TOWARDS THE DEVELOPMENT AND STANDARDISATION OF A MODIFIED HELMINTH EXTRACTION AND QUANTIFICATION METHOD FOR SANITATION SAMPLES

Report to the Water Research Commission

by

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Report no. 2893/1/22

ISBN 978-0-6392-0432-1

May 2022

UNIVERSITY OF KWAZULU-NATAL
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EXECUTIVE SUMMARY

New sludge treatment 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 faecal sludge should be consistent. A highly sensitive, standard helminth isolation and enumeration method is therefore required for application in laboratories globally. Different laboratories and groups have used variations of the standard United States Environmental Protection Agency (US EPA) Method and the Pollution Research Group (PRG) Helminth Method, which was used by the Water, Sanitation and Hygiene Research and Development Centre (WASH R&D Centre or WRDC) for helminth testing, and therefore formed the foundation of this study. This project was aimed at optimising the old PRG Helminth Method, by comparing every step with those of other existing helminth methods, to produce one final, improved procedure that would then be recommended for standardisation internationally. It should be one that is cost- and time-efficient, and adaptable to the sample type, rather than the sample being modified to suit the method.

Existing methods that were selected for comparison included the commonly used US EPA Method (2003), the Mexican modification of the US EPA Method (2006) and the Bailenger Method (1996). After all experimentation was done in this project, the improved PRG Helminth Method, renamed the WRDC Helminth Method, was henceforth adopted and put into practice at our lab. Conventional helminth methods can be broken down into five steps:

1. Washing and sedimentation of the sample to separate eggs from larger particles;
2. Flotation, which involves the use of density gradients to separate eggs from heavier particles;
3. Centrifugation after both washing and flotation;
4. Extraction that involves the use of a buffer and solvent combination to further separate organic material from the eggs; and
5. Analysis using a light microscope.

Some methods also include an incubation step that allows for viability assessment of the eggs.

Every reagent used in all common international helminth methods was tested on *Ascaris suum* eggs for varying time intervals, to determine the effects on egg viability, which included wash, flotation, extraction (both individually and in combination), and incubation solutions. Washing samples under pressure and no pressure were compared for best egg recovery. Different flotation solutions were tested at different specific gravities (SGs) to determine which combination recovered the most eggs. Centrifugation speeds and times were also tested after the washing and flotation steps for optimum egg recovery. Although the extraction step is not included in the PRG Helminth Method, it was sometimes performed on samples that were too dense for microscopic analysis. Therefore, different extraction combinations were tested to determine which of them allowed the most eggs to be recovered. We then tested different wash solutions against various sample types to determine which ones gave the highest eggs recoveries and which solutions facilitated easier microscopic analysis. All the above were

conducted in statistically large enough replicates to provide strong evidence for the best procedure to follow to create an accredited method that will stand up to peer scrutiny.

A final method was then put together, based on the data collected from all experiments (Appendix B). We then attempted to test the reproducibility and repeatability of the method using different technicians of differing levels of expertise and backgrounds. This however proved to be difficult, as we encountered problems with equipment and eggs stocks, resulting in inconsistent results. This step will therefore need to be repeated once we acquire fresh *Ascaris suum* eggs.

Other interesting findings from this study included egg loss during the extraction step. We found that, whilst extraction allowed for the size of the final pellet to be reduced, eggs were actually lost when the supernatant was discarded. We therefore recommend that this step be removed from all helminth methods, and it is not included in the newly improved WRDC method. We also included costing (Appendix C) of setting up a laboratory for helminth testing using the new method, as well as approximate labour costs for running it. Future work on this project includes knowledge dissemination by training laboratories and personnel on the new method and publishing all data in peer-reviewed journals.

ACKNOWLEDGEMENTS

We would like to thank the following Reference Group members for their contribution to the project:

Reference Group	Affiliation
Dr Sudhir Pillay	Water Research Commission (WRC) (Chairperson)
Ms Aileen Manalac Andaya	TÜV SÜD, Singapore
Ms Mei Yee Chan	TÜV SÜD, Singapore
Dr Konstantina Velkushanova	IHE Delft, Netherlands
Ms Lizette Leibach	ERWAT
Mr Nico van Blerk	ERWAT
Ms Alison Chapman	ERWAT
Mr Nkateko Kubayi	WRC, Project Co-Ordinator
Ms Merissa Naidoo	WASH R&D Centre (or WRDC), UKZN

We would like to thank the WRC for funding this project and providing great support throughout. The WRC has also been extremely understanding of the limitations and restrictions imposed on us during the lockdown and the Covid-19 pandemic, and we are very grateful. We would like to thank Miss Merissa Naidoo for her technical support in the laboratory throughout this project. We would also like to thank the entire laboratory team, as well as Ms Estella Jingxi and Mr Arun Kumar Rayavellore Suryakumar, at the WRDC, UKZN, for their participation in the intra-laboratory testing. We are also grateful for the WRDC administrative team's support throughout this project.

DECLARATIONS

All data from this project are currently being written up into four papers for publication in peer-reviewed journals. These articles are still in preparation, thus the work presented here will be cited under them.

1) Naidoo, D and Archer, C. E., in prep 2022. The effect of all reagents used in existing helminth processing and enumeration methods on the viability of *Ascaris suum* eggs.
2) Naidoo, D and Archer, C. E., in prep 2022. The effects of all technical steps used in helminth egg isolation and enumeration methods on egg recovery from pig faeces.
3) Naidoo, D and Archer, C. E., in prep 2022. *Ascaris suum* egg recovery after phase extraction in sludge samples.
4) Naidoo, D and Archer, C. E., in prep 2022. The new WRDC Helminth Test Method: a highly adaptable procedure suitable for sanitation and environmental samples. (Journal aimed at Nature Protocols).

ABBREVIATIONS

AmBic	Ammonium bicarbonate
GIT	Gastrointestinal tract
HAV	Hepatitis A
HEV	Hepatitis E
LAMP	Loop mediated isothermal amplification
MDG	Millennium development goals
PCR	Polymerase chain reaction
PRG	Pollution Research Group (now WASH R&D Centre, UKZN)
qPCR	Real-time PCR
SDG	Sustainable development goals
SG	Specific gravity
SOP	Standard operating procedure
STH(s)	Soil-transmitted helminth(s)
UDDT	Urine diversion dry toilet
UKZN	University of KwaZulu-Natal
VIP	Ventilated improved pit (latrine)
WASH	Water, sanitation, and hygiene
WRDC	WASH Research & Development Centre

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1. LITERATURE REVIEW

1.1 BACKGROUND

Lack of improved water, sanitation, and hygiene (WASH) is directly associated with the silent epidemic of infections that burdens approximately one third of the world's population (Bardosh, 2015). 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) (JMP, 2017; Cooper and Hollingsworth, 2018). *Ascaris lumbricoides*, also known as the human roundworm, is the most common STH of human health importance (Brownell and Nelson, 2006). Infections with *A. lumbricoides* are most prominent in areas that lack a source of potable 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 burdens can lead to symptoms such as diarrhoea, bloating, abdominal blockages and discomfort, malnutrition, and impaired growth and cognitive development (Cooper and 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 5 years (Kotloff, 2017).

One of the United Nations Millennium Development Goals (MDGs) was halving the number of people living without access to sustainable sanitation and potable water (Friedrich et al., 2015; Moe and Rheignans, 2006). With that timeframe having passed, the World Health Organisation (WHO) has developed a post-2015 agenda, in which 17 new goals are outlined. Known as the Sustainable Development Goals (SDGs), they aim to eradicate poverty, protect the planet, and ensure prosperity for all (Joshi et al., 2015). A new target date (2030) has been set by the WHO as part of the 2030 Agenda for Sustainable Development, giving a period of 15 years to accomplish the 17 SDGs (WHO, 2015). The South African government 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.

Diarrhoeal diseases generally manifest as symptoms of bacterial and viral infections but can also be symptoms of infection by parasitic worms (helminths), where *Ascaris lumbricoides* is of importance to human health (Brownell and Nelson, 2006). The lack of improved sanitation has also been linked to malnutrition and poor cognitive development in children, thus retarding educational progress, allowing for a continuing cycle of poverty (Guigas and Molofsky, 2015). The provision of improved sanitation may reduce the prevalence of diarrhoeal diseases by up to 36%. Reduction of the transmission of infectious diseases is, however, impossible without sanitation regimes being combined with education and correct hygiene practices, i.e., washing of hands with soap and thorough washing and cleaning of food (Clasen et al., 2014).

The health impacts of improper sanitation, together with high levels of poverty, drive underprivileged people towards even greater disadvantages. It is impossible to solve the issue of poverty, as well as eradicate exposure to excreta and contamination completely. It is, however, possible to break the link

between humans and their excreta, by implementing basic sanitation and safe sludge disposal techniques (Fewtrell and Bartram, 2001). Stored sludge must be properly treated and decontaminated to reduce the number of viable helminth eggs, and other pathogens, entering the environment.

1.2 PATHOGENS RELATED TO HUMAN WASTE

Pathogens found in sludge can be divided into four groups: bacteria, viruses, protozoans, and helminths (Sidhu and Toze, 2009). It should be noted that, although each pathogen described below (Table 1) is potentially a disease-causing agent, the likelihood of extreme infection is greater for people living in developing countries than for those living in developed nations. Furthermore, a cumulative effect is seen when infection with more than one of the causative agents occurs, especially in immunocompromised patients (Table 1). Large infective dosages are required for most of these pathogens, thus detrimental effects are not necessarily seen in every patient who tests positive for the infection.

Table 1: Summary of three of the four classes of pathogens found in human waste, behaviour during treatment, and source literature

Type of Pathogen	Species	Source / Transmission	Disease or Symptoms Caused	Treatment Options for Inactivation and Survivability	Reference(s)
Bacteria	*Salmonella* spp.	Human faeces	Human gastroenteritis, typhoid fever	Wastewater treatment processes — survives up to 3 months in stored sludge, regrowth is possible under favourable conditions, indicator of faecal contamination	Ashbolt (2004); Gea et al. (2007); Majowicz et al. (2010); Nicholson et al. (2005); Ottoson and Stenström (2003); Sahlström et al. (2004); Sidhu and Toze (2009); Watanabe et al. (1997)
	Escherichia coli	Human gastrointestinal tract (GIT) / excreted in faeces	Foodborne outbreaks, diarrhoeal disease, infantile diarrhoeal complications	Anaerobic digestion — survives 11 weeks in animal manure, 6 months in winter, regrowth is possible.	Ashbolt (2004); Estrada et al. (2004); Sahlström (2003); Sidhu and Toze (2009)
	Campylobacter sp.	Surface water, faecal sludge /animal faeces	Gastroenteritis, traveller's diarrhoea	Aeration or oxygenation — survives up to one month in animal manure, lower infective doses required, regrowth is possible	Ashbolt (2004); Jones (2001); Nicholson et al. (2005); Ottoson and Stenström (2003); Sahlström (2003); Sahlström et al. (2004); Sidhu and Toze (2009)
	Listeria monocytogenes	Human GIT, soil, water / foodborne, zoonotic	Third-highest cause of morbidity and mortality related to foodborne disease	Easily inactivated due to low numbers in environment, can withstand anaerobic digestion, regrowth is possible	Estrada et al. (2004); Sahlström (2003); Sahlström et al. (2004); Sidhu and Toze (2009)
	Vibrio spp.	Estuarine and marine origin / human faeces	Extreme flatulence, watery diarrhoea, possible dysentery, septicaemia, cholera	Little is known about treatment in biosolids but numbers in the environment are low	Ashbolt (2004); Fewtrell and Bartram (2001); Sidhu and Toze (2009)
	Shigella spp.	Human faeces	Shigellosis — diarrhoea, stomach cramps and fever	Behaviour in treated biosolids is mostly unknown	Ashbolt (2004); Fewtrell and Bartram (2001); Sidhu and Toze (2009)
	Yersinia enterocolitica	Faecal sludge, animal faeces	Infantile gastroenteritis	Dewatering – versatile, can survive at 0°C and higher temperatures, survives for prolonged periods at low temperatures	Ashbolt (2004); Fewtrell and Bartram (2001); Sahlström (2003); Sidhu and Toze (2009)
	Aeromonas spp.	Water, wastewater	Opportunistic — diarrhoea, urinary tract infections (UTI), traveller's diarrhoea	Low inactivation rates reported, but occur in high numbers in faecal sludge	Sidhu and Toze (2009); Vila et al. (2003)
Viruses	Enterovirus (Poliovirus; Coxsackievirus and Echovirus)	Excreted in faeces by infected individuals	Polio can lead to eventual paralysis, aseptic meningitis	Anaerobic digestion is not very successful — detection method used plays a critical role in establishing numbers in sludge	Ashbolt (2004); Fewtrell and Bartram (2001); Sidhu and Toze (2009)

	Rotavirus	Excreted in faeces / direct contact	Highest viral cause of diarrhoeal disease – 6% of child deaths worldwide	Low inactivation rates have been reported compared with other viruses, low numbers occur in the environment	Ashbolt (2004); Dennehy (2005); Parashar et al. (2003); Sidhu and Toze (2009)
	Adenovirus	Excreted in faeces / faecal-oral route	Similar diseases caused as rotavirus can either be asymptomatic or flu-like	Occurs in higher numbers than rotaviruses, can withstand treatment, survives in biosolids as infectious particles	Ashbolt (2004); Dennehy (2005); Sidhu and Toze (2009)
	Norovirus	Contaminated water or food, human faeces / direct contact	Very contagious, human gastroenteritis, diarrhoeal disease	Norovirus cannot be cultured, therefore there is very little information available relative to treatment	Dennehy (2005); Sidhu and Toze (2009)
	Hepatitis A (HAV) and Hepatitis E (HEV)	Sewage sludge, poor water supply / faecal-oral route	Infectious hepatitis, possible miscarriages in women, possible death	The exact numbers and behaviour of HAV and HEV in sludge are not well documented	Ashbolt (2004); Dalton et al. (2008); Sidhu and Toze (2009)
Protozoan Parasites	*Giardia duodenalis*	Contaminated food or water	Giardiasis – a form of gastroenteritis	Resilient to treatment efforts but dewatering is promising	Adam (2001); Ashbolt (2004); Fewtrell and Bartram (2001); Sidhu and Toze (2009)
	Cryptosporidium sp.	Contaminated food or water	Opportunistic — respiratory and gastrointestinal disease, watery diarrhoea	Temperature treatment — oocysts can survive for up to 3 months in the soil, more susceptible to treatment than *Giardia*	Ashbolt (2004); Fewtrell and Bartram (2001); Sidhu and Toze (2009)
	Entamoeba histolytica	Human faeces, / contaminated water	Amoebiasis – dysentery, severe abdominal pain, flatulence, and high body temperature	The exact numbers and behaviour of *Entamoeba* in sludge are not well documented, chitin layer of cyst allows for resistance to drying	Ashbolt (2004); Daugschies et al. (2013); Nowak et al. (2015)
	Balantidium coli	Zoonotic (pigs and non-human primates) / faecal-oral route	Balantidiosis, resulting in severe dysentery	Treatment of *Balantidium* in sludge is not well documented	Ashbolt (2004); Schuster and Ramirez-Avila (2008)

1.2.1 Helminths

Taenia spp.

Taenia saginata, the beef tapeworm, and *Taenia solium*, the pork tapeworm, cause taeniasis in human hosts (Buttar et al., 2013; Lustigman et al., 2012). Gravid proglottids, containing infective eggs, are passed out in faeces onto the soil. These eggs of *T. saginata* and *T. solium* must then be ingested by the worms' intermediate hosts, cattle, and pigs, respectively, in which they undergo larval development to the cyst or cysticercus stage in the host's musculature. If undercooked or raw meat is ingested by a human, these infective cysts develop into adult tapeworms in the small intestine of the human host. Humans thus act as definitive hosts for these taeniid tapeworms, which may cause diarrhoea, intestinal blockages, and malnutrition. Ingestion of *T. solium* infective eggs results in cysticercosis, the formation of larval cysts in host tissue (Buttar et al., 2013). When these cysts lodge in the CNS, usually the brain, a condition called neurocysticercosis results that may cause epileptic seizures. Neurocysticercosis is a serious condition with major economic losses both because infected people require hospitalisation and treatment, and because infected pork is condemned and destroyed (Lustigman et al., 2012).

Trichuris trichiura

Trichuris trichiura, the whipworm, causes trichuriasis in human hosts. Heavy infections are especially harmful to children of school-going age (WHO, 2002). This parasite is infective to people following the ingestion of developed eggs from faeces-contaminated soil (Bethony et al., 2006). After ingestion, the eggs hatch in the small intestine, larvae develop to adults, which migrate to the large intestine, where they bury their heads into the intestinal folds, mate and produce eggs. Heavy infections can result in prolapse of the rectum (Lustigman et al., 2012). Chronic trichuriasis is associated with rectal bleeding (dysentery), poor iron status, anaemia, and stunted growth (WHO, 2002). Blood loss is not as severe as that associated with hookworm infection. The prevalence of trichuriasis declines with host age (Bethony et al., 2006).

Hookworms

Ancylostoma duodenale and *Necator americanus*, commonly known as hookworm species, may cause severe infection in humans. The main health complication related to these parasites is intestinal bleeding resulting from the attachment of worms to the wall of the small intestine (Bethony et al., 2006). They may also cause intestinal inflammation and anorexia due to malabsorption of nutrients, leading to stunted growth and poor development (Stephenson et al., 2000). The resultant blood loss leads to iron-deficiency anaemia, eventually impacting school performance, and worker productivity (WHO, 2002). Hookworm infection is also particularly problematic in pregnant women where blood loss and iron deficiency may lead to foetal morbidity. When the iron stores of the host body are low, the haemoglobin content of the blood may also be depleted. It can therefore be said that this infection impedes host metabolism (WHO, 2002).

Infection with this parasite occurs by penetration of the filariform larva through the skin of the host, thus walking barefoot on contaminated soil poses a risk for infection with hookworms (Stephenson et al., 2000). Both *N. americanus* and *A. duodenale* occur in sub-Saharan Africa.

Ascaris lumbricoides

The most prominent and resilient intestinal worm infecting people (and notoriously associated with sludge) is *Ascaris lumbricoides* and will therefore be discussed in detail here. It is also known as the common roundworm, which causes ascariasis (Brownell and Nelson, 2006). It is most prevalent in tropical and sub-tropical regions and infects approximately one third of the worldwide population (Bethony et al., 2006; Schüle et al., 2014). Although ascariasis is seldom lethal, heavy infections are undoubtedly a major contributor to impeded physical and cognitive development in children, particularly those aged < 5 years. These effects may be lifelong (Esrey et al., 1991). Common symptoms associated with ascariasis include abdominal pain and bloating due to intestinal blockages by the worms (Awasthi et al., 2003; Schüle et al., 2014). It may also cause lactose intolerance and insufficient absorption of essential nutrients, such as Vitamin A (Bethony et al., 2006). Digestive complications interfere with schooling as chronic diarrhoeal episodes result in absenteeism, preventing sustained cognitive development (Awasthi et al., 2003). It also affects the immune system such that the patient may become more susceptible to other infections, as well as infection by multiple species of parasitic worms (polyparasitism) (Bethony et al., 2006). According to Eppig et al. (2010), polyparasitism increases these impacts on a child's cognitive development and school performance. Energy that should be used for development is instead used to compensate for malnourishment. On occasion, infection can lead to intestinal perforation and peritonitis, which may be fatal (Bethony et al., 2006). Ascariasis, trichuriasis, and hookworm infection often occur concurrently, thereby advancing the onset of iron-deficiency anaemia (WHO, 2002).

Figure 1 shows the life cycle of *A. lumbricoides*. Eggs excreted by a human host are not immediately infective. There is a latency period of approximately 2-3 weeks from when a fertilised egg is passed onto the soil until it becomes infective. Every female worm lays some unfertilised, or infertile, eggs that cannot undergo any further biological development, even if ingested (O'Lorcain and Holland, 2000). Once ingested, fertilised eggs, containing 2nd stage larvae (L2), hatch and the L2 larvae penetrate the intestinal mucosa to enter the blood stream. They are then carried via the blood to the liver, heart, and lungs, moulting to the 3rd stage (L3) in the lungs. The larvae are coughed up, and re ingested (swallowed), pass to the small intestine, at which point they moult to the 4th stage (L4) and mature into adult worms (Schüle et al., 2014). Mating occurs and the resulting fertilised eggs are passed out onto the soil in faeces. The total duration of the lifecycle is two to three months (O'Lorcain and Holland, 2000). The female worm can produce up to 200 000 fertilised eggs per day, and can remain viable in the soil for up to seven years (Pecson and Nelson, 2005). The infective stage of the life cycle is the L2 stage fertilised egg, with ingestion being the route of transmission from soil to human host.

According to Cruz et al. (2012) (

Figure 2), egg development occurs over twelve stages; however, other studies indicate five to ten developmental stages instead, as the pre-larval stages had not previously been identified. The early-morula stage I is defined as five to 10 visible cells, and the late-morula stage (F) is defined as 11 or

more visible cells within the developing embryo. The blastula and gastrula stages (G and H) are comprised of a spherical layer of cells and a layer of cells surrounding the embryo with a kidney shaped invagination on one side. The pre-larval stages are defined as visible larva-like structures forming concentric rings inside the egg (Cruz et al., 2012).

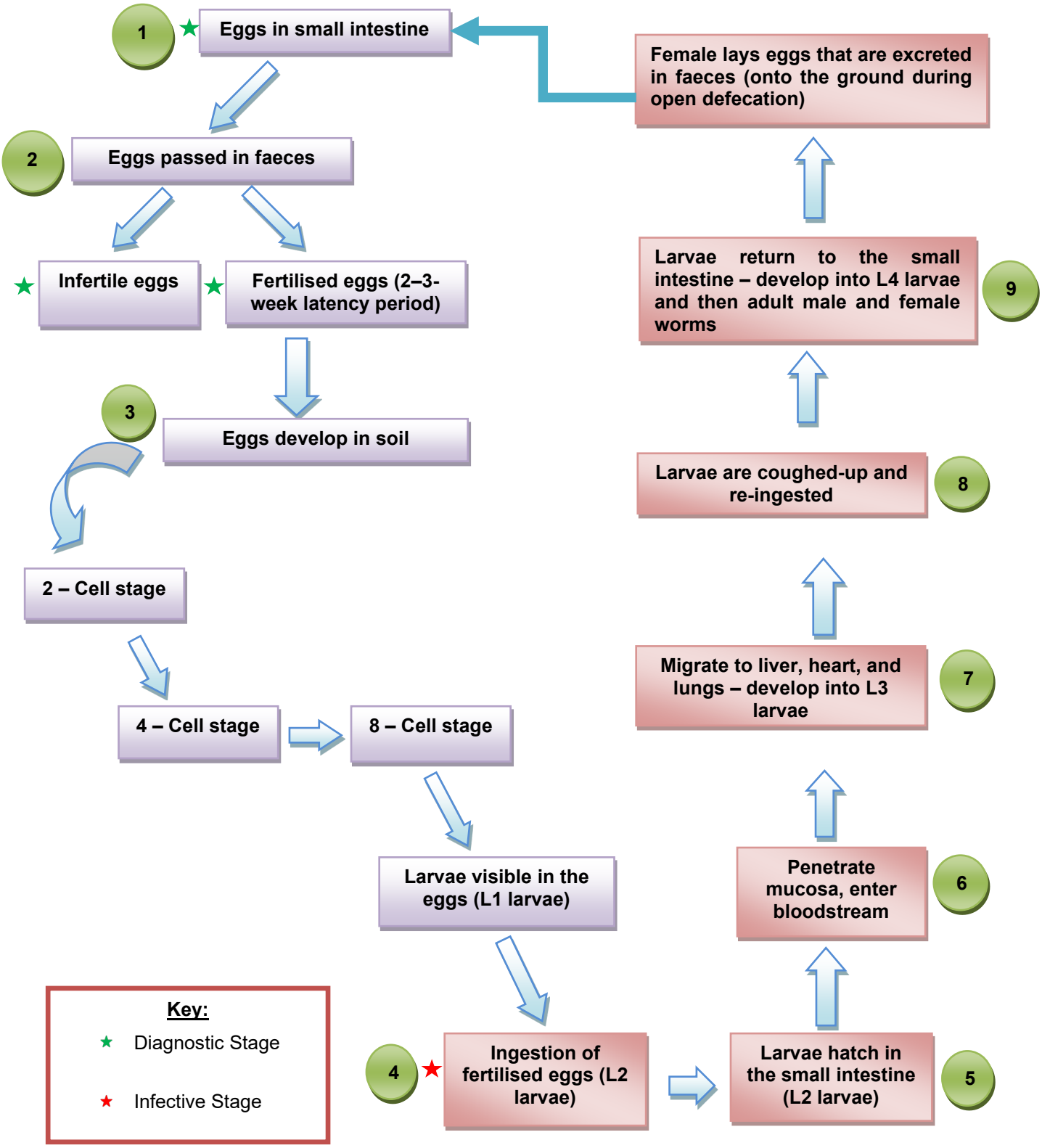

Figure 1: Life cycle of *Ascaris lumbricoides* - lilac blocks indicate development outside the host and pink blocks indicate stages developing within the host.

