The Risk factors of Soil-Transmitted Helminth Infections: A need for Appropriate Measurement methods

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy: Health Sciences in the Faculty of Health Sciences at the Durban University of Technology

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DECLARATION

I confirm that this thesis is composed of my original work and contains no material previously submitted to the Durban University of Technology or any other institution for academic qualifications. The content of my thesis consists of work I have carried out since the commencement of my PhD studies.

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Date: **March 2018**

SUMMARY

Soil-transmitted helminths are a major health concern, especially in tropical and sub-tropical regions. Poor sanitation and poverty are major pre-disposing factors contributing to increase in infections. Infection with STH is mainly through exposure to water, soil and food contaminated with the eggs of these parasites. Accurate detection and quantification of STH eggs in environmental samples is therefore critical for the determination of infection risks from exposure. Accurate detection of these eggs is also important in the adoption of risk reduction strategies. This thesis presents the development of a revised method for the accurate detection and quantification of STH eggs in different environmental matrices, such as wastewater, sludge etc. It further presents the application of this method in the comparative determination of STH egg reduction efficiencies of centralized wastewater treatment plants and decentralized wastewater treatment (DEWATS) plants in Durban, South Africa and Maseru, Lesotho. The concentration of viable STH eggs in dried sludge from Durban, South Africa and Dakar, Senegal was also determined and compared with both WHO guidelines and South African national standards for sludge reuse. The risks of infection with STHs for different populations exposed (directly and indirectly) to wastewater, wastewater contaminated surface water and sludge were determined using both quantitative microbial risks assessment and epidemiological approaches.

Despite the plethora of methods available for the detection and quantification of STH eggs in the environment there is no internationally accepted method, however the most commonly used methods are based on the principles of sedimentation, differential flotation and microscopy. These are mainly adaptations of the WHO and USEPA methods. These methods were found to be similar with a few differences which affected the recovery rates reported. However, the major challenges with the conventional methods are the time needed for sample analysis and the use of reagents that could possibly affect the recovery of viable STH eggs. A new revised method was developed based on review of literature and laboratory experiments. In this method the heterogeneity of environmental samples was accounted for by the development of different pre-processing steps, involving the use of detergents to aid in the separation of eggs from particles in samples such as sludge, UD waste and untreated wastewater. Additionally, the use of sieves of different pore sizes ensured that the number of debris on the microscope slides was reduced considerably. The use of these sieves also reduced the time need for sample analysis, due to the elimination of the spontaneous sedimentation step, which is commonly used. This spontaneous sedimentation step takes between 12-24 hours therefore prolonging the time needed for sample analysis. Reagents such as acetoacetic acid and ethyl acetate were found to result in considerable loss of egg viability after just 5 minutes of exposure. This new method therefore does not involve their usage. The elimination of the use of acetoacetic acid and ethyl acetate step also reduces the number of steps involved in sample analysis. This reduces room for error as well as helping in fast analysis of samples. In addition to a much faster sample analysis the method has recovery percentages of 80.25% to 97.63% in sludge and wastewater samples respectively, with sensitivity of 2-3 eggs per liter in wastewater samples and 5-7 eggs per 20 gram of sludge.

Exposure to STH eggs in the environment is mainly through wastewater, either treated or untreated, this exposure could therefore be eliminated through wastewater treatment. Centralized wastewater treatment systems are the most favored treatment options globally. These centralized treatment systems incur high cost of construction, maintenance and operations which may hamper the robustness in developing countries and rural areas. One of the most widely used alternative means of wastewater treatment is the anaerobic baffled reactors (ABRs) and planted gravel filters

(PGFs) (collectively referred to as DEWATS in this thesis), which have been considered as low cost, effective wastewater treatment options. However, there is lack of comparative assessment of the STH egg removal efficiency of these two different wastewater treatment approaches. Eggs of *Ascaris* spp, hookworm, *Trichuris* spp, *Taenia* spp and *Toxocara* spp were the commonly recorded STH eggs in the untreated wastewater at the inlets of the centralized wastewater treatment plants as well as the DEWATS plants (except for *Toxocara* spp). There was variation in STH egg concentrations between and within the study areas, indicating difference in STH infections among the populations both in Durban and Maseru. STH egg removal varied between and within the different wastewater treatment plants as well. The DEWATS plants achieved 95-100% STH egg removals as compared to the 67 to 100% in the centralized wastewater treatment plants. This could be attributed to the difference in treatment processes. Among the different STHs, reduction in *Ascaris* spp eggs was significantly higher, irrespective of the type of treatment, which is attributed to the high relative density of the egg resulting in a higher settling velocity than the other STH eggs.

Reduction or elimination of STH eggs through wastewater treatment is achieved by removing the eggs from the wastewater into the sludge. STH egg concentration in sludge is therefore mostly higher than in the wastewater. Sludge from Durban and Dakar after 60 days of drying under ambient environmental conditions contained very high concentration of viable STH eggs. *Ascaris* spp, hookworm, *Trichuris* spp, *Taenia* spp and *Toxocara* spp were the commonly recorded STH eggs, except for Dakar were *Taenia* spp and *Toxocara* spp were not detected in the sludge. STH egg concentrations were higher in Dakar than in Durban, with viable STH egg concentrations exceeding both the USEPA regulatory value (≤0.25 eggs/g TS) and the WHO guideline value (≤1 eggs/g TS). This variation in egg concentration could be attributed to the difference in prevalence and intensity of STH infections in the two study areas. Over a ten-month study period concentration of viable eggs in the sludge from Durban varied considerably, probably influenced by the environmental conditions. A decay rate of 0.0056 per day was calculated for egg die-off during drying. The rate of decay is low therefore drying alone cannot produce sludge meeting both local and international standards and guidelines for sludge reuse.

Determination of STH infection risks due to exposure to wastewater and sludge either directly or indirectly is critical in the prevention of infection. Exposure to the effluents during wastewater irrigation is one major route of infection. STH egg concentrations in the final effluents from the centralized and DEWATS wastewater treatment plants were consistently higher than the WHO recommended guideline for unrestricted agricultural use (≤ 1 helminth egg/L), whereby the direct reuse of the effluents for agriculture was found to pose a higher risk than the WHO tolerable risk of infection (1×10^{-2} pppy) for farmers and consumers. Annually the use of effluents from the DEWATS plants poses the least risk of infection (1.9×10^{-2} ($\pm 2.4 \times 10^{-4}$)), which is marginally higher than the WHO tolerable risk value. Well maintained DEWATS plants are more efficient in removing or reducing the concentration of STH eggs in wastewater and therefore pose the least risks of infection compared to centralized wastewater treatment plants. Consumers of vegetables from these farms are also at considerable risks of STH infections.

Probabilistic assessment of the STH infection risks showed that farmers applying sludge from Durban and Dakar without adequate protective measures had risks of infections higher than the WHO tolerable risks figure (1×10^{-2} pppy). Based on the estimated risks of infection after decay, exposure to farm soil after 40-50 days of sludge application may reduce the risks of infection to

levels lower the WHO tolerable risks value. However, this may not be practical due to the need for farmers to attend to their crops frequently. Incorporation of the decay of the eggs into the risks assessment also indicated that, using lettuce as a representative vegetable, harvesting of vegetables in Dakar could be done after 40 days of sludge application to reduce the risks of infection to the WHO tolerable value but in Durban harvesting after 30 days ensures that consumers are protected. Therefore, to protect both the farmers and consumers exposed to STH eggs through wastewater/sludge reuse in agriculture the implementation of the WHO multi-barrier approach to risk reduction is required.

Risks of STH infections could be directly estimated using epidemiological approaches. By using this approach, the concept of STH infection risks for farmers using wastewater was assessed through direct measurements of the concentration of STHs both in wastewater used for irrigation and the farm soil, as well as the actual load of STHs ova in the stool of farmers and their family members. In Kumasi, Ghana, wastewater used for irrigation of vegetables and the farm soil contained high concentration of STH eggs. There was positive correlation between STH concentrations in the wastewater/soil and STH eggs load in stool of the exposed farmers. Stool analysis after 3 months, following deworming, showed a fast re-infection rate. Farmers exposed to the wastewater were three times more likely as compared to the control group of non-farmers to be infected with *Ascaris* spp (OR = 3.9, 95% CI, 1.15-13.86) and hookworm (OR = 3.07, 95% CI, 0.87-10.82). These risks of infection were higher in the rainy season than the dry season. This corresponds to a higher egg concentration in wastewater used for irrigation during this period. This indicates a relationship between STH infection and egg concertation in the environment. This study therefore contributes to the evidence-based conclusion that wastewater irrigation contributes to a higher incidence of STHs infection for farmers.

In conclusion, this thesis therefore presents a new revised method that can be used to determine the STH egg concentration in different environmental samples. The development of this method also provides an opportunity to comparatively assess the STH egg reduction/removal efficiency of the more commonly used centralized wastewater treatment plants and DEWATS plants. The accurate quantification of viable STH eggs provide inputs for the probabilistic assessment of STH infection risks for different populations exposed to effluents from these two wastewater treatment approaches. This assessment of risks provides a public health perspective to the wastewater treatment. Additionally, it was concluded with the used of this method that drying of sludge for 60 days in Durban or Dakar does not produce sludge of good quality for agricultural application. This was confirmed by the estimates of STH infection risks determined using quantitative microbial risks assessment. This thesis therefore shows the importance of accurate quantification of STH eggs in the determination of infection risks either though QMRA or epidemiological approaches.

This work is dedicated to my family

You don't choose your family. They are God's gift to you, as you are to them.

Desmond Tutu

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CONTENTS

LIST OF PAPERS

This thesis is based on the following papers that have been published or under consideration for publication. They are referred to in the text by their Roman numerals.

I. **Isaac Dennis Amoah,** Gulshan Singh, Thor Axel Stenström and Poovendhree Reddy (2017). Detection and quantification of soil-transmitted helminths in environmental samples: A review of current state-of-the-art and future perspectives. *Acta Tropica.* 169:187-201. **DOI:** 10.1016/j.actatropica.2017.02.014

II. **Isaac Dennis Amoah,** Poovendhree Reddy and Thor Axel Stenström (2017). Effect of reagents used during detection and quantification of *Ascaris suum* in environmental samples on egg viability. *Journal of Water Science and Technology.* **DOI**: 10.2166/wst.2017.324

III. **Isaac Dennis Amoah,** Razak Seidu, Poovendhree Reddy, and Thor Axel Stenström (2018). Removal of **s**oil-transmitted helminth egg in selected centralized and decentralized wastewater treatment plants in South Africa and Lesotho: Health implications for direct and indirect exposure to the effluents. *Environmental Science and Pollution Research.* 17:1-13. **DOI**.org/10.1007/s11356-018-1503-7

IV. **Isaac Dennis Amoah,** Razak Seidu, Poovendhree Reddy, and Thor Axel Stenström (2018). Concentration of soil-transmitted helminth eggs in sludge from South Africa and Senegal: A probabilistic estimation of infection risks associated with agricultural application. *Journal of Environmental Management.*206:1020-1027. **DOI**.org/10.1016/j.jenvman.2017.12.003

V. **Isaac Dennis Amoah,** Razak Seidu, Amina Abubakari, Thor Axel Stenström and Robert Clement Abaidoo (2016). Contribution of Wastewater Irrigation to Soil Transmitted Helminths Infection among Vegetable Farmers in Kumasi, Ghana. *PLOS Neglected Tropical Diseases.* 10(12): e0005161. **DOI**.org/10.1371/journal.pntd.0005161

CONFERENCE PRESENTATIONS

Amoah, I.D., Reddy, P. and Stenström, T. A. (2017). Microbial reduction efficiency of decentralized wastewater treatment plants in Lesotho: Health risk implications for effluent reuse. Oral presentation at *The 8TH International Young Water Professional (IYWP) Conference 2017.* Cape Town, South Africa. 10-13th December, 2017

Amoah, I.D. and Stenström, T. A. (2017). Small scale domestic wastewater treatment: Health risk implications for operations and effluent reuse. *3rd Brazil Constructed Wetlands Conference.* Catholic University of Don Bosco (UCDB). Campo Grande. Brazil. 23-26 May, 2017

Amoah, I.D., Reddy, P. and Stenström, T. A. (2017). Decentralized treatment of domestic wastewater: Health risk implications for effluent reuse. Oral Presentation at the The *19th International Symposium on Health-Related Water Microbiology/UNC Water Microbiology Conference in Chapel Hill,* North Carolina. USA. *15 – 19* May, 2017

Amoah, I.D., Reddy, P., Niang, S. and Stenström, T. A. (2017). Method for The Detection and Quantification of Soil Transmitted Helminth Eggs in Faecal Sludge. *Oral Presentation at the 4th Faecal Sludge Management (FSM4) Conference.* Chennai, India. 18-23 February, 2017

Amoah I.D. and Stenström, T.A. (2016). Epidemiological link between exposure and risk of soil transmitted helminths infection: A case for appropriate ova detection methodologies within the Gates Project. Oral Presentation at the *2016 SASM Biennial Congress, Coastlands,* Umhlanga. South Africa. 17-20 January, 2016

Amoah, I.D. and Stenström, T. A. (2016). The role of irrigation type and type of vegetable on E. coli contamination of vegetables irrigated with effluents from a DEWATS wastewater treatment plant. *Water Institute of South Africa Conference and Exhibition 2016,* Durban International Convention Centre, Durban. South Africa. 15-19 May, 2016

Stenström, T. A. and **Amoah, I.D.** (2015). The role of sanitation safety planning in the reclamation and reuse of domestic wastewater in South Africa. *WISA Water Reuse Symposium*. Johannesburg, South Africa. 28 - 29 September, 2015

Amoah, I.D., Seidu**,** R., Abubakari, A., Heistad, A., Larbi, J.A. Stenström, T. A. and Abaidoo, R.C. (2015). Risk of Ascaris infection associated with the use of wastewater in urban agriculture, in Kumasi, Ghana. *18th International Symposium on Health-Related Water Microbiology.* Lisbon, Portugal. 13th -19th September, 2015

AWARDS AND HONOURS

International Water Association award for outstanding performance at the 19th *International Symposium on Health-Related Water Microbiology/UNC Water Microbiology Conference in Chapel Hill, North Carolina. USA. 17TH May, 2017*

3RD Best Oral Presentation at the 2016 *SASM 2 BIENNIAL CONGRESS, 17-20 January, 2016. Coastlands, Umhlanga. South Africa.*

LIST OF ABBREVIATIONS

ABR	Anaerobic baffled reactor
AMBIC	Ammonium bicarbonate
CAMRA	Center for Advancing Microbial Risk Assessment
CDC	Center for Disease Control and Prevention
CFR	Code of Federal Regulations
CONOMA	Conselho Nacional do Meio Ambiente-Brazil
DALYs	Disability-adjusted life years
DEWATS	Decentralized wastewater treatment systems
DHS	Department of Health Services-USA
LAMP	Loop mediated amplification
NOM	Normas Officiales Mexicanas
NRMMC	National Resource Management Ministerial Council-Australia
NTDs	Neglected tropical diseases
PCR	Polymerase chain reaction
pppy	Per person per year
QA/QC	Quality assurance/quality control
QMRA	Quantitative Microbial Risk Assessment
SEMARNAT	Secretarĭa de Medio Ambiente y Recursos Naturales
STHs	Soil-transmitted helminths
USEPA	United States Environmental Protection Agency
WASH	Water, sanitation and hygiene
WHO	World Health Organization
WWTP	Wastewater treatment plant

1.0 INTRODUCTION

1.1 Background

Soil-transmitted helminths (STHs) are a group of parasitic nematode worms infecting humans globally. Infection is through ingestion of parasite eggs in faecally contaminated water, soil and food (Keraita and Amoah, 2011) or skin penetration by infective larvae (hookworms). These eggs/larvae survive mostly in warm and moist soil of tropical and subtropical regions (Katakam et al., 2014). The roundworms (*Ascaris lumbricoides* and *Strongyloides stercoralis*), whipworms (*Trichuris trichuira*) and the hookworms (either *Ancylostoma duodenale* or *Necator americanus*) cause the major STH infections globally (Strunz et al., 2014). The individual infection figures reported are 771.7–891.6 million people for ascariasis (*A. lumbricoides* infections), 429.6–508.0 million people for trichuriasis (*T. trichuira* infections) and 406.3–480.2 million people for hookworm infections (Pullan et al., 2014). The majority of these infections occur in tropical and subtropical countries (Stolk et al., 2016), mainly in sub-Saharan Africa, the Americas, China and East Asia (WHO, 2015). Poverty and poor sanitation are major predisposing conditions for infections (Strunz et al., 2014). One of the major routes of exposure through is through the use of wastewater and sludge in agriculture, which exposes farmers as well as consumers to the parasite eggs/larvae leading to infections (WHO, 2006a; WHO, 2006b; Pham-Duc et al., 2013). Additionally, consumers of the farm produce as well as the communities close to irrigation sites are also at risks of infections (Drechsel et al., 2010; WHO, 2017). In endemic areas, wastewater could contain up to 3000 helminth eggs/L (Kamizoulis, 2008; Mara and Sleigh, 2010) and there are a number of reports demonstrating the link between wastewater reuse in agriculture and STHs infections (Seidu et al., 2008; Blumenthal et al., 2001; Pham-Duc et al., 2013; Rutkowski et al.,

2007; Trang et al., 2006; Habbari et al., 1999). **Paper V** contributes to the knowledge on wastewater irrigation and STH infections within an informal setting.

Wastewater/sludge reuse exposes farmers to high concentration of STH eggs and relatively high infection figures have been reported (Fuhrimann et al., 2016; Pham-Duc et al., 2014; Yajima 2009; Blumenthal et al., 2001). The WHO has developed guidelines for wastewater reuse in agriculture in order to protect the health of farmers and consumers. These guidelines include target values for helminths in wastewater for unrestricted agricultural use, of ≤1 helminth egg per liter to reduce the risk of STHs infections to below tolerable target values. (WHO, 2006). Accurate detection and quantification of STH eggs in environmental samples (such as the irrigation water) is therefore important in determining these STH infection risks. Several methodological variants have been developed over the years, some of which are; hollow-fiber ultrafiltration (HFUF) (Simmons et al., 2001; Ferguson et al., 2004; Hill et al., 2005; Hill et al., 2007), filtration (Maya et al., 2006; Alli et al., 2011) and flotation (Bowman et al., 2003; de Victorica and Galvan, 2003; Bastos et al., 2013). The most widely used methods are the USEPA (USEPA, 1999) and WHO methods (Ayres and Mara, 1996) with several variations depending on the sample matrix and user preference (Yaya-Beas et al., 2016; Yen-Phi et al., 2010; Moodley et al., 2008; Maya et al., 2006; Mes, 2003). Although these methods have similar analytical steps, there are major differences (discussed in **Paper I**) that may affect the number of eggs recovered (Collender et al., 2015). Additionally, some of the chemicals/reagents used in the detection process might affect the viability of the eggs recovered, which could potentially affect the concentration of viable eggs reported. **Paper I** provides a comprehensive review of existing methods with reference to the different sample matrices. This paper also discusses new and emerging methods that are either being used or could be used for the detection of STH eggs in the environment. Despite the perceived effect of different

chemicals/reagents (such as diethyl ether, ethyl acetate, acetoacetic buffer etc.) on the viability of STH eggs, quantitative assessments of the effects of the various reagents are sparse. Therefore **Paper II** provides a quantitative analysis of the effect of some of these reagents on viability of STH eggs. A revised method, presented in **Appendix I**, was developed based on the results and conclusions drawn from **Paper I** and **Paper II.**

One of the ways to reduce STH infections is to limit the fecal contamination of surface waters and soils. Several researchers have established that wastewater/sludge reuse in agriculture plays a major role in increasing the risks of STH infections **(**Habbari et al., 2000; Pham-Duc et al., 2013; Fuhrimann et al., 2016**).** In addition to STH eggs, wastewater contains several other types of pathogenic microorganisms which make treatment before release into the environment imperative. Wastewater treatment must be done for a specified purpose – for example, to produce an effluent suitable for agricultural or aqua-cultural reuse (or both), or to produce an effluent that can be safely discharged into other water bodies (Sanchez-Ramos et al., 2015; Asano, 2002). There are different types of wastewater treatment technologies, generally categorized into centralized and decentralized treatment systems (Eggimann et al., 2016). The main bottlenecks in the establishment and operation of centralized wastewater treatment plants is the requirement for constant supply of energy, the need for pumps to move the wastewater from source of production to the treatment facility, the need for regular maintenance and a high operational cost (Massoud et al., 2009). An alternative is the low cost decentralized wastewater treatment technologies, some of which are constructed wetlands, anaerobic baffled reactors, waste stabilization ponds, aerated lagoons, oxidation ditches etc. (Masi et al., 2015; Istenic et al., 2014; Elmitwalli et al., 2002). The use of the anaerobic baffled reactor (ABR) has increased over the last ten years due to its low maintenance requirements, simple and inexpensive construction and stable operations (Tilley et

al., 2014; Reynaud and Buckley, 2016). Despite the increase in the use of these alternative treatment technologies their pathogen reduction efficiencies are not well documented. A few studies have reported reduction efficiencies for selected microorganisms in different types of decentralized wastewater treatment technologies (Teh et al., 2015; Battilani et al., 2010; Foxon et al., 2004) but there is not enough data on the removal of STH eggs by ABRs. P**aper III** presents the STH egg removal efficiencies of selected decentralized wastewater treatment plants (ABRs and planted gravel filters) as compared to selected centralized wastewater treatment plants.

The STH infection risks for different populations exposed to the effluents from these decentralized and centralized wastewater treatment plants are very important especially in settings where wastewater/sludge reuse in agriculture is encouraged. These infection risks may be estimated through; microbiology laboratory analysis, epidemiological studies and Quantitative Microbial Risk Assessment (QMRA) (Haas et al., 2014). Quantitative microbial risk assessment (QMRA) is emerging as a useful tool for developing standards for human exposure to pathogens (Haas et al., 2014). This technique can be used to determine the risk involved in exposure to final effluents from different wastewater treatment technologies and provide the framework for determination of the best treatment option depending on the required effluent quality. This approach has been used extensively in the estimation of STH infection risks associated with wastewater/sludge reuse (Cutolo et al., 2012; Navarro and Jiménez, 2011; Navarro et al., 2009; O'Connor et al., 2017; Kundu et al., 2014; Schönning et al., 2007; Mara and Sleigh, 2010; Seidu et al., 2008a). One of the main steps in QMRA is exposure assessment which involves the determination of the "amount or number of organisms that correspond to a single exposure (termed the dose) or the total number of STHs eggs that will constitute a set of exposures" (Haas et al., 2014). The correct estimation of risk of infection requires accurate data on the concentration of STHs eggs in the different exposure

mediums (wastewater or sludge in this instance). Using the method developed in this study and presented in **Appendix I**, the STH egg reduction/removal efficiencies of selected centralized wastewater treatment plants was compared with decentralized wastewater treatments (**Paper III**). STH egg concentration in sludge from selected wastewater treatment plants in South Africa and Senegal was also determined with this revised method (**Paper IV**). Using the concentrations of STH eggs detected in the wastewater and sludge the risks of STH infections with their reuse was determined (using QMRA and epidemiological approaches) and compared with the WHO guidelines for wastewater reuse in agriculture. Therefore, by the development of accurate techniques for the detection and quantification of STH eggs in environmental samples such as wastewater, sludge, soil etc., this thesis established the importance of STH egg detection methods as well as different risks determination methods.

1.2 Objectives

The thesis has two broad main objectives, where each have further sub-objectives. These objectives are;

Objective 1: To assess the helminth reduction efficiency of wastewater treatment plants using a developed methodology

- **1a.** To develop and validate adequate methodologies from conventional methods (sedimentation and flotation) to advance molecular methods (qPCR, BacLight Live/Dead Technique) for detection of STHs in spiked environmental samples (**Paper I, II and Appendix I**).
- **1b.** To undertake a comparative assessment of the STHs reduction efficiency of the DEWATS plant at Newlands, Durban and other DEWATS plants in Lesotho (**Paper III**).
- **1c.** To undertake a comparative analysis of the efficiency of DEWATS system, constructed wetlands and conventional wastewater treatment systems in reducing STH eggs in wastewater (**Paper III**).

Objective 2: To determine the risk of infection with STHs for different exposed populations

2a. To determine the risk of STHs infections for workers in the DEWATS plants and constructed wetlands, farmers using the effluents and sludge and consumers of irrigated crops using the Quantitative Microbial Risk Assessment (QMRA) framework (**Paper III and IV**).

2b. To determine the risk of STHs infection associated with the use of surface water for agricultural irrigation by QMRA and epidemiology (**Paper IV and V**).

1.3 Thesis framework

The framework for this thesis is presented in Figure 1, where the link between the objectives of this study is related to the outputs (publications).

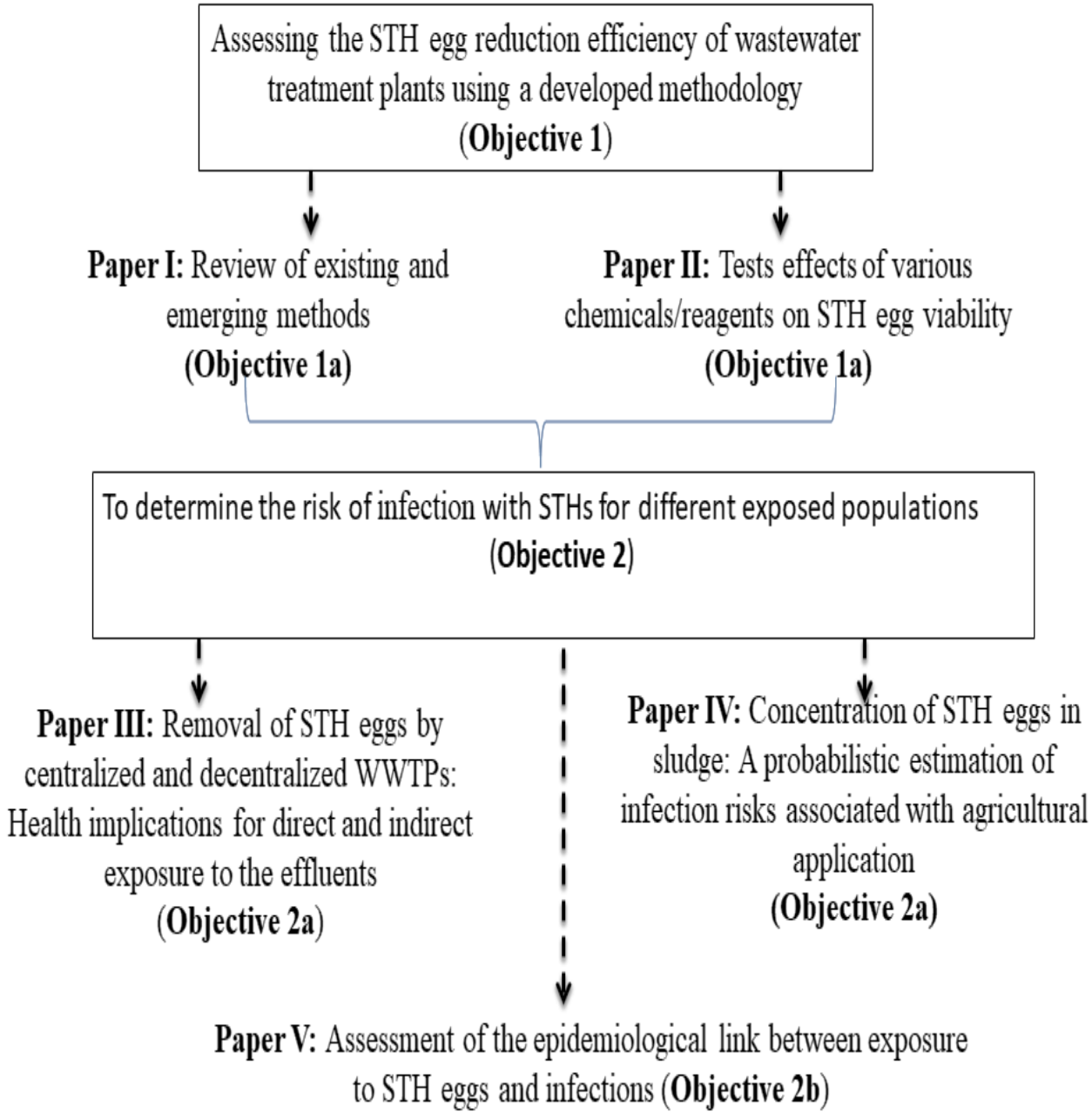

Fig. 1: Layout and interconnectedness of each publication in the thesis.

2.0 LITERATURE REVIEW

2.1 What are helminths and soil-transmitted helminths?

The term "helminth" generally refers to multi-cellular worms, with sizes ranging from 1 mm to several meters in length (Jimenez-Cisneros and Maya-Rendon, 2007). Helminths can be categorized into two groups as illustrated in Figure 2. These worms develop through egg, larval (juvenile stage) and adult stages and knowledge of these different stages is critical in understanding the epidemiology and pathogenesis, diagnoses and treatment of helminth diseases (Castro, 1996; Jimenez-Cisneros and Maya-Rendon, 2007). Additionally, it is important in the diagnosis and treatment of infections that these may cause. Parasitic helminths belong to the Phylum Platyhelminthes (flatworms) with *Taenia* spp, *Hymenolepis* spp, *Schistosoma* spp, *Fasciola hepatica* as major disease causing members (Figure 2). Class Nematoda of the Phylum Aschelminthes (the roundworms) contains the majority of parasitic helminths where *Ascaris lumbricoides*, hookworms (*Ancylostoma duodenale* and *Necator americanus*), *Trichuris trichiura*, etc are some of the common parasitic nematodes (Hotez et al., 2008). The life cycle of these different helminth groups varies, with further variation in the infective stages as well. Adult helminths in the Class Cestoda (Cestodes) release eggs from their gravid segment into the environment where they undergo embryonation and become infective to the intermediate host (organisms supporting the non-reproductive form of the worms) when ingested (Castro, 1996). The embryonated eggs (referred to as oncospheres) then penetrate the tissues of the intermediate hosts and develop into encysted larvae (Castro, 1996; Jimenez-Cisneros and Maya-Rendon, 2007). The encysted larvae (metacestodes) excyst when eaten by a definitive host (organisms supporting the adult or sexually reproductive forms) and develop into adult tapeworms. Eggs of the flukes (trematodes) hatch into free-swimming miracidia upon release into aquatic environments and infect snails (intermediate hosts). The miracidia mature into cercariae and are released back into

the aquatic environments where they either infect new definitive hosts or form encysted metacercariae (Bogitsh and Cheng, 1990). The nematodes (roundworms) release unembryonated eggs in faeces, which undergo development under normal temperature and in moist soil to become infective (embryonated). The duration of environmental exposure required for embryonation varies between the different nematodes, for instance it takes approximately 8-37 days for *Ascaris* spp eggs and 2-14 days for hookworm eggs to reach this infective stage (Brooker et al., 2006; Steinbaum et al., 2016). The embryonated eggs upon ingestion by a definitive host develop and hatch into larvae that matures into adult worms (Jimenez-Cisneros and Maya-Rendon, 2007).

Helminths that release eggs in the unembryonated stage and require periods of development in soil to become infective are referred to as soil-transmitted helminths (STHs). For these STHs, the soil plays a major role in their life cycle and infection is through ingestion of soil, or contaminated water/food (Ziegelbauer et al., 2012). The majority of STHs are found in the Class Nematoda, with *Ascaris* spp (roundworms), hookworms (*Necator americanus* and *Ancylostoma duodenale*) and *Trichuris* spp (whipworms) as the most prevalent of these (CDC, 2013). Hookworms are different from the other common STHs. These release eggs that hatch in the environment into an infective larva that penetrates the skin of humans upon contact (Bogitsh and Cheng, 1990; Jimenez-Cisneros and Maya-Rendon, 2007). Additionally, larvae of *A. duodenale* can also infect through oral ingestion (Bogitsh and Cheng, 1990).

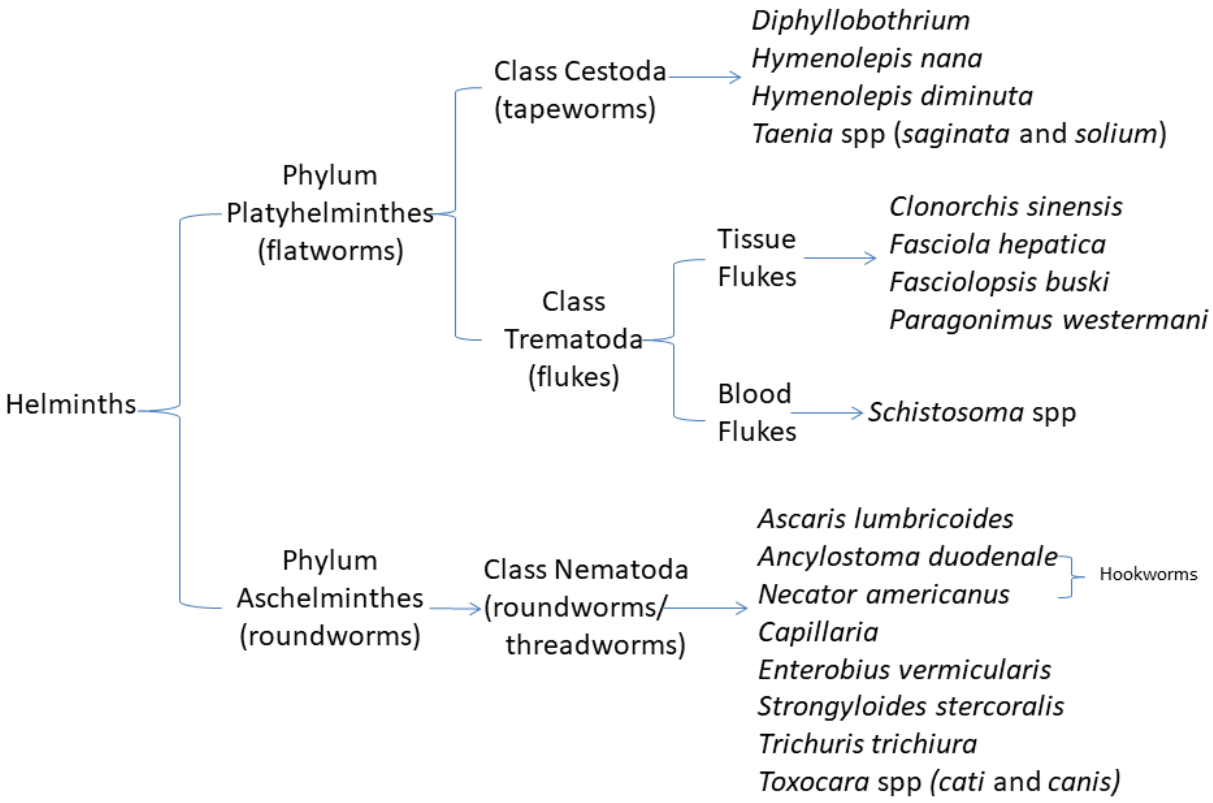

Figure 2: Classification of common helminths of public health importance.

2.2 Global Distribution of soil-transmitted helminth infections

STH infections were historically prevalent in many parts of the world, including Europe, Japan, South Korea, Taiwan, the Caribbean and the southern states of America (Starr and Montgomery, 2011; Pullan and Brooker, 2012). Sustained control efforts have resulted in a decrease and in some instances elimination of infections (Kobayashi et al., 2006; Tikasingh et al., 2011; Pullan and Brooker, 2012). STH infections are still widely distributed in the tropics and subtropical regions of the world. In many parts of sub-Saharan Africa and South and Southeast Asia there has been little change in the prevalence of STH infections over the last half of the 20th century (Brooker et al., 2000; Pullan and Brooker, 2012). Warm temperatures and soil moisture are very essential for

the development of the infective larvae (Bethony et al., 2006), hence the high prevalence in tropical and sub-tropical regions.

STH infection is still potentially endemic in 166 countries, with all countries in Asia, Oceania, Latin America and the Caribbean, North Africa, the Middle East and sub-Saharan Africa included (Pullan et al., 2014) (Figure 3). Many sub-Saharan African countries are endemic with STH infections, although their contribution to the global infection rates is low (between <0.1-5%) as compared to the proportion of infections from Asia (Figure 3.B). The total number of people infected with STHs globally has varied over the years. Bundy (1994) estimated 2.52 billion infections, de Silva et al., (2003) 2.15 billion by and Pullan et al., (2014) reported a global prevalence of 1.45 billion. This sequence of numbers indicates a substantial reduction over the last 20 years. The increase in political and financial support for the control of these infections may have contributed significantly to this reduction.

Despite a higher proportion of global STH infections from Asia, countries in sub-Saharan Africa are among those with the highest prevalence of STH infections (Figure 3A), (Bethony et al., 2006; de Silva et al., 2003). Socioeconomic determinants such as inadequate water supplies, sanitation and poor hygiene play an important role (Strunz et al., 2014; Dunn et al., 2016). Poverty is another important factor in STH infections, either as a cause or a consequence of these infections (Ngui et al., 2011; Ngui et al., 2012).

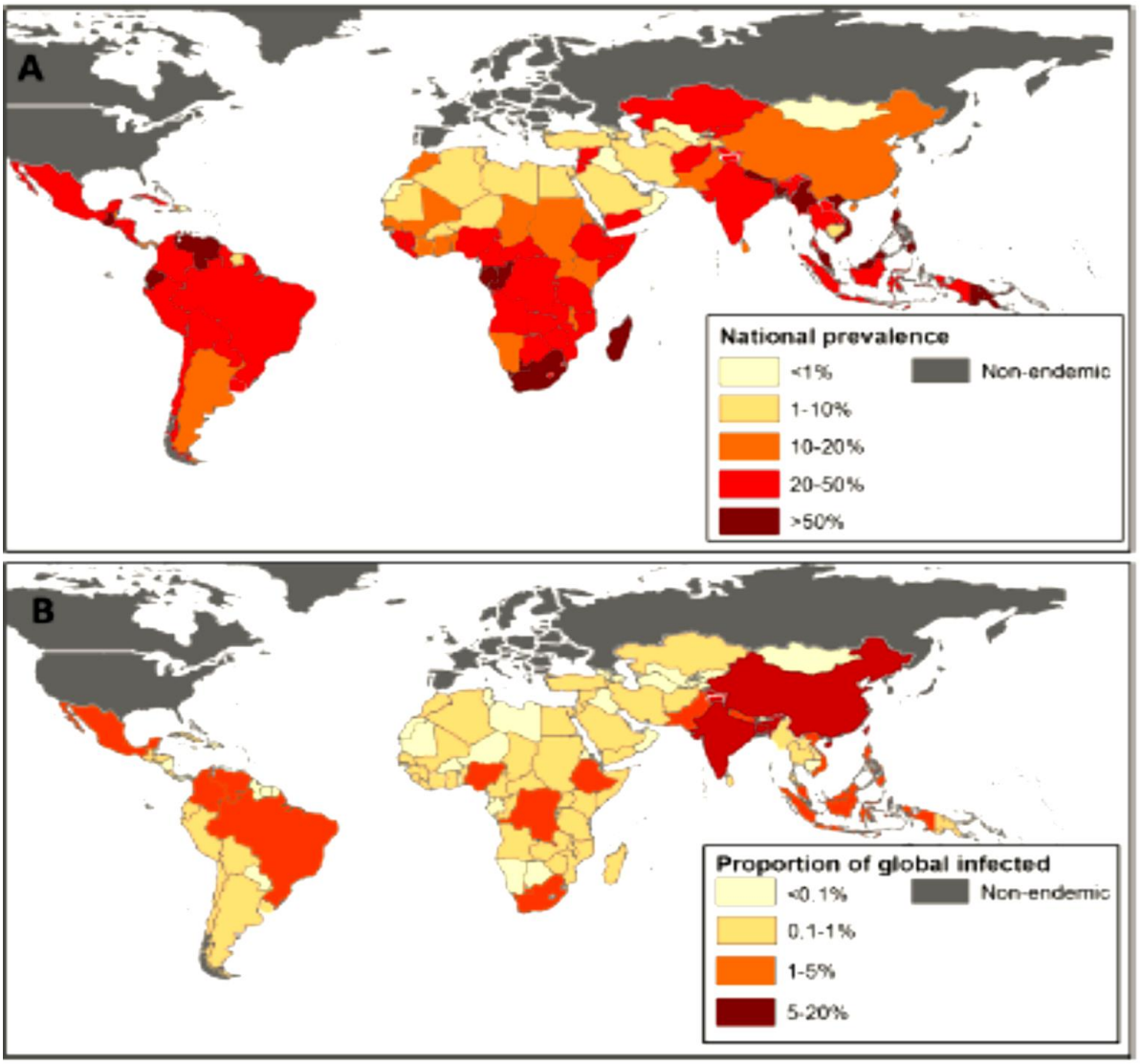

Figure 3: Distribution of STH infection in 2010. (A) Combined prevalence of all STH infections (B) The proportion of the global population infected by country. Source: Pullan et al., 2014.

Overall estimates show that out of the over 800 million people in sub-Saharan Africa, 117.7 million, 117.9 million and 100.8 million are infected with hookworms, *A. lumbricoides* and *T. trichiura* respectively (Pullan et al., 2014). Hookworm infections are most prevalent in Togo and Sierra Leone, while *A. lumbricoides* and *T. trichiura* infections are the highest in Gabon and Rwanda (Karagiannis-Voules et al., 2015).

Approximately 54% of South Africans live in poverty (StatsSA, 2014) where their living conditions increases their risk of STH infections. For instance, in the province of KwaZulu-Natal, a significant proportion of the people live in environments where there is a lack of adequate sanitation (22.7%) and safe water supplies (15.8%) (StatsSA, 2014). With these generally poor living standards, the prevalence of STH infections is high (Mkhize et al., 2017). Overall STH infections in KwaZulu-Natal are between 20% (Kwitshana et al., 2008) to 39% (Mkhize et al., 2017; Molvik et al., 2017) where *A. lumbricoides*, *T. trichiura* and hookworms are the most prevalent (Appleton et al., 1999; Kwitshana et al., 2008; Gwetu et al., 2015; Molvik et al., 2017). Approximately, 10.7-32.3%, 3.2-6.7% and 1.8% of people in the province are infected with *A. lumbricoides, T. trichiuris* and hookworm respectively (Kwitshana et al., 2008; Gwetu et al., 2015).

In the Western Cape Province, especially around Cape Town, the median school-based STH prevalence of 41% has been reported (Adams et al., 2004; Adams et al., 2005; Fincham et al., 2001). This is enhanced in slum-like areas of the city of Cape Town where more than 90% of children were infected with *Ascaris* spp and/or *Trichuris* spp 15-20 years ago (Adams et al., 2005). The combined prevalence of STH infections in South Africa, with high percentages reported along the coastal and rural areas of Kwazulu-Natal as well as the Western Cape are illustrated in Fig 4.

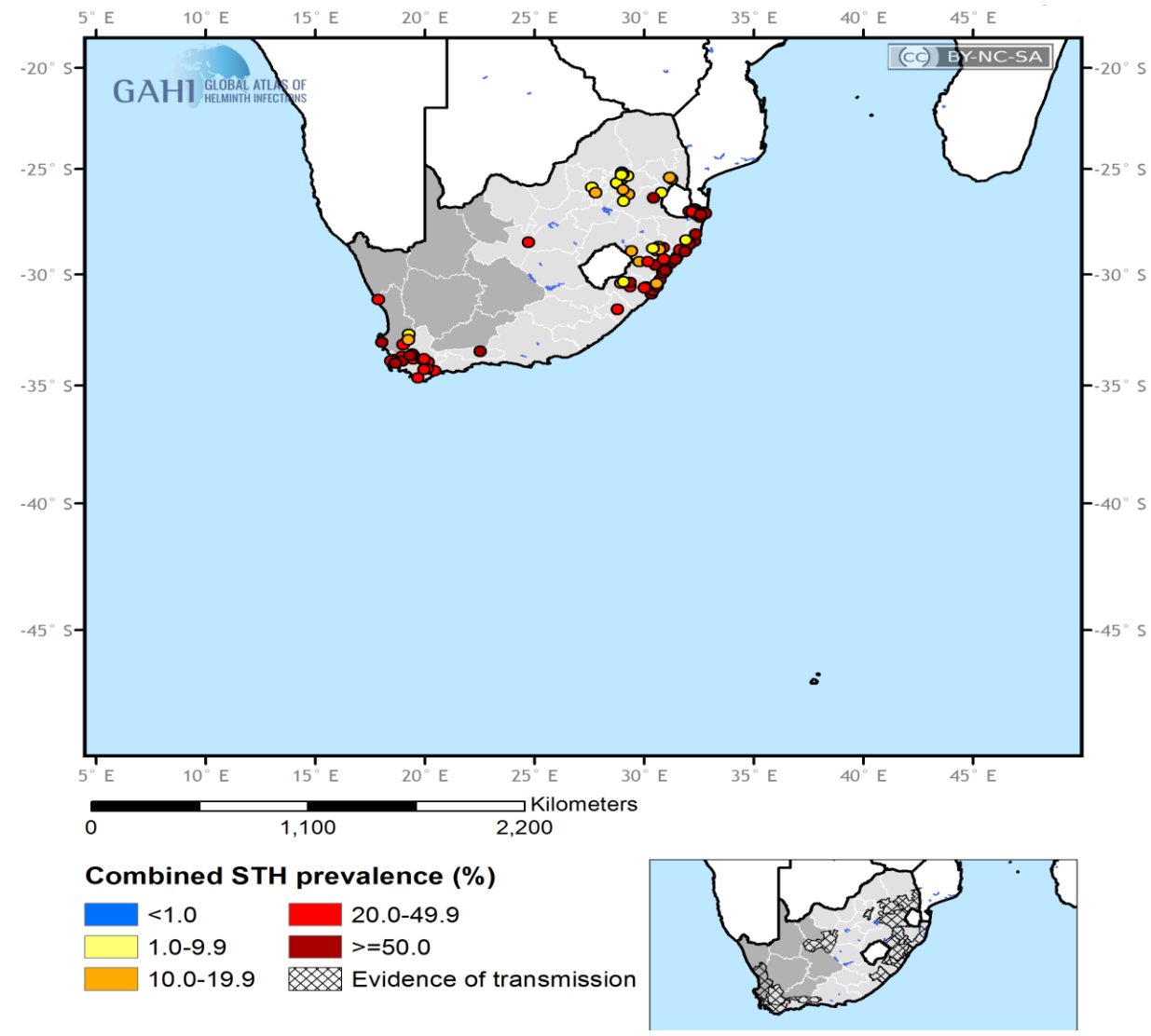

Figure 4: Combined prevalence of STH infections in South Africa. Source: Global Atlas of Helminth Infections (http://www.thiswormyworld.org/).

2.3 Impact of STH infections

STH infections mainly cause morbidity and disability and their global burden is well addressed by the disability-adjusted life years (DALYs) (Bethony et al., 2006). Despite the differences in the DALY estimates quoted since the use of this tool started, human helminthiases are ranked as the largest burden of the neglected tropical diseases (NTDs) in terms of DALYs (GBD, 2010; Hotez and Herricks, 2015). Globally, 5.18 million DALYs are attributed to STH infections, with

hookworm, *A. lumbricoides* and *T. trichiura* contributing 3.23 million DALYs, 1.31 million DALYs and 0.64 million DALYs respectively (Pullan et al., 2014).

Generally, STH infections have been associated with growth impairment (Brooker et al., 1999), in addition to other health impacts. For instance, hookworm infections are a known threat to maternal and child health, contributing to moderate and severe anaemia in school-age (Crompton et al., 1990; Smith and Brooker, 2010; Casmo et al., 2014) and pre-school children (Brooker et al., 1999; Stoltzfus et al., 2000). Approximately 30-54% of moderate to severe anaemia in pregnant women from Africa and Asia is caused by hookworm infections (Stoltzfus et al., 1997; Navitsky et al., 1998). In Kenya, *Ascaris* spp infections were found to be associated with a decrease in weight of 6 month old children (LaBeaud et al., 2015). The target group for anthelminthic treatment is also school age children (Bethony et al., 2006).

To reduce the morbidity caused by STH infections, the World Health Organization (WHO) has set a target of reducing infections in pre-school aged and school aged children to an acceptable level by 2020 (WHO, 2012). Similarly, the 2012 London Declaration on NTDs has set a target to achieve preventive chemotherapy coverage of 75% for all pre-school and school aged children by 2020 (WHO,2013; Dunn et al., 2016).

2.4 Risk factors for STH infection

Preventive chemotherapy has been shown to greatly reduce the morbidity from STH infections (Strunz et al., 2014), but in endemic areas re-infection is rapidly occurring (Jia et al., 2012). This highlights the need to identify the risk factors for STH infections for long-term control and potential elimination. Water, sanitation and hygiene (WASH) has been shown as the main determinants for human STH infections (Bartram and Cairncross, 2010), confounded by poverty (Vandemark et al., 2010; Alemu et al., 2010; Ziegelbauer et al., 2012). Data from the United States

of America, Korea and Japan, shows that improvement in WASH together with deworming was instrumental in the elimination of STH infections (WHO, 2006; Hong et al., 2006; Kobayashi et al., 2006).

2.4.1 Effect of access to water on STH infections

Access to potable water generally result in lower risk of STH infections (Strunz et al., 2014). Access to piped-water decreased the odds of infections with *A. lumbricoides* (OR 0.40, 95% CI 0.39-0.41) and *T. trichiura* (OR 0.57, 95% CI 0.45-0.72) as compared to the use of surface water (Strunz et al., 2014). In Malaysia children with access to clean water had less infections with hookworm (Nasr et al., 2013), whereas inadequate water supply in schools increased STH infection of school children (Hughes et al., 2004). Additionally, the collection of water in dirty and open receptacles is associated with significantly higher risks of STH infections (Quintero et al., 2012; Worrell et al., 2016). The impact of access to potable water on STH infections is mainly due to improved hygiene, for instance handwashing is associated with lower transmission of ascariasis (Bartram and Cairncross, 2010; Fung and Cairncross, 2009). Handwashing before eating and after defecating is associated with lower odds of 0.38 (95% CI 0.26–0.55) and 0.45 (95% CI 0.35–0.58) for *A. lumbricoides* infections (Strunz et al., 2014).

