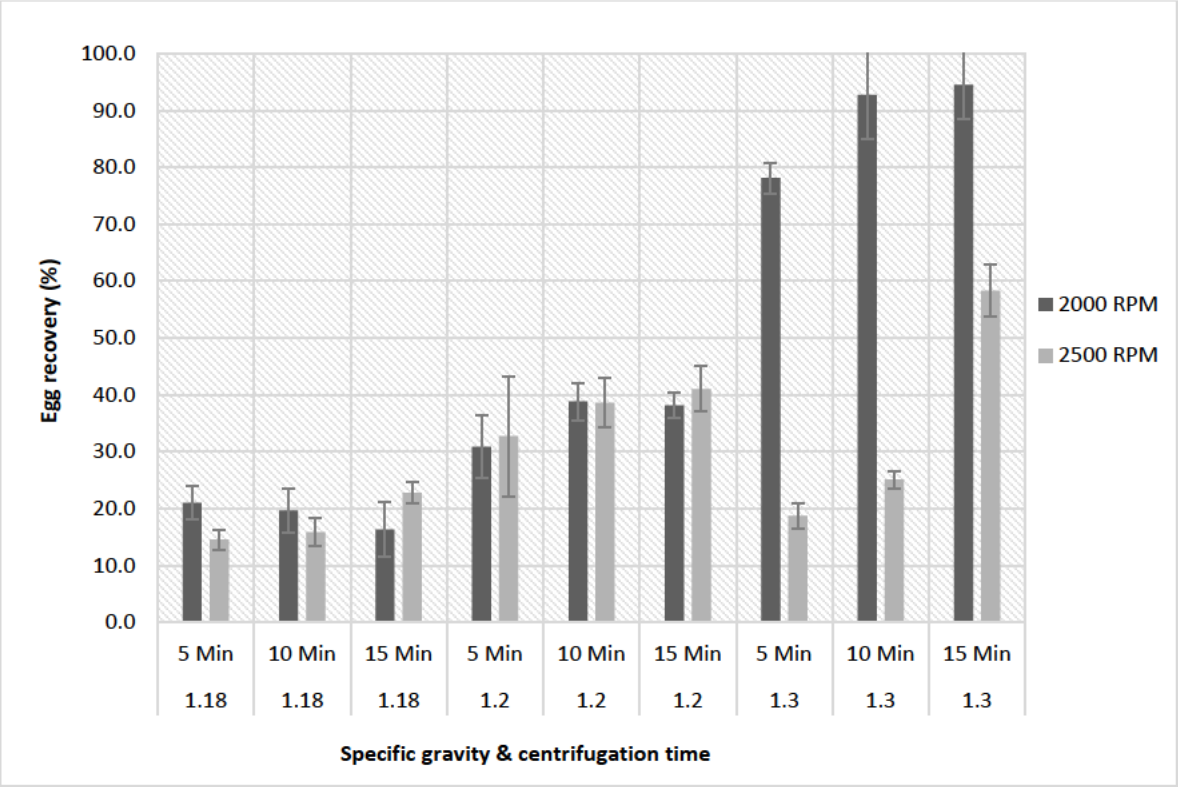

Figure 3: Egg recovery (%) of samples that were floated with zinc sulphate at specific gravities of 1.18, 1.2 and 1.3, and then centrifuged at 2000 and 2500 rpm for 5, 10 and 15 min (n = 5).

Zinc sulphate resulted in the best egg recovery at sp. gr. 1.3 when centrifuged at 2000 rpm for 15 mins (> 90%). It was therefore selected as the ideal flotation solution. It was also noted that eggs required a slower speed and longer centrifugation time to separate from particulate matter and successfully float up the supernatant column. At sp. gr. 1.3, both centrifugation speed and time made a difference

to egg recovery. Eggs require a slower speed (effects of speed alone on egg retrieval: $P < 0.001$) and longer time (effects of time alone on egg retrieval: $P < 0.001$) to separate from denser particles and float up the supernatant column when being centrifuged. Egg recovery with sodium nitrate was very low (< 40%) across all densities, speeds, and times, and was therefore deemed unfit as a flotation solution (Figure 5). Magnesium sulphate was successful at sp. gr. 1.3, and centrifugation at 2000 rpm for 15 min, however, recovery was still < 90% (Figure 4).

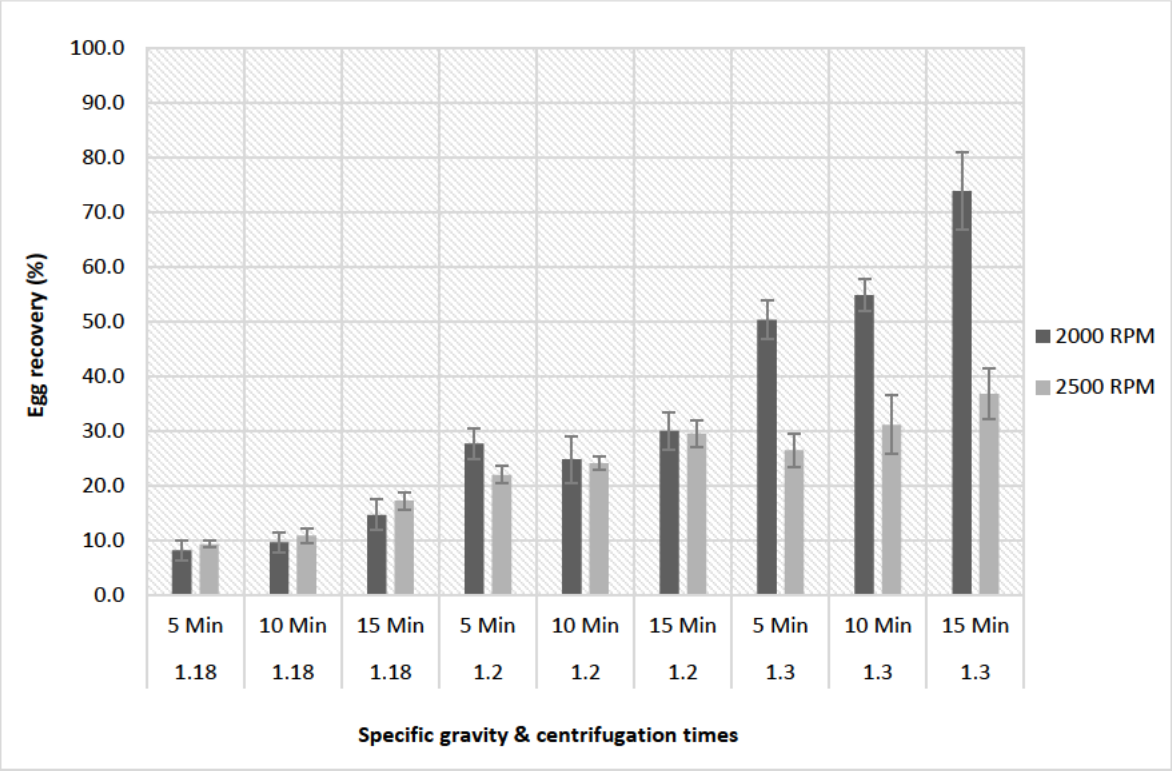

Figure 4: Egg recovery (%) of samples that were floated with magnesium sulphate at specific gravities (sp. gr.) of 1.18, 1.2 and 1.3, and then centrifuged at 2000 and 2500 rpm for 5, 10 and 15 min (n = 5)

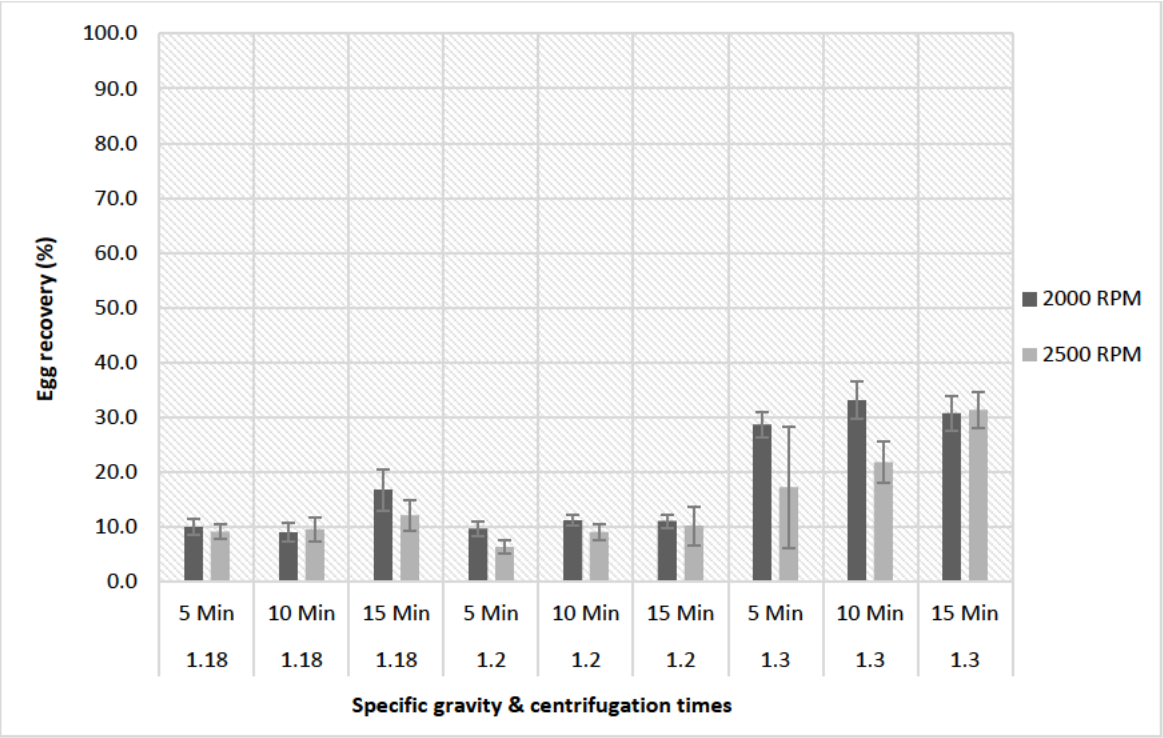

Figure 5: Egg recovery (%) of samples that were floated with sodium nitrate at specific gravities (sp. gr.) of 1.18, 1.2 and 1.3, and then centrifuged at 2000 and 2500 rpm for 5, 10 and 15 min (n = 5)

Determining the most suitable wash solutions for different sample types

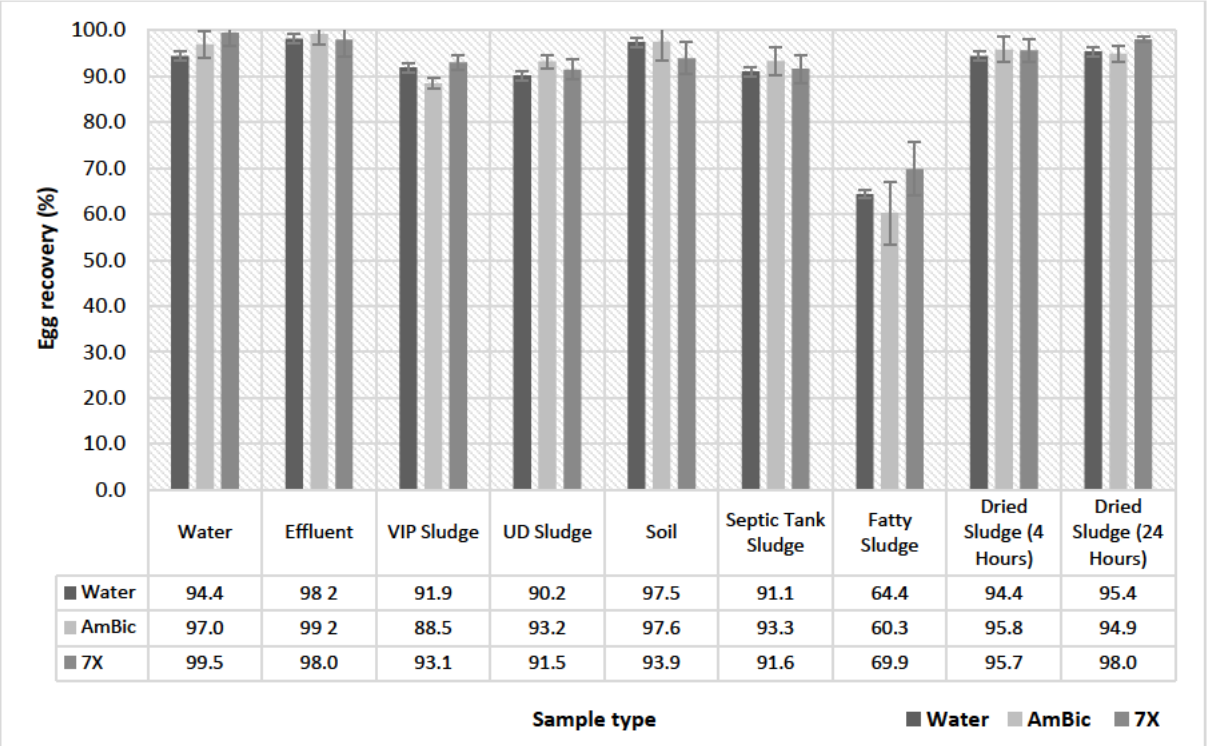

Figure 6: Egg recovery (%) from the 8 different sample types that were processed with water, ammonium bicarbonate and 7X® (n = 5). Abbreviations: ammonium bicarbonate, AmBic.

When looking at the effect of wash solution alone on egg recovery, no significant effect was seen, however, the interactive effect with sample type was significant for egg recovery (P = 0.672 and P = 0.025). Across all 3 wash solutions, egg recovery was ± 90% for each individual sample type, except in the case of fatty sludges, which most likely accounts for the significant interactive effect of the ANOVA. The major difference was not necessarily observed in egg recovery values, but instead when processing and analyzing the different sample types. For water and WWTW effluent samples, washing with water was sufficient, however, if samples had to be incubated, washing with a chemical solution assisted better in preventing an increase in microbial contamination.

For VIP and septic tank sludge, 7X® was the most successful in breaking down the sample and reducing the final pellet size, as well as making microscopy the easiest as the pellet dissociated well, with egg recovery being > 91%. Dried sludge was soaked for 4 and 24 hours (hr), and both resulted in similar egg viability across solutions, indicating that samples can be soaked overnight in either 7X® or ammonium bicarbonate. When comparing all 3 wash solutions, fatty sludge was best broken down by 7X®. These samples were particularly difficult to handle and process, resulting in fatty deposits even after washing with a surfactant. The final pellets for microscopy were large, thus only half the pellet was analyzed, and the values were extrapolated. Egg recovery was highest for UDDT sludge and soil when washed with ammonium bicarbonate. Final pellets were also small enough to microscopically analyze with ease.

DISCUSSION

Nunes et al. (1994) noted that a protocol specific to sample type is important for successful retrieval of helminth eggs from samples. To date, no such method exists that is simple, time- and cost-efficient for all sample types and helminth species (Gyawali, 2017). Analysis of the technical steps was imperative for development of such a method, and the data were used to build towards it.

Filtration using sieves is needed to separate larger particles from helminth eggs after these have been dissociated from particles in the sample matrix using a wash solution (Amoah et al., 2017). The USEPA Method uses a spray bottle to wash samples over the sieve (USEPA, 2003) that could result in residual egg loss, or eggs being trapped in the pores of the 100 µm mesh. The same can be said for the Mexican Standard for Wastewater Analysis, where they recommend that a measured amount be poured over the sample on the sieves (Secretaría de Economía, 2012). Our data indicated that washing under pressure recovers far more eggs than the alternative.

Some studies are in accordance with the results that we found for the extrapolated data, recommending that centrifugation at 2500 rpm for 5 min (Eriksen et al. 1996) and 15 min (Sá et al.,

2017) can sufficiently compact the pellet and recover eggs. Actual recovery data after flotation however, indicated that 3000rpm performed better for egg recovery. Since both 10 and 15 min were successful, we recommend the former, as it means exerting less pressure on the eggs. Manser et al. (2016) reported that egg recovery increased with increasing centrifugation speed and time. Eggs may remain in suspension for prolonged periods of time, thus longer centrifugation speeds resulted in better egg retrieval, however, excess debris interfered with microscopic analyses when eggs were centrifuged at 3500 rpm for 10 min. Allen and Ridley (1970) reported the opposite, that centrifugation at 3000 rpm for 1 min was sufficient for egg recovery. Some kit-methods recommend a slower speed for a longer time, which was found to be unsuccessful in our experiments. Zdybel et al. (2015) centrifuged samples at 3858 rpm (2500 x g), however, the impact of such a high speed on the development of helminth eggs and the eggshell is unknown. No visible degradation of the eggs was evident at 3000 rpm for 10 min (Manser et al., 2016), which is in accordance with our study, and is therefore the recommended speed and time.

Santarem et al. (2009) reported that there was no difference in egg recovery between sp. gr. of 1.2, 1.25 and 1.3 across solutions, but that zinc sulphate and sodium nitrate performed much better than magnesium sulphate. They also stated that sodium nitrate formed crystals that subsequently hindered microscopic analysis. We found that magnesium sulphate precipitated out of solution and formed crystals at the bottom of the bottle when stored and is therefore deemed unfit as a flotation solution. This is in contradiction to the findings of Quinn et al. (1980), who noted that magnesium sulphate performed better than zinc sulphate at sp. gr. 1.3. Smith (1991) reported that prolonged exposure to magnesium sulphate is toxic to eggs, thus supporting its exclusion.

Sá et al. (2017) compared flotation efficacy of zinc sulphate at sp. gr. 1.3 without centrifugation, and at sp. gr. 1.35 at 3500 rpm for 5 min. They found that centrifugation resulted in 57% more eggs recovered than passive flotation. This also indicates that the higher the sp. gr., the better egg retrieval, that is in accordance with our findings. Nunes et al. (1994) investigated the flotation efficacy of zinc sulphate (sp. gr. 1.2) and potassium dichromate (sp. gr. 1.35) for *Toxocara canis* eggs from soil samples. They found that solutions of higher sp. gr. were more successful, despite soil texture, but did not report effects on egg viability. It should however be noted that physical properties, such as viscosity of the solution, affect egg recovery, and the denser the solution, the higher the viscosity (Oge and Oge, 2000). This would create resistance as eggs float up the liquid column and thus impede recovery.

Charitha et al. (2012) investigated flotation efficacy based on 3 different methods that utilized sodium nitrate at sp. gr. 1.35, centrifuged at 1500 and 4000 rpm (327 and 2325 x g) for 3 and 15 min

respectively, and zinc sulphate at sp. gr. 1.2, centrifuged at 2500 rpm (908 x g) for 8 min. It was found that the highest speed recovered 79.6% and 66.5% eggs in sodium nitrate, which is the opposite of what we found in this study, where lower centrifugation speeds (2000 rpm) recovered more eggs (± 95%) in zinc sulphate at sp. gr. 1.3.

Quinn et al. (1980) stated that the flotation step should be repeated up to 4 times to recover eggs that might have been trapped during the previous flotations (10 - 20% more eggs recovered). Gaspard et al. (1995) also reported successful egg recovery when a double flotation was performed. Steinbaum et al. (2017) looked at egg recovery from soil samples when floated with zinc sulphate at specific gravities of 1.2 and 1.25. They reported that egg recovery was better when 2 flotations for 5 min (73.7%) each were performed on a sample as compared with 1 flotation for 10 mins (65.6%), and sp. gr. 1.25 performed better than 1.2.

We found that *A. suum* eggs required a denser solution than sp. gr. 1.18 to float, which was in accordance with both Quinn et al. (1980) and Nunes at al. (1994). Souza et al. (2011) reported 36% *Ascaris* egg recovery when eggs were spiked into wastewater samples and then floated with zinc sulphate at sp. gr. 1.18, also supporting our study's findings. Quinn et al. (1980) found that recovery using zinc sulphate at sp. gr. 1.2 was far lower than magnesium sulphate at sp. gr. 1.27. We found that not only does sp. gr. impact on egg recovery, but so too does flotation solution, with zinc sulphate being the optimum solution at sp. gr. 1.3.