(Derived from http://www.cdc.gov/parasites/images/ascariasis/ascariasis_lifecycle.gif **D. Naidoo *del*)**

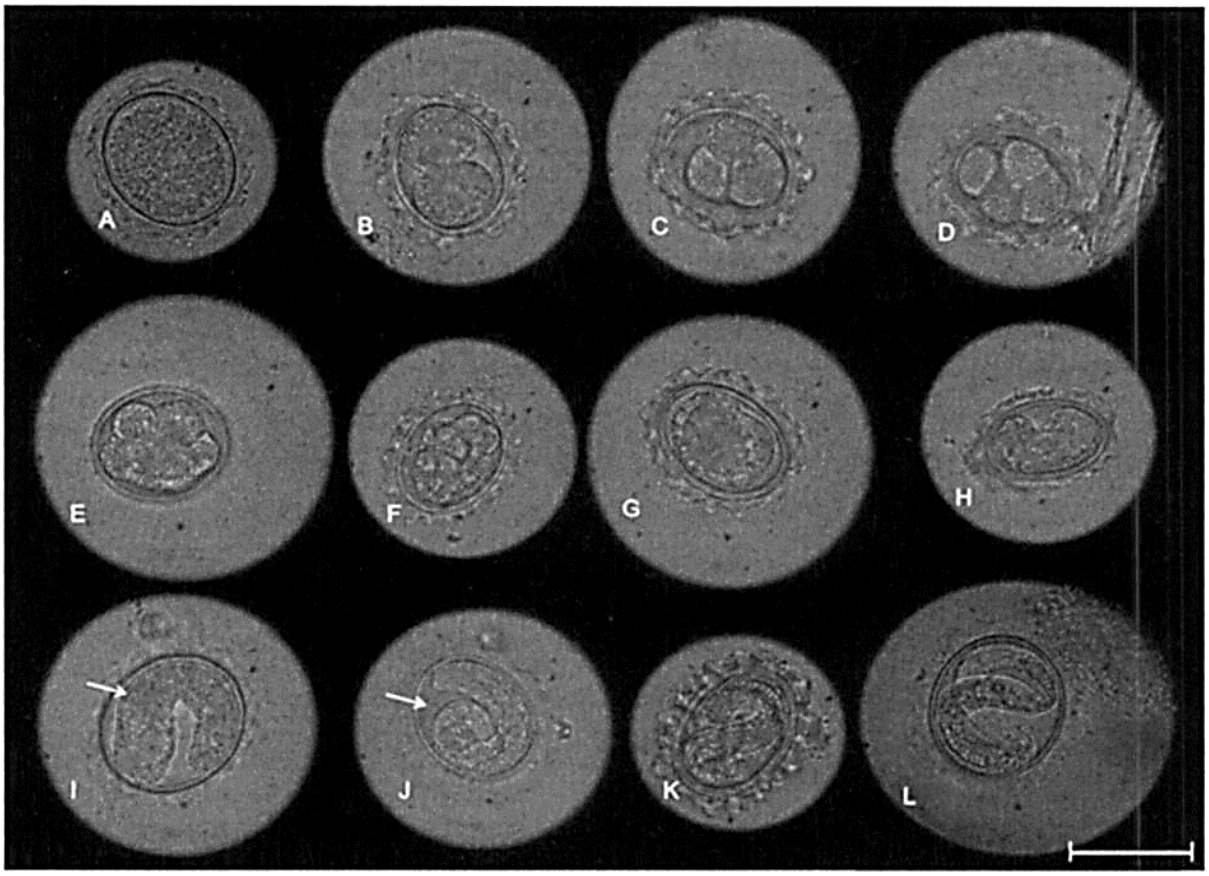

Figure 2: The different developmental stages of *Ascaris* eggs. (A) One-cell; (B) Two-cell; (C) Three-cell (reported in Cruz et al. (2012) but not considered as a plausible developmental stage in the present study); (D) Four-cell; I Early-morula; (F) Late-morula; (G) Blastula; (H) Gastrula; (I) Pre-larva 1; (J) Pre-larva 2; (K) L1 larva and (L) L2 larva. Image sourced from Cruz et al. (2012).

Helminth eggs in faecal sludge may not always be infective. Specific temperature (25°C) and humidity (>55%) levels are required for optimal egg development, and these are generally found in soil. *Ascaris lumbricoides* eggs (40 x 60 µm in size) have a three-layered shell that is approximately 3-4 µm thick. The innermost layer is a lipoprotein layer, followed by a thicker chitin layer, and lastly a vitelline layer (Brownell and Nelson, 2006). The lipoprotein or ascaroside layer surrounds the perivitelline space and is desiccation resistant (Geng et al., 2002). It is made up of 25% proteins and 75% ascarosides that are structurally distinct glycosides that were detected in the family Ascarididae (Quilès et al., 2006). It is impermeable and acts as an osmotic barrier to dissolved ions, preventing diffusion into the egg (Lýsek et al., 1985), but is permeable to oxygen and certain essential proteins (Kaulenas and Fairburn, 1966). The chitinous layer provides the shell with rigidity and shape and is made up of chitin microfibrils within a protein matrix (Quilès et al., 2006). The vitelline layer is made up of glycoproteins, and is characteristically mamillated, developing in the uterus of the female worm (Rogers, 1956). Hypochlorite, and mechanical stress (friction) may be used to remove (or decorticate) the outermost layer of the shell (Murrell et al., 1997). Decortication also facilitates the separation of clumps of eggs (Daugschies et al., 2013). The lipoprotein and chitin layers, however, remain intact (Brownell and Nelson, 2006). Generally, a decorticated egg is still viable, but is more susceptible to inactivation (Rogers, 1956).

Hatching of *Ascaris* eggs can be induced outside the host's body when placed in appropriate solutions (Ransom and Foster, 1919) or in a simulated environment resembling the small intestine. Size and shape are used as differentiating factors between fertilized and unfertilized eggs (Rogers, 1956). An infertile egg may be distinguished from the fertilised egg, as it is slightly narrower and more elongated. According to O'Lorcain and Holland (2000), *Ascaris* eggs are typically sticky, and are therefore able to adhere to a variety of surfaces, such as door handles, furniture, fruits and vegetables, utensils, and the skin. It has been shown that the surface of the egg of *Ascaris suum* (the pig roundworm) has hydrophobic properties due to the dense sticky material that is secreted by the outer vitelline layer of the egg (Capizzi-Banas et al., 2004). According to Pawlowski (1982), the shell of the *Ascaris* egg is permeable to water, rendering it susceptible to inactivation by desiccation and high temperatures. Impermeability is increased upon fertilisation and shell formation (Karkashan et al., 2015); however, during the later stages of embryonic development, larval maturation occurs, and eggshell permeability increases (Ghiglietti et al., 1997).

Due to ethical and logistical issues, it is difficult to source *Ascaris lumbricoides* (human roundworm) 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). They only differ genetically and in terms of the definitive host.

1.2.2 Indicator organisms

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). The principle of indicator organisms was then developed that entails the identification of an organism that is always present in faecal matter, so indicating the presence of other microorganisms and thus contamination of the excreta (Horan, 2003; Sidhu and Toze, 2009). The criteria for an ideal indicator organism are as follows (Horan, 2003; Sidhu and Toze, 2009):

a) It should always be present in faecal matter whenever pathogens are present
b) It should be present in greater numbers than any other pathogens
c) It must be unable to multiply in the environment (or outside of the host)
d) It should display similar survival characteristics to the pathogens found in the sample, in terms of treatment or inactivation processes
e) It should elicit mild to moderate, easily treatable disease only
f) It should be easily detectable using cheap isolation and culturing techniques that are not time-consuming.

No single organism or group of organisms meets all the above criteria; however, faecal coliforms and faecal streptococci are the closest bacterial indicators (Sidhu and Toze, 2009). Faecal coliform bacteria are traditionally defined as microorganisms (generally *E. coli*) that can develop and multiply at 37°C and are able to produce gas and acid from lactose (Horan, 2003). Faecal coliform bacteria are now used as indicators of water and biosolid safety, where low numbers (<1000 per gram of sludge) indicate

the absence of any other pathogens (Sidhu and Toze, 2009). Faecal streptococci are good as indicators because they meet most of the above criteria, but studies have reported false results due to flawed isolation and enumeration techniques (Horan, 2003). Other issues and limitations associated with bacterial indicators 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). The correlation between numbers of pathogens and bacterial indicators may also differ during inactivation or wastewater treatment processes and may not adequately represent the behaviour of pathogens (Horan, 2003; Sidhu and Toze, 2009).

1.2.3 Index organisms

It was recently reported that bacterial indicators may be ineffective for indicating the occurrence or fate of viruses, protozoa and helminths, and the survivability characteristics of bacteria differ compared with other pathogens (Ibarluzea et al., 2007; Sidhu and Toze, 2009). Alternative names for indicator organisms are index or model organisms. These are defined as microorganisms that behave in a similar manner to pathogens when subjected to treatment or inactivation processes (Horan, 2003; Sidhu and Toze, 2009). Somatic coliphages and male-specific coliphages are often used as models of viral contamination. Human adenovirus is said to be thermostable and is therefore also used as a model organism for viral contamination (Sidhu and Toze, 2009). *Giardia* 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 sludge. *Ascaris* eggs are the most resilient of all organisms found in sludge as they can withstand harsh environmental conditions such as desiccation and, according to Pecson and Nelson (2005), can survive in both aerobic and anaerobic environments for up to 7 years. *Ascaris* spp. are therefore deemed fit as index organisms for parasite contamination, as well as overall pathogen contamination and inactivation (Horan, 2003; Sidhu and Toze, 2009). If a treatment process can inactivate *Ascaris* spp. eggs, then it is very likely all other pathogens will be destroyed as well (Daugschies et al., 2013; Maya et al., 2012).

1.3 HELMINTH RECOVERY AND ENUMERATION TECHNIQUES

According to the WHO (2006), the recommended limit for helminth eggs should be one egg per litre of wastewater and one egg per gram of total dried solids (sludge). Counts reported from developing nations lie between 70 and 3000 eggs per litre of wastewater and between 70 and 735 eggs per gram of dried sludge (Maya et al., 2012). Sludge reuse hinges on the complete hygienisation of waste material by pathogen removal or destruction, which is also costly. 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). New sludge treatment and toilet technologies are constantly being developed, where helminth eggs must be spiked into the system to test treatment/ inactivation efficacy. A highly sensitive, standard helminth method is therefore required for application in laboratories globally.

The processing and recovery of helminth eggs in faecal sludge should be consistent. Different laboratories and groups have used variations of the standard United States Environmental Protection Agency (US EPA) method and the Pollution Research Group (PRG) Helminth Method of Hawksworth et al. (2010) adapted for the Water Research Commission report TT322-08 by Moodley et al. (2008), improved by Archer and described by Pebsworth et al. (2012) and Naidoo et al. (2016). The PRG method was the one used by the WASH R&D Centre, UKZN, for helminth testing and formed the foundation for this project. From this method, and after all experimentation done in this project, the newly improved WRDC (WASH R&D Centre) Helminth Method was derived and will be implemented at our lab henceforth.

Recent studies have also aimed to develop more modern identification and enumeration techniques, such as qPCR (real-time PCR) for molecular detection of the presence of helminth eggs (DNA) in sludge, and the BacLight staining technique that requires a specialised confocal microscope for helminth egg quantification. 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. Furthermore, these techniques might have shortfalls that have not yet been solved, e.g., PCR is not specific for egg viability, meaning that it can only give an indication as to the presence or absence of eggs, but cannot determine the viability status of eggs (an indicator of risk to human health). Modern diagnostic techniques, including Polymerase Chain Reaction (PCR), qPCR, digital PCR, loop-mediated isothermal amplification (LAMP), and flow cytometry, were not included in this review.

There is currently an urgent need for the new WRDC Helminth Method to be accredited for acceptance as an internationally recognised standard. The essence of this study was thus the thorough ‘interrogation’ of the existing PRG method to determine the best chemicals to use and optimal exposure times, the optimal wash times, centrifugation times and speeds, and the minimum steps that should be used based on sample-type. To achieve these ends, the following tests were conducted:

1) Every reagent used in all common international helminth methods was tested on fresh, viable *Ascaris suum* eggs for varying time intervals, to determine whether viability of these eggs was affected or not.
2) The most efficient flotation solution and optimum specific gravity (SG) to use, was tested in combination with the best wash solution/s on different types of sanitation/environmental samples.
3) Times and speeds of centrifugation after the washing step, and for flotation and sedimentation, were determined.
4) The WRDC Helminth Method (as it stands) is adaptable to various sample types and the steps to include or exclude in the final method were assessed, considering the nature of the samples.
5) Many methods use an extraction step, some even prior to the flotation step, and 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.

6) All the above were conducted in statistically large 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 of the method for accuracy and reproducibility between technicians of varying capabilities.

1.3.1 The PRG Helminth Method

The PRG helminth method can be broken down into five steps: washing, flotation, centrifugation, analysis, and incubation. Depending on the type of sludge sample, a given amount (either 10-20 g of solid sludge or 200-1000 ml of blackwater or wastewater) is soaked in ammonium bicarbonate. A magnetic stirrer bar is added, and the sample is then placed on a magnetic stirrer plate to allow for dislodgement of helminth eggs from sludge particles. The sample is then poured through a set of drum sieves (a 100 µm sieve over a 20 µm sieve, both 200 mm in diameter) (Belcher et al., 2015; Grego et al., 2018). It is then washed thoroughly on the sieves, using pressure from a hose on a tap, 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 four plastic 15 ml graduated plastic test tubes (called Falcon tubes). These tubes are then centrifuged at 1512 x g (3000 rpm) for 10 minutes and the supernatant discarded.

The principle of the flotation step is to create a density gradient within the test tube, to allow for eggs to float up the column to the surface whilst particulate matter sediments down and gets packed into a pellet when centrifuged. The flotation solution (zinc sulphate [$ZnSO_4$]) is made up to a certain density, i.e., a SG of 1.3. According to David and Lindquist (1982), the relative density of soil-transmitted helminth eggs ranges from 1.05 – 1.27, thus a SG of 1.30 would allow eggs to float up efficiently without exerting too much pressure on the egg wall. Zinc sulphate is 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 are then centrifuged at 672 x g (2000 rpm) for 10 minutes, to allow eggs to float up into the liquid column above the sediment (Belcher et al. 2015; Grego et al. 2018). 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 minutes, 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.

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), immotile (eggs with a fully developed, plump, immotile larva), 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 larva), 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).

1.3.2 US EPA Method

The US EPA Method has been the accepted standard for helminth recovery in water and wastewater samples. This method, however, is not 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) for the recovery of helminth eggs from UDDT waste, that was further modified by Moodley et al. (2008) and then by Archer, who used it to process soil samples for a publication with Pebsworth et al. (2012). The old US EPA Method (US EPA, 1999) also includes 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 (Nelson and Darby, 2001) and the extraction step was therefore excluded from the PRG Helminth Method SOP, however, the step was occasionally included ONLY if the final pellet was too large for microscopic analysis. The US EPA Method was previously the most used method, with different studies using some modification of it (Amoah et al. 2017).

1.3.3 Comparisons between existing methods

A comparison between methods is presented in Table 2 below.

Table 2: Differences between the standard US EPA Method and the PRG Helminth Method

Step	US EPA Method	PRG Method
Sample preparation	Sample is soaked overnight, and then blended in water prior to washing over a set of drum sieves. Wash solution is added, and sample is left overnight to sediment.	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, and then washed over drum sieves.
Apparatus	Glassware is used, all of which must be coated in organosilane. Additional glassware is used, such as Erlenmeyer flasks and large beakers.	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.
	A 180 or 300 µm sieve is used for large particle separation and a 38 µm sieve for small particle separation.	A 100 µm sieve is used for large particle separation and a 20 µm sieve for small particle separation.
Washing & Sedimentation	1% 7X is the only wash solution used. After sedimentation, a vacuum pump is used to suction off the supernatant.	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 centrifugation step is performed.
	Sample is then washed on a 180 or 300 µm sieve placed into a funnel over a beaker, resulting in slow drainage. A second blend,	Soaked sample is washed straight onto a set of drum sieves – 100 µm placed over a 20 µm sieve.

Step	US EPA Method	PRG Method
	wash, sedimentation (for 2 hours) and vacuum separation of the permeate is performed.	
	A wash bottle (only) is used for washing of the samples through the set of sieves.	Washing is done under pressure (using a hose attached to the tap). A spray bottle is used only for retentate collection.
	Sample is washed into 50 ml Falcon tubes.	Sample is pipetted into 15 ml Falcon tubes.
Centrifugation	First and third (before microscopy) centrifugation after washing is conducted at 1000 x g (2500 rpm for 10 minutes). Second centrifugation after flotation is conducted at a similar speed (800 – 1000 x g or 2200-2500 rpm for 5-10 minutes).	First and third centrifugation (before microscopy) after washing is conducted at 1512 x g (3000 rpm for 10 minutes). Second centrifugation after flotation is conducted at much slower speed to allow eggs to float up into the supernatant column (672 x g or 2000 rpm for 10 minutes).
Flotation	Eggs in the washed sample are floated using magnesium sulphate (SG = 1.20).	Eggs in the washed sample are floated using zinc sulphate (SG = 1.30)
Incubation	Sample is suspended in 0.1N sulphuric acid, then incubated first for 3-4 weeks 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.	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 at 25°C.
Microscopy	Sedgwick-Rafter counting chamber is used to examine the concentrated sample after incubation.	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.
Overall Processing Time	Total processing time can take 2-3 days, including overnight soaking and sedimentation steps. Actual results are obtained 3-4 weeks after the incubation step has been completed.	Total processing and initial microscopic analysis time take 1-2 hours. Viability is assessed immediately, and incubation is done only if necessary when assessing viability is difficult.
Overall Chemical Exposure	Eggs in test samples are exposed to more chemicals for prolonged periods.	Chemical exposure is significantly reduced to prevent egg viability being affected.

1.3.4 Overview of the use of various helminth techniques

There have been numerous variations of the US EPA Method that have been implemented in different regions over the years. One such method is the Mexican Helminth Method, which was then further modified and implemented as a standard for helminth testing in Mexico. For the review of studies to follow (Table 3), only those that included wastewater or sludge treatment, or processing were considered. Table 3 shows the versatility of these methods, 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 microscopy counting methods and how data should be reported for publication purposes. There is also an obvious lack of consistency in terms of the actual processing steps. A gold standard method would allow for better cross-comparisons of what does and does not work for sludge treatment technologies, as well as disease prevalence worldwide.

Table 3: Summary of studies that have made use of the PRG Method, the US EPA Method or a variation/modification of either, 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
	Composite VIP sludge sample – liquid VIP sludge mixed with potato flakes.	Ammonium bicarbonate (119 g/l)	Zinc sulphate (SG = 1.3)	No reports of steps of the processing method affecting egg viability and recovery. Eggs were spiked and an equal number of eggs was recovered after treatment and processing.	Belcher et al., 2015
	Blackwater	Ammonium bicarbonate (119 g/l)	Zinc sulphate (SG = 1.3)	*Ascaris* spp., hookworm spp., *Trichuris* spp., *Hymenolepis nana, Hymenolepis diminuta* and other helminth species were recovered.	Grego et al., 2018
US EPA Method (or variations thereof, including UNAM version)	Sludge	0.1% 7X	Magnesium sulphate (SG = 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
	Sludge	0.1% 7X	Zinc sulphate (SG = 1.2)	*Ascaris* spp. 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
	Sludge	0.1% 7X	Zinc sulphate (SG = 1.2)	*Ascaris* spp., *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
	Sludge	0.1% 7X	Magnesium sulphate (SG =1.2)	No report of reagents affecting egg viability. *Ascaris* spp., hookworm spp., *Trichuris* spp., *Hymenolepis* spp., *Toxocara* spp. & *Taenia* spp. were recovered.	Bowman et al., 2003; Capizzi-Banas et al., 2004
	Sludge	0.1% Tween 80	Zinc sulphate (SG = 1.2)	*Ascaris* spp. eggs were recovered. Extraction step with ethyl acetate and acid-alcohol also performed.	Nelson and Darby, 2001
	Pig Faeces	None	Zinc sulphate (SG = 1.2)	*Ascaris suum* eggs were recovered.	Katakam et al., 2014
	Wastewater	None	Sodium chloride (NaCl; SG = 1.27 & 1.18)	*Ascaris* spp., hookworm spp., *Trichuris* spp. & *Toxocara* spp. eggs were recovered	Sengupta et al., 2011; Yaya-Beas et al., 2016

	Wastewater	None	Zinc sulphate (SG = 1.18)	*Ascaris* spp. eggs were recovered. No reporting of reagents affecting egg viability.	de Souza et al., 2011
	Wastewater	0.1% 7X	Zinc sulphate (SG = 1.3)	*Ascaris* sp., hookworm sp., *Trichuris* sp., *Taenia* sp. & *Toxocara* sp. eggs were recovered.	de Victorica and Galvan, 2003
	Wastewater	Tween 80 or Triton X	Zinc sulphate (SG = 1.18)	*Ascaris* sp., hookworm sp., *Trichuris* sp., *Taenia* sp. & *Toxocara* sp. eggs were recovered.	Garcia et al., 2013

1.3.5 Helminth egg quantification techniques for fresh faecal samples

There are also various alternative counting techniques that exist, most of which are for rapid field testing of fresh stool samples, such as the Kato-Katz technique, FLOTAC, and the McMaster Method (Albonico et al., 2013). Even though these techniques are specified for rapid testing, studies have erroneously applied them to environmental samples. This, however, produces incorrect results, as helminths occur in a higher concentration in fresh stool samples of infected individuals rather than in group faecal samples, as in sludges. Thus, while the use of 41.7 mg of sieved fresh stool for the Kato-Katz test is sufficient, it is totally non-representative of a large environmental sample and would result in false negatives being reported.

The FLOTAC test is based on flotation and centrifugation to recover helminth eggs from faecal samples. The FLOTAC apparatus is a cylindrical device comprising three components: the base, the translation disc, and the reading disc. A small faecal sample, usually <1 g, is homogenized, diluted, filtered through a wire mesh, pipetted into the 2 x 5 ml chambers of the FLOTAC device that is then centrifuged (Albonico et al., 2013). The flotation solution is then added, and the device is allowed to stand for 5 minutes, after which it is analysed under a light microscope. As with the Kato-Katz technique, the FLOTAC test was not developed for use on environmental and sludge samples.

The McMaster counting chamber method is used for counting helminth eggs after preparation by flotation, where a faecal sample is homogenized in a flotation solution, then screened through wire mesh and pipetted into the two chambers of the McMaster counting chamber (Dunn and Keymer, 1986). The chamber is left to stand for 5 minutes to allow eggs to float to the surface and is then examined under a microscope (Barda et al., 2014), where one focuses on the grid etched on the top side of the chamber, 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).

There are two types of McMaster-counting-chambers, one with the grid etched on the top of the chamber and one with the grid etched on the bottom surface. This allows samples to be read in a flotation solution, or in water after washing the flotation column through a 20 µm mesh sieve, collecting

the retentate into a test tube, centrifuging it, and suspending the deposit in sufficient water to load the sedimentation-type McMaster chamber (with counting grid at the bottom).

1.3.6 Helminth egg quantification techniques for large samples (water, wastewater, blackwater, sludges, soil, and crops)

1.3.6.1 Step 1: Washing & Sedimentation

Conventional helminth techniques for large volume/mass samples, including the improved PRG Helminth Method, now called the WRDC Helminth Method (that was the subject of this project), involve a series of washing, flotation, and centrifugation steps, and sometimes, an extraction step, followed by the final microscopic analysis of the sample. Each step requires the use of various solutions that could possibly affect the viability and subsequent development of eggs after processing the sample. The first step in validating the WRDC Helminth Method was therefore to test the effects of every possible solution used in helminth egg recovery as per literature and methods employed globally, on egg viability and development after exposure.

The first step of processing a sanitation sample for egg recovery is washing and involves the use of one of a variety of wash solutions. Different wash solutions are used for different sample types to maximise egg recovery from a specific matrix (Amoah et al., 2017). 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). There have however, been reports of wash solutions affecting egg viability and recovery (Jaskoski, 1954). The PRG Helminth Method recommends centrifugation at 3000 rpm for 5-10 minutes, whilst the US EPA Method recommends 2500 rpm for 10 minutes, the Mexican modification of the US EPA Method (2006) recommends 2000 rpm for 5 minutes and the Bailenger Method recommends 2500 rpm for 15 minutes (Ayers et al., 1996; Jimenez-Cisneros & Maya-Rendon, 2006; US EPA, 2003) (see Table 4).

Table 4: Summary of selected wash solutions tested and reasons for selection

Solution	Reason for selection/notes	Reference
Water	Selected for testing as a control. Also, useful to determine if water is as effective in dislodging eggs from particulate matter as a wash solution without exposure to any chemical.	PRG, 2017
Ammonium bicarbonate (AmBic)	Used in the old PRG & in the new WRDC Helminth Method. Has been shown to be effective in the dissociation of bonds formed between eggs and particulate matter.	Moodley et al., 2008; Naidoo and Foutch, 2017; Naidoo et al., 2019; PRG, 2017; Trönnberg et al., 2010
Tween 20	Commonly used as a laboratory disinfectant.	Amoah et al., 2017; Paquet-Durand et al., 2007
Tween 80	One of the three most used detergents for helminth methods. Also used as a laboratory disinfectant.	Ayres and Mara, 1996; Forslund et al., 2010; García et al., 2013; Nelson and Darby, 2001; PRG, 2017
Sunlight Liquid	Commonly used and readily available household detergent in South Africa.	N/A

Solution	Reason for selection/notes	Reference
Triton X-100	One of the three most used detergents for helminth methods. It has cell lysis properties and is used to permeabilise cell membranes.	Ayres and Mara, 1996; Forslund et al., 2010; García et al., 2013
Bentonite	Not a wash solution but has been used to further clean up samples between the washing and flotation steps, to further remove particulate matter when recovering eggs from faeces and possibly sanitation samples.	Burden and Hammet, 1976
7X	The most commonly used detergent in helminth methods – used in the US EPA Method; also used in tissue culture and is low foaming.	Bowman et al., 2003; US EPA, 2003; Capizzi-Banas et al., 2004; Maya et al., 2012; Pecson et al., 2007; PRG, 2017

1.3.6.2 Step 2: Flotation

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 organic matter that was not removed during the washing step (Amoah et al., 2017). The flotation solution is therefore made up to a certain density, so that eggs that are lighter than the other particles and less dense than the flotation solution can float up the liquid column in a test tube. Once centrifuged, the particulate matter packs tightly into a pellet at the bottom of the tube, and the eggs float up towards the surface of the flotation medium. This supernatant (where the eggs are contained) is then poured onto a 20 µm sieve, and the pellet is discarded. The density range of most of the important helminth eggs is 1.05 --1.23, thus a SG of 1.3 is used for our method, to ensure the recovery of all helminth species (David and Lindquist, 1982).

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 too quickly. The PRG Helminth Method recommends the use of zinc sulphate at SG 1.3 and centrifugation at 2000 rpm for 10 minutes, whilst the US EPA Method recommends magnesium sulphate at SG 1.2 and 2200-2500 rpm for 5-10 minutes. The Mexican modification of the US EPA Method (2006) recommends zinc sulphate at SG 1.3 and 2500 rpm for 5 minutes and the Bailenger Method recommends zinc sulphate at SG 1.18 and 2500 rpm for 15 minutes (Ayers et al., 1996; Jimenez-Cisneros & Maya-Rendon, 2006; US EPA, 2003) (see Table 5).