Information on the location of water sources in relation to STH infections is limited, but in Ethiopia Belyhun et al., (2010) showed that the use of an outside water pipe resulted in higher infections of infants than indoor taps. In western Tajikistan, collection of water from rivers/streams gave higher odds of helminth infections as compared to individuals with tap water within their yards (Matthys et al., 2011). Association between the sites/location of water collection points, such as springs or dug wells, and hookworm infections has been established. Infection with hookworm was

significantly higher for individuals collecting water from points far from their households (Echazú et al., 2015). Walking barefooted could result in exposure to the infective larvae of hookworms and thereby increasing the risks of infections (Bogitsh and Cheng, 1990; Jimenez-Cisneros and Maya-Rendon, 2007). Wearing shoes has also as expected been associated with lower odds of hookworm infections (OR 0.29, 95% CI 0.18-0.47) (Strunz et al., 2014).

2.4.2 Sanitation and STH infections

Access to sanitation is associated with reduced STH infection and is a key component of integrated STH control programs (Bartram and Cairncross, 2010; Mara et al., 2010; Ziegelbauer et al., 2012). The likelihood of STH infections with access to sanitation was found to be lower (OR 0.66, 95% CI 0.57–0.76) (Strunz et al., 2014). The highest reduction in STH infection with access to improved sanitation has been reported for *A. lumbricoides*, and *T. trichiura* (Ziegelbauer et al., 2012; Oswald et al., 2017) but did not result in lower hookworm infections (Strunz et al., 2014), which may be due to the different mode of transmission.

In Kenya it was found that the location of toilet facilities outside the household premises led to a significantly higher prevalence of STH infection (Worrell et al., 2013) as compared to facilities within the household premises. Oswald et al., (2017) found no protective association between community sanitation usage and STH infections in Ethiopia and concluded that the association between shared sanitation and STH infections is complex and require further studies. Poorly maintained toilet facilities have been shown to be a focal point for re-infections (Campbell et al., 2014). Therefore, despite the importance of availability of sanitation facilities the transmission could still be high. In Vietnam prevalence of STH was found to be high due to the use of “night

soil" (human excreta) as fertilizer in agriculture, despite a sanitation coverage of about 98.1% (Yajima et al., 2009).

2.4.3 Contamination of soil with STH eggs

Soil contamination with STH eggs presents a potential health hazard, due to the resistant nature of these eggs. Soil contamination has been established as one of the direct ways of STH infections (Rai et al., 2000; Mabaso et al., 2004). Due to the important role that soil plays in the transmission of STH infections, several studies have concentrated on the prevalence and concentration in soil from public areas, such as parks, playgrounds, sandpits, beaches, backyards and gardens, farmyard and other urban and rural defined soil related areas (Blaszkowska et al., 2011). The prevalence of the STH eggs in soil varies greatly depending on the study area, as well as the type of STHs (Steinbaum et al., 2016; Blaszkowska et al., 2011; Horiuchi et al., 2013; Baker and Ensink, 2012). *Toxocara* spp is the most prevalent (Oge and Oge, 2000; Ferré and Dorchies, 2000; Giacometti et al., 2000; de Ybanez et al., 2001) mostly found in parks frequented by animals while in human settlements most infections are caused by *Taenia* spp and *Ascaris* spp (mainly *A. lumbricoides*) (Mabaso et al., 2004). Most of these studies focused on public areas in urban centers but there is evidences of high risks of infections (especially toxocariasis) in rural areas as well (Holland et al., 1995; Uhlikova and Hubner, 1998; Luo et al., 1999; Borecka et al., 2010). These STHs (*Toxocara* spp) are zoonotic infections, infecting dogs, cats and humans, so the presence of animal feces is a major human health concern (Rai et al., 2000). The prevalence of *Toxocara* spp eggs in public areas ranges from 1-78% (Mizgajska, 1997; Matsuo and Najashio, 2005; Dubná et al., 2007; Tavassoli et al., 2008; Klapec, 2009).

The prevalence of STH eggs in soil is also dependent on the type of soil, where studies in KwaZulu-Natal by Appleton and Gouws (1996), Appleton et al., (1999) and Mabaso et al., (2003) found that infections with hookworm were higher in areas with sandy soils of the coastal plains in the region. Although data on the concentration of eggs in the soil were lacking in the above referenced studies, it was assumed that the infection prevalence could be attributed to the suitability of the soil type for development of the eggs.

Contamination of soil with faecal matter (both human and animals) is the main cause for STH egg prevalence in soil but additionally the use of wastewater and sludge in agriculture could also contribute significantly to the contamination of farm soil with STH eggs. Accumulation of STH eggs in farm soil occurs due to multiple applications of wastewater and sludge on the same parcel of land (Amoah et al., 2016; Blaszkowska et al., 2011). Coupled with the persistence of these eggs in the environment, STH egg concentration on farm soils could be higher than concentrations in the wastewater or sludge resulting in higher infection risks (Seidu et al., 2008a; Amoah et al., 2016).

2.5 Wastewater/sludge reuse in agriculture

Wastewater and sludge reuse in agriculture has been promoted as a concept of sustainable development (van der Hoek et al., 2002). Wastewater may be used for agriculture either directly or indirectly through the use of wastewater contaminated surface water. Contamination of surface water with wastewater may occur due to poor infrastructure, such as leaking sewage pipes and faulty wastewater treatment plants, (Rutkowski et al., 2007). Rapid urbanization, especially in cities of developing countries, is one of the major drivers for wastewater irrigation (Drechsel et al., 2006). Increasing water shortage globally, especially in arid regions (van der Hoek et al., 2002),

has also created conditions under which farmers have to find alternative sources of water. Approximately 330 km^3 of municipal wastewater is produced per year (Mateo-Sagasta et al., 2015) which makes wastewater a valid available alternative for irrigation if just volume is accounted for (Rutkowski et al., 2007). This practice is linked to improved livelihood for farmers in urban areas and also contributes to the urban food basket (Amoah et al., 2011). Although wastewater irrigation is most prevalent in developing countries, its use has also been documented in developed countries in Northern America, Europe and Australia (Angelakis et al., 1999; Crook, 2003; Lallana et al., 2001; Rutkowski et al., 2007).

Approximately 5-20 million hectares are irrigated with wastewater worldwide, with China constituting the largest share of this estimate (Drechsel and Evans, 2010). In Mexico alone, approximately 350,000 hectares are irrigated with wastewater (Peasey et al., 2000). Inadequate treatment of wastewater, which results in large-scale pollution of surface water suggest that these areas covered by wastewater irrigation schemes could be substantially larger (WWAP, 2017). Additionally, municipal wastewater alone could potentially irrigate about 40 million hectares of agricultural lands (representing 15% of all irrigated lands) (WWAP, 2017).

Sludge contains high nutrient content and organic material (Pompeo et al., 2016) and is therefore used extensively in agriculture as a soil amendment (Julca-Otiniano et al. 2006). Additionally, it is used to rehabilitate degraded, exhausted and burnt soils (Jiménez and Álvarez 2005; Guerrero et al. 2007). Sludge use has also been reported to help improve water holding capacity of soils (Navarro-Pedreño et al. 2003; Almendro-Candel et al. 2002) and has therefore found widespread application in agriculture to improve crop yields.

2.5.1 Wastewater/sludge reuse and the health implications

Despite the many benefits of wastewater and sludge in agriculture, their use can have adverse impacts on human health (WHO, 2006a; WHO, 2006b; Pham-Duc et al., 2013). Wastewater may contain several contaminants, such as metalloids/metals (Castells, 2012; Lam et al., 2015), excess nutrients, hormones (Burkholder et al., 2007; Dalsgaard,2007; Lam et al., 2015), organic compounds such as pesticides, components of consumer products, pharmaceuticals and personal care products (Yang et al., 2016; Arvaniti et al., 2015; Semblante et al., 2015; Harrison et al., 2006) and most importantly pathogenic microorganisms (Krzyzanowski Jr et al., 2016; Gerba and Smith, 2005; Jiménez et al., 2007; Pepper et al., 2008; Navarro et al., 2009).

STH infections are the most important health concern in wastewater and sludge reuse (WHO, 2006), especially in endemic regions, mainly due to their persistence in the environment and the low infectious dose (Melvin et al., 2001; Nelson and Darby, 2001; Toze, 2006; Crompton and Nesheim, 2002; Stephenson et al., 2000). Concentration of STH eggs in wastewater/sludge is an indication of the health hazard for their application (Zdybel et al., 2015: Gaspard et al., 1995). The prevalence and concentration of these eggs in wastewater and sludge is largely influenced by the infection and carrier-ship status of the population. These concentrations vary between locations (Table 1), with high concentrations generally reported in developing countries where STH infections are endemic.

Direct contact with the wastewater and sludge during the application stage therefore increases the risks of STH infections for farmers, farm workers and communities living close to the application sites (Lam et al., 2015).

Table 1: Concentration of STH eggs in wastewater and sludge from different locations

Country	Wastewater (eggs/L)	Sludge (eggs/g)	References
Egypt	6-42	Mean: 67; Maximum: 735	Stott et al., 1997
Ghana	12.9-15.1	13-94	Jimenez and Wang, 2006; Sengupta et al., 2011; Koné et al., 2007
Morocco	840	3.3-13.3	Jimenez-Cisneros and Maya-Rendon, 2007; Moubarrad and Assobhei, 2007
South Africa	772	25-185	Pillay et al., 2005; Amoah et al., 2018
Tunisia	15-30	0-4	Jimenez-Cisneros and Maya-Rendon, 2007; Saddoud et al., 2007; Riahi et al., 2009; Khouja et al., 2010
Brazil	166-202	75	Jimenez and Wang, 2006; Bastos et al., 2013
United States	1-16	2-776	Jimenez and Wang, 2006; Rose et al., 1996; Bowman et al., 2003; Engohang-Ndong et al., 2015
Mexico	6-98	73-177	Jimenez and Wang, 2006; Pecson et al., 2007
Peru	115-273	60-260	Yaya-Beas et al., 2016; Jimenez-Cisneros and Maya-Rendon, 2007
Japan	80	1-51	Jimenez-Cisneros and Maya-Rendon, 2007
China	840	2300	Jimenez-Cisneros and Maya-Rendon, 2007
Syria	800		Jimenez-Cisneros and Maya-Rendon, 2007
Vietnam	450-16000		Yen-Phi et al., 2010
Pakistan	142-558		Ensink et al., 2007
Ukraine	60	No data	Jimenez-Cisneros and Maya-Rendon, 2007
France	9	5-7	Jimenez and Wang, 2006; Gantzer et al., 2001
Germany	No data	<1	Jimenez and Wang, 2006
Great Britain	No data	<6	Jimenez and Wang, 2006
Spain	0-1	867	Molleda et al., 2008; Abreu-Acosta and Vera, 2011; Reinoso et al., 2008; Reinoso and Becares, 2008

Indirect exposure to wastewater/sludge through the consumption of crops (especially vegetables) fertilized and irrigated with these may also lead to STH infections (Drechsel et al., 2010).

Contamination of vegetables with STH eggs has been reported extensively, especially from wastewater irrigated fields (Al-Megrin, 2010; Adamu et al., 2012; Said, 2012; Adanir and Tasci, 2013; Fallah et al., 2016; Rostami et al., 2016). The consumption of these vegetables, if contaminated, is therefore an exposure route that increase the risks of STH infections for larger populations.

The association between wastewater/sludge use in agriculture and STH infections has been reported by several researchers (Pham-Duc et al., 2013; Yajima et al., 2009; Trang et al., 2007; Uga et al., 2009). Wastewater/sludge reuse is shown to result in significantly higher STH infections (Pham-Duc et al., 2013; Trang et al., 2007; Nguyen et al., 2006; Van der Hoek et al., 2003; Amoah et al., 2016). Some studies have however failed to document an increase in STH infections associated with wastewater/sludge reuse (Pham-Duc et al., 2013; Trang et al., 2006; Trang et al., 2007). Yajima et al., (2009) concluded that direct exposure to sludge does not lead to an increase in STH infections but rather the consumption of vegetables fertilized with faeces. Table 2 summarizes selected studies on the association between wastewater/sludge use in agriculture and STH infections.

Table 2: Studies reporting on the association between wastewater/sludge use in agriculture and STH infections

Author/Year	Target group	Practice	Health risk and Conclusions
Pham-Duc et al., 2013	Farming households	Wastewater and excreta	Contact with wastewater was a significant risk factor for helminth infection (OR = 1.5, 95% CI 1.1–2.2) and *Ascaris lumbricoides* infection (OR = 2.1, 95% CI 1.4–3.2). Significant risk factors for *Trichuris trichiura* infection include the use of human excreta (OR = 1.5, 95% CI 1.0–2.3).
Yajima et al., 2009	Community members	Human excreta only	Consumption of vegetables fertilized with human excreta resulted in high helminth infection rate.
Trang et al. 2006	Farming households	Wastewater	No significant association was found between wastewater exposure and helminth infections.
Trang et al. 2007	Adults and children	Wastewater and human excreta	Wastewater exposure did not pose a significant risk for helminth infection.
Nguyen et al., 2006	Women	Excreta	The use of untreated feces as fertilizer was significantly associated with *A. lumbricoides* (OR = 1.2, 95% CI 1.0–1.6).
van der-Hoek et al., 2003	Community members	Human excreta only	The use of human excreta as fertilizer was a significant risk factor for hookworm infection, especially among adult women.
Pham-Duc et al.,2011	Community members	Wastewater and excreta	Personal hygiene factors determined infection with *E. histolytica*, rather than exposure to human and animal excreta in agricultural activities.
Uga et al., 2009	Community members	Excreta	Vegetables purchased at a market in Vietnam were highly contaminated with parasite eggs excreted by animals and humans
Habbari et al., 2000	Children	Wastewater	Significant increase in prevalence of ascariasis for exposed children.
Bouhoum and Schwartzbrod, 1998	Children	Wastewater	Higher prevalence among exposed of any helminth infection (73% exposed vs. 30% unexposed), *Ascaris* infection (33% vs. 2%), and *Trichuris* infection (17% vs. 2%)
Blumenthal et al., 2001	Agricultural workers and their family members	Wastewater	higher prevalence of *Ascaris* infection among exposed compared to unexposed for children

Cifuentes et al., 1994	Agricultural workers and their family members	Wastewater	higher prevalence of diarrheal disease (30% vs. 23%) and *Ascaris* infection (15% vs. 3%) for exposed children
Amoah et al., 2016	Agricultural workers and their family members	Wastewater	Increased odds of infection for farmers for both *Ascaris* spp and hookworm for farmers and family members as compared to unexposed populations
Fuhrimann et al., 2016a	Community members	Wastewater	High prevalence of intestinal parasite infections for peri-urban farmers, using wastewater for irrigation, as compared to other groups

2.6 Quantification of STH infection risks associated with wastewater/sludge use in agriculture

Health risks associated with wastewater/sludge reuse in agriculture may be assessed either directly (e.g epidemiological studies) or indirectly (e.g quantitative microbial risks assessments) (Dickin et al., 2016). It is important to differentiate between potential risks as well as actual infections when conducting health risk assessments. For instance, individuals involved in wastewater/sludge reuse in agriculture may be exposed to contaminants but not all exposed individuals will be infected and not all infections will lead to illness. Development of illness in infected individuals is dependent on a number of factors, such as, the hosts (e.g. vulnerability, behavior etc.), the environment (e.g., sanitation, provisions of safe water or disposal of excreta) and agent factors (e.g., infectious dose, infection causes disease or furthers transmission) (Lam et al., 2015).

2.6.1 Epidemiological approach

Epidemiological investigations are used to determine the direct link between exposure to wastewater/sludge and the risks of STH infections. Different epidemiological approaches have been applied for the quantification of STH infections risks associated with wastewater/sludge reuse in agriculture (See Table 3 for a summary of these studies), making it an essential tool for quantification of risks. Epidemiological studies are generally more readily understood and accepted by the general public than using theoretical models for risk prediction (Sinclair et al., 2010) but are limited by study design and sensitivity (O'Toole et al., 2012). Additionally, these studies are much more expensive as compared to indirect estimations of risks (Lam et al., 2015).

Table 3: Epidemiological studies used to estimate risks of STH infections associated with wastewater/sludge reuse.

Authors	Location	Health risks	Contamination pathways	Study design
Amahmid and Bouhoum,2005	Marrakech Morocco	Helminth infection (*Ascaris, Trichuris*)	Children living in wastewater irrigation areas, water, sanitation and hygiene, occupational parents)	Cross-sectional study of an exposed group and control group children (n = 610)
Blumenthal et al., 2001	Mezquital Valley, Mexico	Ascariasis and diarrhoeal disease	Occupational (workers and household), water, sanitation and hygiene, consumption of market vegetables	Cross-sectional survey with 850 agricultural households using untreated wastewater for irrigation, 950 households using wastewater stored in a reservoir, and 930 control households (rain-fed agriculture), with a total of 10,489 children and adults
Ensink et al., 2005	Faisalabad, Pakistan	Hookworm infection	Occupational (farmers and their children), water sanitation and hygiene, accidental ingestion of those living in the area, cattle ownership	Cross-sectional survey of wastewater farmers, textile laborer's and farmers using regular irrigation water, with children assigned to their fathers' exposure group (n = 1,704)
Ensink et al., 2008	Hyderabad, India	Intestinal nematode infection	Occupational (entire household), water, sanitation and hygiene, cattle ownership	Cross-sectional survey with 3 exposure groups of farmers exposed to untreated, and partially treated wastewater and river water (n = 1,078)
Gumbo et al., 2010	Malamulele, South Africa	A range of parasitic infections including hookworm and *Giardia lamblia* infection	Occupational (entire household), consumption of contaminated vegetables, water, sanitation and hygiene	Cross-sectional survey of farmers and their children exposed to wastewater irrigation (n = 194) and those not using wastewater for irrigation (n = 249)
Habbari et al., 2000	Beni-Mellal, Morocco	Helminth infection (*Ascaris*, *Trichuris*) in children	Children living near wastewater irrigated areas, water, sanitation and hygiene, occupation of parents	Cross-sectional survey of 740 children in communities using wastewater, and 603 children in communities not using wastewater irrigation
Trang et al., 2006	Nam Dinh city, Vietnam	Helminth infection (*Ascaris*, *Trichuris* and hookworm)	Occupational (rice cultivation), water, sanitation and hygiene, use of human excreta for agriculture	Cross sectional survey, 202 households in a commune where wastewater was used for irrigation and 201 households in a commune that used river water, with a total of 1,088

				individuals >15 years
Trang et al., 2007	Hanoi, Vietnam	Helminth infections (*Ascaris, Trichuris* and hookworm)	Occupational (workers and children) water, sanitation, and hygiene, use of human excreta for agriculture, animal husbandry	A cross-sectional study with 400 agricultural households (620 adults and 187 children)
Fuhrimann et al., 2016a	Kampala, Uganda	Intestinal parasites (*Trichuris, Ascaris*, hookworm)	Urban farmers, slum dwellers at risks of flooding and slum dwellers at no risk of dwelling	A cross-sectional study with 915 adults
Fuhrimann et al., 2016b	Hanoi, Vietnam	Intestinal parasites (hookworm, *Trichuris*)	Workers at wastewater treatment facilities, urban farmers using wastewater and urban dwellers at risks of flooding	A cross-sectional survey of people aged above 18 years

2.6.2. Quantitative Microbial Risk Assessment

Quantitative Microbial Risk Assessment (QMRA) is a modelling process that estimates the potential human health risks from exposure to different pathogens (e.g. human pathogenic viruses, protozoa, and bacteria) (Haas et al., 2014) especially through food and/or water (Beaudequin et al., 2015). This is a structured approach that integrates information and data with mathematical models to examine exposure and spread of microbial pathogens and in this process to characterize the human health risks (CAMRA, 2013; Havelaar, 2012). QMRA is comprised of four interrelated steps as presented in Table 4, where each component is explicitly quantified.

Table 4: Steps involved in Quantitative Microbial Risk Assessment (QMRA) (WHO, 2016.)

Step	Description
Problem formulation	The overall context (reference pathogens, exposure pathways, hazardous events and health outcomes of interest) of the risk assessment is defined and constrained in order to successfully target the specific risk management question that must be addressed
Exposure assessment	The magnitude and frequency of exposure to each reference pathogen via the identified exposure pathways and hazardous events are quantified.
Health effect assessment	Dose-response relationships (linking exposure dose to probability of infection or illness) and probability of morbidity and mortality (depending on the health end-point of the assessment) are identified for each reference pathogen.
Risk characterization	The information on exposure and health effects assessment are combined to generate a quantitative measure of risk.

QMRA could follow two formats or processes. The forward process, most commonly used, involves the selection of a reference pathogen to represent different classes of pathogens that are envisaged to have potential health impacts or prevalent in the study area. In the exposure assessment, all exposure points are considered, for instance, ingestion of wastewater/sludge during the application, consumption of farm produce etc. A dose response model is then chosen to estimate the probability of infection per exposed individual. The final step, risk characterization,

which is the resulting predicted risks is compared to a benchmark probability of infection or converted to DALYs (Murray and Acharya 1997). QMRA could also be conducted in the reverse process, where a health target (either a tolerable risk value or DALY) is the starting point and the dose of pathogen is related to this health target. This reverse approach has been used by the World Health Organization (WHO) and the Australian government (NRMMC, 2004; NRMMC et al., 2006; WHO, 2006) to determine pathogen reductions needed to achieve a health target of 10^{-6} DALYs per person per year (ppy) (Schoen and Garland, 2017). QMRA has been used extensively in the risk estimation of STH infection for different exposed groups related to wastewater reuse and/or sludge application in agriculture (Cutolo et al., 2012; Navarro et al., 2009; Navarro et al., 2008; O'Connor et al., 2017; Kundu et al., 2014; Schönning et al., 2007; Seidu et al., 2008a; Seidu et al., 2008b). The risk estimates from these studies vary, mainly due to differences in the concentration of viable STH eggs ingested by the exposed populations. Additionally, the volume/weight of wastewater/sludge ingested by the different populations also contributes to the variation in risk estimates.

2.7 Guidelines and Regulations to protect public health associated with wastewater/sludge reuse

As discussed previously, the use of wastewater/sludge in agriculture may lead to proliferation of helminthiases and has an adverse effect on public health. The WHO has recommended limits for helminth eggs in wastewater/sludge intended for agricultural purposes, with a limit of 1 viable helminth egg per liter in wastewater and the same number of helminth eggs per gram of total dry solids for sludge as the guidelines (WHO, 2006). The United States Environmental Protection Agency (USEPA) has stricter standards with 0.25 eggs per gram of total dry solids for sludge used in unrestricted agriculture (Class A sludge) (USEPA, 1993). Additionally, wastewater and sludge

regulations have been set by several countries following the WHO guidelines and USEPA standards, as summarized in Table 5. The South African guidelines for wastewater/sludge reuse published in 2009 adopted guideline values as set by the USEPA (Herselman and Moodley, 2009).

Table 5: Regulation and guideline limits for wastewater and sludge reuse in different countries (adopted from Jiménez et al., 2017).

Country/ Body	Regulation standard guidelines	Class/type	Helminth egg limit, eggs per liter/g TS
WHO	WHO, 2006	-	<1 egg
USA	Department of Health Services (DHS), California, 1918. (wastewater); CFR 40 Part 503 /1993(sludge)	N/A (wastewater) Class A (sludge)	0 egg/L 0.25 egg/g TS
Mexico	NOM-001-SEMARNAT-1996 (wastewater); NOM-004-SEMARNAT-2002(sludge)	U(wastewater) R (wastewater) Class A (Sludge) Class B (Sludge) Class C (Sludge)	<1 egg/L <5 egg/L <1 egg/g TS <10 egg/g TS <35 egg/g TS
Brazil	CONAMA 375/2006	Class A(Sludge) Class B(Sludge)	0.25 egg/g TS 10 egg/g TS
Chile	No. 123 (30/08/2006) (sludge); Norma 1333 (1978) (wastewater)	Class A(Sludge) U(wastewater) R(wastewater)	0.25 egg/g TS - <1 egg/L
France	Directive 86/278	-	0.3 egg/g TS
New Zealand	Guidelines for the safe application of biosolids to land in New Zealand	Class A(Sludge)	0.25 egg/g TS
Norway	Regulations for the treatment, use and disposal of sewage sludge	-	Absent
South Africa	Guidelines for the utilization and disposal of wastewater sludge (Vol. 2)	Class A(Sludge) Class B(Sludge)	0.25 egg/g TS 1 egg/g TS

U: Unrestricted irrigation: For agricultural irrigation of all kinds of crops, including those that are eaten uncooked (lettuce, onion); R: Restricted irrigation: For agricultural irrigation of all kinds of crops, except that are eaten uncooked (highly mechanized, labor intensive).

2.8 Removal/inactivation of STH eggs during wastewater/sludge treatment

The development of wastewater/sludge reuse guidelines makes it imperative for treatment of these to meet the set limits before reuse. Unlike many other microorganisms, STH eggs are very resistant (Jiménez et al., 2017) and cannot be inactivated with chlorine, UV light or ozone, which are the processes conventionally used for disinfection (Jiménez et al., 2017).

STH eggs form part of the total solids in wastewater and their removal during wastewater treatment is mainly through sedimentation, flotation and coagulation-flocculation (Jimenez-Cisneros, 2006; Jimenez-Cisneros and Maya-Rendon, 2007). The main approach is to remove the eggs from the wastewater and inactivate them in the sludge (Jimenez-Cisneros, 2006), to achieve the respective guideline values/standards for the respective countries. The STH egg removal efficiencies for different wastewater treatment processes are summarized in Table 6 as removal percentages reported in literature.

Table 6: Wastewater treatment processes and the percentage of STH eggs removed/inactivated (Adopted from Jimenez-Cisneros and Maya-Rendon, 2007)

Treatment Process	Removal percentage
Primary sedimentation	0-<1
Chemically enhanced primary treatment	90-99
Trickling filters + secondary sedimentation	80-90
Activated sludge + secondary sedimentation	85-95
Aerated lagoon or oxidation ditch + settling pond	85-90
Coagulation/flocculation as tertiary treatment	99
High-rate granular or slow-rate sand filtration	>99
Membrane bioreactors	>99,99
Waste stabilization ponds	78-99
Anaerobic up flow sludge blanket reactors	75-96
Wastewater storage and treatment reservoirs	90-99
Constructed wetlands	90
Dual-media filtration	99
Sand dunes infiltration	100

The percentage of eggs removed varies between the different species of STH eggs, largely depending on the sedimentation rate (Mara and Horan, 2003). Generally, *Ascaris* spp eggs have a higher settling velocity (0.77 m/h) compared to the other types of STH eggs, mainly due to a higher specific gravity, 1.2 g/cm^3 as compared to 1.15 g/cm^3 and 1.055 g/cm^3 for *Trichuris* spp and hookworm respectively (Medema et al., 1998; David and Lindquist, 1982; Shuval et al., 1986 and Pike, 1990). Therefore, removal efficiency of *Ascaris* spp eggs during treatment, with most of the conventional technologies may be assumed to be higher than the other common STH eggs.

STH eggs accumulated in sludge during the wastewater treatment must be inactivated to ensure public safety. This inactivation is generally achieved through high temperatures, low moisture content and sometimes the addition of disinfectants (Jimenez-Cisneros and Maya-Rendon, 2007). To achieve the WHO guideline value of 1 helminth egg/g TS, sludge in developing countries will need to undergo treatment processes which inactivate between 98.6 to 99.9% of all viable eggs (Jimenez-Cisneros and Maya-Rendon, 2007). For instance, with STH egg concentrations ranging from 25-185 eggs/g, sludge in South Africa will require further treatment to achieve the 0.25 eggs/g TS for Class A or 1 egg/g TS for Class B sludge. This further treatment could be a combination of treatment technologies or a single technology that can inactivate the viable STH eggs to the required standard value. Table 7 presents some of the sludge treatment processes and the STH egg inactivation achievable.

Table 7: Sludge treatment processes and the STH inactivation percentages achievable.

Process	STH egg inactivation	Reference
Lime post-stabilization	100% from an initial value of 8 HO*/L	Keller et al., 2004
Lime Stabilization	100% with slaked lime heated to 50ºC during 48 min with an initial content of 70 *Ascaris*/g TS	Capizzi-Banas et al., 2004
Quick Lime post-stabilization	At pH 12 for 2 hours 65-92% using 20-40% lime in w/w	Mendez et al., 2002
Mesophilic digestion	With 15 days retention time at 35-55ºC or 60 days at 20ºC less than 30 %	Jimenez and Wang, 2006
Thermophilic aerobic digestion at 48 ºC	Reduction from 4,5 ± 3,2 to <1 viable ovum/10g TS in 30 days, an average efficiency of 78%	Gantzer et al., 2002
Thermophilic anaerobic digestion at 45-65ºC	75 to 78% with HO of around 80 eggs/g TS	Rojas-Oropeza et al., 2002
Thermal treatment at 108ºC	90-93% with an initial content of 9,5 HO/gTS	Gantzer et al., 2002
Irradiation at 750 Gy#	100% inactivation with an initial content of up to 100 HO g-1 TS	Capizzi et al., 1999
Irradiation at 1000 Gy	100% inactivation with an initial content of 88 HO g-1 TS	Capizzi and Schwartzbrod, 2001
Pasteurization at 70ºC	100% with an initial value of 8 HO g-1 TS	Keller et al., 2004
Acid treatment with peracetic acid	95% with an initial content of up to 100 HO g-1 TS	Barrios et al., 2004
Thermal Dry of Sludge Anaerobic Digested	100% sludge with initial high helminth ova content from Brazil	Andreoli et al., 2000
Co-composting	70-100% from initial content of 22-83 eggs/gTS, eggs have 20% viability	Gallizzi, 2003

*HO: Helminth ova

#Gy: Gray (the adsorption of one joule of radiation energy per kilogram of matter)

2.9 Accurate detection and quantification of STH eggs in the environment

The accurate detection and quantification of STH eggs in environmental samples, such as wastewater, sludge and soil, is critical to ensure that the regulatory guidelines are met. Additionally, accurate methods are essential to determine the efficiency of different treatment technologies. Varying amount of moisture, solids and soil particles introduce heterogeneity to environmental samples posing a challenge for accurate detection of STH eggs (Collender et al., 2015). An additional challenge in the detection of STH eggs in environmental samples is the need to recover small number of eggs from large sample volumes (Maya et al., 2006; Mes, 2003).

Over the years different analytical methods have been used (Collender et al., 2015; Bowman et al., 2003; Amoah et al., 2016). These methods are mainly modifications of the USEPA (USEPA, 1999) and WHO methods (Maya et al., 2006) and are largely based on the principles of sedimentation and/or flotation, involving the separation and concentration and a final microscopical identification of the eggs (Amoah et al., 2017; Collender et al., 2015). Despite the plethora of methods available, there is no international standardized method for the detection and quantification of STH eggs in environmental samples. This is mainly due to the lack of quality assurance/quality control (QA/QC) data on the various methods or method variants in use (Bowman et al., 2003). The choice of method is therefore dependent on the type of sample, availability of reagents, skills of the researcher and personal preferences. Most of these conventional or traditional methods (flotation and sedimentation based methods) are time consuming and may possibly lead to false results especially during the microscopy stage.

STH egg detection through microscopy requires trained personnel for correct identification of the eggs (Collender et al., 2015). Debris on the microscope slides may also introduce a further challenge for egg identification. Identification of the different species of STH eggs based on egg morphology is also a major challenge (Collender et al., 2015) but can be solved with the use of the

more advanced molecular techniques. Polymerase chain reaction (PCR)-based methods have made the identification of STH eggs to the species level much easier (Gyawali et al., 2015), by targeting specific gene sequences (Gordon et al., 2011). The high cost involved in most of these PCR–based techniques is the major challenge with their applicability, especially in resource constrained regions. Nucleic acid extraction from environmental samples is another challenge for the applicability of PCR-based techniques. Some of the STH eggs, for example *Ascaris* spp, have tough protective shells, which makes the destruction of the eggs and release of the nucleic material difficult (Salonen et al., 2010). Commercially available DNA extraction kits have been used in the extraction of DNA from STH eggs but these are not optimized for STH eggs. Additionally, environmental samples may contain substances and materials including proteins, lipids etc. which have been shown to be PCR inhibitors (Tebbe et al., 1993; Sørensen et al., 2002; Fortin et al., 2004; Zhou et al., 2002).

To solve the numerous challenges with the more traditional microscopy-based approaches or the PCR-based methods, new techniques such as the Baclight staining assay and image analysis software have been developed (Dabrowaska et al., 2014; Karkashan et al., 2015). Despite the reported applicability of these techniques, they are yet to be widely used and may require further validation. Table 8 bears reference and are included from the comprehensive review paper (**Paper I**) on the various methods and method variants used in the detection and quantification of STH eggs in the environment. This article evaluated the various methods in relation to the different environmental matrices (wastewater, sludge, soil etc.), comparing the advantages and disadvantages in each methodological approach. Based on assessments made in this thesis my recommended method is the one attached in Annex 1.

Table 8: Comparison of different techniques for detection of STHs in environment.

Method category	Techniques	Advantages	Disadvantages	Remarks
Traditional methods	WHO, USEPA, AMBIC, TULANE and several other variations of these methods	Easy to use, does not require sophisticated equipment hence less expensive.	Time consuming and laborious. Leaves too much for errors	These methods mostly consist of sedimentation and flotation using different flotation solutions (e.g. Zinc sulphate, magnesium sulphate, Sodium chloride) and a final microscopy step
Molecular Techniques	PCR, qPCR, LAMP	Rapid, quantitative (for qPCR), specific to species level identification	Expensive and requires skilled personnel	Nucleic acid based techniques are very specific and can provide species level identification that was difficult with conventional techniques. These techniques are laboratory based and can't be used for onsite detection of pathogens
Emerging techniques	digital PCR	More specific, quantitative, no need of reference standards as is the case in other PCR techniques	Expensive and requires skilled personnel	Digital PCR is new technique for absolute quantification, highly prone to PCR inhibitors. This technique needs to be optimized for environmental samples
	BacLight assay	Microscopic method to detect viability of STHs eggs	Expensive and requires skilled personnel	Initially developed for enumerating viable bacteria but can be applied for STHs. Need to validate for different environmental samples

PAPER I

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Detection and quantification of soil-transmitted helminths in environmental samples: A review of current state-of-the-art and future perspectives

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ABSTRACT

It is estimated that over a billion people are infected with soil-transmitted helminths (STHs) globally with majority occurring in tropical and subtropical regions of the world. The roundworm (*Ascaris lumbricoides*), whipworm (*Trichuris trichiura*), and hookworms (*Ancylostoma duodenale* and *Necator americanus*) are the main species infecting people. These infections are mostly gained through exposure to faecally contaminated water, soil or contaminated food and with an increase in the risk of infections due to wastewater and sludge reuse in agriculture. Different methods have been developed for the detection and quantification of STHs eggs in environmental samples. However, there is a lack of a universally accepted technique which creates a challenge for comparative assessments of helminths egg concentrations both in different samples matrices as well as between locations. This review presents a comparison of reported methodologies for the detection of STHs eggs, an assessment of the relative performance of available detection methods and a discussion of new emerging techniques that could be applied for detection and quantification. It is based on a literature search using PubMed and Science Direct considering all geographical locations. Original research articles were selected based on their methodology and results sections. Methods reported in these articles were grouped into conventional, molecular and emerging techniques, the main steps in each method were then compared and discussed. The inclusion of a dissociation step aimed at detaching helminth eggs from particulate matter was found to improve the recovery of eggs. Additionally the selection and application of flotation solutions that take into account the relative densities of the eggs of different species of STHs also results in higher egg recovery. Generally the use of conventional methods was shown to be laborious and time consuming and prone to human error. The alternate use of nucleic acid-based techniques has improved the sensitivity of detection and made species specific identification possible. However, these nucleic acid based methods are expensive and less suitable in regions with limited resources and skill. The loop mediated isothermal amplification method shows promise for application in these settings due to its simplicity and use of basic equipment. In addition, the development of imaging soft-ware for the detection and quantification of STHs shows promise to further reduce human error associated with the analysis of environmental samples. It may be concluded that there is a need to comparatively assess the performance of different methods to determine their applicability in different settings as well as for use with different sample matrices (wastewater, sludge, compost, soil, vegetables etc.).

Contents

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1. Introduction

It is estimated that over 1.5 billion people are infected with at least one species of soil-transmitted helminths (STHs) worldwide (WHO, 2015), with the majority of these infections caused by the roundworms (*Ascaris lumbricoides* and *Strongyloides stercoralis*), whipworms (*Trichuris trichiura*) and hookworms (*Necator americanus* or *Ancylostoma duodenale*) (Strunz et al., 2014). Ascariasis is reported in 771.7–891.6 million people, while 429.6–508.0 million people have trichuriasis and 406.3–480.2 million are infected with hookworm (Pullan et al., 2014). Most of these infections occur in tropical and subtropical regions of the world where poverty results in poor sanitary conditions (Stolk et al., 2016). STHs infections are mostly caused by exposure to faecally contaminated water, soil or contaminated food (Keraita and Amoah, 2011). Wastewater and sludge reuse is reported to contribute significantly to the high risk of infections. In endemic areas, wastewater is estimated to contain up to ~3000 eggs/L (Kamizoulis, 2008; Mara and Sleigh, 2010).

The association between ascariasis and wastewater use among farmers has been reported by several studies (Seidu et al., 2008; Blumenthal et al., 2001; Pham-Duc et al., 2013; Rutkowski et al., 2007; Trang et al., 2006; Habbari et al., 1999), where consumers of the farm produce are also at risk of infection. The highest health risks for consumers of wastewater irrigated produce are with crops which are eaten raw, for example, salad crops and some root crops or crops grown close to the soil surface (e.g. lettuce) (WHO, 2006). Wastewater for unrestricted reuse in agriculture should contain ≤1 helminth egg per liter to reduce the risk of STHs infections to below the WHO guidelines target level (WHO, 2006). This requires sensitive detection and a consistent quantification of STHs eggs in wastewater, sludge or other sample matrices. Accurate detection and quantification of STHs eggs in environmental samples is challenging. The heterogeneity of the occurrence of STHs in environmental samples is problematic for laboratory testing due to varying amounts of moisture, solids, quantity of samples and soil particles (Collender et al., 2015). Another challenge with environmental samples is the need to recover small numbers of STHs eggs from large volumes of samples (Maya et al., 2006; Mes, 2003).

Over the last few years, different techniques for detecting and quantifying the total number and the viable and non-viable fractions of STHs eggs in environmental samples has been developed and applied. The choice of technique used is largely influenced by the different types of samples (Maya et al., 2006). One reason for this may be the lack of published quality assurance/quality control (QA/QC) data on the various methods (Bowman et al., 2003). In addition to the more traditional methods based on sedimentation and/or flotation, which mainly involves the separation and concentration of eggs and the microscopical identification and quantification of these eggs, several new techniques have been developed. These new techniques make the identification and quantification of helminth eggs more efficient and sensitive. The advent of genomic sequencing and the wealth of data generated have markedly increased the feasibility of developing polymerase chain reaction (PCR)-based methods as diagnostic tools for helminth parasites (Gyawali et al., 2015). Defined gene sequences of STHs eggs can be detected with PCR, quantitative PCR (qPCR) and other nucleic acid based methods from small quantities of samples (Gordon et al., 2011). These techniques can also identify STHs eggs to species level (Gordon et al., 2011). Furthermore advanced nucleic acid based techniques (like multiplex PCR) makes it possible to target more than one species at a time (Pontes et al., 2002). The reliable quantification of viable eggs is essential both related to risk based target values and for the validation of sanitation system performance.

The lack of a globally accepted method for the detection and quantification of STHs eggs in environmental samples poses a challenge for comparative assessments of egg concentrations both in different sample matrices as well as between locations. This article presents a review of commonly used methods for the detection and quantification of STHs eggs in environmental samples, with a comparison of the advantages and disadvantages of each technique. We also review new and emerging techniques that may be applied for the detection and quantification of STHs eggs in the environment.

2. Methodology

2.1. Search strategy

The review is based on a literature search using PubMed and Science Direct, with the following keywords and strings (soil-transmitted helminths OR intestinal parasites OR *Ascaris* spp OR hookworm OR *Trichuris* spp OR *Toxocara* spp OR *Taenia*) AND (wastewater OR water). The organism search-string were repeated with AND (sludge OR compost); AND (vegetables OR crops OR plants); as well as with AND (soil OR urine diversion (UD) toilet waste OR biosolids). Original research articles were selected and the methodology section of each article was assessed to determine the method used in the detection and quantification of the STHs. All articles were considered irrespective of the year of publication.

Methodological studies on the concentration of STHs in environmental samples were included but articles and publications related to clinical STHs infections and drug efficiency/administration reports were excluded. All geographical areas were considered for the review, however only articles in English were included. A total of 195 articles were reviewed and information from 164 used, based on a clear method for detection of the STHs. Publications reporting the same method were assessed in relation to methodological variations and the type of sample matrices. When variations were not evident, one major reference to the methodology was considered and the other was captured as an additional method.

3. Conventional methods

Most methods used for the detection and quantification of STHs in environmental samples involve the recovery of STHs eggs from the sample matrix and quantification of STHs eggs or larvae using microscopy. These methods are collectively referred to as 'conventional methods' due to the application of basic techniques that includes sedimentation, followed by an extraction phase and then flotation with zinc sulphate (Ayres and Mara, 1996) before viewing under a microscope. The methods reviewed are categorized based on the major steps involved in the analysis. These steps mostly are the source of method variability, relating to sample types and quantity, pre-treatment, separation of STHs eggs, microscopy and viability determination.

3.1. Sample types and quantity

This section is focused primarily on the sample volumes studied and reported in the reviewed literature, with the subsequent sections below dealing with the various method variations and the influence of the different steps on the recovery of STHs eggs from the various sample matrices.

3.1.1. Wastewater/water

Helminth egg concentrations in wastewater (especially treated wastewater and water) are normally very low (Mara and Silva, 1987; Schwartzbrod et al., 1987; Ayres et al., 1991), mainly due to the dilution of egg concentration in the environment. Therefore, most methods use large sample volumes for detection. The concentration of the eggs is largely dependent on the prevalence of infection in a study area (see Table 1 below). When the prevalence of STH infections in a study area is low larger sampling volumes applies, where volumes up to 200 L has been reported (Levantesi et al., 2010). Most methods used for the detection of STHs eggs in environmental samples are a modification of the United States Environmental Protection Agency (USEPA) (Schwartzbrod, 1998) and the World Health Organization (WHO) methods (Ayres and Mara, 1996). These methods recommend the use of one liter samples (Yaya-Beas et al., 2016; de Souza et al., 2011; Sengupta et al., 2011; Ajeagah 2013). However, there are reports of the use of 10 L samples (Abreu-Acosta and Vera, 2011; Molleda et al., 2008). Furthermore, the volume of water analyzed is directly related to the level of detection achieved (Bowman et al., 2003). For instance, the WHO guidelines for wastewater reuse in agriculture recommends <1 viable helminth egg/Liter for unrestricted irrigation (WHO, 2006). Therefore methods that aim to determine the quality of wastewater for irrigation must consider volume sufficient to reach the limit of detection. High solid content in the sample may result in lower rates of egg recovery due to interference of the solids (Rocha et al., 2016). Data presented in Table 1 below shows that even when sample volumes are the same, the amount of helminth egg in the wastewater differs greatly from one region to the other, which is dependent on prevalence of infection in the study area.

3.1.2. Sludge/Compost/Biosolids

Sludge, biosolids and compost may contain high concentration of STHs eggs since these will attach and concentrate together with particulate material and accumulate in the sludge (Shamma and Al-Adawi, 2002) during wastewater treatment. Eggs of STHs settle much easier into sludge during primary treatment than bacteria and viruses due to their larger sizes (Yeager and O'Brien, 1983). The concentration of STHs eggs in sludge samples varies greatly depending on the prevalence of infection in the relevant population. Table 2 shows concentration of eggs in sludge from different locations, with a higher concentration in sludge samples from developing countries irrespective of the sample size. The amount of sludge sampled is often related to the dry weights, which are in the interval from 2 g (Scaglia et al., 2014; Pecson et al., 2007; Nelson and Darby, 2001) to 5 g (Bowman et al., 2003). This contrasts with the USEPA method that recommends 300 g as a dry weight sample size (Cappizzi-Banas et al., 2004; Maya et al., 2012; Gantzer et al., 2001). The variation in the weight of solid sample, as well as is the case for liquid samples, is mainly influenced by prevalence of the STHs in the study area based either on literature or past experiences. Large samples of sludge have been found to lower the rate of egg recovery, mainly due to the interference of solids. Bean and Brabants (2001) observed that the recovery of *Ascaris* spp eggs from a 50 g sludge sample was twice as high as from a 300 g of sludge and biosolids. It is generally recommended that samples should contain between 5 g (Reimers et al., 1989) to 50 g (USEPA, 2003) of total solids. Table 2 contains information of the sample weight and the study area which may play a critical role in the amount of eggs recovered. Studies performed in developed countries (France, Italy etc.) reported lower concentration of STHs eggs (Scaglia et al., 2014; Gantzer et al., 2001), as compared to studies in developing countries (Ghana, Mexico, Burkina Faso) (Buitrón and Galván, 1998; Koné et al., 2007; Bastos et al., 2013). With larger sample sizes the concentration of reported STHs egg is high irrespective of the location of study (Reinoso and Becares, 2008; Engohang-Ndong et al., 2015; Bowman et al., 2003).

3.1.3. Soil

The concentration of STHs eggs in soil can be very high depending on the sampling site especially in unsanitary environments or where open defecation is practiced. Soil sampling for other helminths in addition to STHs eggs is important due to the possibility of zoonotic infections from infected animals, particularly dogs and cats (e.g. *Toxocara canis, Toxocara cati* and *Ancylostoma caninum*). Direct contact with dogs or cats that harbor adult *Toxocara* worms is unlikely to cause an infection in humans because once shed, the eggs must undergo a period of development in the environment before they can become infective (Paquet-Durand et al., 2007). The sample size for soil is measured in dry weights, with sample sizes between 10 and 50 g of dry soil (Paquet-Durand et al., 2007; Muna et al., 2009; Oge and Oge, 2000; Lindgren et al., 2008;

Table 1
STHs egg concentration in wastewater/water/septage from different countries and volume of sample analyzed.

Matrix	Volume	STHS	Concentration (eggs/L)	Location	Reference
Wastewater	1 L	All STHs	194 ± 79	Peru	Yaya-Beas et al., 2016
Wastewater	1 L	All STHs	15 ± 4	Tunisia	Saddoud et al., 2007
Wastewater	1 L	All STHs	15 ± 0.3	Tunisia	Riahi et al., 2009
Wastewater	10 L	All STHs	<1	Spain	Molleda et al., 2008
Wastewater	20 L	All STHs	2	Europe	Levantesi et al., 2010
Wastewater	500 mL	All STHs	16	USA	Rose et al., 1996
Wastewater	5 L	*Ascaris* spp Hookworm	142 (*Ascaris* spp) 558 (Hookworm)	Pakistan	Ensink et al., 2007
Wastewater	10 L	All STHs	0	Spain	Abreu-Acosta and Vera, 2011
Wastewater	10 L	All STHs	0.96 ± 0.10	Spain	Reinoso et al., 2008
Irrigation water	1 L	All STHs	12.9–15.1	Ghana	Sengupta et al., 2011
Irrigation water	10 L	All STHs	0	Italy, Serbia	Forslund et al., 2010
Stream Water	1 L	*Ascariss* pp	11	Cameroun	Ajeagah, 2013
Septage sludge	1 L	All STHs	16000	Vietnam	Yen-Phi et al., 2010
Untreated septage	1 L	All STHs	450	Vietnam	Yen-Phi et al., 2010

Table 2
STHs egg concentration in sludge/compost from different countries and sample weights analyzed.

Matrix	Weights	STHs[a]	Concentration (eggs/g)	Location	Reference
Sludge	2–5 g	All STHs	0	Italy	Scaglia et al., 2014
Sludge	5 g	*Ascaris* spp	776 ± 13.4	USA	Bowman et al., 2003
Sludge	2 g	All STHs	173.5	Mexico	Pecson et al., 2007
Sludge	4 g	*Ascaris* spp *Trichuris* spp	13–94 (*Ascaris* spp) 2–24 (*T. trichiura*)	Ghana	Koné et al., 2007
Sludge	500 mL	*Ascaris* spp	312 ± 24	USA	Engohang-Ndong et al., 2015
Sludge	10 g	All STHs	0.27 ± 0.15	France	Gantzer et al., 2001
Sludge	100 g	*Ascaris* spp *Toxocara* spp *Trichuris* spp	52.2 (*Ascaris* spp) 27.2 (*Toxocara* spp) 12.4 (*Trichuris* spp)	Czechoslovakia	Horak, 1992
Sludge	100 g	*Ascaris* spp *Hymenolepis* spp	1.8–9.3 (*Ascaris* spp) 1.5–6 (*Hymenolepis* spp)	Morocco	Moubarrad and Assobhei, 2007
Sludge	100 g	*Ascaris* spp	3.79 ± 1.96	Brazil	Bastos et al., 2013
Slurry	75 mL	All STHs	2910 eggs/L	Mexico	Buitrón and Galván, 1998
Pig slurry	500 g	All STHs	867	Spain	Reinoso and Becares, 2008
Compost	10 g	All STHs	0.15 ± 0.20	France	Gantzer et al., 2001

[a] Although the main focus was on STHs, other helminths were added for additional information.