Bowman et al (2003) reported that the choice of wash solution affects the dissociation of eggs from the sample, and that 7X® was superior to Triton®X-100 and Tween®80 for egg recovery. It should be noted that 7X® also does not form a precipitate when it comes into contact with the salts from flotation solution (Ravindran et al., 2019). Figure 6 indicates that 7X® recovered the most eggs for VIP and septic tank sludge, and is therefore recommended for these sample types. It is also recommended for fatty sludge samples; however, egg recovery was not as efficient. Manser et al. (2016) reported that helminth eggs can be trapped by fats in a sludge sample, and Satchwell (1986) stated that the greatest loss of eggs generally occurs prior to the flotation step. In this study, eggs were lost during sample processing, most likely due to the wash solution not being able to fully dissociate eggs from lipids in fatty sludge.

Ammonium bicarbonate facilitates the dissociation of bonds formed between eggs and silica particles, and UDDT sludge is known to contain soil (Hawksworth et al., 2010), thus final pellets were easily dislodged, and small particles well dispersed for easier microscopy. It is therefore recommended for use on UDDT and soil samples. Santarem et al. (2009) reported that samples can be washed twice with Tween®80 to release eggs trapped in the first washing, however we found that one wash with

ammonium bicarbonate was sufficient to recover 97.6% of eggs. Eggs can adhere to a variety of components in fecal sludge, such as humic, and fulvic, acids that are commonly found in soil (Rocha et al., 2016). Ammonium bicarbonate would therefore facilitate dissociation of these bonds and allow separation of silica particles from eggs, with Hawksworth et al. (2010) reporting 77.28% egg recovery from spiked UDDT sludge samples.

Maya et al. (2006) compared *Ascaris* egg recoverability of various wash and flotation solution combinations from well water and wastewater samples. They found that Tween®80 and zinc sulphate (sp. gr. 1.3) were able to retrieve 80% and 83% eggs from well water and wastewater, respectively, whilst no wash solution combined with zinc sulphate at sp. gr. 1.2 resulted in 86% and 63% respectively. This indicates that both a wash solution, and a flotation solution of higher sp. gr., are required for successful egg recovery from samples with some solids content (wastewater in this case).

CONCLUSION

To ensure dissociation of any bonds formed between helminth eggs and particulate matter, samples must be homogenized in a wash solution prior to processing. We conclude that water and liquid samples can either be processed without the use of a wash solution, or with 7X®. For sludge samples, we recommend the use of 7X® as a surfactant to break down lipids. For soil (or soil-containing) samples, ammonium bicarbonate should be used for bond dissociation. Dried sludge can be soaked for a minimum of 4 hr and up to 24 hr if necessary in either 7X® or ammonium bicarbonate. Mode of washing is important. Water under pressure is required when washing samples over the sieves to facilitate breaking up of the sample matrix and pushing eggs through the coarser mesh of the top sieve (100 µm in the PRG Helminth Method). Samples should then be centrifuged at 3000 rpm (1512 x g) for 10 min for optimal egg recovery and a well compacted pellet at the bottom of the test tube to allow for the supernatant to be easily discarded prior to floating. Zinc sulphate at sp. gr. 1.3 and centrifugation at 2000 rpm (672 x g) for 15 min recovered the most eggs and is therefore recommended for flotation. Based on data collected, the final method at the end of this study can recover > 90% eggs in most sample types, except fatty sludge.

Future research includes further testing of steps that could be included in the protocol to enhance recovery of helminth eggs from fatty sludge, and the testing of multiple flotations to determine at which point all eggs are recovered. Testing the efficacy of the phase extraction step for egg recovery must also be undertaken, thus concluding the testing of every step in conventional sanitation and environmental helminth test methods.

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CHAPTER 4: PUBLICATION 3

ASCARIS SUUM EGG RECOVERY FROM FECAL SLUDGE SAMPLES AFTER PHASE EXTRACTION

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ASCARIS SUUM EGG RECOVERY FROM FECAL SLUDGE SAMPLES AFTER PHASE EXTRACTION

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ABSTRACT

Processing sanitation samples for helminth egg recovery sometimes includes a phase extraction step to further reduce lipid content prior to microscopy. Both hydrophilic and lipophilic solutions are used to create two phases, between which a disc / plug of organic material, often referred to as debris, forms, whilst eggs are compacted at the bottom of the test tube. We tested 10% formalin, acetoacetic buffer and acid-alcohol as the hydrophilic solutions, and ethyl acetate and diethyl ether as the lipophilic solvents, for egg recoverability in water, primary sludge, and fatty sludge. The supernatant and plug of debris are normally discarded and the eggs that sediment in the pellet are counted. We, however, collected the entire supernatant and plug of debris to determine where the eggs were trapped. We found that eggs are lost when samples are extracted with 10% formalin + ethyl acetate, 10% formalin + diethyl ether, aceto-acetic buffer + ethyl acetate and acetoacetic buffer + diethyl ether combinations (< 50% egg recovery). Acid-alcohol + ethyl acetate resulted in 93.2%, 89.8% and 57.3% egg recovery in water, primary sludge, and fatty sludge respectively, however, the size of the final pellet was not reduced, defeating the purpose of the extraction step. We thus recommend that this step be excluded.

Keywords: *Ascaris suum*; helminth eggs; hydrophilic; lipophilic; phase extraction; recovery; solvent

INTRODUCTION

Typical helminth isolation and enumeration methods used for sanitation and environmental samples generally comprise of the same basic steps: washing of the sample over a set of sieves (filtration), flotation using density gradients for separation of eggs from heavier particles, phase extraction to further remove debris, microscopic analysis, and incubation of the eggs to determine viability (Amoah et al., 2017a; Pakdad et al., 2018; Ravindran et al., 2019; Rocha et al., 2016; USEPA, 2003). Two of

these steps, namely, flotation and phase extraction, were derived from stand-alone parasitology methods for concentration of parasites from fresh faecal samples (Cheeseborough, 1981).

Phase extraction involves the use of a hydrophilic solution and a (generally) lipophilic solvent to create an interphase that traps lipids and proteins from the sludge test sample (Collender et al., 2015; Nelson and Darby, 2001; Rocha et al., 2016). Specific volumes of each chemical are added to a sample in a test tube that is vigorously shaken and centrifuged. The sample separates into two phases, light and heavy, with a disc or plug of organic material that forms in between. After centrifugation, helminth eggs are said to be concentrated in the pellet at the bottom of the tube, and the entire supernatant (that includes both phases and the middle disc) is discarded (Amoah et al., 2017a; Collender et al., 2015; Nelson and Darby et al., 2001; Rocha et al., 2016).

Allen and Ridley (1970) originally presented 'the extraction step' as a pathology laboratory method for isolating helminth eggs and larvae, and protozoan cysts from individual stool samples using a combination of 10% formalin and diethyl ether. For wastewater and sanitation samples, aceto-acetic buffer, formalin, or acid-alcohol, which is a mixture of sulphuric acid and ethanol (Ayres and Mara, 1996; Nelson and Darby, 2001; Secretaría de Economía, 2012; Velkushanova et al., 2021) are commonly used to form the hydrophilic phase, whilst ethyl acetate or diethyl ether form the lipophilic phase (Amoah et al., 2017a; Nelson and Darby, 2001; Rocha et al., 2016; Rude et al., 1987; USEPA, 1999). Some methods recommend that extraction be performed before the flotation step (Bailenger Method) whilst others recommend that it be performed afterwards (PRG Helminth Method, old United States Environmental Protection Agency (USEPA) Method and the Mexican Standard for Wastewater Analysis) (Ayers et al., 1996; Satchwell, 1986; Secretaría de Economía, 2012; USEPA, 1999; Velkushanova et al., 2021).

Some studies have indicated that extraction solutions can negatively impact egg viability (Nelson and Darby, 2001; Rocha et al., 2016), and recommend that this step should not be included in sample processing; however, if it is included, then the exposure time should be minimal (Nelson and Darby, 2001). It has been reported that whilst organic matter is removed upon extraction, it can also result in the loss of approximately 95% of eggs due to their distortion (Satchwell, 1986). Furthermore, Nelson and Darby (2001) reported that eggs were lost after extraction, either by complete destruction due to activity of the solvent or when discarding the layers above the pellet. The original USEPA Method (1999) recommended that extraction be performed on samples, however, the updated version (USEPA, 2003) excludes the extraction step. Nelson and Darby (2001) recommended that it be replaced with an additional filtration step over a 400 - mesh sieve (38 µm) to remove excess debris. The disadvantage of such a step though, is the potential loss of smaller egg species such as *Trichuris*

and *Taenia*. Gaspard and Schwartzbrod (1995) used ethyl acetate to emulsify the sample and break down organic material after the addition of a detergent, but prior to the flotation step. The PRG Helminth Method suggests extraction after flotation with 10% formalin and diethyl ether or ethyl acetate, only when the pellet is very large and impedes analysis by microscopy.

Naidoo and Archer looked at the single and combination effects of various extraction solutions used in existing methods on the viability of *Ascaris suum* eggs. The present study therefore aimed at determining egg recoverability from samples that were subjected to a phase extraction. Various combinations of hydrophilic solutions and lipophilic solvents were tested against different sample types spiked with a known number of *A. suum* eggs to determine which combination allowed for the highest recovery of eggs (whilst not damaging them in the process).

MATERIALS AND METHODS

Chemical exposure

The efficacy of all extraction combinations for egg recovery was tested in water, primary sludge collected from a wastewater treatment plant, and fatty sludge from a different wastewater treatment works in replicates of 5. Water acted as a control, and primary and fatty sludge samples were chosen as commonly tested sanitation samples. While primary sludge is not too problematic to process and examine microscopically, fatty sludge is the most difficult sample type to process and results in a large pellet that some analysts feel requires further processing to reduce the pellet size and make microscopy less cumbersome.

A. suum eggs were isolated from feces of research pigs. Approximately 250 eggs / milliliter (ml) stock solution was spiked into 10 g of primary sludge, 10 g of fatty sludge, and 14 ml of water in plastic, graduated, conical, test tubes, and these were thoroughly homogenized. For the sludge samples, 0.1% 7X was added and the samples were mixed well. Each sample was then washed using tap water under pressure over a set of pan sieves (each 200 mm diameter and 50 mm deep, with the 100 µm mesh sieve placed on top of the 20 µm mesh sieve). The retentate on the 20 µm sieve was collected into 2 x 15 ml test tubes. The tubes were centrifuged at 3000 rpm (1512 x g-force) for 10 minutes (min). The supernatant was discarded, and the deposits floated with zinc sulphate of specific gravity (sp. gr.) 1.3 and centrifuged at 2000 rpm (672 x g-force) for 15 min. The supernatant was poured onto a 100mm diameter 20 µm mesh sieve and thoroughly rinsed with water, after which the retentate was collected into a 15 ml test tube and centrifuged at 3000 rpm for 10 min. The water samples were also centrifuged.

After the final centrifugation step, all the supernatants were discarded, and the extraction solutions were added in the following combinations, each using 7 ml of buffer + 4 ml of solvent: 10% formalin + ethyl acetate, 10% formalin + diethyl ether, aceto-acetic buffer + ethyl acetate, aceto-acetic buffer + diethyl ether, and acid-alcohol + ethyl acetate. The tubes were thoroughly and vigorously shaken for 1 min, ensuring that the pellets were completely dislodged. A disc or plug of organic material (debris) formed between the buffer and solvent layers (Figure 1). The samples were centrifuged at 3000 rpm for 10 min. The supernatant (including the 2 phases and the disc at the interphase) would normally be discarded, but instead it was removed with a 3 ml plastic Pasteur pipette and expelled onto a 20 µm sieve (samples were pipetted rather than poured off to ensure that egg loss was not due to dislodgement of the pellet) and rinsed thoroughly to remove all chemicals. All the retentate remaining on the sieve was collected into a clean test tube and centrifuged at 3000 rpm for 10 min. The pellet that remained in the original test tube was immediately analysed for egg recovery using a light microscope. The washed and centrifuged supernatant portion was also analysed using light microscopy for egg recovery, and the sum of both analyses gave the total egg recovery.

Statistical analyses

The data from all experiments were statistically analysed using the Kolmogorov-Smirnov test for normality of data, followed by two-way ANOVAs together with the Shapiro-Wilk test for normality of residuals and Levene's test for homogeneity of variance of residuals from the ANOVA. The analyses were run on IBM SPSS Statistics (version 25, IBM Corp., Armonk, NY, USA) and R (version 3.5.2. R Core Team 2018). Egg recovery was calculated as follows:

*Pellet recovery (%) = (Total number of eggs recovered in pellet/Total number of eggs spiked) *100* *(equation 1)*

*Supernatant recovery (%) = (Total number of eggs recovered in supernatant/Total number of eggs spiked) *100* *(equation 2)*

Total egg recovery (%) = Percentage of eggs recovered in pellet + percentage of eggs recovered in disc/supernatant *(equation 3)*

Pellet recovery refers to the percentage of eggs recovered and counted in the pellet after the phase extraction contents were pipetted off, relative to the number of eggs spiked. This figure is representative of the actual egg recovery after phase extraction, as the supernatant would normally be discarded, and only the contents of the pellet would be analysed. Supernatant recovery refers to the percentage of eggs recovered in both phases, plus the disc or plug of organic matter in between (Figure 1), relative to the number of eggs spiked. Total egg recovery refers to the sum of what was

counted in the pellet and the supernatant and should be equal to what was spiked (± 90%, which was the criterion set for successful egg recovery for this study).

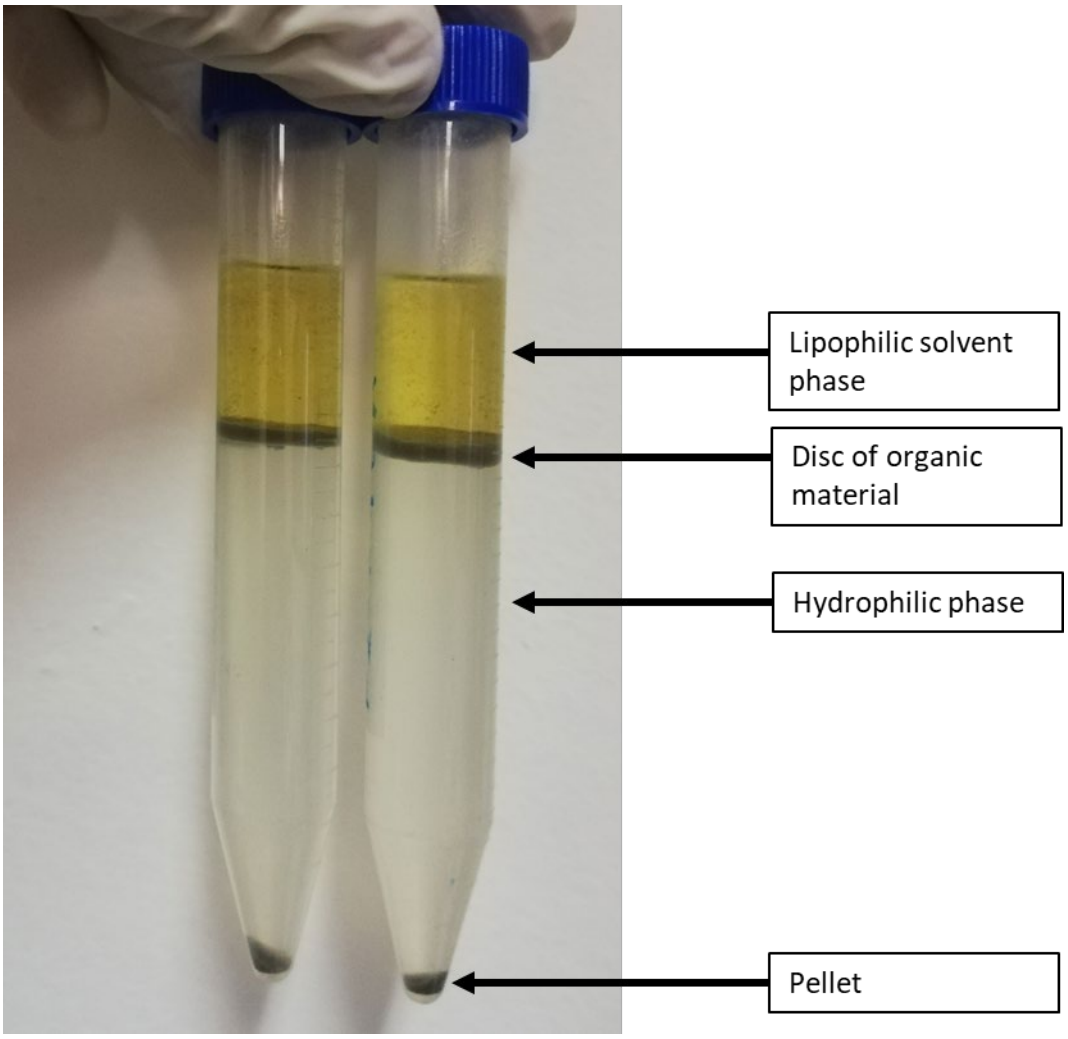

Figure 1. Samples after phase extraction performed with acetoacetic buffer and diethyl ether. Separation into two distinct phases can be seen, with the disc of organic material between the two phases, and the pellet, ideally containing the helminth eggs, at the bottom of the test tube.

RESULTS

The pellet egg recovery is a true representation of the efficacy of the phase extraction with the different chemical combinations. When looking at the effect of the extraction combination alone on egg recovery, a significant effect was seen ($P < 0.001$). The same can be said for sample type alone ($P < 0.001$), meaning that sample type affected egg recovery after extraction. The number of eggs in the pellet was also significantly impacted when looking at the interactive effects of extraction combination and sample type ($P < 0.001$), indicating that both the ability of the extraction solutions to separate

eggs from organic material (including their respective synergistic effects) and the sample matrix play a role in egg recovery. Figures 2 - 4 show the egg recovery in both the pellet and supernatant for each extraction combination per sample type and Figure 5 represents total egg recovery post extraction. The mean total egg recovery (the sum of eggs in the pellet and supernatant) in water, primary sludge and fatty sludge was 95.1%, 90.3% and 70.1% respectively.

Table I. Specific gravity (sp. gr.) measurements of the 5 extraction solutions

Extraction solution	Sp. gr.
10% Formalin	1.011
Acetoacetic buffer	1.091
Acid-alcohol	0.957
Ethyl acetate	0.900
Diethyl ether	0.720

Figure 2 shows data from phase extractions conducted in water samples. Whilst total egg recovery across the 5 combinations was high (> 85%), the actual egg recovery for formalin + ethyl acetate, formalin + diethyl ether, acetoacetic buffer + ethyl acetate and acetoacetic buffer + diethyl ether was very low, at 8.2%, 13.7%, 0.8% and 0.5% respectively. The acid-alcohol + ethyl acetate combination, however, showed promising results, with an egg recovery of 93.2%. Extractions performed on primary sludge samples produced slightly better results (Figure 3), however egg recovery was still far too low (22.0 - 46.1%) for the first four combinations. The acid-alcohol + ethyl acetate pairing once again resulted in sufficient egg recovery of 89.8%, however, it was found that this combination did not actually remove much of the organic matter, thus the pellet size was not reduced. Extractions with formalin (48.7% and 32.6%) allowed for more eggs to be recovered than those with acetoacetic buffer (1.3% and 0.4%), and acid-alcohol + ethyl acetate retrieved 57.3%. but was unable to reduce the pellet size. In general, total egg recovery was much lower in fatty sludge samples, which were much more difficult to process and analyse microscopically. Across the first 4 extraction combinations and 3 sample types, eggs did end up in the supernatant, either being unable to sediment but instead floating in the hydrophilic phase, or trapped in the disc, or floated above the disc in the solvent, being unable to travel back down the supernatant column to sediment into the pellet. We then removed the solvent separately from the disc using a pipette and did not find eggs present. It was therefore concluded that the eggs were trapped with the organic material in the disc. The acid-alcohol flotation was the most successful in terms of egg recovery across sample types, but was unable to further remove organic matter, resulting in a pellet of the same size as pre-extraction.