Table 5: Summary of selected flotation solutions tested and reasons for selection

Solution	Reason for selection/notes	Reference
Zinc sulphate	Used in the PRG Helminth Method to float eggs after washing of the sample (SG = 1.3).	Ayres and Mara, 1996; Jimenez-Cisneros & Maya-Rendon, 2006; PRG, 2017
Magnesium sulphate	Used in the US EPA Method to float eggs after washing of the sample (SG = 1.2).	US EPA, 2003
Sodium nitrate	Commonly used as an adaptation of the US EPA Method for egg flotation.	Blaszowska et al., 2013; Dubná et al., 2007; Mizgajska, 1997
Brine	Cheap and readily available, has been reported as efficient for floating eggs.	Forslund et al., 2010; Gaspard et al., 1996; Sengupta et al., 2011; Yaya-Beas et al., 2016
Sucrose	Cheap and readily available, has been reported as successful for floating eggs.	Fallah et al., 2016; Horiuchi et al., 2013; Maikai et al., 2012

1.3.6.3 Step 3: Extraction

Once centrifuged, eggs and debris are contained in a pellet at the bottom of a test tube, and the supernatant fluid is discarded. The principle behind the extraction step is to get rid of further debris and this is achieved by trapping sample debris between an acidic aqueous and a lipophilic phase of the mixture. Both extraction reagents are thus added to the pellet in the test tube, and the tube is shaken vigorously to allow phase separation to occur. It has been reported that whilst approximately 40% of organic matter is removed upon extraction, it can also result in the loss of approximately 95% of the eggs due to egg distortion (Satchwell, 1986). Some studies have indicated that extraction solutions can have detrimental effects on the eggs (Nelson and Darby, 2001; Rocha et al., 2016), thus recommending that it should not be included in sample processing. If it is however included, then the exposure time should be minimal (Nelson and Darby, 2001). The PRG Helminth Method suggests extraction after flotation with 10% formalin and diethyl ether or ethyl acetate ONLY when the pellet is very large so as to impede microscopic analysis thereof. The Mexican modification of the US EPA Method (2006) recommends extraction after flotation using acid-alcohol and ethyl acetate, whilst the original US EPA Method (1999) and the Bailenger Method recommend extraction before flotation using acetoacetic buffer and ethyl acetate (Ayers et al., 1996; Jimenez-Cisneros & Maya-Rendon, 2006; US EPA, 2003) (see Table 6).

Table 6: Summary of selected extraction solutions tested and reasons for selection

Solution	Reason for selection/notes	Reference
10% Formalin	Used as the buffer (the hydrophilic extraction phase) in the PRG Helminth Method, following the formal ether concentration (FEC) method commonly used for faecal samples.	Allen & Ridley, 1970; PRG, 2017
Acetoacetic buffer	Commonly used as the buffer (the hydrophilic extraction phase) in the US EPA Method and its variations.	Ayres and Mara, 1996; US EPA, 2003; Verbyla et al., 2013
Ethyl acetate	Used as the solvent (the lipophilic extraction phase) in the PRG Helminth Method and the US EPA Method.	Ayres and Mara, 1996; Jimenez-Cisneros & Maya-Rendon, 2006; PRG, 2017; US EPA, 2003
Diethyl ether	Used as the solvent (the lipophilic extraction phase) in the PRG Helminth Method, following the faecal egg counts (FEC) method commonly used for faecal samples.	Allen & Ridley, 1970; PRG, 2017
Acid-alcohol	Used as the buffer in the extraction step of the Mexican modification of the US EPA Method.	Jimenez-Cisneros & Maya-Rendon, 2006

1.3.6.4 Step 4: Incubation

Incubation solutions are used to suspend and contain the eggs and allow growth and development over a 28-day incubation period (see Table 7). An ideal incubation solution should therefore possess antimicrobial and antifungal properties to prevent the growth of contaminants, but it should also be mild enough not to affect the development, survivability, and viability of the eggs. Many methods do not

include the incubation step, but it is imperative when determining egg viability, especially when testing sewage treatment technologies. Six incubation solutions were identified and tested for this deliverable.

Table 7: Summary of selected incubation solutions tested and reasons for selection

Solution	Reason for selection/notes	Reference
Water	Sometimes used in the PRG Method, however contamination becomes a problem; also used as a control in the incubation solutions experiment.	PRG, 2017
Physiological saline (0.9%)	Cheap and readily available, providing a natural environment for egg development.	N/A
0.1N Sulphuric acid	Commonly used in the US EPA & US EPA-based methods for incubating eggs.	PRG, 2017; US EPA, 2003
0.5%; 2% & 5% Formalin	Used in the PRG Helminth Method for incubating eggs. Various concentrations were therefore selected for testing, to determine the optimum solution that ensures egg development but also minimises contamination.	Bowman et al., 2003; PRG, 2017

2. EXPERIMENTATION

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). They only differ genetically and in terms of definitive host. *Ascaris suum* eggs were sourced from Ukulinga Research Farm, where the life cycle of this helminth is being maintained in two Kolbroek pigs, for research purposes. The pig faeces were washed with water through a 100 µm sieve onto a 20 µm sieve and the retentate on the latter was collected into a beaker (WRDC Helminth Method). Based on previous data and a rapid count of 1 ml of washed sample, egg stock solutions were made up and stored in deionised water, with an approximate egg count of 250 - 300 per millilitre of suspension.

2.1 EXPERIMENT 1: THE EFFECTS OF ALL REAGENTS ON EGG VIABILITY (Naidoo and Archer, in prep 2022)

2.1.1 Wash solutions:

As mentioned above, wash solutions were chosen based on methods used globally, literature and availability. Exposure times were selected based on level of experience of the laboratory personnel practicing the method - the shortest times correspond to our level of expertise and how long we would take to process a sample. Other exposure times were selected based on sample type and possible soaking times needed prior to processing (for dried/ pelletised sludge samples for example). Wash solutions that were tested included Water, ammonium bicarbonate, Tween 20 (0.1%), Tween 80 (0.1%), Sunlight Liquid (0.1%), Triton X-100 (1%), 7X (0.1%) and bentonite (1%), and were made up with deionised water. Exposure times included 10 minutes, 30 minutes, 2 hours, 6 hours, and 24 hours. Each wash solution and exposure time combination was tested in replicates of five.

Wash solutions were made up to the recommended dilutions used in the various methods. Approximately 13 ml of each wash solution was pipetted into 15 ml Falcon tubes, and 1 ml of the egg stock solution was added (spiked). Spiking was staggered by 2-minute intervals allowing for efficient processing of each sample whilst preventing prolonged exposure to the solutions. Immediately after the respective exposure times, samples were passed through a 20 µm sieve. The sample was washed thoroughly to ensure that all residue from the wash solution was removed, and it was then collected back into a 15 ml Falcon tube, centrifuged at 1512 x g (3000 rpm) for 5 minutes. The supernatant was discarded, and the pellet was analysed via light microscopy, after which the sample was washed back into the test tube and incubated for 28 days at 25°C and reanalysed. Eggs are categorised as potentially viable: undeveloped (one-celled), developing (two-cell to gastrula), motile (developed larva that moves) and immotile (developed larva that does not move); and potentially non-viable: necrotic (shrivelled larva that has pulled away from the egg wall); dead (globular, ruptured, or irregular contents) and infertile (unfertilised) eggs.

Incubation is necessary to determine if undeveloped eggs can develop further to infective second stage (L2) larvae after treatment, or, in this case, exposure to wash solutions. Egg damage may not necessarily be visible, but egg development may halt. For the purposes of data analysis, eggs were categorised under **potential viability** (including undeveloped and developing eggs after incubation) and **actual viability** (including only those eggs that developed to a larval stage after incubation). After incubation, eggs should be at the larval stage of development, however, delayed development could be a result of chemical exposure. Consequently, eggs cannot be confidently classified as dead or non-viable if still at a developing or undeveloped stage, especially if they appear to be healthy, hence the discrepancy between potential and actual viability figures.

2.1.2 Flotation solutions

Flotation solutions were chosen based on methods used globally, literature, and availability. Exposure times were selected based on level of experience of laboratory personnel practicing the method (the shortest times chosen correspond to our level of expertise and how long we would take to process a sample). Flotation solutions that were tested included: zinc sulphate, magnesium sulphate, sodium nitrate, brine, and sucrose (at SGs usually used in all methods previously mentioned); and exposure times included 15 minutes, 1 hour and 2 hours. Flotation solutions were made up to prescribed densities or SGs as per respective helminth method. Zinc sulphate, magnesium sulphate and sodium nitrate were made up to a SG of 1.3, whilst brine became saturated at 1.18 and sucrose at 1.2. We were unable to increase the SG of these solutions any further, even when the solution was heated. Sengupta et al. 2011, however, reported that they were able to make-up a brine solution of SG 1.27. Samples were then set up, exposed, and processed as per wash solutions above.

2.1.3 Extraction solutions

Extraction is commonly performed using a combination of two solutions: one solvent and one buffer (or water-soluble reagent/s). Before testing the combined effects of solvent + buffer, the individual effect of each solution was tested on egg viability. Formalin (10%), acetoacetic buffer, acid-alcohol, ethyl acetate and diethyl ether were tested independently, and in combinations of: formalin + ethyl acetate, formalin + diethyl ether, acetoacetic buffer + ethyl acetate, acetoacetic buffer + diethyl ether and acid-alcohol + ethyl acetate. Exposure times for both individual and combined solutions included 15 minutes, 30 minutes and 1 hour. Extraction solutions are either used neat (ethyl acetate and diethyl ether) or made up (10% formalin, acetoacetic buffer, and acid-alcohol). The 10% formalin was made up to 1 *ℓ by* mixing 100 ml formaldehyde with 900 ml deionised water. Acetoacetic buffer was made up by mixing 15 g sodium acetate trihydrate with 3.6 ml glacial acetic acid, and this was then made up to 1 *ℓ with* deionised water. Acid alcohol was made up by combining 650 ml of 0.1N sulphuric acid and 350ml ethanol. Sulphuric acid (0.1N) was made up by combining 2.7 ml concentrated H_2SO_4 with 1 *ℓ of* deionised water. (Important note: Always add the acid to water, and not water to acid). Physiological saline was made up by mixing 8.5 g NaCl in 1 *l* of deionised water.

For individual exposure of eggs to extraction solutions, samples were set up, exposed, and processed as per wash solutions above. For the combination extractions, eggs were exposed exactly how a sample extraction would be done. The buffer solutions (formalin or acetoacetic buffer) were poured into test tubes first (7 ml) followed by 4 ml of the solvent (ethyl acetate, diethyl ether or acid-alcohol). The eggs were then spiked into the test tube by pipetting 1 ml of the egg suspension; tubes were plugged with rubber bungs and shaken for one minute to allow phase extraction to occur. The samples were then stood for the remainder of the exposure period and were processed as described above. The samples were immediately analysed under a light microscope and categorised as previously mentioned, then washed back into the test tubes, and analysed again after incubation.

2.1.4 Incubation solutions:

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. Eggs (1 ml aliquots of the stock solution) were pipetted into 15 ml Falcon tubes. The tubes were centrifuged at 1512 x g (3000 rpm) for 5 minutes, the supernatant discarded, and 2 ml of each incubation solution added to the respective tubes. Orange sticks were used to dislodge the pellets to allow eggs to be fully exposed to the incubation solution. The tubes were then incubated for 28 days at 25 - 27°C and analysed thereafter to determine egg development and viability. Eggs were categorised as previously described.

2.2 EXPERIMENT 2: THE EFFICACY OF DIFFERENT TYPES OF WASHING ON EGG RECOVERY (Naidoo and Archer, in prep 2022)

Different types of washing procedures were tested on spiked pig faeces i.e., washing the spiked samples under pressure and washing without pressure using a wash bottle. A slurry was made up by

blending pig faces with water, to facilitate efficient spiking and mixing of eggs as pig faeces alone is dry. Approximately 200 eggs were spiked into 10 g of pig slurry and samples were either washed using running tap water and a shower nozzle (for pressure) or using a spray bottle, onto a set of drum sieves (100 µm over 20 µm). The retentate on the 20 µm sieve was then collected into 15 ml Falcon tubes and centrifuged at 2500 and 3000 rpm for 5, 10 and 15 minutes.

Since we were testing the efficacy of the actual washing step, and to avoid confounding results, we could not perform flotations on the samples. After centrifugation, the supernatants of all samples were discarded (thus testing the efficacy of speeds and times at compacting the pellet), after which the samples were topped up with water to 5 ml. The pellet was dislodged, the sample homogenized and 1 ml was removed for immediate analysis. The egg recovery was then extrapolated for a 5 ml sample. This was to establish an estimated egg recovery without any possible egg loss during the flotation step.

The remaining 4 ml was stored in the cold room at 4°C for analysis at a later point, after various flotation solutions, respective SGs and centrifugation speeds and times, post flotation, had been tested. After determining the optimal flotation solution, SG, centrifugation speeds and times, the 4 ml samples were then floated using zinc sulphate, and subsequently taken through the entire method process, after which they were analysed for egg recovery. This figure was then added to the recovery from the 1 ml sample to establish total egg recovery.

2.3 EXPERIMENT 3: TESTING THE EFFICACY OF DIFFERENT FLOTATION SOLUTIONS, SPECIFIC GRAVITIES AND CENTRIFUGATION SPEEDS & TIMES ON EGG RECOVERY (Naidoo and Archer, in prep 2022)

Zinc sulphate, magnesium sulphate and sodium nitrate were tested for optimal egg recovery at SGs of 1.18, 1.2 and 1.3 each. Centrifugation speeds and times after the flotation step were also tested for egg recovery – 2000 and 2500 rpm for 5, 10 and 15 minutes. Pig slurry was made up as described above, 10 g samples were weighed out and approximately 200 eggs were spiked per sample. The sample was then washed under pressure using tap water over the set of drum sieves (100 over 20 µm). The retentate on the 20 µm sieve was then collected into 15 ml Falcon tubes and centrifuged at 3000 rpm for 10 minutes (the optimum type of washing and centrifugation speed and time was selected based on the previous experiment). The supernatant was discarded, and orange sticks were used to dislodge the pellet whilst simultaneously pipetting 3 ml of the tested flotation solution into the tube and vortexing to ensure homogenisation. The sample was taken up to 14 ml with the flotation solution and then centrifuged. The supernatant was poured onto a 20 µm sieve and rinsed thoroughly with tap water. It was then pipetted back into 15 ml Falcon tubes and centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded, and the sample analysed using light microscopy for egg recovery.

2.4 EXPERIMENT 4: TESTING THE EFFICACY OF DIFFERENT WASH SOLUTIONS ON EGG RECOVERY FROM DIFFERENT SAMPLE TYPES (Naidoo and Archer, in prep 2022)

Various sample types were tested against different wash solutions to determine which solution resulted in the best egg recovery. Sample types included water, effluent, VIP sludge, UDDT sludge, dried sludge (soaked for both 4 and 24 hours), fatty sludge, septic tank sludge and soil. Selection of wash solutions was based on the data from Experiment 1 on egg viability combined with what is commonly used in laboratories according to literature. We therefore tested ammonium bicarbonate (used in the PRG and WRDC Helminth Methods), 7X (used in the US EPA Method) and water as a control. Samples were processed in full according to the WRDC Helminth Method in quintuplicate.

Each sludge type was weighed out into a beaker according to the consistency and approximate moisture content: water – 1 *ℓ effluent* – *1 ℓ* VIP sludge – 10 g, UDDT sludge – 10 g, dried sludge – 5 g, fatty sludge – 10 g, septic tank sludge – 250 ml and soil – 50 g. Approximately 50 ml of each test wash solution was added to the 10 and 50 g, and 250 ml samples. For the 1 ℓ measured samples, the wash solution was made up directly into the sample – 1 ml of 7X and 119 g of ammonium bicarbonate were added directly to the water and effluent. The sample was thoroughly homogenised with the wash solution and left to stand for 10 minutes. This was then washed over a set of drum sieves (100 over 20 µm) under pressure using tap water. The retentate on the 20 µm sieve was collected into as many 15 ml Falcon tubes as required, depending on sample type. The tubes were centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded, and the deposits floated with zinc sulphate (SG = 1.3) as mentioned above and centrifuged for 2000 rpm for 15 minutes. The supernatant was then poured onto a 100 mm 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 minutes. The final pellet was then analysed under a light microscope to determine egg recovery. The final pellets of the fatty sludge samples were quite large, and required an extraction step, but eggs were being loss in the process. The fatty sludge samples were then re-run a few times until we were able to find an effective way of counting the final pellet. Eventually, the pellet was taken up to 1 ml with water in the test tube and homogenised, half the drops were counted, and the total number of eggs was doubled to calculate total egg recovery.

2.5 EXPERIMENT 5: TESTING THE EFFICACY OF DIFFERENT EXTRACTION SOLUTIONS ON EGG RECOVERY FROM DIFFERENT SAMPLE TYPES (Naidoo and Archer, in prep 2022)

Preliminary testing and results of the previous experiment showed that the extraction step caused loss of eggs. We therefore tested the efficacy of all extraction combinations for egg recovery in water, primary sludge (collected from a wastewater treatment plant), and fatty sludge (as this was the most difficult sludge type to handle and resulted in a large pellet that required further processing). The best wash solutions were selected based on data from the previous experiment, and samples were

processed as mentioned above. After the final centrifugation step, all the supernatant was discarded, and the extraction solutions were added in combination using 7 ml of the buffer + 4 ml of solvent: 10% formalin + ethyl acetate, 10% formalin + diethyl ether, acetoacetic buffer + ethyl acetate, acetoacetic buffer + diethyl ether, and acid-alcohol + ethyl acetate. The tubes were thoroughly and vigorously shaken for 1 minute, ensuring that the pellets were completely dislodged. A disc of organic material would appear between the buffer and solvent layers. The samples were then centrifuged at 3000 rpm for 10 minutes. The supernatant would normally be discarded, but instead it was poured onto a 20 µm sieve and rinsed thoroughly to remove all chemical. All the retentate, that included the disc between the two chemical layers, was collected back into the test tube and centrifuged at 3000 rpm for 10 minutes. The pellet was immediately analysed for egg recovery using a light microscope. The supernatant portion was also analysed using light microscopy for egg recovery, and the sum of both analyses gave the total egg recovery after processing the samples using the complete method.

At this point, samples were fully processed according to the new improved WRDC Helminth Method, thus attesting to the recoverability of the procedure for helminth eggs in sludge samples (whether naturally occurring or spiked).

2.6 EXPERIMENT 6: TESTING THE EFFICACY OF DIFFERENT STAINING PROTOCOLS

A series of experiments were conducted on various stains to establish a staining technique for helminth eggs, particularly for spiking experiments, to differentiate between naturally occurring helminth eggs in the sludge, and those that had been spiked. Crystal violet, trypan blue, methyl violet and safranin-o were tested at different concentrations, for various exposure times, based on literature. These tests were done as preliminary runs, to determine an initial protocol to work with and develop further. Eggs were exposed to the different stains in test tubes and looked at directly with the stain still present. They were subsequently washed to remove the stain and then analysed again using light microscopy. The best performing stains, respective concentrations and exposure times were selected, and eggs were stained, washed, and spiked into UDDT sludge samples, to determine if the stains could withstand the processing method without fading.

2.7 EXPERIMENT 7: TESTING THE REPRODUCIBILITY AND REPEATABILITY OF THE METHOD VIA INTRA-LABORATORY TESTING (BETWEEN DIFFERENT TECHNICIANS)

A final standard operating procedure (SOP) was written up, taking into consideration data from every experiment run and selecting the best options with the greatest egg survival and recovery (see attached). The method then needed to be run by various technicians, preferably of different levels of expertise and backgrounds. This experiment was repeated several times, due to egg stocks having been damaged during the lockdown periods. Initially, the entire process was run on effluent, VIP sludge, UDDT sludge and fatty sludge in quintuplicate. Egg recovery was poor, and we had to troubleshoot to

determine where eggs were being lost. It was determined that the sieves were possibly damaged and so the entire process had to be repeated. Egg recovery was again low, and the experiment was repeated a few times with different technicians, each time with us troubleshooting at every step and attempting to optimise the method.

3. DATA COLLATION & RESULTS

This chapter presents all data collected from the seven experiments described above.

3.1 EXPERIMENT 1: EFFECTS OF ALL REAGENTS ON EGG VIABILITY (Naidoo and Archer, in prep 2022)

3.1.1 Wash solutions

Figures 3 to 7 present the potential viability and actual viability of samples exposed to all eight wash solutions at different time intervals from 10 minutes to 24 hours.

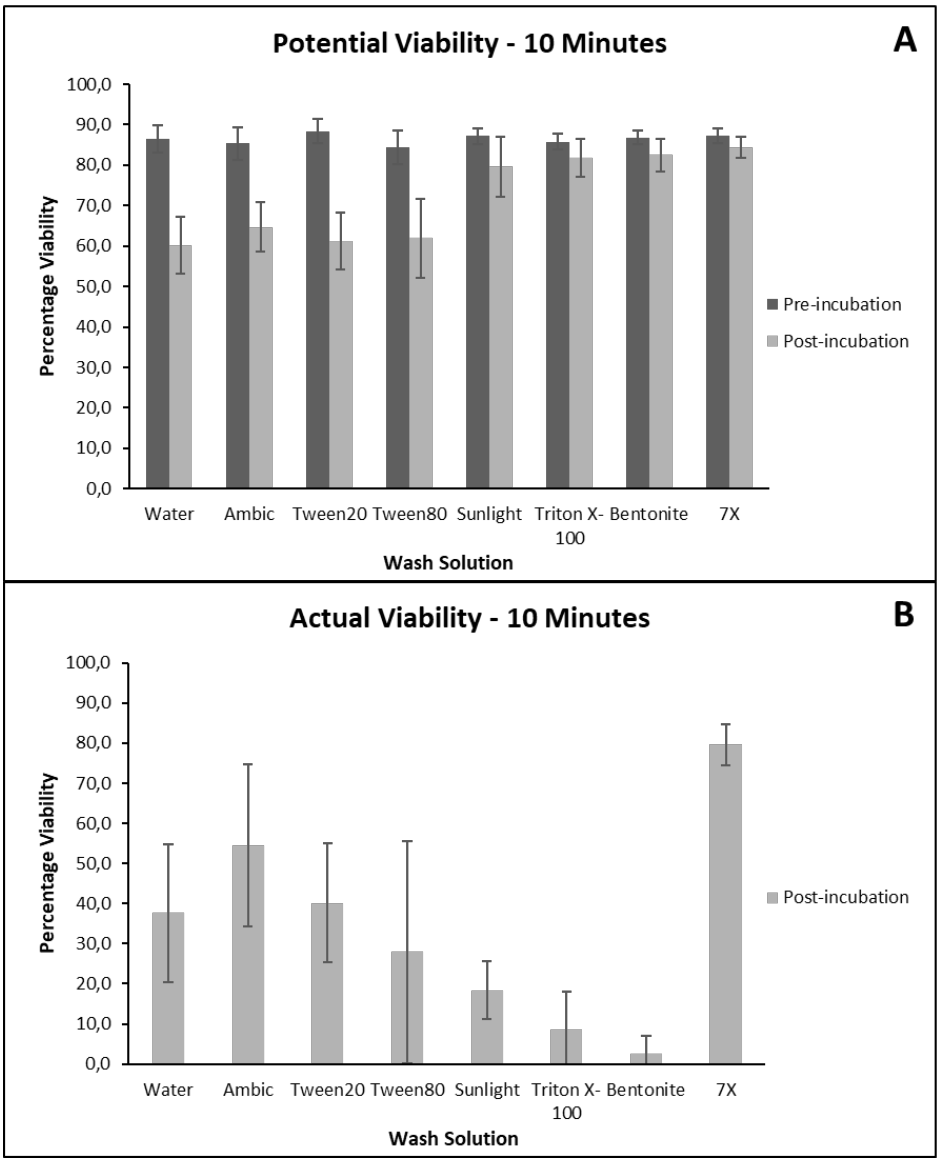

Figure 3: Potential viability (A) and actual viability (B) of samples exposed to all eight wash solutions for 10 minutes, pre- and post-incubation (N = 5). N.B. Actual viability includes only those eggs that developed a healthy larva inside.

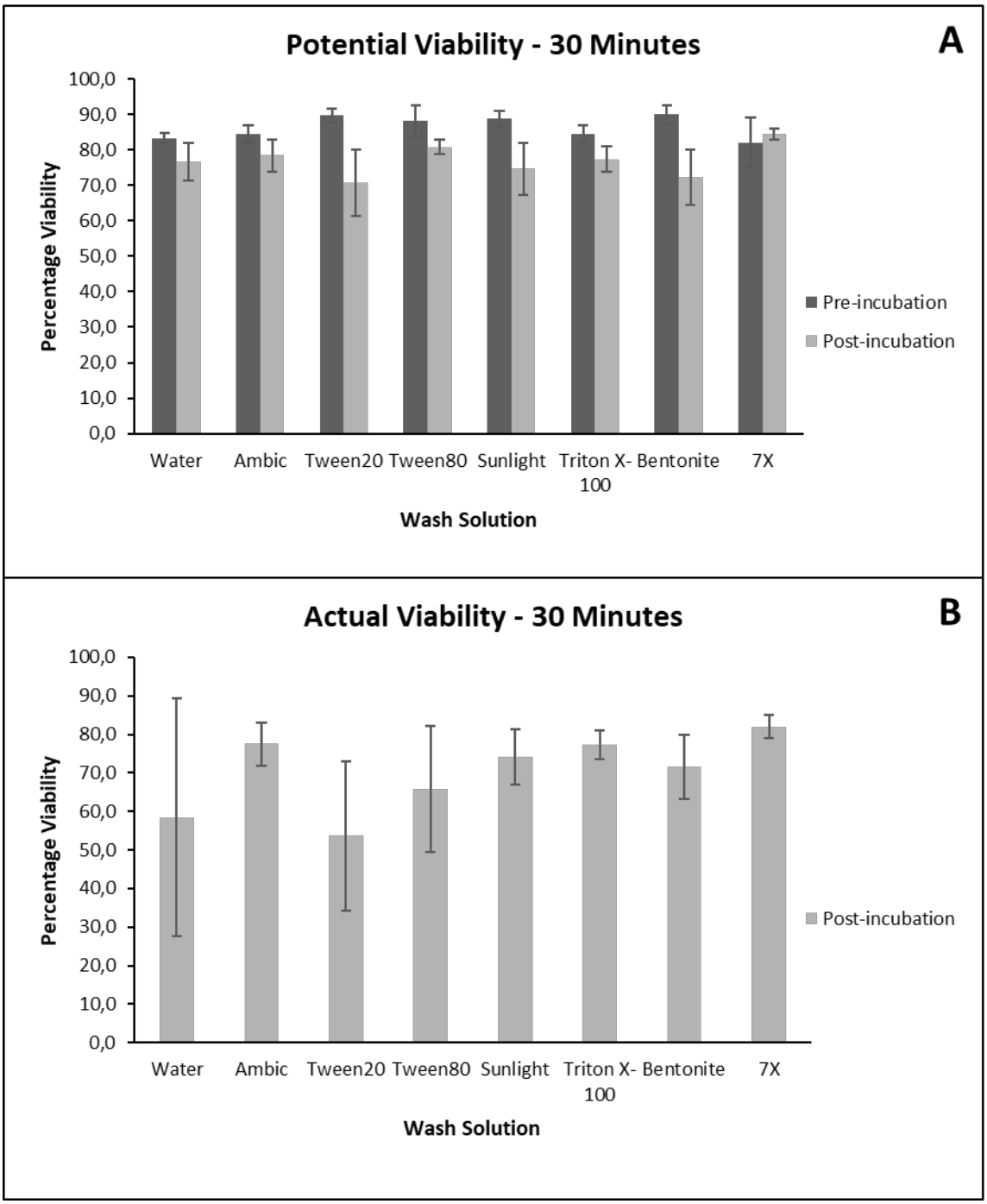

Figure 4: Potential viability (A) and actual viability (B) of samples exposed to all eight wash solutions for 30 minutes, pre- and post-incubation (N = 5). N.B. Actual viability includes only those eggs that developed a healthy larva inside.