Table 3
STHs egg concentration in soil/sand from different countries and sample weights analyzed.

Matrix	Weights	STHs[a]	Concentration (eggs/g)	Location	Reference
Sand	20 g	*Toxocara* spp.	0.8% (16 samples)	Costa Rica	Paquet-Durand et al., 2007
Soil	40 g	All STHs	3.25 (*Toxocara* spp) 2.9 (*T. vulpis*) 1.75 (*Ascaris* spp) 0.2 (*Trichuris* spp)	Poland	Mizgajska, 1997
Soil	2 g	*Toxocara*	2	Philippines	Fajutag and Paller, 2013
Soil	2 g	All STHs	454.5 (*A. lumbricoides*), 150.5 (*Toxocara* spp) 120 (*Trichuris* spp)	Philippines	Horiuchi et al., 2013
Soil	100 g	All STHs	99	Brazil	Rocha et al., 2016
Soil	50 g	*Toxocara* spp	5.8 ± 2.6	Czech Republic	Dubná et al., 2007
Soil	20 g	All STHs	1.1	Iraq	Nooraldeen, 2015
Soil	20 g	All STHs	0.063	Poland	Blaszkowska et al., 2013
Soil	30 g	All STHs	3.3%	Brazil	Mandarino-Pereira et al., 2010

[a] Information regarding *Toxocara* has been included as a references.

Mizgajska, 1997; Fajutag and Paller, 2013). Samples mostly represent the top 0–5 cm layer of the soil (Paquet-Durand et al., 2007; Mizgajska, 1997; Fajutag and Paller, 2013; Nooraldeen, 2015).

Important factors that influence the recovery of helminth eggs from soil include soil type and texture (Nunes et al., 1994). Sand and sandy soils are reported to result in higher recoveries of helminths than from clay, silt and silty clay-soils (Oge and Oge, 2000). Sandy soils also result in less variation in the number of eggs recovered, which might be due to the homogeneity of sandy soils based on the greater particle sizes held loosely together compared to other soil types (Oge and Oge, 2000). In addition to the investigated soil type, the study location also plays a critical role in the determination of soil weight to sample, as shown in Table 3. Since the prevalence of STHs eggs in soil samples vary from one location to the other, sampling should account for the local prevalence of the STH where possible.

3.1.4. Plants

With an increase in the use of wastewater for irrigation of crops, as well as the use of sludge, biosolids and compost as fertilizers or soil conditioners in agriculture, there is an enhanced likelihood of plants becoming contaminated with pathogens with the further

Table 4
STHs egg prevalence (%) in grass/vegetables in different countries and weights of sample analyzed.

Matrix	Weights	STHs	Prevalence	Location	Reference
Vegetables	250 g	All STHs	50 (of 96) imported vegetables 71 (of 45) native vegetables	Iran	Daryani et al., 2008
Vegetables	200 g	All STHs	3.5 (of 1130)	Nigeria	Adamu et al., 2012
Vegetables	250 g	All STHs	16.2 (of 270)	Saudi Arabia	Al-Megrin, 2010
Vegetables	200–300 g	All STHs	14.9 (of 772)	Iran	Rostami et al., 2016
Vegetables	250 g	All STHs	57.8 (of 199)	Nigeria	Maikai et al., 2012
Vegetables	100 g	All STHs	6.3 (of 111)	Turkey	Adanir and Tasci, 2013
Vegetables	250 g	All STHs	25.2 (of 453)	Iran	Fallah et al., 2016
Vegetables	200 g	All STHs	31.7 (of 300)	Egypt	Said, 2012
Vegetables	100 g	All STHs	58 (of 126)	Libya	Abougrain et al., 2010
Vegetables	200 g	*Toxocara* spp. and *Ascaris lumbricoides*	5.9 (of 203)	Turkey	Kozan et al., 2005
Vegetables	100 g	All STHs	44.2 (of 172)	India	Gupta et al., 2009
Vegetables	200 g	All STHs	32.6 (of 304)	Iran	Fallah et al., 2016
Vegetables and fruits	100 g	*Ascaris* spp	1–12 eggs/100 g	Turkey	Erdoğrul and Şener, 2005
Grass	41 g	*Toxocara* spp., Ancylostomidae	68 (of 69)	Costa Rica	Paquet-Durand et al., 2007

transmission of STHs to consumers (WHO, 2006). Different types of plants irrigated with wastewater or fertilized with sludge have different likelihoods for transmission (e.g. differences between salad crops and foliage plants). This may be reflected in the sampling strategy as well as in the contamination levels. The amount/weight of vegetable sampled or analyzed varies between methods. Preferred weights of plant samples normally falls between 100 and 250 g (Adamu et al., 2012; Maikai et al., 2012; Adanir and Tasci, 2013; Fallah et al., 2016). These weights are mostly of vegetables, with lower weights for grass (Paquet-Durand et al., 2007; Robertson et al., 2002). Several reported studies fail to report on the weight of plant material analyzed (Su et al., 2012; Hassan et al., 2012; Said, 2012; Rostami et al., 2016). The specific weight is essential for risk calculation for quantitative microbial risk assessment (QMRA) and risk of infections based on consumption. However, the concentration of STHs eggs reported from different studies is largely influenced by the quality of wastewater used for irrigation, as well as the STHs egg concentration in sludge or compost used as fertilizers. Table 4 presents the prevalence of positive STHs in vegetable or plant samples from different countries.

3.2. Egg recovery

The variation in recovery is influenced by methodological approaches in each of the analytical stages as well as the sample matrix, type of STHs eggs, available reagents/materials and the discretion of the researcher. Table 5 summarizes some of these variations reported in literature. The sampling strategy for STHs eggs should reflect the uneven distribution in the environment. Their recovery will be enhanced through homogenization. Homogenization is most relevant for solid samples such as sludge, biosolids, compost and the solid waste fraction from source separation toilets (Bowman et al., 2003). It is usually achieved through blending or mixing (Bowman et al., 2003; Cappizzi-Banas et al., 2004; Pecson et al., 2007). Blending also breaks down coarse materials into finer particles which improves the recovery of the eggs (Koné et al., 2007).

3.2.1. Separation of eggs from particles

Separation of STHs eggs from solids in the sample is of major concern especially when considering sludge, compost and biosolids, due to their heterogeneity. Samples with a high concentration of particles (including soil particles) could easily trap the eggs, or become attached to the eggs which will result in lower recovery. Different solutions have been used to recover eggs from particulate matter. These solutions are mainly anionic detergents that break the bonds between the STHs eggs and particles in the samples. The most commonly used detergent solutions are Tween 80, Triton X-100 (Molleda et al., 2008; Forslund et al., 2010), 7X (Sengupta et al., 2011; Saddoud et al., 2007; Konaté et al., 2013) and ammonium bicarbonate (Moodley et al., 2008; Trönnberg et al., 2010). Although most researchers favor the use of detergents for dissociation, some have reported a lower recovery for treated versus untreated samples (Rocha et al., 2016). Earlier reports showed that the use of detergents reduced egg viability due to damage of egg integrity (Jaskoski, 1954).

Although there is a lack of comparative data to determine the best detergent for dissociation of helminth eggs from environmental samples, the use of 7X has been reported to give better recoveries (Bowman et al., 2003). This detergent is water soluble at all concentrations and comprises of anionic surface active agent and special solvents which may account for the improved recovery. In addition 7X does not form precipitates when in contact with salt solutions used during the flotation step (Rocha et al., 2016). The first report of the recovery of STHs eggs from soil was in 1928 by Caldwell and Caldwell (David, 1977), where antiformin resulted in a good recovery (especially in clay soils) (David, 1977). Saturated ammonium bicarbonate has been used to improve recovery of eggs from samples with a high content of soil (Moodley et al., 2008; Collender et al., 2015). A variety of solutions have been used in the analysis of vegetable or plant samples. Table 5 includes the different separation solutions used in the analysis of wastewater or sludge. Solutions such as physiological saline (Adanir and Tasci, 2013; Maikai et al., 2012; Hassan et al., 2012; Adamu et al., 2012), phosphate buffered saline (PBS) (Said, 2012), Tween 80 (Kozan et al., 2005; Pacquet-Durand et al., 2007) and water (Su et al., 2012; Kłapeć, 2009) are commonly used in contrast to Tween 80/20 or 1% 7X that is more common in other sample matrices. Again, due to a lack of comparative data on the best solutions to use for washing of plant samples, the choice is mostly reliant on the discretion of the researcher.

3.2.2. Filtration of samples

After dissociation of eggs from larger particles within samples, there is a need to separate the eggs from these particles. One step used to achieve this separation is filtration or the use of sieves to retain larger particles whiles allowing the eggs of interest to pass into the filtrate for further analysis (Bowman et al., 2003; Katakam et al., 2014; Engohang-Ndong et al., 2015). The choice of pore size for this step is the most important factor to consider. Most parasite

Table 5
STHs species reported with different dissociation solutions, pore sizes and flotation solutions of sieves from different sample matrices.

Matrix	Dissociation	Filtration/pore size (μm)	Flotation (S.G)[a]	STHs recovered[b]	References
Wastewater	None	None	NaCl (1.18)	All STHs	Yaya-Beas et al., 2016
Wastewater	None	500, 212, 90, 38, 35, 31.5 and 30	NaCl (1.27)	*Ascaris* spp., *Trichuris* spp.	Sengupta et al., 2011
Wastewater	None	None	$ZnSO_4$ (1.18)	*Ascaris* spp.	de Souza et al., 2011
Wastewater	1% 7X	500, 212, 90, 38, 35, 31.5 and 30	$MgSO_4$ (1.20)	*Ascaris* spp. *Trichuris* spp	Sengupta et al., 2011
Wastewater	1% 7X	50 and 20	$MgSO_4$ (not stated)	*Ascaris* spp. *Hymenolepis* spp.	Verbyla et al., 2016
Wastewater	1% 7X	400	$MgSO_4$ (1.20)	*Ascaris* spp, *Trichuris* spp	Riahi et al., 2009
Wastewater	Triton X-100 or Tween 80,	None	$ZnSO_4$ (1.18)	*Trichuris*	Molleda et al., 2008
Wastewater	1% 7X	400	$MgSO_4$ (1.20)	All STHs	Saddoud et al., 2007
Wastewater	Triton X-100 or Tween 80,	None	$ZnSO_4$ (1.18)	All STHs	García et al., 2013
Wastewater	Triton X-100 or Tween 80,	None	$ZnSO_4$ (1.18)	All STHs	Reinoso et al., 2008
Wastewater	Triton X-100 or Tween 80,	None	NaCl (not stated)	All STHs	Forslund et al., 2010
Wastewater	Triton X-100 or Tween 80,	None	$ZnSO_4$ (1.18)	All STHs	Abreu-Acosta and Vera, 2011
Wastewater	1% 7X	None	$ZnSO_4$ (not stated)	*Ascaris* spp.	Ajeagah, 2013
Wastewater	1% 7X	160	$ZnSO_4$ (1.3)	All STHs	de Victorica and Galvan, 2003
Wastewater	1% 7X	400, 50 and 20	$MgSO_4$ (1.20)	*Ancylostoma* spp. *Ascaris* spp. *Trichuris* spp.	Konaté et al., 2013
Wastewater	Triton X-100 or Tween 80,	None	$ZnSO_4$ (1.18)	*Ascaris* spp. *Hymenolepis* spp. Hookworm.	Yen-Phi et al., 2010
Sludge	1% 7X	400, 50 and 20	$MgSO_4$ (1.20)	All STHs	Bowman et al., 2003
Sludge	1% 7X	20, 50 and 400	$MgSO_4$ (1.20)	All STHs	Cappizzi-Banas et al., 2004
Sludge	1% 7X	20, 50 and 400	$ZnSO_4$ (1.20)	All STHs	Pecson et al., 2007
Sludge	1% 7X	80	$MgSO_4$ (1.29)	*Ascaris* spp. *Trichuris* spp.	Koné et al., 2007
Sludge	1% 7X	35 and 20	$ZnSO_4$ (1.20)	*Ascaris* spp *Toxocara* spp, *Trichuris* spp, *Hymenolepis* spp	Maya et al., 2012
Pig Slurry	None	500, 212, 90 and 36	$ZnSO_4$ (1.20)	*Ascaris* spp.	Katakam et al., 2014
Sludge	0.1% Tween 80	160	$ZnSO_4$ (1.20)	*Ascaris* spp.	Nelson and Darby, 2001
Sludge	0.1% SDS	500 and 100	NaCl (1.19)	*Ascaris* spp. *Toxocara* spp.	Gaspard et al., 1996
Sludge	1% 7X	20, 50 and 400	$MgSO_4$ (1.20)	*Ascaris* spp	Engohang-Ndong et al., 2015
Sludge	1% 7X	20, 50 and 400	$ZnSO_4$ (1.30)	*Ascaris* spp. *Trichuris* spp. *Toxocara* spp.	Gantzer et al., 2001
Sludge	None	90 and 32	$ZnSO_4$ (1.33)	*Ascaris* spp	Gaasenbeek and Borgsteede, 1998
Sludge	1% 7X	20, 50 and 400	$MgSO_4$ (1.20)	All STHs	Reinoso and Becares, 2008
Soil	None	2 mm	$NaNO_3$ (1.39)	*Toxocara* spp. *Ascaris* spp. *Trichuris* spp.	Mizgajska, 1997
Archeological samples	0.5% trisodium phosphate	300, 160 and 20	None	*Trichuris* spp. *Ascaris* spp.	Yeh et al., 2015
Soil	Tween-80	150	sucrose solution (1.20)	*Ascaris* spp, *Trichuris* spp, *Toxocara* spp,	Horiuchi et al., 2013
Soil	Tween	None	$NaNO_3$ (1.30)	*Toxocara* spp	Dubná et al., 2007
Soil	5% NaOH	4 mm	$NaNO_3$ (1.30)	*Toxocara spp. Trichuris* spp . *Ascaris* spp . *Ancylostoma* spp.	Blaszkowska et al., 2013
Soil	Tween 80	None	$NaNO_3$ (1.22)	*Toxocara* spp . *Trichuris spp. Ancylostoma* spp.	Mandarino-Pereira et al., 2010
Soil	Triton-X100	None	$ZnSO_4$ (1.45)	*Ascaris* spp, *Trichuris* spp,	Wang and Bundy, 1990
Soil	Tween 80	Gauze (pore size not specified)	$NaNO_3$ (1.20)	*Toxocara* spp.	Rosa Xavier et al., 2010
Soil	None	0.1 mm	Not specified	*Toxocara* spp. *Trichuris* spp.	Zenner et al., 2002
Vegetables	physiological saline	None	sucrose (1.21)	*Toxocara* spp. *Strongyloides* spp . *Ancylostoma* spp. *Trichuris* spp.	Maikai et al., 2012
Vegetables	physiological saline	None	None	*Toxocara* spp. *Ascaris* spp.	Adanir and Tasci, 2013
Grass	Tween 20	None	Hyper saturated sugar solution.	*Toxocara* spp. *Toxascaris* spp. *Trichuris* spp.	Paquet-Durand et al., 2007
Vegetables	normal saline	None	Sucrose solution (1.21)	*Ascaris* spp . *Strongyloides* spp. *Toxocara* spp.	Fallah et al., 2016
Vegetables	None	None	None	*Ascaris* spp . *T. trichiura*, hookworm	Su et al., 2012
Vegetables	PBS and Tween 80	Gauze (pore size not specified)	None	*Ascaris* spp. *Toxocara* spp. *Hymenolepis* spp.	Said, 2012
Vegetables	physiological saline	None	None	*Ascaris* spp . *Toxocara* spp,	Abougrain et al., 2010
Vegetables	physiological saline	None	None	*Ascaris* spp . *T. trichiura*, Hookworms	Gupta et al., 2009
Vegetables	physiological saline	None	None	*Ascaris* spp ., *Toxocara* spp.	Fallah et al., 2016
Fruits and vegetables	physiological saline	None	None	*Enterobius. vermicularis* *Ascaris* spp,	Erdoğrul and Şener, 2005
Vegetables	physiological saline	None	None	*Ascaris* spp.	Amahmid et al., 1999

[a] S. G. values given in this table refer to stated values in the respective paper.
[b] Helminth species other than the STHs have been retained, since the method recover these as well.

eggs have dimensions between 25 μm–150 μm (Yeh et al., 2015; Bouchet et al., 2003). Therefore, to allow eggs to pass through, pore sizes from 4 μm to125 μm (sizes varying based on the helminth egg of interest), are commonly used (Gaspard et al., 1995; Gantzer et al., 2001; Mizgajska, 1997; Blaszkowska et al., 2013; Horiuchi et al., 2013; Fajutag and Paller, 2013). In some instances the interest is to retain the eggs on the sieves instead and therefore smaller pore sizes such as 20 μm (Maya et al., 2010; Landa-Cansigno et al., 2013) or even as low as 8 μm are chosen (Buitrón and Galván, 1998). In instances where the STHs of interest are restricted to nematodes, sieves of pore sizes between 32 and 36 μm are used (Katakam et al., 2014; Maya et al., 2010; Gaasenbeek and Borgsteede, 1998). Table 5 gives examples of pore sizes of sieves that have been used to analyze different samples. It is evident that the variation in the pore size is mostly influenced by the helminth species of interest, as well as the sample matrix. For instance, most researchers working with vegetable or plant samples do not include this step due to decreased amounts of particulate matter. The influence of pore sizes of sieves on the recovery of eggs from different sample matrices has not been reported but filtration may reduce particulate material in the sample which would make the microscopy step easier, while also enhancing the accuracy in identification and quantification of the eggs. Efficiency in microscopy stage is improved due to a reduction in the particles that may obstruct the eggs on the slides. However, filtration may also result in a lower recovery of eggs due to the trapping of particle associated eggs or clumped eggs.

3.3. Concentration

3.3.1. Sedimentation

The eggs in the filtrate need to be separated from the liquid phase. Separation of the solid particles (which includes the eggs) in samples from the liquid phase is mostly achieved through sedimentation. Sedimentation could be passive or with the use of centrifuge where different speeds are applied. This difference in the centrifugation speeds may introduce variation to the concentration of eggs recovered, however there is no data to ascertain the best speed. The time used for passive sedimentation varies between methods applied with setup time ranging from 1 h (Molleda et al., 2008; Reinoso et al., 2008; Yen-Phi et al., 2010) to overnight (Riahi et al., 2009; Saddoud et al., 2007; de Victorica and Galvan, 2003). The effect of passive sedimentation on the recovery of eggs could be influenced by several factors. These include the sample matrix and volume, the dimension of the container used for the sedimentation and the duration of the sedimentation. A high solid content in the sample may interfere with the settling of the eggs with a potential loss of some eggs (Rocha et al., 2016). The sedimentation rate is also greatly influenced by the dimension of the container used (Shuval et al., 1986). The WHO method (Ayres and Mara, 1996) recommends the use of an open-topped, straight-sided container of at least 10 L volume, aimed at making the removal of the supernatant easier and to permit thorough rinsing. In addition, the duration of the sedimentation is crucial and the sedimentation rates differ from one helminth species to the other. Adequate time is necessary for the settling of the eggs, accounting for the viscosity of the sample as well as the dimension of the container. For instance, *Ascaris* spp eggs (relative density of 1.13) have a settling velocity of 0.65 m/h, *Trichuris* spp eggs (relative density of 1.15) of 1.53 m/h while *Taenia* spp eggs (relative density of 1.23) will have a settling velocity of almost 2 m/h (David and Lindquist, 1982; Dryden et al., 2005). Inadequate time for sedimentation would result in the loss of some eggs, whereby overnight sedimentation as with the USEPA and Tulane methods would be an advantage (Mara and Sleigh, 2010).

3.3.2. Flotation

A critical step that results in method variation is the flotation step, where the main aim is to separate eggs from other materials in the sample that were not removed during the filtration or sedimentation steps. Flotation achieve separation by creating a gravity gradient that allows particles of interest (in this case the eggs) to float while heavier particles (like soil or other heavier particles) settle and are discarded. In this step the loss of STHs eggs relates to the specific gravity of the solution versus the density range for eggs, ranging from 1.05 to 1.23 (David and Lindquist, 1982). A variety of flotation solutions used include zinc sulphate (Trönnberg et al., 2010; Zamudio-Pérez et al., 2013; Amahmid et al., 2002), magnesium sulphate (Karkashan et al., 2014), sodium nitrate (Blaszkowska et al., 2013,) and sucrose solutions (Kouraa et al., 2002). For the recovery of all STH eggs, independent of species, the flotation solutions used should be heavier than 1.25 specific gravity (s.g) (David, and Lindquist, 1982). Since some methods use flotation solutions of specific gravity lower than 1.3 only some of the STHs species eggs will be recovered in consistent numbers. For example the USEPA and the Tulane method both use magnesium sulphate of specific gravity of 1.2 (Bowman et al., 2003; Saddoud et al., 2007; Konaté et al., 2013; Sengupta et al., 2011). Flotation solutions (e.g. sodium chloride and zinc sulphate) with lower specific gravity (1.18) are used in other methods (Yaya-Beas et al., 2016; Molleda et al., 2008; García et al., 2013). In addition to the STHs, *Taenia* spp eggs, with a specific density of 1.23 (David and Lindquist, 1982) is of main interest in environmental sampling and may not be recovered with low specific gravity solutions. Other adverse effects with flotation solutions are found with the use of sucrose, where increased viscosity has been shown to hinder the recovery of eggs. This interferes with the movement of the eggs as well as other particles (Bowman et al., 2003). Saturated sucrose has been shown to deform STHs eggs (Collender et al., 2015), and sodium nitrate forms crystals that would interfere with the quantification (Santarém et al., 2009).

Nunes et al. (1994) found that sodium dichromate gave the best recovery ratio, which most certainly is due to the high specific gravity (s. g = 1.34) of the sodium dichromate used. Quinn et al. (1980) found magnesium sulphate (s. g = 1.27) plus 5% potassium iodide (KI) to be the most efficient solutions in the recovery of helminth eggs. Dada and Lindquist, (1979), found that zinc sulphate gives a better recovery of STHs eggs than sodium dichromate of the same specific gravity (1.20). This was later confirmed by Oge and Oge (2000). Table 5 shows some of the flotation solutions used and the variation in specific gravity and the corresponding STHs eggs that were recovered.

3.3.3. Phase extraction

Some recovery methods include a phase extraction step after the flotation. This aims at removing lipid-soluble and ether-soluble material from the sample. It results in the partitioning of the sample into acidic aqueous and lipophilic phases (Beaver et al., 1984), where a plug of waste (remaining particles) material is formed at the interphase between these. Pellets containing the eggs are deposited at the bottom of the centrifuge tubes. The commonly used reagents for the lipophilic extraction include ethyl acetate and diethyl ether (Bornay-Llinares et al., 2006; Horiuchi et al., 2013; Verbyla et al., 2016; de Victorica and Galvan, 2003; Ayres and Mara, 1996; Gaspard et al., 1996; Rude et al., 1987; USEPA, 1999). Reagents for the hydrophilic extraction include a mixture of sulfuric acid and acetoacetic buffer (Verbyla et al., 2016; de Victorica and Galván, 2003; Ayres and Mara, 1996; Fuhrimann et al., 2015). Satchwell (1986) found that the inclusion of a phase extraction step removes about 40% of contaminants (such as proteins and lipids) but results in the loss of 95% of the eggs. This may be due to distortion of the eggs or toxic effects. Several studies have documented

the detrimental effects of some of these chemicals, such as ethyl acetate and acetoacetic buffer, on egg integrity and subsequently viability (Nelson and Darby, 2001; Rocha et al., 2016). Amoah et al. (submitted manuscript) found acetoacetic acid to result in the loss of egg viability. Due to the effect of this step on egg integrity it has been recommended to replace it with a sieving step, to remove proteins, lipids and other contaminant molecules (USEPA, 2003). However if the step is still used, then the exposure of the eggs to the chemicals must be within the shortest time possible in order to reduce their adverse impact (Nelson and Darby, 2001).

3.4. Viability determination

For the purposes of risk estimation and in accordance with some international and national standards and guidelines (USEPA, 2003; WHO, 2006), it is important to determine the number of viable STH eggs per amount of sample. This is, however, excluded in some studies which did not differentiate between viable and non-viable eggs (Fugazzola and Stancampiano, 2012; Bornay-Llinares et al., 2006; Pacquet-Durand et al., 2007; Hassan et al., 2012; Maikai et al., 2012). Viability assessment was excluded from these studies due to difficulties in the assessment process and time constraints. The most widely used viability assessment method is the time consuming incubation to achieve the development of the larvae. Other authors report viability based on morphological integrity of the eggs and their response to staining with vital dyes. The viability assessment method is influenced by factors such as materials and available equipment, experience of the researcher and personal preferences. The morphological determinations include size, shape and the presence of visible larvae and are used as a criterion for viability of eggs during microscopy. Viability determination based on the presence of visible motile larvae could be subjective based on the experience of the microscopists.

Incubation of recovered eggs could reduce these errors, but selection of the incubation solution also varies between method applications. Solutions such as sulphuric acid (Maya et al., 2012; Katakam et al., 2014; Nordin et al., 2009; USEPA, 1999; Reinoso and Barnes, 2008) and formalin (Bowman et al., 2003; Eriksen et al., 1995; Shamma and Al-Adawi, 2002) are frequently used, with incubation temperatures varying between 22 °C and 26 °C and duration of incubation period between 21 and 30 days (Bowman et al., 2003; Buitrón and Galván, 1998; Pecson et al., 2007). Sulphuric acid (0.1N) has been found to be the best incubation solution reporting 83–92% (Yanko, 1987) or 75–80% viability (USEPA, 2003) followed by formalin (75–80%) (Reimers et al., 1989; Bowman et al., 2003). The use of 0.1% formalin has however also been reported to show retarded development compared to those incubated in water or sulfuric acid (Oksanen et al., 1990).

The limitation with the incubation to determine viability is due to its time consuming nature. When applied as part of verification monitoring, for example, in instances such as the reuse of wastewater or sludge in agriculture, the time lag for data may be a deterrent. This warrants the use of stains differentiating viable and non-viable eggs based on the permeability of the egg shells. Commonly used stains include Lugol's iodine (Benti and Gemechu, 2014; Ajeagah, 2013; Adanir and Tasci, 2013; Su et al., 2012), safranin O (Koné et al., 2007; Konaté et al., 2013) trypan blue (Maya et al., 2012; Buitrón and Galván, 1998) and eosin Y (de Victorica and Galvan, 2003). Karkashan et al. (2015) compared different stains against conventional incubation and found that conventional incubation detects 86% of viable eggs which is lower than the viable eggs determine through safranin stain (97%), crystal violet stain (92%) and methylene blue (87%). The use of BacLight stain gave between 78 and 85% viability and the lowest viability (39%) was reported with trypan blue staining (Karkashan et al., 2015). The use of vital stains therefore presents an opportunity to determine viability of eggs without the prolonged incubation time required. Despite their potential advantages, some stains are toxic to embryos and require sample examination within a few minutes of application (Karkashan et al., 2015). Karkashan et al. (2015) found that only the BacLight stain is not toxic to helminth eggs.

3.4.1. BacLight Dead/Live method

This method is based on the BacLight LIVE/DEAD Bacterial Viability Kit (Molecular Probes, Invitrogen, Eugene, USA) and was initially developed for the enumeration of viable bacteria. It is based on detecting the difference in the membrane integrity of viable and non-viable cells. These have a differential intake of two specialized membrane permeable DNA-labelling dyes, Syto 9 which fluoresces green with a maximum emission of 498 nm and propidium iodide (PI) which fluoresces red with a maximum emission of 617 nm. The detection of viable eggs using this technique is based on the differential staining of viable eggs with Syto 9 whiles the PI stains the non-viable eggs (Lisle et al., 1998). Dabrowaska et al. (2014) found 58% live, 38% dead and 3.7% as insufficiently stained eggs of *Ascaris* spp, *Toxocara* spp and *Trichuris* spp in sludge samples. The authors concluded that the method was suitable for viability determination. These results were obtained from seeded samples and its performance was not compared with other methods. However Karkashan et al. (2015) reported between 78 and 85% viability of *Ascaris* spp eggs using the BacLight LIVE/DEAD staining technique. They also concluded that staining with this kit was the only procedure that does not affect the viability of eggs after analysis. Therefore, the use of the BacLight LIVE/DEAD staining is emerging as a rapid way of determining of the viability of STHs eggs without the need for incubation.

4. Nucleic acid based techniques

Advances in molecular technology provide an opportunity for an accurate and fast detection and quantification of STHs eggs in different sample matrices. PCR techniques have emerged as very specific, sensitive and rapid methods for the detection of different pathogens in a variety of matrices, from wastewater to soil and food items (Gomez-Couso et al., 2004; Ishiwata et al., 2004; Le Cann et al., 2004; Deisingh and Thompson, 2004). Several different PCR based methods are available for detection of pathogens, for example quantitative polymerase chain reaction (qPCR), multiplex polymerase chain reaction (mPCR), droplet/digital polymerase chain reaction (ddPCR). Despite the advancements in the area of molecular diagnostics only a few studies have considered their use for the detection of STHs eggs in environmental samples, even though they have found widespread use in clinical settings (Rocha et al., 2016; Pecson et al., 2006; Raynal et al., 2012).

4.1. Nucleic acid extraction

One of the main hindrances for the effective use of molecular methods in the detection of STHs eggs is the extraction of nucleic material of good quantity and quality. The extraction is hindered by the tough egg shell (van Frankenhuyzen et al., 2011). Furthermore the presence of high amounts of suspended solids in most of the sample matrices also impedes nucleic acid extraction (Bass et al., 2015) and may inhibit PCR reactions. Separation of eggs from these solid particles is mostly done before the extraction of DNA (Bass et al., 2015) using flotation and/or sedimentation steps. The method of choice for DNA extraction varies between studies (Raynal et al., 2012; Khouja et al., 2010; Mugambi et al., 2015; Loreille et al., 2001). Table 6 presents commonly used nucleic acid extraction methods, including commercial nucleic acid extraction kits and the molecular application of the product. The main component in these commercial kits is a proteinase enzyme which helps in the lysis of the egg

Table 6
Common nucleic acid extraction methods and molecular techniques used.

Matrix	Extraction method	Technique	STHs[a]	References
Sludge	DNeasy Blood and Tissue Kit (Qiagen) MO Bio Power Soil® DNA Kit (Mo Bio, Carlsbad, CA)	real-time PCR	*Ancylostoma caninum*	Gyawali et al., 2015
Wastewater	MO Bio Power Max® Soil DNA Extraction Kit	qPCR	*Ancylostoma caninum*	Gyawali et al., 2015
Wastewater	UltraClean microbial DNA and RNA kits (MoBio Laboratories, Carlsbad, CA)	qPCR	*Ascaris suum*	Pecson et al., 2006
Archeological samples	Ultra-sonication, DNA extracted with phenol/chloroform/isoamyl alcohol	PCR	*Ascaris* spp	Loreille et al., 2001
Sand	UltraClean Soil DNA Isolation kit (MoBio Laboratories, USA)	real-time PCR	*Baylisascaris procyonis*	Gatcombe et al., 2010
Wastewater and Sludge	FastDNA SPIN Kit for Soil (Qbiogene, Irvine, CA, USA)	nested PCR	*Ascaris* spp., *Enterobius. vermicularis, Hymenolepis nana, Taenia saginata*	Khouja et al., 2010
Water	QIAamp DNA mini kit (Qiagen, Valencia, CA) and Mobio Ultraclean Fecal DNA isolation kit (Mo Bio Laboratories, Inc. USA,)	qPCR	*Ascaris* spp	Raynal et al., 2012
Copro samples	PSP Spin Stool DNA Plus extraction Kit (Invitek, Berlin, Germany)	LAMP	*Enterobius granulosus*	Salant et al., 2012
Water	QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany)	mPCR and LAMP	*Taenia* spp	Nkouawa et al., 2009
Water	EZNA DNA extraction kit (Omega Bio-Tek, Norcross, GA, USA)	qPCR	*Fasciola hepatica*	Rodríguez et al., 2012

[a] Where helminths other than STHs are mentioned it is done since the combined matrix, extraction method and technique have been considered to add valuable supplementary information.

shell and finally an elution in different solutions depending on the manufacturer. Most of these kits are optimized for extraction of bacterial genome but the egg shells of STHs eggs are much tougher than the cell walls of bacteria. Therefore the use of such kits may result in a lower nucleic acid yield. Destruction of the egg shells to release the nucleic material could result in a higher nucleic acid yield, which has been reported with methods using sonication steps (Loreille et al., 2001) and beads (Raynal et al., 2012; Gyawali et al., 2015; Gatcombe et al., 2010).

4.2. Polymerase chain reactions

The development of PCR has transformed the detection of microorganisms in different sample matrices. However, the major limitation with conventional PCR is its inability to quantify the microorganisms detected (Zhang and Fang, 2004). There are other interferences with PCR analysis, some of which include the quality of the samples (purity, quantity and integrity of DNA); the inadequate homogenization of reagents used; the inaccurate setting of the baseline and calibration curves; and pipetting and dilution of standards (Rocha et al., 2016). Despite the limitations of PCR methods, they have proven to be more sensitive than microscopy based methods in detecting low numbers of eggs (Oliveira et al., 2010; Guy et al., 2003). Since PCR methods are based on species or group specific gene sequences, the outcome is more specific than morphological methods. The latter sometimes limit species differentiation, for example of hookworm or *Ascaris* eggs (Valero et al., 2009; Ai et al., 2010). Advances in PCR technology has also made it possible to detect multiple parasites using multiplex PCR by amplifying more than one target of interest using multiple primer pairs (Henegariu et al., 1997). The design and selection of the multiple primer pairs can make the reaction specific for the target organism (Gordon et al., 2011). qPCR has the ability to amplify and simultaneously detect and quantify the DNA amplification in real time (Bass et al., 2015) which is a major advancement over conventional PCR. This real-time detection, important for the absolute quantification of targeted DNA sequences, depends on the fluorescence generated as the reaction proceeds. The level of fluorescence is directly proportional to the quantity of target amplicons accumulated (van Frankenhuyzen et al., 2011). There are several reagents or chemicals available such as intercalating dyes, hydrolysis and hybridization probes (Gyawali et al., 2015), that could be added to facilitate the detection of the end product and aid in the quantification of the parasites. The main advantages associated with qPCR are its sensitivity, specificity, reproducibility and wide quantification range (Gyawali et al., 2015).

Although qPCR emerged as a suitable and powerful method for quantification, its use in environmental sample analysis has limitations. The quantification of low concentration of STH eggs from environmental samples is a challenge and the potential presence of PCR inhibitors in the samples may interfere with the PCR reactions (Toze, 1999; Shannon et al., 2007). Specificity of group-specific primers, florescent probes and nucleic acid extraction efficiency are also critical points (Durant et al., 2012). In addition, the quality of template nucleic acid and amplification of DNA of non-viable STHs eggs are also important factors that affect the analysis (Zeehaida et al., 2011).

Despite the advantages, there are limited environmental studies that have utilized qPCR for detection of STHs. A qPCR method was developed by Pecson et al. (2006) to determine the levels of total and viable *Ascaris* eggs using the first internally transcribed spacer (ITS-1) region of ribosomal DNA (rDNA) and rRNA. The transcription product of the ITS-1 region is short lived within the cell due to rapid enzymatic degradation and absent in non-viable eggs resulting in an estimate of viability (Pecson et al., 2006). The detection limit of the rDNA-based method was approximately one larvated egg or 90 single-celled eggs whereas the detection limit for the rRNA-based method was 968 single-celled eggs. This is a limitation in areas where the prevalence of infection is very low. This study promotes the applicability of the qPCR method in the detection of viable STHs eggs due to its ability to detect a single larvated egg. Gyawali et al. (2015) also reported a qPCR method for the rapid concentration, sensitive and specific detection of hookworm (*Ancylostoma caninum*) eggs from wastewater matrices. The

morphological similarity of *A. caninum* to the human hookworms (*Ancylostoma duodenale* and *Necator americanus*) makes it a preferred choice to determine the specificity of qPCR in quantification of closely related STHs eggs. The detection limit of this developed qPCR was found to be less than one *A. caninum* egg per 1 L of secondary treated wastewater, four *A. caninum* eggs per 1 L of raw wastewater and per ~4 g of treated sludge. A duplex qPCR assay targeting the ribosomal RNA gene internal transcribed spacer (ITS2) for the detection and differentiation of the eggs of *Toxocara canis* and *Toxocara cati* (Nematoda, Ascaridoidea) in soil and fecal samples was reported by Durant et al. (2012). The detection limit of the assay in spiked samples was 2 eggs/g of soil. This study reported that the duplex qPCR could be used for the detection of *T. canis* and/or *T. cati* eggs in fecal samples as well as in soil samples. Several other studies have shown that qPCR is much more sensitive than the conventional microscopy based methods in the detection of helminths from different environmental samples (Zeehaida et al., 2011; Sultana et al., 2013; Saugar et al., 2015; Kramme et al., 2011; Schär et al., 2013).

4.3. Loop-mediated isothermal amplification

LAMP is a nucleic acid amplification method with extremely high sensitivity and specificity, able to discriminate between single nucleotide differences (Parida et al., 2008). It is characterized by the use of a DNA polymerase that has low sensitivity to inhibitors and a set of four primers specially designed to recognize six different sequences on the target gene (Paris et al., 2007). In this technique amplification occurs only when all primers bind, thus forming a product. It can amplify a few copies of genetic material to 10^9 within an hour (Notomi et al., 2000) thereby reducing the time required for analysis. LAMP is based on an isothermal reaction and only a heating block or hot water bath is required for the reaction to progress as outlined in Fig. 1. There is no need for a thermocycler. Additionally, using white magnesium pyrophosphate results in precipitation of the DNA fragments (Mori et al., 2001) and the turbidity caused by this reaction is proportional to the amount of DNA synthesized. As a result, it is possible to evaluate the reaction in real time by measuring the turbidity or, more importantly, by visualization with the naked eye, making it a suitable candidate technique for field applications (Nkouawa et al., 2009). As illustrated in Fig. 1, LAMP is a simple method that does not require sophisticated equipment and is therefore a cheaper alternative than other nucleic acid based methods (especially the PCRs). It could have potential in developing regions of the world where laboratory resources are scarce. The LAMP method has been used in the detection of *T. saginata*, *T. solium* and *T. asiatica* DNAs in human faecal samples with higher sensitivity (88.4%) than multiplex PCR (37.2%) (Gordon et al., 2011). Liang et al. (2009) also reported a success rate of 92% in using LAMP method to detect *Entamoeba histolytica* in faecal samples as compared to nested PCR. The method has been applied in other applications in the detection of different parasites in a variety of sample matrices, such as *Necator americanus* in faecal samples (Mugambi et al., 2015) and detection of *Echinococcus granulosus* (Salant et al., 2012).

5. Emerging techniques

5.1. Digital PCR

Droplet digital PCR (ddPCR), a third generation PCR technology, was introduced to provide absolute quantification of targeted genes applicable for pathogens. Digital droplet PCR uses microwells or microfluidic chambers, which are simply referred to as wells, that split samples into several nanoliter partitions (Hindson et al., 2013). The advantages of ddPCR over qPCR-based assays are that ddPCR is based on endpoint PCR (efficiency of primer/probe annealing is minimized) and does not require the use of standards for accurate quantification. Most importantly, ddPCR is a high throughput assay with an approximate 15,000–20,000 PCR reactions per well (Baker and Ensink, 2012).

Baker and Ensink (2012) described commercially available digital PCRs based on either a chip or on droplets. The application of droplet digital PCR in the detection of zoonotic pathogens in poultry processing water samples was reported by Rothrock et al. (2013). The results showed that ddPCR out-performed qPCR and culture based methods used for processing poultry zoonotic pathogens. The frequent application of digital PCR (chip-based or droplets) for detection of STHs in environmental samples has not been explored yet. Further research is needed to optimize this method in terms of sensitivity and precision for detection of STHs in environmental samples.

5.2. Parasite identification using image analysis software

The final step involved in most conventional methods is microscopy which may be time consuming and dependent on the technician/microscopists' experience. The identification of STHs eggs including all the processing steps and microscopy takes

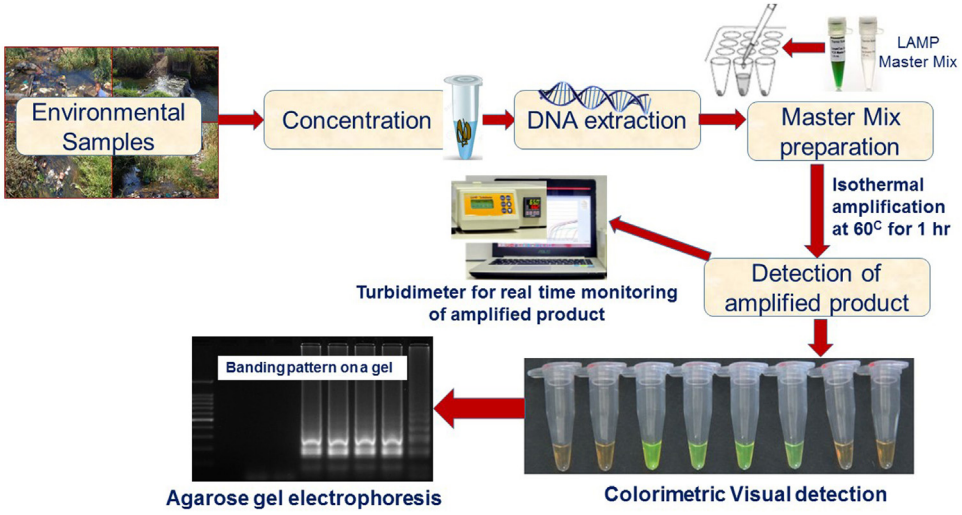

Fig. 1. Flow diagram for the use of LAMP in detection of microorganisms.

Table 7
Comparison of different techniques for detection of STHs in environment.

Methodological category	Techniques	Advantages	Disadvantages	Remarks
Conventional Techniques	WHO, USEPA, AMBIC, TULANE and several other variations of these methods	Easy to use, does not require sophisticated equipment hence less expensive.	Time consuming and laborious. Leaves too much for errors	These methods mostly consist of sedimentation and flotation using different flotation solutions (e.g. Zinc sulphate, magnesium sulphate, Sodium chloride) and a final microscopy step
Molecular Techniques	PCR, qPCR, LAMP	Rapid, quantitative (for qPCR), specific to species level identification	Expensive and requires skilled personnel	Nucleic acid based techniques are very specific and can provide species level identification that was difficult with conventional techniques. These techniques are laboratory based and can't be used for onsite detection of pathogens
Emerging techniques	digital PCR	More specific, quantitative, no need of reference standards as is the case in other PCR techniques	Expensive and requires skilled personnel	Digital PCR is new technique for absolute quantification, highly prone to PCR inhibitors. This technique needs to be optimized for environmental samples
	BacLight assay	Microscopic method to detect viability of STHs eggs	Expensive and requires skilled personnel	Initially developed for enumerating viable bacteria but can be applied for STHs. Need to validate for different environmental samples

approximately 2–5 h for samples with high suspended solids content (TSS) (wastewater sludge, biosolids, or excreta) or 30–60 min for clean samples (less than 15 mg/L TSS) (Jiménez et al., 2016). Recently, an automated microscopy step has been developed, where the use of software to identify and count the number of STHs eggs is included (Jiménez et al., 2016; Gomes et al., 2015). This automation technique still requires the separation and concentration of the eggs from the environmental samples although they eliminate human involvement in the final detection and quantification of eggs. In addition, the images of these eggs must be captured with cameras with good resolution for easy analysis with the software's. Jiménez et al. (2016) reports sensitivities between 80 and 100% and close to 100% specificity, while in an earlier publication using software called Parasitology DSS, Gomes et al. (2015) reported that false results may occur. The study by Gomes et al. (2015) reported that 81.86% of parasites were correctly identified by the software and 18.13% could not be identified. The image analysis technique needs further validation with different sample matrices as the presence of debris and poor picture qualities were found to be the main issues leading to false reports (Gomes et al., 2015). An alternative approach that uses fluidic geometries to concentrate eggs into a single field of view (FOV) and combining it with a mobile phone (Nokia Lumia 1020) with digital photo-microscopy has been reported by Sowerby et al. (2016). Combined with an additional objective lens, this photo-microscopy system provided sufficient resolution for single FOV images of nematode eggs due to the extended field of depth which enhances the quality of the images. However, these software identification systems still relies on effective sample processing just as is the case in the more conventional methods. In addition, improved optics are needed to be able to provide good images that would make the use of mobile phones a feasible option in the detection of STHs eggs, which naturally would be very beneficial for low income areas.

6. Future prospects

6.1. Flow cytometry

Flow cytometry simultaneously measures and analyzes multiple physical properties of a single particle, such as cells and potentially eggs/cysts, as they flow in a fluid stream through a beam of light. Properties such as relative size, relative granularity or internal complexity and relative fluorescence intensity are used in the differentiation of one cell from the other. Any suspended particle or cell from 0.2–150 micrometers in size is suitable for analysis (Vesey et al., 1997). STHs eggs could be analyzed using flow cytometry, but there is no report of its use in the detection of STHs eggs either from clinical or environmental samples. However, flow cytometry has been used in the detection of *Cryptosporidium parvum* oocysts in water samples (Vesey et al., 1994; Hoffman et al., 1997; Power et al., 2003) to determine experimental parasite loads in mice (Arrowood et al., 1995) and to enumerate viable oocysts (Vesey et al., 1997; Campbell et al., 1993).

Since STHs eggs fall within the cell sizes that could, in theory, be analyzed with flow cytometry, it may be possible to use this technique in the detection and quantification of these eggs. Flow cytometry is able to analyze cells based on size whereas the different STHs species could be differentiated based on their sizes. In addition, it may be possible to determine the viability of eggs. This technique is able to differentiate between cells based on complexity which is dependent on the internal structures of the cell. As STHs eggs mature there is an increase in cell numbers in each egg. This increases the complexity and it may be possible to estimate the stage of development of the egg and therefore predict its viability. Further analysis could be achieved through staining with fluorescent dyes (El-Kowrany et al., 2015; Beers et al., 2015), which could make it possible to differentiate viable eggs from non-viable eggs through differential staining. The BacLight Live/Dead staining procedure has been coupled with flow cytometry to determine viability of bacterial cells (Schumann et al., 2003; Kramer et al., 2009; Berney et al., 2007), and since this technique has been used in the determination of viable STHs eggs, (Dabrowaska et al., 2014; Karkashan et al., 2015) it could be coupled with flow cytometry to adequately determine the viability of STHs eggs. The main limitation to the widespread use of flow cytometry is the cost involved in the purchase and use of the equipment which hinders its routine use especially in developing countries.

7. Conclusion

Environmental samples pose a challenge for the detection and quantification of STHs eggs. The development of methods/techniques which are applicable to the variety of sample matrices is imperative for uniformity. Many of the methods in use have analytical steps that are similar from method to method. It can be concluded from this review that some of the major steps that influence egg recovery are, but not limited to, filtration/sieving, egg recovery from particles, sedimentation, flotation and microscopy. Microscopy which is the last step in most conventional methods could also introduce an element of variability due to human subjectivity and experience. Other main challenges of conventional methods as presented in this review is the time needed to process

and analyze samples, the additional identification of STHs eggs of similar species or differentiation of eggs of the same genus into different species. The development of molecular techniques has shown the potential to solve many of the challenges of the conventional techniques, but these techniques have shortfalls as well. The high cost involved in sample analysis is the main challenge with the use of molecular techniques for routine use. However the LAMP method promises to be a fast and cost-effective technique for the molecular detection of STHs eggs in environmental samples. In addition, there are new and emerging techniques that potentially could be used for the efficient and sensitive detection and quantification of STHs eggs in environmental samples. Some of these new techniques are flow cytometry, LAMP, digital droplet PCR and the use of image analysis softwares. There is the need to validate and compare all techniques to determine their applicability in environmental samples. For instance, the use of conventional methods is cheap and more suited to laboratories found in low-income countries. However, the conventional methods as shown in Table 7 are time consuming, laborious and may result in systematic errors, which could be reduced with molecular methods. These can be rapid, sensitive and identify the STHs down to species level; a feature that is difficult with conventional methods. Despite the aforementioned advantages of the molecular methods, these are expensive due to the high cost of equipment and consumables and the need for constant supply of electricity, which is a challenge in developing countries. The improvements in the molecular techniques such as the LAMP method could considerably reduce the cost involved in analysis and provides an opportunity for more rapid, sensitive and cheap means of analysis.

The validation and subsequent comparison of techniques would help determine the most cost-effective and efficient technique for uniform detection and quantification of STHs eggs in environmental samples.