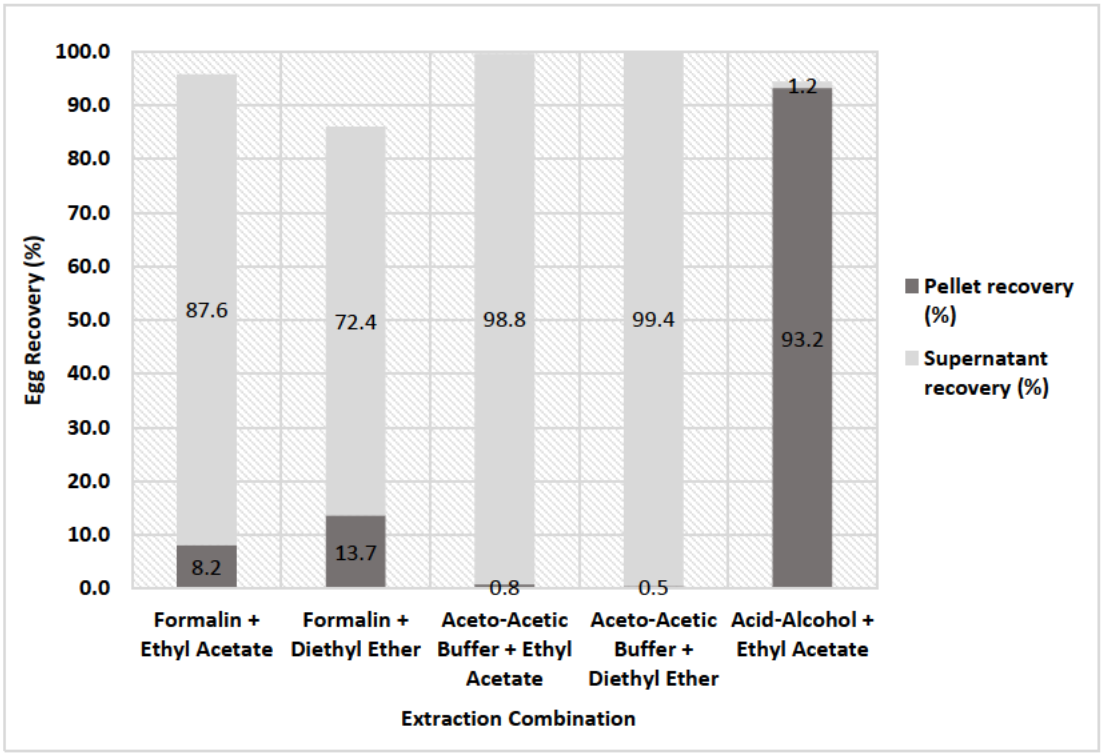

Figure 2. Egg recovery from both the pellet and supernatant of the control water samples after phase extraction with 5 different chemical combinations (n = 5).

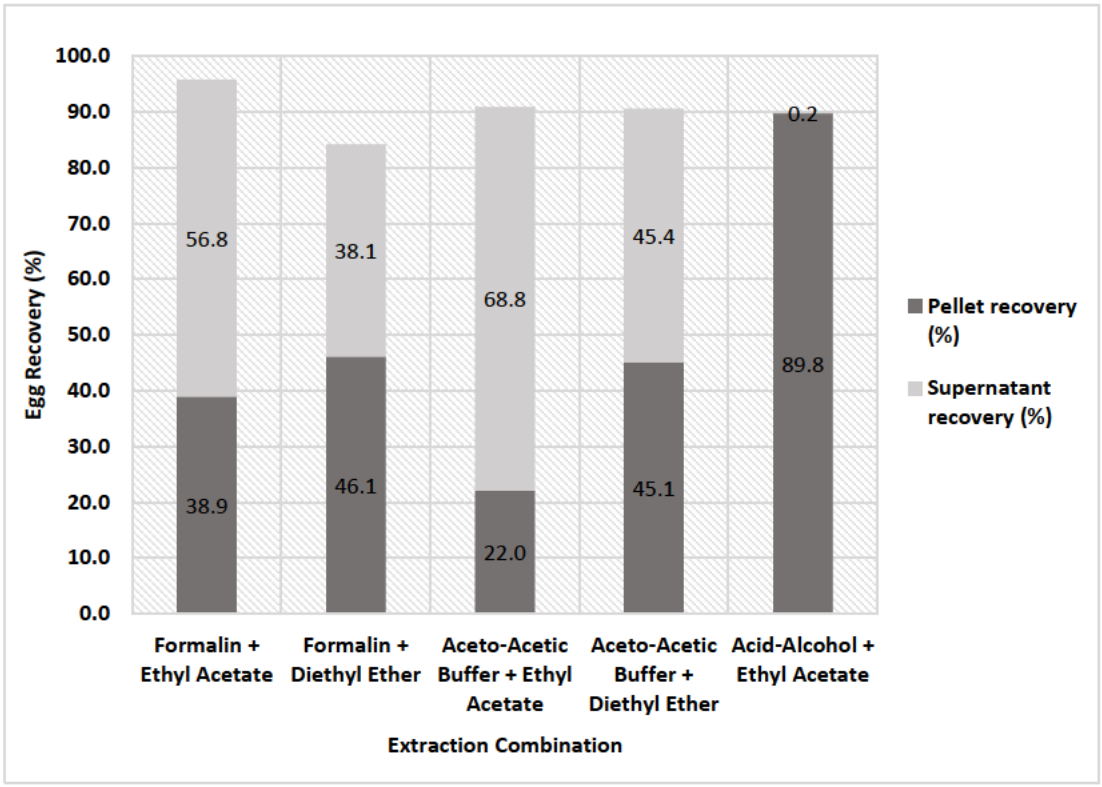

Figure 3. Egg recovery from both the pellet and supernatant of primary sludge samples after phase extraction with 5 different chemical combinations (n = 5).

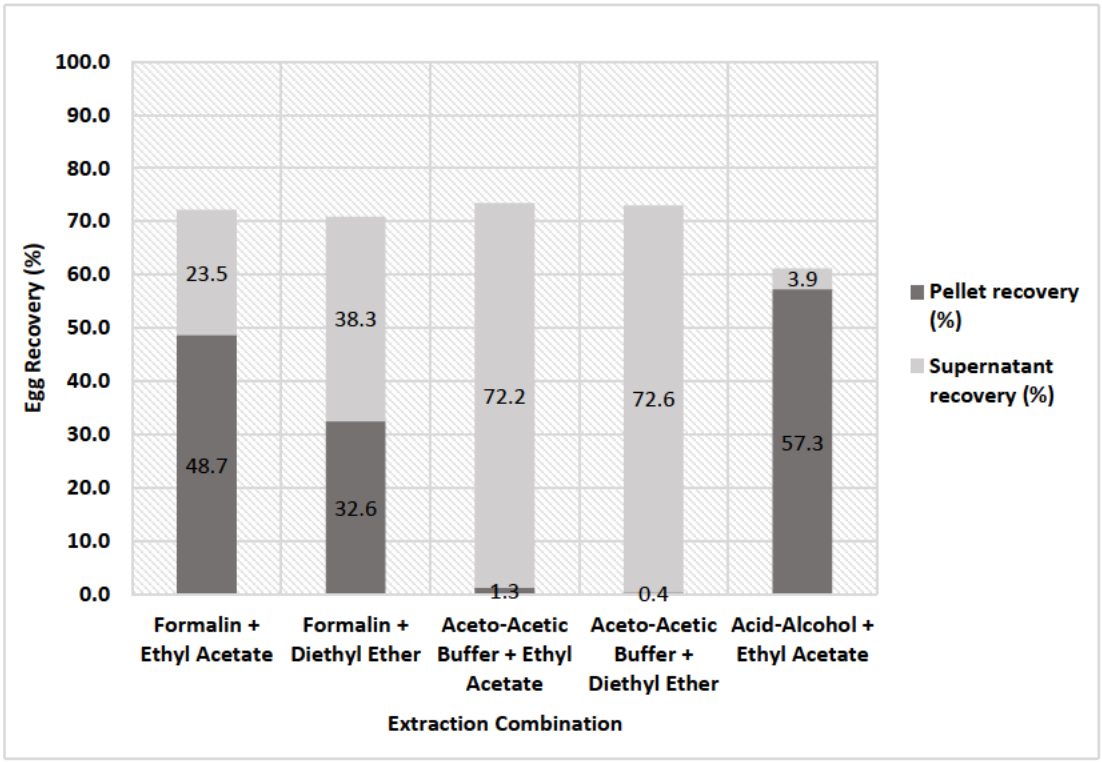

Figure 4. Egg recovery from both the pellet and supernatant of fatty sludge samples after phase extraction with 5 different chemical combinations (n = 5).

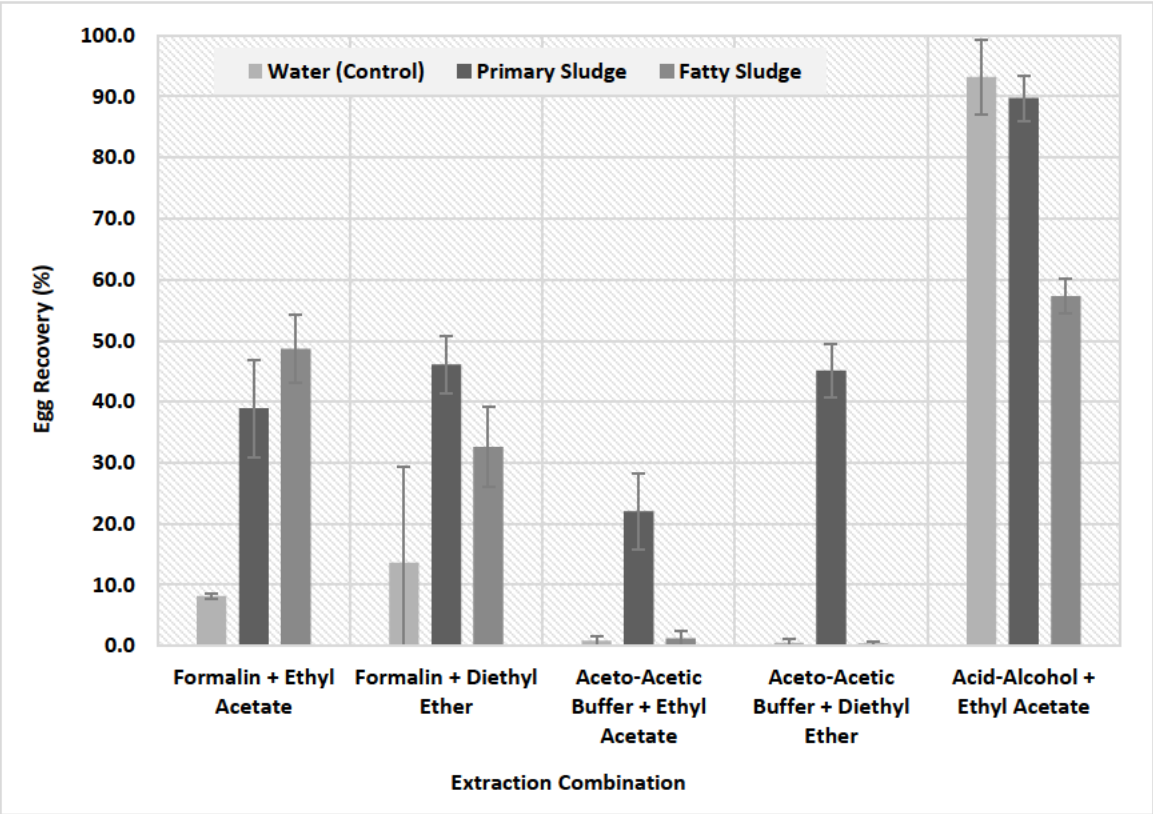

Figure 5. Total egg recovery (eggs in the pellet) from water, primary and fatty sludge samples after phase extraction with 5 different chemical combinations (n = 5).

DISCUSSION

Extraction is effective in the removal of organic material and purification of samples; however, the reagents have toxic effects on the viability of the eggs (Rocha et al., 2016). One of the steps involved in fecal sludge processing to recover helminth eggs is flotation. This involves the use of density gradients to separate eggs from particulate matter, where the solution must be denser than the eggs to allow for them to float up the supernatant column to the surface of the sample (Rocha et al., 2016). The same principle can be used to explain the differences in egg recovery across the different extraction solutions. The sp. gr. of *Ascaris* eggs is 1.13 (David and Lindquist, 1982). Table I shows the specific gravity of all 5 tested extraction solutions, and clearly, *Ascaris* eggs are denser than all solutions. This would mean that eggs should settle and not float and would therefore compact into the pellet after centrifugation. Acid-alcohol and ethyl acetate are both much less dense than the other solutions, resulting in the thick pellet that forms after extraction. When looking at the hydrophilic phase, both 10% formalin and acetoacetic buffer are quite dense (1.011 and 1.091) and similar to the relative density of *Ascaris* eggs. This could account for the eggs being trapped in the disc, as eggs would require time to be pushed down into the pellet. Since the disc forms quickly after the sample is shaken, eggs could have instead adhered to organic matter and thus been trapped in the disc.

Manser et al. (2016) reported a lower egg recovery when 10% formalin was used, with formalin made up in water being less dense, thus less effective at retaining eggs when compared with formalin made up in saline, which is denser. Acetoacetic buffer is slightly denser than formalin and would therefore allow eggs to remain in solution, thus being trapped in or above the fatty layer or debris disc. Formalin made up in water is slightly less dense, thus allowing eggs to settle better when shaken fast or centrifuged and could account for the difference in performance in terms of egg recovery, where formalin samples recovered more eggs than acetoacetic buffer.

Ritchie's formal-ether concentration was originally developed as a diagnostic method for stool samples to determine total fecal egg counts (FEC) (Wyhoff and Ritchie, 1952). It was then simplified (Ridley and Hawgood, 1956) and optimized (Allen and Ridley, 1970) and is used to concentrate parasites in fresh or pre-weighed, formalin-preserved fecal samples. Other methods for detection of helminth eggs in stool samples include the Kato-Katz technique (Ash et al., 1994) and the FLOTAC test, which is based on flotation and centrifugation (Albonico et al., 2013). Allen and Ridley (1970) stated that the formal-ether concentration, which ideally results in parasites sedimenting at the bottom of the test tube, was preferred over the zinc-sulphate (sp. gr. 1.18) flotation method for recovering eggs, where eggs rise to the surface of the supernatant due to a difference in densities between the eggs and the suspension medium. The study also stated that heavier eggs were lost during flotation but were recovered with sedimentation, with the latter resulting in clearer microscopy such that structural details of eggs can be observed more easily, and recovery of a greater number of egg species. When comparing egg recoveries between formal-ether and the Kato-Katz technique, it was found that 50% more eggs were lost with the former, and the latter was more sensitive for egg quantification (Ebrahim et al., 1997). The formal-ether concentration is commonly used in routine testing laboratories for stool samples, however the difference in egg recovery between these two methods has not been questioned and thoroughly investigated. The data from our study aids in understanding such differences in egg recovery in terms of egg loss during extraction.

Polarity of a solution determines its solubility. Ethyl acetate is more polar than diethyl ether, causing the latter to be a better emulsifying agent (Manser et al., 2016). Diethyl ether, therefore, can attract eggs towards the lipophilic layer, due to the high fat content of the cell membrane and lipid layer of the eggshell, thus being trapped in or above the disc. Ethyl acetate is a less effective emulsifier, thus resulting in a larger pellet but containing more eggs. According to Manser et al. (2016), extractions with diethyl ether resulted in a final deposit that was free of fat, but eggs were trapped in the fats in the debris disc, thus being discarded along with the supernatant layer. Acid-alcohol is made up of sulphuric acid, that is polar, and ethanol, that possesses both polar and non-polar ends, resulting in a more polar solution when combined to form acid-alcohol. This could further explain the large deposit

formed with the acid-alcohol and ethyl acetate combination, as both are less effective emulsifying agents. For fatty sludge samples, ethyl acetate resulted in better egg recovery than diethyl ether (Figure 5), that could also be due to less effective emulsification of the lipids resulting in reduced trapping of eggs in the disc or in the solvent above.

Amoah et al. (2017b) reported a loss in egg viability when samples were exposed to acetoacetic acid alone, and in combination with ethyl acetate, possibly due to the destruction of the lipid membrane of the eggs by the solvent. Nelson and Darby (2001) reported a 70% average loss of egg viability when samples were exposed to extraction solutions. Furthermore, all eggs were not recovered, but possibly lost or destroyed during phase extraction. They found a loss in viability of eggs exposed to acid-alcohol, and a synergistic effect when combined with a solvent.

Diethyl ether is flammable, explosive, and hazardous to human health; thus, ethyl acetate is preferred for extraction (Rude et al., 1987). These authors found that a larger plug of debris formed after centrifugation, and egg recovery was greater with ethyl acetate as compared with diethyl ether in both instances. Young et al. (1979) reported similar findings, thereby recommending the use of ethyl acetate over diethyl ether. Pakdad et al. (2018) reported a 43.3% *Ascaris* egg recovery with a 10% formalin and diethyl ether combination. We found that the diethyl ether recovered similar amounts of eggs in primary sludge samples (± 45.5%) when combined with both 10% formalin and acetoacetic buffer, but recovery was still far too low. It has also been recommended that exposure time should be kept to the minimum if extraction is being performed (Amoah et al., 2017b; Nelson and Darby, 2001). Ethyl acetate performed better with 10% formalin than with acetoacetic buffer, but egg recovery was still too low, and performed the best in combination with acid-alcohol (Figures 2 - 4), but the pellet size was not reduced, and microscopy still proved difficult. Replacing diethyl ether with ethyl acetate for phase extraction, even though the latter is less harmful, would not be ideal as egg loss is not preventable.

CONCLUSION

Exposure to extraction solutions can negatively impact the development and viability of *Ascaris* eggs (Amoah et al., 2017b; Nelson and Darby, 2001; Ravindran et al., 2019; Rocha et al., 2016). To prevent such egg-damage, it is recommended that the extraction step be removed completely, and possibly be replaced with an additional sieving step that can remove proteins and lipids (Rocha et al., 2016). We support the removal of the extraction step, primarily due to egg loss in the supernatant phase (including the disc of organic matter), and the fact that the combination that produced the best egg recovery (acid-alcohol and ethyl acetate) did not actually reduce the size of the final pellet for microscopy. This defeats the purpose of the phase extraction step and causes unnecessary exposure

of eggs to potentially harmful chemicals, without facilitating easier microscopy. We recommend that instead of extraction, samples that have a thick final pellet after the supernatant is removed, should be resuspended in a little water and half the sample be analysed. The egg recovery can then be extrapolated accordingly. Further research needs to be done into a possible additional sieving and rinsing step that allows for recovery of smaller egg species as well as those > 38 µm. Also, a comparative study on egg recovery between the formal-ether concentration and the Kato-Katz technique needs to be conducted. This study concludes the work conducted by Naidoo and Archer, that aimed at optimizing the existing PRG Helminth Method upon comparison with the steps of other helminth test methods. Based on data from this study and the previous two, a final optimized method will be renamed the WRDC Helminth Method and published.

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CHAPTER 5: PUBLICATION 4

THE WRDC HELMINTH METHOD: AN OPTIMISED AND HIGHLY ADAPTABLE PROCEDURE FOR HELMINTH EGG RECOVERY THAT IS SUITABLE FOR SANITATION AND ENVIRONMENTAL SAMPLES

To be submitted to a methods journal

THE WRDC HELMINTH METHOD: AN OPTIMISED AND HIGHLY ADAPTABLE PROCEDURE FOR HELMINTH EGG RECOVERY THAT IS SUITABLE FOR SANITATION AND ENVIRONMENTAL SAMPLES

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ABSTRACT

A variety of helminth test methods are currently employed globally for the recovery of eggs; however, a single standard procedure that is adaptable to various sample types does not exist. We propose the WRDC Helminth Method be implemented as it recovers > 90% eggs. It is applicable to solid, semi-solid and solid sludges, soil, and plant material samples. It involves a series of steps, including washing the sample in a wash solution over a set of particle-size separation pan sieves, floating the sample to separate eggs from heavier particles, microscopic analysis to quantify eggs and determine their condition, and incubation to confirm their viability. The amount of sample to process, and wash solution to use, depends on sample type. Zinc sulphate must be used as the flotation solution, ensuring that it is made up to a specific gravity of 1.3. Examples of helminth species that this method can detect include *Ascaris* sp., *Trichuris* sp., *Taenia* sp., and hookworm species. This method is quicker than most others, requires fewer resources, and is thus less costly. It is applicable to laboratories in developing nations, where financial resources and power supply are limited. The average time required to carry out this entire protocol, from processing of a sample to microscopic analysis of recovered eggs, is approximately 2.5 hr. Whilst specialised expertise is not required for carrying out of the procedure, it is required for microscopy. Determination of egg species and assessment of viability can be subjective and therefore requires a 'trained eye'.

INTRODUCTION

Background

Diarrhoeal diseases are generally symptomatic of bacterial and viral infections but can also be due to infection by parasitic worms (helminths). Human helminths commonly seen in faecal sludge are *Ascaris lumbricoides, Trichuris trichiura, Taenia* sp., hookworm sp. and *Schistosoma* sp.[1,2], and non-human helminth species include *Toxocara* sp., several *Trichuris* sp., *Colodium* sp. (Bancroft, 1893) (previously *Capillaria* sp.) and occasionally other cestode and trematode species. The provision of improved sanitation and safe sludge disposal may reduce the prevalence of diarrhoeal diseases by up to 36%, by minimising contact between humans and their excreta[3]. Stored faecal sludge must be properly treated and decontaminated to reduce the number of viable helminth eggs, and other pathogens, entering the environment.