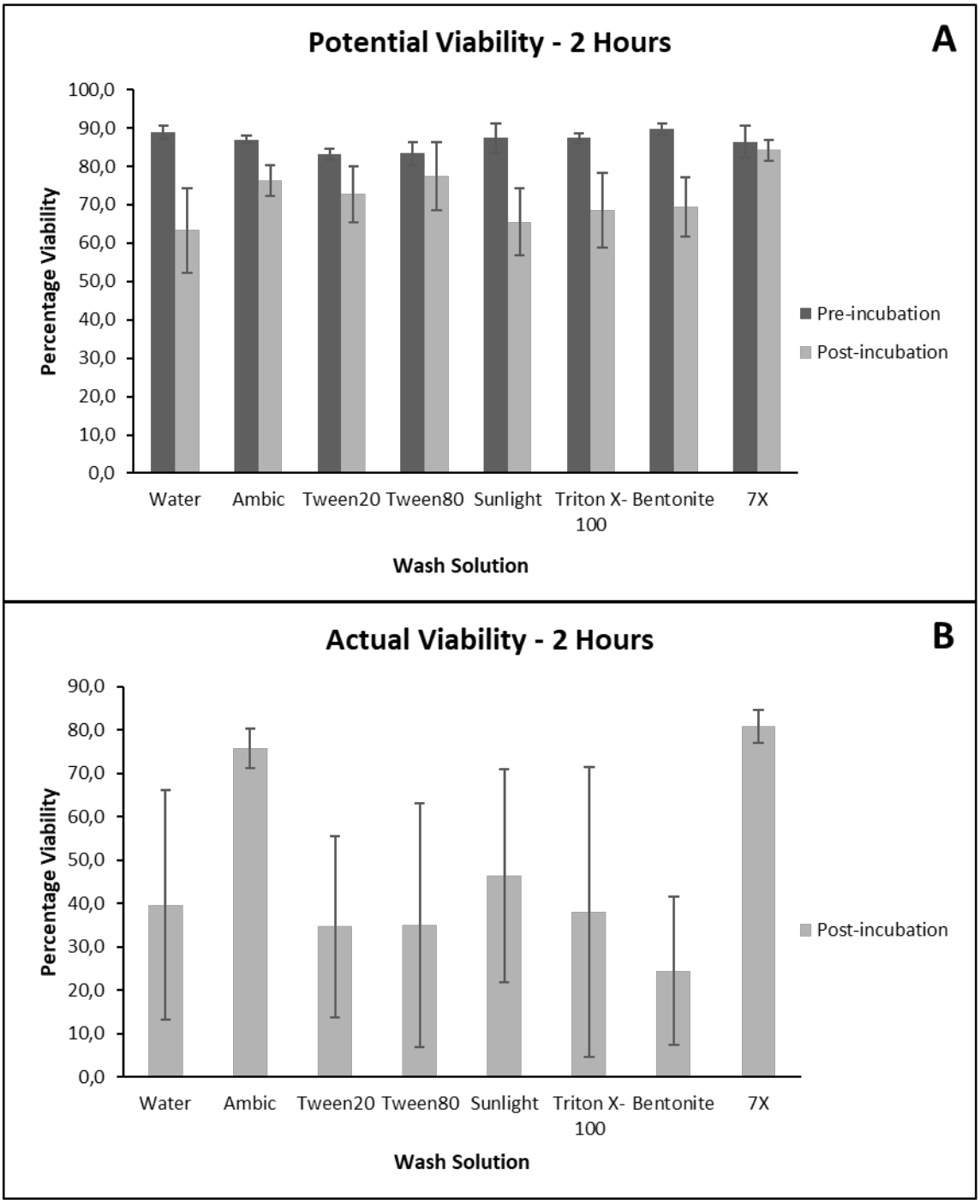

Figure 5: Potential viability (A) and actual viability (B) of samples exposed to all eight wash solutions for 2 hours, pre- and post-incubation (N = 5). N.B. Actual viability includes only those eggs that developed a healthy larva inside.

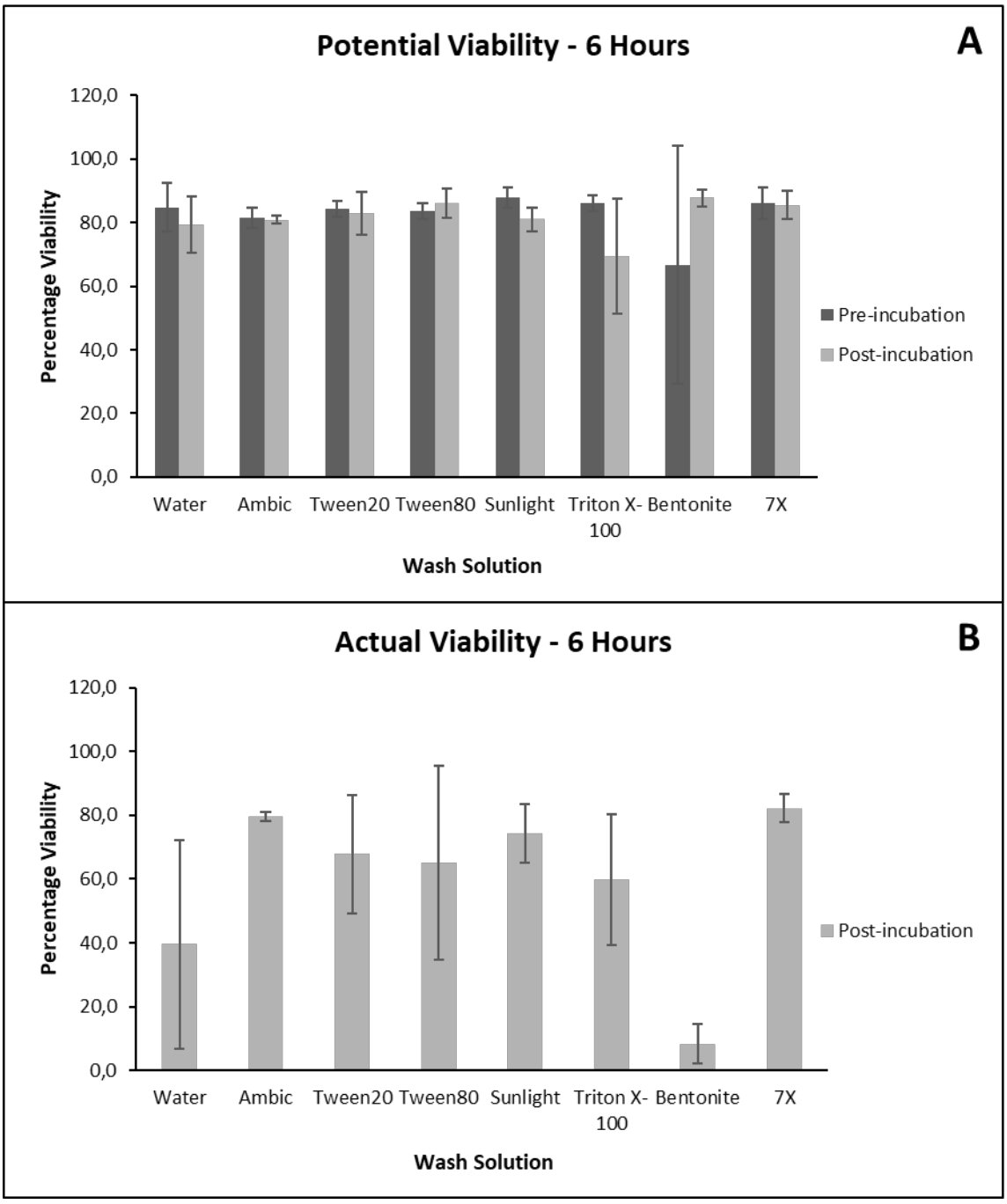

Figure 6: Potential viability (A) and actual viability (B) of samples exposed to all eight wash solutions for 6 hours, pre- and post-incubation (N = 5). N.B. Actual viability includes only those eggs that developed a healthy larva inside.

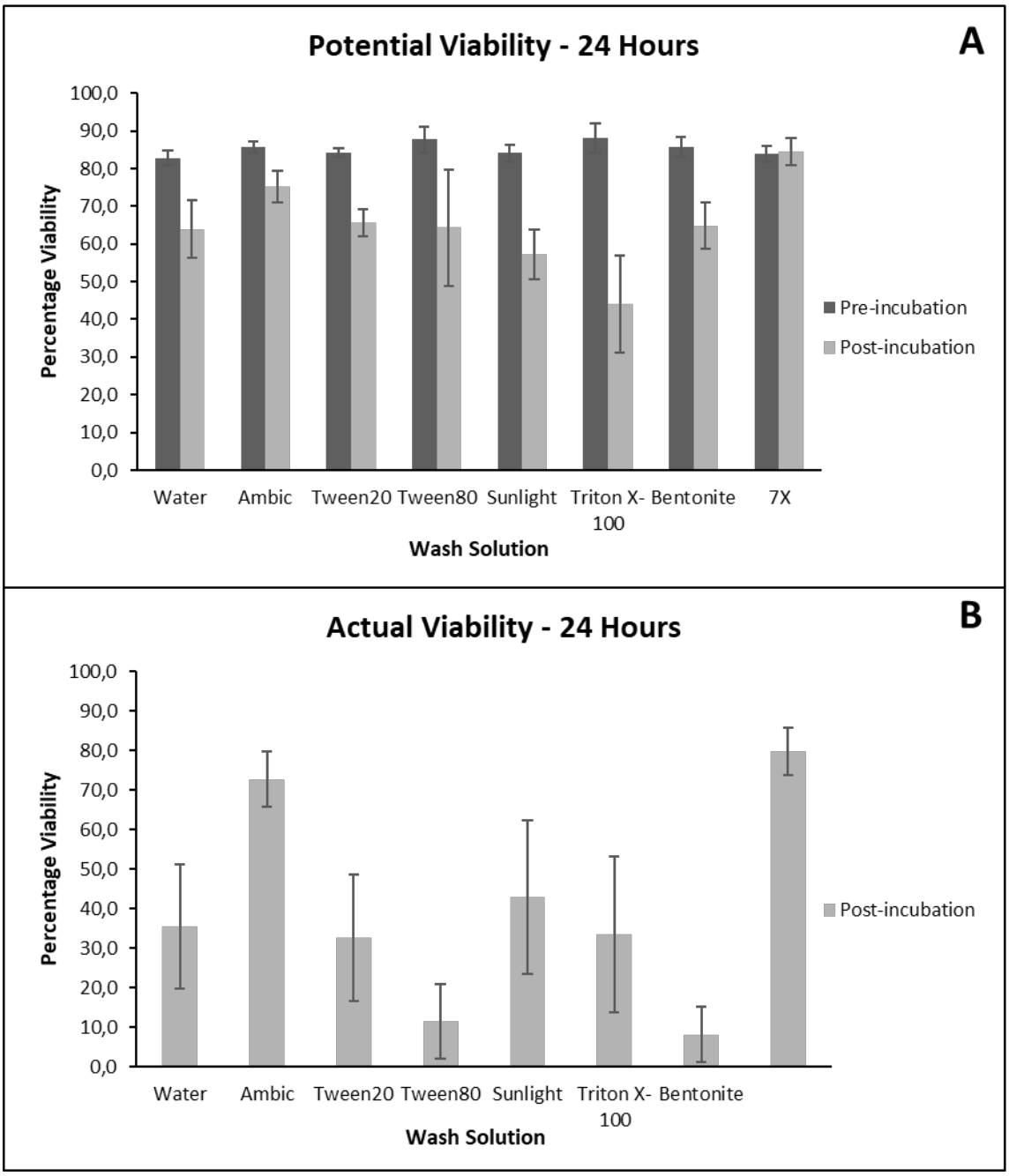

Figure 7: Potential viability (A) and actual viability (B) of samples exposed to all eight wash solutions for 24 hours, pre- and post-incubation (N = 5). N.B. Actual viability includes only those eggs that developed a healthy larva inside.

3.1.2 Observations and conclusions

10 minutes

When looking at potential viability, physical damage was not evident, thus pre-incubation viability was high across all solutions. Post-incubation, bentonite, Triton X-100, Sunlight Liquid and 7X had the least effect on egg viability. When looking at actual viability however, ammonium bicarbonate and 7X showed the highest larval development. This indicates that either arrested or slower development occurred for bentonite, Triton X-100, and Sunlight Liquid.

30 minutes

In terms of potential viability, all solutions showed viability above 70%, with 7X, ammonium bicarbonate, Tween 80 and water allowing for the highest viability. Actual viability figures support these findings, with 7X and ammonium bicarbonate allowing for the highest larval development. Triton X-100 and Sunlight Liquid were also successful.

Two hours

Potential viability patterns were similar to the previous exposure times, across all wash solutions. Numbers were higher pre-incubation as compared with post-incubation, again indicating that damage was not necessarily visible after chemical exposure. The actual viability figures, however, differed greatly, with 7X and ammonium bicarbonate demonstrating the highest larval development, followed by Sunlight Liquid and Triton X-100. For longer soaking of samples, 7X and ammonium bicarbonate are the most successful for development.

Six hours

Potential viability was similar to the above exposure times across all wash solutions, and pre-incubation values were higher than post-incubation. Actual viability indicated that 7X and ammonium bicarbonate allowed for the highest larval development once again, followed by Sunlight Liquid, Tween 80 and Triton X-100. Bentonite showed very poor larval development.

24 hours

Potential viability was once again high across all wash solutions pre-incubation, but there was a greater decline in overall potential viability post-incubation. Actual viability showed that both 7X and ammonium bicarbonate were the most favourable for larval development when samples need to be soaked overnight, followed by Sunlight Liquid and Triton X-100. Bentonite and Tween 80 were the least successful.

Overall, ammonium bicarbonate and 7X performed the best across all exposure times for larval development, indicating the lowest impact on egg viability after processing and incubation. For both ammonium bicarbonate and 7X, samples were clear to analyse under the microscope before incubation. It should be noted that bacterial and fungal contamination was observed in samples after incubation, since the initial pig faeces was not processed with any chemicals.

Fungi and bacteria would therefore not have been destroyed during initial processing and egg recovery. Egg morphology varied across contaminated samples, where some samples were less affected by the contamination and resulted in good larval development, whilst others looked very old and damaged. Although fungal contamination was observed across the board (all wash solutions and exposure times), it was evident that to avoid severe microbial contamination and resultant egg death, a surfactant or chemical is required for processing of samples where incubation will follow. The contamination persisted particularly in samples that were exposed to non-soapy solutions, such as water and bentonite (a clay

solution), accounting for poor larval development and very damaged eggs. Studies have shown that certain strains of fungi can affect egg viability due to ovicidal properties.

Data indicated that ammonium bicarbonate and 7X can be used for processing various sludge types, including those that require prolonged soaking periods prior to processing. Samples exposed to 7X allowed for the best larval development. Triton X-100 and Sunlight liquid also produced clear samples for microscopy and larval development was good up until 6 hours. The 24-hour exposure however, showed a decline in larval development, suggesting a direct chemical impact on egg viability by these two solutions.

3.1.3 Flotation solutions

Figures 8 to 10 present the potential viability and actual viability of samples exposed to all five flotation solutions at different time intervals from 15 minutes to 2 hours.

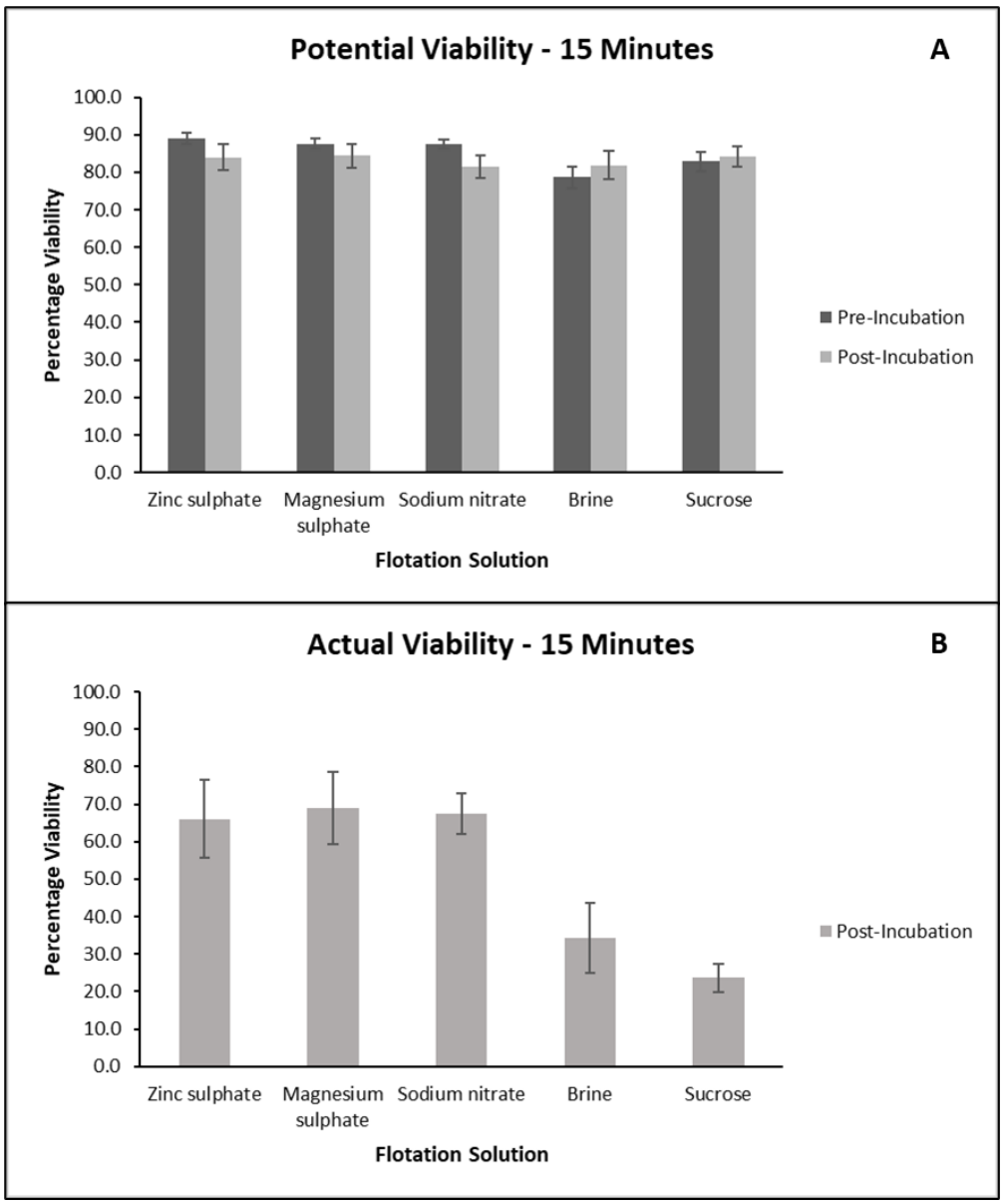

Figure 6: Potential viability (A) and actual viability (B) of samples exposed to all five flotation solutions for 15 minutes, pre- and post-incubation (N = 5). N.B. Actual viability includes only those eggs that developed a healthy larva inside.

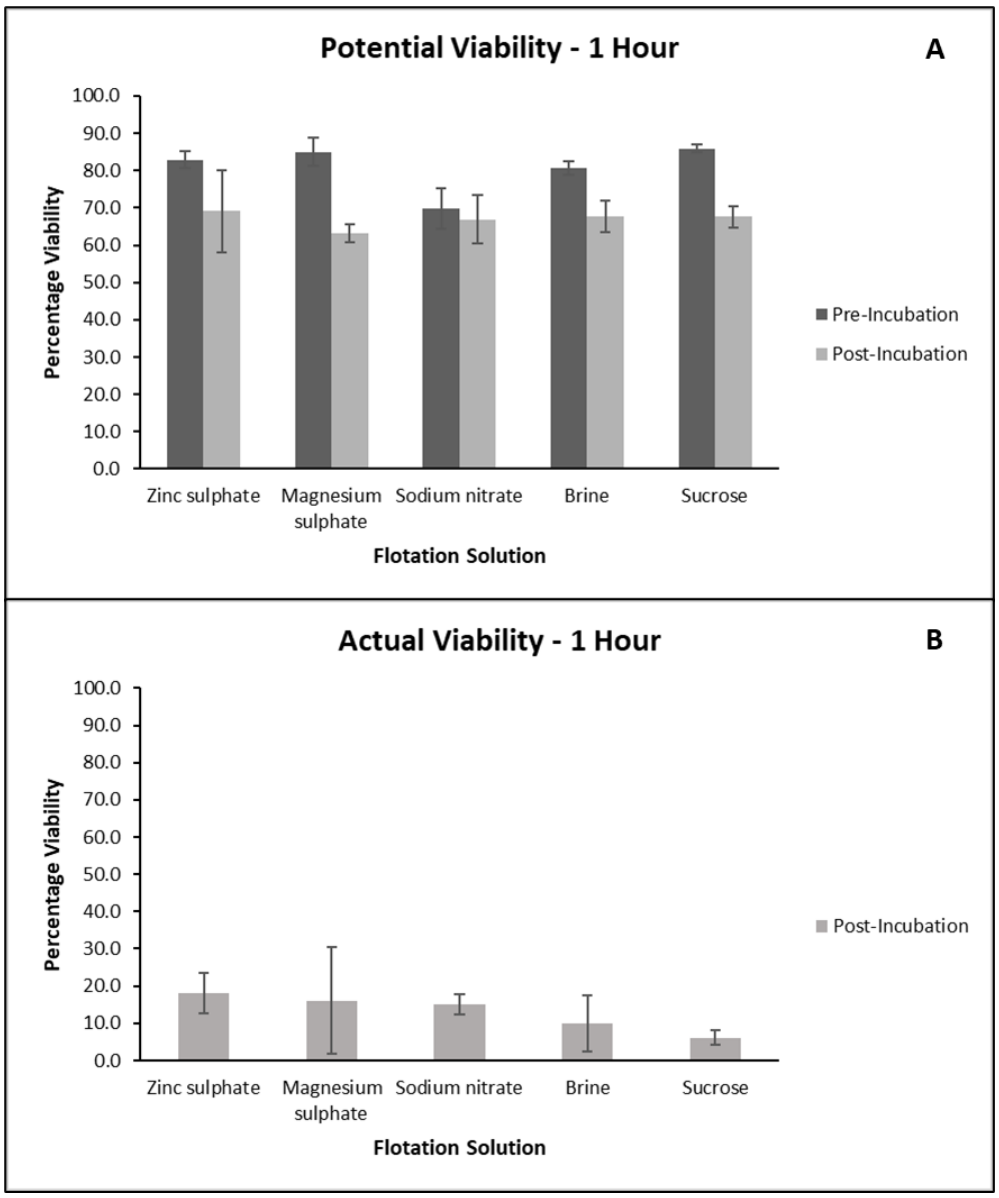

Figure 7. Potential viability (A) and actual viability (B) of samples exposed to all five flotation solutions for 1 hour, pre- and post-incubation (N = 5). N.B. Actual viability includes only those eggs that developed a healthy larva inside.

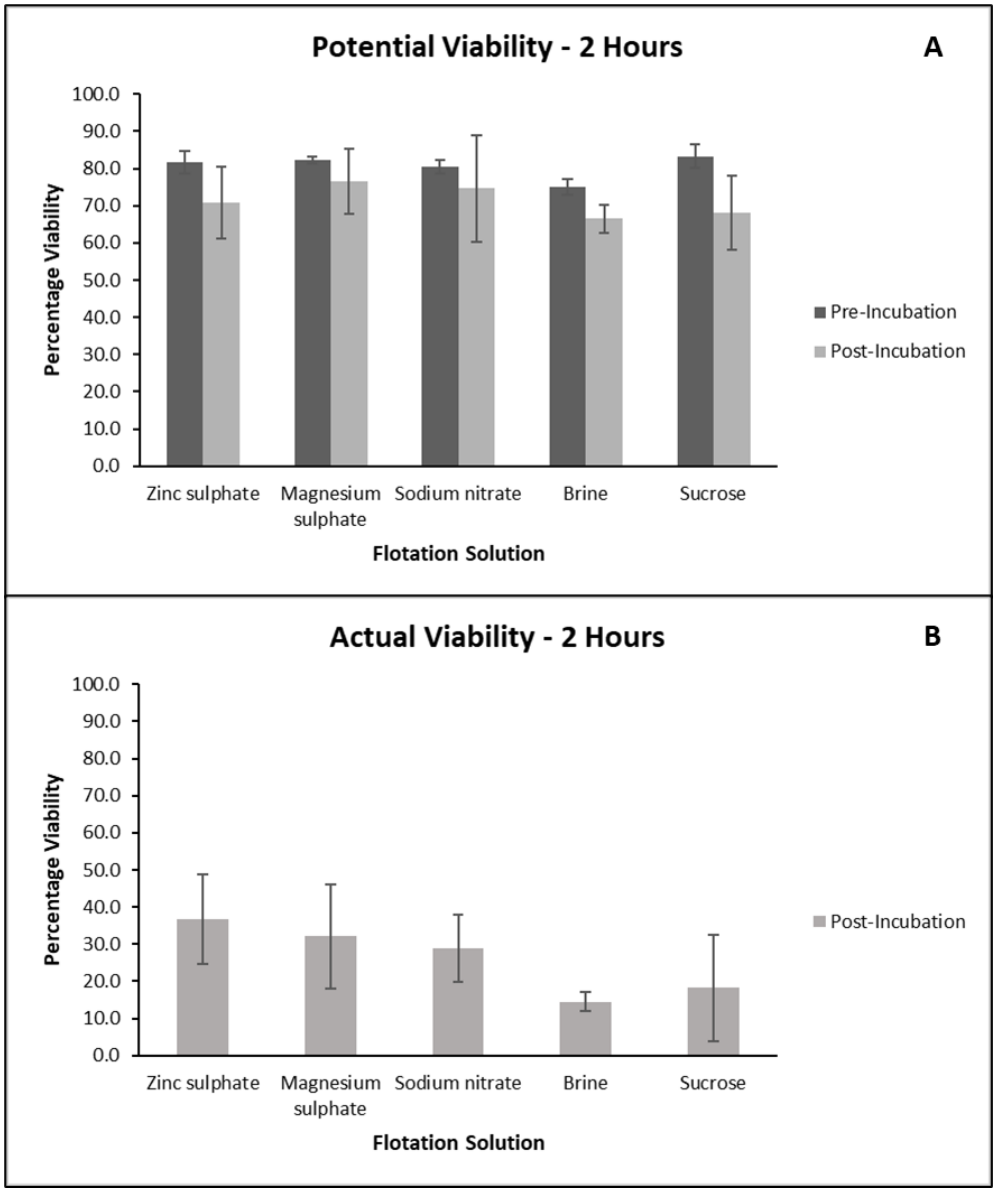

Figure 8. Potential viability (A) and actual viability (B) of samples exposed to all five flotation solutions for 2 hours, pre- and post-incubation (N = 5). N.B. Actual viability includes only those eggs that developed a healthy larva inside.