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References

Abougrain, A.K., Nahaisi, M.H., Madi, N.S., Saied, M.M., Ghenghesh, K.S., 2010. Parasitological contamination in salad vegetables in Tripoli-Libya. Food Control. 21, 760–762.
Abreu-Acosta, N., Vera, L., 2011. Occurrence and removal of parasites enteric bacteria and faecal contamination indicators in wastewater natural reclamation systems in Tenerife-Canary Islands, Spain. Ecol. Eng. 37, 496–503.
Adamu, N.B., Adamu, J.Y., Mohammed, D., 2012. Prevalence of helminth parasites found on vegetables sold in Maiduguri, Northeastern Nigeria. Food Control. 25, 23–26.
Adanir, R., Tasci, F., 2013. Prevalence of helminth eggs in raw vegetables consumed in Burdur, Turkey. Food Control. 31, 482–484.
Ai, L., Dong, S.J., Zhang, W.Y., Elsheikha, H.M., Mahmmod, Y.S., Lin, R.Q., 2010. Specific PCR-based assays for the identification of Fasciola species: their development, evaluation and potential usefulness in prevalence surveys. Ann. Trop. Med. Parasitol. 104, 65–72.
Ajeagah, G.A., 2013. Occurrence of bacteria, protozoans and metazoans in waters from two semi-urbanized areas of Cameroon. Ecohydrol. Hydrobiol. 13, 218–225.
Al-Megrin, W.A.I., 2010. Prevalence of intestinal parasites in leafy vegetables in Riyadh, Saudi Arabia. Int. J. Zool. Res. 5, 20–23.
Amahmid, O., Asmama, S., Bouhoum, K., 1999. The effect of wastewater reuse in irrigation on the contamination level of food crops by Giardia cysts and Ascaris eggs. Int. J. Food Microbiol. 49, 19–26.
Amahmid, O., Asmama, S., Bouhoum, K., 2002. Urban wastewater treatment in stabilization ponds: occurrence and removal of pathogens. Urban Water. 4, 255–262.
Arrowood, M.J., Hurd, M.R., Mead, J.R., 1995. A new method for evaluating experimental cryptosporidial parasite loads using immunofluorescence flow cytometry. J. Parasitol. 81, 404–409.
Ayres, R.M., Mara, D.D., 1996. Analysis of Wastewater for Use in Agriculture: a Laboratory Manual of Parasitological and Bacteriological Techniques. World Health Organization, Geneva, Switzerland.
Ayres, R.M., Stott, R., Lee, D.L., Mara, D.D., Silva, S.A., 1991. Comparison of techniques for the enumeration of human parasitic helminth eggs in treated wastewater. Environ. Technol. 12, 617–623.
Baker, S.M., Ensink, J.H.J., 2012. Helminth transmission in simple pit latrines. Trans. R. Soc. Trop. Med. Hyg. 106, 709–710.
Bass, D., Stentiford, G.D., Littlewood, D.T.J., Hartikainen, H., 2015. Diverse applications of environmental DNA methods in parasitology. Trends Parasitol. 31, 499–513.
Bastos, V.K., Cutolo, S.A., Doria, M.C.O., Razzolini, M.T.P., 2013. Detection and quantification of viable Ascaris sp. and other helminth eggs in sewage sludge. Int. J. Environ. Health Res. 23, 352–362.
Bean, C.L., Brabants, J.J., 2001. Lab analyzes Ascaris ova recovery rates using revised protocols. Biosolids Technol. Bull. 7, 12–14.
Beaver, P.C., Jung, R.C., Cupp, E.W., 1984. Clinical Parasitology, 9th ed. Lea & Febiger, Philadelphia, PA.
Beers, K., Ferguson, J., Park, S.H., Cook, P., Baker, C.A., Miller, M., Caldwell, D., Ramsaroop, S., Ricke, S.C., 2015. Comparison of the RAPID-B® flow cytometer and the BAX® system for the detection of non-O157 shiga toxin-producing Escherichia coli (STEC) in beef products. Food Control. 50, 72–75.
Benti, G., Gemechu, F., 2014. Parasitic contamination on vegetables irrigated with Awash River in selected farms Eastern Showa, Ethiopia. J. Parasitol. Vector Biol. 6, 103–109.
Berney, M., Hammes, F., Bosshard, F., Weilenmann, H.-U., Egli, T., 2007. Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight Kit in combination with flow cytometry. Appl. Environ. Microbiol. 73, 3283–3290.
Blaszkowska, J., Wojcik, A., Kurnatowski, P., Szwabe, K., 2013. Biological interactions between soil saprotrophic fungi and Ascaris suum eggs. Vet. Parasitol. 196, 401–408.
Blumenthal, U.J., Cifuentes, E., Bennett, S., Quigley, M., Ruiz-Palacios, G., 2001. The risk of enteric infections associated with wastewater reuse; the effect of season and degree of storage of wastewater. Trans. R. Soc. Trop. Med. Hyg. 95, 131–137.
Bornay-Llinares, F.J., Navarro-i-Martínez, L., García-Orenes, F., Araez, H., Pérez-Murcia, M.D., Moral, R., 2006. Detection of intestinal parasites in pig slurry: a preliminary study from five farms in Spain. Livest. Sci. 102, 237–242.
Bouchet, F., Guidon, N., Dittmar, K., Harter, S., Ferreira, L.F., Chaves, S.M., Reinhard, K., Araújo, A., 2003. Parasite remains in archaeological sites. Mem. Inst. Oswaldo Cruz. 98, 47–52.
Bowman, D.D., Little, M.D., Reimers, R.S., 2003. Precision and accuracy of an assay for detecting Ascaris eggs in various biosolid matrices. Water Res. 37, 2063–2072.
Buitrón, G., Galván, M., 1998. Effect of compression ± decompression on helminth eggs present in sludge of a settling tank. Water Res. 32, 1708–1712.
Campbell, A.T., Robertson, L.J., Smith, H.V., 1993. Novel methodology for the detection of Cryptosporidium paroum? a comparison of cooled charge couple devices (ccd) and flow cytometry. Water Sci. Technol. 21, 89–92.
Cappizzi-Banas, S.C., Deloge, M., Remy, M., Schwartzbrod, J., 2004. Liming as an advanced treatment for sludge sanitization: helminth eggs elimination—Ascaris eggs as model. Water Res. 38, 3251–3258.
Collender, P.A., Kirby, A.E., Addiss, D.G., Freeman, M.C., Remais, J.V., 2015. Methods for quantification of soil-transmitted helminths in environmental media: current techniques and recent advances. Trends Parasitol. 31, 623–639.
Dabrowaska, J., Zdybel, J., Karamon, J., Kochanowski, M., Stojecki, K., Cencek, T., Klapec, T., 2014. Assessment of viability of the nematode eggs (Ascaris, Toxocara, Trichuris) in sewage sludge with the use of LIVE/DEAD Bacterial Viability Kit. Ann. Agric. Environ. Med. 21, 35–41.
Dada, B.J.O., Lindquist, W.D., 1979. Studies on flotation techniques for the recovery of helminth eggs from soil and the prevalence of eggs of Toxocara spp in some Kansas public places. J. Am. Vet. Med. Assoc. 174, 1208–1210.
Daryani, A., Ettehad, G.H., Sharif, M., Ghorbani, L., Ziaei, H., 2008. Prevalence of intestinal parasites in vegetables consumed in Ardabil, Iran. Food Control. 19, 790–794.
David, E.D., Lindquist, W.D., 1982. Determination of the Specific Gravity of Certain Helminth Eggs Using Sucrose Density Gradient Centrifugation. J. Parasitol. 68, 916–919.
David, E.D., 1977. Evaluation of a modification of the Dada Technique for the Recovery of Helminth eggs from soil, and determination of the specific gravity of certain helminth eggs using sucrose density gradient centrifugation. In: A Master's Thesis. Department of Laboratory Medicine, Kansas State University, Manhattan, Kansas.
Deisingh, A.K., Thompson, M., 2004. Strategies for the detection of Escherichia coli O157:H7 in foods. J. Appl. Microbiol. 96, 419–429.
Dryden, M.W., Payne, P.A., Ridley, R., Smith, V., 2005. Comparison of common fecal flotation techniques for the recovery of parasite eggs and oocysts. Vet. Ther. 6, 15–29.
Dubná, S., Langrova, I., Jankovska, I., Vadlejch, J., Pekar, S., Napravnik, J., 2007. Contamination of soil with Toxocara eggs in urban (Praque) and rural areas in the Czech Republic. Vet. Parasitol. 144, 81–86.
Durant, J.F., Irenge, L.M., Fogt-Wyrwas, R., Dumont, C., Doucet, J.P., 2012. Duplex quantitative real-time PCR assay for the detection and discrimination of the

eggs of Toxocara canis and Toxocara cati (Nematoda, Ascaridoidea) in soil and fecal samples. Parasit. Vectors 5, 1–9.
El-Kowrany, S.I., El- Zamarany, E.A., El-Nouby, K.A., El-Mehy, D.A., Abo Ali, E.A., Othman, A.A., Salah, W., El-Ebiary, A.A., 2015. Water pollution in the Middle Nile Delta, Egypt: an environmental study. J. Adv. Res. 7, 781–794.
Engohang-Ndong, J., Uribe, R.M., Gregory, R., Gangoda, M., Nickelsen, M.G., Loar, P., 2015. Effect of electron beam irradiation on bacterial and Ascaris ova loads and volatile organic compounds in municipal sewage sludge. Radiat. Phys. Chem. 112, 6–12.
Ensink, J.H.J., Mahmood, T., Dalsgaard, A., 2007. Wastewater-irrigated vegetables: market handling versus irrigation water quality. Trop. Med. Int. Health 12, 2–7.
Erdoğrul, Ö., Şener, H., 2005. The contamination of various fruit and vegetable with Enterobius vermicularis, Ascaris eggs, Entamoeba histolytica cysts and Giardia cysts. Food Control. 16, 557–560.
Eriksen, L., Andreasen, P., Ilsoe, B., 1995. Inactivation of Ascaris suum eggs during storage in time treated sewage sludge. Water Res. 30, 1026–1029.
Fajutag, A.J., Paller, V.G., 2013. Toxocara egg soil contamination and its seroprevalence among public school children in Los Banos, Laguna, Philippines. Southeast Asian J. Trop. Med. Public Health 44, 551–560.
Fallah, A.A., Makhtumi, Y., Pirali-Kheirabadi, K., 2016. Seasonal study of parasitic contamination in fresh salad vegetables marketed in Shahrekord, Iran. Food Control 60, 538–542.
Forslund, A., Ensink, J.H.J., Battilani, A., Kljujev, I., Gola, S., Raicevi, V., Jovanovic, Z., Stikic, R., Sandei, L., Fletcherg, T., Dalsgaard, A., 2010. Faecal contamination and hygiene aspect associated with the use of treated wastewater and canal water for irrigation of potatoes (Solanum tuberosum). Agric. Water Manage. 98, 440–450.
Fugazzola, M.C., Stancampiano, L., 2012. Host social rank and parasites: plains zebra (Equus quagga) and intestinal helminths in Uganda. Vet. Parasitol. 188, 115–119.
Fuhrimann, S., Stalder, M., Winkler, M.S., Niwagaba, C.B., Babu, M., Masaba, G., Kabatereine, N.B., Halage, A.A., Schneeberger, P.H.H., Utzinger, J., Cissé, G., 2015. Microbial and chemical contamination of water sediment and soil in the Nakivubo wetland area in Kampala, Uganda. Environ. Monit. Assess. 187, 475–489.
Gaasenbeek, C., Borgsteede, F., 1998. Studies on the survival of Ascaris suum eggs under laboratory and simulated field conditions. Vet. Parasitol. 75, 227–234.
Gantzer, C., Gaspard, P., Galvez, L., Huyard, A., Dumouthier, N., Schwartzbrod, J., 2001. Monitoring of bacterial and parasitological contamination during various treatment of sludge. Water Res. 35, 3763–3770.
García, J.A., Paredes, D., Cubillos, J.A., 2013. Effect of plants and the combination of wetland treatment type systems on pathogen removal in tropical climate conditions. Ecol. Eng. 58, 57–62.
Gaspard, P.G., Wiart, J., Shwartzbrod, J., 1995. Urban sludge reuse in agriculture: waste treatment and parasitological risk. Biores. Technol. 52, 37–40.
Gaspard, P., Wiart, J., Schwartzbrod, J., 1996. A method for assessing the viability of nematode eggs in sludge. Environ. Technol. 17, 415–420.
Gatcombe, R.R., Jothikumar, N., Dangoudoubiyam, S., Kazacos, K.R., Hill, V.R., 2010. Evaluation of a molecular beacon real-time PCR assay for detection of Baylisascaris procyonis in different soil types and water samples. Parasitol. Res. 106, 499–504.
Gomes, A.P., Noguerol, L., Bez, M.R., Tavares, R.G., 2015. Performance analysis of software for identification of intestinal parasites. J. Bras. Patol. Med. Lab. 51, 218–223.
Gomez-Couso, H., Freire-Santos, F., Amar, C.F.L., Grant, K.A., Williamson, K., Ares-Mazas, M.E., McLauchlin, J., 2004. Detection of Cryptosporidium and Giardia in molluscan shellfish by multiplexed nested-PCR. Int. J. Food Microbiol. 91, 279–288.
Gordon, C.A., Gray, D.J., Gobert, G.N., McManus, D.P., 2011. DNA amplification approaches for the diagnosis of key parasitic helminth infections of humans. Mol. Cell. Probes 25, 143–152.
Gupta, N., Khan, D.K., Santra, S.C., 2009. Prevalence of intestinal helminth eggs on vegetables grown in wastewater-irrigated areas of Titagarh West Bengal, India. Food Control 20, 942–945.
Guy, R.A., Payment, P., Krull, U.J., Horgen, P.A., 2003. Real-time PCR for quantification of Giardia and Cryptosporidium in environmental water samples and sewage. Appl. Environ. Microbiol. 69, 5178–5185.
Gyawali, P., Ahmed, W., Sidhu, J.P.S., Jagals, P., Toze, S., 2015. Comparison of concentration methods for rapid detection of hookworm ova in wastewater matrices using quantitative PCR. Exp. Parasitol. 159, 160–167.
Habbari, K., Tifnouti, A., Bitton, G., Mandil, A., 1999. Helminthic infections associated with the use of raw wastewater infections associated with the use of raw wastewater for agricultural purposes in Beni Mellal, Morocco. East Mediterr. Health J. 5, 912–921.
Hassan, A., Farouk, H., Abdul-Ghani, R., 2012. Parasitological contamination of freshly eaten vegetables collected from local markets in Alexandria, Egypt : A preliminary study. Food Control 26, 500–503.
Henegariu, O., Heerama, N.A., SDlouhy, S.R., Vg, H., Vogt, P.H., 1997. Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques 23, 504–511.
Hindson, C.M., Chevillet, J.R., Briggs, H.A., Gallichotte, E.N., Ruf, I.K., Hindson, B.J., Vessella, R.L., Tewari, M., 2013. Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat. Methods 10, 1003–1005.
Hoffman, R.M., Standridge, J.H., Prieve, A.F., Cucunato, J.C., Bernhardt, M., 1997. Using flow cytometry to detect protozoa. J. Am. Water Works Assoc. 89, 104–111.
Horak, P., 1992. Helminth eggs in the sludge from three sewage plants in Czechoslovakia. Folia Parasitol. 39, 153–157.
Horiuchi, S., Paller, V.G.V., Uga, S., 2013. Soil contamination by parasite eggs in rural village in the Philippines. Trop. Biomed. 30, 495–503.
Ishiwata, K., Shinohara, A., Yagi, K., Horii, Y., Tsuchiya, K., Nawa, Y., 2004. Identification of tissue-embedded ascarid larvae by ribosomal DNA sequencing. Parasitol. Res. 92, 50–52.
Jaskoski, B.J., 1954. A comparative study of detergent effects on ascarid development. Am. Midl. Nat. 52, 142–148.
Jiménez, B., Maya, C., Velásquez, G., Torner, F., Arambula, F., Barrios, J.A., Velasco, M., 2016. Identification and quantification of pathogenic helminth eggs using a digital image system. Exp. Parasitol. 166, 164–172.
Kłapeć, T., 2009. Contamination of soil with geohelminth eggs on organic farms of vegetable production profile in the Lublin Region, Poland. Wiad. Parazytol. 55, 405–409.
Kamizoulis, G., 2008. Setting health based targets for water reuse (in agriculture). Desalination 218, 154–163.
Karkashan, A., Khallaf, B., Morris, J., Thurbon, N., Rouch, D., Smith, S.R., Deighton, M., 2014. Comparison of methodologies for enumerating and detecting the viability of Ascaris eggs in sewage sludge by standard incubation-microscopy, the BacLight live/dead viability assay and other vital dyes. Water Res. 68, 533–544.
Karkashan, A., Khallaf, B., Morris, J., Thurbon, N., Rouch, D., Smith, S.R., Deighton, M., 2015. Comparison of methodologies for enumerating and detecting the viability of Ascaris eggs in sewage sludge by standard incubation-microscopy, the BacLight Live/Dead viability assay and other vital dyes. Water Res. 68, 533–544.
Katakam, K.K., Mejer, H., Dalsgaard, A., Kyvsgaard, N.C., Thamsborg, S.M., 2014. Survival of Ascaris suum and Ascaridia galli eggs in liquid manure at different ammonia concentrations and temperatures. Vet. Parasitol. 204, 249–257.
Keraita, B., Amoah, P., 2011. Fecal Exposure Pathways in Accra: A Literature Review with Specific Focus on IWMI's Work on Wastewater Irrigated Agriculture (IWMI). Literature Review Report. submitted to the Centre for Global Safe Water, Emory University.
Khouja, L.B., Cama, V., Xiao, L., 2010. Parasitic contamination in wastewater and sludge samples in Tunisia using three different detection techniques. Parasitol. Res. 107, 109–116.
Konaté, Y., Maiga, A.H., Basset, D., Casellas, C., Picot, B., 2013. Parasite removal by waste stabilization pond in Burkina Faso, accumulation and inactivation in sludge. Ecol. Eng. 50, 101–106.
Koneí, D., Cofie, O., Zurbrügg, C., Gallizzi, K., Moser, D., Drescher, S., Strauss, M., 2007. Helminth eggs inactivation efficiency by faecal sludge dewatering and co-composting in tropical climates. Water Res. 41, 4397–4402.
Kouraa, A., Fethi, F., Fahde, A., Lahlou, A., Ouazzani, N., 2002. Reuse of urban sewage water treated by combined stabilization pond system in Benslimane (Morocco). Urban Water 4, 373–378.
Kozan, E., Gonenc, B., Sarimehmetoglu, O., Aycicek, H., 2005. Prevalence of helminth eggs on raw vegetables used for salads. Food Control 16, 239–242.
Kramer, M., Obermajer, N., Matijašić, B., Rogelj, I., Kmetec, V., 2009. Quantification of live and dead probiotic bacteria in lyophilised product by real-time PCR and by flow cytometry. Appl. Microbiol. Biotechnol. 84, 1137–1147.
Kramme, S., Nissen, N., Soblik, H., Erttmann, K., Tannich, E., Fleischer, B., Panning, M., Brattig, N., Nissen, N., Soblik, H., 2011. Novel real-time PCR for the universal detection of Strongyloides species. J. Med. Microbiol. 60, 454–458.
Landa-Cansigno, O., Durán-Álvarez, J.C., Jiménez-Cisneros, B., 2013. Retention of Escherichia coli, Giardia lamblia cysts and Ascaris lumbricoides eggs in agricultural soils irrigated by untreated wastewater. J. Environ. Manag. 128, 22–29.
Le Cann, P., Ranarijaona, S., Monpoeho, S., Le Guyader, F., Ferre, V., 2004. Quantification of human astroviruses in sewage using real-time RTPCR. Res. Microbiol. 155, 11–15.
Levantesi, C., La Mantia, R., Masciopinto, C., Böckelmann, U., Ayuso-Gabella, M.N., Salgot, M., Tandoi, V., Van Houtte, E., Wintgens, T., Grohmann, E., 2010. Quantification of pathogenic microorganisms and microbial indicators in three wastewater reclamation and managed aquifer recharge facilities in Europe. Sci. Total Environ. 408, 4923–4930.
Liang, S.Y., Chan, Y.H., Hsia, K.T., Lee, J.L., Kuo, M.C., Hwa, K.Y., Chan, C.W., Chiang, T.Y., Chen, J.S., Wu, F.T., Ji, D.D., 2009. Development of loop-mediated isothermal amplification assay for detection of Entamoeba histolytica. J. Clin. Microbiol. 47, 1892–1895.
Lindgren, K., Ljungvall, Ö., Nilsson, O., Ljungström, B.-L., Lindahl, C., Höglund, J., 2008. Parascaris equorum in foals and in their environment on a Swedish stud farm, with notes on treatment failure of ivermectin. Vet. Parasitol. 151, 337–343.
Lisle, J.T., Broadaway, S.C., Prescott, A.M., Pyle, B.H., Fricker, C., McFeters, G.A., 1998. Effects of starvation on physiological activity and chlorine disinfection resistance in Escherichia coli O157:H7. Appl. Environ. Microbiol. 64, 4658–4662.
Loreille, O., Roumat, E., Verneau, O., Bouchet, F., Hänni, C., 2001. Ancient DNA from Ascaris: extraction amplification and sequences from eggs collected in coprolites. Int. J. Parasitol. 31, 1101–1106.
Maikai, B., Elisha, I., Baba-Onoja, E., 2012. Contamination of vegetables sold in markets with helminth eggs in Zaria metropolis, Kaduna State-Nigeria. Food Control 28, 345–348.

Mandarino-Pereira, A., de Souza, F.S., Lopes, C.W.G., Pereira, M.J.S., 2010. Prevalence of parasites in soil and dog feces according to diagnostic tests. Vet. Parasitol. 170, 176–181.
Mara, D., Silva, S.A., 1987. Removal of intestinal nematodes in tropical waste stabilisation ponds. Am. J. Trop. Med. Hyg. 89, 71–74.
Mara, D., Sleigh, A., 2010. Estimation of Ascaris infection risks in children under 15 from the consumption of wastewater irrigated carrots. J. Water Health 8, 35–38.
Maya, C., Jimenez, B., Schwartzbrod, J., 2006. Comparison of techniques for the detection of helminth ova in drinking water and wastewater. Water Environ. Res. 78, 118–124.
Maya, C., Ortiz, M., Jiménez, B., 2010. Viability of Ascaris and other helminth genera non larval eggs in different conditions of temperature: lime (pH) and humidity. Water Sci. Technol. 62, 616–2624.
Maya, C., Torner-Morales, F.J., Lucario, E.S., Hernández, E., Jiménez, B., 2012. Viability of six species of larval and non-larval helminth eggs for different conditions of temperature, pH and dryness. Water Res. 46, 4770–4782.
Mes, T.H.M., 2003. Technical variability and required sample size of helminth egg isolation procedures. Vet. Parasitol. 115, 311–320.
Mizgajska, H., 1997. The role of some environmental factors in the contamination of soil with Toxocara spp. and other geohelminth eggs. Parasitol. Int. 46, 67–72.
Molleda, P., Blanco, I., Ansola, G., de Luis, E., 2008. Removal of wastewater pathogen indicators in a constructed wetland in Leon, Spain. Ecol. Eng. 33, 252–257.
Moodley, P., Archer, C., Hawksworth, D., 2008. Standard methods for the recovery and enumeration of helminth ova in wastewater, sludge, compost, and urine diversion waste in South Africa, Water Research Commission. In: WRC Report No. TT322/08.
Mori, Y., Nagamine, K., Tomita, N., Notomi, T., 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. 289, 150–154.
Moubarrad, F.Z.L., Assobhei, O., 2007. Health risks of raw sewage with particular reference to Ascaris in the discharge zone of El Jadida (Morocco). Desalination 215, 120–126.
Mugambi, R.M., Agola, E.L., Mwangi, I.N., Kinyua, J., Shiraho, E.A., Mkoji, G.M., 2015. Development and evaluation of a Loop Mediated Isothermal Amplification (LAMP) technique for the detection of hookworm (Necator americanus) infection in fecal samples. Parasit. Vectors 8, 574–581.
Muna, S., Cho, S., Kim, T., Oh, B., Yoon, J., 2009. Inactivation of Ascaris eggs in soil by microwave treatment compared to UV and ozone treatment. Chemosphere 77, 285–290.
Nelson, K.L., Darby, J.L., 2001. Inactivation of viable ascaris eggs by reagents during enumeration. Appl. Environ. Microbiol. 67, 5453–5459.
Nkouawa, A., Sako, Y., Nakao, M., Nakaya, K., Ito, A., 2009. Loop-mediated isothermal amplification method for differentiation and rapid detection of Taenia species. J. Clin. Microbiol. 47, 168–174.
Nooraldeen, K., 2015. Contamination of public squares and parks with parasites in Erbil city, Iraq. Ann. Agric. Environ. Med. 22, 418–420.
Nordin, A., Nyberg, K., Vinnerås, B., 2009. Inactivation of Ascaris eggs in source-separated urine and faeces by ammonia at ambient temperatures. Appl. Environ. Microbiol. 75, 662–667.
Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, 63–69.
Nunes, C.M., Sinhorini, I.L., Ogassawara, S., 1994. Influence of soil texture in the recovery of Toxocara canis eggs by a flotation method. Vet. Parasitol. 53, 269–274.
Oge, H., Oge, S., 2000. Quantitative comparison of various methods for detecting eggs of Toxocara canis in samples of sand. Vet. Parasitol. 92, 75–79.
Oksanen, A., Eriksen, L., Roepstorff, A., Ilsoe, B., Nansen, P., Lind, P., 1990. Embryonation and infectivity of Ascaris-suum eggs e a comparison of eggs collected from worm uteri with eggs isolated from pig feces. Acta. Vet. Scand. 31, 393–398.
Oliveira, L.M.A., Santos, H.L.C., Gonçalves, M.M.L., Barreto, M.G.M., Peralta, J.M., 2010. Evaluation of polymerase chain reaction as an additional tool for the diagnosis of low-intensity Schistosoma mansoni infection. Diagn. Microbiol. Infect. Dis. 68, 416–420.
Paquet-Durand, I., Herna' ndez, J., Dolz, G., Zun˜ iga, J.J.R., Schnieder, T., Epe, C., 2007. Prevalence of Toxocara spp., Toxascaris leonina and ancylostomidae in public parks and beaches in different climate zones of Costa Rica. Acta Trop. 104, 30–37.
Parida, M., Sannarangaiah, S., Dash, P.K., Rao, P.V., Morita, K., 2008. Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. Rev. Med. Virol. 18, 407–421.
Paris, D.H., Imwong, M., Faiz, A.M., Hasan, M., Yunus, E.B., Silamut, K., Lee, S.J., Day, N.P., Dondorp, A.M., 2007. Loop-mediated isothermal PCR (LAMP) for the diagnosis of falciparum malaria. Am. J. Trop. Med. Hyg. 77, 972–976.
Pecson, B.M., Barrios, J.A., Johnson, D.R., Nelson, K.L., 2006. A realtime PCR method for quantifying viable Ascaris eggs using the first internally transcribed spacer region of ribosomal DNA. Appl. Environ. Microbiol. 72, 7864–7872.
Pecson, B.M., Barrios, J.A., Jimenez, B.E., Nelson, K.L., 2007. The effects of temperature, pH, and ammonia concentration on the inactivation of Ascaris eggs in sewage sludge. Water Res. 41, 2893–2902.
Pham-Duc, P., Nguyen-Viet, H., Hattendorf, J., Zinsstag, J., Phung-Dac, C., Zurbrügg, C., Odermatt, P., 2013. Ascaris lumbricoides and Trichuris trichiura infections associated with wastewater and human excreta use in agriculture in Vietnam. Parasitol. Int. 62, 172–180.
Pontes, L.A., Dias-Neto, E., Rabello, A., 2002. Detection by polymerase chain reaction of Schistosoma mansoni DNA in human serum and feces. Am. J. Trop. Med. Hyg. 66, 157–162.
Power, M.L., Shanker, S.R., Sangster, N.C., Veal, D.A., 2003. Evaluation of a combined immunomagnetic separation/flow cytometry technique for epidemiological investigations of Cryptosporidium in domestic and Australian native animals. Vet. Parasitol. 112, 21–31.
Pullan, R.L., Smith, J.L., Jasrasaria, R., Brooker, S.J., 2014. Global numbers of infection and disease burden of soil transmitted helminth infections in 2010. Parasit. Vectors 7, 37–56.
Quinn, R., Smith, H.V., Bruce, R.G., Girdwood, R.W.A., 1980. Studies on the incidence of Toxocara and Toxascaris spp. ova in the environment: a comparison of flotation procedures for recovering Toxocara spp. ova from soil. J. Hyg. 84, 83–89.
Raynal, M., Villegas, E.N., Nelson, K.L., 2012. Enumeration of viable and non-viable larvated Ascaris eggs with quantitative PCR. J. Water Health 10, 594–604.
Reimers, R.S., Little, M.D., Akers, T.G., Henriques, W.D., Badeaux, R.C., McDonnell, D.B., Mbela, K.K., 1989. Persistence of pathogens in lagoon stored sludge. In: EPA 600/2-89/015, NTIS Report PB 89-190359.
Reinoso, R., Becares, E., 2008. The occurrence of intestinal parasites in swine slurry and their removal in activated sludge plants. Bioresour. Technol. 99, 6661–6665.
Reinoso, R., Torres, L.A., Bécares, E., 2008. Efficiency of natural systems for removal of bacteria and pathogenic parasites from wastewater. Sci. Total Environ. 395, 80–86.
Riahi, K., Mammou, A.B., Thayer, B.B., 2009. Date-palm fibers media filters as a potential technology for tertiary domestic wastewater treatment. J. Hazard. Mater. 161, 608–613.
Robertson, L.J., Johannessen, G.S., Gjerde, B.K., Loncarevic, S., 2002. Microbiological analysis of seed sprouts in Norway. Int. J. Food Microbiol. 75, 119–126.
Rocha, M.C.V., Barés, M.E., Braga, M.C.B., 2016. Quantification of viable helminth eggs in samples of sewage sludge. Water Res. 103, 245–255.
Rodríguez, D.C., Pino, N., Peñuela, G., 2012. Microbiological quality indicators in waters of dairy farms: detection of pathogens by PCR in real time. Sci. Total Environ. 427–428, 314–318.
Rosa Xavier, I.G., Ramos, B.C., Santarém, V.A., 2010. Recovery threshold of Toxocara canis eggs from soil. Vet. Parasitol. 167, 77–80.
Rose, J.B., Dickson, L.J., Farrah, S.R., Carnahan, R.P., 1996. Removal of pathogenic and indicator microorganisms by a full-scale water reclamation facility. Water Res. 30, 2785–2797.
Rostami, A., Ebrahimi, M., Mehravar, S., Omrani, V.F., Fallahi, S., Behniafar, H., 2016. Contamination of commonly consumed raw vegetables with soil transmitted helminth eggs in Mazandaran province, Northern Iran. Int. J. Food Microbiol. 225, 54–58.
Rothrock Jr., M.J., Hiett, K.L., Kiepper, B.H., Ingram, K., Hinton, A., 2013. Quantification of zoonotic bacterial pathogens within commercial poultry processing water samples using droplet digital PCR. Adv. Microbiol. 3, 403–411.
Rude, R.A., Peeler, J.T., Risty, N.G., 1987. Comparison of diethyl ether and ethyl acetate as extracting agents for recovery of Ascaris spp. and Trichuris spp eggs. J. Assoc. Offic. Anal. Chem. 70, 1000–1002.
Rutkowski, T., Raschid-Sally, L., Buechler, S., 2007. Wastewater irrigation in the developing world—two case studies from the Kathmandu Valley in Nepal. Agric. Water Manag. 88, 83–91.
Saddoud, A., Ellouze, M., Dhouib, A., Sayadi, S., 2007. Anaerobic membrane bioreactor treatment of domestic wastewater in Tunisia. Desalination 207, 205–215.
Said, D.E., 2012. Detection of parasites in commonly consumed raw vegetables. Alexandria Med. J. 48, 345–352.
Salant, H., Abbasi, I., Hamburger, J., 2012. The development of a loop-Mediated isothermal amplification method (LAMP) for Echinococcus granulosis coprodetection. Am. J. Trop. Med. Hyg. 87, 883–887.
Santarém, V.A., Magoti, L.P., Sichieri, T.D., 2009. Influence of variables on centrifuge-flotation technique for recovery of toxocara canis eggs from soil. Rev. Inst. Med. Trop. S. Paulo. 51, 163–167.
Satchwell, M.G., 1986. An adaptation of concentration technique for the enumeration of parasitic helminth eggs from sewage sludge. Water Res. 20, 813–816.
Saugar, J.M., Merino, F.J., Martín-Rabadán, P., Fernández-Soto, P., Ortega, S., Gárate, T., Rodríguez, E., 2015. Application of real-time PCR for the detection of Strongyloides spp. in clinical samples in a reference center in Spain. Acta Trop. 142, 20–25.
Scaglia, B., D'Imporzano, G., Garuti, G., Negri, M., Adani, F., 2014. Sanitation ability of anaerobic digestion performed at different temperature on sewage sludge. Sci. Total Environ. 466–467, 888–897.
Schär, F., Odermatt, P., Khieu, V., Panning, M., Duong, S., Muth, S., Marti, H., Kramme, S., 2013. Evaluation of real-time PCR for Strongyloides stercoralis and hookworm as diagnostic tool in asymptomatic schoolchildren in Cambodia. Acta Trop. 126, 89–92.
Schumann, R., Schiewer, U., Karsten, U., Rieling, T., 2003. Viability of bacteria from different aquatic habitats II. Cellular fluorescent markers for membrane integrity and metabolic activity. Aquat. Microb. Ecol. 32, 137–150.
Schwartzbrod, J., Mathieu, C., Thevenot, M.T., Baradel, J.M., Schwartzbrod, L., 1987. Wastewater sludge: parasitological and virological contamination. Water Sci. Technol. 19, 30–40.

Schwartzbrod, J., 1998. Methods of Analysis of Helminth Eggs and Cysts in Wastewater, Sludge, Soils and Crops. University Henri Poincareí, Nancy, France.
Seidu, R., Heistad, A., Amoah, P., Drechsel, P., Jenssen, P.D., Stenström, T.A., 2008. Quantification of the health risk associated with wastewater reuse in Accra, Ghana : a contribution toward local guidelines. J. Water Health 6, 461–471.
Sengupta, M.E., Keraita, B., Olsen, A., Boateng, O.K., Thamsborg, S.M., Pálsdóttir, G.R., Dalsgaard, A., 2011. Use of Moringa oleifera seed extracts to reduce helminth egg numbers and turbidity in irrigation water. Water Res. 46, 3646–3656.
Shamma, M., Al-Adawi, M.A., 2002. The morphological changes of Ascaris lumbricoides ova in sewage, sludge water treated by gamma irradiation. Radiat. Phys. Chem. 65, 277–279.
Shannon, K., Lee, D.Y., Trevors, J., Beaudette, L., 2007. Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment. Sci. Total Environ. 382, 121–129.
Shuval, H.I., Adin, A., Fattal, B., Rawitz, E., Yekutiel, P., 1986. Wastewater Irrigation in Developing Countries: Health Effects and Technical Solutions. World Bank, Washington DC.
Sowerby, S.J., Crump, J.A., Johnstone, M.C., Krause, K.L., Hill, P.C., 2016. Smartphone microscopy of parasite eggs accumulated into a single field of view. Am. J. Trop. Med. Hyg. 94, 227–230.
Stolk, W.A., Kulik, M.C., le Rutte, E.A., Jacobson, J., Richardus, J.H., de Vlas, S.J., 2016. Between-country inequalities in the neglected tropical disease burden in 1990 and 2010, with projections for 2020. PLoS Negl. Trop. Dis. 10 (5), e0004560, http://dx.doi.org/10.1371/journal.pntd.0004560.
Strunz, E.C., Addiss, D.G., Stocks, M.E., Ogden, S., Utzinger, J., Freeman, M.C., 2014. Water, sanitation, hygiene, and soil-transmitted helminth infection: a systematic review and meta-analysis. PLoS Med. 11, e1001620.
Su, G.L.S., Mariano, C.M.R., Matti, N.S.A., Ramos, G.B., 2012. Assessing parasitic infestation of vegetables in selected markets in Metro Manila, Philippines. Asian Pac. Trop. Dis. 12, 51–54.
Sultana, Y., Jeoffreys, N., Watts, M.R., Gilbert, G.L., Lee, R., 2013. Real-time polymerase chain reaction for detection of Strongyloides stercoralis in stool. Am. J. Trop. Med. Hyg. 88, 1048–1051.
Toze, S., 1999. PCR and the detection of microbial pathogens in water and wastewater. Water Res. 33, 3545–3556.
Trönnberg, L., Hawksworth, D., Hansen, A., Archer, C., Stenström, T.A., 2010. Household-based prevalence of helminths and parasitic protozoa in rural KwaZulu-Natal, South Africa, assessed from faecal vault sampling. Trans. R. Soc. Trop. Med. Hyg. 104, 646–652.
Trang, D.T., van der Hoek, W., Cam, P.D., 2006. Low risk for helminth infection in wastewater-fed rice cultivation in Vietnam. J. Water Health. 4, 321–331.
U.S. Environmental Protection Agency, 1999. Control of Pathogens and Vector Attraction in Sewage Sludge EPA/625/R-92/013. Revised Edition. U.S. Environmental Protection Agency, Washington, D.C.
USEPA—U. S. Environmental Protection Agency., 2003. Control of Pathogens and Vector Attraction in Sewage Sludge. EPA 815-R-06-002.
Valero, M.A., Perez-Crespo, I., Periago, M.V., Khoubbane, M., Mas-Coma, S., 2009. Fluke egg characteristics for the diagnosis of human and animal fascioliasis by Fasciola hepatica and F gigantica. Acta. Trop. 111, 150–159.
Verbyla, M.E., Iriarte, M.M., Guzmán, A.M., Coronado, O., Almanza, M., Mihelci, J.R., 2016. Pathogens and fecal indicators in waste stabilization pond systems with direct reuse for irrigation: fate and transport in water, soil and crops. Sci. Total Environ. 551–552, 429–437.
Vesey, G., Hutton, P.E., Champion, A.C., Ashbolt, N.J., Williams, K.L., Warton, A., Veal, D.A., 1994. Application of flow cytometric methods for the routine detection of Cryptosporidium and Giardia in water. Cytometry 16, l–6.
Vesey, G., Griffith, K.R., Gauci, M.R., Deere, D., Williams, K.L., Veal, D.A., 1997. Simple and rapid measurement of cryptosporidium excystation using flow cytometry. Int. J. Parasitol. 21, 1353–1359.
WHO- World Health Organization, 2006. Guidelines for the Safe Use of Wastewater Excreta and Greywater, vol. 4. World Health Organization, Geneva.
WHO-World Health Organization, 2015. Investing To Overcome The Global Impact Of Neglected Tropical Diseases Third Who Report On Neglected Tropical Diseases. WHO Document Production Services, Geneva, Switzerland (WHO/HTM/NTD/2015.1).
Wang, M.S., Bundy, D.A.P., 1990. Quantitative assessment of contamination of soil by the eggs of Ascaris lumbricoides and Trichuris trichiura. Trans. R. Soc. Trop. Med. Hyg. 84, 567–570.
Yanko, W.A., 1987. Occurrence of Pathogens in Distribution and Marketing Municipal Sludges. EPA-600/1-87-014 National Technical Information Service, Springfield, Virginia.
Yaya-Beas, R., Cadillo-La-Torre, E., Kujawa-Roeleveld, K., van Lier, J.B., Zeeman, G., 2016. Presence of helminth eggs in domestic wastewater and its removal at low temperature UASB reactors in Peruvian highlands. Water Res. 90, 286–293.
Yeager, J.G., O'Brien, R.T., 1983. Irradiation as a means to minimize public health risks from sludge-borne pathogens. J. Water Pollut. Control Fed. 55, 977–983.
Yeh, H., Prag, K., Clamer, C., Humbert, J., Mitchella, P.D., 2015. Human intestinal parasites from a Mamluk Period cesspool in the Christian quarter of Jerusalem: potential indicators of long distance travel in the 15th century AD. Int. J. Paleopath. 9, 69–75.
Yen-Phi, V.T., Rechenburg, A., Vinneras, B., Clemens, J., Kistemann, T., 2010. Pathogens in septage in Vietnam. Sci. Total Environ. 408, 2050–2053.
Zamudio-Pérez, E., Rojas-Valencia, N., Chairez, I., Torres, L.G., 2013. Coliforms and helminth eggs removals by coagulation-flocculation treatment based on natural polymers. J. Water Resour. Prot. 5, 1027–1036.
Zeehaida, M., Zairi, N.Z., Rahmah, N., Maimunah, A., Madihah, B., 2011. Strongyloides stercoralis in common vegetables and herbs in Kota Bharu, Kelantan, Malaysia. Trop. Biomed. 28, 188–193.
Zenner, L., Gounel, J.M., Chauve, C.M., 2002. A standardized method for detecting parasite eggs and oocysts in soils. Revue Méd. Vét. 153, 729–734.
Zhang, T., Fang, H.H., 2004. Quantification of Saccharomyces cerevisiae viability using BacLight. Biotechnol. Lett. 26, 989–992.
de Souza, G.S.M.B., Rodrigues, L.A., de Oliveira, W.J., Chernicharo, C.A.L., Guimarães, M.P., Massara, C.L., Grossi, P.A., 2011. Disinfection of domestic effluents by gamma radiation: effects on the inactivation of ascaris lumbricoides eggs. Water Res. 45, 5523–5528.
de Victorica, J., Galvan, M., 2003. Preliminary testing of a rapid coupled methodology for quantitation/viability determination of helminth eggs in raw and treated wastewater. Water Res. 37, 1278–1287.
van Frankenhuyzen, J.K., Trevors, J.T., Lee, H., Flemming, C.A., Habash, M.B., 2011. Molecular pathogen detection in biosolids with a focus on quantitative PCR using propidium monoazide for viable cell enumeration. J. Microbiol. Methods 87, 263–272.

Paper II

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Effect of reagents used during detection and quantification of *Ascaris suum* in environmental samples on egg viability

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ABSTRACT

Soil-transmitted helminths (STHs) are a major health concern globally. Infection is mostly through contact with contaminated water, food or soil. Therefore to break the cycle of transmission STH eggs must be quantitatively detected in the environment. The effect of different reagents on the viability of *Ascaris suum* eggs during laboratory detection and quantification was assessed and different incubation solutions compared. Sulfuric acid gave a slightly higher recovery percentage of viable eggs (91.2%) than distilled water (90.0%) and 0.5% formalin (87.6%), although the difference was not statistically significant ($p > 0.05$). Acetoacetic acid, ethyl acetate, ammonium bicarbonate, zinc sulphate, magnesium sulphate and Tween 80, are reagents widely used in test protocols for the detection and quantification of STH eggs. Eggs were exposed to these reagents for different time durations. Acetoacetic acid resulted in the highest loss of viability ($3.4 \pm 0.7\%$ viable), while magnesium sulphate resulted in the least effect ($88.5 \pm 1.2\%$ viable). In conclusion the use of the selected reagents in the detection of these eggs in was found to affect the viability of exposed eggs especially during prolonged exposures. Therefore we recommended that eggs be exposed for $\leq$5 minutes, to reduce the risk of viability loss.

Key words | *Ascaris suum*, inactivation, sludge, soil-transmitted helminths, viability, wastewater

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INTRODUCTION

In soil-transmitted helminth (STH) infection endemic regions it is estimated that wastewater may contain up to ~3,000/L (Kamizoulis 2008; Mara & Sleigh 2010) of STH eggs, while documented counts in faecal sludge from public toilets are between 2,500 to 60,000 eggs/L (Yen-Phi *et al.* 2010). From septic tanks the concentrations are reported to be from 600 to 16,000 eggs/L (Yen-Phi *et al.* 2010). Trönnberg *et al.* (2010) assessed the concentrations of STHs in the fecal vaults of urine-diverting (UD) toilets in the KwaZulu Natal Province of South Africa. Counts varied from below detection limit to a maximum of 1,425 eggs per gram (EPG) of faeces for *Ascaris lumbricoides*, 147 EPG for *Trichuris trichiura* and 703 EPG for *Taenia* spp. These STH eggs can survive for a long period of time in the environment, where fertilized eggs of *Ascaris* spp for instance have been reported to survive for up 7 years under favorable temperatures of 25 °C and humidity greater than 55% (WHO 2016). Due to the highly varying numbers, the detection and quantification of STHs eggs is important for the determination of health risk due to exposure during the collection, conveyance, treatment and possible reuse of faecal waste. It is also important in the validation of wastewater treatment technologies (Peterson *et al.* 2011).

Ingestion of fertilized eggs of STHs may result in asymptomatic diarrhea (Brownell & Nelson 2006). The most common STH infection is ascariasis, infecting between 771.7–891.6 million people worldwide (Strunz *et al.* 2014), with the majority of these infections occurring in tropical and subtropical countries (Stolk *et al.* 2016). Although infection with STH is not lethal it may result in impairment of physical and cognitive development (Naidoo *et al.* 2016).

The greatest risk to public health in relation to STHs infection is the reuse of wastewater, sludge, compost etc.

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in agriculture (Habbari *et al.* 1999; Amahmid & Bouhoum 2005; Ensink *et al.* 2005). To ensure public safety several international and national agencies have recommended guidelines for wastewater reuse in agriculture (USEPA 1992; WHO 2006). These guidelines have been adopted by various national and regional bodies, such as the Union of National Associations of Water Suppliers and Waste Water Services from countries within the EU (Angelakis & Bontoux 2001). The guidelines do not only account for the quality for reuse but also the treatment steps needed to achieve the desired quality. The World Health Organization (WHO 2006) recommends ≤1 helminth egg per liter for unrestricted agriculture as against no detectable helminth egg per litre of irrigation water by the US Environmental Protection Agency (USEPA 1992). To determine the suitability of wastewater or sludge etc. for reuse or the validation of treatment technologies there is the need for accurate determination of viable STHs eggs in the various samples. However there are a variety of methods, with the major ones being the United States Environmental Protection Agency (USEPA) (Schwartzbrod 1998) and the World Health Organization (WHO) methods (Ayres & Mara 1996), or variants of these (Amoah *et al.* 2017).

The variety in the techniques used is influenced by several factors, with key among these being the variability in the sample matrices, reagents available and personal preferences of the researcher (Mes 2003). Sludge, compost and UD wastes are often blended with a detergent solution, which could be Tween 20 or Tween 80, ammonium bicarbonate or others with the intention to increase the separation of the egg from solid materials in the samples (Zenner *et al.* 2002; Trönnberg *et al.* 2010). In addition to these detergents, there are several other reagents used with different purposes. For instance flotation solutions, such as zinc sulphate, magnesium sulphate, sodium chloride, are used to further separate the eggs from other particulate matter based on differential flotation of these materials (Maya *et al.* 2006; Rosa Xavier *et al.* 2010). A phase extraction step is included in some of the methods for the removal of lipid-soluble and ether-absorbing material from the samples (Rude *et al.* 1987; Nelson & Darby 2001). Diethyl ether, ethyl acetate and acetoacetic buffer are commonly used for this purpose.

This variety of reagents used may inadvertently affect the viability of the STHs eggs. Long-term exposure to $ZnSO_4$ has been shown to be toxic to STHs eggs (Gaspard *et al.* 1996) as well as soaking eggs in $MgSO_4$ overnight that may also inactivate embryonated eggs (Smith 1991). One percent (1%) of formalin is mainly used as incubation solution for STHs eggs but Oksanen *et al.* (1990) stated that it retarded their development as compared to water or 0.1 N H_2SO_4 (Nelson & Darby 2001). Phase extraction with acid-alcohol and diethyl ether also decreases egg viability (Nelson & Darby 2001). Inactivation of eggs may be due to the damage of the egg shell of the parasites, which protect the egg and enhance the survival ability of STHs. A change in the permeability of the cell-wall layer of eggs may result in the loss of viability (de Souza *et al.* 2011).

Despite the perceived effect of different chemicals/reagents on the viability of helminth eggs, few quantitative assessments of the effects of the various reagents have been made. As part of an ongoing project aimed at developing a uniform method for the detection and quantification of STH eggs in environmental samples, a series of experiments were carried out to determine the effect of selected reagents (commonly used) on the viability of STH eggs, using *Ascaris suum* eggs as a surrogate.

METHODOLOGY

Preparation of stock solution

Ascaris suum eggs were purchased from Excelsior Sentinel Inc. (Ithaca, N.Y.). The eggs were collected from the intestinal contents of infected pigs and concentrated through series of sieves. A working egg solution with approximately 1,000 eggs/mL was prepared by suspending the eggs in distilled water. Stock solution of eggs was stored at 4 °C to prevent any egg development. Individual samples were prepared by placing 1.0 mL of the working solution in 50-mL centrifuge tubes.

Initial screening and comparison of incubation solutions

Viability of the eggs was determined, after initial screening, to ascertain their stage of development. Initial viability was determined by incubation in three different solutions; distilled water, 0.5% formalin and 0.1 N sulfuric acid. Eggs were viewed microscopically (X40) after 28 days of incubation and categorized as potentially viable or non-viable based on morphology. Undeveloped (at different cell stages) and embryonated eggs with a visible motile larva were considered potentially viable. Eggs with broken egg shell, visible internal globules and necrotic or immotile larvae were considered to be potentially non-viable (Moodley *et al.* 2008). Percentage viability was calculated for each incubation solution by dividing viable eggs by the

total eggs counted and multiplied by 100, based on a minimum of 200 eggs counted per each microscopic reading. The solution that gave the highest percentage viability was then selected for the inactivation tests (Section 2.3).

Inactivation tests

Acetoacetic acid (Sigma Aldrich, Germany), ethyl acetate (ACS reagent, ≥99.5%; Sigma Aldrich, Germany), ammonium bicarbonate (ACS reagent, ≥99.5%;Sigma Aldrich, Germany), zinc sulphate (99.9%; Promark Chemicals, South Africa), magnesium sulphate (≥99.0%;Promark Chemicals, South Africa), Tween 80 (Promark Chemicals, South Africa) and a combination of acetoacetic acid and ethyl acetate as well as a combination of acetoacetic acid and formalin, were the reagents and combinations selected for the experiments. These were chosen based on their widespread use in several methods for the detection and quantification of STH eggs in environmental samples (Bornay-Llinares *et al.* 2006; Horiuchi *et al.* 2013; Fuhrimann *et al.* 2015; Rocha *et al.* 2016; Verbyla *et al.*, 2016). The concentrations of these reagents were the same as recommended by the various methods. These are, 100% for acetoacetic acid and ethyl acetate, 0.5% Formalin, zinc sulphate of specific gravity of 1.3 (56.8%), saturated ammonium bicarbonate at 11.9% and 0.1% of Tween 80. These concentrations were not varied so as to determine their effect on *A. suum* viability based on the recommended concentrations in the methods.