It is impossible to isolate every microorganism that is found in faecal sludge, as detection methods lack specificity, are costly, and time consuming[2]. Indicator organisms are therefore targeted based on the principle that their presence is indicative of other microorganisms in the excreta. *Ascaris* eggs are thus deemed fit as indicators for parasite and overall microbial and viral contamination, as they are the most resilient of all pathogens found in faecal sludge[2]. If a treatment process can inactivate *Ascaris* spp. eggs, then it is very likely all other pathogens will be destroyed[4]. New faecal sludge treatment and toilet technologies are constantly being developed, where helminth eggs must be spiked into the system to test treatment efficacy. Furthermore, waste material (sludge) is considered a resource; but reuse is dependent on complete hygienisation by pathogen removal or destruction. According to the WHO (2006), the recommended limit for helminth eggs should be: 1 egg per litre (L) of wastewater and 1 egg per gram of total dried solids[4]. A highly sensitive, standard helminth test method, that can recover eggs from various sample types, is therefore required for application in laboratories globally, so that samples and systems may be tested. This ensures that data are comparable in terms of what does and does not work for faecal sludge treatment technologies, and disease prevalence worldwide.

Development of the method

Conventional helminth techniques for samples of high volume or large mass, including the improved PRG Helminth Method (to be renamed the WRDC Helminth Method in this article), involve a series of steps. These include the use of a wash solution and pan sieves to facilitate dislodging of eggs from, and breaking down of, the sample matrix, flotation using density gradients to further separate heavier particles from eggs, centrifugation to either compact eggs in the pellet or to float eggs out of the sample, and sometimes, an extraction step using hydrophilic and lipophilic solutions to remove proteins and lipids and ideally reduce the size of the final pellet for analysis (not recommended for

inclusion in the method presented here). The final step is microscopic analysis of the processed sample, that can include viability assessment of eggs.

Different laboratories and groups have implemented the PRG Helminth Method[5], the standard United States Environmental Protection Agency (USEPA) Method[6], the Mexican Standard for Wastewater Analysis[7] or the Bailenger Method[8]. There is, however, no single, universal, standard helminth test method. This protocol was therefore developed to fill the niche, being more cost- and time-efficient as well as adaptable to different sample types, rather than adapting samples to fit the method.

The USEPA Method[6] has been the most used and accepted standard for helminth recovery in water and wastewater samples. It is not, however, the most suitable for more solid sludge samples as found in on-site sanitation systems, e.g., Ventilated Improved Pit (VIP) Latrines or Urine Diversion Dry Toilets (UDDTs). This led to the development of the method used in Hawksworth et al. (2010)[9] for the recovery of helminth eggs from UDDT waste (this work originated in 2005 but was only publicly presented in 2010), that was further modified by Moodley et al. (2008)[10] and then by Archer, who used it to process soil samples for a publication with Pebsworth et al. (2012)[11]. The basic principles of these methods are the same as what is presented in this paper; however, major differences were, no sieves were used in the washing step and the flotation step was done in test tubes alone and not poured over a sieve after centrifugation. This made the test cumbersome, time consumable and used many laboratory consumables. The PRG Helminth Method[5] then adapted the protocol by pouring off the supernatant from all floatation tubes of one sample over a sieve and collecting the retentate into one final test tube for centrifugation. During this evolution of the method[5], adaptations were made to accommodate a variety of sample types. The WRDC Helminth Method was then derived by comparing steps in the PRG Helminth Method[5], the USEPA Method[6], the Mexican Standard[7] and the Bailenger Method[8] to present one modified, optimised procedure for helminth testing. It can accommodate liquid samples (water samples, effluent, septic tank sludge and runny fatty sludge), semi-solid samples (from VIPs and UDDTs), thick fatty and primary sludges (from wastewater treatment works (WWTWs)) and other faecal sludge types, solid samples (dried or pelletised sludge, or ash from incineration toilets), soil samples, compost, and plant material.

Comparison with other methods

The USEPA Method[6] reports a 75 - 80% egg recovery, whereas the WRDC Helminth Method can recover > 90% helminth eggs in a given sample. The Mexican Standard[7] and Bailenger Method[8] do not indicate their respective average recoverability. Furthermore, our method is adaptable to suit specific sample types, whereas some methods classify samples as just solid, liquid or compost, some do not take sample size into consideration at all, and others are designed for wastewater samples and require

that any other sample type be manipulated to fit the method. The USEPA Method was specifically designed to isolate and microscopically identify *Ascaris* spp. eggs, and not all helminth species.

The old USEPA Method[12], the Mexican Standard[7] and the Bailenger Method[8] include an extraction step to clean up the sample and make it easier to examine microscopically. It is reported that solvents affect the wall of *Ascaris* eggs[13], therefore the extraction step was excluded from the PRG Helminth Method Standard Operating Procedure (SOP). The step was however, occasionally included only if the final pellet was too large for microscopic analysis. We then found that eggs are lost during the extraction step and recommended that the step be removed completely from helminth testing procedures.

Other differences between our method and others include extra steps that can be avoided, such as the use of glassware only, which can add to cost, the use of organosilane (a monomeric silicone-based chemical) to coat the glassware, vacuum aspiration of samples, which can result in sample loss if the technician is inexperienced, and prolonged soaking and gravitational sedimentation periods that are not necessary for successful egg recovery.

Modern test methods have also been developed for helminth detection in samples, such as conventional polymerase chain reaction (PCR), real-time PCR (qPCR) and the BacLight staining technique. Whilst conventional PCR can detect the presence and absence of a particular helminth species, it is unable to quantify or assess viability of the eggs. Quantification is possible with qPCR; however, the test does not determine whether eggs are viable or non-viable. The BacLight staining technique requires the purchase of various stains and a specialised confocal microscope for helminth egg quantification[14]. These more sophisticated methods are not always applicable to small laboratories in developing countries, where a constant supply of electricity, financial support, or specialised equipment and reagents, may be lacking. We therefore aimed for test specificity, cost-effectiveness, and minimal processing and turnaround time.

Experimental design

This project was separated into four studies, the first three of which formed the experimental element and were published as peer-reviewed articles, and the current study being the fourth that describes the final optimised method in detail. The first study looked at the effects of all reagents and chemicals used in existing helminth test methods on the viability of *Ascaris suum* eggs. The optimum solutions were then selected for use in the experiments to follow. The second study looked at the technical aspects of these methods, including the mode of washing samples, different wash solutions relative to sample type, flotation solutions and their respective specific gravities, and centrifugation speeds and times after each step, all in terms of egg recovery. The third study looked at the efficacy of the

phase extraction step on the retrieval of eggs from the pellet that remained after sample processing by washing and flotation. The least harmful chemicals and the most efficient recovery steps (from all three studies) were then used to produce the final method presented in this paper.

Expertise required to implement the method

The actual implementation of the method is simple and requires good laboratory practice and basic laboratory skills. The need for expertise arises at the microscopy step, particularly with regards to viability assessment. Also, certain biological specimens can be mistaken for helminth eggs, such as spores and pollen grains. Sometimes, free-living nematode and other organisms' eggs, can be mistaken for hookworm eggs.

Limitations

This method is currently employed at the WRDC helminth lab and can retrieve parasite eggs of varying sizes, from *Taenia* sp., one of the smallest helminth eggs, to *Toxocara* sp., one of the larger ones. Limitations of this method include that it is unable to recover protozoan parasites, such as *Giardia* sp. and *Cryptosporidium* sp., as mesh sizes are too large to contain them. Also, use of a 100 µm sieve above the 20 µm, means that larger eggs, such as *Fasciola* sp., would be trapped on the top sieve and consequently discarded. In countries where large trematode eggs are common, it is suggested that either the top sieve be of a larger mesh size, to allow these eggs to pass through it onto the bottom 20 µm sieve, or an extra larger sieve be placed on top of the 100 µm mesh sieve and that the retentate on the middle 100 µm mesh sieve be processed further in parallel with the retentate collected from the 20 µm sieve. It should be noted that the former option would result in increased debris collection on the bottom sieve that also retains the eggs. Diagnostic accuracy too, is subjective to the person analysing the sample and their level of expertise in parasitology and experience with microscopy.

MATERIALS:

Reagents

- Ammonium bicarbonate
- 0.1% 7X® (MP Biochemicals)
- Zinc sulphate ($ZnSO_4$) specific gravity (sp. gr.) 1.3
- 0.1N Sulphuric acid (H_2SO_4)
- Commercial (3.3 – 3.5%) sodium hypochlorite, diluted to 50% (± 1.75% NaClO)

Equipment

- Compound microscope with 10X and 40X objectives (and preferably, a camera)
- Bench-top centrifuge with a swing-out rotor that can spin a minimum of 8 x 15 ml graduated, conical, plastic test tubes (Falcon tubes) and, if possible, buckets that can spin a minimum of 4 x 50 ml Falcon tubes
- Sink with hose attached to the tap, or a shower head, for washing using pressure
- Top-pan balance for weighing quantities up to 1200 g and accurate to 2 decimal places
- Magnetic stirrer plate
- Stirrer bars
- Vortex mixer
- Hydrometer that can measure sp. gr. of 1.2 - 1.3
- 100 µm mesh stainless steel pan sieve, height 50 mm x diameter 200 mm (a larger mesh sieve can be used instead of, or on top of, the 100 µm mesh sieve if samples are expected to contain eggs > 100 µm in size).
- 20 µm mesh stainless steel pan sieve, height 50 mm x diameter 200 mm
- 20 µm mesh stainless steel pan sieve, height 40 – 50 mm x diameter 100 mm
- Plastic test tube racks (minimum of 2) to hold the 15 ml Falcon tubes and 2 for 50 ml tubes
- Plastic 1000 ml measuring cylinder
- Plastic 250 ml beakers
- Silicon kitchen spatula – with medium to long handle but small spatula (on stirring side)
- Plastic disposable 3 ml Pasteur pipettes (non-sterile)
- Non-sterile gloves (good quality, size important, must fit well)
- Wooden applicator sticks
- Wooden tongue depressors
- Microscope slides (76 x 26 x 1.2 mm)
- Cover glasses (22 x 40 mm)

Reagent Setup

- **Ammonium bicarbonate**

Dissolve 119 g ammonium bicarbonate in 1 L deionised water, mix on a magnetic stirrer plate using a bar magnet, and store in a glass bottle. Cap bottle tightly and store at room temperature.

- **0.1% 7X®**

Pipette 1 ml 7X® into 1L deionised water and mix by inverting several times. Cap bottle tightly and store at room temperature.

- **Zinc sulphate**

Dissolve 500 g zinc sulphate in 700 ml deionised water and mix on a magnetic stirrer plate using a bar magnet. Use a hydrometer to measure the SG and alter accordingly. If the sp. gr. is < 1.3, add more chemical and if the sp. gr. > 1.3, add more water. Cap bottle tightly and store at room temperature.

- **0.1N Sulphuric acid**

Add 500 ml deionised water to a 1 L bottle, measure 3ml concentrated sulphuric acid in a 10ml graduated cylinder and pour this into the bottle containing water, cap and mix slowly by inversion. Uncap, add 497 ml of deionised water to the plastic bottle, re-cap, mix slowly by inversion, and store safely.

PROCEDURE

Total solids (TS): solid sludges contain > 25% TS; semi-solid sludges contain 15 – 25% TS; liquid sludges contain < 5% TS.

For solid sludges (pelletised, dried, or ash from incineration toilets):

1) Weigh 10 g of sample into a 250 ml beaker.
2) Add 80 – 100 ml ammonium bicarbonate or 7X® and soak for 4 hr to soften. If the sample is very hard, it can be soaked for up to 24 hr. Use a tongue depressor to break up the sample and proceed to step 3 below.

For semi-solid sludges (including VIP, UDDT & other thick sludges) and soil samples:

1) Weigh 10 g of the sludge sample or 50 g of the soil sample into a 250 ml plastic beaker.
2) Add 80 – 100 ml ammonium bicarbonate to the UDDT or soil samples and 7X® to VIP and solid fatty sludges. Add a bar magnet to the beaker and mix on a magnetic stirrer for 10 min.
3) Wet the 2 x 200 mm diameter sieves with tap water, ensuring the 100 µm is placed on top of the 20 µm mesh sieve. Pour the wash solution-sludge mixture over the top sieve. Rinse the beaker with tap water and pour over the top sieve, repeating several times until the beaker is clean.
4) Using the hose attachment or shower head on the tap, wash the sample on the 100 µm mesh sieve (keeping it on top of the 20 µm). Use a silicon spatula or the back of a gloved hand to break up clumps and aid separation of eggs from particulate matter. Regularly check the bottom sieve for fluid build-up. When this occurs, use the same spatula to stir the sample on the 20 µm sieve while holding the 100 µm sieve directly above so as not to lose any sample. When the 20 µm sieve has drained sufficiently, place the 100 µm sieve back on top and continue washing. Repeat this until the sample on the 100 µm sieve is well washed.
5) Separate the sieves and set aside the 100 µm sieve, the retentate on this can be discarded later.

6) Wash the retentate on the 20 µm sieve well, then wash it to one side of the sieve for collection. If there is not a large quantity of retentate, skip step 7, and using a plastic 3 ml Pasteur pipette, collect the retentate from step 6 directly into test tubes as described in step 8; otherwise, proceed to step 7.
7) Rinse the total retentate off the 20 µm filter into the original rinsed-out, beaker. If there is a lot of water, allow the contents of the beaker to settle for at least 2 hr, then pipette off some of the supernatant fluid without disturbing the sediment.
8) Pour the beaker contents into 4 x 15 ml appropriately labelled Falcon tubes (or as many as necessary), or if the retentate is large, use 50 ml tubes. After the next step, the aim is to have ≤ 1 ml deposit in a 15 ml tube / ≤ 5 ml in a 50 ml tube.
9) Centrifuge the tubes at 1512 x g (3000 rpm) for 10 minutes (min). If the pellet is well-compacted, pour off the supernatant in one smooth movement. If the pellet is 'loose,' use a 3 ml plastic Pasteur pipette to suction off the supernatant.
10) Place the test tubes in a rack with an applicator stick in each (to act as a stirring rod) and pipette in $ZnSO_4$, 3 ml at a time, mixing on a vortex stirrer in between addition of the chemical, until the tubes are filled to the 14 ml mark for 15 ml tubes / 40 ml mark for 50 ml tubes.
11) Centrifuge the tubes at 672 x g (2000 rpm) for 15 min. Wet the 100 mm diameter, 20 µm sieve with tap water and pour the supernatant from all the tubes of one sample over the sieve. Discard all the deposits left in all the test tubes and keep one empty test tube aside for re-use.
12) Wash the retentate well with tap water and rinse it to one side of the sieve for collection. Using a 3 ml plastic pipette, transfer the retentate back into the test tube kept aside.
13) Centrifuge the tubes at 1512 x g (3000 rpm) for 10 min and pour off the supernatant. The final pellet is now ready for microscopy.
14) Pipette up the deposit, dispense it onto one or more microscope slides, but make one slide at a time so they don't stand for long periods and dry out. Place a 22 x 40 mm cover-glass on top and examine under a compound microscope using the 10X objective for counting, and the 40X objective to confirm identifications and morphological states for viability assessment.
15) Examine the entire preparation and count every *Ascaris* egg, classifying them as viable (plump motile larva in the egg), potentially viable (egg undeveloped; developing, from a two celled to gastrula stage; or containing a plump immotile larva), necrotic (egg containing a shrivelled, dead larva), or dead (egg globular, empty, or with wall damage). Also count *Trichuris*, *Taenia*, *Toxocara,* hookworm spp. and other helminth species eggs and assess simply as potentially viable or dead (using the same features as used for *Ascaris*).

16) When done, discard cover glasses into a 'sharps' container, and soak slides in a beaker of 50% commercial NaClO for 1 hr in a sealable container with airtight lid to ensure inactivation of eggs[15]. All retentate and deposits in the tubes meant for discard must also be soaked in 50% commercial NaClO prior to discard.

For liquid or slurry samples (including water, WWTW effluent, septic tank & liquid fatty sludge):

1) The amount of sample required for processing is dependent upon the solids content:
    1.1) For clean samples with low total suspended solids (TSS < 5%): 5 – 10 L of sample. No wash solution is needed, but 7X® can be used if desired (added as in 1.3 below).
    1.2) For dirty water samples with low to moderate suspended solids: 1-5 L of sample. No wash solution is needed, but 7X® can be used if desired (added as in 1.3 below).
    1.3) For septic tank sludge and black water with high solids content: 200 – 500 ml and make up a 0.1% 7X® solution directly in the sample, by pipetting in the relative amount of 7X®.
    1.4) For liquid fatty sludge samples 200 – 500 ml and make up a 0.1% 7X® solution directly in the sample, by pipetting in the relative amount of 7X®.
2) In the case of using a wash solution, mix samples well using a tongue depressor until well homogenised. For samples without a wash solution, be sure to swirl the beaker to resuspend the sediment prior to poring it over the sieves. Proceed to steps 3 - 16 of the semi-solid sludge method above.

For plant material and green compost:

1) Weight 50 g of plant material into a 500 ml (or larger) beaker and cut it up into small pieces using scissors.
2) Add ammonium bicarbonate to the sample until fully submerged and mix well with a tongue depressor. Place the scissors and tongue depressor into a beaker of water.
3) Wet the 2 x 200 mm diameter sieves with tap water, place the 100 µm on top of the 20 µm mesh sieve. Pour the sample and the water from the beaker through the sieves. Rinse both beakers several times and wash the tongue depressor and scissors over the sieves as well. Proceed to steps 4 - 16 of semi-solid sludge method above.

For incubating samples for viability testing:

1) If viability cannot be definitively determined on initial microscopy of the sample (due to no motile larvae present in the eggs), and the client requires viability to be determined and

reported, then an extra sample must be processed as per the correct procedure per sample type and the deposit resuspended in 1.5 times the volume of the pellet using 0.1N H_2SO_4. Mark the test tube at the fluid level.

2) Loosely cap the tube to allow air into the sample. Incubate for 21 - 28 days at 2 5- 28°C. Aerate the sample weekly by gently swirling the tube and, if the fluid level has dropped, top up to the mark with 0.1N H_2SO_4.
3) After 28 days, remove from the incubator, centrifuge at (1512 x g) 3000 rpm for 10 min, remove the supernatant fluid, examine, and report on egg viability as for the initial test sample.

TIMING

Total processing time, excluding centrifugation, is approximately 60 – 90 min, depending on level of expertise, as well as whether samples will be incubated or not. Centrifugation after washing is 10 min, after flotation is 15 min and the last step to compact the final pellet prior to microscopy is 10 min, bringing the total centrifugation time to 35 min. Microscopy is estimated at 60 min per sample thus the total test time is approximately 2 – 2.5 hr.

TROUBLESHOOTING

Quality control – QA/QC

For QA/QC testing, uninfected faecal sludge, wastewater, or relative sample type is to be used such that a known quantity of eggs may be spiked. Furthermore, it should be of similar consistency to samples being tested.

1) One uninfected sample weighed in grams and/or one sample measured in litres as a negative control (that is not spiked).
2) For the positive controls, spike a known number of eggs into weighed amounts from the same uninfected sample used for the negative control.
3) Process the samples according to the methods listed above, relative to sample type.
4) Run a negative and a positive control in parallel with a batch of similar consistency samples per test run.
5) Control samples should be prepared and re-examined (after the technician has completed their analysis) by a senior, experienced analyst as a control for the microscopy readings.
6) An egg recovery of > 80% is considered successful (USEPA, 2003).

ANTICIPATED RESULTS

A variety of helminth eggs are expected to be found, depending on sample type and source, including round worms (nematodes), tapeworms (cestodes), and flukes (trematodes). Species that are expected to be found are: *Ascaris* sp. (*A. suum* and *A. lumbricoides* are morphologically identical to one another but have different hosts and genetic compositions), *Trichuris* sp. (of humans, dogs, rats, etc.), *Taenia* sp. (*T. solium* and *T. saginata* cannot be morphologically distinguished), *Hymenolepis diminuta, Hymenolepis nana, Toxocara* sp., *Schistosoma mansoni, Schistosoma haematobium*, *Colodium* sp., *Fasciola* sp., *Dicrocoelium dendriticum, Enterobius vermicularis* and *Strongyloides stercoralis* larvae. It is also extremely difficult to morphologically distinguish hookworm species eggs, and they are therefore reported as 'hookworm sp.'.