3.1.4 Observations and conclusions

15 minutes:

When looking at potential viability, there was no significant difference seen between the flotation solutions, both pre- and post-incubation. Actual viability however, showed lower larval development in both brine and sucrose, indicating that these solutions are not ideal for egg recovery.

One hour:

In terms of potential viability, pre-incubation data showed high viability percentages (±80%) and post-incubation data showed a drop in egg viability (approximately 20% lower), indicating a low effect of exposure time of eggs to flotation solutions on egg viability and the ability to develop. Actual viability figures were much lower, indicating that exposure time hinders larval development of eggs. Again, larval development was the lowest for brine and sucrose. \

Two hours:

A similar viability pattern was seen at 1 and 2 hours of exposure to all flotation solutions, across the board, both pre- and post-incubation, with similar development between each flotation solution. After incubation, a reduction in egg viability was seen, indicating an effect of exposure time. Actual viability again showed lower larval development, with brine and sucrose being the lowest.

Overall, it is recommended that eggs should be exposed to flotation solutions for the least amount of time possible to prevent egg damage and decreased larval development. Zinc sulphate and magnesium sulphate appeared to be the best solutions, overall, for flotation. It was however noted that magnesium sulphate tended to sediment out of solution and form crystals at the bottom of the bottle, thus zinc sulphate is the recommended flotation solution.

3.1.5 Extraction solutions (individual exposures)

Figures 11 to 13 present the potential viability and actual viability of samples exposed to all five individual extraction solutions for different exposure times from 5 minutes to 1 hour.

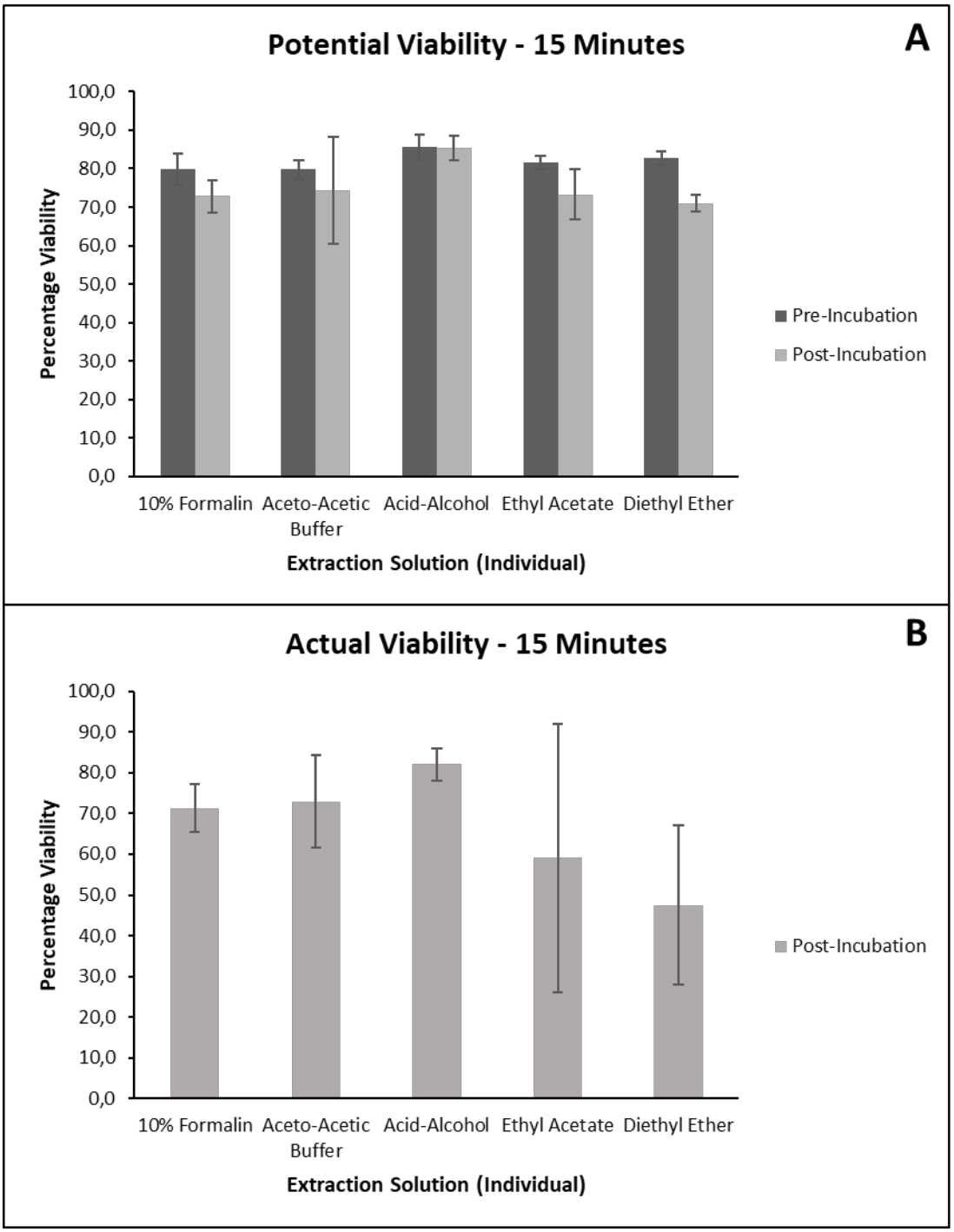

Figure 11: Potential viability (A) and actual viability (B) of samples exposed to all five extraction solutions (individual extraction solutions) for 15 minutes, pre- and post-incubation (N = 5). N.B. Actual viability includes only those eggs that developed a healthy larva inside.

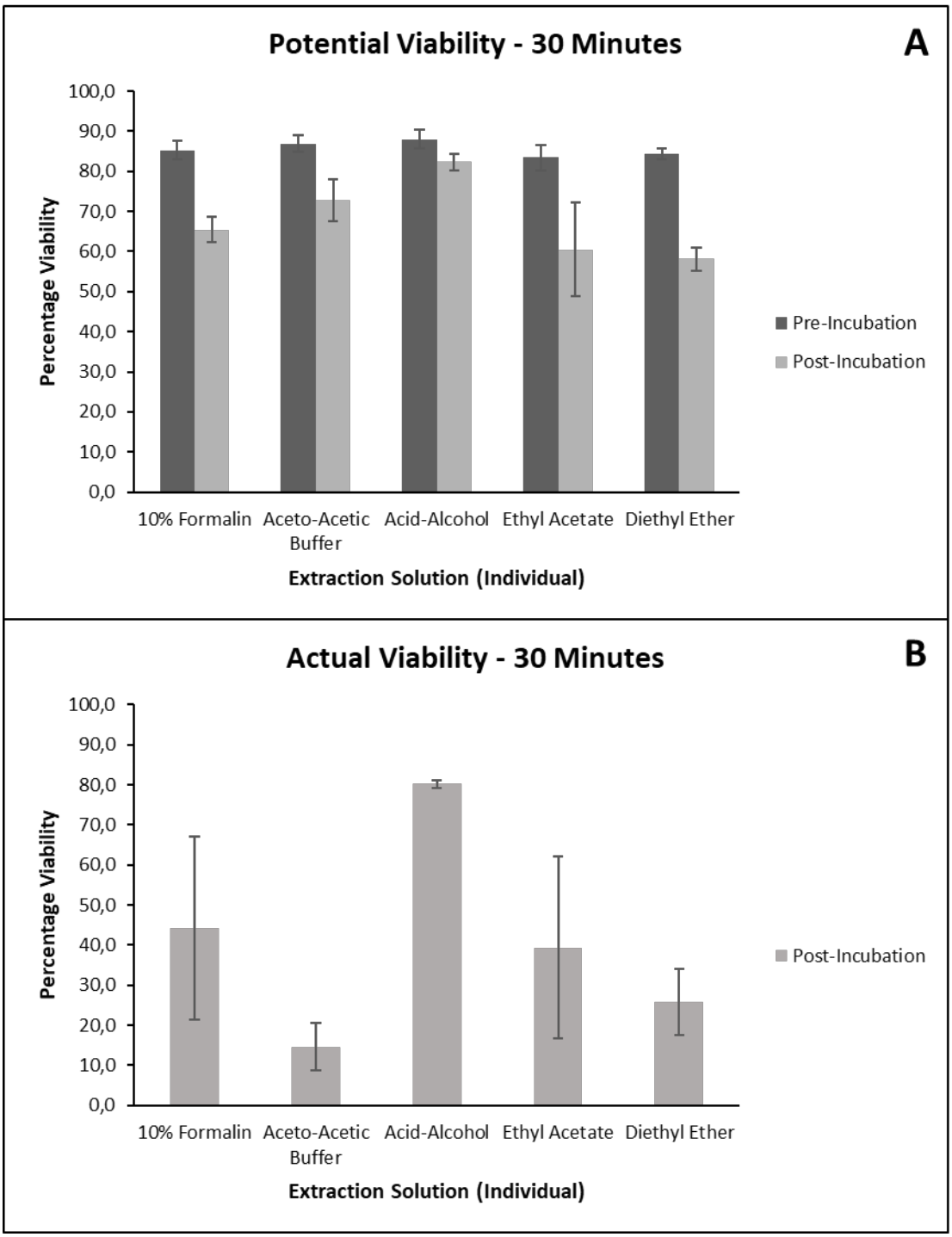

Figure 9: Potential viability (A) and actual viability (B) of samples exposed to all five extraction solutions (individual extraction solutions) for 30 minutes, pre- and post-incubation (N = 5). N.B. Actual viability includes only those eggs that developed a healthy larva inside.

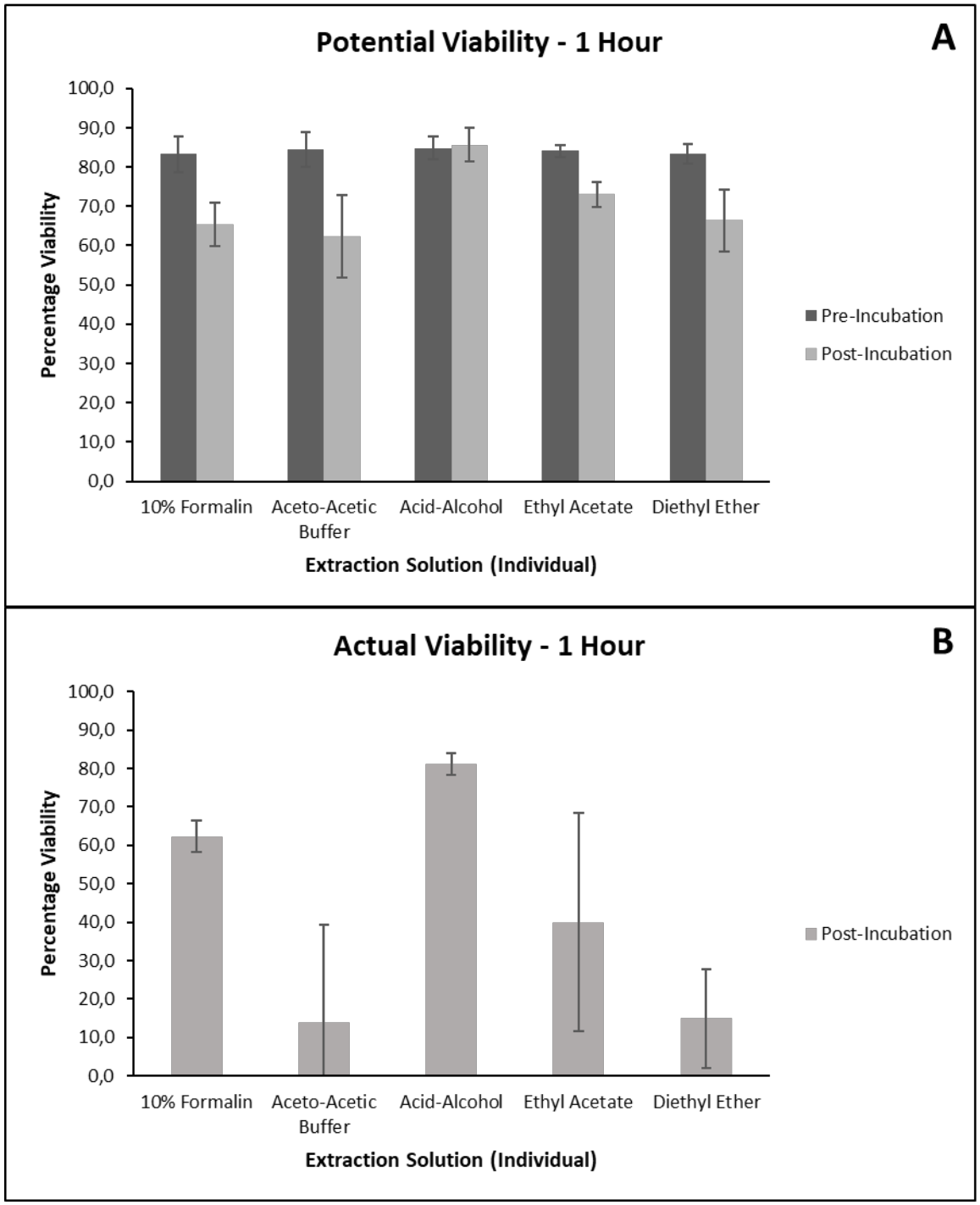

Figure 10: Potential viability (A) and actual viability (B) of samples exposed to all five extraction solutions (individual extraction solutions) for 1 hour, pre- and post-incubation (N = 5). N.B. Actual viability includes only those eggs that developed a healthy larva inside.

3.1.6 Observations and conclusions: extraction solutions (individual exposures)

15 minutes

Potential viability was approximately 80% across all extraction solutions pre-incubation, and post-incubation showed approximately 70% viability, with acid-alcohol performing the best. There was no significant difference in egg viability between the other four solutions. Actual viability showed a

reduction in larval development when eggs were exposed to the solvents (ethyl acetate and diethyl ether).

30 minutes

Potential viability showed that egg viability was similar across all extraction solutions, pre-incubation with acid-alcohol performing the best again. The solvents indicated lower viability post-incubation. Actual viability indicated lower larval development, except in the case of acid-alcohol, showing that exposure time influenced egg development. Acetoacetic buffer and diethyl ether resulted in the lowest larval development.

One hour

Pre-incubation figures under potential viability were similar to the 30-minute exposure figures seen above. Acetoacetic buffer resulted in the lowest egg viability post-incubation, and together with diethyl ether, again showed low larval development. Larval development was better in samples exposed to acid-alcohol, 10% formalin and ethyl acetate. Extensive contamination and hatching of fully developed eggs were noted across all acid-alcohol exposures, making this extraction solution not ideal.

3.1.7 Extraction solutions (combination exposures)

Figures 14 to 16 present the potential viability and actual viability of samples exposed to all five extraction solutions (in combination) for different exposure times from 5 minutes to 1 hour.

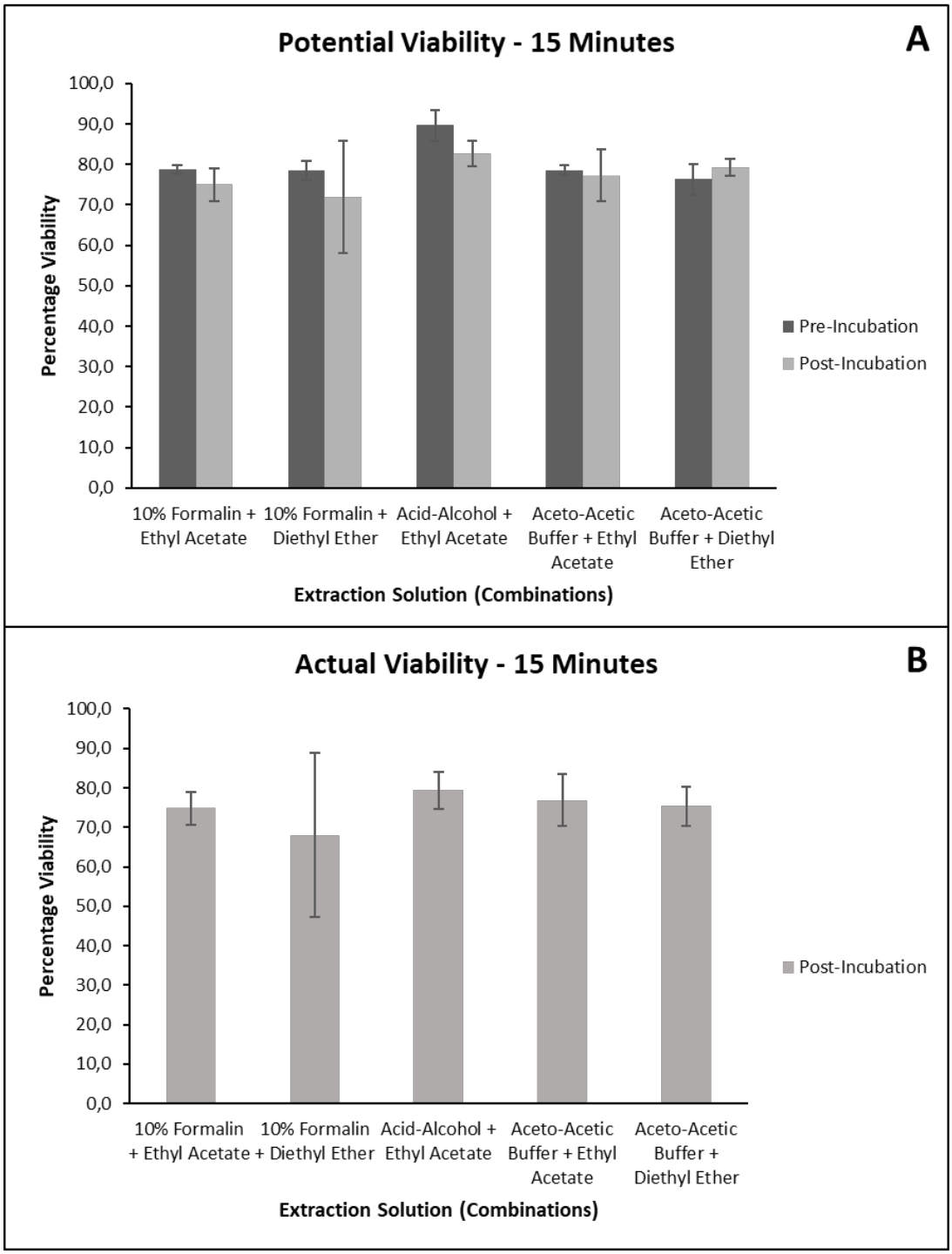

Figure 11: Potential viability (A) and actual viability (B) of samples exposed to all five extraction solutions (in combination) for 15 minutes, pre- and post-incubation (N = 5). N.B. Actual viability includes only those eggs that developed a healthy larva inside

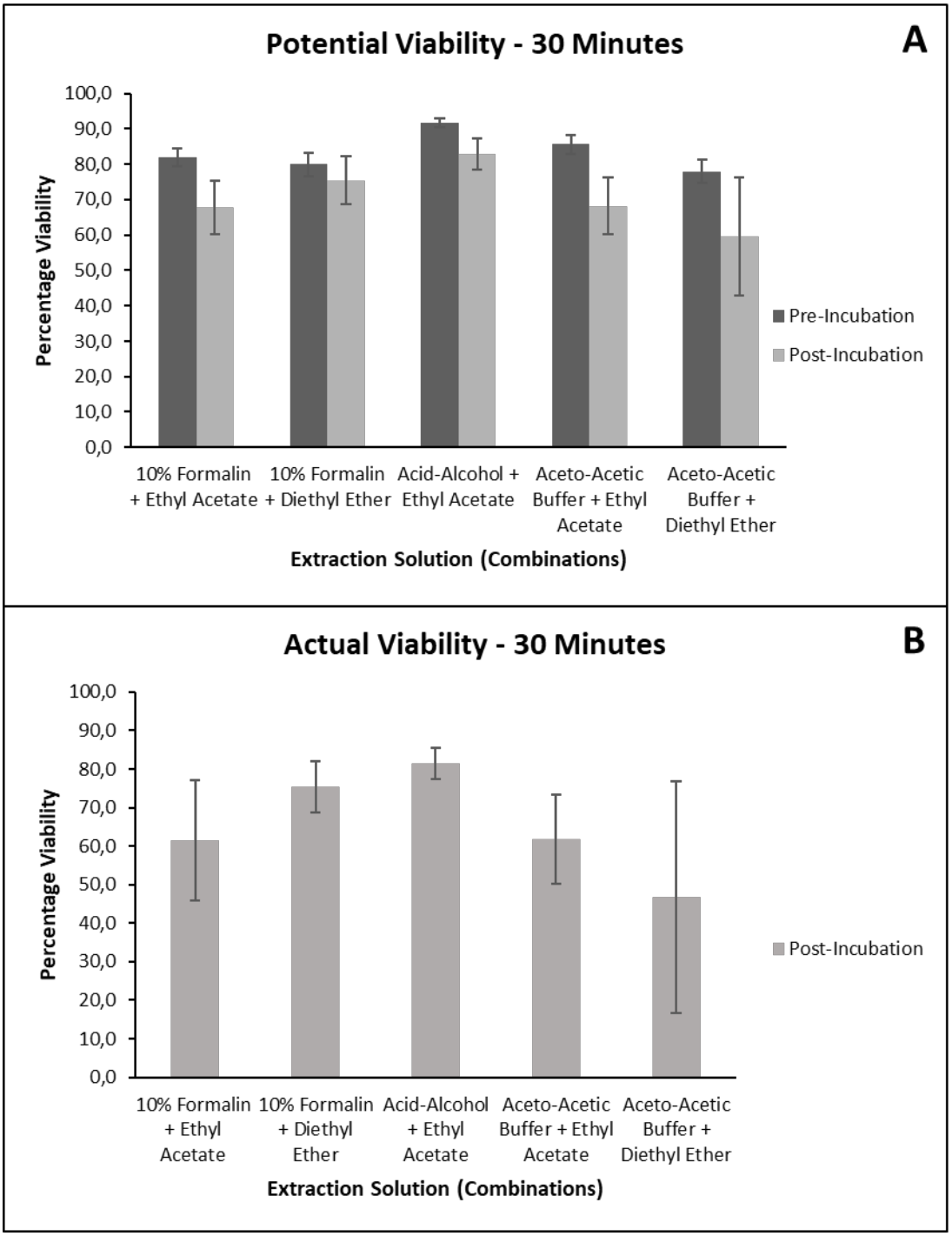

Figure 12: Potential viability (A) and actual viability (B) of samples exposed to all five extraction solutions (in combination) for 30 minutes, pre- and post-incubation (N = 5). N.B. Actual viability includes only those eggs that developed a healthy larva inside.

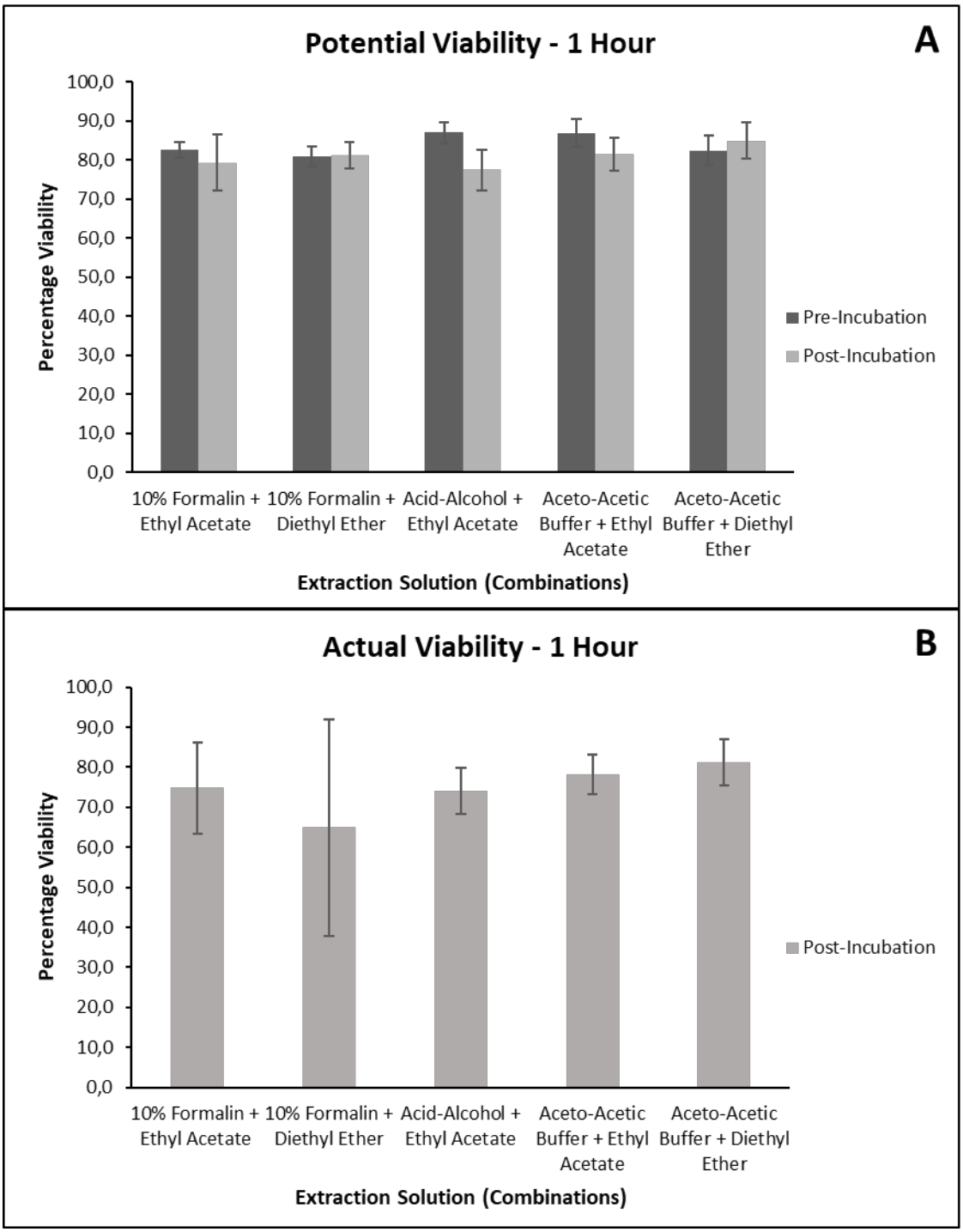

Figure 13: Potential viability (A) and actual viability (B) of samples exposed to all five extraction solutions (in combination) for 1 hour, pre- and post-incubation (N = 5). N.B. Actual viability includes only those eggs that developed a healthy larva inside.