Approximately 1,000 *A. suum* eggs were exposed in 20 mL of each reagent for 5, 10, 30, 60, 90 and 120 minutes (each treatment was in triplicates). After the duration of exposure, the solution, containing the eggs, was poured through a 20 μm sieve and the eggs washed four (4) times with distilled water to remove any remaining residues of the reagents. The contents collected on the sieve were then washed into 50 mL centrifuge tubes. Eggs where incubated at room temperature (with temperature ranging from 20 °C to 26 °C), using 0.1 N sulfuric acid, based on the results of the incubation tests stated above. Viability was determined after 18 days and finally confirmed after 28 days of incubation. For enumeration, an aliquot from each well-mixed sample was placed on a glass microscope slide with coverslip and a minimum of 200 eggs counted under the microscope and categorized into potentially viable or non-viable based on the criteria above (Section 2.2). A visible egg shell without a larvae or egg contents was considered as potential viable based on the assumption that the eggs may have hatched during the course of the incubation. Percentage viability for eggs exposed to each reagent or combination of reagents was calculated by dividing the number of viable eggs by the total number of eggs observed and multiplying by 100.

Spiking of wastewater and sludge with *A. suum* eggs

Approximately 100 mL of raw untreated wastewater and 30 g of sludge were sampled from the inlet of a wastewater treatment plant for spiked exposure assessment. The stock solution containing 1,000 eggs was inoculated into containers containing the wastewater and sludge samples. For the sludge samples, 100 mL distilled water was added and mixed thoroughly before spiking. A control tests was run with wastewater and sludge samples without exposure to the reagents, by spiking eggs into the respective volumes and weights of wastewater and sludge. Samples were then filtered through a 100 μm sieve, washed thoroughly with running water, unto a 20 μm sieve. The process was thereafter continued as detailed in Section 2.3 above.

Statistical analysis

Descriptive analysis was undertaken to assess the mean percentage viability of eggs for each treatment (Stata, Statacorp, Texas, USA). One tail t-test was performed to determine the statistical difference between mean percentage viability for each of the treatments used against the percentage viability as determined for the untreated *A. suum* eggs, as well as between eggs spiked into the different sample media. Pearson correlation analysis was done to determine the effect of duration of exposure on the viability. Data from reagents that were found to correlate negatively with longer duration of exposure was then used in regression analysis to determine the association between reagent and duration of exposure (GraphPad Software, Inc. USA). The best regression model for each treatment was chosen using the Akaike Information Criterion (AIC) and r-squared values.

RESULTS AND DISCUSSION

Effect of different incubation solutions on viability of *A. suum* eggs

Initial microscopic analysis of the eggs showed that the eggs generally were in the pre-larval stage prior to any

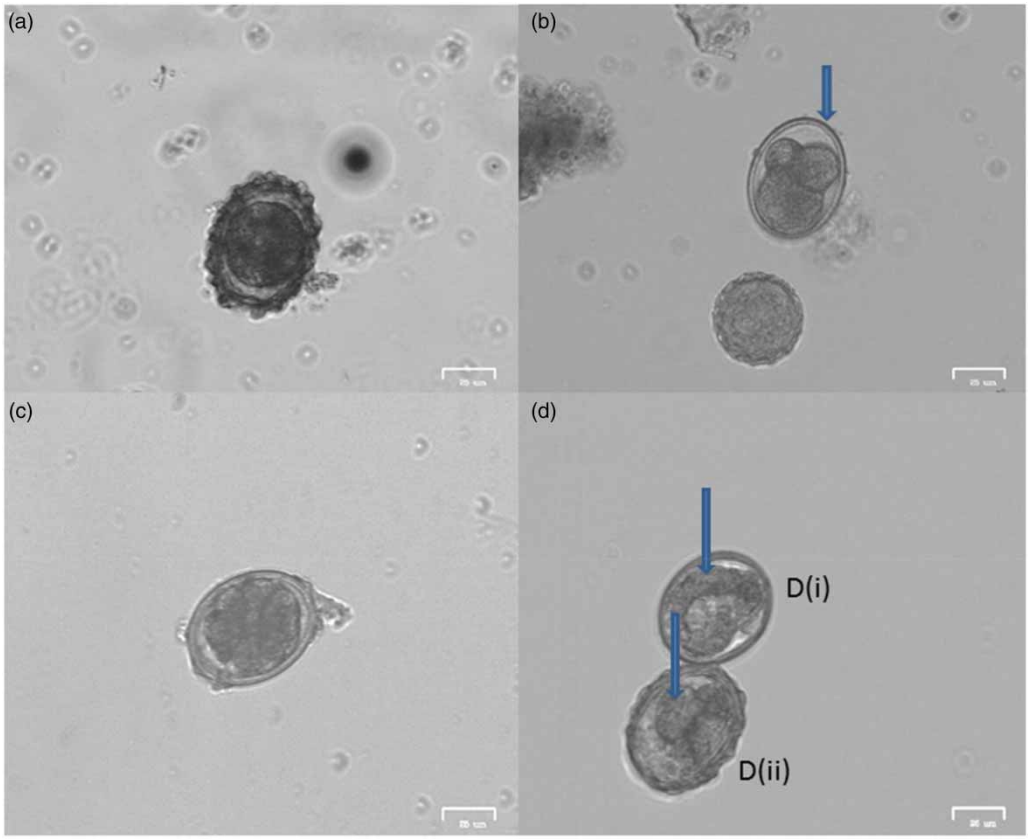

Figure 1 | Images of potentially viable at different stages of development; (a) Egg at the one cell stage; (b) Decorticated egg undergoing embryonation; (c) Decorticated egg at multicellular stage (>7 cells); (d) Eggs with visible larvae (indicated by the arrow).

experiment. After 28 days of incubation viable eggs were quantified based on stated criteria (Section 2.2). Figures 1 and 2 shows the stages of egg development. Incubation with the three solutions (distilled water, 0.5% formalin and 0.1 N sulfuric acid) gave viability between 90.0 to 91.2% of the analyzed eggs. The highest percentage of viability after the 28 days of incubation was recorded with 0.1 N sulfuric acid (91.2 ± 0.6%), followed by distilled water (90.0 ± 3.7%) and the least percentage viability was recorded with 0.5% formalin solution (87.6 ± 0.5%) (Figure 3). The difference in the percentage viabilities between these three solutions was not statistically

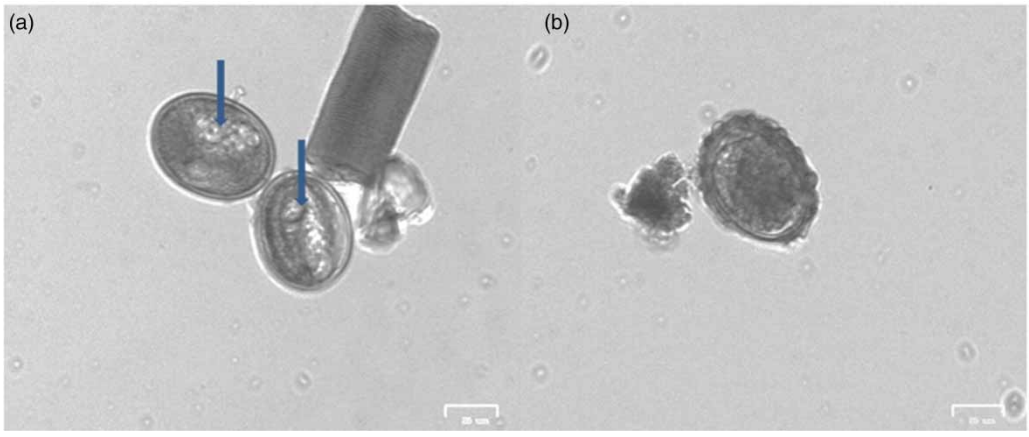

Figure 2 | Images of non-viable eggs after incubation; (a) Decorticated eggs with visible globules (indicated by arrows); (b) Dead egg.

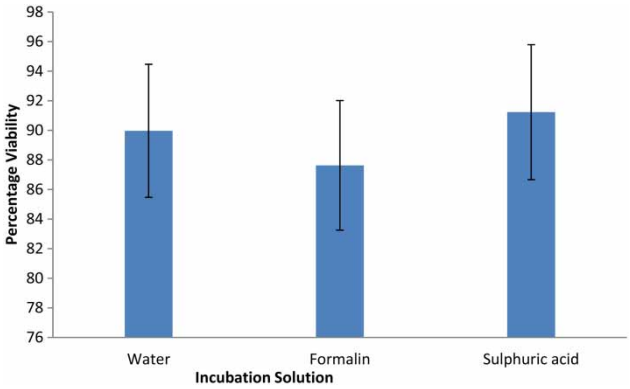

Figure 3 | Viability of *A. suum* eggs with different incubation solutions ($n = 6$).

significant at 95% confidence level ($p > 0.05$). These results are in line with results obtained by other researchers earlier (Nelson & Darby 2001; Karkashan *et al.* 2015). Investigations by Pecson & Nelson (2005) also showed that only 5% of *A. suum* eggs were inactivated during incubation in sulfuric acid. Oksanen *et al.* (1990) reported a decrease in egg viability when formalin is used as an incubation solution, which might be due to the ability of formalin to penetrate into cells by diffusion. The penetration of formalin and other chemicals into the eggs may be enhanced during hatching of the eggs, when an increase in permeability of the eggs occurs (Clarke & Perry 1988). The egg shell of *Ascaris spp* has four layers, the outermost uterine layer (a glycoprotein), followed by a thin vitelline layer, a chitinous layer and then the innermost lipid layer (termed ascaroside membrane) (Quilès *et al.* 2006), which becomes permeable during hatching.

Distilled water showed a good performance as an incubation solution, although sulphuric acid gave a slightly higher percentage of viability, however not statistically significant. The results confirm the findings by Nelson & Darby (2001). Distilled water, thus, could replace other solutions that are more expensive and toxic. However the use of distilled water as an incubation solution could also, on the other hand, result in the loss of egg viability due to growth of bacteria and fungi which may result in egg inactivation (Ciarmela *et al.* 2002).

Sulfuric acid is very corrosive and therefore poses health concern for laboratory personnel, especially when there is direct exposure to sufficient concentrations (PHE 2015). Exposure to formalin may also result in irritation and corrosive effects (Pandey *et al.* 2000). Therefore their replacement with distilled water as an incubation solution would result in accurate viability results whiles ensuring health safety of laboratory personnel.

Effect of reagents on viability of *A. suum* eggs spiked into distilled water

Exposure of *A. suum* eggs to the different reagents had effect on the viability of the egg as determined by incubation. Out of the nine reagents or combination of reagents, the highest negative effect in viability was observed due to exposure to acetoacetic acid, with only 3.4 (±1.68) % of the eggs remaining viable, followed by combination of acetoacetic acid and ethyl acetate (13.2 ± 14.4%). Ethyl acetate as well as the combination of acetoacetic acid and formalin also gave statistically significant loss in viability as compared to the mean viability determined by incubation in sulphuric acid without any treatment (Table 1). Statistically, ethyl acetate, acetoacetic acid, combination of ethyl acetate and acetoacetic acid and then the combination of acetoacetic acid and formalin resulted in mean percentage viabilities that were significantly lower than the mean percentage viability of untreated (un-exposed) eggs (Table 1). This might be due to the effect of the acid on the lipid layer of the eggs thereby increasing permeability of the egg shell which subsequently lead to the inactivation (Clarke & Perry 1988). Although formalin and ethyl acetate did not significantly affect the viability of the eggs as individual components, their combinations with acetoacetic acid lead to a significant reduction in viability. However, the individual effect of acetoacetic acid was higher. Table 1 shows that the reagents used as detergents for the dissociation of eggs from particulate matter (Tween 80 and ammonium bicarbonate) as well as solutions used for the flotation steps (magnesium sulphate and zinc sulphate) did not significantly affect the viability of the *A. suum* eggs. However reagents used during the phase extraction step (ethyl acetate and acetoacetic acid) significantly affected the egg viability. These phase extraction reagents are mostly lipid soluble and ether-absorbing chemicals and their negative effect might be due to the degradation of the lipid layer of the egg (which is the last defense for the eggs) thereby inactivating them.

The dose of a toxic substance that an organism is exposed to, as well as the duration of exposure to the said substance, is very important in the determination of the dose-response relationships (Nelson & Darby 2001). This is used here to evaluate the inactivation of *A. suum* eggs as a result of prolonged exposure to these reagents. With a fixed concentration of these reagents the duration of exposure is the main factor in the inactivation of *A. suum* eggs. It was found that prolonged exposure had a destructive effect on the viability of the *A. suum* eggs. There was a negative correlation for ethyl acetate, acetoacetic acid,

Table 1 | Detailed descriptive analysis of the mean percentage of viable eggs for each treatment ($N = 200$)

	Ethyl acetate	Acetoacetic acid	Formalin	Ammonium bicarbonate	Zinc Sulphate	Magnesium Sulphate	Tween 80	Acetoacetic acid + Ethyl acetate	Acetoacetic acid + Formalin
Minimum	60.8	1.8	75.0	1.8	83.1	84.9	82.8	2.2	52.6
25% Percentile	67.5	1.9	86.0	50.9	83.5	85.3	84.5	2.9	53.1
Median	75.9	3.3	90.6	88.1	85.9	89.1	89.0	5.6	55.5
75% Percentile	80.4	4.2	95.5	90.4	94.9	90.7	90.8	29.7	68.8
Maximum	83.3	6.5	97.2	90.8	98.2	92.3	92.6	34.9	72.9
Mean (SD)	74.4 (8.3)	3.4 (1.7)	89.7 (7.8)	71.1 (35.1)	88.5 (6.3)	88.5 (2.8)	87.9 (3.6)	13.2 (14.4)	59.1 (9.3)
95% Confidence Interval of mean	64.1–84.7	1.6–5.2	81.5–97.8	34.2–107.9	80.7–96.4	85.5–91.5	83.4–92.4	−1.8–28.3	44.3–73.9
T test*	**0.0109**	**<0.0001**	0.6878	0.2228	0.4329	0.0818	0.1343	**<0.0001**	**0.0064**

*p value < 0.05 was considered as statistically significant.

magnesium sulphate, Tween 80 and then the combination of ethyl acetate and acetoacetic acid as well combination of acetoacetic acid and formalin with percentage viability (shown in Table 2). Although Tween 80 and magnesium sulphate did not show any significant reduction in mean percentage viability, an increase in the duration of exposure results in a reduction in viable eggs. This makes it necessary to reduce the exposure of eggs to the reagents to the shortest time possible. The best correlation between duration of exposure and inactivation was found for the combination of acetoacetic acid and ethyl acetate, probably due to the individual effect of acetoacetic acid, as treatment with this reagent alone resulted in the highest loss of viability, with only 3.4 (±1.68) % of eggs remaining viable. This is confirmed by the correlation results presented in Table 2.

Although ethyl acetate recorded a mean percentage of viable eggs of 73.4 (±8.3%), which was not significantly different from the untreated eggs (determined during the initial screening phase with sulfuric acid, formalin and distilled), an increase in the duration of exposure lead to a greater reduction in viability. Increase in duration of exposure correlated negatively with percentage viability (−0.63), which was the highest negative correlation for all the reagents studied.

This shows that although exposure for short periods (≤5 minutes) might not affect the viability of exposed eggs, prolonged exposure (≥30 minutes) might result in the loss of viability. The loss in viability might be due to the increase in permeability during the hatching of the eggs. Permeability of egg shells increases during hatching which might be the reason for the increase in the inactivation effect of the reagents with prolonged duration (Clarke & Perry 1988). Similar relationship between prolonged exposure and percentage viability was seen for exposure to the combination

Table 2 | Correlation between duration of exposure and the mean percentage viability

	Time	Ethyl acetate	Acetoacetic acid	Formalin	Ammonium bicarbonate	Zinc Sulphate	Magnesium Sulphate	Tween 80	Acetoacetic acid + Ethyl acetate	Acetoacetic acid + Formalin
Time	1									
Ethyl acetate	−0.24	1								
Acetoacetic acid	−0.47	0.54	1							
Formalin	0.15	0.86	0.19	1						
Ammonium bicarbonate	0.35	−0.04	0.24	−0.11	1					
Zinc Sulphate	0.28	−0.13	0.30	−0.27	0.98	1				
Magnesium Sulphate	−0.18	−0.05	0.58	−0.11	0.43	0.43	1			
Tween 80	−0.28	−0.31	0.37	−0.70	0.30	0.49	0.06	1		
Acetoacetic acid + Ethyl acetate	−0.72	0.62	0.93	0.21	−0.05	0.02	0.37	0.33	1	
Acetoacetic acid + Formalin	−0.61	0.43	0.80	0.05	−0.28	−0.15	0.14	0.48	0.89	1

Table 3 | Percentage viability (±SEM) of *A. suum* eggs spiked into wastewater samples

Wastewater samples

	Ethyl acetate	Acetoacetic acid	Formalin	Ammonium bicarbonate	Zinc Sulphate	Magnesium Sulphate	Tween 80	Acetoacetic acid + Ethyl acetate	Acetoacetic acid + Formalin
5	80.5 ± 1.5	8.5 ± 1.2	92.1 ± 6.1	92.5 ± 5.1	92.5 ± 5.2	92.1 ± 4.6	92.6 ± 6.4	56.6 ± 4.3	79.1 ± 4.9
10	81.2 ± 2.6	6.8 ± 2.0	91.3 ± 3.6	90.2 ± 5.2	91.6 ± 4.6	89.2 ± 7.6	90.1 ± 6.1	55.1 ± 3.2	64.2 ± 6.4
30	79.5 ± 1.6	6.0 ± 1.4	91.2 ± 4.5	90.6 ± 4.6	94.6 ± 7.8	88.6 ± 8.4	93.1 ± 4.2	55.9 ± 4.2	57.6 ± 5.7
60	77.6 ± 2.8	5.4 ± 1.3	90.4 ± 7.1	90.8 ± 6.5	97.8 ± 6.9	90.5 ± 6.1	90.1 ± 5.3	45.2 ± 6.2	55.6 ± 8.7
90	76.9 ± 3.6	5.5 ± 1.2	90.9 ± 5.5	89.9 ± 8.7	92.5 ± 8.4	89.6 ± 2.9	89.9 ± 6.4	40.1 ± 4.8	56.7 ± 6.7
120	76.2 ± 5.4	4.9 ± 3.1	91.5 ± 4.2	89.5 ± 9.4	97.9 ± 6.4	90.5 ± 4.6	90.1 ± 4.6	28.9 ± 7.5	54.9 ± 2.9

of acetoacetic acid and formalin with acetoacetic acid seen as the main contributor to the effect. This is based on the correlation between the effects of the combined treatment against treatment with the reagents individually (Table 2). Although the exposure to magnesium sulphate did not show a significant loss in viability as compared to the untreated eggs, prolonged exposure negatively correlated with the percentage of viability (−0.53) which means that as duration of exposure increases there is a decrease in the percentage of eggs that retain their viability. Magnesium sulphate is used as a flotation solution in most literature as recommended by the USEPA method (USEPA 1999), but there is no maximum time of exposure recommended. In this case the duration of exposure of samples (which presumably contains the *Ascaris* spp eggs or other helminths) is left to the discretion of the researcher. The results obtained indicate that with prolonged exposure there is a decrease in the number of viable eggs recovered thereby resulting in false results. The same trend was seen for Tween 80, but its influence was very weak (−0.0027). Acetoacetic acid exposure for 5 minutes (the minimum time of exposure studied) resulted in only 3.4 (±1.7) % of the eggs remaining viable and the prolonged exposure had a negative correlation with the percentage of viable egg recovered. Based on this the use of acetoacetic acid or reagents that contain this chemical should not be recommended for use as it results in the loss of over 95% of viable eggs, which might result in inaccurate results if viability is determined after exposure of eggs.

For every one minute increase in duration of exposure the viability of the exposed *A. suum* eggs reduces. The greatest decrease in percentage viability with every one minute increase in exposure time was for the combined exposure to acetoacetic acid and ethyl acetate (−0.72), followed by exposure to acetoacetic acid (−0.47), then a combined exposure to acetoacetic acid and formalin (−0.61). The reduction in percentage viability for the six treatments with negative correlation between duration of exposure and reduction in percentage viability are shown in Figures 4–6.

The exponential regression model was the best fit model for treatments with acetoacetic acid and the combined treatment with acetoacetic acid and ethyl acetate (Figure 4). Exponential models are best in describing a process where the rate of an event occurring depends on the amount present (Shestopaloff 2010). Therefore inactivation of *A. suum* eggs with prolonged exposure to these two reagents (acetoacetic acid and the combination of acetoacetic acid and ethyl acetate) may depend on the amount of these remaining at a given time. This exponential rate of inactivation of the eggs might be due to reduction in the potency of the reagents as a result of evaporation or other unknown factors. Polynomial regression models were found to be the best fit model for treatments with ethyl acetate (cubic), magnesium sulphate (4th order), Tween 80 (cubic), and combined treatments with acetoacetic acid and formalin (cubic). Polynomial models best predict relationship between variables based on data present but they have poor interpolatory properties (Shestopaloff 2010). However none of the regression models fitted for the data as explained above were statistically significant at 95% confidence level.

Inactivation of eggs spiked into wastewater and sludge

The percentage of viability for eggs spiked into the wastewater samples as compared to the eggs spiked into the distilled water was not significantly different (at 95% confidence interval), except for exposure to acetoacetic acid (p value was 0.0001). The eggs in wastewater would thus be affected in a similar manner as eggs in the distilled

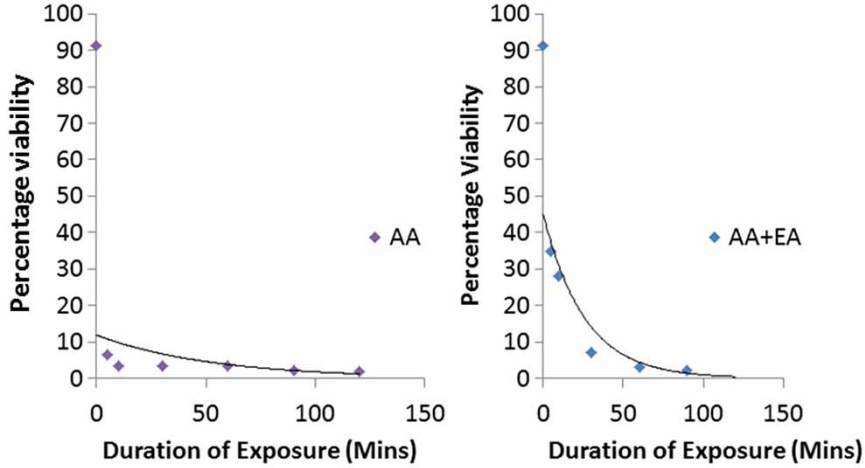

Figure 4 | Reduction in percentage viability of eggs after prolonged exposure to treatments of acetoacetic acid (AA) and combination of acetoacetic acid and ethyl acetate (AA + EA).

water. Acetoacetic acid was found in the previous sections to result in the highest loss of viability (Table 1), but the results from the wastewater spiking tests indicates that its effect there was due to the properties of the wastewater. Wastewater contains a lot of particles (Bougrier *et al.* 2005) that might have had a shielding effect against the impact of this reagent. In addition there might be components within the wastewater that may be antagonistic to the effect of acetoacetic acid. For instance, wastewater from domestic sources, as well some industrial sources, may contain basic compounds, such as soaps and other detergents (Lajeunesse *et al.* 2008; Gracia-Lor *et al.* 2010) that react with the acetoacetic acid.

A major shielding effect was seen for eggs spiked into the sludge samples, with a higher percentage of viable egg in these samples as compared to distilled water spiked eggs. Reagents that had a negative impact on egg spiked in distilled water, such as ethyl acetate, acetoacetic acid, combination of these two and combination of acetoacetic acid and formalin still resulted in the loss of egg viability but not as much as was seen in the distilled water (Table 4). The impact of these reagents was mostly severe when exposure time exceeded 30–60 minutes. After 30 minutes of exposure to acetoacetic acid, about 64.2 (±6.1) of the eggs were still viable which is higher than the viability of eggs in the distilled water (See Table 4 below).

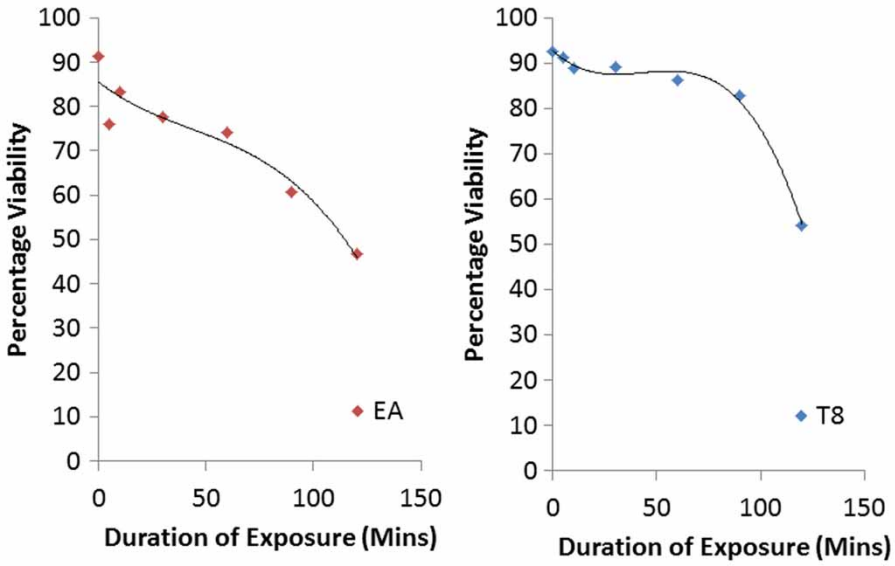

Figure 5 | Reduction in percentage viability of eggs after prolonged exposure to treatments of ethyl acetate (EA) and tween 80 (T8).

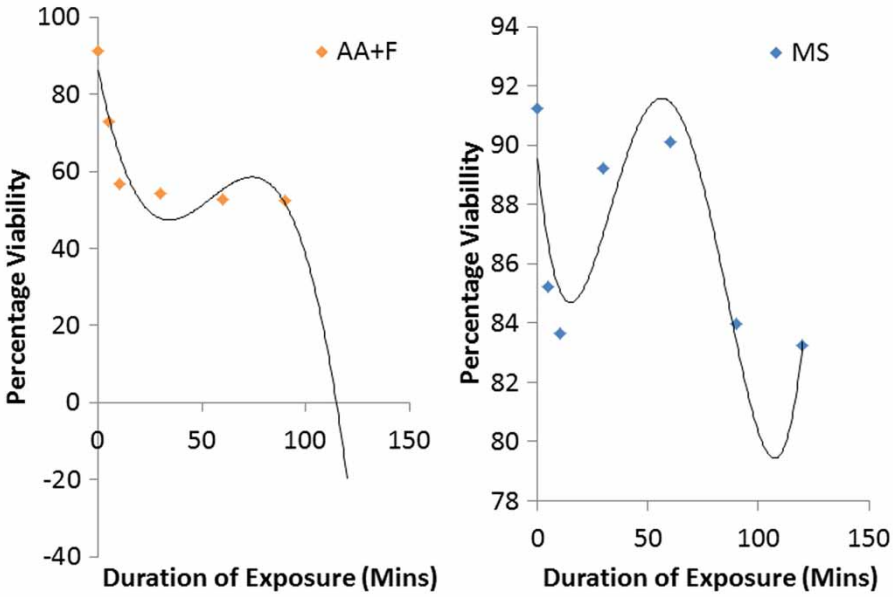

Figure 6 | Reduction in percentage viability of eggs after prolonged exposure to treatments of magnesium sulphate (MS) and combination of acetoacetic acid and formalin (AA + F).

Table 4 | Percentage viability (±SEM) of *A. suum* eggs spiked into sludge samples

Sludge

	Ethyl acetate	Acetoacetic acid	Formalin	Ammonium bicarbonate	Zinc Sulphate	Magnesium Sulphate	Tween 80	Acetoacetic acid + Ethyl acetate	Acetoacetic acid + Formalin
5	92.6 ± 7.8	78.2 ± 3.4	93.0 ± 6.1	91.2 ± 4.3	95.6 ± 6.1	93.1 ± 2.3	92.6 ± 6.2	80.1 ± 2.3	84.2 ± 4.6
10	90.5 ± 6.5	74.6 ± 5.2	91.3 ± 3.2	91.5 ± 5.1	94.2 ± 1.6	91.2 ± 1.9	90.1 ± 6.1	68.5 ± 4.1	86.2 ± 5.1
30	89.6 ± 4.2	65.6 ± 3.2	91.6 ± 1.8	90.3 ± 2.6	93.1 ± 5.4	90.1 ± 5.2	93.1 ± 4.1	73.5 ± 2.1	72.0 ± 7.1
60	88.8 ± 2.1	66.1 ± 2.8	92.1 ± 2.5	91.0 ± 4.6	92.1 ± 1.2	90.3 ± 4.2	90.1 ± 2.1	70.5 ± 3.5	63.0 ± 1.6
90	90.2 ± 3.1	64.2 ± 6.2	91.1 ± 6.1	90.1 ± 8.1	93.5 ± 3.8	90.2 ± 6.2	89.9 ± 3.1	72.3 ± 4.9	56.7 ± 2.9
120	87.6 ± 1.6	35.5 ± 1.5	90.1 ± 4.3	89.9 ± 7.0	95.6 ± 7.4	91.2 ± 7.6	90.1 ± 2.6	64.2 ± 5.6	55.1 ± 2.4

A similar trend was seen for almost all the other reagents indicating a shielding effect. The shielding effect found in the sludge tests could be attributed to several factors, one major factor could be the presence of larger particles within the samples (Shimizu *et al.* 1997; Zorpas *et al.* 2002; Bougrier *et al.* 2005) with the potential to protect the eggs from the reagents limiting their impact. In addition, the heterogeneity of sludge means that there may be particles within the sludge that might absorb the reagents (Hörsing *et al.* 2011; Stevens-Garmon *et al.* 2011) thereby reducing the concentration of these in the samples. A comparison of the number of viable eggs for each exposure time for eggs spiked into the wastewater and sludge showed that, sludge has a much higher shielding effect than wastewater. Eggs spiked into wastewater and sludge and exposed to ethyl acetate, acetoacetic acid, combination of these two and combination of acetoacetic acid and formalin recorded significantly different percentage of viable eggs after incubation (p values <0.05). The increase in loss of viability with increase in exposure time might be attributed to the assumption that as the time of exposure increase more of the eggs got exposed to the reagents therefore resulting in greater impact.

CONCLUSION

Even though sulphuric acid had the best performance and was used in the further inactivation tests, it is important to note that the difference in percentage viability as assessed

by sulphuric acid and distilled water were not statistically significant. Distilled water performed very well (90.0 ± 3.7%) as an incubation solution, better than 0.5% formalin (87.6 ± 0.5%) which is widely used. However there could be the loss of egg viability due to bacterial or fungal growth when distilled water is used but this effect was not determined in the current study. Therefore in conclusion distilled water may be used to reduce the cost involved in STHs analysis without compromising results.

Exposure to some of the reagents used in the detection and quantification of *A. suum* eggs in environmental samples may affect egg viability. Acetoacetic acid was found to result in the highest loss of egg viability upon exposure, however eggs in wastewater and sludge were shown to be least impacted. This therefore indicates that direct exposure of eggs in these samples may not result in the viability loss, however the use of these reagents, as recommended by many of the methods (Ayres & Mara 1996; Schwartzbrod 1998; Collender *et al.* 2015; Amoah *et al.* 2017), is after the eggs have been concentrated from the samples therefore the shielding effect might be lower than was seen.

Techniques that require the use of acetoacetic acid should therefore be modified to ensure that helminth eggs are not inactivated. This is especially important if quantity of viable eggs is needed. The best approach would be to select methods that do not make use of this chemical.

Although most of the reagents studied did not affect egg viability within short time frames (5 minutes) of exposure, an increase in the duration of exposure adversely affected the percentage of viable eggs recovered at the end. Therefore the use of reagents such as ethyl acetate, magnesium sulphate and Tween 80 should be limited to the shortest possible time (ideally 5 minutes or below). In situations where these reagents are used, care must be taken to limit the exposure time. Exposure may be reduced by thoroughly rinsing the eggs after steps involving these reagents so as to completely remove any residual concentration of the reagents on the eggs before incubation.

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REFERENCES

Amahmid, O. & Bouhoum, K. 2005 Assessment of the health hazards associated with wastewater reuse: transmission of geohelminthic infections (Marrakech, Morocco). *International Journal of Environmental Health Research* **15** (2), 127–133.

Amoah, I. D., Singh, G., Stenström, T. A. & Reddy, P. 2017 Detection and quantification of soil-transmitted helminths in environmental samples: a review of current state-of-the-art and future perspectives. *Acta Tropica* **169**, 187–201. 10.1016/j.actatropica.2017.02.014.

Angelakis, A. N. & Bontoux, L. 2001 Wastewater reclamation and reuse in Eureau countries. *Water Policy* **3** (1), 47–59.

Ayres, R. M. & Mara, D. D. 1996 *Analysis of Wastewater for Use in Agriculture: A Laboratory Manual of Parasitological and Bacteriological Techniques.* World Health Organization, Geneva, Switzerland.

Bornay-Llinares, F. J., Navarro-i-Martínez, L., García-Orenes, F., Araez, H., Pérez-Murcia, M. D. & Moral, R. 2006 Detection of intestinal parasites in pigslurry: a preliminary study from five farms in Spain. *Livestock Science* **102** (3), 237–242.

Bougrier, C., Carrère, H. & Delgenès, J. P. 2005 Solubilisation of waste-activated sludge by ultrasonic treatment. *Chemical Engineering Journal* **106** (2), 163–169.

Bowman, D. D., Little, M. D. & Reimers, R. S. 2003 Precision and accuracy of an assay for detecting Ascaris eggs in various biosolid matrices. *Water Research* **37** (9), 2063–2072.

Ciarmela, M. L., Minvielle, M. C., Lori, G. & Basualdo, J. A. 2002 Biological interaction between soil fungi and *Toxocara canis* eggs. *Veterinary Parasitology* **103** (3), 251–257.

Clarke, A. J. & Perry, R. N. 1988 The induction of permeability in egg-shells of *Ascaris suum* prior to hatching. *International Journal for Parasitology* **18** (7), 987–990.

Collender, P. A., Kirby, A. E., Addiss, D. G., Freeman, M. C. & Remais, J. V. 2015 Methods for quantification of soil-transmitted helminths in environmental media: current techniques and recent advances. *Trends in Parasitology* **31** (12), 623–639.

de Souza, G. S. M. B., Rodrigues, L. A., de Oliveira, W. J., Chernicharo, C. A. L., Guimarães, M. P., Massara, C. L. & Grossi, P. A. 2011 Disinfection of domestic effluents by gamma radiation: effects on the inactivation of *Ascaris lumbricoides* eggs. *Water Research* **45** (17), 5523–5528.

Ensink, J. H. J., Van Der Hoek, W., Mukhtar, M., Tahir, Z. & Amerasinghe, F. P. 2005 High risk of hookworm infection among wastewater farmers in Pakistan. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **99** (11), 809–818.

Fuhrimann, S., Stalder, M., Winkler, M. S., Niwagaba, C. B., Babu, M., Masaba, G., Kabatereine, N. B., Halage, A. A., Schneeberger, P. H. H., Utzinger, J. & Cissé, G. 2015 Microbial and chemical contamination of water sediment

and soil in theNakivubo wetland area in Kampala, Uganda. *Environmental Monitoring and Assessment* **187** (7), 475–489.

Gaspard, P., Wiart, J. & Schwartzbrod, J. 1996 A method for assessing the viability of nematode eggs in sludge. *Environmental Technology* **17** (4), 415–420.

Gracia-Lor, E., Sancho, J. V. & Hernández, F. 2010 Simultaneous determination of acidic, neutral and basic pharmaceuticals in urban wastewater by ultra high-pressure liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* **1217** (5), 622–632.

Habbari, K., Tifnouti, A., Bitton, G. & Mandil, A. 1999 Helminthic infections associated with the use of raw wastewater infections associated with the use of raw wastewater for agricultural purposes in Beni Mellal, Morocco. *Eastern Mediterranean Health Journal* **5** (5), 912–921.

Horiuchi, S., Paller, V. G. V. & Uga, S. 2013 Soil contamination by parasite eggs in rural village in the Philippines. *Tropical Biomedicine* **30** (3), 495–503.

Hörsing, M., Ledin, A., Grabic, R., Fick, J., Tysklind, M., Jansen, J. C. & Andersen, H. R. 2011 Determination of sorption of seventy-five pharmaceuticals in sewage sludge. *Water Research* **45** (2011), 4470–4482.

Hotez, P. J., Brooker, S., Bethony, J. M., Bottazzi, M. E., Loukas, A. & Xiao, S. 2004 Hookworm infection. *New England Journal of Medicine* **351** (8), 799–807.

Hotez, P. J., Brindley, P. J., Bethony, J. M., King, C. H., Pearce, E. J. & Jacobson, J. 2008 Helminth infections: the great neglected tropical diseases. *Journal of Clinical Investigation* **118** (4), 1311–1321.

Karkashan, A., Khallaf, B., Morris, J., Thurbon, N., Rouch, D., Smith, S. R. & Deighton, M. 2015 Comparison of methodologies for enumerating and detecting the viability of *Ascaris* eggs in sewage sludge by standard incubation-microscopy, the BacLight Live/Dead viability assay and other vital dyes. *Water Research* **68** (1), 533–544.

Keraita, B. & Amoah, P. 2011 *Fecal Exposure Pathways in Accra: A Literature Review with Specific Focus on IWMI's Work on Wastewater Irrigated Agriculture.* (IWMI). Literature Review Report submitted to the Centre for Global Safe Water, Emory University.

Lajeunesse, A., Gagnon, C. & Sauvé, S. 2008 Determination of basic antidepressants and their N-desmethyl metabolites in raw sewage and wastewater using solid-phase extraction and liquid chromatography-tandem mass spectrometry. *Analytical Chemistry* **80** (14), 5325–5333.

Maya, C., Jimenez, B. & Schwartzbrod, J. 2006 Comparison of techniques for the detection of helminth ova in drinking water and wastewater. *Water Environment Research* **78** (2), 118–124.

Mes, T. H. M. 2003 Technical variability and required sample size of helminth egg isolation procedures. *Veterinary Parasitology* **115** (4), 311–320.

Moodley, C. L., 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 Report no. TT 322/08, Water Research Commission, Pretoria.

Naidoo, D., Archer, C., Louton, B. & Rodda, N. 2016 Testing household disinfectants for the inactivation of helminth eggs on surfaces and in spills during pit latrine emptying. *Water SA*. **42** (4), 560–570.

Nelson, K. L. & Darby, J. L. 2001 Inactivation of viable *Ascaris* eggs by reagents during enumeration. *Applied Environmental Microbiology* **67** (12), 5453–5459.

Oksanen, A., Eriksen, L., Roepstorff, A., Ilsøe, B., Nansen, P. & Lind, P. 1990 Embryonation and infectivity of *Ascaris suum* eggs: a comparison of eggs collected from worm uteri with eggs isolated from pig faeces. *Acta Veterinaria Scandinavica* **31** (4), 393–398.

Pandey, C. K., Agarwal, A., Baronia, A. & Singh, A. 2000 Toxicity of ingested formalin and its management. *Human and Experimental Toxicology* **19** (6), 360–366.

Pecson, B. M. & Nelson, K. L. 2005 Inactivation of *Ascaris suum* eggs by ammonia. *Environmental Science and Technology* **39** (20), 7909–7914.

Peterson, J. D., Murphy, R. R., Jin, Y., Wang, L., Nessl, M. B. & Ikehata, K. 2011 Health effects associated with wastewater treatment, reuse, and disposal. *Water Environment Research* **83** (10), 1853–1875.

PHE, Public Health England 2015 *Compendium of Chemical Hazards: Sulfuric Acid*. Publication Number 2014790.

Quilès, F., Balandier, J. & Capizzi-Banas, S. 2006 In situ characterisation of a microorganism surface by Raman microspectroscopy: the shell of *Ascaris* eggs. *Analytical and Bioanalytical Chemistry* **386** (2), 249–256.

Rocha, M. C. V., Barés, M. E. & Braga, M. C. B. 2016 Quantification of viable helminth eggs in samples of sewage sludge. *Water Research* **103**, 245–255.

Rosa Xavier, I. G., Ramos, B. C. & Santarém, V. A. 2010 Recovery threshold of *Toxocara canis* eggs from soil. *Veterinary Parasitology* **167** (1), 77–80.

Rude, R. A., Peeler, J. T. & Risty, N. G. 1987 Comparison of diethyl ether and ethyl acetate as extracting agents for recovery of *Ascaris* spp. and *Trichuris* spp. eggs. *Journal of the Association of Official Analytical Chemists* **70** (6), 1000–1002.

Schwartzbrod, J. 1998 *Methods of Analysis of Helminth Eggs and Cysts in Wastewater, Sludge, Soils and Crops.* University Henri Poincaré, Nancy, France.

Shestopaloff, Y. K. 2010 *Properties and Interrelationships of Polynomial, Exponential, Logarithmic and Power Functions with Applications to Modeling Natural Phenomena*. AKVY Press, USA.

Shimizu, Y., Uryu, K., Okuno, Y., Ohtubo, S. & Watanabe, A. 1997 Effect of particle size distributions of activated sludges on cross-flow microfiltration flux for submerged membranes. *Journal of Fermentation and Bioengineering* **83** (6), 583–589.

Stevens-Garmon, J., Drewes, J. E., Khan, S. J., McDonald, J. A. & Dickenson, E. R. V. 2011 Sorption of emerging trace organic compounds onto wastewater sludge solids. *Water Research.* **45** (10), 3417–3426.

Stolk, W. A., Kulik, M. C., le Rutte, E. A., Jacobson, J., Richardus, J. H. & de Vlas, S. J. 2016 Between-Country inequalities in the neglected tropical disease burden in 1990 and 2010, with

projections for 2020. *PLOS Neglected Tropical Diseases*. **10** (5), e0004560. doi:10.1371/journal.pntd.0004560.

Strunz, E. C., Addiss, D. G., Stocks, M. E., Ogden, S., Utzinger, J. & Freeman, M. C. 2014 Water, sanitation, hygiene, and soil-transmitted helminth infection: a systematic review and meta-analysis. *PLOS Medicine* **11** (3), e1001620. doi:10.1371/journal.pmed.1001620.

Trönnberg, L., Hawksworth, D., Hansen, A., Archer, C. & Stenström, T. A. 2010 Household-based prevalence of helminths and parasitic protozoa in rural KwaZulu-Natal, South Africa, assessed from faecal vault sampling. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **104** (10), 646–652.

U.S. Environmental Protection Agency 1999 *Control of Pathogens and Vector Attraction in Sewage Sludge EPA/625/R-92/013*. Appendix, I. Revised edition. U.S. Environmental Protection Agency, Washington, DC.

Verbyla, M. E., Iriarte, M. M., Guzmán, A. M., Coronado, O., Almanza, M. & Mihelci, J. R. 2016 Pathogens and fecal indicators in waste stabilization pond systems with direct reuse for irrigation: fate and transport in water, soil and crops. *Science of the Total Environment* **551–552**, 429–437.

World Health Organization 2015 *Investing to Overcome the Global Impact of Neglected Tropical Diseases Third WHO Report on Neglected Tropical Diseases*. WHO Document Production Services, Geneva, Switzerland. WHO/HTM/NTD/2015.1.

Zenner, L., Gounel, J. M. & Chauve, C. M. 2002 A standardized method for detecting parasite eggs and oocysts in soils. *Revue de Médecine Vétérinaire* **153** (11), 729–734.

Zorpas, A. A., Vassilis, I., Loizidou, M. & Grigoropoulou, H. 2002 Particle size effects on uptake of heavy metals from sewage sludge compost using natural zeolite Clinoptilolite. *Journal of Colloid and Interface Science* **250** (1), 1–4.

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Paper III

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RESEARCH ARTICLE

Removal of helminth eggs by centralized and decentralized wastewater treatment plants in South Africa and Lesotho: health implications for direct and indirect exposure to the effluents

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Abstract
Wastewater may contain contaminants harmful to human health; hence, there is the need for treatment before discharge. Centralized wastewater treatment systems are the favored treatment options globally, but these are not necessarily superior in reduction of pathogens as compared to decentralized wastewater treatment systems (collectively called DEWATS). This study was therefore undertaken to assess the soil-transmitted helminth (STH) and *Taenia* sp. egg reduction efficiency of selected anaerobic baffled reactors and planted gravel filters compared to centralized wastewater treatment plants in South Africa and Lesotho. The risk of ascariasis with exposure to effluents from the centralized wastewater treatment plants was also assessed using the quantitative microbial risk assessment (QMRA) approach. Eggs of *Ascaris* spp., hookworm, *Trichuris* spp., *Taenia* spp., and *Toxocara* spp. were commonly detected in the untreated wastewater. The DEWATS plants removed between 95 and 100% of the STH and *Taenia* sp. eggs, with centralized plants removing between 67 and 100%. Helminth egg concentrations in the final effluents from the centralized wastewater treatment plants were consistently higher than those in the WHO recommended guideline (≤ 1 helminth egg/L) for agricultural use resulting in higher risk of ascariasis. Therefore, in conclusion, DEWATS plants may be more efficient in reducing the concentration of helminth eggs in wastewater, resulting in lower risks of STH infections upon exposure.

Keywords Wastewater treatment · South Africa · Lesotho · Wastewater irrigation · Soil-transmitted helminths · QMRA

Introduction

Municipal wastewater contains a variety of pathogens, reflecting the carrier state and infection levels in the community (Carr et al. 2011; Hanjra et al. 2012). The contamination of surface water with untreated or partially treated wastewater may as a consequence lead to adverse health implications (Ahmed et al. 2016; Petterson et al. 2016). There is an epidemiological link between gastro-intestinal diseases and contact with fecally contaminated surface water (Thurston et al. 2001; Amoah et al. 2016). Treatment of wastewater before discharge into surface water bodies will therefore function as a barrier; efficiency of treatment however differs impacting on the reduction of risks achievable (Hussain et al. 2001; Hussain et al. 2002; Qadir et al. 2015). Centralized wastewater treatment plants (WWTPs) are the main wastewater treatment option globally, especially in developed countries. The major bottleneck in the establishment of these centralized WWTPs is the exorbitant costs associated with their construction, operation, maintenance (Massoud et al. 2009), and cost of transportation of the wastewater (UN-Water 2015). According to the UN World Water Development Report 2015, these costs could be reduced considerably by treating wastewater close to the

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source using simple technologies. The issue of costs of constructing and operating of wastewater treatment plants is mainly a challenge in poor settings (Massoud et al. 2009); access to finance for these investments therefore acts as the main stumbling block (Hanjra et al. 2015; Duchin 2016).

Some of the widely used decentralized wastewater treatment technologies are constructed wetlands, anaerobic baffled reactors (ABRs), upflow anaerobic sludge blankets (UASBs), waste stabilization ponds, aerated lagoons, and oxidation ditches (Elmitwalli et al. 2002; Istenic et al. 2014; Masi et al. 2015). The use of ABRs has increased over the last 10 years due to their low maintenance requirements, simple and inexpensive construction, and stable operational conditions (Tilley et al. 2014; Reynaud and Buckley 2016). Although decentralized wastewater treatment plants, such as the ABRs, have the potential to eliminate some of the challenges associated with centralized wastewater treatment, there is limited information on the achievable pathogen reduction, especially the soil-transmitted helminths (STHs) and other helminths (von Sperling et al. 2003; Foxon et al. 2004; Nasr et al. 2009). In fact, STHs are recognized as a major public health problem affecting over 1.5 billion people worldwide (WHO 2015), with *Ascaris* spp., hookworm, and *Trichuris* spp. infections the most common (Pullan et al. 2014). These infections are associated with low-income countries, mainly occurring in Sub-Saharan Africa, Asia, and South America (WHO 2015).

The increasing reuse of wastewater is making it very important to determine the concentration of these pathogens in effluents from ABR systems. An essential public health concern with wastewater reuse is the health treat from STH infections (WHO 2006) especially in endemic regions. The WHO guidelines for wastewater reuse proposed a guideline value of < 1 helminth egg/L for wastewater intended for unrestricted agriculture, aimed at reducing risks of infections (WHO 2006). Reuse of wastewater and sludge has been associated with elevated STH infections globally (Fuhrimann et al. 2014; Fuhrimann et al. 2016; Contreras et al. 2017; Gyawali 2017). In South Africa, Gumbo et al. (2010) reported a higher prevalence of hookworm infections among farmers using wastewater for irrigation. STHs are a major health concern due to their long periods of persistence in the environment, from a few months up to years (Bethony et al. 2006).

In this study, the STH and *Taenia* spp. egg reduction efficiency of selected centralized and decentralized (ABR coupled with planted gravel filters (PGFs)) plants in South Africa and Lesotho was assessed and compared. In addition, a comparison of the risk of *Ascaris* spp. (as a surrogate for STHs) infections for different exposed populations was estimated to provide a public health perspective to the choice of treatment approach, especially within the context of water reuse.