Helminth eggs are considered potentially viable (and thus potentially infective) if they are in the undeveloped stage (zygote), developing stages (cleavage, resulting in blastomeres, i.e., 2-, 4-, 8-cell stage), morula (16- to 32-cell stage), blastula (> 32-cell stage) and gastrula (when a foetal shape starts to form), then an L1 and finally an infective L2 larva. If an L2 larva is moving in the egg then it is considered viable and infective. When eggs die, they may become globular, have broken shells, collapsed walls or they appear empty inside, and are collectively termed 'dead'. If a formed larva dies inside the egg, it appears shrivelled and occupies much less space than a plump, healthy viable larva and is termed necrotic. Eggs that have never been fertilised are termed 'infertile' and cannot develop and are therefore classified under non-viable eggs. *Ascaris* sp., are categorised as such under viable, potentially viable and non-viable.

Cestodes, like *Taenia* spp., contain an oncosphere within the egg that does not develop further, thus we describe it as potentially viable if it looks in good condition and the hooklets are visible, and dead if the contents are globular or have no structure. *Taenia* eggs are counted, assessed for potential viability, and reported as *Taenia* sp. Except for *Taenia* spp., all other cestode and trematode eggs are simply counted and recorded, however recording viability status is optional and not a requirement unless specifically requested. Final data should be reported as number of eggs per litre or per gram dry weight of sample.

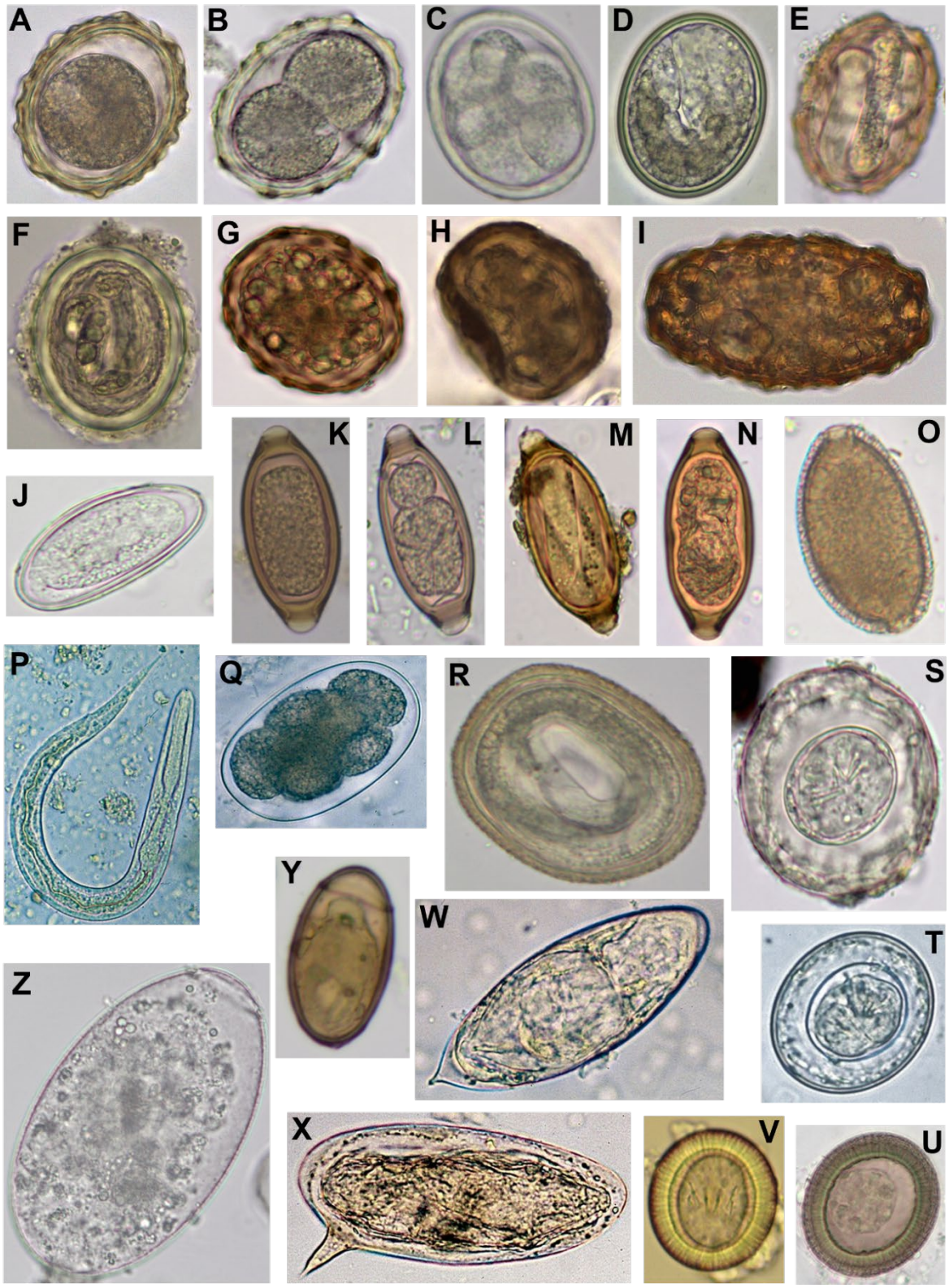

Figure 1: Plate of photographs of helminth eggs and larvae found in sanitation and soil samples (taken from ([5]) where the method was published as the PRG Helminth Method). (A) *Ascaris* sp. – undeveloped; (B) *Ascaris* sp. – 2-cell & developing; (C) *Ascaris* sp. – developing; (D) *Ascaris* sp. – gastrula; (E) *Ascaris* sp. – viable larva; (F) *Ascaris* sp. – necrotic (dead) larva; (G) *Ascaris* sp. – dead &

globular; (H) *Ascaris* sp. – dead with wall collapsing & globular; (I) *Ascaris* sp. – infertile; (J) *Enterobius vermicularis* (Linnaeus, 1758) – dead; (K) *Trichuris* sp. – undeveloped; (L) *Trichuris* sp. – developing; (M) *Trichuris* sp. – viable larva; (N) *Trichuris* sp. – dead; (O) *Colodium* sp. (previously *Capillaria* sp.) – dead; (P) *Strongyloides stercoralus* (Bavay, 1876) – rhabditiform larva; (Q) Hookworm sp. – developing; (R) *Toxocara* sp. – developing; (S) *Hymenolepis diminuta* – potentially viable; (T) *Hymenolepis nana* – potentially viable; (U) *Taenia* sp. – dead; (V) *Taenia* sp – viable; (W) *Schistosoma haematobium* – dead; (X) *Schistosoma mansoni* – dead; (Y) *Dicrocoelium dendriticum* (Rudolphi, 1819) – potentially viable; (Z) *Fasciola* sp. – dead.

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AUTHOR CONTRIBUTIONS

D. Naidoo was the principal investigator on this project, was involved in project design, conducted all laboratory work and was the primary author on papers. C.E. Archer was the project leader, was involved in project design, laboratory work, and paper writing and editing.

ETHICS

Ethical approval for this study was granted by both the Animal Research Ethics Committee (AREC) and the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (AREC/071/018 and BREC/00002794/2021).

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CHAPTER 6: GENERAL SUMMARY & CONCLUSIONS

There is no universally accepted standard helminth test method in existence (Gyawali, 2017). Furthermore, most methods are designed to accommodate samples categorised as either liquid or solid, and in some cases, compost. This means that all environmental sample types are not considered for helminth testing by the methods developed for effluent testing of discharges from traditional wastewater treatment works. These methods therefore adapt non-liquid type samples to suit the method instead of adapting the method to accommodate the various sample matrices. These methods are also long and allow for chemical exposure to eggs that could be avoided. This project was therefore aimed at filling this niche, by optimising the existing PRG Helminth Method to formulate a robust, quick, and cost-efficient method recommended for application in laboratories globally. Even though chapter 5 (publication 4) presented the new, optimised WRDC Helminth Method, this chapter collates the data from publications 1, 2 and 3, and outlines this information in publication 4 that make up the individual steps of the method, relative to sample type. The table below summarises these steps and compares them with what was done in the original PRG Helminth Method and presents alternative options.

Table III: Summary table describing differences between the original PRG Helminth Method and the final, optimised WRDC Helminth Method and alternate options for each step

Processing step	Steps in PRG Helminth Method	Recommendation for WRDC Helminth Method	Reason for recommendation	Alternative option and reason
Sample type	Considers liquid, VIP, UDDT, thick sludges, and soil samples. Failed to document many other items/media tested, e.g., masks.	The method must be adjusted to fit all sample types, including those mentioned, as well as septic tank, fatty and dried sludges, composts and plant matter.	Each sample type will need to be handled differently in terms of the appropriate wash solution to break down the sample matrix, and possible soaking times.	None
Washing and sedimentation	Pour sample over sieves for separation of eggs from sample matrix	Pour sample over sieves for separation of eggs from sample matrix	The use of sieves facilitates separation of lager particles from eggs.	Adding a wash solution to the sample in test tubes and mixing on a vortex to break up large particles according to Hawksworth et al. 2010, but this is tedious.
	Wash under pressure	Wash under pressure	Pressure is needed to separate eggs from particulate matter.	None

Processing step	Steps in PRG Helminth Method	Recommendation for WRDC Helminth Method	Reason for recommendation	Alternative option and reason
Washing and sedimentation	Use of water to wash water and WWTW effluent samples by pouring sample straight through a set of sieves and immediately washing.	Use of water to wash water and WWTW effluent samples by pouring sample straight through a set of sieves and immediately washing.	Water is sufficient for clear, liquid samples.	Can use ammonium bicarbonate, 7X®, Triton®X-100 or Sunlight® Liquid can be used (common dishwashing liquid), IF particulate matter is present in sample.
	Ammonium bicarbonate for all thick or solid sludges, except fatty sludge.	Ammonium bicarbonate for UDDT and dried sludges and soil (or soil-containing) samples.	Breaks bonds between soil particles and eggs, and results in a clear pellet for microscopy.	Can use 7X®, Triton®X-100 or Sunlight® Liquid, but microscopy might not be as clear.
	Ammonium bicarbonate for VIP sludge and 0.1% Tween®80 for fatty sludges.	7X® for VIP, septic tank, fatty and dried sludge samples.	Soapiness can break down lipids in the sample and separate eggs from particles or particles from one another.	Triton®X-100 or Sunlight® Liquid can be used, but not for overnight soaking.
	Soak in physiological saline overnight for dried sludges, then wash with 0.1% Tween®80.	Dried sludge samples can be soaked from 4 - 24 hr in either ammonium bicarbonate or 7X®.	Soaking time is selected based on how dry the sludge is, and both wash solutions were effective.	None
	Centrifuge at 3000 rpm for 10 min.	Centrifuge at 3000 rpm for 10 min.	Resulted in > 90% egg recovery and a compact pellet.	None
Flotation	Zinc sulphate at sp. gr. 1.3	Zinc sulphate at sp. gr. 1.3	Zinc sulphate was the best flotation solution. The solution must be dense enough for all eggs to float, thus sp. gr. 1.3.	None – magnesium sulphate precipitated out and could affect the density of the solution. Sodium nitrate was ineffective in floating all eggs and is also reported to form crystals.
	Centrifuge at 2000rpm for 10 min.	Centrifuge at 2000 rpm for 15 min.	2000 rpm is a slow enough speed to allow for eggs to travel slowly up the supernatant column without causing egg damage. 15 min resulted in > 90% egg recovery.	None
Extraction	Extract **if necessary** using 10% formalin and ethyl acetate or diethyl ether.	Step to be removed completely.	Eggs are lost in the disc and supernatant when discarded after extraction. Eggs were	None

Processing step	Steps in PRG Helminth Method	Recommendation for WRDC Helminth Method	Reason for recommendation	Alternative option and reason
Extraction			only fully recovered with acid-alcohol where the disc size didn't reduce at all, making extraction pointless.	
Microscopy	Light microscopy (100X & 400X magnification) - samples to be centrifuged at 3000 rpm for 10 min.	Light microscopy (100 & 400X magnification) - samples to be centrifuged at 3000 rpm for 10 min.	Light microscopy at these magnifications is sufficient to count and categorise eggs. 3000 rpm for 10 min results in a compact final pellet and > 90% egg recovery.	None
Incubation	Water, sulphuric acid or 1% formalin	Sulphuric acid	Resulted in best larval development and minimal biological contamination of the sample.	0.5% and 2% formalin can be used; however, some contamination is likely after incubation.

This final method has been adapted from the original PRG Method (Appendix A) and is now optimised to include all sample types for maximum egg recovery (Table III). The best reagents, times of exposure, mode of washing, density for flotation, centrifugation speeds and times were chosen based on data, and a final method was drawn up as a SOP for implementation in our laboratory and then as a manuscript for publication in Nature Protocols (Chapter 5). The final method also recommends the elimination of the extraction step. It was found that eggs were lost during extraction, and the combination that recovered > 90% eggs did not reduce the final pellet size for microscopy, making it futile. This also has implications for routine parasitological stool sample testing using the formol-ether concentration (Allen & Ridley, 1970). Total egg recovery after implementing this method was, on average, > 90% for all sample types except fatty sludge, thus attesting to its success.

Table IV: Advantages and disadvantages of the 4 methods explored in this study

Method	Advantages	Disadvantages
WRDC Helminth Method	• Fewer processing steps than the other methods, thus requiring less specialised equipment. • Fewer steps also mean it is easier to carry out. • Quicker processing time than the other methods.	• Expertise is required for microscopy, particularly for correct viability assessment. • Processing of fatty sludge was not fully optimised (common problem in most methods). • Method has been optimised according to South African parasite prevalence and the use

Method	Advantages	Disadvantages
WRDC Helminth Method	• Minimal exposure to chemicals, and chemicals are readily available and inexpensive. • Does not require an extraction step, preventing egg loss. • Exposure to fewer chemicals. • Use of plasticware only, and ability to reuse apparatus reduces costs. • > 90% egg recovery for most sample types. • The entire final pellet is analysed and not just a representative aliquot (except in the case of fatty sludge). • Assessment of the state of eggs done after processing, thus viability can be reported immediately instead of after weeks of incubation. • Is adaptable to specific sample type and not simply broad categories such as liquids or solids. The method is thus adapted to sample type and not vice versa.	of a 100µm sieve is recommended. It is therefore possible to miss helminth eggs > 100µm in length, e.g., *Fasciola* sp. • Sieves are expensive to purchase.
USEPA Method	• Most commonly implemented method and is a USA national standard. • Known to recover 75 - 80% eggs. • Does not incorporate a phase extraction step, thus circumventing egg loss (present in the 1999 protocol, removed from the 2003 protocol).	• Developed for *Ascaris* sp. eggs only and NOT all helminth species. • Does not take all sample types into account. • Includes many (unnecessary) steps, making the method very long, thus increasing labour costs. • Additional steps mean additional apparatus and equipment, increasing setup and maintenance costs. • Includes numerous gravitational sedimentation steps, increasing processing and chemical exposure time. • Use of glassware also increases costs. • All glassware must be coated in organosilane (difficult and costly to source). • Use of glassware could result in breakages, (dangerous and increases costs). • Washing is done using a spray bottle, and not water under pressure. • Use of magnesium sulphate at sp. gr. 1.2, that was shown in our study to be inefficient for total egg recovery.

Method	Advantages	Disadvantages
USEPA Method		• All centrifugation times and speeds were far less than those demonstrated necessary in our study for egg recovery. • Microscopy is done using a Sedgewick-Rafter counting chamber, which limits magnification. Assessing egg viability then becomes difficult at 100X, (400X magnification is needed). • Microscopy is only done after incubation for a minimum of 18 days.
Mexican Standard for Wastewater Analysis	• Similar to the USEPA Method, but much quicker. • The entire final pellet is analysed and not just a representative aliquot.	• Method was designed for wastewater and treated wastewater samples, thus not accounting for all sample types. • Washing is not done under pressure. • All centrifugation times and some speeds are far lower than those demonstrated necessary in our study for egg recovery. • Centrifugation after flotation is too fast for successful egg recovery. • Incorporates an extraction step, therefore eggs are lost. • Microscopy is done using a Sedgewick-Rafter or Doncaster Disc counting chamber, which limits magnification. Assessing egg viability then becomes difficult (400X magnification is necessary). • Exact egg recoverability is unknown.
Bailenger Method	• Few processing steps thus the method is not long. • Specialised containers are not required for sedimentation. • Minimal laboratory equipment needed for processing. • Few chemicals are required, all of which are readily available and inexpensive. • Microscopy is done using a McMaster slide, which is reportedly viewed in 1-2 minutes per slide.	• The method was designed to accommodate raw or treated wastewater samples, and not for all sample types. • Exact egg recovery percentages are not known for this method. • Sieves are not used, thus separation of particles of different sizes from eggs is not very efficient. • Incorporates an extraction step, therefore eggs are lost. • Flotation is done using zinc sulphate at sp. gr. 1.18, demonstrated in this study to not be dense enough for successful egg recovery.

Method	Advantages	Disadvantages
Bailenger Method		• An aliquot of flotation fluid containing randomly dispersed eggs is counted, thus extrapolation of data for a full sample is likely inaccurate. • Helminth eggs are counted, but not assessed and categorised for viability. • Also, 1 - 2 min for egg assessment per sample is insufficient time to differentiate helminth species. • Incubation of eggs is not performed to confirm egg viability.

Although the methods have yet to be run in parallel with one another on different sample types with a known, spiked number of eggs, analysis of the actual protocols, as well as the comparative analyses were performed on steps from each method, allowing us to compare the pros and cons of each. From Table IV, it is evident that the WRDC Helminth Method is more robust and adaptable to sample type and does not require the sample to be adapted to the method. It is also more time- thus labour- and cost-efficient than the other 3 methods and designed to recover all helminth eggs of sanitation importance (except large trematode eggs > 100µm), with the best egg recoverability.

Table V: Estimated time taken to process and analyse a sample using the WRDC Helminth Method, the USEPA Method, the Mexican Standard for Wastewater Analysis and the Bailenger Method, in terms of labour.

	WRDC Helminth Method	USEPA Method	Mexican Standard	Bailenger Method
Processing time	1.5 hr including centrifugation steps.	Solid samples: approx. 5 workdays (± 38 hr) Liquid samples: approx. 2.5 workdays (± 22 hr).	Gravitational sedimentation: 12 hr Centrifugation: 3 hr.	3.5 hr including gravitational sedimentation and centrifugation.
Microscopy: mode of analysis	Wet mount preparation – entire sample is analysed, and all helminth eggs are categorised (except for fatty sludges with large deposits).	Sedgewick-Rafter counting chamber – entire sample is analysed and ONLY *Ascaris* eggs are categorised, after 18 days (minimum) of incubation.	Sedgewick-Rafter counting chamber or Doncaster disc – entire sample is analysed, and helminth eggs are counted ONLY.	McMaster slide – an aliquot of the sample is analysed, and helminth eggs are counted ONLY.
Microscopy time	8 samples/ day (8 hr) = 1 sample/ hr..	2 samples/ hr = 30 min per sample.	4 samples/ hr = 15 min per sample.	2 min/slide – can assess more than 1 slide per sample.

Table V details the actual processing time needed to perform the WRDC Helminth Method, the USEPA Method, the Mexican Standard and the Bailenger Method on a single sample, inclusive of all gravitational sedimentation and centrifugation steps. It is difficult to cost a sample according to each method without having set up the laboratory for running it, and fully processing and analysing samples respectively. It should however be noted that increased processing steps and time results in increased labour costs, thus individual sample costing would increase too.

With respect to implementation of the WRDC Helminth Method and costing, processing steps such as soaking and washing can be done on multiple samples simultaneously. Individual samples are therefore not processed at once, and costing is estimated accordingly, where a sample is estimated to take 30 minutes, if, for example, 8 samples are processed in 4 hours. The same can be applied to the other methods, however, overnight soaking cannot be costed for the test, but prolonged gravitational sedimentation times during the working day can be. All the above-mentioned factors need to be considered when calculating the cost of testing a single sample, relative to any of these methods. Table V indicates that the WRDC Method is the quickest in terms of processing times and steps, and microscopy is used to count all helminth species' eggs and assess viability. The USEPA Method takes days for a single liquid or solid sample to be processed, and only *Ascaris* sp. eggs are then quantified after a period of incubation.