3.1.8 Observations and conclusions: extraction solutions (combination exposures)

15 minutes

When examining potential viability, similar viability patterns were seen across the extraction combinations – approximately 80% viability. High larval development was also seen across the combinations at 15 minutes of exposure, indicating that this step could be included when necessary for enumerating helminth eggs.

30 minutes

All extraction combinations showed high egg viability pre-incubation, with ± 20% reduction in egg viability post-incubation. A reduction in larval development was seen when looking at the actual viability figures, with acetoacetic buffer and diethyl ether allowing for the lowest development. This indicates that prolonged exposure to these solutions could result in egg damage. The acid-alcohol and ethyl acetate combination, however, resulted in excellent larval development.

One hour

One hour of exposure indicated some contradictory results when compared with 30 minutes of exposure to the extraction combinations. High viability was seen both pre- and post-incubation across all combinations. An approximate 20% reduction in egg viability was seen when looking at actual viability, but larval development was relatively high (65% and above). Once again, larval development was good in the acid-alcohol combinations. This again indicated that eggs could withstand the extraction step and exposure to these chemicals, when necessary, for successful recovery of eggs from sanitation samples.

3.1.9 Incubation solutions

Figure 14 presents the potential viability and actual viability of samples exposed to all 6 incubation solutions.

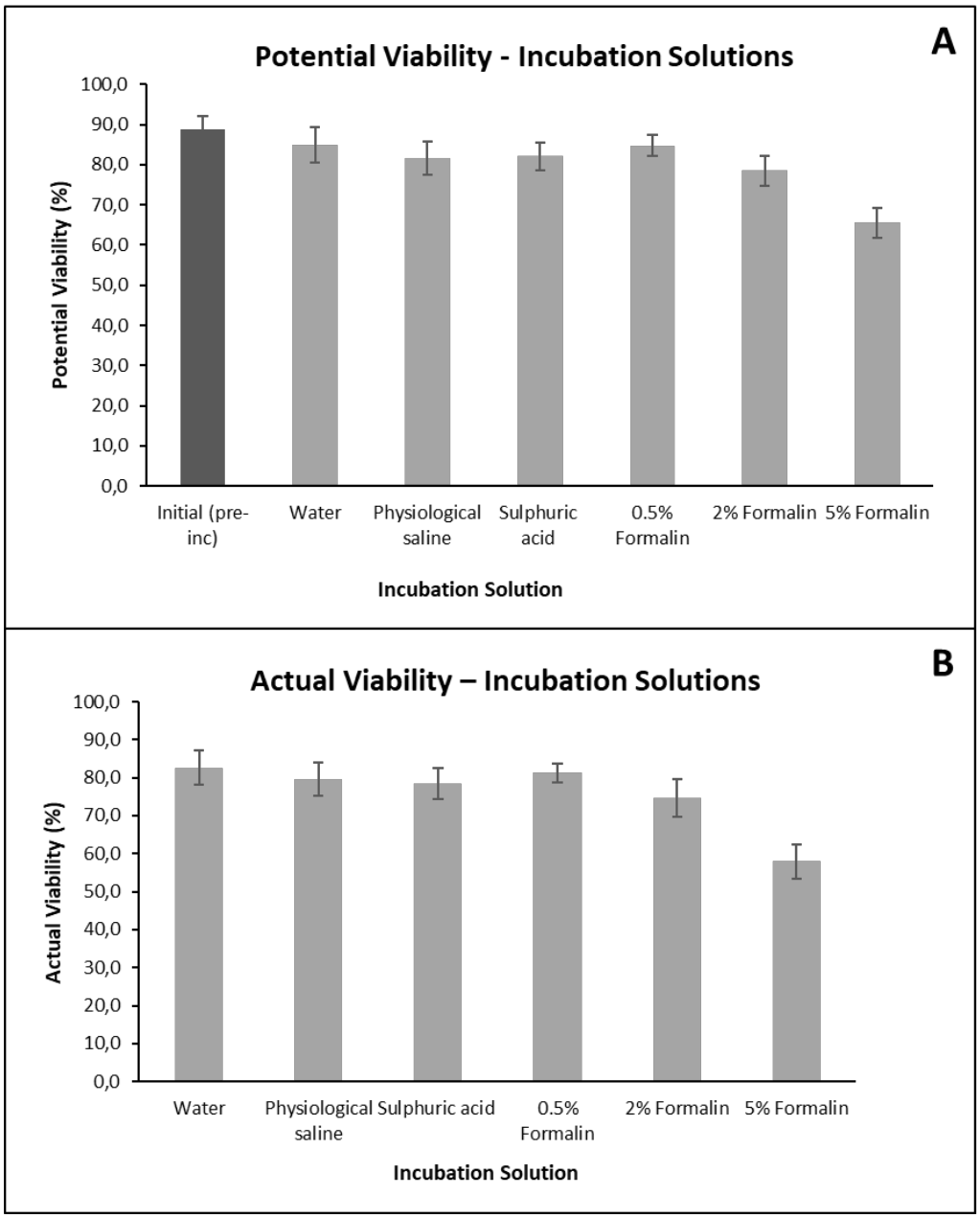

Figure 14: Potential viability (A) and actual viability (B) of samples exposed to all 6 incubation solutions, incubated for 28 days at 25°- 27°C (N = 5) Only the initial sample was analysed pre-incubation in Figure 4A (dark grey bar). N.B. Actual viability includes only those eggs that developed a healthy larva inside.

3.1.10 Observations and conclusions

When looking at actual development, all incubation solutions, except for 5% formalin, produced similar potential viability figures of approximately 80%. Potential viability for 5% formalin, however, dropped down to approximately 60%. This indicated that 5% is too concentrated and harsh for survivability of eggs over time. The actual viability figures indicated a similar pattern, where all incubation solutions except 5% formalin, resulted in approximately 80% larval development. The 5% formalin solution resulted in under 60% larval development, indicating that formalin concentrations of ≥5% hinder egg development and thus potential infectivity.

Incubation in water resulted in extensive contamination when analysing samples after incubation, which was expected, as water possesses no antimicrobial properties. Physiological saline also resulted in 'webby' contamination across all samples. Sulphuric acid showed contrasting results, as samples appeared very clean with little evidence of contamination, and eggs looked in very good condition. All formalin samples (0.5%, 2% and 5%) resulted in some contamination, with 0.5% showing the most and 5% showing the least. Sulphuric acid appeared to be the best incubation solution, both in terms of egg development and in hindering growth of microbial contaminants.

3.2 EXPERIMENT 2: THE EFFICACY OF DIFFERENT MODES OF WASHING ON EGG RECOVERY (Naidoo and Archer, in prep 2022)

Figure 15 presents the egg recovery for samples washed under pressure and no pressure, and then centrifuged at 2500 and 3000 rpm for 5, 10 and 15 minutes, respectively.

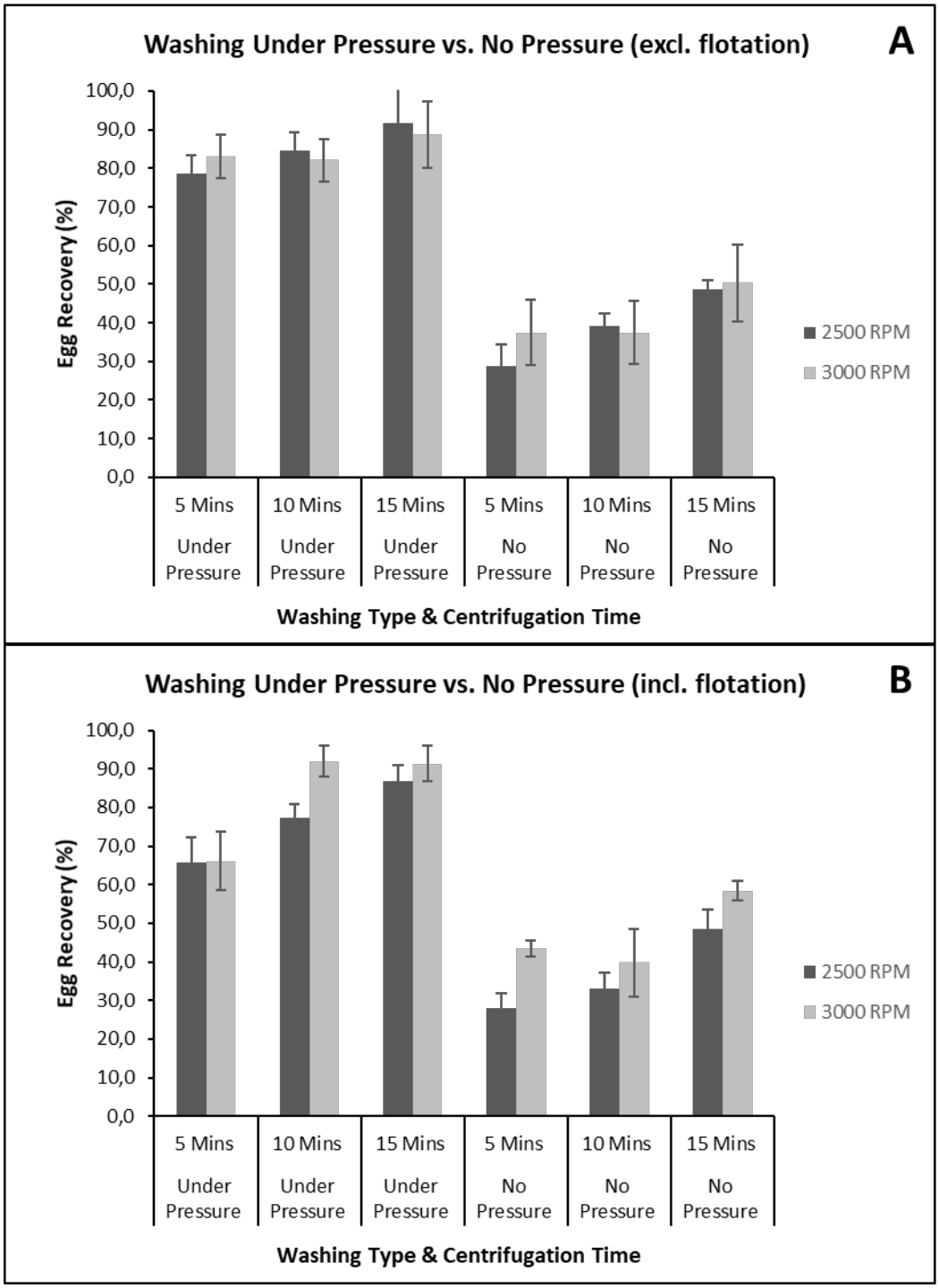

Figure 15: Egg recovery (%) of samples that were washed under pressure and no pressure, and then centrifuged at 2500 and 3000 rpm for 5, 10 and 15 minutes (N = 5). Figure 18 (A) represents extrapolated figures that did not include a flotation step and Figure 18 (B) includes flotation.

3.2.1 Observations & conclusions

After the sample has been homogenised with a wash solution, it must be passed through a set of sieves to separate larger particles and allow for sedimentation of the eggs.

Figure 15 clearly indicates that samples need to be washed under pressure over a set of drum sieves. The pressure pushes eggs through the 100 µm mesh of the top sieve, and physically separates the

larger particles on the 100 µm sieve whilst eggs pass through onto the 20 µm sieve. When using the wash bottle to wash the sample on the 100 µm sieve, it was difficult to break up larger particles, and push this through the pores with the finer debris. The sample never quite looked adequately washed, where the water run-off eventually runs clear from the 100 µm sieves, indicating that nothing further can be pushed through onto the 20 µm sieve.

The figures excluding the flotation step (Figure 18 A), indicated that both 10 and 15 minutes resulted in >90% egg recovery. It also indicated that 2500 rpm resulted in better recovery at 10 and 15 minutes, but in both cases, these were extrapolated figures. The actual recovery after flotation indicated that 3000 rpm allowed for better egg recovery than 2500 rpm, for 15 minutes. 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 microscopy.

3.3. EXPERIMENT 3: TESTING THE EFFICACY OF DIFFERENT FLOTATION SOLUTIONS, SPECIFIC GRAVITIES AND CENTRIFUGATION SPEEDS & TIMES ON EGG RECOVERY (Naidoo and Archer, in prep 2022)

Figures 19 to 21 present the results from testing the efficacy of different flotation solutions, SGs and centrifugation speeds & times on egg recovery, respectively.

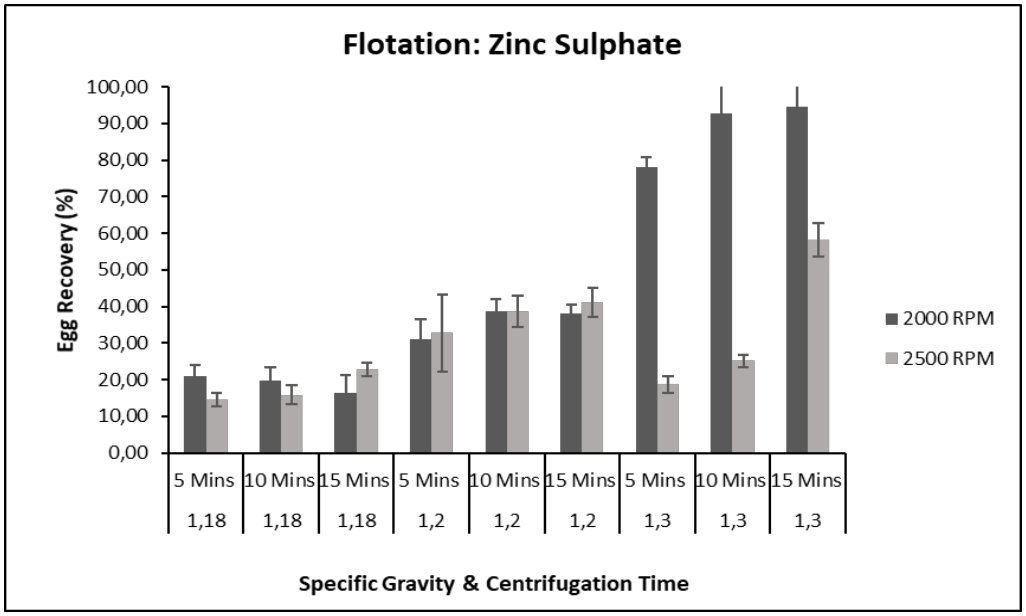

Figure 16: Egg recovery (%) of samples that were floated with zinc sulphate at *Specific Gravities* (SG) of 1.18, 1.2 and 1.3, and then centrifuged at 2000 and 2500 rpm for 5, 10 and 15 minutes (N = 5).

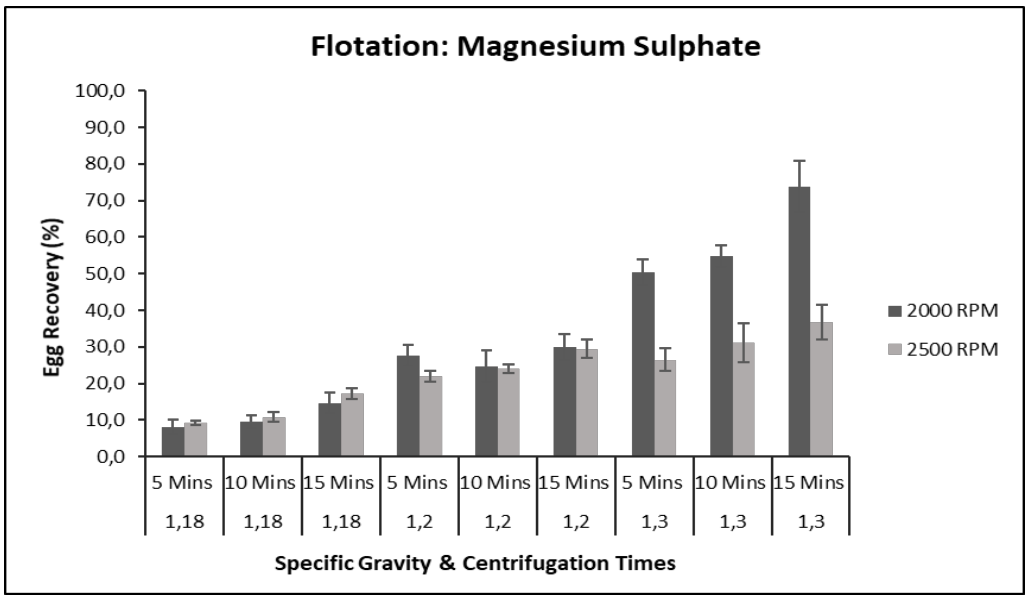

Figure 17: Egg recovery (%) of samples that were floated with magnesium sulphate at *Specific Gravities* (SG) of 1.18, 1.2 and 1.3, and then centrifuged at 2000 and 2500 rpm for 5, 10 and 15 minutes (N = 5)

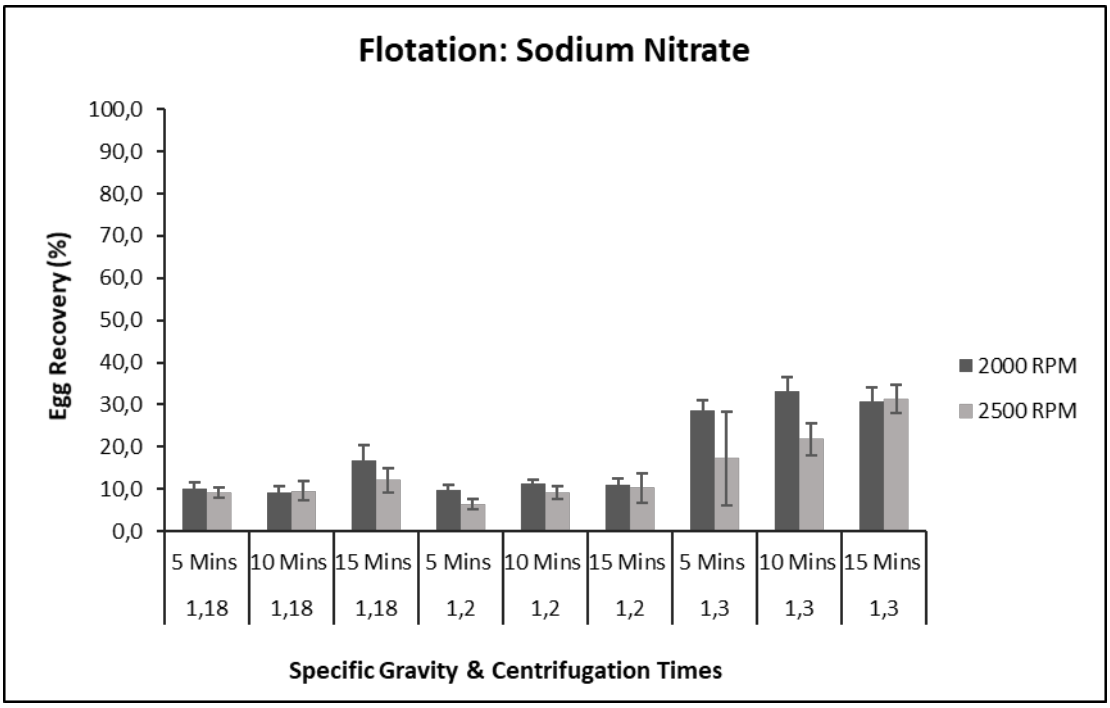

Figure 18: Egg recovery (%) of samples that were floated with sodium nitrate at specific gravities (SG) of 1.18, 1.2 and 1.3, and then centrifuged at 2000 and 2500 rpm for 5, 10 and 15 minutes (N = 5)

3.3.1 Observations & conclusions

Across all three flotation solutions, egg recovery increased as the SG of solutions increased. Egg recovery at SG 1.18 was extremely low for all three flotation solutions, indicating that eggs require a denser solution for better separation from particles. At SG 1.2, egg recovery was still very low, with all three solutions recovering < 50% eggs. This indicated that the density of the solutions plays a more

important role in egg recovery than centrifugation speeds and times. Even when samples are spun down for a longer period, separation from particles was not completely successful. At SG 1.3, both centrifugation speed and time made a difference to egg recovery. Eggs require a slower speed and longer time 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. Magnesium sulphate was successful at a SG of 1.3, and centrifugation at 2000 rpm for 15 minutes, however, recovery was still < 90%. It also precipitated out and formed crystals at the bottom of the bottle when stored and is therefore also deemed unfit as a flotation solution. Zinc sulphate resulted in the best egg recovery at SG 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 successfully float up the supernatant column and separate from the particulate matter.

3.4 EXPERIMENT 4: TESTING THE EFFICACY OF DIFFERENT WASH SOLUTIONS ON EGG RECOVERY FROM DIFFERENT SAMPLE TYPES (Naidoo and Archer, in prep 2022)

presents the egg recovery from the eight different sample types that were processed with water, ammonium bicarbonate and 7X.

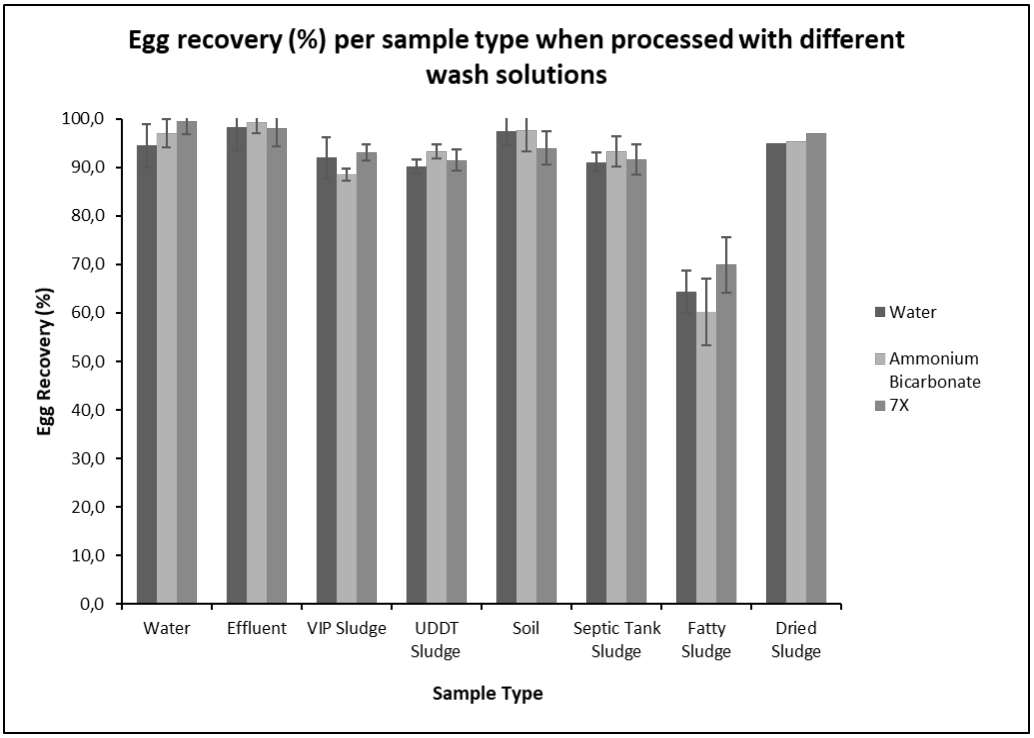

Figure 19: Egg recovery (%) from the 8 different sample types that were processed with water, ammonium bicarbonate and 7X (N = 5).

3.4.1 Observations & conclusions

Across all three wash solutions, egg recovery was ± 90% for each individual sample type, except in the case of fatty sludges. The major difference was not necessarily in egg recovery values, but instead was

observed when processing and analysing the samples. For water and effluent samples, washing with water was sufficient, however, if samples had to be incubated, washing with a chemical solution assisted better in preventing contamination. For VIP, septic tank, fatty, and dried sludges, 7X was the most successful in breaking down the sample and reducing the size of the final pellet, as well as making microscopy the easiest as the pellet dissociated well. Egg recovery was highest for UDDT sludge and soil when washed with ammonium bicarbonate. It facilitates the dissociation of bonds formed between eggs and soil particles, and UDDT sludge is known to contain soil, thus final pellets were easily dislodged and well dispersed for microscopy. Final pellets were also small enough to analyse easily via microscopy. Dried sludge was soaked for 4 and 24 hours, and both resulted in similar egg viability across solutions, indicating that samples can be soaked overnight.

3.5 EXPERIMENT 5: TESTING THE EFFICACY OF DIFFERENT EXTRACTION SOLUTIONS ON EGG RECOVERY FROM DIFFERENT SAMPLE TYPES (Naidoo and Archer, in prep 2022)

Figure 20 to 25 present the egg recovery from water, primary sludge, and fatty sludge samples, respectively, that were fully processed and extracted with the five different combinations.