Material and methods

Study area and sampling points

Centralized wastewater treatment plants

Wastewater samples were taken from three (3) centralized wastewater treatment plants (WWTPs), all within the eThekwini Municipality of KwaZulu-Natal province in South Africa. This municipality is known globally for its achievements in the field of water and sanitation; therefore, the results from this study will add to the efforts of the municipality in the provision of safe sanitation. The treatment steps within these WWTPs are similar, with the main stages being mechanical grit removal trap, flow division chamber, raw sewage pump station, reaction tank/biological reactor/biological filters, clarifiers, chlorine contact tank, and chemical dosing facilities. Table 1 summarizes the characteristics of the centralized WWTPs studied.

Decentralized wastewater treatment plants

The decentralized treatment plants are ABRs, with planted gravel filters (PGFs) for final treatment and collectively referred to as decentralized wastewater treatment system (DEWATS). The South African DEWATS plant is at an experimental site in Durban, designed to treat domestic wastewater from about 80 households, with a design capacity for a total of 462 persons. The DEWATS plant is part of a research site managed by the eThekwini municipality; this is aimed at studying the performance of DEWATS systems in treating domestic wastewater under different hydraulic conditions. This plant has an initial two-chamber settling step (also serving as a biogas collection point), and from this, the wastewater is distributed into three parallel ABR treatment trains. Two chambers of anaerobic filters (AFs) follow each ABR train. The final polishing steps are planted gravel filters (PGFs), both vertical and horizontal. Samples were taken from the inlet, after the AFs and finally after the PGFs.

Sampling

Approximately five (5) liters of wastewater was taken from each sampling point within the WWTPs studied. Composite samples (in triplicates) were taken based on consecutive subsamples at intervals till the required volume is reached. For a five (5)-liter sample, samples were taken ten times in approximate volumes of 500 mL each.

Sampling at the centralized WWTPs and the DEWATS plant in Durban was done monthly from January to October 2016, and sampling of the DEWATS plants in Lesotho was in June 2015 (five plants) and August 2016 (ten plants). Ten samples were taken from each treatment step for each of the plants to account for variability.

Table 1 Characteristics of centralized wastewater treatment plants studied

Plant	Capacity (mL/day)	Size of population served	Characteristics of population served	Sampling points
WWTP A	10.98	30,200	Low- and middle-income individuals	Influent, outlet of rotating biological filters (RBF), outlet of settling tank and outflow of the maturation ponds
WWTP B	4.69	13,800	High- and middle-income households	Influent, outlet of clarifier and effluent (after chlorination)
WWTP C	10.08	29,800	Middle-income households	Influent, outlet of clarifiers, outlet of the chlorination tanks, and outflow of the maturation ponds

Laboratory analysis

Sample analysis for the STH eggs was carried out using a new revised methodology (developed in our laboratory) based on the principles of sedimentation and flotation. Briefly, samples were poured through a 100-μm sieve onto a 20-μm sieve (Wirsam Scientific ad Precision Equipment (Pty) Ltd). The contents on the 20-μm sieve were carefully washed into 50-mL centrifuge tubes and centrifuged for 10 min at 3000 rpm. The supernatants were discarded and $ZnSO_4$ solution (specific gravity of 1.30) added to a total volume of 50 mL. After resuspension, the mixture was then centrifuged again at 2000 rpm for 10 min. The supernatant was poured through the 20-μm sieve, and the contents of the sieve washed under running water into a 50-mL centrifuge tube and centrifuged at 3000 rpm for 10 min. Supernatants were discarded and the pellets incubated in 0.1 N sulfuric acid for 28 days. The pellets were re-suspended after incubation screened under the microscope at × 100 magnification and further examined at × 400 to determine the stage of development (necessary for the determination of potential viability). Only potentially viable eggs, based on morphology and presence of motile larvae, were counted.

Statistical analysis

Descriptive analysis to assess the mean concentration and distribution of eggs in the samples was performed using GraphPadPrism version 7.0 (GraphPad Software Inc). Analysis of variance as well as *t* test was performed to determine the statistical difference between the concentrations of the STH eggs and removal efficiencies between/within the WWTPs at 95% confidence interval. Probability distribution functions (PDFs) were fitted to the concentration of STH eggs detected in the different samples analyzed using @Risk version 4.5.2 professional edition (Palisade Corporation) added on to Microsoft Excel. The best PDF that described the data was determined by assessing the Akaike information criteria (AIC). The STH removal efficiencies were calculated using the following formula:

$$\%\mathrm{Eff} = \frac{Cinf - Ceff}{Cinf} \times 100$$

where "*Cinf*" is the concentration of eggs in the influent and "Ceff" is the concentration of eggs in the effluent of the respective plants.

Assessment of risk of *Ascaris* sp. infection

The quantitative microbial risk assessment (QMRA) approach was used to estimate the infection risks associated with direct and indirect exposure to effluents from the centralized WWTPs. This was performed for only the centralized WWTPs due to limited data for accurate assessment of risks for the DEWATS systems. The approach involved the interlink steps of the following: (a) hazard identification, (b) exposure assessment, (c) dose-response assessment, and (d) risk characterization (Haas et al. 2014).

Hazard identification

Ascaris spp. was chosen as the main organism for the assessment of risk of infections associated with exposure to the effluents. Several studies have shown a significant relationship between direct/indirect exposure to wastewater (e.g., wastewater irrigation and consumption of wastewater irrigated vegetables) and STH infections (especially ascariasis) (Navarro and Jimenez 2011; Amoah et al. 2016; Amoah et al. 2018). *Ascaris* spp. eggs can survive for long periods of time under adverse environmental conditions (Feachem et al. 1983) and has therefore been suggested as the index organism for QMRAs in developing countries by the WHO (2006). In addition, *Ascaris* spp. is the only STH with a dose-response model.

Exposure assessment

Exposure assessment involves the determination of the "number of organisms that correspond to a single exposure (termed the dose) or the total number of *Ascaris* spp. eggs that will constitute a set of exposures" (Haas et al. 2014). In this study, two main exposure groups were assessed, namely, (a) occupational exposure and (b) community exposure.

Occupational exposure scenario Irrigation of crops especially vegetables, on small scale/household level, could expose the farmers to *Ascaris* spp. eggs in the irrigation water (treated wastewater). The risk of infection for the farmers using the effluent from these WWTPs for irrigation was therefore quantified and compared between the WWTPs. In this study, the volume ingested was assumed to be uniformly distributed from 1 to 5 mL per irrigation event (WHO 2006). The dose of *Ascaris* spp. eggs ingested by the farmers per day ("λ") was therefore determined using the following formula:

$$\lambda = C_{raw} \times V$$

where "C_{raw}" is the concentration of *Ascaris* spp. eggs per milliliter of the final effluents (irrigation water) and "V" is the volume (mL/day) of water accidentally ingested by farmers. Frequency of exposure was also assumed to be uniformly distributed from 120 to 140 days per year based on information from farmers in the study area.

Community exposure scenario Community exposure to the final effluents from the investigated WWTPs could also lead to STH infections through the following exposure routes:

1) Recreational/accidental exposure to the effluents: Exposure to the final effluents either intentionally or unintentionally was considered assuming eggs are in the infective stage. Immersion in the maturation ponds (in the case of some of the centralized WWTP) was the main exposure scenario. In some of the WWTPs, the maturation ponds are not fenced and therefore accessible by the general community. In addition, the final effluents are discharged into surface water bodies that run through communities where exposure might occur. In this instance, it is assumed that the concentration of the STH eggs remains constant irrespective of dilution or egg die-off (assuming a worst case scenario). The different exposure scenarios and the volume of water ingested are presented in Table 2.
2) Consumption of wastewater irrigated vegetables: The risk of STH infection for consumers of crops irrigated with effluents from the WWTPs was modeled using lettuce as a surrogate for all vegetables. The dose (λ) of *Ascaris* spp. eggs (λ; no. ingested per person per day) resulting from consumption of the effluent irrigated lettuce was modeled as follows:

$$\lambda = VIc$$

where "V" is the volume of effluent (irrigation water) caught on the surface of the lettuce plant following irrigation (mL g^{-1}), "I" is the mean per capita intake of lettuce (g $person^{-1}$ day^{-1}), and "c" is the concentration of *Ascaris* spp. eggs in the final effluents being used for irrigation (no. mL^{-1}). There is a large variation on the volume of irrigation water caught on the surface of vegetables following irrigation, and for this study, this volume was assumed to be normally distributed as reported by Hamilton et al. (2006). In addition, it was assumed that there would not be any reduction in the concentration of these eggs either through natural die-off or washing.

Dose-response assessment

The *Ascaris* spp. infection risk associated with the different exposure pathways was assessed using the exponential dose-response model (Westrell 2004, Seidu et al. 2008), which is given as follows:

$$P_{inf} = 1 - e^{-rd}$$

where P_{inf} is the *Ascaris* infection risk associated with the ingestion of d number of infectious *Ascaris* spp. and r is a dimensionless infectivity constant. In this study, r value of 0.039 was used (Navarro et al. 2008). The dose of *Ascaris* spp. egg per exposure scenario was modeled by fitting probability distribution functions (PDFs) to the concentration of these eggs as determined in this study. Table 8 in the Appendix describes the various PDFs that best described the *Ascaris* spp. egg concentrations in the effluents from the WWTPs.

Risk characterization

In the risk characterization, all the outcomes of the hazard identification, exposure assessment, and dose-response assessment were combined to characterize the severity of *Ascaris* spp. infection. The annual infection risk (P_A) associated with multiple exposures was determined using the following formula:

$$P_A = 1 - (1 - P_{inf})^n$$

where P_{inf} is the risk of infection from a single exposure to a dose d of *Ascaris* spp. and n being the number of days of exposure to the single dose d (Sakaji and Funamizu 1998). For the scenario of farmers' ingesting both irrigation water and crops, the combined annual risk of infection was determined by using the following formula:

$$\pi_t = 1 - (1 - \pi_i)(1 - \pi_x)$$

where "π_t" is the combined annual risk of infection from exposures to irrigation water and crops, "π_i" is the *Ascaris* spp. infection risk resulting from accidental ingestion of irrigation water, and "π_x" is the *Ascaris* spp. infection risk resulting from consumption of wastewater irrigated crops (Haas et al. 2014). All risk models were subjected to Monte Carlo simulations of 10,000 iterations for probability of infections. These models were constructed in Microsoft Excel using the @Risk 7.5 (Palisade Corporation) software add-on to Excel.

Table 2 Points of exposure with assumptions based on volume ingested and frequency of exposure

Exposure scenario/assumptions for dosage	Volume ingested (mL or g)	Frequency	Reference
(Un)intentional immersion/swimming at maturation ponds or effluent contaminated surface water	Uniform distribution (10, 15)	Uniform distribution (64,128)*	Dorevitch et al. 2011
Volume caught on lettuce	Normal distribution (0.108, 0.019)		Hamilton et al. (2006)
Daily per capita intake of vegetables	Pert distribution (25, 50, 75)	Uniform distribution (156,160)*	Sant'Ana et al. 2014

*Assumption

Results

Occurrence and removal of STHs and *Taenia* spp. in centralized WWTPs and DEWATS

Concentration of STH and *Taenia* sp. eggs in raw wastewater at the centralized WWTPs

Different species of STHs at varying concentrations were detected in the influent of the centralized WWTPs (Table 3). *Ascaris* spp. was the most prevalent STH detected, with concentrations ranging from 0 to 201 eggs/L (Table 3). Samples from WWTP A had higher concentrations of almost all the STH eggs (except for *Trichuris* spp.) than the other two WWTPs. Concentration of *Ascaris* spp. eggs did not vary statistically between the WWTP A and WWTP B; same trend was observed for the non-STH, *Taenia* spp. (Table 3). However, concentrations of *Ascaris* spp. and hookworm varied significantly between WWTP B and WWTP C.

Variation in the mean concentration of eggs was recorded for the various months throughout the study. Irrespective of the WWTP, these variations followed a similar trend with no significant difference between the WWTPs (p value > 0.05) at a said month. Therefore, the concentrations were combined and the mean values are presented in Fig. 1a, b. *Ascaris* spp. and hookworm eggs recorded high concentrations in January and October (Fig. 1a), with the other STH and *Taenia* spp. eggs peaking in January and again steadily from July to October. However, *Taenia* spp. egg concentrations reduced in October (Fig. 1b).

Concentration of STH and *Taenia* spp. eggs in raw wastewater at the DEWATS treatment plants

Raw wastewater at the DEWATS plant in Durban only contained eggs of *Ascaris* spp. and hookworm. These were only detected during one (September) month throughout the 10-month study period, in relatively low concentrations. These as well as the corresponding concentrations for the Lesotho treatment plants, for the occurrence of *Ascaris* spp., hookworm, *Taenia* spp., and *Trichuris* spp., are given in Table 3. During the first sampling in June 2015, in Lesotho, only two out of the five DEWATS plants contained *Ascaris* spp. and hookworm eggs in the raw wastewater. In the second sampling in August 2016, additional plants were included with STH eggs occurring more in the raw wastewater. A direct comparison of mean concentrations in the original five (5) DEWATS plants did not give any statistical differences (p value ≥ 0.05) between the two sampling rounds. *Ascaris* spp. egg concentrations varied significantly between the raw wastewaters from Durban and Lesotho, except for hookworm concentrations that did not show any statistical significant difference (Table 3). STH egg concentrations varied between the individual DEWATS plants in Lesotho, but the differences were not statistically significant here either.

Concentration of STH and *Taenia* spp. eggs in effluents from centralized wastewater treatment plants

All helminth species detected in the raw wastewater ("Concentration of STH and *Taenia* spp. eggs in raw

Table 3 Concentration of STH and *Taenia* spp. eggs in the influent and effluent of the wastewater treatment plants studied

	WWTP A		WWTP B		WWTP C		DEWATS—Durban		DEWATS—Lesotho	
	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
Ascaris spp.*	91 (± 101.5)	2.2 (± 8.4)	16 (± 24.8)	2.4 (± 8.0)	55 (± 45.2)	3.8 (± 2.6)	0.4 (± 0.9)	N/D	87 (± 96)	2.3 (± 1.5)
Hookworm	61 (± 52.1)	3.8 (± 12.2)	15 (± 16.2)	2.5 (± 5.2)	18 (± 18.5)	2.6 (± 4.5)	13 (23.4)	N/D	26 (± 32)	0.19 (± 0.19)
Trichuris spp.*	16 (± 12.2)	1.6 (± 8.0)	4.6 (± 1.2)	1.2 (± 1.0)	23 (± 21.7)	13 (± 6.0)	N/D	N/D	12 (± 8)	0.25 (± 0.25)
Taenia spp	29.6 (± 9.8)	8.4 (± 8.0)	6.4 (± 2.4)	1.4 (± 2.1)	9.8 (± 8.8)	3.2 (± 8.0)	N/D	N/D	2.3 (± 2.4)	0.25 (± 0.17)
Toxocara spp	14 (± 20.1)	1.3 (± 3.1)	7.8 (± 6.1)	1.3 (± 2.2)	9.2 (± 8.9)	3.0 (± 8.0)	N/D	N/D	N/D	N/D

*Significant difference in egg concentrations (p < 0.05)

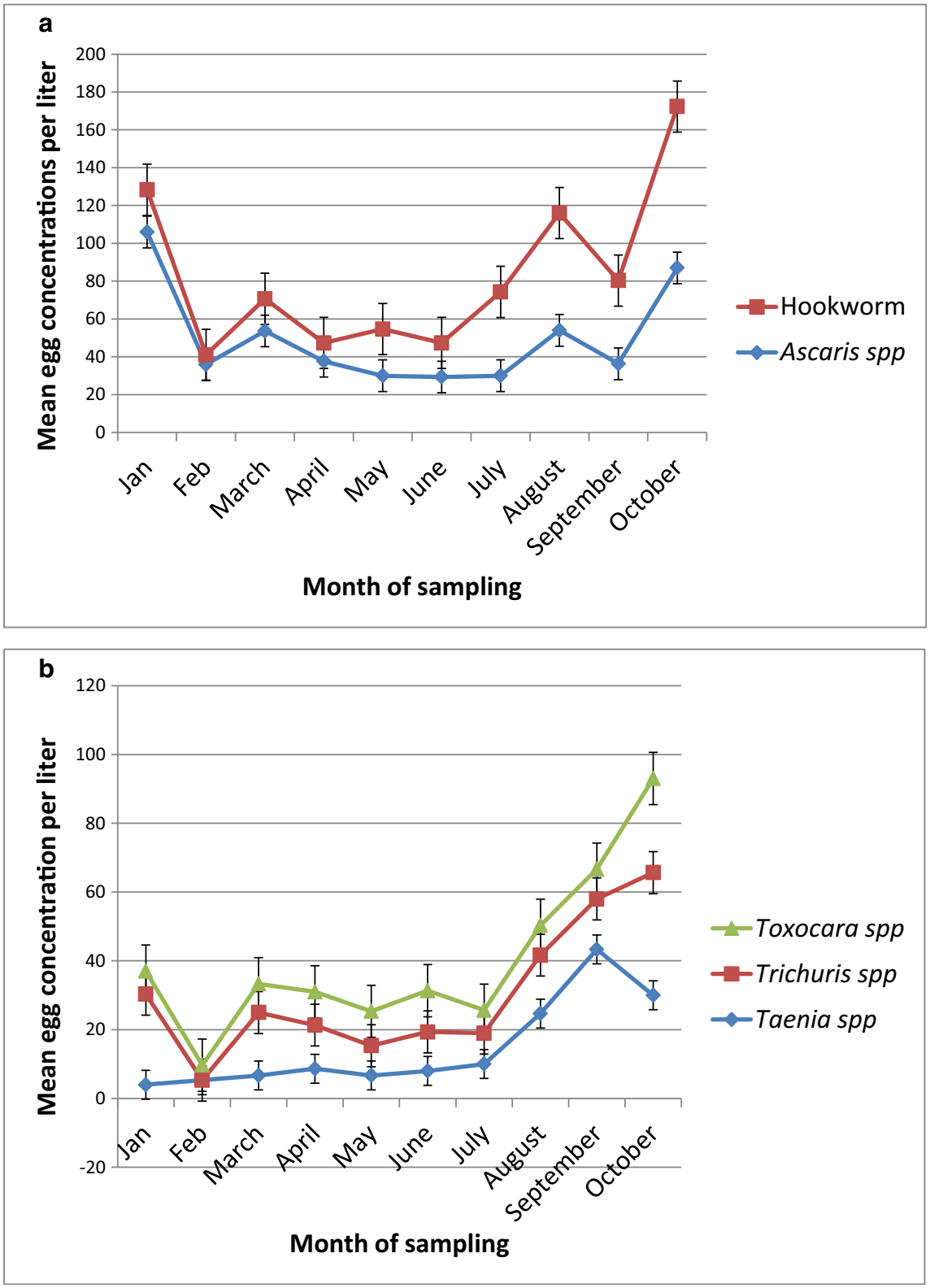

Fig. 1 **a** Variation in mean *Ascaris* spp. and hookworm egg concentrations in the raw wastewater at the centralized WWTPs over the study period ($n = 10$). **b** Variation in mean *Toxocara* spp., *Trichuris* spp., and *Taenia* spp. concentration in the raw wastewater at the centralized WWTPs over the study period ($n = 10$)

wastewater at the centralized WWTPs") were recorded in the final effluents of the centralized WWTPs. However, the concentrations varied between the plants, exemplified by *Ascaris* spp. with the highest values in effluents from WWTP C (3.8 (± 2.6) eggs/L), while effluents from WWTP A contained the highest concentrations of both hookworm (3.8 (± 12.2) eggs/L) and *Taenia* spp. (8.4 (± 8.0) eggs/L). Although there were differences in the concentration of STH eggs in the final effluents between the WWTPs, these were not significant except for *Trichuris* spp. concentrations (p value ≤ 0.05) (Table 4). Within the individual WWTPs there was variation in the concentrations of the various STH eggs detected (Table 3). The difference in the STH egg concentrations between the influent and effluent was statistically significant (p value ≤ 0.05).

Concentration of STH eggs in effluents from the DEWATS plants

There were no STH eggs detected in the final effluents from the DEWATS plant in Durban. However in Lesotho, eggs of all the STH groups, except *Toxocara* spp., which occurred in the untreated wastewater, were found in low numbers, in the effluents (Table 3). For instance, the highest STH egg found was *Ascaris* spp. (2.3 (± 1.5) eggs/L). These concentrations varied between the various DEWATS plants in Lesotho, but during June 2015 sampling, there was no STH egg in the final effluents. The mean concentrations in the final effluents (Table 3) are from the ten DEWATS plants sampled in August 2016. There was no statistically significant variation in the STH egg concentrations in the final effluents from the individual DEWATS plants that had positive samples in the second sampling.

STH and *Taenia* sp. egg removal efficiency of the wastewater treatment plants

The overall removal efficiency for the various centralized WWTPs and DEWATS varied greatly, with difference in the removal achieved for the different STHs and *Taenia* spp. as well. WWTP A had mean removal percentages from 80 (± 9.9) % to 96 (± 1.8) %, 72 (± 12.0) % to 96 (± 3.7) % for WWTP B, and 56 (± 8.7) % to 90 (± 3.5) % for WWTP C. The DEWATS in Lesotho recorded removal efficiencies from 98 (± 2.1) % to 100 (± 0.29) %; a complete removal of STH and *Taenia* spp. eggs in the DEWATS plant in Durban was recorded.

The percentage of the individual helminth eggs removed varied; however, *Ascaris* spp. egg removal was consistently high irrespective of the treatment plant. For instance in WWTP A and C, removal of *Ascaris* spp. eggs was the highest (96 (± 1.8) % and 90 (3.5) % respectively). In WWTP B, removal of *Trichuris* spp. eggs was highest (96 (± 3.7) %) (Table 4). Within the DEWATS plants with positive samples, the removal percentages varied. Plants with accumulation of biogas within the treatment system reported significantly lower helminth egg removals. However, the DEWATS plants achieved a consistently higher STH and *Taenia* spp. egg removal than the centralized WWTPs (Table 4), with removal of *Ascaris* spp. being the highest (99 (± 0.35) %).

The efficiency of the various WWTPs in removing STH and *Taenia* spp. eggs varied within the WWTPs depending on the treatment step. In WWTP A, the highest egg removal occurred in the maturation ponds for *Ascaris* spp. (86 (± 19) %). The settling tanks (both primary and secondary) also contributed to the removal of the STH and *Taenia* spp. eggs, with 44 (± 38) % and 51 (± 44) % removal of *Taenia* spp. and *Trichuris* spp. respectively in the primary settling tanks. The secondary settling tanks removed 44 (± 38) % and 51 (± 44) % of hookworm and *Toxocara* spp. eggs respectively. For WWTP B, the highest reduction was achieved during the clarifier stage with 50 (± 40) % of hookworm eggs. Additionally, *Ascaris* spp. and *Taenia* spp. eggs were best removed at the post-clarifier stage, with 48 (± 47) % and 30 (± 48) % removal respectively. STH egg removal in WWTP C maturation ponds ranged from 44 (± 43) % for hookworm to 53 (± 50) % for *Toxocara* spp. The secondary settling tanks also resulted in an additional egg removal, with 50 (± 29) % for *Ascaris* spp. eggs, 57 (± 36) % for hookworm and 63 (± 34) % for *Trichuris* spp.

In the DEWATS plants, the highest reduction was achieved during the anaerobic treatment step (ABR section), with removals ranging between 72 (± 24) % (*Taenia* spp) to 90 (± 38) % (*Ascaris* spp).

Quantitative risk assessment according to exposure scenarios

Probability of *Ascaris* sp. infection for farmers using treated wastewater for irrigation (occupational exposure)

Reuse of the effluents from the centralized WWTPs for irrigation poses risks of *Ascaris* sp. infections, with effluents from WWTP C giving the highest mean risks of 4.8×10^{-4} ($\pm 9.9 \times 10^{-6}$). The variation in infection risk from one-time exposure during irrigation was found to be statistically insignificant ($p \leq 0.005$) (Table 5). Multiple/annual exposure/s to the effluents would result in increased risks of infections (Table 5), using the assumptions stated in Table 2. This risk of infection due to annual or multiple exposures was not significant for reuse associated with the different WWTPs ($p > 0.005$).

Probability of *Ascaris* spp. infection for communities exposed to the treated wastewater directly and indirectly

Direct exposure to the final effluents from the centralized WWTPs either through (un)intentional immersion or

Table 4 Mean percentage (± SD) removal of STH and *Taenia* spp. eggs from the wastewater treatment plants studied

	Ascaris spp.		Hookworm		*Trichuris* spp.		*Taenia* spp.		*Toxocara* spp.	
	Mean (±SD)	Range	Mean (± SD)	Range	Mean (± SD)	Range	Mean (± SD)	Range	Mean (± SD)	Range
WWTP A	96 (± 1.8)	88–100	86 (± 6.4)	33–100	80 (± 9.9)	20–100	96 (± 4.4)	60–100	89 (± 6.1)	40–100
WWTP B	89 (± 4.4)	67–100	72 (± 12)	0.0–100	96 (± 3.7)	67–100	82 (± 8.8)	20–100	82 (± 9.6)	25–100
WWTP C	90 (± 3.5)	67–100	83 (± 8.7)	20–100	56 (± 11)	0.0–100	73 (± 10)	25–100	71 (± 11)	20–100
DEWATS Lesotho	99 (± 0.35)	95–100	100 (± 0.29)	95–100	98 (± 2.1)	67–100	100 (± 0.15)	98–100	–	–

SD standard deviation

swimming poses a high risk of infection. The highest mean risk of infection (2.0×10^{-3} ($\pm 3.7 \times 10^{-5}$)) was recorded with exposure to effluents from WWTP C, as was the case for the risk of infection for the farmers. Immersion in effluents from WWTP A resulted in the least probability of infection (9.6×10^{-4} ($\pm 1.8 \times 10^{-5}$)). With multiple exposures, the risk of infection increased for each of the WWTPs, where the annual risks ranged from 8.3×10^{-2} ($\pm 1.4 \times 10^{-3}$) (WWTP A) to 1.6×10^{-1} ($\pm 2.5 \times 10^{-3}$) (WWTP C) (refer to Table 6).

Combined probability of infection for farmers exposed to treated wastewater as well as consumption of vegetables

Exposure to the effluents during irrigation and consumption of the vegetables (lettuce) from the farm annually would result in a much higher probability of infection (Table 7). Combined risks were higher in populations using effluents from WWTP C (1.0 ($\pm 5.6 \times 10^{-2}$)), with the least probability (7.3×10^{-1} ($\pm 2.4 \times 10^{-2}$)) with exposure to effluents from WWTP B. This difference in probability of infection was statistically significant ($p \leq 0.05$). Based on these estimations, farmers using effluents from WWTP C who also consume their own produce are all at risk of infection with *Ascaris* spp.

Discussion

Ascaris spp., hookworm, *Trichuris* spp., and *Toxocara* spp. (except in Lesotho) were the soil-transmitted helminth (STH) eggs detected in this study, including the non-STH, *Taenia* spp., with *Ascaris* spp. and hookworm the most prevalent. These are the most common helminth infections in South Africa (Appleton et al. 2009; Mkhize-Kwitshana and Mabaso 2014; Molvik et al. 2017). *Toxocara* spp. are mainly infections of animals such as dogs and cats (Chen et al. 2012; Pereira et al. 2016; Kostopoulou et al. 2017); their presence in the wastewater may therefore be from animal feces. A high prevalence and level of infection of helminths exacerbated by poor sanitation, poverty, and low water usage per capita (Chan 1997; Mara and Horan 2003) and the potentially high number of eggs excreted per day by infected individuals (10^2–10^4 eggs/g) (Smith and Rose 1998) all contribute to the occurrence of high concentration of helminth eggs in untreated wastewater. The variation in the concentration of these eggs in the untreated wastewater between the WWTPs is an indication of the difference in infection patterns within the cities of Durban and Maseru, mainly influenced by the factors mentioned above. Additionally, the temporal variations seen in helminth egg concentrations may be attributed to the variation in infection levels influenced by either environmental or human factors. The areas served by these treatment plants vary in terms of population size and demographic distribution. Wastewater from poor neighborhoods is expected to contain higher concentration of helminth eggs than wastewater from middle- or high-income areas (Stolk et al. 2016). Untreated wastewater at the DEWATS plant in Durban contained low concentration of these eggs, which may be attributed to the fact that this DEWATS plant treats wastewater from middle-income households. A similar trend was observed for concentrations in untreated wastewater in Lesotho, where the DEWATS plants are privately financed and treat domestic wastewater from middle- and higher-income households. A few plants were treating wastewater from schools and orphanages, and these contained higher concentrations of helminth eggs, reflecting differences in infection patterns. STH and *Taenia* spp. infections are much more prevalent in children than adults due to different exposure patterns (Anderson et al. 2015; Lo et al. 2017). The concentrations of STH and *Taenia* spp. eggs in this study are in a similar range as those from other studies in developing countries with similar socio-economic settings as South Africa and Lesotho. For instance in Brazil, Ayres (1991) reported concentrations of up to 700 eggs/L for *Ascaris* spp., 19 eggs/L for *Trichuris* spp., and 8

Table 5 Mean probability (± 90% CI) of infection for farmers using final effluents for irrigation

	Onetime exposure	Multiple exposure
WWTP A	2.3×10^{-4} ($\pm 4.7 \times 10^{-6}$)	2.9×10^{-2} ($\pm 5.7 \times 10^{-4}$)
WWTP B	2.5×10^{-4} ($\pm 5.2 \times 10^{-6}$)	3.1×10^{-2} ($\pm 6.3 \times 10^{-4}$)
WWTP C	4.8×10^{-4} ($\pm 9.9 \times 10^{-6}$)	5.8×10^{-2}($\pm 1.1 \times 10^{-3}$)

Table 6 Mean probability (± 90% CI) of infection with *Ascaris* spp. due to (un)intentional exposures to final wastewater effluents

	WWTP A		WWTP B		WWTP C	
	Onetime exposure	Annual	Onetime exposure	Annual	Onetime exposure	Annual
(Un)intentional immersion/swimming at maturation ponds or effluent contaminated surface water	9.6×10^{-4} ($\pm 1.8 \times 10^{-5}$)	8.3×10^{-2} ($\pm 1.4 \times 10^{-3}$)	1.0×10^{-3} ($\pm 1.9 \times 10^{-5}$)	9.0×10^{-2} ($\pm 1.6 \times 10^{-3}$)	2.0×10^{-3} ($\pm 3.7 \times 10^{-5}$)	1.6×10^{-1} ($\pm 2.5 \times 10^{-3}$)
Consumption of vegetables	4.1×10^{-4} ($\pm 8.0 \times 10^{-6}$)	6.1×10^{-2} ($\pm 1.1 \times 10^{-3}$)	4.5×10^{-4} ($\pm 8.9 \times 10^{-6}$)	6.6×10^{-2} ($\pm 1.2 \times 10^{-3}$)	6.9×10^{-4} ($\pm 1.2 \times 10^{-5}$)	9.7×10^{-2} ($\pm 1.6 \times 10^{-3}$)

eggs/L for hookworm. In Tunisia, concentrations of 15 eggs/L were found (Riahi et al. 2009), and in Vietnam, 450 eggs/L were reported (Yen-Phi et al. 2010).

Performance of the WWTPs in removing STH and *Taenia* spp. eggs varied greatly but was expected to be high, due to the egg sizes, in well performing plants. Removal of *Ascaris* spp. was higher than the removal of the other helminth eggs in almost all the WWTPs with similar results reported elsewhere (Panicker and Krishnamoorthi 1978; Rose et al. 1996; Jimenez et al. 2000). The effective removal of *Ascaris* spp. eggs is probable partly due to sedimentation (Mara and Horan 2003). Eggs of *Ascaris* spp. have a specific gravity of 1.2 g/cm^3 as compared to 1.15 g/cm^3 and 1.055 g/cm^3 for *Trichuris* spp. and hookworm respectively. This result in a higher settling velocity (0.77 m/h) for *Ascaris* spp. eggs than that for *Trichuris* spp. (0.73 m/h) and hookworm (0.39 m/h) (Medema et al. 1998; David and Lindquist 1982; Shuval et al. 1986; and Pike 1990). This differential terminal settling velocity based on specific gravity and other factors such as dimensions of the egg and liquid density (and temperature) are explained by Stoke's law for discrete particle settling in sedimentation basins (Mara and Horan 2003). Additionally, the eggs may attach to particles in the wastewater aiding in their rapid sedimentation; this is most common for *Ascaris* spp. eggs (Capizzi-Banas et al. 2002; Sengupta et al. 2011). This attachment of the *Ascaris* spp. eggs to particles might have contributed to the higher removal.

In comparison, the removal of the STH and *Taenia* spp. eggs was higher in the DEWATS plants, with an average of 95–100% reduction in Lesotho, and 100% in Durban, compared to that of the centralized WWTPs where a high variation occurred. Generally, centralized WWTPs with activated sludge and trickling filter processes remove between 75 and 100% of STH eggs (Rose et al. 1996; Chaoua et al. 2017) mainly due to sedimentation. The activated sludge process has no or little effect on egg viability (Mayer and Palmer 1996; Dowd et al. 1998). In this study, the removal of viable STH eggs was recorded, resulting in lower and variable reduction figures. A high removal percentage in the DEWATS plants may be attributed to several factors. Influent wastewater is forced through the sludge bed/blanket due to the upflow baffles, whereby removal of the eggs would be enhanced by filtration and aggregation (Mara and Horan 2003). The anaerobic digestion processes (especially within the biogas digesters) may also have contributed to the inactivation of the STH eggs. Johansen et al. (2013) reported a 0.5 log reduction in viable *Ascaris suum* eggs in a mesophilic anaerobic digester at 34 °C. Hailu (2006) also reported a 50–60% reduction in STH eggs during anaerobic digestion processes from studies in Ethiopia. Additionally, the planted gravel filters (both horizontal and vertical) would further contribute to the egg removal where horizontal subsurface constructed wetlands alone have been reported to remove over 90% of STH eggs (Stott et al. 2002).

The presence of the STH and *Taenia* spp. eggs in the final effluents poses potential risk of infections. In this regard, intentional exposures, through swimming or playing nearby, pose different degrees of risk depending on the efficiency of the WWTPs. As expected, (un)intentional immersion in the final effluents from WWTP C resulted in the highest probability of infection based on the concentrations found. Exposure to the final effluents might occur in situations where the maturation ponds or final effluents are easily accessible to the community. Under such circumstances, children or even adults may swim in these and therefore exposing them to risk of infections. In other instances, workers within the WWTPs are exposed to these effluents during maintenance; for instance, it was observed in some of the WWTPs that algae and other aquatic plants grow in the ponds and therefore have to be removed, during which accidental immersion might occur exposing them to infections. Exposure to large quantities of the final effluents might not be a situation in the DEWATS plants since these are household level WWTPs and are mainly within the compounds of these houses; however, children may play close to or even within the planted gravel filters therefore exposing them to the effluents. *Ascaris* spp. eggs have a latency period of between 2 and 4 weeks, at temperatures between 15.5 and 38 °C, before they become infectious (Bogitsh et al. 2012). Therefore, the risk of infections would differ (considerably lower) from the estimates reported here. It has even been reported that at temperatures of 25 °C, *Ascaris* spp. eggs could reach the infectious stage within 10 days (Maya et al. 2012). For instance, on-site wastewater treatment systems, such as the DEWATS, increase the level of exposure to STH egg contaminated surfaces; therefore, the likelihood of infection as a result of exposure to eggs in their infective stage is

Table 7 Mean (±90% CI) probability of infection with *Ascaris* spp. from combined exposure to irrigation water and consumption of farm produce

Treatment plant	Probability of infection(±90% CI)*
WWTP A	8.8×10^{-1} ($\pm 8.3 \times 10^{-3}$)
WWTP B	7.3×10^{-1} ($\pm 2.4 \times 10^{-2}$)
WWTP C	1.0 ($\pm 5.6 \times 10^{-2}$)

*$p \leq 0.05$

enhanced and might increase the risks beyond what we reported for centralized WWTPs. In addition, the reuse of final effluents (from both the centralized WWTPs and DEWATS) for irrigation may result in the accumulation of STH eggs in the soil (Seidu et al. 2008) which allows the eggs to develop to the infective stage under the right environmental conditions, thereby also increasing the risk of infection.

WHO as part of its guidelines for safe wastewater reuse in agriculture suggested that for unrestricted agriculture, wastewater should have ≤1 helminth egg per liter (WHO 2006). Only final effluents from some of the DEWATS plants met the guideline. Therefore, the use of the effluents from the centralized WWTPs needs to be looked into in line with additional barriers to reduce the risks of STH infections for farmers as well as consumers of the farm produce. For instance, further treatments with storage, elevated pH, etc. may reduce the egg concentrations to safe limits (Jimenez-Cisneros and Maya-Rendon 2007).

Despite the low concentrations of STH and *Taenia* spp. eggs in the effluents from the DEWATS plants, the infection risk from the reuse of the effluents may still be higher than that of the WHO tolerable infection risk of 1×10^{-2} (Mara et al. 2007). This might be most likely for the few DEWATS plants where effluent quality was compromised due to system failures. The frequency and durability of such failures are determinants of the risk. It was observed in some of these plants that the biogas was not being used which led to its accumulation within the system; this reduces the hydraulic retention time which in turn reduces the treatment efficiency.

Consumption of farm produce would expose the populations to additional risk of infections, with varying probability based on the effluent quality. Except for reuse of effluents from WWTP C, the rest of the centralized WWTPs gave lower annual risk of infections as compared to the WHO tolerable risk figure for consumers. However, the combined exposure to the wastewater during irrigation and consumption of the farm produce leads to an increased risk above the tolerable risk guideline by the WHO. It was observed that wastewater reuse for irrigation was on a small scale, mainly for household consumption, whereby the possibility of a combined risk of infection due to exposure to irrigation water and consumption of the farm produce is very high (especially for the farmers). The risks of ascariasis due to exposure (either intentionally or unintentionally) to the final effluents from these WWTPs vary greatly depending on the WWTP as well as point of exposure. This variation is largely dependent on the concentration of the *Ascaris* spp. eggs in the exposure medium, which is solely dependent on the STH egg reduction efficiency of the various treatment plants and the volume/weight of exposure medium ingested.

Conclusion

Soil-transmitted helminth and *Taenia* spp. prevalence and concentration were found to be consistent with other reports. Wastewater from low-income communities was found to be high in STH and *Taenia* spp. eggs; additionally, decentralized wastewater treatment plants located in schools and orphanages also reported high concentrations of these eggs. The link between poor communities and helminth infections needs to be studied further. The removal of STH and *Taenia* spp. eggs by the different WWTPs varied greatly depending on the type of treatment between WWTPs and also type of STH. It can be concluded that wastewater treatment achieves higher removal of *Ascaris* spp. eggs as compared to the other STH eggs reported in this study. The DEWATS plants were also found to give the highest removal efficiency of STH and *Taenia* spp. eggs as compared to the centralized WWTPs, with some of the DEWATS plants meeting the WHO guideline for wastewater reuse in irrigation. Direct or indirect exposure to effluents from these WWTPs (especially the centralized treatment plants) would therefore increase the risk of STH infections.

In conclusion, DEWATS plants in addition to their robust, cost-effective, and easy maintenance are also more efficient in removing STH eggs from wastewater, therefore making them a good option for domestic wastewater treatment, especially where effluent reuse is planned. These findings have important implications for public and environmental health protection and emerging approaches like the WHO sanitation safety planning (Hanjra et al. 2012; Winkler et al. 2017). The results obtained calls for continuous monitoring of wastewater treatment systems so as to ensure their efficiency.

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Appendix

Table 8 Probability distribution functions for concentrations of STH eggs in final effluents of centralized treatment plants

	Ascaris spp.	Hookworm	*Trichuris* spp.	*Taenia* spp.	*Toxocara* spp.
Plant A	Exponential ($\lambda = 0.45455$)	Exponential ($\lambda = 0.26316$)	Exponential ($\lambda = 0.625$)	Exponential ($\lambda = 0.11905$)	Exponential ($\lambda = 0.76923$)
Plant B	Exponential ($\lambda = 0.41667$)	Exponential ($\lambda = 0.4$)	Exponential ($\lambda = 0.83333$)	Exponential ($\lambda = 0.71429$)	Exponential ($\lambda = 0.76923$)
Plant C	Gen. Extreme Value ($k = 0.23432$; $\sigma = 2.2296$; $\mu = 1.8484$)	Exponential ($\lambda = 0.34615$)	Levy ($\sigma = 5.9093$)	Exponential ($\lambda = 0.3125$)	Uniform ($a = 2.9442$; $b = 8.9442$)

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References

Ahmed W, Sidhu JPS, Smith K, Beale DJ, Gyawali P, Toze S (2016) Distributions of fecal markers in wastewater from different climatic zones for human fecal pollution tracking in Australian surface waters. Appl Environ Microbiol 82:1316–1323

Amoah ID, Abubakari A, Stenström TA, Abaidoo RC, Seidu R (2016) Contribution of wastewater irrigation to soil transmitted helminths infection among vegetable farmers in Kumasi, Ghana. PLoS Negl Trop Dis 10(12):e0005161. https://doi.org/10.1371/journal.pntd.0005161

Amoah ID, Reddy P, Seidu R, Stenström TA (2018) Concentration of soil-transmitted helminth eggs in sludge from South Africa and Senegal: a probabilistic estimation of infection risks associated with agricultural application. J Environ Manag 206:1020–1027

Anderson RM, Turner HC, Truscott JE, Hollingsworth TD, Brooker SJ (2015) Should the goal for the treatment of soil transmitted helminth (STH) infections be changed from morbidity control in children to community-wide transmission elimination? PLoS Negl Trop Dis 9: e0003897

Appleton CC, Mosala TI, Levin J, Olsen A (2009) Geohelminth infection and reinfection after chemotherapy among slum-dwelling children in Durban, South Africa. South Africa. Ann Trop Med Parasitol 103: 249–261

Ayres RM (1991) On the removal of nematode eggs in waste stabilization ponds and consequent potential health risks from effluent reuse. PhD. Thesis, University of Leeds, UK

Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D, Hotez PJ (2006) Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet 367:1521–1532

Bogitsh BJ, Carter CE, Oeltmann TN (2012) General characteristics of the Nematoda (chapter 15), intestinal nematodes (chapter 16). Human Parasitology. Academic Press, UK, pp 269–345

Capizzi-Banas S, Maux M, Schwartzbrod J (2002) Surface hydrophobicity of Ascaris eggs and Giardia cysts. Helminthologia 39:197–204

Carr G, Potter RB, Nortcliff S (2011) Water reuse for irrigation in Jordan: perceptions of water quality among farmers. Agric Water Manag 98: 847–854. https://doi.org/10.1016/j.agwat.2010.1012.1011

Chan MS (1997) The global burden of intestinal nematode infections-fifty years on. Parasitol Today 13:438–443

Chaoua S, Boussaa S, Khadra A, Boumezzough A (2017) Efficiency of two sewage treatment systems (activated sludge and natural lagoons) for helminth egg removal in Morocco. J Infect Public Health (in press). doi: https://doi.org/10.1016/j.jiph.2017.07.026

Chen J, Xu MJ, Zhou DH, Song HQ, Wang CR, Zhu XQ (2012) Canine and feline parasitic zoonoses in China. Parasit Vectors 5:152–160

Contreras JD, Meza R, Siebe C, Rodríguez-Dozal S, López-Vidal YA, Castillo-Rojas G, Amieva RI, Solano-Gálvez SG, Mazari-Hiriart M, Silva-Magaña MA, Vázquez-Salvador N, Pérez IR, Romero LM, Cortez ES, Riojas-Rodríguez H, Eisenberg JNS (2017) Health risks from exposure to untreated wastewater used for irrigation in the Mezquital Valley, Mexico: a 25-year update. Water Res 123:834–850

David ED, Lindquist WD (1982) Determination of the specific gravity of certain helminth eggs using sucrose density gradient centrifugation. J Parasitol 68:916–919

Dorevitch S, Pathi S, Huang Y, Li H, Michalek AM, Pratap P, Wroblewski M, Liu L, Scheff PA, Li A (2011) Water ingestion during water recreation. Water Res 45:2020–2028

Dowd SE, Gerba CP, Pepper IL (1998) Confirmation of the human-pathogenic microsporidia *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis* and *Vittaforma corneae* in water. Appl Environ Microbiol 64:3332–3335

Duchin F (2016) A global case-study framework applied to water supply and sanitation. J Ind Ecol 20:387–395

Elmitwalli TA, Oahn KLT, Zeeman G, Lettinga G (2002) Treatment of domestic sewage in a two-step anaerobic filter/anaerobic hybrid system at low temperature. Water Res 36:2225–2232

Feachem RG, Bradley DJ, Garelick H, Mara DD (1983) Sanitation and disease: health aspects of excreta and wastewater management. John Wiley, Chicester

Foxon KM, Pillay S, Lalbahadur T, Rodda N, Holder F, Buckley CA (2004) The anaerobic baffled reactor (ABR): an appropriate technology for on-site sanitation. Water SA 30:44–50

Fuhrimann S, Winkler MS, Schneeberger PHH, Niwagaba CB, Buwule J, Babu M, Medlicott K, Utzinger J, Cissé G (2014) Health risk assessment along the wastewater and faecal sludge management and reuse chain of Kampala, Uganda: a visualization. Geospat Health 9: 251–255

Fuhrimann S, Winkler MS, Pham-Duc P, Do-Trung D, Schindler C, Utzinger J, Cissé G (2016) Intestinal parasite infections and associated risk factors in communities exposed to wastewater in urban and peri-urban transition zones in Hanoi, Vietnam. Parasit Vectors 9: 537. https://doi.org/10.1186/s13071-016-1809-6

Gumbo JR, Malaka EM, Odiyo JO, Nare L (2010) The health implications of wastewater reuse in vegetable irrigation: a case study from Malamulele, South Africa. Int J Environ Health Res 20:201–211

Gyawali P (2017) Infectious helminth ova in wastewater and sludge: a review on public health issues and current quantification practices. Water Sci Technol. https://doi.org/10.2166/wst.2017.619

Haas CN, Rose JB, Gerba CP (2014) Quantitative microbial risk assessment (second ed.). John Wiley and Sons, New York

Hailu T (2006) Assessment of intestinal parasites in the effluent slurry of toilet-linked biogas digesters. Addis Ababa University; 2006. M.Sc thesis

Hamilton AJ, Stagnitti F, Premier R, Boland A-M, Hale G (2006) Quantitative microbial risk assessment models for consumption of raw vegetables irrigated with reclaimed water. Appl Environ Microbiol 72:3284–3290

Hanjra MA, Blackwell J, Carr G, Zhang FH, Jackson TM (2012) Wastewater irrigation and environmental health: implications for water governance and public policy. Int J Hyg Environ Health 215:255–269

Hanjra MA, Drechsel P, Mateo-Sagasta J, Otoo M, Hernandez-Sancho F (2015) Assessing the finance and economics of resource recovery and reuse solutions across scales. In: Drechsel, P., Qadir, M., Wichelns, D. (Eds.), Wastewater: economic asset in an urbanizing world. Springer

Hussain I, Raschid L, Hanjra MA, Marikar F, van der Hoek W (2001) A framework for analyzing socioeconomic, health and environmental impacts of wastewater use in agriculture in developing countries, Working Paper 26, Colombo, Sri Lanka, International Water Management Institute

Hussain I, Raschid L, Hanjra MA, Marikar F, Van Der Hoek W (2002) Wastewater use in agriculture: review of impacts and methodological issues in valuing impacts. (with an extended list of bibliographical references). Working Paper 37, Colombo, Sri Lanka, International Water Management Institute

Istenic D, Bodík I, Bulc T (2014) Status of decentralised wastewater treatment systems and barriers for implementation of nature-based systems in central and eastern Europe. Environ Sci Pollut Res 22: 12879–12884

Jimenez B, Chavez A, Leyva A, Tchobanoglous G (2000) Sand and synthetic medium filtration of advanced primary treatment effluent from Mexico City. Water Res 34:473–480

Jimenez-Cisneros BE, Maya-Rendon C (2007) In: Méndez-Vilas A (ed) Helminths and sanitation. Communicating current research and educational topics and trends in applied microbiology. Formatex Research Center, Badajoz, pp 60–71

Johansen A, Nielsen HB, Hansen CM, Andreasen C, Carlsgart J, Hauggard-Nielsen H, Roepstorff A (2013) Survival of weed seeds and animal parasites as affected by anaerobic digestion at meso- and thermophilic conditions. Waste Manag 33:807–812

Kostopoulou D, Claerebout E, Arvanitis D, Ligda P, Voutzourakis N, Casaert S, Sotiraki S (2017) Abundance, zoonotic potential and risk factors of intestinal parasitism amongst dog and cat populations: the scenario of Crete, Greece. Parasit Vectors 10:43–55

Lo NC, Addiss DG, Hotez PJ, King CH, Stothard JR, Evans DS, Colley DG, Lin W, Coulibaly JT, Bustinduy AL, Raso G, Bendavid E, Bogoch II, Fenwick A, Savioli L, Molyneux D, Utzinger J, Andrews JR (2017) A call to strengthen the global strategy against schistosomiasis and soil-transmitted helminthiasis: the time is now. Lancet 17:64–69

Mara D, Horan N (2003) Handbook of water and wastewater microbiology. Academic Press, London

Mara DD, Sleigh PA, Blumenthal UJ, Carr RM (2007) Health risks in wastewater irrigation: comparing estimates from quantitative microbial risk analyses and epidemiological studies. J Water Health 5:39–50

Masi F, Rochereau J, Troesch S, Ruiz I, Soto M (2015) Wineries wastewater treatment by constructed wetlands: a review. Water Sci Technol 71:1113–1127

Massoud MA, Tarhini A, Nasr JA (2009) Decentralized approaches to wastewater treatment and management: applicability in developing countries. J Environ Manag 90:652–659

Maya C, Torner-Morales FJ, Lucario ES, Hernández E, Jiménez B (2012) Viability of six species of larval and non-larval helminth eggs for different conditions of temperature, pH and dryness. Water Res 46: 4770–4782