Microscopy for both the Mexican Standard and the Bailenger Method only focuses on quantifying the total number of eggs per helminth species. Egg viability is not considered thus eggs are not categorised as undeveloped, developing, containing a motile larva, containing a necrotic larva, dead, or infertile. Also, the Bailenger Method claims to be able to read a single McMaster slide and count eggs in 2 minutes. It is highly questionable how accurately egg species are classified and counted, especially considering that accurate microscopy is time-consuming, even for highly experienced parasitologists.

Appendix C highlights the costs for setting up a laboratory for the WRDC Helminth Method, and these would most likely be once-off costs (except in the case of disposable consumables such as gloves, tongue depressors, applicator sticks, and coverslips) that will need to be constantly replaced). Whilst direct comparisons were not completed between the 4 methods, the average time needed to perform each method on a single sample was estimated (Table V). It can be seen that the WRDC method is quicker and thus cheaper than the other 3 procedures. Table IV further highlights advantages and disadvantages of each method, thus how the WRDC Helminth Method outperforms the rest, and we therefore recommend it as an international standard for helminth testing.

Recommendations for future work

Intra- and inter-laboratory testing of the method needs to be completed in order to verify its success in terms of accuracy, reproducibility, repeatability, and egg recovery between experienced laboratories and those with varying experience in parasitology. In order for this to be done, a fresh stock of eggs is needed, so that testing is done on healthy eggs that have not been exposed to any factors that can influence recoverability and viability. A known number of eggs must be spiked into faecal sludge samples, and these must be processed using the method. This must be done between different technicians of varying levels of expertise, as well as between different laboratories. Only once this is completed can it be recommended for international standardisation. The WRDC laboratory is currently working towards obtaining ISO-17025 accreditation for this method.

The processing of and successful recovery of helminth eggs from fatty sludge needs to be explored. This was the most difficult sample type to handle and resulted in ± 65% eggs recovered from spiked samples. A protocol to break down and reduce fats in a sample and reduce the final pellet size needs to be determined. Phase extraction should also be further explored to understand why other studies have found it successful, and if and how it can be optimised such that eggs are not lost.

Furthermore, the WRDC Helminth Method needs to be run in parallel with the USEPA Method, the Mexican Standard and the Bailenger Method for direct comparison, taking into consideration egg recovery, costs involved, time taken and expertise and equipment needed. The aim is to have the final method published in Nature Protocols or Nature Methods, to ensure accessibility and acceptance within the science community. Extensive training is also required for all South African wastewater laboratories and labs in other developing countries where helminth testing is done. Knowledge and skills dissemination are key to ensuring that the method is reproducible and comparable between labs and technicians.

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APPENDIX A: ORIGINAL PRG HELMINTH METHOD STANDARD OPERATING PROCEDURE (SOP)

Standard Operation Procedure – Helminth testing on samples

1. Scope and application

The prevalence of helminth infections in people living with (or without) basic water and sanitation in developing countries, is generally high. Due to the extreme hardiness of the eggs of the roundworm, *Ascaris lumbricoides*, they are used in the waste and sanitation field as a 'marker' for the safe re-use of human waste. It is generally accepted that if any of the various waste treatments used are successful in inactivating *Ascaris* eggs, then most pathogenic gastrointestinal bacteria and viruses should also be killed.

Other commonly found helminths are *Trichuris trichiura*, and *Taenia* spp. Various animal parasites are also commonly encountered. In countries where piped water is not chlorinated, the presence of free-living soil and water organisms (regularly found in municipal sewage works) must be differentiated from pathogens.

2. Summary

Helminth eggs are thought to adhere to soil particles, possibly as a result of charge interactions with or adsorption of eggs to the particles. Many waste samples, even if not from Urine-Diversion Dry Toilets, are often contaminated with silica particles, hence the use of ammonium bicarbonate as a wash solution. Water samples that have a high fat content, commonly found in places like India, need to be treated differently from black-water and other water and wastewater samples. Here it is suggested that a surfactant such as Tween 80 or 7X is used to break up the fats rather than ammonium bicarbonate (AmBic). Laboratory testing for helminths is based on four main principals: washing, filtration, centrifugation and flotation of eggs to remove them from various types of waste.

AmBic is used as both a wash solution and to dissociate helminth eggs from soil particles, whereas surfactants like Tween 80 or 7X are used to degrease fatty samples. Filtration, through 100µm and/or 20µm sieves, is used to separate larger and smaller particles from the eggs both after washing and after flotation. Centrifugation is used to sediment the deposit and remove the water before flotation, aid the separation process during flotation, and sediment the final sieved and washed eggs retrieved

after flotation. Flotation, using a solution of zinc sulphate at a specific gravity (SG) of 1.3 is used to float eggs (with a relative density of <1.3) out of the matter retained (i.e., retentate) on the 20µm sieve.

3. Apparatus and glassware

- Compound microscope with 10x and 40x objectives (and preferably, a camera)
- Bench-top centrifuge with a swing-out rotor that can spin a minimum of 8 x 15ml plastic conical test tubes (Falcon tubes) and, if possible, buckets that can also spin a minimum of 4 x 50ml Falcon tubes
- Sink with hose attached to tap for washing using pressure
- Top-pan balance (scale, for weights up to 200gm and accurate to 2 decimal places)
- Magnetic stirrer and bar magnets
- Vortex mixer
- Hydrometer that can measure SG between 1.2 and 1.3
- 100µm mesh stainless steel pan sieve, height 50mm x diameter 200mm
- 20µm mesh stainless steel pan sieve, height 50mm x diameter 200mm
- 20µm mesh stainless steel flat sieve, height 40 - 50mm x diameter 100mm
- Plastic test tube racks (minimum of 2) to hold the 15ml Falcon tubes (and one or two for 50ml tubes)
- Plastic 1000ml measuring cylinder
- Plastic 250ml beakers (8 – 16)
- Silicon kitchen spatula – with medium to long handle
- Plastic 3ml Pasteur pipettes (non-sterile)
- Non-sterile gloves (good quality, size important, must fit well)
- Applicator sticks and wooden tongue depressors
- Microscope slides (76 x 26 x 1.2mm) and Cover glasses (22 x 40mm)

4. Collection and storage

- After taking samples from various waste materials, store at approximately 4^{o}C. Processing is best done as soon after sampling as possible, but providing that there is sufficient moisture and the samples are fairly large, the eggs should be unharmed and development will be arrested at this temperature.

5. Safety precautions

- Always wear gloves, laboratory coat, plastic apron and mask while processing samples.
- After testing, wash and rinse sieves and beakers, leave to drain on draining rack.
- Spray gloves with 3.3% NaClO once samples are processed and dispose into biological waste box.
- All soiled cover glasses must be disposed of into a sharps-container.
- Soak wooden applicator sticks and tongue depressors in 3.3% NaClO in a beaker for ≥1hr, then discard into biological waste box.
- Soak plastic pipettes and glass slides for ≥1hr in 3.3% NaClO, then wash, rinse well and dry.

- Wipe centrifuge inside and out with cloth and 3.3% NaClO and allow to dry. (For spills, refer to Helminth SOP 002).
- When done, wipe all work surfaces with 3.3% NaClO and wash hands using antiseptic soap.

6. Reagents

- **Physiological Saline (8.5g/lt NaCl)**
  Dissolve 8.5gm sodium chloride in distilled or deionized water. Make small amounts to use up at one time or if large amounts are made, preferably decant into smaller containers, autoclave for 15 min at 121°C, cool to room temperature and store.
- **Ammonium Bicarbonate (AmBic)**
  Dissolve 119gm ammonium bicarbonate in 1lt deionized water (use magnetic stirrer and bar magnet) – store in glass jar.
- **Tween 80 or 7X**
  Use neat – see method
- **Zinc Sulphate ($ZnSO_4$ $7H_2O$)**
  Dissolve 500gm zinc sulphate in approximately 700ml deionized water (use magnetic stirrer and bar magnet) and adjust SG using more of the chemical or water to raise or lower the SG to 1.3
- **0.1N Sulphuric acid (H_2SO_4)**
  Add 500ml deionized water to a 1lt plastic bottle, pour 3ml concentrated sulphuric acid into a 10ml graduated cylinder, then pour the H_2SO_4 into the plastic bottle containing the water, re-cap and shake. Uncap, add 497ml of deionized water to the plastic bottle, re-cap and shake.

7. Method

Procedure for VIP, UDDT, Thick Sludges

- Place a 200ml plastic beaker (labelled with sample number) on top-pan balance, zero balance, weigh 10 or 20gm of sample into beaker.
  NOTE: IF waste material is very dry (e.g. pelletised or totally desiccated), then soak weighed sample for 12 – 24 hours in ±80ml physiological saline to soften. Next, break up and mix sample well in the saline. Stand to sediment solids for ≥4 hours. Remove as much supernatant as possible without disturbing deposit, continue with next step below.
- Add 50-80ml AmBic and a magnetic stirring bar, mix on magnetic stirrer for 10 minutes.
- Wet the 2 x 200mm diameter sieves with tap water, place the 100µm on top of the 20µm mesh sieve.
- Pour the AmBic-sludge mixture over the top sieve.
- Rinse beaker with tap H_2O and pour over the sieves.
- Wash magnet well over sieves and set aside.
- Using spray from hose on tap, wash 100µm sieve well, keeping it over the 20µm sieve at all times (use silicon spatula, or doubled-gloved hand, to aid separation of eggs from particulate matter). Regularly check bottom sieve for fluid build-up. When this occurs, use the same spatula to stir sample on 20µm sieve while holding 100µm sieve directly above so as not to lose any sample. When 20µm sieve has drained sufficiently, place the 100µm sieve back on top and continue washing. Repeat this until sample on 100µm sieve is well washed.

- Separate sieves and set aside the 20µm sieve. Wash retentate from 100µm sieve into a small bucket containing 3.3% NaClO to disinfect. Place lid on top and set aside.
- Wash retentate on 20µm sieve well, then wash it to one side of sieve to make collection easier.
- Rinse total retentate off 20µm filter into original rinsed-out labelled beaker. If there is a lot of water, allow contents of beaker to settle for at least 2 hours, then pipette off some of the supernatant fluid without disturbing the sediment.
- Pour beaker contents into 4 x 15ml Falcon tubes labelled with sample number, or if retentate is large, use 50ml tubes. (After next step, the aim is to have ±1ml deposit in a 15ml tube / ±5ml in a 50ml tube.)
- Centrifuge at 3000 rpm [1512g-force or 1512 RCF (Relative Centrifugal Force)] in centrifuge with swing-out rotor for 10 minutes.
- Pour off supernatant, sedimented deposits remain in the test tubes.
- Place test tubes in rack with applicator stick in each (as a stirring rod) and pipette in $ZnSO_4$, 3ml at a time, mixing on a vortex in between addition of the chemical, until tubes are filled to 14ml mark for 15ml tubes / 40ml mark for 50ml tubes.
- Centrifuge at 2000 rpm (672g-force) for 10 minutes.
- Pour supernatant flotation fluid over smaller diameter 20µm sieve. Collect remaining deposits into one test tube, add 3.3% NaClO to this tube and stand ≥1hr before washing out into municipal drain. Keep one empty test tube aside, wash and set aside for re-use.
- Wash retentate well with tap water and rinse it down to one side of the sieve for collection. Using a 3ml plastic pipette, transfer the retentate back into the test tube kept aside.
- Centrifuge tube at 3000 rpm (1512g-force) for 10 minutes to obtain the final deposit.
- Pour off supernatant water and pipette up the deposit, place it on one or more microscope slides (but make one slide at a time so they don't stand for long periods and dry out), place a 22x40mm cover-glass on top, examine the entire preparation and count every *Ascaris* egg, classifying them as viable, potentially viable or dead. Also count *Trichuris*, *Taenia*, hookworm spp. eggs and assess simply as potentially viable or dead.

Procedure for Liquid Samples

- If the water is effluent from a waste-water treatment plant and is fairly clean with low suspended solids, then it is preferable to use a large sample of 5 – 10lt, measured out using a large, graduated measuring jug (5lt) or a measuring cylinder. If the sample is dirty water with low to moderate suspended solids, then measure out a sample between 1 – 5lt.

  NOTE 1: IF sample is black water with a high concentration of solids, then use amounts of between 200 and 500ml. The sample should be measured out and then stood for 4 hours or overnight to sediment the solids. Then, discard the supernatant fluid and treat as in second step above of: **7. *Procedure for VIP, UDDT, and thick sludges***

  NOTE 2: IF sample is fatty, then measure out a sub-sample, size 200 – 500ml (depending on solids content – visually assessed), pour into plastic beaker large enough to contain the sample with at least 5-10cm above it, so that it does not spill when mixing on magnetic stirrer. Add 1ml of neat Tween 80 or 7X per litre of sample, directly into the sample (to make a ± 0.1% solution in the liquid sludge). Mix well using magnetic stirrer and magnet in beaker for 20 minutes. Then proceed as for next step below.

- Pour the measured sample slowly through a 100µm sieve placed on top of a 20µm sieve and wash well, checking bottom sieve for fluid build-up. Wash well using hose on tap.
- Separate sieves and set aside the 20µm sieve. Wash retentate from 100µm sieve into a small bucket containing 3.3% NaClO to disinfect. Place lid on top and set aside.
- Now, rinse 20µm sieve well and wash retentate to one side for collection.
- Rinse total retentate off 20µm sieve into 2 or 4 x 15ml Falcon tubes (OR 50ml tubes).
- Centrifuge at 3000 rpm (1512g-force) in centrifuge with swing-out rotor for 10 minutes.
- Pour off supernatant and retain deposits in 15ml (or 50ml) Falcon tubes.
- Place test tubes in rack with applicator stick in each (as stirring rod), pipette in $ZnSO_4$, 3ml at a time while mixing on vortex stirrer, until tubes are filled to 14ml (or 40ml) mark.
- Centrifuge at 2000 rpm (672g-force) for 10 minutes.
- Pour supernatant over 100mm diameter 20µm sieve. Collect remaining deposits into one test tube, add 3.3% NaClO to this tube and stand ≥1 hour before washing out into the municipal drain. Keep one empty test tube aside, wash and set aside for re-use.
- Wash retentate on sieve with tap water and rinse down to one side of sieve for collection. Using a 3ml plastic pipette, transfer retentate back into test tube kept aside.
- Centrifuge at 3000 rpm (1512g-force) for 10 minutes to obtain final deposit.
- Pour off supernatant water, pipette up deposit, place on microscope slide, place a 22x40mm cover-glass on top, examine and count every *Ascaris* egg, classifying them as viable, potentially viable or dead. Also count *Trichuris*, *Taenia* and hookworm spp. eggs, and assess simply as potentially viable or dead.

Procedure for incubating samples for viability testing

- Weigh 10 or 20gm into a 200ml plastic beaker, on a top-pan balance.
- Add approximately 10 – 20ml deionized water; 0.1N H_2SO_4, or 1% formalin to sample.
- Cover with parafilm and prick holes in it to allow air into sample, or instead, use a plastic Petri dish as a loose lid on top of the beaker.
- Incubate for 21 - 28 days at 25-28°C, checking regularly to see that the sample has not dried out. If necessary, add more water, 0.1N H_2SO_4 or 1% formalin as necessary to keep sample moist. Aerate the samples daily by swirling carefully.
- After 28 days, remove from incubator, stand for 4 hours or overnight to sediment the sample, remove the supernatant fluid, and then proceed as for step 2 onwards described above in: **7. Method: *Procedure for VIP, UDDT, and thick sludges*.**

Quality Control – QA / QC

- To make up QA/QC samples, you will need uninfected sludge (preferably of a consistency very similar to the samples being tested) or uninfected wastewater.
- Use one uninfected sample, weighed (in grams) and/or one sample measured (in litres) as a negative control.
- For the positive controls, spike a known number of *A. suum* eggs into a weighed sample and/or another known number into a measured sample (see PRG Helminth SOP_004 on PRG website (see footer).
- Then, proceed as for **7. Method: *Procedure*,** using the appropriate procedure for the sample type, i.e. ***VIP, UDDT, Thick Sludges*** **(this includes faeces), or *liquid Samples*.**

- Run a negative and a positive in parallel with a batch of similar consistency samples per day.
- Control samples should be re-examined by a senior, experienced analyst as a control for the microscopy part of the analysis.
- Most sludge and wastewater methods consider recovering > 80% of spiked eggs to be extremely satisfactory.

8. Calculations

Count all eggs, then calculate results to report number of eggs per litre or per gram.

Example 1: If 2.5lt of liquid sample was analysed and there were 500 *Ascaris* eggs found, then use simple proportions:

$$\frac{500 \text{ eggs}}{2{,}5\text{lt}} : \frac{X}{1\text{lt}}$$

$$= \frac{500 \times 1}{2{,}5\text{lt}}$$

$$= 200 \text{ eggs/lt}$$

Example 2: If 15gm of solid sample was analysed and 3450 *Ascaris* eggs were counted, then using proportions again:

$$\frac{3450 \text{ eggs}}{15\text{gm}} : \frac{X}{1\text{gm}}$$

$$= \frac{3450 \times 1}{15}$$

$$= 230 \text{ eggs/gm}$$

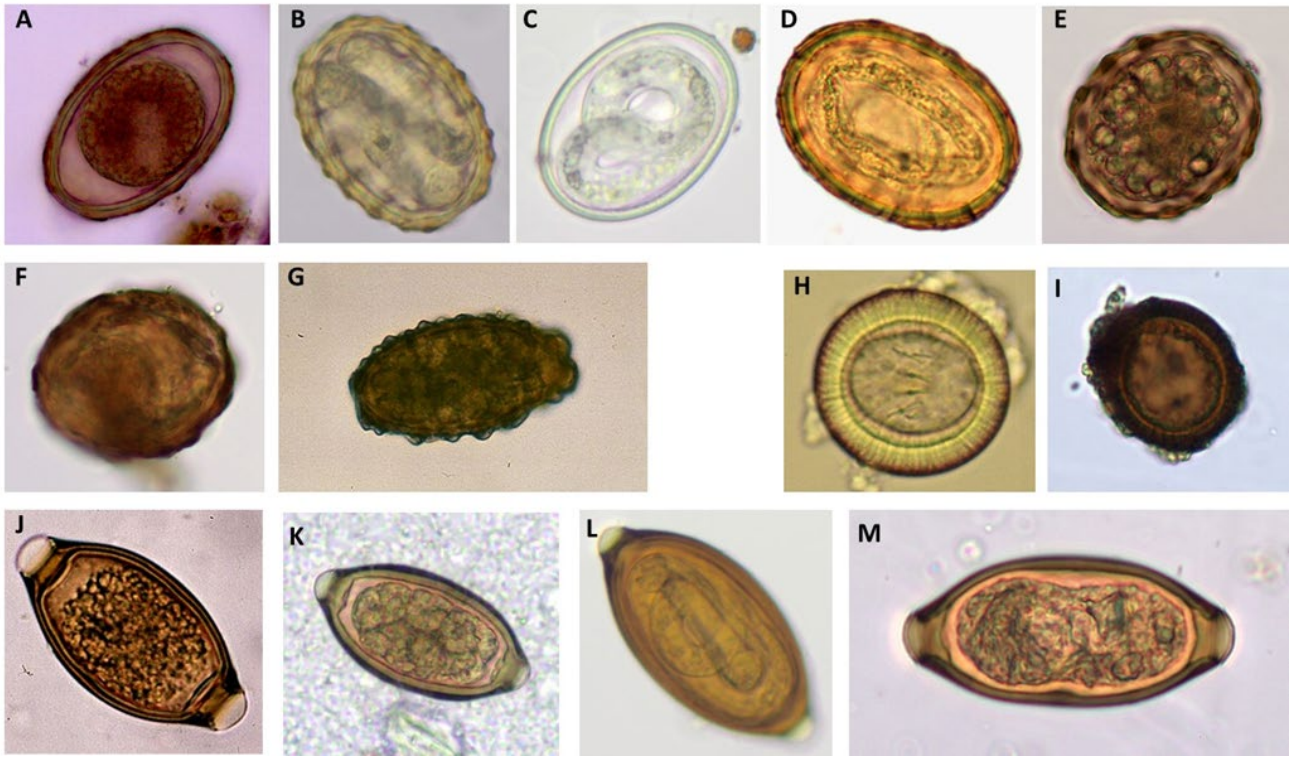
Note: This can be adjusted to eggs per dry gram mass (using proportions) if a sample of the sludge has been tested for moisture content.