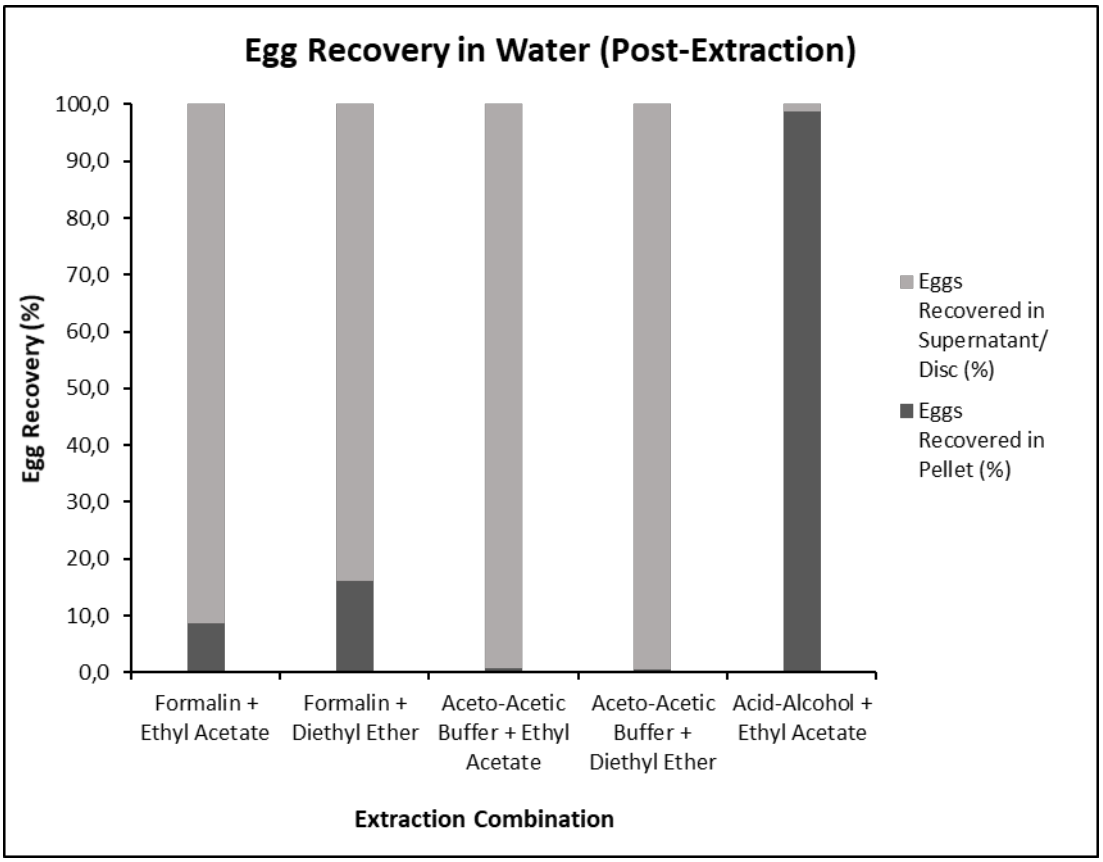

Figure 20: Egg recovery (%) from water samples that were fully processed and extracted with the five different combinations. Each bar represents the average of total eggs per sample, with the lower half representing the percentage of eggs recovered in the pellet and the upper half, the percentage of eggs in the supernatant and/or trapped in the disc (N = 5).

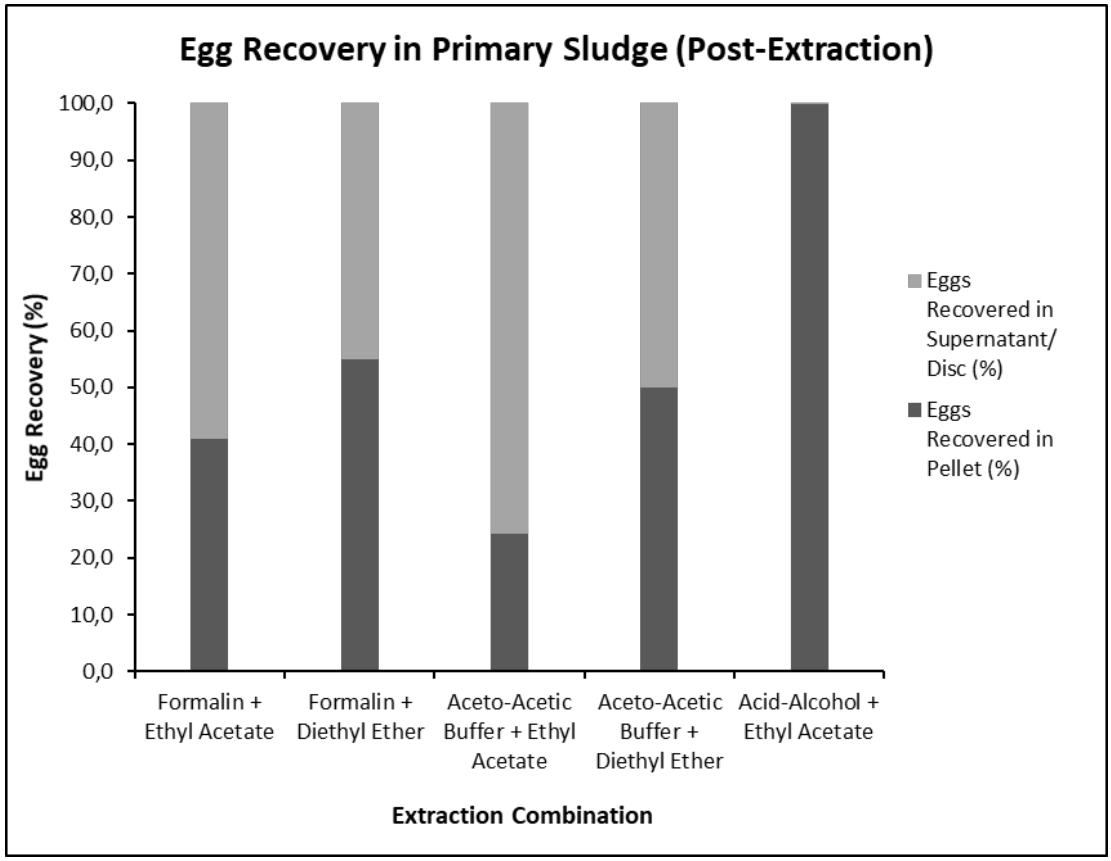

Figure 21: Egg recovery (%) from primary sludge samples that were fully processed and extracted with the five different combinations. Each bar represents the average of total eggs per sample, with the lower half representing the percentage of eggs recovered in the pellet and the upper half, the percentage of eggs in the supernatant or trapped in the disc (N = 5).

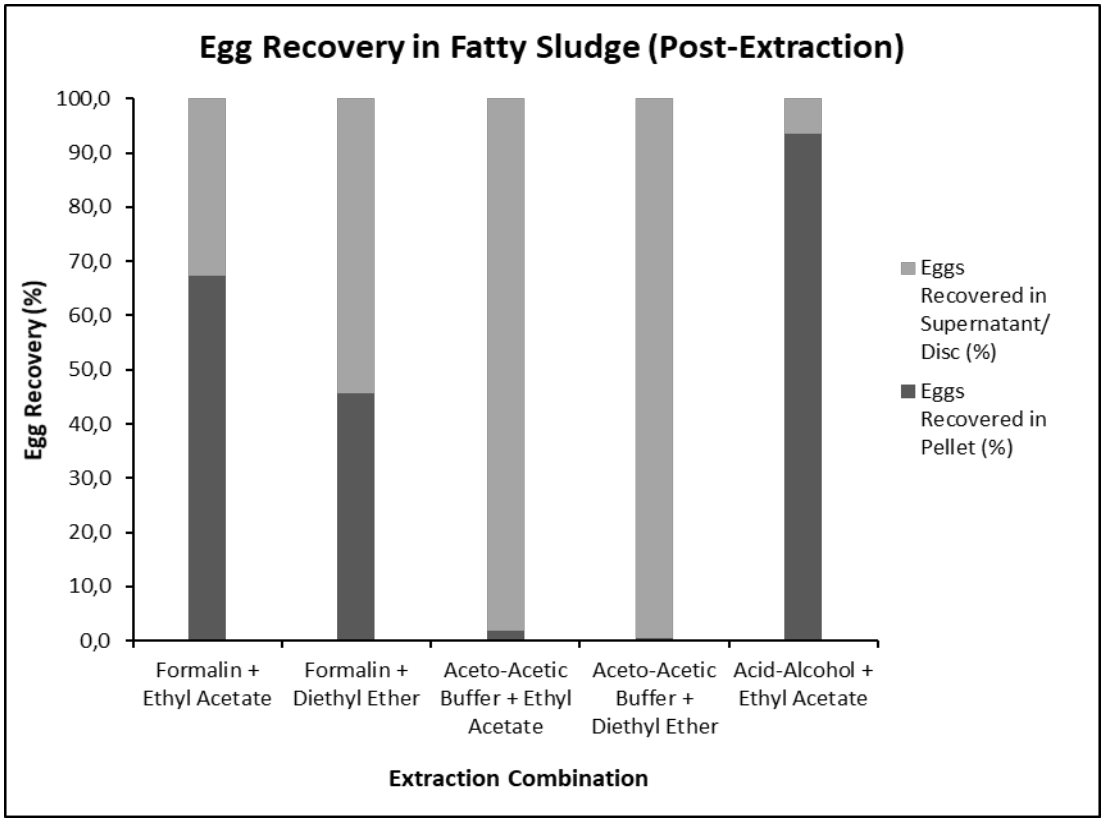

Figure 22: Egg recovery (%) from fatty sludge samples that were fully processed and extracted with the five different combinations. Each bar represents the average of total eggs per sample, with the lower half representing the percentage of eggs recovered in the pellet and the upper half, the percentage of eggs in the supernatant or trapped in the disc (N = 5).

3.5.1 Observations & conclusions

When an extraction is performed on a sample, the sample separates into 2 phases, and organic matter is trapped in a disc between the two. The entire supernatant, including the disc, is discarded, and the pellet at the bottom of the test tube is microscopically analysed. It has always been assumed that helminth eggs separate from organic matter during the extraction and sediment in this pellet. Data from the extraction experiments suggested otherwise, i.e., that eggs are lost when the supernatant is discarded as they do not sediment in the pellet. Across all three sample types, most of the eggs were found in the supernatant and would therefore be lost if an extraction were performed (and the supernatant was discarded), except for the acid-alcohol and ethyl acetate extraction. Although almost all eggs were found in the pellet, the actual extraction process did not work with this combination, as the size of the pellet did not reduce at all – no disc formed between the two phases, indicating that acid-alcohol was an unfit buffer for the extraction process. From this, we learnt that the extraction process does not work and recommend that it should not be included as a step in any helminth recovery method.

3.6 OVERALL RECOVERABILITY OF THE ENTIRE HELMINTH METHOD (Naidoo and Archer, in prep 2022)

Figure 23 presents the egg recovery from primary sludge samples that were fully processed via the newly improvised WRDC Helminth Method.

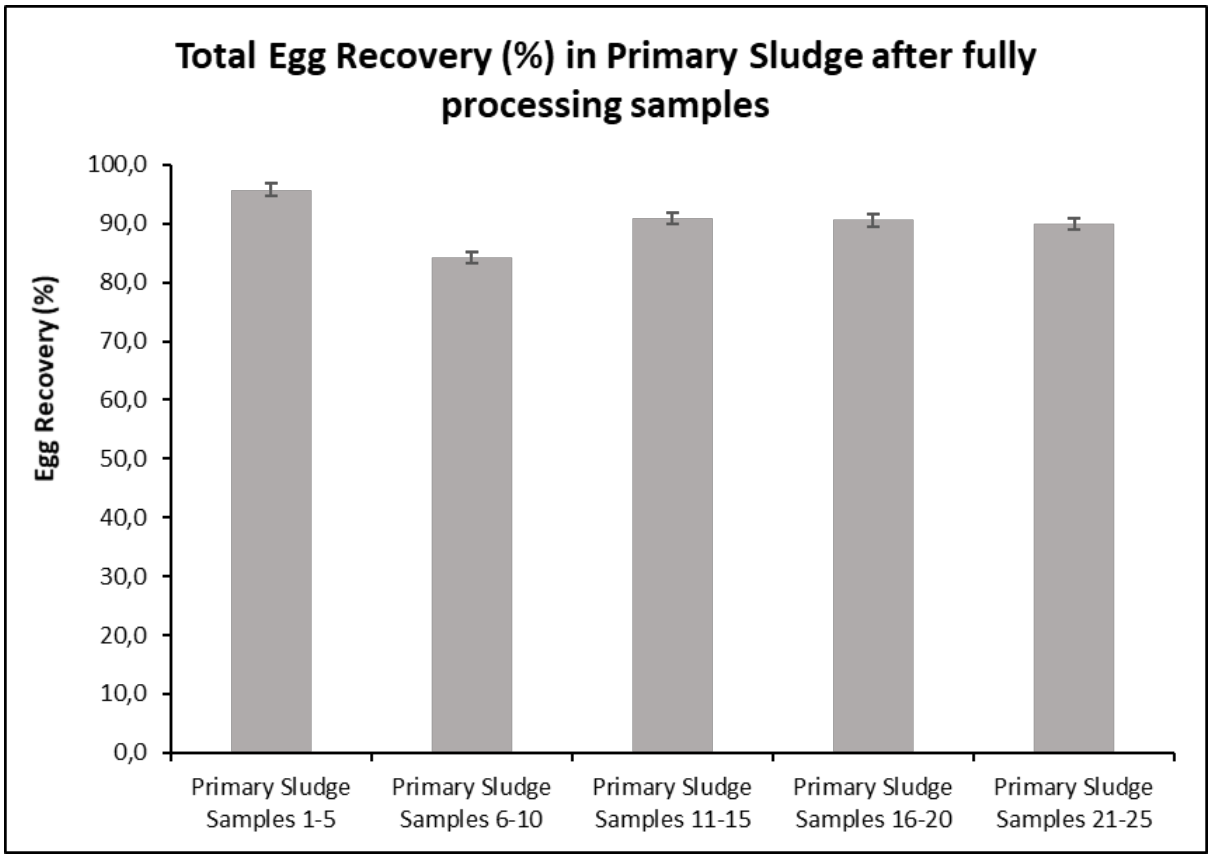

Figure 23: Egg recovery (%) from primary sludge samples that were processed by the new improvised WRDC Helminth Method, with the addition of the extraction step. Each bar represents the average of total eggs recovered per 5 samples (total of eggs recovered in the pellet and eggs in the supernatant and trapped in the disc, indicative of total percentage egg recovery) (N = 5).

3.6.1 Observations & conclusions

From the above egg recovery percentages, it can be concluded that on average, egg recoverability was > 90% when the final modified WRDC method was applied to sludge samples, and they were subsequently extracted, and the supernatant fluid and extraction disc collected and analysed instead of discarding. This is indicative of the accuracy of the test method, where a minimal number of eggs are lost during processing and analysis of samples.

3.7 EXPERIMENT 6: TESTING THE EFFICACY OF DIFFERENT STAINING PROTOCOLS

Results from the staining experiments were inconclusive as we were unable to develop a protocol that worked, where eggs were able to hold the stain throughout the method processing. It was also found that most stains were light sensitive, thus the intensity of the stain on the eggs decreased in samples

that stood on the benchtop waiting to be analysed. Eggs that were stained and washed (without first being spiked into sludge samples) and then analysed were able to hold the stain better than those that were spiked and processed. This indicated that most likely all washing steps caused the stain to reduce in intensity. The purple stains (methyl violet and crystal violet) were found to be the only two successful stains where eggs were recognisably and differentially coloured. Only corticated eggs were able to take up the stain properly, while decorticated eggs either appeared a very light lilac colour or did not take up the stain at all. We need to determine if stain concentrations and exposure times need to be optimised and if a pre-preparation step is needed for eggs prior to staining, for it to work.

3.8 EXPERIMENT 7: TESTING THE REPRODUCIBILITY AND REPEATABILITY OF THE METHOD VIA INTRA-LABORATORY TESTING (between different technicians in the same lab)

This experiment initially began with five technicians of differing levels of expertise and backgrounds – from the head of the parasitology laboratory to a student, and from a biology background to an engineering one.

Egg recovery results varied widely, and it was found that data from samples run by the head of the laboratory and the experienced technician were very low, indicating these results were not due to inexperience. Troubleshooting was carried out on the method, and samples were re-run with modifications made to steps where errors could have been made or equipment could have malfunctioned.

Egg recovery rates were still too low, and we then looked at chemical makeup and the potential of losing eggs due to errors in that regard. Samples were again re-run, this time with different technicians (due to availability), but data was still inconsistent. We then looked at the sieves that were used and realised the mesh had stretched and could have resulted in loss of eggs due to an increase in pore size. New sieves were used, and sample types were adjusted and reduced to avoid too many repeats.

Data was still flawed, so number of replicates was reduced to three and the method was then run on primary sludge, as the previous data showed excellent egg recovery (Figure 23 above). Egg recovery was still low, and it was concluded that the egg stocks had been damaged (and had fungal contamination) due to prolonged storage without aeration during the Covid-19 lockdown. Fresh egg stocks are thus required to complete the intra- and inter-laboratory testing of the method prior to recommendation for standardisation.

4. FINAL SOP DEVELOPMENT AND RECOMMENDATIONS FOR ALTERNATIVE CHEMICALS AND EQUIPMENT

Table 8: Summary table describing differences between the old PRG Helminth Method and final WRDC Helminth Method, recommendations for the SOP and alternate options for reagents

Processing step	Steps in PRG Helminth Method	Recommendation for WRDC Helminth Method	Reason for recommendation	Alternative option and reason
Washing	Wash under pressure	Wash under pressure	Pressure needed to separate particles and eggs	None
	Use of water to wash water and effluent samples by pouring sample straight through set of sieves and immediately washing	Use of water to wash water and effluent samples by pouring sample straight through set of sieves and immediately washing	Water is sufficient for clear, liquid samples	Can use ammonium bicarbonate, 7X, Triton X-100 or Sunlight Liquid (common dishwashing liquid)
	Ammonium bicarbonate for all thick or solid sludges, except fatty sludge.	Ammonium bicarbonate for UDDT sludge and soil samples	Breaks bonds between soil particles and eggs, and results in clear pellet for microscopy	Can use 7X, Triton X-100 or Sunlight Liquid, but samples may not be as clear for microscopy
	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 sample and separate particles	Triton X-100 or Sunlight Liquid, but not for overnight soaking
	Soak in physiological saline overnight for dried sludges, then washed with 0.1% Tween 80.	Dried sludge samples can be soaked from 4 to 24 hours	Soaking time is selected based on how dry the sludge is	N/A
	Centrifuge at 3000 rpm for 10 minutes	Centrifuge at 3000 rpm for 10 minutes	Resulted in > 90% egg recovery and a compact pellet	None
Flotation	Zinc sulphate at SG 1.3	Zinc sulphate at SG 1.3	Zinc sulphate was the best flotation solution. The solution must be dense enough for all eggs to float, thus SG 1.3	None – magnesium sulphate precipitated out and could affect the density of the solution. Sodium nitrate was ineffective in floating all eggs.
	Centrifuge at 2000 rpm for 10 minutes	Centrifuge at 2000 rpm for 15 minutes	2000 rpm is a slow enough speed to allow eggs to travel slowly up the supernatant column without rupturing due to pressure gradients.	None

			15 minutes is sufficient time for eggs to slowly float to surface of supernatant column	
Extraction	Extract if **necessary** using 10% formalin and ethyl acetate or diethyl ether	Step to be removed completely	Eggs are lost in disc and supernatant when the pellet size is reduced after extraction. Eggs were only fully recovered with acid-alcohol where the disc size did not reduce at all, making extraction pointless.	None
Microscopy	Light microscopy (100 & 400X magnification) -samples to be centrifuged at 3000 rpm for 10 minutes	Light microscopy (100 & 400X magnification) -samples to be centrifuged at 3000 rpm for 10 minutes	Light microscopy at these magnifications is sufficient to count and categorise eggs. 3000 rpm for 10 minutes results in a compact final pellet	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, however some contamination is likely after incubation

5. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

This final method differs from the original PRG Method and is now optimised per sample type for maximum egg recovery. The best reagents, times of exposure, type of washing, density for flotation, centrifugation speeds and times were chosen based on data, and a final method was drawn up. It has also eliminated an entire step (extraction) that was previously assumed effective in recovering eggs, whilst reducing the size of the final pellet for microscopy. Total egg recovery after implementing this method on samples resulted in excellent egg recovery, speaking to the success of the method. It is more robust and adaptable to the sample type and does not require the sample to be adapted to the method. It is also more time- and cost-efficient than the commonly employed US EPA Method, and we therefore recommend it as a standard for helminth testing.

There is, however, work that still needs to be completed with regards to this project and in relation to optimisation of the method:

1) Staining of eggs, particularly for spiking experiments: A thorough investigation into stain concentrations and exposure times, pre-preparation of egg stocks needs to be conducted to develop a successful staining protocol to assist in differentiating between eggs occurring naturally occurring in sludge samples and spiked eggs. Decorticated eggs tend not to take up the stain, thus ways of circumventing poor stain quality for microscopy should be assessed.
2) Intra- and inter-laboratory testing: Waiting for fresh, healthy egg stocks to repeat this work on the WRDC Helminth Method, to ensure accuracy, reproducibility, and repeatability of the method, between different technicians of varying levels of expertise, as well as between different laboratories. Only once this is completed can the method be recommended for international standardisation. The WRDC laboratory is currently working towards obtaining ISO-17025 accreditation for the method.
3) The WRDC Helminth Method and the US EPA Method need to be run in parallel in the same lab, by the same technicians, for a direct comparison.
4) Data from this study are being collated into papers for publishing in good peer-reviewed journals.
5) The aim is to have the final method published in a peer-reviewed methods journal, to ensure accessibility and acceptance of the method within the science community.
6) Extensive training is 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 SOP

UNIVERSITY OF KWAZULU-NATAL	*Standard Operating Procedure* PRG pollution research group	**Effective Date:** 14 April 2015	**Version:** ***006***
		Reviewed: 05 February 2020	
SOP_Parasites_005 Helminth testing on samples			Page #: ***1 of* 6**

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 because 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 100 µm and/or 20 µm 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 20 µm 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 15 ml plastic conical test tubes (Falcon tubes) and, if possible, buckets that can also spin a minimum of 4 x 50 ml Falcon tubes
- Sink with hose attached to tap for washing using pressure
- Top-pan balance (scale, for weights up to 200 gm and accurate to 2 decimal places)
- Magnetic stirrer and bar magnets
- Vortex mixer
- Hydrometer that can measure SG between 1.2 and 1.3
- 100 µm mesh stainless steel pan sieve, height 50 mm x diameter 200 mm
- 20 µm mesh stainless steel pan sieve, height 50 mm x diameter 200 mm
- 20 µm mesh stainless steel flat sieve, height 40 – 50 mm x diameter 100 mm
- Plastic test tube racks (minimum of 2) to hold the 15 ml Falcon tubes (and one or two for 50 ml tubes)
- Plastic 1000ml measuring cylinder
- Plastic 250 ml beakers (8 – 16)
- Silicon kitchen spatula – with medium to long handle
- Plastic 3 ml 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 1.2 mm) and Cover glasses (22 x 40 mm)

4. Collection and storage

- After taking samples from various waste materials, store at approximately 4°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 ≥1 hr, and then discard into biological waste box.
- Soak plastic pipettes and glass slides for ≥1 hr in 3.3% NaClO, and 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 (8.5 g/ℓ NaCl)**

  Dissolve 8.5 gm sodium chloride in distilled or deionised 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 119 gm ammonium bicarbonate in 1 ℓ deionised 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 500 gm zinc sulphate in approximately 700 ml deionised 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 500 ml deionised water to a 1 ℓ plastic bottle, pour 3 ml concentrated sulphuric acid into a 10 ml graduated cylinder, and then pour the H_2SO_4 into the plastic bottle containing the water, re-cap and shake. Uncap, add 497 ml of deionised water to the plastic bottle, re-cap and shake.

7. Method

Procedure for VIP, UDDT, Thick Sludges

- Place a 200 ml plastic beaker (labelled with sample number) on top-pan balance, zero balance, weigh 10 or 20 gm 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 ±80 ml 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 50-80 ml AmBic and a magnetic stirring bar, mix on magnetic stirrer for 10 minutes.
- 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 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 100 µm sieve well, keeping it over the 20 µm 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 20 µm sieve while holding 100 µm sieve directly above so as not to lose any sample. When 20 µm sieve has drained sufficiently, place the 100 µm sieve back on top and continue washing. Repeat this until sample on 100 µm sieve is well washed.
- Separate sieves and set aside the 20 µm sieve. Wash retentate from 100 µm sieve into a small bucket containing 3.3% NaClO to disinfect. Place lid on top and set aside.
- Wash retentate on 20 µm sieve well, and then wash it to one side of sieve to make collection easier.
- Rinse total retentate off 20 µm 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 15 ml Falcon tubes labelled with sample number, or if retentate is large, use 50 ml tubes. (After next step, the aim is to have ±1 ml deposit in a 15 ml tube / ±5 ml in a 50 ml tube.)
- Centrifuge at 3000 rpm [1512 g-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 $ZnSO_4$, 3 ml at a time, mixing on a vortex in between addition of the chemical, until tubes are filled to 14 ml mark for 15 ml tubes / 40 ml mark for 50 ml tubes.
- Centrifuge at 2000 rpm (672 g-force) for 10 minutes.
- Pour supernatant flotation fluid over smaller diameter 20 µm sieve. Collect remaining deposits into one test tube, add 3.3% NaClO to this tube and stand ≥1 hr 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 3 ml plastic pipette, transfer the retentate back into the test tube kept aside.
- Centrifuge tube at 3000 rpm (1512 g-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 22x40 mm

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

  NOTE 1: IF sample is blackwater with a high concentration of solids, then use amounts of between 200 and 500 ml. 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 – 500 ml (depending on solids content – visually assessed), and pour into plastic beaker large enough to contain the sample with at least 5-10 cm above it, so that it does not spill when mixing on magnetic stirrer. Add 1 ml 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 100 µm sieve placed on top of a 20 µm sieve and wash well, checking bottom sieve for fluid build-up. Wash well using hose on tap.
- Separate sieves and set aside the 20 µm sieve. Wash retentate from 100 µm sieve into a small bucket containing 3.3% NaClO to disinfect. Place lid on top and set aside.
- Now, rinse 20 µm sieve well and wash retentate to one side for collection.
- Rinse total retentate off 20 µm sieve into 2 or 4 x 15 ml Falcon tubes (OR 50 ml tubes).
- Centrifuge at 3000 rpm (1512 g-force) in centrifuge with swing-out rotor for 10 minutes.
- Pour off supernatant and retain deposits in 15 ml (or 50 ml) Falcon tubes.
- Place test tubes in rack with applicator stick in each (as stirring rod), pipette in $ZnSO_4$, 3 ml at a time while mixing on vortex stirrer, until tubes are filled to 14 ml (or 40 ml) mark.
- Centrifuge at 2000 rpm (672 g-force) for 10 minutes.
- Pour supernatant over 100 mm diameter 20 µm 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 3 ml plastic pipette, transfer retentate back into test tube kept aside.
- Centrifuge at 3000 rpm (1512 g-force) for 10 minutes to obtain final deposit.