Mayer CL, Palmer CJ (1996) Evaluation of PCR, nested PCR and fluorescent antibodies for detection of giardia and cryptosporidium species in wastewater. Appl Environ Microbiol 62:2081–2085

Medema GJ, Schets FF, Tuenis PFM, Havelaar AH (1998) Sedimentation of free and attached cryptosporidium oocysts and giardia cysts in water. Appl Environ Microbiol 64:4460–4466

Mkhize-Kwitshana ZL, Mabaso MLH (2014) The neglected triple disease burden and interaction of helminths, HIV and tuberculosis: an opportunity for integrated action in South Africa. SAMJ 104:258–259

Molvik M, Helland E, Zulu SG, Kleppa E, Lillebo K, Gundersen SG, Kvalsvig JD, Taylor M, Kjetland EF, Vennervald BJ (2017) Co-infection with Schistosoma haematobium and soil-transmitted helminths in rural South Africa. S Afr J Sci 113, doi: https://doi.org/10.17159/ sajs.2017/20160251

Nasr FA, Doma HS, Nassar HF (2009) Treatment of domestic wastewater using an anaerobic baffled reactor followed by a duckweed pond for agricultural purposes. Environmentalist 29:270–279

Navarro I, Jimenez B (2011) Evaluation of the WHO helminth eggs criteria using a QMRA approach for the safe reuse of wastewater

and sludge in developing countries. Water Sci Technol 63:1499–1505

Navarro I, Jiménez B, Cifuentes E, Lucario S (2008) A quantitative microbial risk assessment of helminth ova in reusing sludge for agricultural production in developing countries. WIT Trans Inf Comput 39:65–74

Panicker PVRC, Krishnamoorthi KP (1978) Elimination of enteric parasites during sewage treatment processes. IAWPC Technol Annu 5: 130–138

Pereira A, Martins A, Brancal H, Vilhena H, Silva P, Pimenta P, Diz-Lopes D, Neves N, Coimbra M, Alves AC, Cardoso L, Maia C (2016) Parasitic zoonoses associated with dogs and cats: a survey of Portuguese pet owners' awareness and deworming practices. Parasit Vectors 9:245–254

Petterson SR, Stenström TA, Ottoson J (2016) A theoretical approach to using faecal indicator data to model norovirus concentration in surface water for QMRA: Glomma River, Norway. Water Res 91:31–37

Pike EB (1990) The removal of Cryptosporidium oocysts during sewage treatment. In: Cryptosporidium in water supplies. Dept. of Env and Dept. of Health Report of the Group of experts. Chair Sir John Badenoch. Part II Paper X, PP. 205–208. HMSO, London

Pullan RL, Smith JL, Jasrasaria R, Brooker SJ (2014) Global numbers of infection and disease burden of soil transmitted helminth infections in 2010. Parasit Vectors 7:37–56

Qadir M, Mateo-Sagasta J, Jiménez B, Siebe C, Siemens J, Hanjra MA (2015) Environmental risks and cost-effective risk management of wastewater. In: Drechsel P, Qadir M, Wichelns D (eds) Wastewater: economic asset in an urbanizing world. Springer, Dordrecht

Reynaud N, Buckley CA (2016) The anaerobic baffled reactor (ABR) treating communal wastewater under mesophilic conditions: a review. Water Sci Technol 73:463–478

Riahi K, Mammou AB, Thayer BB (2009) Date-palm fibers media filters as a potential technology for tertiary domestic wastewater treatment. J Hazard Mater 161:608–613

Rose JB, Dickson LJ, Farrah SR, Carnahan RP (1996) Removal of pathogenic and indicator microorganisms by a full-scale water reclamation facility. Water Res 30:2785–2797

Sakaji RH, Funamizu N (1998) Microbial risk assessment and its role in the development of wastewater reclamation policy. In: Asano T (ed) Wastewater reclamation and reuse, vol 10. CRC Press, Boca Raton, pp 705–756

Sant'Ana AS, Franco BDGM, Schaffner DW (2014) Risk of infection with Salmonella and Listeria monocytogenes due to consumption of ready-to-eat leafy vegetables in Brazil. Food Control 42:1–8

Seidu R, Heistad A, Amoah P, Drechsel P, Jenssen PD, Stenström TA (2008) Quantification of the health risk associated with wastewater reuse in Accra, Ghana: a contribution toward local guidelines. J Water Health 6:461–471

Sengupta ME, Thamsborg SM, Andersen TJ, Olsen A, Dalsgaard A (2011) Sedimentation of helminth eggs in water. Water Res 45: 4651–4660

Shuval HI, Adin A, Fattal B, Rawitz E, Yekutiel P (1986) Wastewater irrigation in developing countries: health effects and technical solutions. World Bank, Washington DC

Smith H, Rose JB (1998) Waterborne cryptosporidiosis: current status. Parasitol Today 14:14–22

von Sperling M, Chernicharo CAL, Soares AME, Zerbini AM (2003) Evaluation and modelling of helminth eggs removal in baffled and unbaffled ponds treating anaerobic effluent. Water Sci Technol 48: 113–120

Stolk WA, Kulik MC, le Rutte EA, Jacobson J, Richardus JH, de Vlas SJ (2016) Between-country inequalities in the neglected tropical disease burden in 1990 and 2010, with projections for 2020. PLoS Negl Trop Dis 10(5):e0004560. https://doi.org/10.1371/journal.pntd.0004560

Stott R, May E, Mara DD (2002) Parasite removal by natural wastewater treatment systems: performance of waste stabilization ponds and constructed wetlands. Proceedings of the 5th IWA Waste Stabilization Ponds, 2–5 April. Auckland, New Zealand

Thurston J, Gerba C, Foster K, Karpiscak M (2001) Fate of indicator microorganisms, Giardia and Cryptosporidium in subsurface flow constructed wetlands. Water Res 35:1547–1551

Tilley E, Ulrich L, Luthi C, Reymond P, Zurbrüg C (2014) Compendium of sanitation systems and technologies. Dübendorf: Swiss Federal Institute of Aquatic Sciences and Technology (EAWAG) 2nd Edition

UN-WATER-United Nations World Water Assessment Programme (2015) The United Nations World Water Development Report 2015: water for a sustainable world. Paris, UNESCO

Westrell T (2004) Microbial risk assessment and its implications for risk management in urban water systems. PhD Thesis, Department of Water and Environmental Studies, Linköpings University, Sweden

WHO- World Health Organization (2006) Guidelines for the safe use of wastewater, excreta and greywater, vol 4. World Health Organization, Geneva

WHO-World Health Organization (2015) Investing to overcome the global impact of neglected tropical diseases third who report on neglected tropical diseases. WHO Document Production Services, Geneva, Switzerland (2015) (WHO/HTM/NTD/2015.1)

Winkler M, Jackson D, Sutherland DP, Lim J, Srikantaiah V, Fuhrimann S, Medlicott K (2017) Sanitation safety planning as a tool for achieving safely managed sanitation systems and safe use of wastewater. WHO S East Asia J Public Health 6:34–40

Yen-Phi VT, Rechenburg A, Vinneras B, Clemens J, Kistemann T (2010) Pathogens in septage in Vietnam. Sci Total Environ 408:2050–2053

Paper IV

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Research article

Concentration of soil-transmitted helminth eggs in sludge from South Africa and Senegal: A probabilistic estimation of infection risks associated with agricultural application

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ABSTRACT

The use of sludge in agriculture has been encouraged as a means of increasing soil nutrient content and improving the water holding capacity. On the negative side, major public health concerns with sludge application prevail, mainly due to the high concentration of pathogenic microorganisms. Soil-transmitted helminths (STHs) are of major health concern in this regard, especially in endemic regions, mainly due to the high environmental resistant of the eggs combined with a low infectious dose. In this study the concentration of STH eggs in two months dried sludge from Durban, South Africa and Dakar, Senegal was determined and compared. Sampling was carried out from January to October 2016 and in September 2016 for Dakar. *Ascaris* spp, hookworm, *Trichuris* spp, *Taenia* spp and *Toxocara* spp were the commonly recorded STH eggs. STH egg concentrations were higher in Dakar than in Durban, with viable STH egg concentrations exceeding both local and international guidelines. Due to the high concentration of viable STH eggs, risks of *Ascaris* spp infection was very high for farmers applying this sludge on their farms in both Durban (7.9×10^{-1} ($\pm 1.7 \times 10^{-2}$)) and Dakar (9.9×10^{-1} ($\pm 1.3 \times 10^{-5}$)). Consumption of lettuce grown on sludge amended soil will result in probable infections but harvest after 30 days between sludge application and harvest in Durban gave median probability infection risks with a risk level similar to the WHO tolerable risk value (10^{-4}). This time period need to be prolonged to harvest in Dakar to 40 days to reduce the risks of infection to the tolerable risks values. Further treatment of the sludge either through composting or drying for longer periods of time is thus recommended from a public health perspective.

1. Introduction

Application of faecal sludge on agricultural lands is a major component of resource recovery, aimed to ensure that vital plant nutrients are returned to the soil. Sludge is high in organic matter and nutrients (Hernández et al., 2017), making it a valuable amendment in restoring degraded, exhausted (Jiménez and Álvarez, 2005) and burned soils (Guerrero et al., 2007), as well as improving fertility and crop productivity (Bittencourt et al., 2013). It may also increase the water holding capacity of soil (Navarro-Pedreño et al., 2003; Almendro-Candel et al., 2014).

Despite the recognition of faecal sludge application globally, there are public health concerns regarding its quality and impact on human health. The physico-chemical and biological processes involved in wastewater and sludge treatment will concentrate several harmful substances, such as metals/metalloids (Castells, 2012) and most importantly pathogenic bacteria (Krzyzanowski et al., 2016; Pepper et al., 2008; Jiménez et al., 2007) and helminths (Gerba and Smith, 2005; Navarro et al., 2009; Murcia Navarro, 2013).

The most important microbial health risk when applying sludge to agricultural soils are the soil-transmitted helminths (STHs) (WHO, 2006). The presence of these parasite eggs is considered as an indication of the health hazard of sludge after application, due to the very high resistance of the eggs (Zdybel et al., 2015: Gaspard

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et al., 1995) to adverse environment conditions (Melvin et al., 2001; Nelson and Darby, 2001), resulting in long-term environmental contamination (Johnson et al., 1998). In addition, the occurrence of these eggs correspond to a very low infectious dose (Toze, 2006; Crompton and Nesheim, 2002; Stephenson et al., 2000). Poor sanitation and faecal contamination result in an enhanced transmission and a high prevalence in developing countries, where faecal sludge is a main contributing factor (67–735 eggs/g total solids (TS)) (Feachem et al., 1983; Strauss et al., 2003; Jiménez and Wang, 2006) as compared to more developed countries (2–13 eggs/g TS) (Jiménez et al., 2002).

To protect humans from the health impact of STH infections as a result of sludge application in agriculture, many countries have set up national regulations to treat and reuse sludge (Navarro et al., 2009). These regulations are adopted from the limits for STH eggs stated by the United States Environmental Protection Agency (USEPA) (1993) and expressed as a guideline value by World Health Organization (WHO) (2006), namely 0.25 and 1 helminth egg/g TS respectively. Countries such as Brazil, Chile, New Zealand and South Africa adopted the regulations based on the USEPA limits (Jiménez et al., 2002) for Class A sludge that is intended for use in unrestricted agriculture, but monitoring is very rarely done. To meet these international and national guidelines, sludge undergoes further treatments, such as air-drying, composting or long-term storage (O'Connor et al., 2017). However long-term storage (>3 years) results in loss of key plant nutrients (Grant et al., 2012). Reduction in viable STH egg concentrations during air-drying is dependent on ambient temperature and duration of drying in addition to several environmental factors, such as irradiation, rainfall etc. (Grant et al., 2012).

The regulations proposed by the USEPA and the WHO were based on sparse epidemiological data rather than risk assessment estimates (Navarro et al., 2009). The use of the quantitative microbial risk assessment (QMRA) approach to establish the health risks involved in the application of sludge in agriculture was done after the development of these guidelines and have been applied in Australia (NWQMS, 2006). QMRA is a probabilistic approach to predict human health risks from exposure to pathogenic microorganisms and it has been used extensively in assessing the transmission of water (Smeets et al., 2010; McBride et al., 2013; Bichai, and Smeets, 2013) and food borne (Janevska et al., 2010; Romero-Barrios et al., 2013; Membré and Boué, 2017) infections. QMRA has also been used in estimating STH infection risks from the reuse of wastewater and sludge in agriculture (Jiménez et al., 2002; Navarro and Jiménez, 2011; Navarro et al., 2008; Kundu et al., 2014; Seidu et al., 2008a, 2008b).

This study contributes to the knowledge on STH egg concentration in sludge from different populations by comparing the total and viable STH egg concentrations in sludge from two large scale wastewater treatment plants in Durban, South Africa and one plant in Dakar, Senegal. The suitability of sludge, after approximately 60 days of drying (without mixing) under ambient environmental conditions, for agricultural application was determined using the QMRA approach. This study therefore contributes to the evidence necessary for the revision of sludge application guidelines depending on the type of treatment.

2. Methodology

2.1. Study area and sampling

Sludge samples were taken from two centralized wastewater treatment plants (WWTPs) in Durban, South Africa. One has an operational capacity of 10.98 Ml/d (WWTP A) and the second smaller one with a capacity of 4.69 Ml/d (WWTP B). Each of these plants have sludge drying beds were sludge is dried for a duration of 60–90 days (depending on demand and availability of drying beds) before disposal. Sludge sampling in Senegal was from a WWTP treating wastewater in the city of Dakar, which has an operational capacity of about 13 Ml/d. Composite samples were taken in triplicates from the drying beds from January to October 2016 for the two WWTPs in Durban and in September 2016 for the plant in Dakar.

The decay of viable STH eggs during drying over a 90 day period was also determined by sampling from an experimental bed at WWTP A, starting from the first day of drying to 90 days. Composite samples were taken in triplicates from different depths of the same sludge bed, by dividing the depth into three, top, middle and bottom layers.

2.2. Laboratory analysis

Sludge analysis for STH eggs was performed using a revised method based on the principles of flotation and sedimentation, as compared in Amoah et al. (2017). Briefly, samples were homogenized and approximately 20 g sludge portions measured into 250 mL to which 50 mL of ammonium bicarbonate (119 g/L) (Sigma Aldrich, Germany) was added and allowed to soak for 10 min. The samples were then blended at top speed, with a laboratory blender (Isolab Laborgeräte GmbH) and poured through a 100 μm sieve onto a 20 μm sieve. The contents on the 100 μm sieve were washed carefully with tap water under pressure, to separate all STH eggs from particulate matter and enable the eggs pass through the pores. The contents collected on the 20 μm sieve were then carefully washed and collected into a 50 mL centrifuge tube and centrifuged for 10 min at 3000 rpm. The supernatants were discarded and $ZnSO_4$ (Promark Chemicals, South Africa) solution of specific gravity 1.30 added to the pellets. The pellets were then re-suspended in the $ZnSO_4$ by vortexing and centrifuged again at 2000 rpm for another 10 min. The supernatants was poured through the 20 μm sieve and washed carefully with tap water, to remove all residual $ZnSO_4$, and the contents collected into 50 mL centrifuge tubes and centrifuged at 3000 rpm for 10 min. The supernatants were finally discarded and pellets viewed under ×10 magnification (Leica DM2000) to determine the total STH eggs per gram of the sludge analyzed. STH eggs on the positive slides were carefully washed into a petri dish for incubation at room temperature with 0.1N sulphuric acid (Promark Chemicals, South Africa) as an incubation solution for 28 days, after which the viable STH eggs were counted by viewing under ×40 magnification. STH eggs with a visible motile larva were considered as viable.

2.3. Statistical analysis

Descriptive analysis of the STH egg concentrations and distribution was performed using Excel (Microsoft Corporation). Difference in concentration of the various types of STH eggs was determined using the Kruskal-Wallis tests, with the Mann-Whitney *U* test used to compare the difference in concentrations between the total and viable STH eggs as well as difference in egg concentrations between the sampling sites. All statistical analysis was performed in Graphpad Prism 7 software (GraphPad Software, Inc. USA).

2.4. Assessment of STH infection risks

Risk of infection with STH was determined using the QMRA approach, which involves four interrelated steps: a) hazard identification; b) exposure assessment; c) dose-response assessment and d) risk characterization (Haas et al., 2014).

2.4.1. Hazard identification

For the purpose of this assessment *Ascaris* spp was chosen as an index for STHs. The link between ascariasis and wastewater/sludge reuse in agriculture has been established by several reports (Ensink et al., 2008; Fuhrimann et al., 2014, 2016; Amoah et al., 2016; Contreras et al., 2017), with risks successfully estimated using the QMRA approach (Seidu et al., 2008a, 2008b; Navarro et al., 2009). Additionally *Ascaris* spp is the only STH with a dose-response model.

2.4.2. Exposure assessment

This step involves the determination of number of *Ascaris* spp eggs that will be ingested per single exposure. Accidental ingestion of the sludge during the application was considered the first point of exposure. Westrell et al. (2004) used an ingestion weight of 2 g, but in this study we assumed that the weight of sludge ingested will be uniformly distributed from 1-2 g. Using lettuce as a surrogate for vegetables grown on sludge amended soils, it was further assumed that the sludge will be spread before each growing season which translates to 7–12 times per year, considering that most lettuce varieties take 30–50 days to mature (CTAHR, 2000).

Ingestion of soil amended with sludge was also considered, where it was expected that there will be a dilution of the sludge in the soil in addition to decay of the *Ascaris* spp eggs over time. Therefore the concentration of STH eggs in the sludge amended soil on the day of application was calculated using the formula;

$$N_o = C_{sludge} \times Df \quad (1)$$

where "N_o" is the concentration of STH per gram of soil after spreading on the day of application, "C_{sludge}" the concentration of STH eggs in the sludge, as determined in this study and "*Df*" the dilution factor, which was taken to be 0.001, assuming a sludge to soil ratio of 1:100 (Schönning et al., 2007). Taking into consideration the decay of the *Ascaris* spp eggs over time, the concentration ingested days after application was determined using the formula:

$$N_t = No{*}e^{-kt} \quad (2)$$

where N_t is the concentration of viable *Ascaris* spp eggs at time *t* (measured in days), N_o is the concentration of viable eggs on the day of sludge application as determined in equation 1 and *k* is the decay constant of *Ascaris* spp eggs, which was calculated to be 0.0056 based on results obtained during the egg decay analysis (See Table A1 in Appendix for Data).

It was assumed that farmers will ingest 0.03–0.1 g of soil per day (Schönning et al., 2007). Additionally, frequency of ingestion was assumed to be uniformly distributed from 64 to 128 days in a year to account for the labour-intensive nature of agriculture in developing countries.

We also hypothesized that consumption of the lettuce may result in *Ascaris* spp infections. Westrell et al. (2004) used an assumption of 1 g of sludge per each serving of raw vegetables grown on sludge amended soils but in this study we assumed that the amount of sludge ingested through this route would be uniformly distributed from 0.5 g to 1 g per serving with an annual frequency of 156–160 per year. This risk was however calculated for 30–50 days after application of the sludge, to account for time needed for lettuce to mature. Table 1 below presents the various assumptions made in the estimation of risk based on the exposure scenarios.

2.4.3. Dose-response assessment

The exponential dose-response model as proposed by Navarro et al. (2008) was used. This model has been used in estimation of *Ascaris* spp infection risks by other researchers (Westrell et al., 2004; Seidu et al., 2008a). Therefore the risk of *Ascaris* spp infection was assessed using the formula

$$P_{inf} = 1 - e^{-rd} \quad (3)$$

where "P_{inf}" is the infection risk per exposure event, "*d*" the number of *Ascaris* spp eggs ingested per that event and "*r*" the dimensionless infectivity constant. An "*r*" value of 0.039 as reported by Navarro et al. (2008) was used in this study.

2.4.4. Risk characterization

All the outcomes of the hazard identification, exposure assessment and dose response assessment were combined to determine the severity of *Ascaris* spp infection from the different exposure scenarios considered. Risk of infection due to multiple exposures or annual risk (P_A) was determined using the formula:

$$P_A = 1 - \left(1 - P_{inf}\right)^n \quad (4)$$

P_{inf} is the risk of infection from a single exposure event and *n* being the frequency of exposure per year (Sakaji and Funamizu, 1998). All models used for the risk of infection determination was constructed in Microsoft Excel using @Risk 7.5 (Palisade Corporation) software add-on to Excel and subjected to Monte-Carlo simulations of 10, 000 iterations.

Table 1
Points of exposure with assumptions based on weights of sludge ingested and frequency of exposure.

Exposure scenario	Weight of sludge ingested (g) per day	Frequency	Reference
Spreading on sludge	Uniform distribution (1,2)	Uniform distribution (7,12)[a]	Westrell et al., 2004 (Maximum weight of 2 g per event)
Ingestion of sludge amended soil	Uniform distribution (0.03,0.1)	Uniform distribution (64,128)[a]	Schönning et al., 2007
Ingestion of sludge through raw vegetables	Uniform distribution (0.5,1)	Uniform distribution (156,160)[a]	Westrell et al., 2004 (Maximum weight of 1g per day)

[a] Assumptions made in this study.

Table 2
Mean (±SD) concentration of STH eggs per gram of sludge from Durban, South Africa.

	Mean (±SD)total STH eggs/g		Mean (±SD)viable STH eggs/g	
	Durban	Senegal	Durban	Senegal
Ascaris spp	722 (±534)	1079(±114)	369(±260)	769(±107)
Hookworm	334(±246)	257(±72)	146(±154)	186(±172)
Trichuris spp	154(±148)	1647(±270)	49(±49)	84(±17)
Taenia spp	54(±62)	N/A	20(±27)	N/A
Toxocara spp	43(±57)	N/A	22(±32)	N/A

3. Results

3.1. Concentration of STH eggs in sludge from Durban, South Africa

Ascaris spp, hookworm, *Trichuris* spp, *Taenia* spp and *Toxocara* spp were detected in sludge from the two wastewater treatment plants (WWTPs) in Durban. The difference in egg concentrations between these two WWTPs was not statistically significant, therefore these were combined and their means used to represent STH egg concentrations in sludge from Durban. *Ascaris* spp was the most abundant (722 (±534)/g), with *Toxocara* spp being the least (43(±57)/g) (Table 2). The difference between the total and viable STH egg concentrations was statistically significant for all the types of eggs detected except for *Taenia* spp and *Toxocara* spp. Table 2 presents the viable STH egg concentrations. Viable egg concentrations varied significantly over the duration of the study. As shown in Fig. 1, *Ascaris* spp and hookworm eggs showed the largest variation, with high concentrations for these STHs (*Ascaris* spp and hookworm) in January with decrease in concentrations from February to March. There was an increase again in *Ascaris* spp and hookworm egg concentrations from July to September in the case of *Ascaris* spp and August in the case of hookworm.

Ascaris spp, *Trichuris* spp and hookworm were the only STH egg detected in the sludge samples from Dakar, with *Trichuris* spp eggs the most abundant (1647(±270) eggs/g), the variation in egg concentration of these STHs was statistically significant (p value $\leq$ 0.05). Viable STH eggs were significantly lower (p value $\leq$ 0.05) than the total STH egg counts as expected. Table 2 presents the mean concentrations and variation of the STH eggs recorded.

STH egg concentrations in sludge from Dakar were significantly higher (p value $\leq$ 0.05) as compared to concentrations in Durban for STHs for *Ascaris* spp and *Trichuris* spp. However, the concentration of hookworm was higher in Durban (334(±246)/g) than in Dakar (257 (±72)/g).

3.2. Reduction viable STH egg concentration over a 90 day drying period

It was observed that mean concentrations of viable STH eggs reduced over the 60 day drying period (maximum days of sludge drying at the WWTPs), with mean viable STH egg concentrations of 826(±93) eggs/g in the fresh sludge. After 60 days of drying, concentration of viable STH eggs was 625(±30) eggs/g, however extension of the drying by approximately a month resulted in further reduction in viable STH eggs. After 90 days of drying, 406(±15) eggs/g were viable. A linear decay model was fitted to this data to derive a decay rate of 0.0056 per day. Table A1 in Appendix presents the results of the decay assessment.

3.3. Risk of Ascaris spp infection for farmers during the spread of sludge

Application of the sludge (after 60 days of drying) resulted in varying degrees of *Ascaris* spp infection risks, with a higher mean

Table 3
Mean risk (±90% CI) if *Ascaris* spp infection for farmers spreading sludge on their farms.

	Probability of infection from one time exposure	Annual probability of infection
Durban	7.9×10^{-1} ($\pm 1.7 \times 10^{-2}$)	1.0 ($\pm 1.1 \times 10^{4}$)
Dakar	9.9×10^{-1} ($\pm 1.3 \times 10^{-5}$)	1.0 ($\pm 3.5 \times 10^{-10}$)

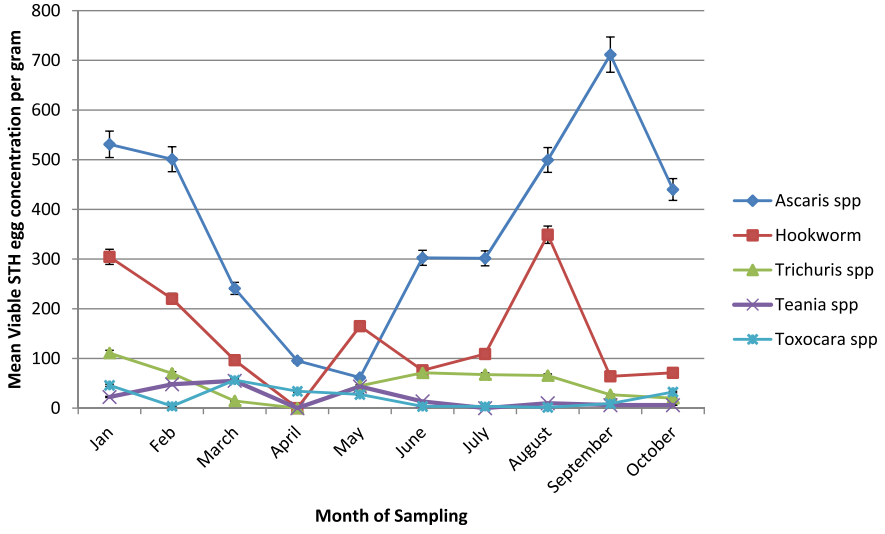

Fig. 1. Variation in viable STH egg concentrations during the study in Durban, South Africa.

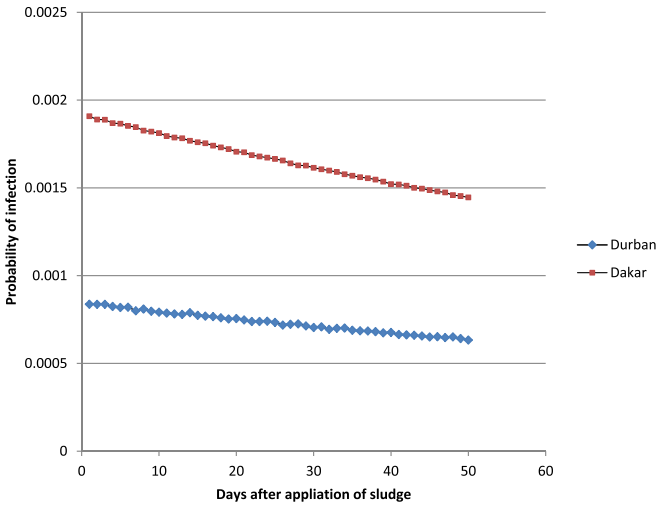

Fig. 2. Median probability of infection with *Ascaris* spp from ingestion of sludge amended soil on different days after sludge application.

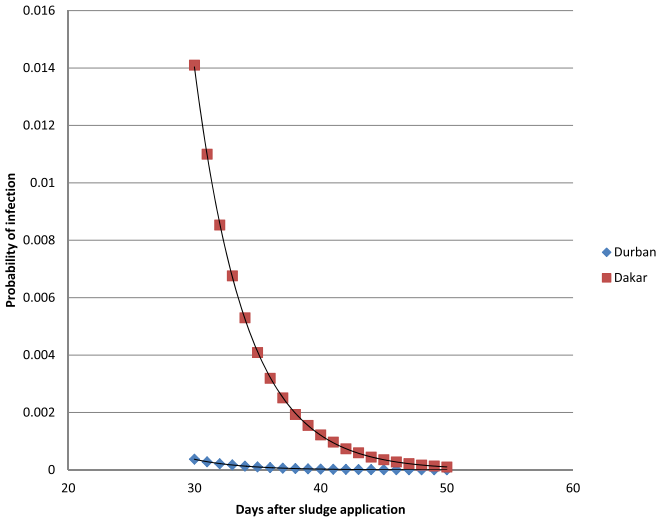

Fig. 3. Median probability of infection with *Ascaris* spp from consumption of lettuce contaminated with sludge on different days after sludge application.

risk for farmers in Dakar (9.9×10^{-1} ($\pm 1.3 \times 10^{-5}$)) compared to farmers in Durban due to a lower concentration of *Ascaris* spp eggs in the sludge (Table 3). Multiple exposures to the sludge during the application resulted in a much higher risk of *Ascaris* spp infection annually, as presented in Table 3, irrespective of the study site. Variation in the viable *Ascaris* spp egg concentrations over the duration of the study as shown in Fig. 1 did not significantly affect the estimated risks of infections, hence the mean risk was reported.

3.4. Risk of Ascaris spp infection farmers associated with ingestion of sludge amended soil

Ingestion of sludge amended soil will result in varying risks of infection depending on how many days after sludge application the exposure occurred. One day after sludge application leads to a median risks of 8.36×10^{-4}($\pm 1.13 \times 10^{-5}$) for farmers in Durban and 1.91×10^{-3} ($\pm 1.09 \times 10^{-5}$) in Dakar. Accounting for decay of the eggs the risks of infection reduced with time. Approximately one month (30 days) after application, risks were 7.14×10^{-4} ($\pm 9.61 \times 10^{-6}$) in Durban and 1.62×10^{-3} ($\pm 9.27 \times 10^{-6}$) in Dakar. Fig. 2 shows the median probability of infection due to ingestion of sludge amended soil depending on the day of exposure, with a maximum duration of 50 days after application.

3.5. Risk of Ascaris spp infection for consumers of lettuce grown on sludge amended soil

Harvest of lettuce after 30 days of sludge application resulted in risks estimates of 2.7×10^{-4} ($\pm 1.24 \times 10^{-4}$) for consumers in Durban and 4.5×10^{-3} ($\pm 1.9 \times 10^{-4}$) for consumers in Dakar and by the 50th day the risks are expected to be reduce further to 1.21×10^{-6} ($\pm 1.18 \times 10^{-4}$) in Durban and 6.24×10^{-5} ($\pm 1.91 \times 10^{-4}$) for consumers in Dakar. Fig. 3 shows the probability of infection depending on the day of harvest, indicating that risks of ascariasis in Durban are very low as compared to Dakar.

4. Discussion

Ascaris spp, hookworm, *Trichuris* spp, *Taenia* spp and *Toxocara* spp were the common soil-transmitted helminth eggs in the sludge, except for Dakar, where *Taenia* spp and *Toxocara* spp were not detected. The difference in prevalence and concentration of these STH eggs is reflective of the prevalence of infections within the respective populations. STH eggs detected in sludge from Durban correspond to STH infections within this study area (Mkhize-Kwitshana and Mabaso, 2014; Molvik et al., 2017; Appleton et al., 2009). STH egg concentration in fresh faeces collected from urine-diverting latrines within the same province (KwaZulu Natal) contained from 1–1425 eggs of STH per gram (Trönnberg et al. (2010) which represent both a high concentration and a high variability of STH eggs in faecal matter from infected individuals. In Senegal, the most common STH infections are *Ascaris* spp, hookworm and *Trichuris* spp (Tine et al., 2013), similar to the STHs detected in this study. Globally, *Ascaris* spp, hookworm and *Trichuris* spp are the most common STH infections, accounting for approximately 1.5 billion infections mainly in sub-Saharan Africa, the Americas, China and East Asia (WHO, 2017). STH egg concentration in sludge from main wastewater treatment plants is mainly dependent on their concentration in raw wastewater, which in turn is dependent on infection prevalence in the population. STH eggs are particles which forms part of the total suspended solid component of wastewater and therefore the reduction and sedimentation of suspended solids in wastewater treatment processes result in their (STH eggs) removal as well (Jimenez-Cisneros, 2006). This results in an accumulation of STH eggs in the sludge. The concentration of STH eggs found in this study is similar to concentrations in Mexico (Pecson et al., 2007) and Ghana (Koné et al., 2007). In other studies lower concentrations have been found, e.g in France (Gantzer et al., 2001), Brazil (Bastos et al., 2013) and Morocco (Moubarrad and Assobhei, 2007).

With total viable STH eggs ranging from 25-185 per gram, the sludge from both study areas exceeded the USEPA standards and WHO guideline values or the South African regulations (which are an adaptation of the USEPA regulations). These high viable STH egg concentrations after approximately 2 months of drying could be attributed to the conditions under which the sludge was dried. The sludge was air dried without mixing for periods ranging from 60 to 90 days depending on demand from farmers and other users.

The ambient temperatures of 24 °C in Durban and 28 °C in Dakar (www.climate-data.org) and the duration of drying is thus inadequate to inactivate a greater proportion of the eggs. Composting at

ambient temperatures of 20–30 °C for 1–4 months has been shown to have no effect on *Ascaris* spp egg viability, however elevated temperatures of 40–50 °C decreases egg viability within hours to two weeks (Berendes et al., 2015; McKinley et al., 2012; Szabova et al., 2010).

Viscous heating at temperatures over 70 °C and 80 °C can achieve complete inactivation of *Ascaris* spp eggs within 15 and 5 s respectively (Belcher et al., 2015; Naidoo et al., 2017), additionally, UV irradiation of about 200 to 2000 J/m^2 (using a UV lamp) results in the activation of between 0 and 1.5 log of *Ascaris suum* eggs (Brownell and Nelson, 2006).

With the assumption that sludge will be collected for land application after 60 days of drying in both study sites, the risks of ascariasis was found to be very high for farmers involved in the practice and above the WHO tolerable risks value (10^{-4}) for sludge application in agriculture (WHO, 2006). The mean risks of infection for farmers in Durban (10^{-4}) during the application stage was found to be similar to risks estimates from Ghana, however the estimates from Dakar were one magnitude higher (10^{-3}) (Seidu et al., 2008b). Due to the labour-intensive nature of farming in many African countries including Senegal and parts of South Africa, ingestion of soil is a likely route of further risks but accounting for the decay of *Ascaris* spp eggs over time, the risks of infection will reduce.

Despite these anticipated reductions in viable *Ascaris* spp eggs, the annual risks of infections still exceed the WHO tolerable risks value in both study areas. Based on the decay of the viable eggs determined in this study further treatment is needed to reduce the risks of infections. The decay rates determined might be due to the ability of these eggs to survive for longer periods under favourable conditions (WHO, 2015). Additionally multiple application of sludge on the same piece of land may result in egg accumulation in soil (Seidu et al., 2008a), which may increase the doses ingested by the farmers. All these factors may result in higher risks of ascariasis than were estimated here.

Comparatively consumers of vegetables grown on sludge amended soils in Durban are less likely to get infected with ascariasis after 30 days of sludge application, compared to Senegal. In Senegal harvest of lettuce after 30 days of sludge application will however still result in risks of infections. To protect consumers in Senegal from *Ascaris* spp infections, it is suggested that harvesting be done at least 40 days after the sludge application and most preferably after further treatment. It must be noted that these risks estimates are based on the assumption of decay of the *Ascaris* spp eggs, however these eggs are fairly resistant and survive for longer periods of time. In addition, contamination of lettuce with soil might also result in higher contamination levels than have been estimated due to egg accumulation in soil.

To reduce the estimated risks of ascariasis there is the need for further treatment of the sludge to protect public health, composting using a pH elevation 12 (Gantzer et al., 2001), as well as thermophilic composting in a vessel (aerobic/anaerobic) (Haug, 1993; Eller et al., 1996), may all result in 3 log reductions in helminth egg concentration within 1–5 days. Low cost options such as composting in pH > 9 may also result in 3 log reductions but after a storage duration of 6 months (Chien, 2001). Windrow thermophilic composting for 3 months may also achieve 1.5–2 log reduction (Koné and Struass, 2004). Additionally, viscous heating may result in over 90% to complete inactivation of STH eggs providing an additional inactivation barrier before composting. These additional treatment barriers may reduce the viable STH egg concentrations to levels that will result in probability of infection within the WHO guideline value (10^{-4}) translating into 10^{-6} Disability Adjusted Life Years, rendering the sludge safe for land application.

5. Conclusion

Soil-transmitted helminth egg concentration in sludge from both Durban and Dakar were found to be high, which is consistent with prevalence of infection within the study areas. These concentrations are also similar to results from other geographical locations with similar socio-economic status as the study areas. Although viable STH egg concentrations were significantly lower than the total STH egg concentrations, these were above the WHO guideline value as the USEPA limit for sludge intended for unrestricted agricultural application. Due to this high concentration of viable STH eggs, risks of *Ascaris* spp infection for farmers spreading the sludge was found to be higher than the WHO tolerable risk value. Additionally consumption of lettuce grown on sludge amended soil was also found to result in higher risks of infection especially in Dakar, however delay of harvest till 40 days after sludge application reduces the risks below the WHO tolerable risk value. Therefore sludge from both study sites needs further treatment, such as composting under elevated pH or heat treatment, to reduce the concentration of viable STH eggs and therefore protect public health.

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Appendix I

Table A1
Reduction of viable STH egg count over a 91 day drying period at different depths on sludge.

	Day1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Day 63	Day 70	Day 77	Day 84	Day 91
Top	598.16	570.12	569.23	468.59	412.23	303.01	308.33	309.34	302.15	300.18	299.48	298.2	301.24	289.45
Middle	658.46	639.78	640.23	590.12	610.12	598.79	568.79	565.78	490.12	482.45	440.59	438.79	419.12	420.12
Bottom	620.45	623.12	602.89	597.45	548.97	578.98	580.26	576.45	560.45	561.04	559.78	520.15	512.23	408.15
AVG	625.69	611.01	604.12	552.05	523.77	493.59	485.79	483.86	450.91	447.89	433.28	419.05	410.86	372.57
SD	30.49	36.38	35.52	72.37	101.32	165.35	153.79	151.23	133.54	133.82	130.30	112.28	105.74	72.24

References

Almendro-Candel, M.B., Navarro-Pedreño, J., Jordán, Vidal M.M., Gómez, I., Meléndez-Pastor, I., 2014. Use of municipal waste compost to reclaim limestone quarries mine spoils as soil amendments: effects on cd and Ni. J. Geochem. Explor. 144, 263–366.

Amoah, I.D., Singh, G., Stenström, T.A., Reddy, P., 2017. Detection and quantification of soil-transmitted helminths in environmental samples: a review of current state-of-the-art and future perspectives. Acta Trop. 169, 187–201.

Amoah, I.D., Abubakari, A., Stenström, T.A., Abaidoo, R.C., Seidu, R., 2016. Contribution of wastewater irrigation to soil transmitted helminths infection among vegetable farmers in Kumasi, Ghana. PLoS Neglected Trop. Dis. 10, 1–12.

Appleton, C.C., Mosala, T.I., Levin, J., Olsen, A., 2009. Geohelminth infection and reinfection after chemotherapy among slum-dwelling children in Durban, South Africa. South Africa. Ann. Trop. Med. Parasitol. 103, 249–261.

Bastos, V.K., Cutolo, S.A., Doria, M.C.O., Razzolini, M.T.P., 2013. Detection and quantification of viable Ascaris sp. and other helminth eggs in sewage sludge. Int. J. Environ. Health Res. 23, 352–362.

Belcher, D., Foutch, G.L., Smay, J., Archer, C., Buckley, C.A., 2015. Viscous heating effect on deactivation of helminth eggs in ventilated improved pit sludge. Water Sci. Technol. 72, 1119–1126.

Berendes, D., Levy, K., Knee, J., Handzel, T., Hill, V.R., 2015. Ascaris and Escherichia coli Inactivation in an Ecological Sanitation System in Port-au-Prince, Haiti. PLoS ONE 10, 1–14.

Bichai, F., Smeets, P.W.M.H., 2013. Using QMRA-based regulation as a water quality management tool in the water security challenge: experience from The Netherlands and Australia. Water Res. 47, 7315–7326.

Bittencourt, S., Serrat, B.M., Andreoli, C.V., Moura, E.N., Togny, F.L., Silva, L.A.T.P., 2013. Sewage sludge subjected to mixed: effect on total solids, pH and viable helminth eggs. Rev. Acad. Ciências Agrárias eAmbientais PUCPR Impresso 11, S191–S200. In Portuguese.

Brownell, S.A., Nelson, K.L., 2006. Inactivation of single-celled Ascaris suum eggs by low-pressure UV radiation. Appl. Environ. Microbiol. 72, 2178–2184.

Castells, X.E., 2012. Vías de tratamiento y valorización de fangos dedepuradora: Reciclaje de residuos industriales. Ed. Díaz de Santos, Madrid class B biosoloids J. Environ. Qual. 3437, S58–S67.

Chien, B.T., 2001. Biological study on retention time of microorganisms in faecal material in urine-diverting eco-san latrines in Vietnam. In: Proceedings of the 1st International Conference on Ecological Sanitation, Nanning, China, 5-8 November, pp. 120–124.

Contreras, J.D., Meza, R., Siebe, C., Rodríguez-Dozal, S., López-Vidal, Y.A., Castillo-Rojas, G., Amieva, R.I., Solano-Gálvez, S.G., Mazari-Hiriart, M., Silva-Magaña, M.A., Vázquez-Salvador, N., Pérez, I.R., Romero, L.M., Cortez, E.S., Riojas-Rodríguez, H., Eisenberg, J.N.S., 2017. Health risks from exposure to untreated wastewater used for irrigation in the Mezquital Valley, Mexico: a 25-year update. Water Res. 123, 834–850.

Crompton, D.W.T., Nesheim, M.C., 2002. Nutritional impact of intestinal helminthiasis during the human life cycle. Annu. Rev. Nutr. 22, 35–59.

CTAHR, 2000. Lettuce for the Home Garden. Cooperative Extension Service, College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa. . (Accessed 23 August 2017).

Eller, G., Norin, E., Stenström, T.A., 1996. Aerobic thermophilic treatment of black-water mixed with organic waste and liquid manure. Persistence of selected pathogens and indicator organisms. Environ. Res. Forum 5–6, 355–358.

Ensink, J.H.J., Blumenthal, U.J., Brooker, S., 2008. Wastewater quality and the risk of intestinal nematode infection in sewage farming families in Hyderabad, India. Am. J. Trop. Med. Hyg. 79, 561–567.

Feachem, R.G., Bradley, D.J., Garelick, H., Mara, D.D., 1983. World Bank studies in water supply and sanitation. Sanitation and Disease: Health Aspects of Excreta and Wastewater Management, vol. 3. John Wiley & Sons, New York, NY, USA.

Fuhrimann, S., Winkler, M.S., Pham-Duc, P., Do-Trung, D., Schindler, C., Utzinger, J., Cissé, G., 2016. Intestinal parasite infections and associated risk factors in communities exposed to wastewater in urban and peri-urban transition zones in Hanoi, Vietnam. Parasites Vectors 9, 537–551.

Fuhrimann, S., Winkler, M.S., Schneeberger, P.H.H., Niwagaba, C.B., Buwule, J., Babu, M., Medlicott, K., Utzinger, J., Cissé, G., 2014. Health risk assessment along the wastewater and faecal sludge management and reuse chain of Kampala, Uganda: a visualization. Geospat. Health 9, 251–255.

Gantzer, C., Gaspard, P., Galvez, L., Huyard, A., Dumouthier, N., Schwartzbrod, J., 2001. Monitoring of bacterial and parasitological contamination during various treatment of sludge. Water Res. 35, 3763–3770.

Gaspard, P.G., Wiart, J., Shwartzbrod, J., 1995. Urban sludge reuse in agriculture: waste treatment and parasitological risk. Bioresour. Technol. 52, 37–40.

Gerba, C.P., Smith Jr., J.E., 2005. Sources of pathogenic microorganisms and their fate during land application of wastes. J. Environ. Qual. 34, 42–48.

Grant, E.J., Rouch, D.A., Deighton, M., Smith, S.R., 2012. Pathogen risks in land-applied biosolids. Evaluating risks of biosolids produced by conventional treatment. AWA Water J. 39, 72–78.

Guerrero, C., Gómez, I., Mataix-Solera, J., 2007. El uso de enmiendas en la restauración de suelos quemados. In: Incendios forestales, Suelos yErosión hídrica, pp. 119–154. Caja Mediterráneo CEMACAM, Alcoy.

Haas, C.N., Rose, J.B., Gerba, C.P., 2014. Quantitative Microbial Risk Assessment, second ed. John Wiley and Sons, New York, NY.

Haug, R.T., 1993. The Practical Handbook of Compost Engineering. FL,Lewis Publishers, Boca Raton, FL.USA.

Hernández, J.R.R., Gómez-Lucas, I., Navarro-Pedreño, J., Jordán, M.M., Bech, J., Asencio, V.M.N., Iñiguez, N.P., 2017. Environmental consequences from the use of sewage sludge in soil restoration related to microbiological pollution. J. Soils Sediments. https://doi.org/10.1007/s11368-017-1721-5.

Janevska, D.P., Gospavic, R., Pacholewicz, E., Popov, V., 2010. Application of a HACCP–QMRA approach for managing the impact of climate change on food quality and safety. Food Res. Int. 43, 1915–1924.

Jiménez, B., Austin, A., Cloete, E., Plasha, C., Beltran, N., 2007. Biological risks to foods crops fertilezed with Ecosan sludge. Water Sci. Technol. 55, S21–S29.

Jiménez, B., Maya, C., Sánchez, E., Romero, A., Lira, L., Barrios, J.A., 2002. Comparison of the quantity and quality of the microbiological content of sludge in countries with low and high content of pathogens. Water Sci. Technol. 46, 17–24.

Jiménez, B., Wang, L., 2006. Sludge treatment and management. Chap. 10. In: Ujang, Z., Henze, M. (Eds.), Municipal Wastewater Management in Developing Countries: Principles and Engineering. IWA Publishing, London, UK, ISBN 1-84339-030-2, pp. 237–292.

Jiménez, R., Álvarez, A.M., 2005. Control de la degradación de suelos. Universidad Autónoma de Madrid, Madrid.

Jimenez-Cisneros, B.E., 2006. Helminth ova control in wastewater and sludge for agriculture reuse. Water Health II. ©Encyclopedia of Life Support Systems (EOLSS).

Johnson, P.W., Dixon, R., Ross, A.D., 1998. An in-vitro test for assessing the viability of Ascaris suum eggs exposed to various sewage treatment processes. Int. J. Parasitol. 28, 627–633.

Koné, D., Cofie, O., Zurbrügg, C., Gallizzi, K., Moser, D., Drescher, S., Strauss, M., 2007. Helminth eggs inactivation efficiency by faecal sludge dewatering and co-composting in tropical climates. Water Res. 41, 4397–4402.

Koné, D., Struass, M., 2004. Low cost options for treating faecal sludges (FS) in developing countries-challenges and performance. In: Proceedings of the 9th International IWA Specialists Group Conference on Wetlands Systems for Water Pollution Control and the 6th International IWA Specialists Group Conference on Waste Stabilization Ponds, Avignon, France, 27September-1 October.

Krzyzanowski Jr., F., Lauretto, M.S., Nardocci, A.C., Sato, M.I.Z., Razzolini, M.T.P., 2016. Assessing the probability of infection by Salmonella due to sewage sludge use in agriculture under several exposure scenarios for crops and soil ingestion. Sci. Total Environ. 568, 66–74.

Kundu, A., Poma, H.R., Jenkins, M.W., Rajal, V.B., Wuertz, S., 2014. QMRA of intestinal nematode infection via multimedia exposure pathways. In: Ames, Daniel P., Quinn, Nigel W.T., Rizzoli, Andrea E. (Eds.), International Environmental Modelling and Software Society (iEMSs) 7th Intl. Congress on Env. Modelling and Software, San Diego, CA, USA.

McBride, G.B., Stott, R., Miller, W., Bambic, D., Wuertz, S., 2013. Discharge-based QMRA for estimation of public health risks from exposure to storm waterborne pathogens in recreational waters in the United States. Water Res. 47, 5282–5297.

McKinley, J.W., Parzen, R.E., Guzmán, Á.M., 2012. Ammonia inactivation of Ascaris Ova in ecological compost by using urine and ash. Appl. Environ. Microbiol. 78 (15), 5133–5137.

Melvin, D.M., Brooke, M.M., Sadun, E.H., 2001. Common Intestinal Helminths of Humans. DHEW Publication No. (CDC) 80–8286, Atlanta, Georgia, USA.

Membré, J.-M., Boué, G., 2017. Quantitative microbiological risk assessment in food industry: theory and practical application. Food Res. Int. https://doi.org/10.1016/j.foodres.2017.11.025.

Mkhize-Kwitshana, Z.L., Mabaso, M.L.H., 2014. The neglected triple disease burden and interaction of helminths, HIV and tuberculosis: an opportunity for integrated action in South Africa. S. Afr. Med. J. 104, 258–259.

Molvik, M., Helland, E., Zulu, S.G., Kleppa, E., Lillebo, K., Gundersen, S.G., Kvalsvig, J.D., Taylor, M., Kjetland, E.F., Vennervald, B.J., 2017. Co-infection with Schistosoma haematobium and soil-transmitted helminths in rural South Africa. South Afr. J. Sci. 113, 1–6.