9. Reference 1 below was used to formulate the PRG method, and the others, 2 – 9, were publications where this method was used

1. Moodley CL, Archer C, Hawksworth D, Leibach L. (2008) Standard methods for the recovery and enumeration of helminth ova in wastewater, sludge, compost and urine diversion waste in South Africa. *WRC TT Report* No*.* 322/08, Water Research Commission, Pretoria
2. Trönnberg L, Hawksworth D, Hansen A, Archer C, Stenstrom TA. (2010) Household-based prevalence of helminthes and parasitic protozoa in rural KwaZulu-Natal, South Africa, assessed from faecal vault sampling. *Transactions of the Royal Society of Tropical Medicine & Hygiene* 104: 646-654
3. Pebsworth PA, Archer CE, Appleton CC and Huffman MA (2012) Parasite transmission risk from geophagic and foraging behavior in Chacma baboons. *American Journal of Primatology* 74: 940–947

4. Belcher D, Foutch GL, Smay J, Archer C, Buckley CA. (2015) Viscous heating effect on deactivation of helminth eggs in ventilated improved pit sludge. *Water Science & Technology* 72 (7): 1119-1126
5. Naidoo D, Archer C, Louton B, Rodda N. (2016) Testing household disinfectants for the inactivation of helminth eggs on surfaces and in spills during pit latrine emptying *Water SA* 42(4): 560-570
6. Grego S, Barani V, Hegarty-Craver M, Raj A, Perumal P, Berg AB, Archer C. (2018) Soil-transmitted helminth egg assessment in wastewater in an urban area in India. *Journal of Water and Health* 16(1): 34-43
7. Naidoo D, Appleton CC, Archer CE, Foutch GL. (2019) The inactivation of *Ascaris suum* eggs by short exposure to high temperatures. *Journal of Water, Sanitation and Hygiene for Development* 9(1): 19-27
8. Naidoo D, Archer CE, Appleton CC, Septien S, Buckley CA. (2020) Inactivation of *Ascaris* for thermal treatment and drying applications in faecal sludge. *Journal of Water, Sanitation and Hygiene for Development* (IN PRESS)
9. LINKS to IHE-Delft lecture & method demonstration: to be included as soon as they are available.

10. Photographs of some helminth eggs



A: Undeveloped *Ascaris* egg; **B:** *Ascaris* with a motile larva; **C:** motile larva in decorticated *Ascaris* egg; **D:** *Ascaris* egg containing necrotic (dead) larva; **E:** dead *Ascaris* egg containing globules; **F:** dead *Ascaris* egg, empty with collapsing wall; **G:** infertile *Ascaris* egg. **H:** *Taenia* sp. egg in good condition (probably viable); **I:** dead *Taenia* sp. egg. **J:** undeveloped *Trichuris* egg; **K:** developing *Trichuris* egg; **L:** *Trichuris* egg containing a viable, motile larva; **M:** dead *Trichuris* egg. [Pictures provided curtesy of PRG Helminth Lab.]

APPENDIX B: COSTING FOR LABORATORY SETUP TO IMPLEMENT THE WRDC HELMINTH METHOD

Table VI: Costing (including VAT) for laboratory setup in relation to the implementation of the WRDC Method, and a list of our distributors/suppliers (costing relates to cheapest options available on the market and not necessarily what we purchased from our suppliers).

Equipment/Consumable	Distributor/ Supplier	Cost (ZAR/unit)	Total Cost (ZAR)
Laboratory Equipment – Long Term Equipment			
Microscope + camera	Zeiss (Carl Zeiss Pty LTD); Leica (SMMI); Olympus (Wirsam Scientific)	74 000.00	74 000.00
Cooling incubator	Wirsam Scientific	40 000.00	40 000.00
Centrifuge (swing out rotor with 15 & 50ml bucket capacity)		46 000.00	46 000.00
Top-pan balance		2 000.00	2 000.00
Vortex mixer		3 000.00	3 000.00
Magnetic stirrer plate		2 000.00	2 000.00
Sieve – 200mm diameter; 100µm mesh	Reliance Laboratory Equipment	1092.50	1092.50
Sieve – 200mm diameter; 20µm mesh		3818.00	3818.00
Sieve – 100mm diameter; 20µm mesh		7986.75	7986.75
Smaller Laboratory Equipment			
Multichannel tally counter	Lasec SA	2 668.00	2 668.00
Single tally counter		150.00	150.00
Beakers (250ml) x 10	Lichro Chemical and Laboratory Supplies	30.00	300.00
Beakers (2l) x 2		109.25	218.50
Hydrometer (1.2 -1.3)	United Scientific SA	170.20	170.20
Magnetic stirrer bars (10/pack)	Lasec SA	131.10	131.10
Schott bottle (1l) x 5	Lichro Chemical and Laboratory Supplies	194.54	972.70
15ml graduated plastic test tubes (1000/pack)		1622.50	1622.50
15ml test tube rack x 3		98.54	295.62
50ml graduated plastic test tubes (50/pack) x 2		327.75	655.50
50ml test tube rack x 3		78.00	234.00
Consumables & Reagents			
Ammonium bicarbonate (500g) x 3	United Scientific SA	174.25	522.75
*7X (3.8l)	Lichro Chemical and Laboratory Supplies	4246.28	4246.28
Zinc sulphate (25kg)	United Scientific SA	4249.25	4249.25
Sulphuric acid		157.55	157.55
Coverslips (22 x 40mm) – 100/pack x 20	Lasec SA	65.50	1310.00
Coverslips (22 x 22mm) – 100/pack x 10		33.92	339.20
Microscope slides (50/pack) x 5		20.00	100.00
Wooden tongue depressors (100/pack)	Lichro Chemical and Laboratory Supplies	30.00	30.00
Wooden applicator sticks (1000/pack)		103.00	103.00
3ml plastic Pasteur pipettes (500/pack)		677.35	677.35
Disposable gloves (100/pack) x 10		92.00	920.00
TOTAL		**195 296.23**	**199 970.25**

*7X – Although expensive to import, it lasts for a very long time as only 1ml is needed per litre of 0.1% 7X solution

APPENDIX C: BIOMEDICAL RESEARCH ETHICS COMMITTEE (BREC) APPROVAL LETTER

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

09 June 2021

Ms Danica Naidoo (209512429)
School of Engineering
Howard College

Dear Ms Naidoo,

Protocol reference number: BREC/00002794/2021
Project title: Towards the development and standardisation of a modified helminth extraction and quantification method for sanitation samples.
Degree: PhD

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 09 June 2021. Please ensure that outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations, see (http://research.ukzn.ac.za/Libraries/BREC/BREC_Lockdown_Level_2_Guidelines_1.sflb.ashx). Based on feedback from some sites, we urge PIs to show sensitivity and exercise appropriate consideration at sites where personnel and service users appear stressed or overloaded.

This approval is valid for one year from 09 June 2021. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 13 July 2021.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

APPENDIX D: ANIMAL RESEARCH ETHIC COMMITTEE (AREC) APPROVAL LETTER

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

04 March 2019

Dr Colleen Edith Archer (609)
School of Chemical Engineering
Howard College Campus

Dear Dr Archer,

Protocol reference number: AREC/071/018
Project title: Maintaining *Ascaris suum* life-cycle in pigs

Approval Notification – Research Application

With regards to your revised application received on 27 November 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted with the following conditions:

CONDITIONS:

1. The study can only start once the renovations at the Ukulinga facility is complete and ready to use. Please provide certificate / letter.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 04 March 2020.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

.....

Dr Sanil D Singh, PhD
Deputy Chair: Animal Research Ethics Committee

/ms

Cc Academic Leader Research: Dr Akshay Kumar Saha

Cc Registrar: Mr Simon Mokoena

Animal Research Ethics Committee (AREC)
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Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

APPENDIX E: GATEKEEPER'S LETTER FOR SAMPLING

Water and Sanitation Unit

3 Prior Road, Durban, 4001 PO Box 1038, Durban, 4000

www.durban.gov.za

2nd February 2021

To whom it may concern

We, as the eThekwini Water and Sanitation Department (EWS), are aware of, and give permission to, the WASH R&D Centre (formerly the Pollution Research Group) within the University of KwaZulu-Natal, and affiliated post-graduate students and researchers (both local and international) to undertake field testing of new sanitation technologies within local communities as identified by EWS in consultation with ward councillors and community leaders. This includes testing at communal ablution facilities, households, and municipal treatment works, including operation, maintenance, trouble-shooting, sampling from these systems and analysis of collected samples. Field testing of these systems will be undertaken in close collaboration with EWS staff and the community leadership with informed consent being obtained prior to any interventions.

In light of COVID-19, risk assessments to mitigate transmission to research staff and students have been prepared and will be adhered to, as per the following guidelines:

- SA Department of Health (DoH) COVID-19 Infection Prevention and Control Guidelines
- UKZN BREC Studies Guidelines for Level 3 Lockdown (published 5 June 2020)
- eThekwini Municipality Revised Guidelines for the Containment/Management of the Coronavirus (COVID-19) in the eThekwini Municipality (Version 5, updated 24 August 2020)

The EWS and the WASH R&D Centre have worked closely together for the past 10 years and a Memorandum of Understanding (MOU) exists between UKZN and EWS. EWS provides funds to the WASH R&D Centre through a MOA to provide research support on water and sanitation service delivery.

We are fully aware of these projects and we have informed relevant stakeholders about the work.

Regards,

[redacted]

Ms Lungi Zuma

Professional Engineer

eThekwini Water and Sanitation

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APPENDIX F: PAPER 0 – FIRST PUBLISHED PAPER TOWARDS PHD DEGREE

Naidoo, D., Archer, C. E., Septien, S., Appleton, C. C., & Buckley, C. A. (2020). Inactivation of Ascaris for thermal treatment and drying applications in faecal sludge. *Journal of Water, Sanitation and Hygiene, for Development*, 10(2), 209-218.

Research Paper

Inactivation of *Ascaris* for thermal treatment and drying applications in faecal sludge

D. Naidoo, C. E. Archer, S. Septien, C. C. Appleton and C. A. Buckley

ABSTRACT

Ascaris lumbricoides is the most common helminth of human health importance, and the most resilient helminth found in faecal sludge. There are numerous types of sludge treatments; however, heating and drying are most commonly used for pathogen inactivation. *Ascaris suum* eggs were heated in a water bath at 40–55 °C for 10 seconds to 60 minutes in water, as well as heated in both urine diversion dry toilet and ventilated improved pit latrine sludge at 40 °C, 60 °C and 80 °C for times ranging from 5 seconds to 120 minutes. Eggs were also spiked into sludges of different moisture contents and incubated over 12 weeks at 25 °C, with samples analysed weekly. Overall, we concluded that eggs were inactivated at temperatures >50 °C, that the temperature–time relationship directly impacted the efficacy of heat treatment, that suspension medium had no effect, and that eggs survived better in wet rather than dry sludges.

Key words | *Ascaris*, exposure time, heating, inactivation, moisture content, temperature

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INTRODUCTION

Approximately 2.3 billion people globally lack access to basic sanitation facilities and one-third of the world's population is infected with soil-transmitted helminths (STHs) (JMP 2017; Cooper & Hollingsworth 2018). *Ascaris lumbricoides*, also known as the human roundworm, is the most common STH of human health importance (Brownell & Nelson 2006). Infection with *A. lumbricoides* is most prominent in areas that lack a potable source of water, improved sanitation and proper hygiene practices, with an estimated 804 million people infected worldwide (Jourdan *et al.* 2018). Mild infections may be asymptomatic; however, heavy worm infections can lead to symptoms such as diarrhoea, bloating, abdominal blockages and discomfort, malnutrition, and impaired growth and cognitive development (Cooper & Hollingsworth 2018). Diarrhoeal diseases are the cause of 1.3 million deaths per year, of which one in eight are children under the age of five years (Kotloff 2017).

Ascaris eggs can withstand harsh environmental conditions and are considered the most resilient organisms found in faecal sludge, as they are able to survive for up to seven years in the soil (Pecson & Nelson 2005). *Ascaris* spp. eggs are therefore commonly used as indicator organisms of faecal contamination and for inactivation experiments (Maya *et al.* 2012).

Heat treatment technologies for sludge have become common practice due to increased pathogen inactivation success (Belcher *et al.* 2015). There have been a number of studies globally that have focused on heating or drying for pathogen inactivation and sludge sanitisation (Brownell & Nelson 2006; Maya *et al.* 2010; Szabová *et al.* 2010; Buttar

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et al. 2013; Andes & Paller 2018). Composting systems and drying beds are feasible treatment options that operate at lower temperatures over prolonged periods of time, and are common practice in Africa. Koné et al. (2007) reported that heat generation due to the composting process in a drying bed resulted in 90–100% *Ascaris* inactivation after 80 days at ≥55 °C. Septien et al. (2018) reported that pelletisation and infrared drying of faecal sludge resulted in the inactivation of *Ascaris* eggs.

Naidoo & Foutch (2017) collated results from various heat treatment studies in order to establish a temperature–time relationship relative to *Ascaris* inactivation. Few studies have investigated the isolated effects of heat on *Ascaris* eggs, as most include the effects of other treatment factors, such as pH and relative humidity (Pecson & Nelson 2005; Capizzi-Banas et al. 2004), or involve some type of treatment technology, such as a viscous heater (Belcher et al. 2015). There are also gaps in the literature in terms of isolated heat treatment of helminth eggs at lower temperatures (40–50 °C), where such data may be useful to sanitation practitioners employing drying beds, composting systems and low heat technologies for sludge treatment. This study therefore aimed at extending the temperature–time relationship profile towards the lower end of the temperature scale, based on work previously investigated by Naidoo & Foutch (2017) and Naidoo et al. (2019) that focused on heat treatment at 60–80 °C in water. Eggs were heated in water at 40, 45, 50 and 55 °C for times ranging from 10 seconds to 60 minutes.

According to Jebri et al. (2013), the suspension medium of the eggs plays a role in the efficacy of heat treatment. Buttar et al. (2013) went on to report that sludge, or any similar suspension medium, may act as insulation against heat, thus higher temperatures and longer exposure times might be needed for successful inactivation. The role of the suspension medium on the inactivation of *Ascaris* eggs was therefore investigated in this study, testing water, urine diversion dry toilet (UDDT, to be referred to as UD from henceforth) sludge and ventilated improved pit (VIP) latrine sludge where eggs were heated at 40, 60 and 80 °C, for various exposure times that were selected based on previous data (Naidoo et al. 2019).

Drying leads to a decrease in moisture content of treated sludge. While previous studies have focused on the effects of reduced sludge moisture on bacterial and viral inactivation (Romdhana et al. 2009) and the effects of dryness in combination with heating and pH alteration (Maya et al. 2012), none have explored the effects of dryness alone on the survival of helminth eggs. These effects were thus explored in the present study for both UD and VIP sludge.

Ascaris eggs are used as indicator organisms for overall pathogenic contamination of sludge, as it is assumed that if all *Ascaris* eggs have been killed off, then all other pathogens would have died as well (Sidhu & Toze 2009). Due to ethical and logistical issues it is often difficult to source *Ascaris lumbricoides* eggs, thus eggs of the pig roundworm, *Ascaris suum*, are often used as a surrogate. Both species are morphologically identical in all developmental stages (Daugschies et al. 2013), thus *A. suum* eggs were used for inactivation testing in this study.

MATERIALS AND METHODS

Sample collection and sludge characterisation

The effects of heating were tested in three separate experiments. UD sludge was collected from a stockpile that was collected from UD toilets within the eThekwini Municipality (Durban, South Africa) and transported to the Isipingo Wastewater Treatment Plant in KwaZulu-Natal for treatment. VIP sludge was collected directly from household latrines in Bester (within the eThekwini Municipality), and wet VIP sludge was collected directly from the vacuum trucks that were emptying pits in the Bester area at the time of sampling. Water was added to the UD sludge to match the moisture content of the wet VIP sludge, for the purposes of consistency during treatment. The sludge characteristics are summarised in Table 1. *Ascaris suum* eggs were purchased from Excelsior Sentinel Inc. (USA) and were stored at 4 °C until needed.

Experiment 1: Heat treatment of eggs in water

The first experiment was a continuation of Naidoo et al. (2019) that focused on heating at 60, 65, 70, 75 and 80 °C. Eggs were heated at 40, 45, 50 and 55 °C for 10 and 30 seconds, and at 1, 2, 5, 10 and 60 minutes. Plastic 15 mL test tubes containing tap water were preheated in a water

Table 1 | Characteristics of the collected UD, VIP (from latrines) sludge, wet VIP (from vacuum tanker, used for Experiment 2) and wet UD (diluted UD sludge used for Experiment 2)

Sludge	Moisture content (g/g wet sample)	Total solids (g/g wet sample)	Volatile solids (g/g dry sample)	Ash content (g/g dry sample)
UD	0.789 ± 0.005	0.211 ± 0.005	0.576 ± 0.045	0.424 ± 0.045
VIP	0.877 ± 0.033	0.123 ± 0.033	–	–
Wet VIP	0.901 ± 0.045	0.099 ± 0.045	0.644 ± 0.016	0.356 ± 0.016
Wet UD	0.902 ± 0.030	0.098 ± 0.030	–	–

UD, urine diversion; VIP, ventilated improved pit latrine.

bath to the respective test temperature. Approximately 500 *Ascaris* eggs (suspended in 1 mL of water) were spiked into each heated test tube and exposed for the test time. The tubes were then removed from the water bath and the contents brought back to room temperature immediately by emptying into iced tap water, in order to prevent prolonged heat exposure. Samples were treated, processed and analysed using light microscopy, both before and after incubation according to the methodology used by Naidoo *et al.* (2019).

Experiment 2: The effects of the suspension medium on the efficacy of heat treatment

The second experiment involved the heating of *Ascaris* eggs in both VIP and UD sludge, and water as a control. Test parameters included 30, 60 and 120 minutes at 40 °C, 30 seconds, 2 minutes and 5 minutes at 60 °C, and 5 and 10 seconds and 1 minute at 80 °C. The UD sludge was diluted to match the moisture content of the wet VIP sludge. Sludge samples of approximately 10 g each were weighed out into aluminium cups (70 mm × 40 mm) and covered with sheets of aluminium foil. The water bath was preheated to the respective test temperature, after which the aluminium cups containing the sludge were inserted into holes cut into a polystyrene sheet (350 mm × 250 mm × 15 mm), being fully immersed in the water. Once the entire system reached the test temperature, eggs were spiked into the sludge and mixed, and the cups were immediately covered again to prevent moisture loss. The samples were exposed for the respective test times, after which the cups were removed and placed into plastic bowls containing iced water, and approximately 30 mL iced water was poured into the sludge sample and mixed to allow the sample to return to room temperature and prevent prolonged heating.

The samples were then processed according to the PRG Helminth Method (2018). Ammonium bicarbonate was poured into the aluminium cups until the sludge was just covered and mixed well. The contents of the cup were then poured over a set of drum sieves (100 µm over a 20 µm sieve). The sample was washed thoroughly in the sieve, using pressure from a hose on the tap, and by breaking any clumps using the back of a gloved hand. The 100 µm sieve was then removed and the retentate discarded. The retentate (containing the *Ascaris* eggs) on the 20 µm sieve was then washed thoroughly and collected into four plastic 15 mL test tubes. These were centrifuged at 1,512 × *g* (3,000 rpm) for 10 minutes and the supernatant discarded. Zinc sulphate ($ZnSO_4$) was added to each tube in 3 mL aliquots to a total of 14 mL while vortexing to break up the pellet and homogenise the suspension. The test tubes were then centrifuged at 672 × *g* (2,000 rpm) for 10 minutes, to allow eggs to float up into the liquid column above the sediment. The supernatant was then poured onto a smaller 20 µm sieve, washed with water, and collected into a single 15 mL tube. The final samples were centrifuged at 1,512 × *g* (3,000 rpm) for 3 minutes, after which the supernatant was discarded and the final pellet microscopically analysed. The samples were then washed back into the test tubes, incubated for 28 days at 25 °C and re-analysed microscopically. Eggs were scored and categorised according to morphology and viability, as per Naidoo *et al.* (2019).

Experiment 3: The effects of moisture content on *Ascaris* survivability

The third experiment involved the incubation of eggs in sludge samples of different moistures. Both VIP and UD sludges were dried down to different approximate moistures – 60, 50, 40, 30 and 20%, and undried sludge was used as a

control. Sludge was dried in an oven at 105 °C until the respective moisture content was reached. These dried samples were further weighed out into 5 g samples, spiked with approximately 400 *Ascaris* eggs per sample and incubated at 25 °C for a period of 12 weeks. Each week, samples were processed using the PRG Helminth Method described in Experiment 2, then analysed microscopically.