- Pour off supernatant water, pipette up deposit, place on microscope slide, place a 22x40 mm 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 20 gm into a 200 ml plastic beaker, on a top-pan balance.
- Add approximately 10-20 ml deionised 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, and then calculate results to report number of eggs per litre or per gram.

Example 1: If 2.5 ℓ of liquid sample was analysed and 500 *Ascaris* eggs were 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 15 gm 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. References

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

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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

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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

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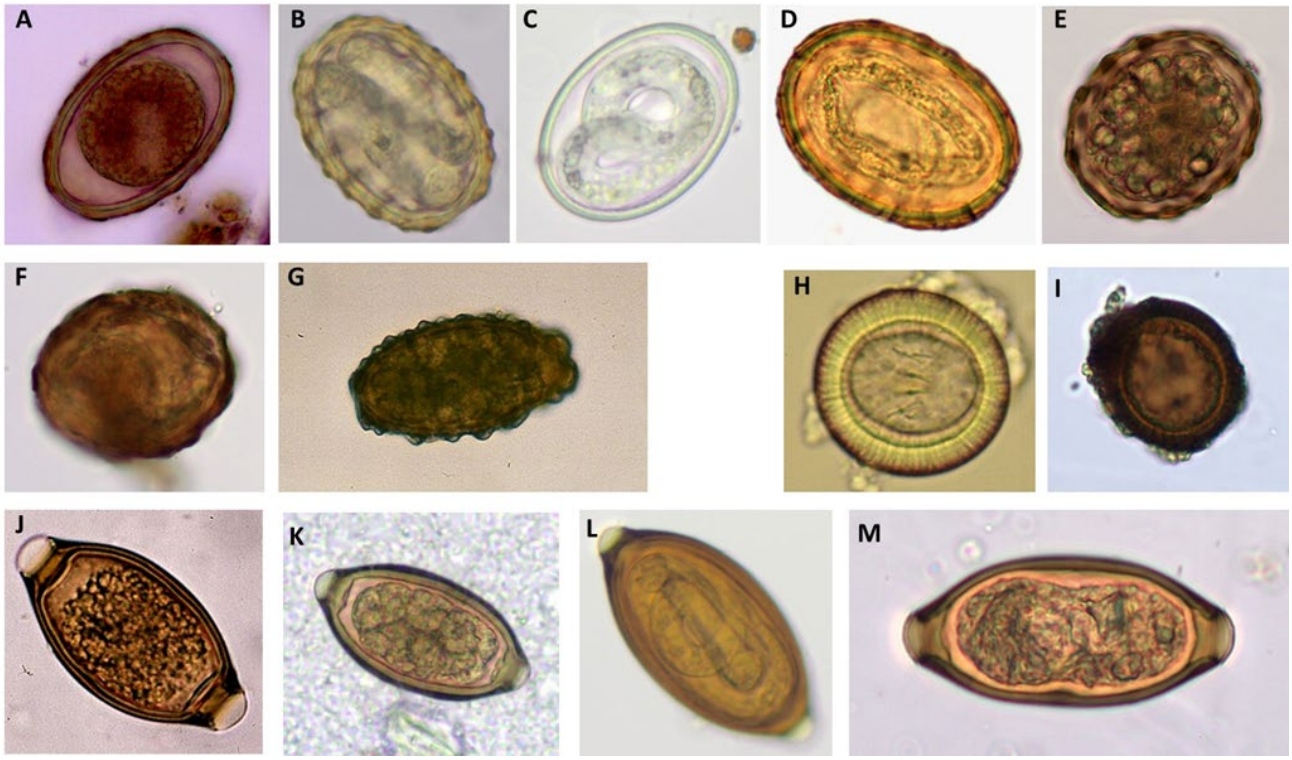
9. LINKS to IHE-Delft lecture & method demonstration: to be included as soon as they are available.

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: FINAL WRDC HELMINTH METHOD SOP

SOP_Helminths_HL-05: The WRDC Helminth Method for Sample Testing

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 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 in South Africa are *Trichuris trichiura*, and *Taenia* spp. A variety of animal parasites is also 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 due to charge interactions with, or adsorption of, eggs to the particles. Many waste samples, even if not from Urine-Diversion Dry Toilets (UDDTs), 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 wastewater treatment work servicing communities that eat oily food, e.g., Chinese, and Indian communities, need to be treated differently from black-water and other water and wastewater samples. Here it is suggested that a surfactant such as 7X or Triton X-100 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/ environmental samples.

AmBic is used as both a wash solution and to dissociate helminth eggs from soil particles, whereas surfactants like 7X or Triton X-100 are used to degrease fatty samples. Filtration, through 100 µm and/or 20 µm 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 SG of 1.3 is used to float eggs (that have a relative density of <1.3) out of the matter (retentate) retained on and subsequently collected from the 20 µm 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 15 ml plastic conical 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 tap for washing using pressure
- Top-pan balance for weighing quantities up to 1200 gm and accurate to 2 decimal places
- Magnetic stirrer and bar magnets
- Vortex mixer
- Hydrometer that can measure SG between 1.2 and 1.3
- 100 µm mesh stainless steel pan sieve, height 50 mm x diameter 200 mm
- 20 µm mesh stainless steel pan sieve, height 50 mm x diameter 200 mm
- 20 µm mesh stainless steel flat 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 1000ml measuring cylinder
- Plastic 250 ml beakers (8- 16)
- Silicon kitchen spatula – with medium to long handle but small spatula (stirring) side
- Plastic 3 ml 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 1.2 mm) and cover glasses (22 x 40 mm)

4. Collection and storage

After taking samples from various waste materials, store at 4°C. Processing is best done as soon after sampling as possible, but providing that there is sufficient moisture and the samples are large enough not to dry out, the eggs should be unharmed, and development will be arrested at this temperature.

5. Safety precautions

- All staff who work with faecal waste are required to be vaccinated against Hepatitis A, Hepatitis B, tetanus, and typhoid fever.
- 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 ≥1 hr, and then discard into biological waste box.
- Soak plastic pipettes and glass slides for ≥1 hr 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.
  N.B. IF there is a spill in the centrifuge, due to a Falcon tube leaking, remove the bucket containing the leaking tube directly into a containment tray and transfer to the sink. Add 3.3% NaClO to the bucket and tube, leave to stand in a containment tray ≥1 hr for eggs to be killed, wash out the centrifuge bucket, discard broken tube into a *Sharps* container and drain the bucket.
- Any spills in the centrifuge itself should be wiped with a cloth, wet with 3.3% NaClO, and the cloth soaked in 3.3% NaClO for ≥1 hr, then rinsed and allowed to dry. Allow centrifuge to air dry, then close.
- When done, wipe all work surfaces with 3.3% NaClO and wash hands using antiseptic soap.

6. Reagents

- **Ammonium Bicarbonate (AmBic)**
  Dissolve 119 gm ammonium bicarbonate in 1 ℓ deionised water (use magnetic stirrer and bar magnet) – store in a **glass** jar.
- **7X or Triton X-100 (or Sunlight® dishwashing liquid)**
  Use neat in liquid samples.
  Other sludges make up a 0.1% solution: measure out 1 ℓ deionised water into a Schott bottle (or other suitable storage container), pipette 1 ml 7X, or Triton X-100, or Sunlight® dishwashing liquid, into the water in the bottle, cap and invert several times to mix, store safely.
- **Zinc Sulphate ($ZnSO_4$ $7H_2O$)**
  Dissolve 1000 gm zinc sulphate in approximately 1400 ml deionised water (use magnetic stirrer and bar magnet) and adjust SG using more of the chemical or water to raise or lower the SG to exactly 1.3 – cap and store in a 2.5 ℓ polypropylene bottle or 2 ℓ glass bottle.
- **0.1N Sulphuric acid (H_2SO_4)**
  Add 500 ml deionised water to a 1 ℓ plastic bottle, pour 3 ml concentrated sulphuric acid into a 10 ml graduated cylinder, and then pour the H_2SO_4 into the plastic bottle containing the water, re-cap, and shake. Uncap, add 497 ml of deionised water to the plastic bottle, re-cap, shake, and store safely.

7. A: Method - *Procedure for VIP, UDDT, Thick Sludges, Dry Sludges, Soil*

[NOTE: If **'solid'** waste material is very dry, (e.g., pelletised or totally desiccated), total solids (TS) will be >25%. So, weigh 10 gm of sample into a 250 ml plastic beaker (labelled with the sample number), add ±80 ml AmBic and soak for four hrs to soften. If very hard and dry, it is safe to soak the sample for up to 24 hours without adversely affecting the helminth eggs. Next, using a tongue depressor or applicator stick/s, break up and mix the sample well and proceed to step 3 below.

For other 'solid' (TS >25%) or 'semi-solid' (TS 15-25%) sludges, and soil:

1. Weigh 10 gm of any type of sludge sample or 50 gm of soil sample into a 250 ml labelled plastic beaker.
2. Wash solution will depend on sample type: Add ±80 ml AmBic to UDDT, dry sludge and soil samples; and ±80 ml 7X (or Triton X-100, or Sunlight dishwashing liquid) to VIP and solid fatty sludge. Mix well with a tongue depressor or applicator stick/s until homogenised.
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 wash solution-sludge mixture over the top sieve. Rinse the beaker with tap H_2O and pour over the top sieve.
4. Using water spray from a hose attached to the tap, wash the 100 µm sieve well, always holding it over the 20 µm sieve (use a silicon spatula, or doubled-gloved hand, to 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 20 µm sieve. Wash the retentate from the 100 µm sieve into a small bucket containing 3.3% NaClO to disinfect. Place the lid on top and set aside.
6. Wash 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 total retentate off the 20 µm filter into the original rinsed-out labelled beaker. If there is a lot of water, allow the contents of the beaker to settle for at least two hrs, 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 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 at 3000 rpm [1512 g-force or 1512 RCF (Relative Centrifugal Force)] for 10 min. Pour off the supernatant, the sedimented deposits remain in the test tubes.
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 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 at 2000 rpm (672 g-force) for 15 min. Wet the 100 mm diameter, 20 µm sieve with tap water and pour the supernatant flotation fluid from all the tubes of one sample over the sieve. Collect the deposits left in all the test tubes into one tube, add 3.3% NaClO to this tube and stand ≥1 hr before washing it out into the municipal drain. Keep one empty test tube, wash and set aside for re-use.
12. Wash the retentate well with tap water and rinse it down 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 3000 rpm (1512 g-force) for 10 min to obtain the final deposit. Pour off the supernatant and pipette up the deposit, dispense 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 22x40 mm cover-glass on top and examine under a compound microscope using the 10x objective for counting, and the 40x objective to confirm the identifications.
14. Examine the entire preparation and count every *Ascaris* egg, classifying them as viable (plump motile larva in the egg), **potentially viable** (egg undeveloped; developing; or containing a plump immotile larva), necrotic (egg containing a shrivelled, dead larva), or **dead** (egg globular or with wall damage). Also, count *Trichuris*, *Taenia*, hookworm spp. eggs and assess simply as potentially viable or dead.
15. When done, discard cover glasses into *Sharps* container, and soak slides in a beaker of 3.3% NaClO.

7. B: Method - *Procedure for Liquid Samples (water, effluent, septic tank, liquid fatty sludge)*

1. **(i)** For clean samples with low suspended solids (**'liquid' TS <5%**), 5 – 10 ℓ of sample is sufficient. For dirty water samples with low to moderate suspended solids, 1 – 5 ℓ of sample is sufficient.
   (ii) For septic tank liquid sludge and black water with a high solids content (**'slurry', TS 5-15%**), use amounts of 200 - 500 ml. Measure out the sample and add the equivalent ratio of 1 ml (to make a 0.1% solution in the sample) of 7X, Triton X-100 or Sunlight® dishwashing liquid, to the measured amount of sludge (e.g., 0.2 ml to 200 ml, or 0.5 ml to 500 ml).
   (iii) For fatty samples (**'slurry', TS 5-15%**), use amounts of 200 – 500 ml (depending on solids content – visually assessed), and pour into a plastic beaker large enough to avoid spills when mixing. Add 0.2-0.5 ml of neat 7X, Triton X-100, or Sunlight® dishwashing liquid per litre of sample, directly into the sample (to make a ± 0.1% solution in the liquid sludge).
   Mix well, using a tongue depressor or applicator stick/s, until well homogenised.
2. From here, proceed as for steps **3 - 15** in **Method 7. A** above.

7. C: Method - *Procedure for Plants and Green Compost*

1. Weigh 50 gm of plant material into a 500 ml (or larger) labelled plastic beaker and cut it up into small pieces using scissors.
2. Place the scissors into a beaker of water and add AmBic to the cut-up plant material to completely submerge it. Mix well, using a wooden tongue depressor, then place it into the beaker of water with the scissors.
3. Wet the 2 x 200 mm diameter sieves with tap water, place the 100 µm on top of the 20 µm mesh sieve.
4. Pour the plant-AmBic mixture through the sieves. Then pour the water from the beaker with the scissors and tongue depressor over the sieves and wash the scissors and tongue depressor

well over the sieves, then set aside. From here, proceed as for steps **4 – 15** of method **7. A** above.

8. ***Procedure 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 *Ascaris* eggs), the client may require viability to be determined and reported. If so, an extra sample must be processed as per the correct procedure per specimen type and the deposit is then resuspended in 1.5X 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 25-28°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 3000 rpm for 10 min, remove the supernatant fluid, examine, and report on egg viability as for the initial test sample (see **7. A** step number 14.)

9. ***Quality Control – QA/QC***

1. Use **uninfected** sludge (preferably of a consistency very similar to the samples being tested) or uninfected wastewater, or other relative sample type, for QA/QC testing.
2. Use one uninfected sample, weighed (in grams) and/or one sample measured (in litres) as a negative control.
3. For the positive controls, spike a known number of *Ascaris suum* eggs into a weighed and/or measured portion of the same uninfected sample used for a negative control.
   Then, proceed as for **7. Method: *Procedure*,** using the appropriate procedure for the sample type, i.e., ***VIP, UDDT, Thick Sludges, Dry Sludges, Soil*, *Liquid Samples*, or *Plants and Green Compost*.**
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. Most sludge and wastewater methods consider recovering >80% of spiked eggs to be excellent.
7. Intra-laboratory controls must be run monthly – two different technicians should run three positive control sample each with a known number of *A. suum* eggs per sample. Presently, interlaboratory controls are not possible as there are no other environmental labs in South Africa with the necessary experience.

10. Calculations, Example of Data Sheet Results, & Interpretation

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

EXAMPLE 1:	**EXAMPLE 2:**
If 2.5 ℓ of liquid sample was analysed and there were 500 *Ascaris* eggs found, then use simple proportions:	If 15 gm of solid sample was analysed and 3450 *Ascaris* eggs were counted, then using proportions:
$\frac{500\ \text{eggs}}{2{,}5\text{lt}} : \frac{X}{1\text{lt}}$ $= \frac{500 \times 1}{2{,}5\text{lt}}$ $= 200\ \text{eggs/lt}$	$\frac{3450\ \text{eggs}}{15\text{gm}} : \frac{X}{1\text{gm}}$ $= \frac{3450 \times 1}{15}$ $= 230\ \text{eggs/gm}$
Note: If a sample of sludge was tested for moisture content, adjust egg counts to 'per gram dry mass' (using ratios).	

Example of Data Sheet showing Results

ID No.	Sample type	Sample quantity	Ascaris – DEAD			Ascaris – potentially VIABLE				Trich	Trich	Taen	Taen	Other
			Inf	Dead	Nec	Imm	Mot	Devel	Undev	Pot vi	Dead	Pot vi	Dead	
01	Effluent	10 ℓ	1	261	12	3	9	15	8	2	26	1	7	0
	Results per litre		<1	26.1	1.2	<1	<1	1.5	<1	<1	2.6	<1	<1	0
02	Sludge	10 gm	6	543	28	23	19	267	399	88	54	49	9	1 E v
	Results per gram		<1	54.3	2.8	2.3	1.9	26.7	39.9	8.8	5.4	4.9	<1	<1 E v

Abbreviations:

ID No. = the sample identification number;

Inf = infertile, i.e. eggs that were not fertilised;

Nec = necrotic, i.e. egg contains a dead, shrivelled larva;

Imm = immotile larva, healthy looking, but not moving;

Mot = a motile larva;

Devel = embryo in egg in ≥2-cell stage of development;

Undev = embryo in single cell stage;

Trich = *Trichuris* sp.

Taen = *Taenia* sp.

Pot vi = potentially viable as for *Ascaris* (Imm, Mot, Devel, Undev);

Dead = as for *Ascaris* (Inf, Dead, Nec);

Other = any other helminth eggs found (count and record number of eggs only);

E v = *Enterobius vermicularis*.

Interpretation of Results

The term 'helminths' encompasses round worms (nematodes), tapeworms (cestodes) and flatworms (trematodes). The nematodes that are a concern in sanitation are those that lay eggs or produce larvae in an undeveloped stage and require time in the soil to develop to infective larvae

(geohelminths), e.g., *Ascaris* spp., *Trichuris* spp., hookworm spp. and *Strongyloides stercoralis*. Other nematode eggs that rapidly develop larvae and are infective for humans in a few hours, e.g., *Enterobius vermicularis*, may also be a concern.

Geohelminth eggs are considered as potentially viable (and thus potentially infective) if they are in the **undeveloped** stage, **developing** through a 2-, 4-, 8-, 16-, 32-cell stage or more (blastula stages) to an immature larva (gastrula), 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 that are undeveloped or in early cleavage (one of the blastula stages) die, they may become globular or have broken shells or collapsed walls or appear empty inside – these are termed **dead**. If a formed larva dies inside the egg, it appears shrivelled, occupies much less space than a plump, healthy viable larva, and is termed **necrotic**. Eggs that have never been fertilised are **infertile** and cannot develop, and are therefore classified under “non-viable” eggs.

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. Cestode eggs (except for *Hymenolepis nana*) require an intermediate host to ingest them before that host passes on the infection to humans, however ingesting *Taenia solium* eggs poses a serious risk for humans as they become the intermediate host (like the pig) and develop cysts in the brain resulting in neurocysticercosis, (*T. saginata* poses no risk). The eggs of these two species are indistinguishable and therefore all ***Taenia*** eggs are counted, assessed for potential viability, and reported as *Taenia* sp.

Some trematode eggs are excreted in the undeveloped stage and a miracidium develops in about two weeks, others contain a miracidium when laid – these eggs are only a concern if fresh sanitation waste is dumped into water bodies, as they require aquatic plants to encyst on or an aquatic intermediate host to develop within, for transmission to occur. Except for *Taenia* spp., all trematode and cestode eggs are thus counted and recorded, however recording viability status is optional and not a requirement unless specifically requested by the client.

11. References

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
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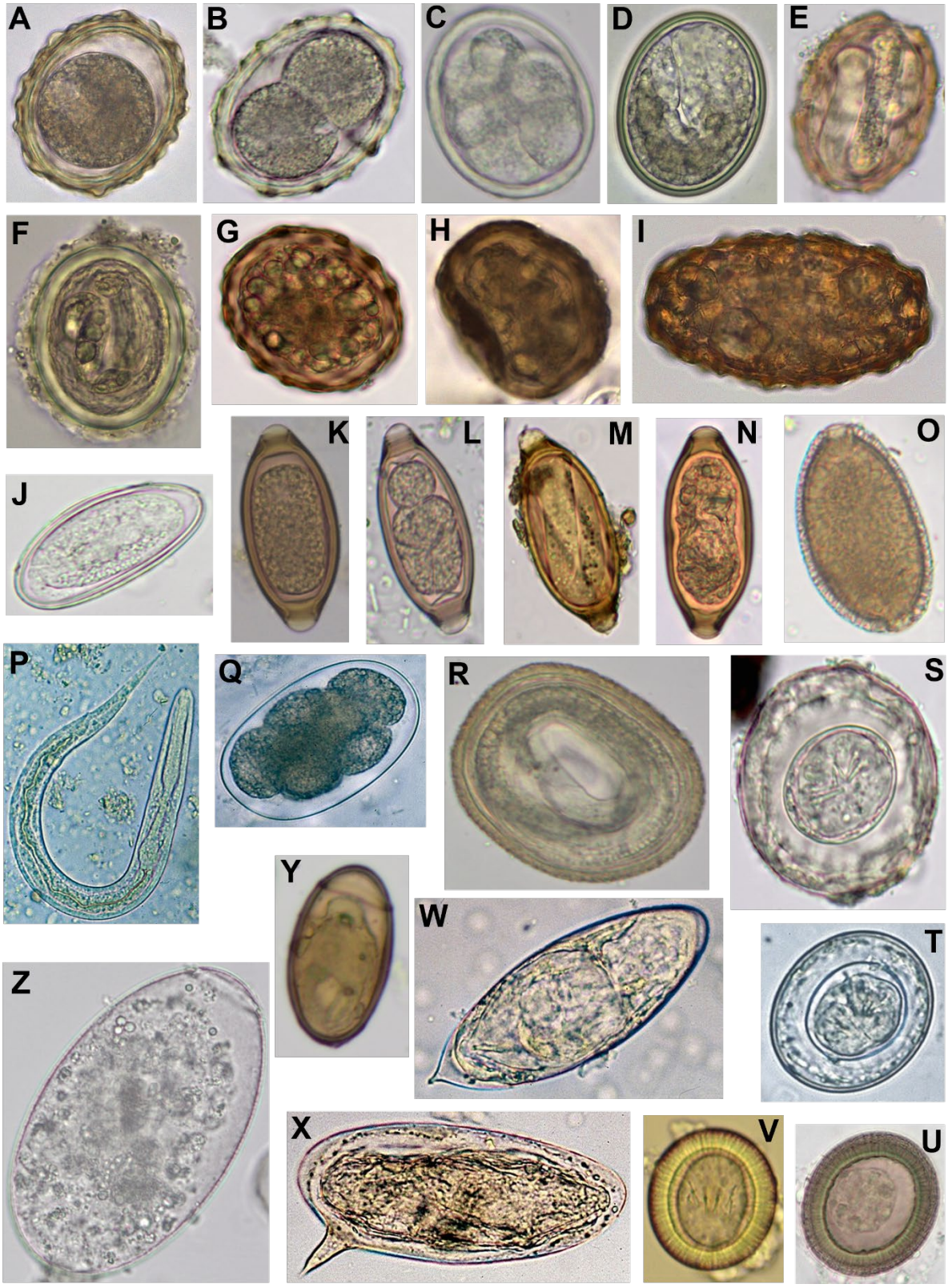
6. Naidoo D, Archer CE, Appleton CC, Septien S, Buckley CA. (2020) Inactivation of *Ascaris* for thermal treatment and drying applications in faecal sludge. *Journal of Water, Sanitation and Hygiene for Development*

12. Photographs of some helminth eggs (on next page) table below provides identification

PLATE OF MICRO-PHOTOGRAPHS OF HELMINTH EGGS AND LARVAE			
A	*Ascaris* – undeveloped	**N**	*Trichuris* - dead
B	*Ascaris* 2-cell – developing	**O**	*Capillaria* sp.- dead
C	*Ascaris* multiple cells - developing	**P**	*Strongyloides stercoralis* - rhabditiform larva
D	*Ascaris* - gastrula	**Q**	Hookworm sp. - developing
E	*Ascaris* - viable larva	**R**	*Toxocara* sp. - developed larva
F	*Ascaris* - necrotic (dead) larva	**S**	*Hymenolepis diminuta* - possibly viable
G	*Ascaris* dead – globular	**T**	*Hymenolepis nana* - possibly viable
H	*Ascaris* dead – wall collapsing, globular	**U**	*Taenia* sp. - dead
I	*Ascaris* – infertile	**V**	*Taenia* sp. - viable
J	*Enterobius vermicularis* – dead	**W**	*Schistosoma haematobium* - dead
K	*Trichuris* – undeveloped	**X**	*Schistosoma mansoni* - dead
L	*Trichuris* – developing	**Y**	*Dicrocoelium dendriticum* - possibly viable
M	*Trichuris* - viable larva	**Z**	*Fasciola* sp. - dead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APPENDIX C: COSTING FOR LABORATORY SETUP & FOR LABOUR PER SAMPLE

Table 9: 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 Vision SA 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 & 50 ml 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 – 200 mm diameter; 100 µm mesh	Reliance Laboratory Equipment	1092.50	1092.50
Sieve – 200 mm diameter; 20 µm mesh		3818.00	3818.00
Sieve – 100 mm diameter; 20 µm 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 (250 ml) x 10	Lichro Chemicals and Laboratory Supplies	30.00	300.00
Beakers (2 ℓ) 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 (1 ℓ) x 5	Lichro Chemicals and Laboratory Supplies	194.54	972.70
15 ml graduated plastic test tubes (1000/pack)		1622.50	1622.50
15 ml test tube rack x 3		98.54	295.62
50 ml graduated plastic test tubes (50/pack) x 2		327.75	655.50
50 ml test tube rack x 3		78.00	234.00
Consumables & Reagents			
Ammonium bicarbonate (500 g) x 3	United Scientific SA	174.25	522.75

*7X (3.8 ℓ)	Lichro Chemicals and Laboratory Supplies	4246.28	4246.28
Zinc sulphate (25 kg)	United Scientific SA	4249.25	4249.25
Sulphuric acid		157.55	157.55
Coverslips (22 x 40 mm) – 100/pack x 20	Lasec SA	65.50	1310.00
Coverslips (22 x 22 mm) – 100/pack x 10		33.92	339.20
Microscope slides (50/pack) x 5		20.00	100.00
Wooden tongue depressors (100/pack)	Lichro Chemicals and Laboratory Supplies	30.00	30.00
Wooden applicator sticks (1000/pack)		103.00	103.00
3 ml plastic Pasteur pipettes (500/pack)		677.35	677.35
TOTAL		**195 204.23**	**199 050.25**

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

Table 10: Costing of labour for the WRDC Helminth Method

Description	**Total cost or time**	**Total/hour or Total/sample**
Average pay per technician	R1 600/day for 8 hours	R200/hour
Microscopy	8 samples/day (8 hours)	1 sample/hour
Sample processing	8 samples/half day (4 hours)	30 minutes/sample
Total Labour Costs	**1.5 hours**	**R300/sample**