For this study, the criterion set for successful inactivation was <10% viable eggs recovered after treatment (Ayçiçek *et al.* 2001; Naidoo *et al.* 2019). Statistical analyses included the Kolmogorov–Smirnov test for normality of data, followed by a nested analysis of variance (ANOVA), the Shapiro–Wilk test for normality of residuals and the Levene's test for homogeneity of variance of residuals, both from the nested ANOVA. The analyses were run on IBM SPSS Statistics (version 25) and R (version 3.5.2). Percentage viability was calculated as follows:

$$\textit{Percentage viable eggs recovered} = (\textit{Total viable eggs recovered} / \textit{Total number of eggs recovered}) * 100$$

RESULTS AND DISCUSSION

Table 2 presents the statistical analyses of the results of the nested ANOVA and compares the percentage of viable eggs recovered between each variable and respective combinations. The in-depth description of the statistical analyses (including the exact nested designs and p values for specific variable combinations) were not included in this article, but can be made available upon request.

Experiment 1

Results from the nested ANOVA (Table 2) for Experiment 1 indicate that alone, temperature had a significant effect on *Ascaris* egg inactivation ($p < 0.001$). In combination with the second and third levels of nestedness (exposure time and point of analysis, respectively), significant effects were also observed ($p < 0.001$). Experiment 1 indicated that 40 and 45 °C was not sufficient for successful *Ascaris* inactivation within the tested exposure time frame ($p = 0.844$ and $p = 0.866$). After 60 minutes of heat exposure, egg viability was 81.4% before incubation and 76.6% after incubation at 40 °C, and 83.2% before incubation and 83.5% after incubation at 45 °C, thus not meeting our inactivation criterion (<10% viable eggs recovered) (Figure 1(a) and 1(b)). Treatment at 50 and 55 °C led to a statistically significant egg inactivation after 60 minutes ($p < 0.001$) and 5 minutes ($p < 0.001$) of exposure, respectively. At 50 °C for 10 minutes of heat exposure, egg viability before incubation was 85.5%, and a decline in viability was seen after incubation (65.5%; $p = 0.762$), but this did not meet the inactivation criterion for this study. After 60 minutes of heat exposure, egg viability was 86.5% before incubation and 1.9% after incubation ($p < 0.001$). Treatment at 55 °C rendered an egg viability of 89, 92 and 84% before incubation, and 3.8, 0.2 and 0% after incubation, for 5 minutes ($p < 0.001$), 10 minutes ($p < 0.001$) and 60 minutes ($p < 0.001$) of heat exposure, respectively, meeting the inactivation criterion for this study (Figure 1(d)). The 5 °C difference between heat treatment at 50 and 55 °C appears to be crucial in terms of inactivation efficacy, as the most effective exposure time for each temperature ranges from 60 down to 5 minutes. It should also be noted that complete inactivation was not observed after 1 hour of treatment at temperatures below 50 °C.

Table 2 | Statistical results of the general ANOVA from the nested ANOVA design for Experiments 1, 2 and 3

Experiment	Primary variable	$F_{(df)}$	p-Value	[a]Variable combinations	p-Value
1	Temperature	$F_{(6,\ 174)} - 10.58$	<0.001	Temperature/Exposure time	<0.001
				Temperature/Exposure time/point of analysis	<0.001
2	Temperature	$F_{(5,\ 90)} - 10.58$	<0.001	Temperature/Exposure time	0.001
				Temperature/Exposure time/suspension medium	0.860
3	Time (week)	$F_{(1,\ 216)} - 10.58$	<0.001	Time/Moisture content	<0.001
				Time/Moisture content/suspension medium	0.223

[a]Variable combinations represent the nestedness of the statistical design. Point of analysis refers to whether eggs were examined before or after incubation.

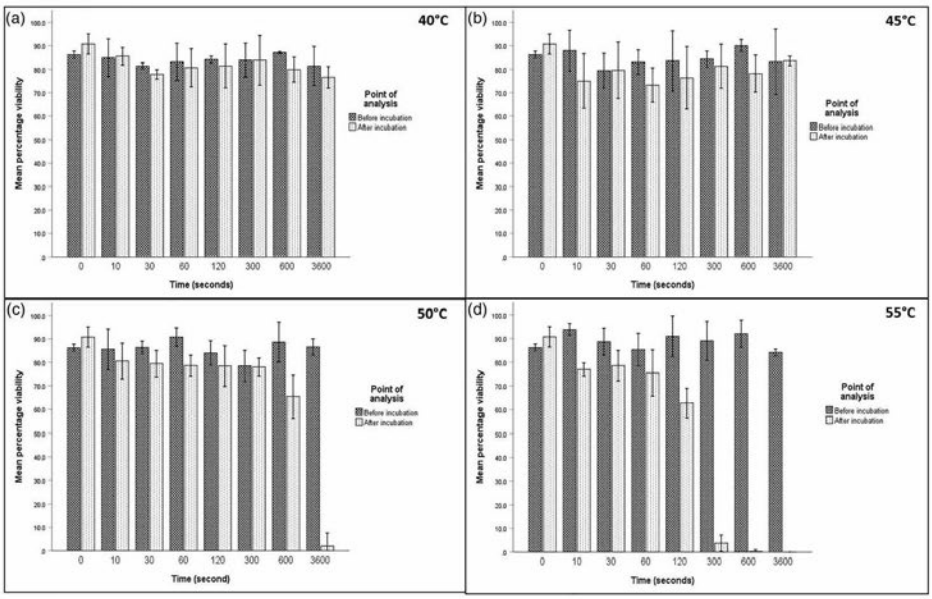

Figure 1 | The effects of heat treatment on *Ascaris* eggs at: (a) 40 °C, (b) 45 °C, (c) 50 °C and (d) 55 °C, both before and after incubation at 25 °C for 28 days, for exposure times of 10, 30 and 60 seconds; 2, 5, 10 and 60 minutes; 0 seconds represents the control tests in water ($n = 3$).

Brannen *et al.* (1975) and Brandon (1978) both investigated the effects of direct heating of *Ascaris* eggs in a water bath. The former reported almost 100% inhibition of egg development when exposed to 55 °C for 4 minutes and the latter reported that egg viability was reduced to almost 0% when heated at 55 °C for 60 minutes. Pecson & Nelson (2003) reported that complete *Ascaris* egg inactivation was achieved after treatment for 24 hours at 48 °C, and 1 hour at 54 °C, in various buffered solutions. These studies support the findings of the current study in terms of the temperature–time relationship for *Ascaris* egg inactivation.

The major difference in egg viability between analyses conducted before and after incubation, particularly for 50 and 55 °C, should be noted. Naidoo *et al.* (2019) reported that at higher temperatures (≥60 °C), morphological damage to eggs was easily identifiable and egg death very apparent with a globular embryo or disintegrated larva. Damage was, however, not as visible at lower temperatures. Drawing on our experience, we know that eggs sometimes appear healthy at the one-celled undeveloped stage after treatment at low temperatures; however, upon incubation, no further development occurs, indicating successful inactivation. This scenario explains the difference in viability seen in Figure 1(c) at 50 °C and Figure 1(d) at 55 °C, where eggs appeared healthy before incubation, but did not develop further when incubated, and were thereafter scored as dead. The incubation step (at 25 °C for 28 days, which is the duration of the environmental development steps of the life cycle of *Ascaris* spp.) is therefore imperative, especially for *Ascaris* inactivation studies conducted at low temperatures. Note that the opposite could also occur, where an egg sometimes appears somewhat damaged, but upon incubation, it would develop further, indicating that inactivation was not successful.

Experiment 2

Results from the nested ANOVA (Table 2) for Experiment 2 indicated that alone, temperature had a significant effect on

Ascaris egg inactivation ($p < 0.001$). In combination with time (second level of nestedness), significant differences in egg viability were observed ($p < 0.001$). However, when taking into consideration the suspension medium (third level of nestedness), no significant differences were noted in egg viability after treatment ($p = 0.860$). Based on data from Naidoo *et al.* (2019) and from Experiment 1, it is known that at lower temperatures, damage to the eggs after heat treatment may not always be visible, thus the importance of incubation has already been established. Statistical and graphical analyses therefore only included egg viability after incubation (Table 2; Figure 2).

At 40 °C, significant inactivation was not achieved after the maximum exposure time of 2 hours in water (66.5%; $p = 0.981$), UD sludge (56.9%; $p = 0.261$) and VIP sludge (65.5%; $p = 1.000$), thus none of the above met the inactivation criterion for this study (Figure 2(a)). Figure 2(b) shows that significant inactivation was achieved at 60 °C after 30 seconds of exposure in water (5.6%; $p < 0.001$), UD sludge (12.7%; $p < 0.001$) and VIP sludge (3.6%; $p < 0.001$). A near complete inactivation of *Ascaris* eggs was obtained at 2 minutes' exposure ($p < 0.001$) and complete inactivation was achieved at 5 minutes' exposure ($p < 0.001$), at 60 °C in all three suspension media. At 80 °C, significant inactivation was achieved after 5 seconds' exposure in water (3.3%; $p < 0.001$), UD sludge (0.0%; $p < 0.001$) and VIP sludge (3.6%, $p < 0.001$). After 10 seconds of treatment, egg viability was almost negligible and at 60 seconds, complete die-off was observed (Figure 2(c)). It should be noted that the suspension medium did not impact treatment and resultant inactivation efficacy. This implies that laboratory testing of *Ascaris* inactivation for technology design could be done in water rather than sludge or faecal simulants, saving time and avoiding the handling of hazardous material.

Popat *et al.* (2010) investigated the effects of thermophilic anaerobic digestion on *Ascaris* eggs in a sludge of 98% moisture content. A 2-log reduction in egg viability was reported after 2 hours of exposure at 55.5 °C. Thomas *et al.* (2015) investigated the effects of shear and temperature, both together and independently, on the inactivation of *Ascaris* eggs, using methods similar to the current study. It was found that after 60 seconds at 47, 51 and 55 °C, there was 94.7%, 91.1% and 89.7% egg development, respectively, after treatment. A similar inactivation pattern was seen in the current study, where successful inactivation was only observed after treatment at 55 °C for ≥5 minutes, and at 60 °C after ≥30 seconds, in sludges of similar moisture content.

Neither Brannen *et al.* (1975) nor Brandon (1978) reported any significant difference in *Ascaris* inactivation between treatment in water or sludge, suggesting that inactivation follows a similar pattern, irrespective of the suspension medium of the eggs. Similar data were observed in the current study (Experiment 2, Figure 2), where inactivation patterns were similar across all three suspension media – water, UD sludge and VIP sludge.

Experiment 3

Results from the nested ANOVA (Table 2) for Experiment 3 indicated that alone, time (week), significantly affected egg survivability and development ($p < 0.001$). In combination with moisture content (second level of nestedness), there was a significant effect on survivability as well ($p < 0.001$). Egg development (at the expected developmental rate as per the *Ascaris* spp. life cycle) was the most consistent in sludges of 50% moisture (Figure 3) and much slower in drier sludges (20% and 30% moisture), indicating that *Ascaris* eggs survived longer in wetter sludges (Figure 3). There was however, no significant difference between incubation of eggs in UD sludge and VIP sludge (suspension medium; third level of nestedness; $p = 0.223$), meaning that eggs developed or died in a similar manner in both sludges. The findings from this study indicate that drying sludge to a low moisture content creates unfavourable conditions for egg survival and development. Long-term storage of sludge, which may result in a reduction of moisture, can promote the inactivation of *Ascaris* eggs.

Maya *et al.* (2012) investigated the effects of temperature, pH and dryness on the inactivation of *A. suum* and *A. lumbricoides* eggs (together with four other parasite species), where eggs were spiked into sterilised sludge. They stated that at 80 °C, 3 hours' exposure was sufficient to inactivate undeveloped eggs in dry sludges (5–10% moisture), and at 60 °C, 1 hour was sufficient in sludges of 5–15% moisture. It was also reported that drier conditions resulted in increased inactivation, and that larval eggs were more

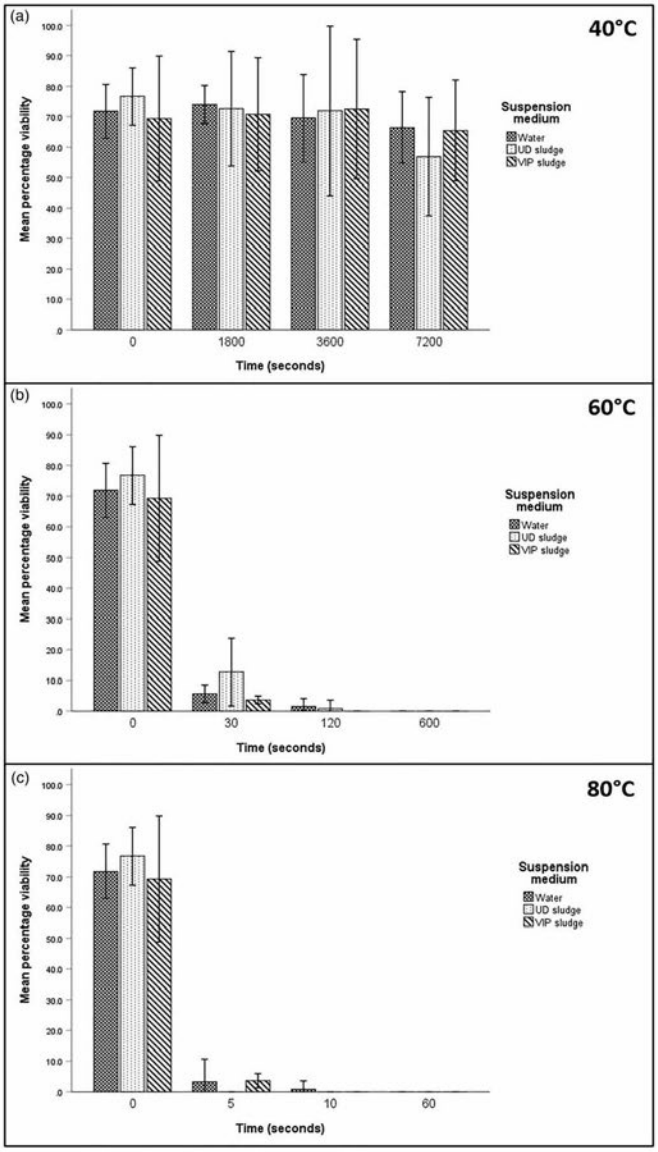

Figure 2 | Testing the role of the suspension medium on *Ascaris* egg inactivation by heat treatment at: (a) 40 °C for 30, 60 and 120 minutes, (b) 60 °C for 30, 120 and 600 seconds and (c) 80 °C for 5, 10 and 60 seconds, in water, UD sludge and VIP sludge after incubation at 25 °C for 28 days; 0 seconds represents the controls ($n = 3$).

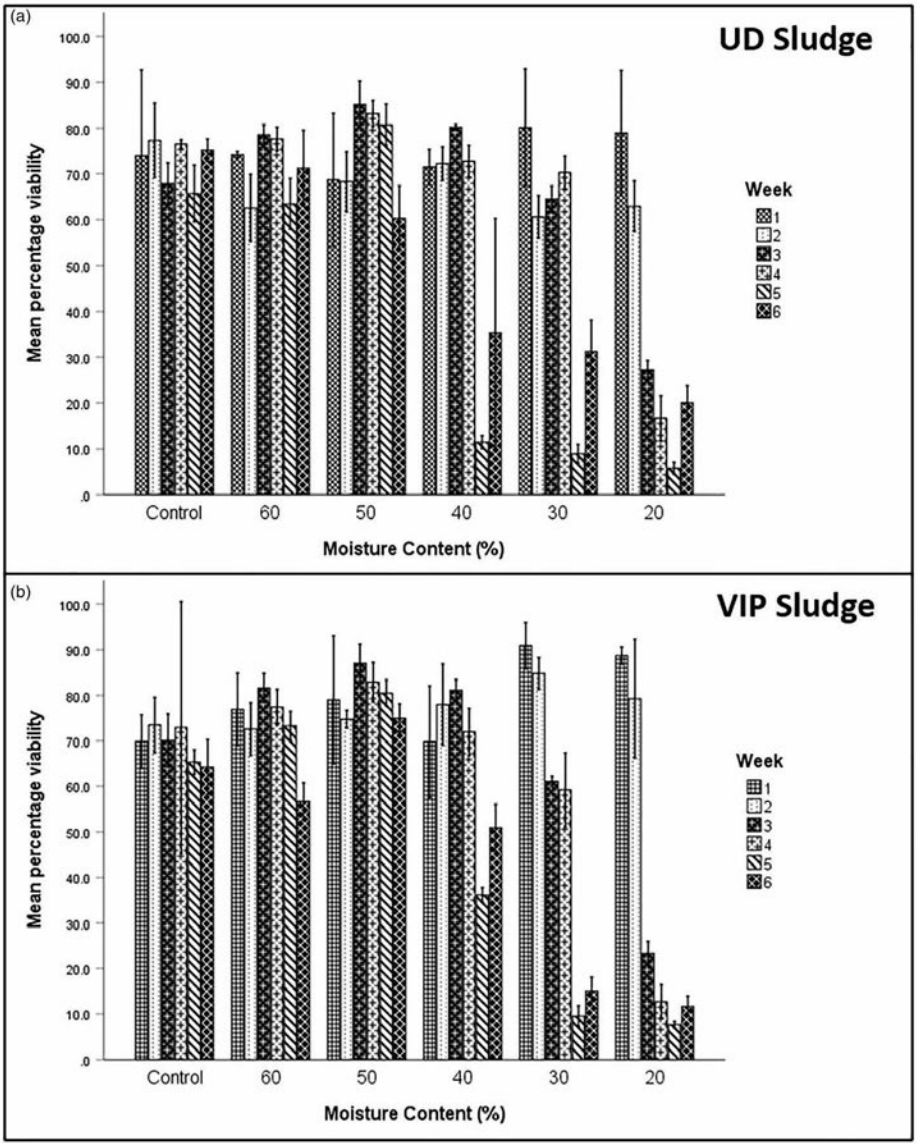

Figure 3 | The effects of different levels of moisture of the suspension medium on the development and survival of *Ascaris* eggs when incubated at 25 °C for 6 weeks, in (a) UD sludge and (b) VIP sludge ($n = 3$).

susceptible to environmental stressors as opposed to undeveloped eggs, supporting the findings of this study.

During the 12-week incubation period, there was development of fungal contamination within the samples. Data from weeks 7 to 12 were therefore excluded from analyses, as studies have found that certain strains of fungi may have ovicidal properties (Ferreira *et al.* 2011; Blaszkowska *et al.* 2014). Filamentous growth was observed around eggs isolated from sludge samples that had visible contamination and, in most cases, these eggs were very damaged and dead.

CONCLUSION AND RECOMMENDATIONS FOR FUTURE WORK

The conclusion and recommendations for future work are as follows:

- This study was aimed at determining the effects of low temperatures, suspension medium and storage conditions on *Ascaris* eggs.
- Heat treatment at 40 and 45 °C after 60 minutes, and treatment at 40 °C after 120 minutes were insufficient for *Ascaris* inactivation, providing critical data for the planning related to slow heating technologies such as drying beds.
- At 50 and 55 °C, 60 minutes and 5 minutes, respectively, are sufficient for successful *Ascaris* inactivation.
- Furthermore, morphological damage was not necessarily visible before incubation. The incubation step is therefore imperative when testing any kind of heat treatment in order to verify inactivation.
- The suspension medium did not play a role in the efficacy of heat treatment of the eggs – inactivation patterns were the same for water and UD and VIP sludge, meaning that heat treatment of *Ascaris* eggs can be done in water rather than having to source sludge
- Time had an effect on *Ascaris* egg survival and development in sludge, indicating the potential for egg inactivation during long-term storage.
- In combination with moisture content, a significant effect was also observed. Eggs preferred wetter sludges for development, with 50% moisture being the optimum condition, and development was much slower in drier sludges (20–30% moisture).
- Fungal growth is inevitable over time, supporting egg inactivation during long-term storage of sludge.

The exact exposure time for successful inactivation at low temperatures (40–49 °C) is still unknown (it fell outside the time frame of this project), in terms of isolated heat treatment. Further work is therefore necessary in order to complete the full temperature–time relationship for *Ascaris* egg inactivation. Fungal development in stored sludge could be advantageous in terms of developing biological control protocols for *Ascaris*. Studies have indicated the total ovicidal potential of certain strains of fungus, which have been used for the control of plant nematodes in agriculture. Further research is needed for the application to faecal sludge management.

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