Moubarrad, F.Z.L., Assobhei, O., 2007. Health risks of raw sewage with particular reference to Ascaris in the discharge zone of El Jadida (Morocco). Desalination 215, 120–126.

Murcia Navarro, F.J., 2013. Lodos de depuradora: una visión integral parasu posible aplicación a suelos desde una perspectiva agrícola. PhD Thesis. Universidad de Murcia, Murcia.

Naidoo, D., Foutch, G.L., Archer, C.E., Appleton, C.C., 2017. The inactivation of Ascaris suum eggs by short exposure to high temperatures for the purpose of sanitizing VIP latrine sludge by viscous heating. In: Oral Presentation at the 4th International Conference on Faecal Sludge Management (FSM4), pp. 19–23. Febraury, 2017, Chennai-India.

Navarro, I., Jiménez, B., 2011. Evaluation of the WHO helminth eggs criteria using a QMRA approach for the safe reuse of wastewater and sludge in developing countries. Water Sci. Technol. 63, 1499–1505.

Navarro, I., Jiménez, B., Lucario, S., Cifuentes, E., 2009. Application of helminth ova infection dose curve to estimate the risks associated with biosolid application on soil. J. Water Health 7, 31–44.

Navarro, I., Jiménez, B., Cifuentes, E., Lucario, S., 2008. A quantitative microbial risk assessment of helminth ova in reusing sludge for agricultural production in developing countries. Risk Anal. 6, 65–74.

Navarro-Pedreño, J., Almendro-Candel, M.B., Jordán-Vidal, M.M., Mataix- Solera, J., Garcia-Sánchez, E., 2003. Mobility of cadmium, chromium, and nickel through the profile of a calcisol treated with sewage sludge in the southeast of Spain. Environ. Geol. 44, 545–553.

Nelson, K.L., Darby, J., 2001. Inactivation of viable Ascaris eggs by reagents during enumeration. Appl. Environ. Microbiol. 67, 5453–5459.
NWQMS-National Water Quality Management Strategy, 2006. Australian guidelines for water recycling: managing health and environmental risks (phase 1). In: Environment Protection and Heritage Council, the Natural Resource Management Ministerial Council and the Australian Health Ministers' Conference. Canberra, Australia.
O'Connor, N.A., Surapaneni, A., Smith, D., Stevens, D., 2017. Occurrence and fate of Ascaris lumbricoides ova in biosolids in Victoria, Australia: a human health risk assessment of biosolids storage periods. Water Sci. Technol. https://doi.org/10.2166/wst.2017.222.
Pecson, B.M., Barrios, J.A., Jimenez, B.E., Nelson, K.L., 2007. The effects of temperature, pH, and ammonia concentration on the inactivation of Ascaris eggs in sewage sludge. Water Res. 41, 2893–2902.
Pepper, I.L., Zerzghi, H., Brooks, J.P., Gerba, C.P., 2008. Sustainability of land application of class B biosoloids. J. Environ. Qual. 3437, S58–S67.
Romero-Barrios, P., Hempen, M., Messens, W., Stella, P., Hugas, M., 2013. Quantitative microbiological risk assessment (QMRA) of food-borne zoonoses at the European level. Food Contr. 29, 343–349.
Sakaji, R.H., Funamizu, N., 1998. Microbial risk assessment and its role in the development of wastewater reclamation policy. In: Asano, T. (Ed.), Wastewater Reclamation and Reuse, vol. 10. CRC Press, Boca Raton, Fla, USA, pp. 705–756.
Schönning, C., Westrell, T., Stenström, T.A., Arnbjerg-Nielsen, K., Hasling, A.B., Høibye, L., Carlsen, A., 2007. Microbial risk assessment of local handling and use of human faeces. J. Water Health 5, 117–128.
Seidu, R., Heistad, A., Amoah, P., Drechsel, P., Jenssen, P.D., Stenström, T.A., 2008a. Quantification of the health risk associated with wastewater reuse in Accra, Ghana: a contribution toward local guidelines. J. Water Health 6, 461–471.
Seidu, R., Drechsel, P., Amoah, P., Löfman, O., Heistad, R., Fodge, M., Jenssen, P., Stenström, T.A., 2008b. Quantitative microbial risk assessment of wastewater and faecal sludge reuse in Ghana. In: 33rd WEDC International Conference, Accra, Ghana, 2008.
Smeets, P.W.M.H., Rietveld, L.C., van Dijk, J.C., Medema, G.J., 2010. Practical applications of quantitative microbial risk assessment (QMRA) for water safety plans. Water Sci. Technol. 61, 1561–1568.
Stephenson, L.S., Latham, M.C., Ottesen, E.A., 2000. Malnutrition and parasitic helminth infections. Parasitology 121, 23–38.
Strauss, M., Drescher, S., Zurbrügg, C.H., Montangero, A., 2003. Co-composting of Faecal Sludge and Municipal Organic Waste. A Literature and State-of-knowledge Review. Dübendorf Switzerland: Swiss Federal Institute of Environmental Science and Technology (EAWAG) and IMWI, Duebendorf, Switzerland and Accra, Ghana.
Szabová, E., Juriš, P., Papajová, I., 2010. Sanitation composting process in different seasons. *Ascaris suum* as model. Waste Manage 30, 426–432.
Tine, R.C.K., Faye, B., Ndour, C.T., Sylla, K., Sow, D., Ndiaye, M., Ndiaye, J.L., Magnussen, P., Alifrangis, M., Bygbjerg, Ib C., Gaye, O., 2013. Parasitic infections among children under five years in Senegal: prevalence and effect on anaemia and nutritional status. ISRN Parasitol. 2013, 272701 https://doi.org/10.5402/2013/272701, 6 pages.
Toze, S., 2006. Reuse of effluent water-benefits and risks. Agric. Water Manage. 80, 147–159.
Trönnberg, L., Hawksworth, D., Hansen, A., Archer, C., Stenström, T.A., 2010. Household-based prevalence of helminths and parasitic protozoa in rural KwaZulu-Natal, South Africa, assessed from faecal vault sampling. Trans. Royal Soc. Trop. Med. Hyg. 104, 646–652.
U. S. EPA, 1993. 40 code of federal regulations. Part 503. Fed. Regist. 58, 9248–9415.
Westrell, T., Schönning, C., Stenström, T.A., Ashbolt, N.J., 2004. QMRA (quantitative microbial risk assessment) and HACCP (hazard analysis and critical control points) for management of pathogens in wastewater and sewage sludge treatment and reuse. Water Sci. Technol. 50, 23–30.
WHO-World Health Organization, 2017. Soil-transmitted Helminth Infections Fact Sheet. Available on. http://www.who.int/mediacentre/factsheets/fs366/en/. (Accessed 6 September 2017).
WHO-World Health Organization, 2015. Investing to Overcome the Global Impact of Neglected Tropical Diseases Third Who Report on Neglected Tropical Diseases. WHO Document Production Services, Geneva, Switzerland (WHO/HTM/NTD/2015.1).
WHO - World Health Organization, 2006. Guidelines for the Safe Use of Wastewater Excreta and Greywater, vol. 4. World Health Organization, Geneva, 2017. www.climate-data.org. (Accessed 30 August 2017).
Zdybel, J., Cencek, T., Karamon, J., Kłapéc, T., 2015. Effectiveness of selected stages of waste water treatment in elimination of eggs of intestinal parasites. Bull. Vet. Inst. Pulawy 59, 51–57.

Paper V

Isaac Dennis Amoah, Razak Seidu, Amina Abubakari, Thor Axel Stenström and Robert Clement Abaidoo (2016). Contribution of Wastewater Irrigation to Soil Transmitted Helminths Infection among Vegetable Farmers in Kumasi, Ghana. *PLOS Neglected Tropical Diseases.* 10(12):e0005161. **DOI**.org/10.1371/journal.pntd.0005161

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RESEARCH ARTICLE

Contribution of Wastewater Irrigation to Soil Transmitted Helminths Infection among Vegetable Farmers in Kumasi, Ghana

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Abstract

Wastewater irrigation is associated with several benefits but can also lead to significant health risks. The health risk for contracting infections from Soil Transmitted Helminths (STHs) among farmers has mainly been assessed indirectly through measured quantities in the wastewater or on the crops alone and only on a limited scale through epidemiological assessments. In this study we broadened the concept of infection risks in the exposure assessments by measurements of the concentration of STHs both in wastewater used for irrigation and the soil, as well as the actual load of STHs ova in the stool of farmers and their family members (165 and 127 in the wet and dry seasons respectively) and a control group of non-farmers (100 and 52 in the wet and dry seasons, respectively). Odds ratios were calculated for exposure and non-exposure to wastewater irrigation. The results obtained indicate positive correlation between STH concentrations in irrigation water/soil and STHs ova as measured in the stool of the exposed farmer population. The correlations are based on reinfection during a 3 months period after prior confirmed deworming. Farmers and family members exposed to irrigation water were three times more likely as compared to the control group of non-farmers to be infected with *Ascaris* (OR = 3.9, 95% CI, 1.15–13.86) and hookworm (OR = 3.07, 95% CI, 0.87–10.82). This study therefore contributes to the evidence-based conclusion that wastewater irrigation contributes to a higher incidence of STHs infection for farmers exposed annually, with higher odds of infection in the wet season.

Author Summary

Wastewater irrigation in agriculture is a common reality in many developing cities, linked to rapid urbanization. Approximately 50%-90% of urban dwellers in West Africa consume wastewater/ polluted surface water irrigated-vegetables within cities with 10% of the population involved in the practice. Viral, bacterial and parasitic pathogens can all be found in

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wastewater putting exposed populations at risk of pathogenic infections. The biggest risk however is to helminth infections, due to their long survival time in the environment. Wastewater irrigation has been practiced in Ghana for many years, however few studies have investigated the epidemiological link between the practice and helminth infections among the farmers. In this study the authors measured the helminth ova concentration in wastewater used for irrigation and the infection loads of farmers as well as a non-farmer control group in Ghana. They reported high concentrations of helminth ova in the wastewater as well as soil on the farms, above the World Health Organization (WHO) guidelines for wastewater irrigation, which resulted in a three times higher probability of infections with helminths for farmers as compared to the non-farmer control group. This research provides information on the direct link between wastewater irrigation and helminth infection in exposed individuals.

Introduction

Wastewater use in agriculture has been promoted as part of the concept of sustainable development. In many cities in developing countries, wastewater irrigation is a common reality linked to rapid urbanization. The practice improves farmers' livelihoods, contributes to the urban food basket and slightly improves the urban environment by diverting wastewater to agricultural fields [1]. In Sub-Saharan Africa (SSA), it is estimated that 10% of the population in cities are involved in the practice of wastewater irrigation, with 50% to 90% of urban dwellers in West Africa reported to consume vegetables irrigated with wastewater or polluted surface water within or close to cities [2]. In Ghana, a significant proportion of untreated wastewater is discharged into drains and nearby water bodies, which is then used by farmers for irrigation. Wastewater irrigation in the cities of Ghana is mainly for the production of vegetables, such as cabbage, lettuce, spring onion and carrots [3].

Although there are many benefits associated with wastewater irrigation, the practice can lead to significant health risk if not undertaken in a safe manner [4]. All enteric pathogens of viral, bacterial and parasitic (helminthic and protozoan) origins can be found in wastewater; and can be transmitted to farmers using the wastewater for irrigation, consumers of wastewater-irrigated vegetables and communities close to wastewater irrigated fields [5]. Several studies have shown a significant relationship between *Ascaris* infection and exposure to wastewater (either treated or untreated) [6,7,8]. This is because soil transmitted helminths (STHs) (such as *Ascaris*) can survive for long periods of time under severe adverse environmental conditions [9] contributing to their high risk of infection. STHs are common worldwide with more than a billion people infected [10, 11]. Estimates suggest that *Ascaris lumbricoides* infects over 1 billion people, *Trichuris trichiura* 795 million, and hookworms (*Ancylostoma duodenale* and *Necator americanus*) 740 million [12]. Farmers in Pakistan using wastewater for irrigation have been reported to be five times more likely to be infected with hookworms than others using canal water [13] and in Dakar, Senegal the reported incidence of amoebiasis and ascariasis is 60% in farmers involved in wastewater irrigation [14]. In a study in Mexico, irrigation with untreated or partially treated wastewater was directly responsible for 80% of all *A. lumbricoides* infections and 30% of diarrheal disease in farm-workers and their families [15]. The health risk can differ depending on age and gender. An epidemiological study by Habbari *et al.* [16] undertaken in Morocco to determine possible health risks associated with raw wastewater use in agriculture found ascariasis infection to be approximately five times higher, especially in children in wastewater impacted regions compared to control regions. In another study

Fuhrimann *et al* [35] found that farmers exposed to wastewater in Uganda were more likely to be infected with helminths than slum dwellers and workers involved in sludge collection. However, in Vietnam, Trang *et al*. [17] found no evidence that rice cultivation with wastewater posed any significant helminth infection risk to farmers, even though they were exposed to wastewater containing 40–200 helminth eggs/L. Prevalence of and risk factors for helminth infections have been studied in Ghana [18]. Although wastewater irrigation has been the practice for many years in Ghana, especially in major cities (e.g. Accra, Kumasi, Tamale), there are no studies that have investigated the epidemiological link between the practice and helminth infections among the farmers. Studies in Ghana have reported a mean helminth ova concentration of 5–10 helminth ova per liter in water used for irrigation by farmers [19,20].

In this regard this study aimed at determining the association between STHs ova concentration in wastewater used for irrigation as well as in farm soil that farmers are exposed to and the actual infection loads in order to ascertain the epidemiological link between wastewater irrigation and risk of STHs infection for farmers. The aim of this study was achieved as it was deduced that farmers had a higher probability of infection than non-farmers.

Methods

Study Area

The study was conducted in wastewater irrigated vegetable farms in the Kumasi Metropolitan Area of Ghana (Fig 1). The Metropolis has two major seasons, the rainy (April to October) and the dry one (November to March). Relative humidity ranges from 60–84% with daily minimum and maximum temperatures of 21.5˚C and 30.7˚C, respectively [21]. The majority of vegetable farms in Kumasi are irrigated with wastewater which is most predominant in the dry season. Wastewater from domestic and small-scale industrial (e.g vehicle garages, saw mills, welding shops, tanneries etc) sources are discharged directly into stormwater drains and streams and collected for irrigation by farmers.

Inclusion and Exclusion Criteria

An initial survey was carried out in the Kumasi Metropolis to identify the farms using wastewater for irrigation. This included a detailed explanation of the purpose of the study and farmers and control-group who gave consent to be part of the study were recruited. Non-farmer (control group) inhabited the same areas as the farmers and recruitment was made concomitantly. The control group thereby constituted members of families of their communities who did not take part in the practice of wastewater irrigation but stayed in the same neighborhood (as can be seen in Fig 1 below). An initial prevalence survey was undertaken after which participants were dewormed and the efficiency of the deworming exercise assessed directly afterwards. The farmer group consisted of 165 (in the wet season) farmers and family members dropping to 127 in the dry season, while the control group originally consisted of 100 individuals (in the wet season), dropping to 52 in the dry season.

The exclusion criteria was arrived at after the administration of the questionnaires to all participants, afterwards any participant who did not fall within the criteria set (based on self-reporting) was not included in the final data used for analysis, hence the dropout rate, but the project team still visited them and administered antihelminthic drugs when needed so as to encourage participation in subsequent studies.

Wastewater farmers recruited into the cohort met the following inclusion criteria: a) did not consume vegetable salad irrigated with wastewater from their farms; b) used improved toilet facilities at home and at work; c) did not use protective clothing during farm work; and d) had access to treated drinking water in their homes/communities.

Ethics Statement

The Committee on Human Research, Publications and Ethics (CHRPE) of the Kwame Nkrumah University of Science and Technology (KNUST) approved the study (No. CHRPE/RC/051/12) with additional informed oral consent received from all participants. Informed oral consent of parents or guardians was received for all children who participated in the study, which was written on the field questionnaire administered to each person. The purpose and details of the study was explained to all participants in Twi (a local dialect) in the presence of a witness and those willing to participate gave their consent, which was noted on the questionnaires. Each participant was given a unique identifier which was used throughout the study for confidentiality. After the initial deworming exercise all participants who became re-infected were treated again with 400 mg of albendazole (XL Laboratories PVT Ltd).

Sampling

Irrigation water was collected from August 2012 to October 2012 to represent the wet season and December 2012 to March 2013 to represent the dry season. Irrigation water samples were

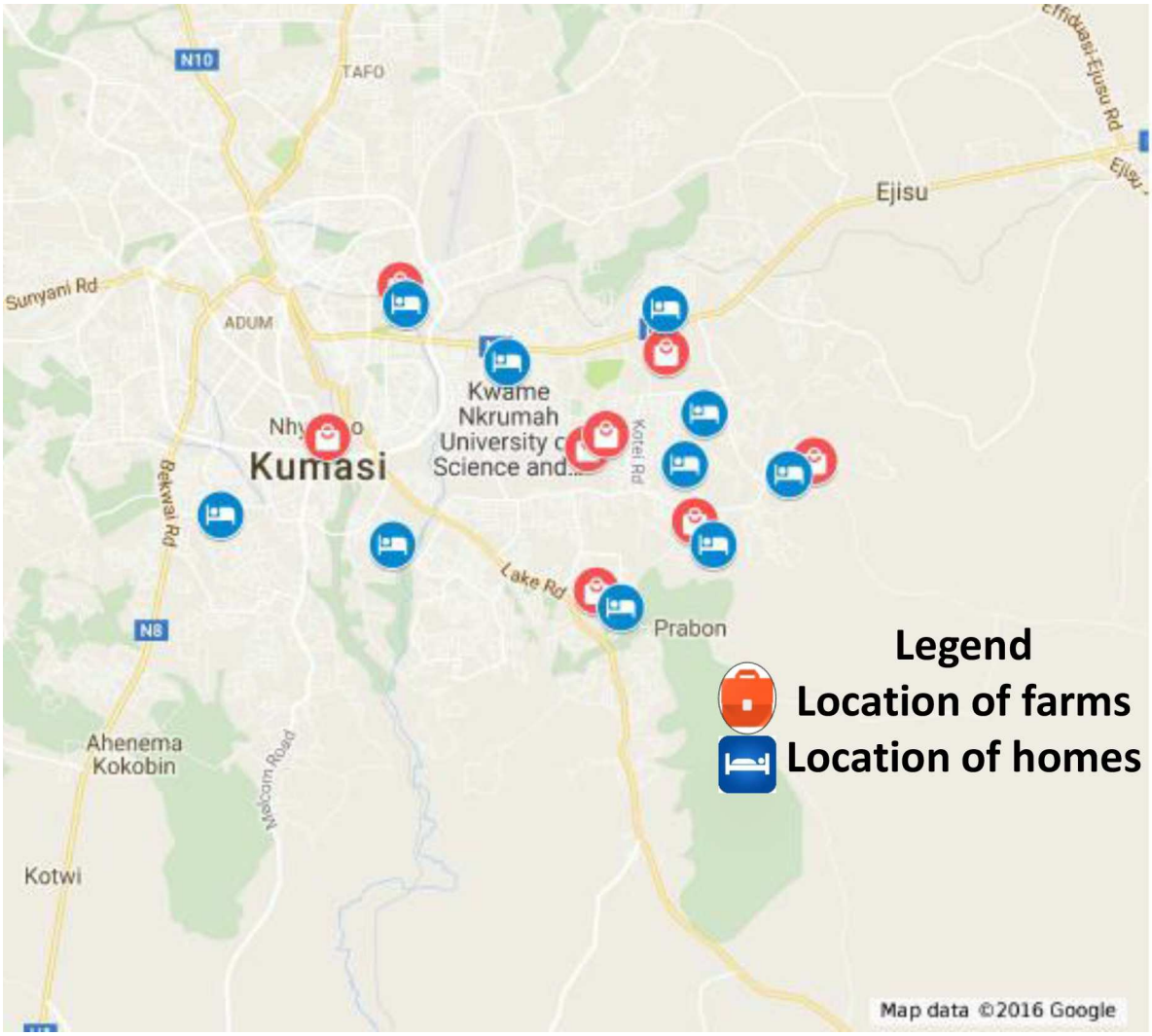

Fig 1. A map of Kumasi showing the location of the wastewater farms and homes of the farmers and control (courtesy Google Maps).

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taken from storm drains, streams, shallow wells and potable water pipes (in a few instances), which represented the sources of water in use by the farmers. Soil samples were taken from the vegetable beds that were irrigated with the water types sampled as stated above. In all, 214 and 156 samples (for soil and irrigation water) were taken during the wet and dry seasons, respectively. All samples were collected in the morning between 06.00 and 10.00 (Greenwich Mean Time (GMT) on each day of sampling. Irrigation water and soil samples were collected in triplicates into sterile pre-labeled sample bottles (about 4 L for the wastewater) and plastic re-sealable bags (30 g composite soil sample each) and kept in a cooling box and transported to the laboratory where they were processed and analyzed for helminth eggs using the Modified EPA Method [22]. The helminths eggs were identified on the basis of their shape and size with the aid of bench aids for the Diagnosis of Intestinal Parasites [23]. Only viable helminth eggs were counted, viability was assessed based on the presence of motile larvae within the eggs. Stool samples were collected from farmers and family members as well as the non-farmer control group and analyzed using the formal-ether concentration method [23]. After three months, stool samples were taken again from the participants for assessment of re-infections.

Statistical Analysis

Descriptive analysis was undertaken to assess the mean concentration and distribution of ova in the irrigation water and soil and described by box plots (Stata, Statacorp, Texas, USA). Analysis of variance was performed to determine the statistical difference between the concentrations of *Ascaris* spp and hookworm in the dry and wet seasons. The relationship between STHs loads in irrigation water/soil and actual STHs ova per gram of faecal matter from the farmers was determined using Poisson regression analysis (Stata, Statacorp, Texas, USA). The odds ratio (OR), its standard error and 95% confidence intervals were calculated according to Altman [24].

Results

STHs Ova Concentration in Irrigation Water and Soil

Ova of *Ascaris* spp, hookworm and *Schistosoma* spp (*Schistosoma* spp was found only in the irrigation water and is not further reported in this article) were identified in the irrigation water and soil in the vegetable farms. In general ova concentrations were higher in the wet season than the dry season for both irrigation water and soil samples (Refer to Table 1). Statistically there was difference in the concentration of hookworm ova in the two seasons and *Ascaris* spp concentration in the soil for between the seasons (Table 1). Figs 2 and 3 shows the distribution of the ova of *Ascaris* spp and hookworm in the irrigation water and soil for both the wet and dry seasons.

Prevalence of *Ascaris* spp and Hookworms among Farmers

Infection with the two parasites differed between seasons and between the farmers and the control group. The prevalence of *Ascaris* spp infection in the wet season was 15.77% (n = 165)

Table 1. Mean concentration (±S.D) of *Ascaris* spp and hookworm ova in irrigation water and soil for the dry and wet season in Kumasi, Ghana.

	Water			Soil		
	Wet season (n = 107)	**Dry season (n = 78)**	**P value**	**Wet season (n = 107)**	**Dry season (n = 78)**	**P value**
Ascaris spp (ova/L)	2.82 (±0.25)	2.62 (±0.13)	0.41	3.70 (±0.23)	2.90 (±0.21)	0.01
Hookworm (ova/L)	2.05 (±0.23)	1.38 (±0.10)	0.01	2.01(±0.16)	1.67 (±0.14)	0.16

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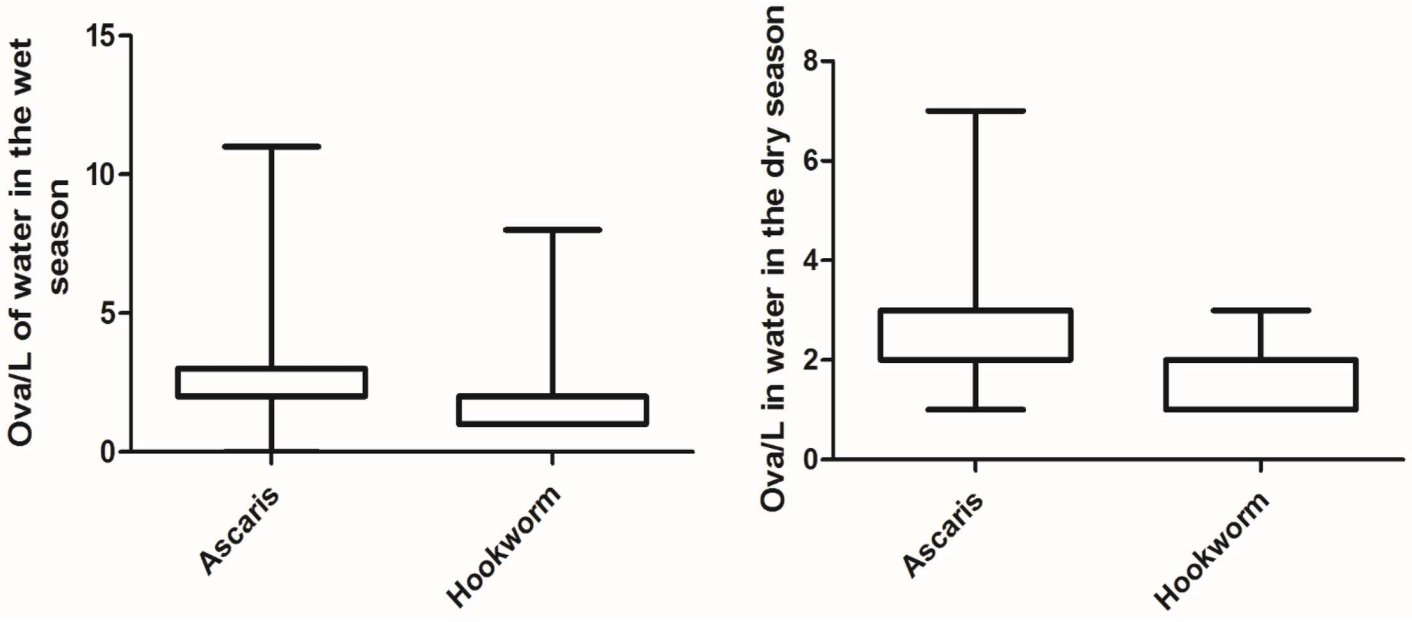

Fig 2. Distribution of *Ascaris* spp and hookworm ova in irrigation water in the dry (n = 71) and wet (n = 107) seasons.

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for the farmers and 6.00% (n = 100) for the control group. Similarly, the prevalence of hookworm infection in the wet season was 12.73% (n = 165) for the farmers and 2.00% (n = 100) for the control group. In the dry season prevalence of *Ascaris* spp. was lower for both groups, the farmers had a prevalence of 11.02% (n = 127) and 5.74% (n = 52) for the control group. A much lower prevalence was recorded for hookworm infections for farmers with 4.72%

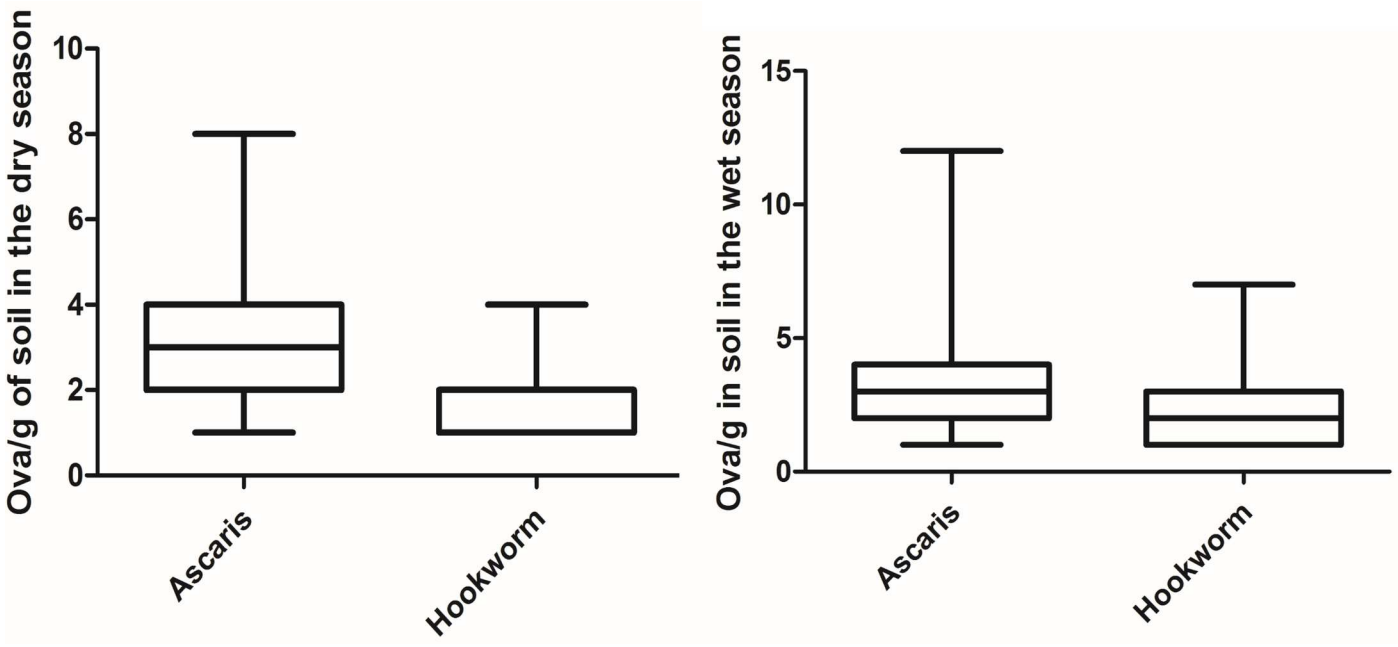

Fig 3. Distribution of *Ascaris* spp and hookworm ova in farm soil in the dry (n = 71) and wet (n = 107) seasons.

doi:10.1371/journal.pntd.0005161.g003

Table 2. Range of eggs per gram (epg) of *Ascaris* spp and hookworm infection intensity in wastewater farmers and a control group in Kumasi, Ghana.

	Farmers			Non-farmers (Control group)		
	Wet season (n = 165)	Dry season (n = 127)	P value	Wet season (n = 100)	Dry season (n = 52)	P value
Ascaris spp (epg)	4–223	3–124	0.01	8–112	19–68	0.35
Hookworm (epg)	2–84	4–20	0.06	9–54	15–57	0.40

doi:10.1371/journal.pntd.0005161.t002

(n = 127) however same prevalence as reported for *Ascaris spp* was reported for hookworm infections of the control group, but with different mean infections. Table 2 above shows the details of the range and significant difference and Figs 4 and 5 distribution of infection intensity.

Relationship between Parasite Ova in the Irrigation Water and Soil and the Incidence of Infection in Farmers

The concentration of the ova of the two reported helminths in both the irrigation water and soil and the intensity of infection of the exposed farmers showed a significantly positive relationship ($p < 0.05$) in the wet season (regression coefficient of 0.04; 95% CI: 0.203–0.69). The opposite was the case in the dry season (regression coefficient of -.0023; 95% CI: -.020 - .016), however this was not statistically significant ($p > 0.05$).

Odds of Infection for Farmers and Non-Exposed Populations

The probability of farmers getting infected with STHs compared to the control group as a result of exposure to the ova in the irrigation water and the soil was higher in the wet season than in the dry season for the two STHs (Table 3). In the wet season, farmers exposed to irrigation water and soil were more likely than the control group to be infected with *Ascaris* spp (OR = 3.99, 95% CI: 1.15–13.86) and Hookworm (OR = 3.07, 95% CI: 0.87–10.82). However, there was lesser probability of infection in the dry season as shown in Table 3.

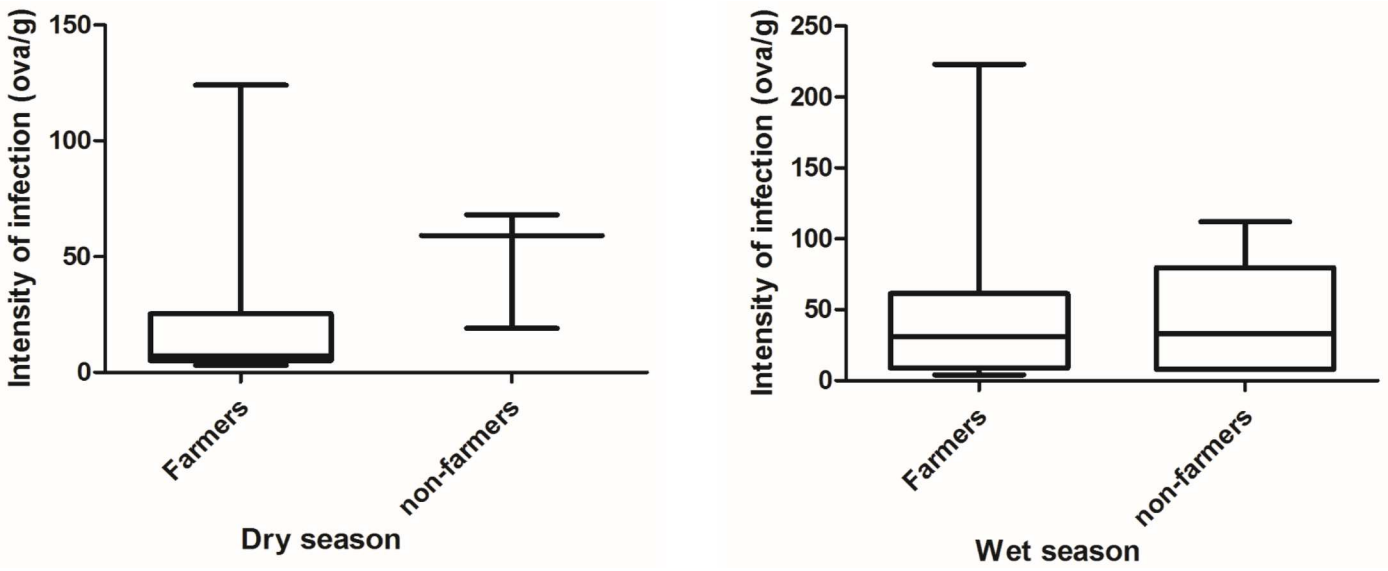

Fig 4. Distribution of *Ascaris* spp for farmers and non-farmers in both seasons.

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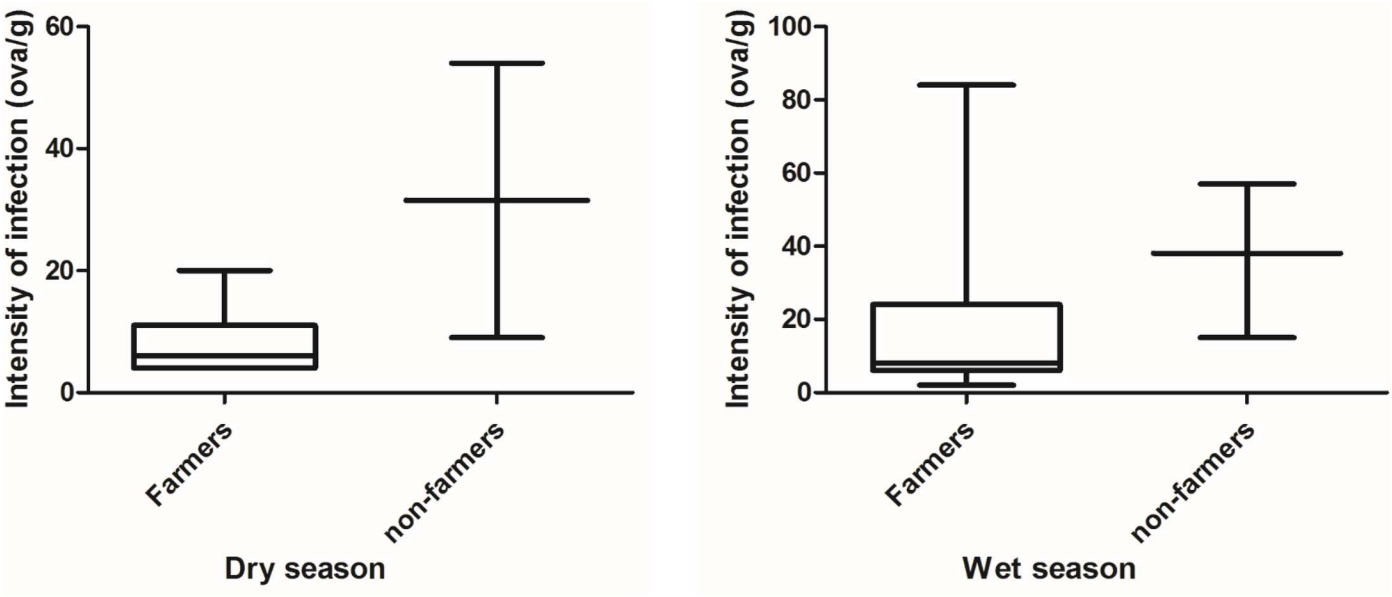

Fig 5. Distribution of hookworm infection intensity for farmers and non-farmers in both seasons.

doi:10.1371/journal.pntd.0005161.g005

Discussion

Helminth contamination of irrigation water is a serious public health issue, due to their persistence in the environment and their low infective dose. To safeguard human health, the WHO formulated guidelines for the use of wastewater in unrestricted agriculture [4], with a guideline value of <1 ova/L aimed at reducing the risk of infection. In this study, the mean concentration of STHs ova was higher than the recommended guideline value, especially for *Ascaris* spp (2.62 ova/L and 2.82 ova/L for dry and wet seasons, respectively) and hookworm (2.05 ova/L in the wet season), in line with similar results reported from studies in Ghana [3, 19, 20, 29] as well as other countries [25, 30, 35]. There was seasonal variation in the mean concentration of the STHs ova in the irrigation water, with the wet season showing higher concentrations than the dry season. Keraita *et al.* [26] reported similar patterns of helminth ova concentration in irrigation water from studies conducted in Kumasi. This occurrence could be attributed to rainfall and reduced temperatures which extend the survival period of the ova. However in general, helminth ova are resistant to many types of inactivation, with ova of *Ascaris* spp and *Taenia* spp having the highest resistance and survival rates [27, 28]. In addition to the lower temperatures and much lesser UV irradiation in the wet season, ova concentration could be increased during this period of the year due to run-offs from agricultural fields and other surrounding areas. Open defecation in areas close to these wastewater irrigated farms could also potentially lead to higher STHs ova concentrations after rainfalls. Wastewater irrigation does not only increase risk of STH infection due to exposure to the irrigation water but also exposure to the farm soil. Exposure to the farm soil in wastewater irrigated farms may result in higher risk of infection with STHs than risk attributable to the wastewater alone [29, 30]. This is due to a higher concentration of STHs ova in the soil as was seen in this study. Irrigation with wastewater result in accumulation of STHs ova in the soil, and therefore accounts for the higher concentration of ova. *A. lumbricoides* eggs have been found to attach to soil particles (especially clay) thereby contributing to their high concentrations in the soil samples [31]. In addition, contamination of soils could serve as a source for re-introduction of eggs into the irrigation water channels.

Table 3. Odds of infection with *Ascaris* spp and hookworm for farmers involved in wastewater irrigation compared with a control group in Kumasi, Ghana.

	DRY SEASON*	WET SEASON[$]
Ascaris spp	0.92 (95% CI; 0.33–2.56)	3.99 (95% CI; 1.15–13.86)
Hookworm	1.21 (95% CI; 0.24–6.20)	3.07 (95% CI; 0.87–10.82)

*In the dry season the study population was 127 for farmers and 52 for the control group

[$]In the wet season the study population was 165 for farmers and 100 for the control group

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The elevated concentrations of STHs ova, above the WHO guideline levels, in the irrigation water and soil pose a risk of infection for farmers involved in the practice of wastewater irrigation. However, there is always a difference between the estimated risk and actual infections. The potential health risk is based on the number of pathogens in the wastewater or soil, while the actual health risk depends on an expansion of this concept, including: i) the period pathogens survive in water or soil; ii) the dose in which pathogens are infective to a human host and iii) host immunity for pathogens circulating in the environment [32]. The seasonal variation in STHs ova concentration in the irrigation water and soil was also apparent in the STHs infection intensity of the farmers, reflected in higher infection frequency in the wet season. There are other factors such as, climate, types of soils and hygiene behavior, which might have also contributed to this variation in infection rate [33]. The interrelationship with other confounding factors was seen with the correlation analysis where there was a weak association between the load or concentration in the irrigation water/soil and the intensity of the STH infection for the farmers, especially in the dry season.

To quantify the actual contribution of wastewater irrigation to STHs infection a control group of non-farmers who had no exposure to the irrigation water and soil but used same sanitation and portable water infrastructure as the farmers (staying in the same suburbs as the farmers) was needed. The increased probability of infection for farmers was expected due to a higher exposure to STHs ova over the course of the year as compared to the non-farmers. Infection with *Ascaris* spp and hookworm for the farmers is three times more likely than it is for non-farmers. This clearly indicates that wastewater use in irrigation contributes significantly to the incidence of helminthiases, as reported by many other studies [13, 29, 34, 35], especially in the wet season (Table 3). In the dry season the odds of infection for both farmers and non-farmers is not significantly different. This could be attributed to the lower concentrations of ova recorded in the irrigation water and soil during this time of the year.

It can be concluded from the results obtained in this study that exposure to STHs ova in irrigation water and soil contributes to infections in farmers and that farmers involved in the practice are three times likely to be infected with *Ascaris* spp and hookworm than unexposed populations. This is particularly so during the wet season where there is an increase in the concentration of the STHs ova. The results obtained show an epidemiological link between wastewater irrigation and helminth infection in Ghana, therefore emphasizing the need for regulations and interventions aimed at making the practice safer for the farmers which in turn would contribute significantly in breaking the cycle of infection.

Supporting Information

S1 Checklist.
(DOC)

S1 Appendix. Tables containing descriptive analysis of data.
(DOCX)

S1 Data Tables. Tables containing the raw data recorded for each sample of irrigation water, soil and stool.
(XLSX)

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Author Contributions

Conceived and designed the experiments: IDA RS AA RCA TAS.

Performed the experiments: IDA AA.

Analyzed the data: IDA RS TAS.

Contributed reagents/materials/analysis tools: RS.

Wrote the paper: IDA RS AA RCA TAS.

References

1. van der Hoek W, Hassan MUl, Ensink JHJ, Feenstra S, Raschid-Sally L, Munir S, et al. Urban Wastewater: A Valuable Resource for Agriculture: a Case Study from Haroonabad, Pakistan'. Research Report 63, Colombo, Sri Lanka. International Water Management Institute. 2002. pp 2–5.
2. Drechsel P, Graefe S, Sonou M, Cofie O. Informal irrigation in urban West Africa: An overview, Research Report 102, International Water Management Institute, Colombo, Sri Lanka. 2006
3. Andoh L. Helminth contamination of lettuce and associated risk factors at production sites, markets, and street food vendor sites in urban and peri-urban Kumasi. [M.Sc thesis presented to the Department of Theoretical and Applied Biology], Kwame Nkrumah University Science and Technology. Kumasi. 2008
4. WHO (World Health Organization). Guidelines for the safe use of wastewater, excreta and grey water: Wastewater use in agriculture (Volume 2). Report of a WHO Scientific Group. WHO Technical Report Series 778. World Health Organization, Geneva, Switzerland. 2006. pp 74–219.
5. Asano T. Wastewater Reclamation and Reuse: Water Quality Management Library, Volume 10. CRC Press LLC. Florida. 1998.
6. Mara D, Sleigh A. Estimation of norovirus and *Ascaris* infection risks to urban farmers in developing countries using wastewater for crop irrigation. J Water Health 2010; 8:572–576. doi: 10.2166/wh.2010.097 PMID: 20375486
7. Cutolo SA, Piveli RP, Santos JG, Martes CR, Sundefeld G, Campos F, et al. Parasitological risk assessment from wastewater reuse for disposal in soil in developing countries. Water Sci Technol. 2012; 65:1357–1367. doi: 10.2166/wst.2012.012 PMID: 22466580
8. Seidu R, Sjölander I, Abubakari A, Amoah ID, Larbi JA, Stenström TA. Modeling the die-off of *E. coli* and *Ascaris* in wastewater-irrigated vegetables: implications for microbial health risk reduction associated with irrigation cessation. Water Sci Technol 2013; 68:1013–1021. doi: 10.2166/wst.2013.335 PMID: 24037151
9. Katakam KK, Majer H, Dalsgaard A, Kyvsgaard NC, Thamsborg SM. Survival of *Ascaris suum* and *Ascaridia galli* eggs in liquid manure at different ammonia concentrations and temperatures. Vet Parasitol 2014; 264: 249–257.
10. WHO (World Health Organization). Prevention and control of Schistosomiasis and soil-transmitted helminthiasis: report of a WHO expert committee. World Health Organization Technical Report Series; 2002; 912:1–57.
11. Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D. Soil-transmitted helminth infections: ascariasis, trichuriasis and hookworm. Lancet 2006; 367:1521–1532. doi: 10.1016/S0140-6736(06)68653-4 PMID: 16679166

12. de Silva NR, Brooker S, Hotez PJ, Montresor A, Engels D, Savioli L. Soil-transmitted helminth infections: updating the global picture. Trends Parasitol 2003; 19: 547–551. PMID: 14642761
13. Ensink JH, van der Hoek W, Mukhtar M, Tahir Z, Amerasinghe FP. High risk of hookworm infection among wastewater farmers in Pakistan. T Roy Soc Trop Med H 2005; 99:809–818.
14. Faruqui N, Niang S, Redwood M. Untreated wastewater reuse in market gardens: a case study of Dakar, Senegal. In: Scott CA, Faruqui NI, Raschid-Sally L, eds. Wastewater use in irrigated agriculture: Confronting the livelihood and environmental realities. Wellingon, CAB International in association with IWMI and IDRC. 2004. pp 113–125.
15. Cifuentes E, Blumenthal U, Ruiz-Palacios G, Bennett S, Quigley M. Health risks in agricultural villages practising wastewater irrigation in Central Mexico: perspectives for protection In *Water Sanitation and Health* (eds Chorus I., Ringelband U., Schlag G. and Schmoll O.), IWA Publishing, London. 2000. pp. 249–256.
16. Habbari K, Tifnouti A, Bitton G, Mandil A. Geohelminth infections associated with raw wastewater reuse for agricultural purposes in Beni-Mallal, Morocco. Parasitol Int. 2000; 48:249–254. PMID: 11227765
17. Trang DT, van der Hoek W, Cam PD. Low risk for helminth infection in wastewater-fed rice cultivation in Vietnam. J Water Health. 2006; 4:321–331. PMID: 17036840
18. Humphries D, Mosites E, Otchere J, Twum WA, Woo L, Jones-Sanpei H, et al. Epidemiology of Hookworm Infection in Kintampo North Municipality, Ghana: Patterns of Malaria Coinfection, Anemia, and Albendazole Treatment Failure. Am J Trop Med Hyg. 2011; 84:792–800. doi: 10.4269/ajtmh.2011.11-0003 PMID: 21540391
19. Obiri-Danso K, Weobong CAA, Jones K. Aspects of health-related microbiology of the Subin, an urban river in Kumasi, Ghana. J Water Health. 2005; 3:69–76. PMID: 15952454
20. Amoah P, Keraita B, Akple M, Drechsel P, Abaidoo RC, Konradsen F. Low-cost Options for Reducing Consumer Health Risks from Farm to Fork where Crops Are Irrigated with Polluted Water in West Africa International Water Management Institute. Research Report 141, 2011. pp. 4
21. MOFA (Ministry of Food and Agriculture). Kumasi Metropolitan Assembly, Ministry of Food and Agriculture.http://mofa.gov.gh/site/?page_id=859 Date accessed: 16 April 2015.
22. Schwartzbrod J. Methods of Analysis of Helminth Eggs and Cysts in Wastewater, Sludge, Soils and Crops. Université Henry Poincare, Nancy, France. 1998.
23. WHO (World Health Organization). Basic laboratory methods in medical parasitology. Geneva, Switzerland. 1991. pp. 3.
24. Altman DG. Practical Statistics for Medical Research, Chapman and Hall, UK. 1991.
25. Fuhrimann S, Stalder M, Winkler MS, Niwagaba CB, Babu M, Masaba G, Kabatereine N B, Halage AA, Schneeberger PHH, Utzinger J, Cissé G. Microbial and chemical contamination of water, sediment and soil in the Nakivubo wetland area in Kampala, Uganda. Environ Monit Assess. 2015; 187: 474–489
26. Keraita B, Konradsen F, Drechsel P, Abaidoo RC. Effect of low-cost irrigation methods on microbial contamination of lettuce irrigated with untreated wastewater. Trop Med Int Health. 2007; 12:15–22.
27. Feachem RG, Bradley DJ, Garelick H, Mara D. Sanitation and Disease: Health Aspects of Excreta and Wastewater Management. Wiley, New York. 1983.
28. Gaasenbeek CPH, Borgsteede FHM. Studies on the survival of *Ascaris suum* eggs under laboratory and simulated field conditions. Vet Parasitol 1998; 75: 227–234. PMID: 9637224
29. Seidu R, Heistad A, Amoah P, Drechsel P, Jenssen P D, Stenström TA. Quantification of the health risk associated with wastewater reuse in Accra, Ghana: a contribution toward local guidelines. J Water Health 2008; 6:461–471. doi: 10.2166/wh.2008.118 PMID: 18401111
30. Verbyla ME, Iriarte M, Mercado A, Coronado O, Almanza M, Mihelcic JR. Pathogens and fecal indicators in waste stabilization pond systems with direct reuse for irrigation: Fate and transport in water, soil and crops. Sci Total Environ. 2016; 551–552:429–437 doi: 10.1016/j.scitotenv.2016.01.159 PMID: 26881733
31. Landa-Cansigno O, Durán-Álvarez JC, Jiménez-Cisneros B, Retention of Escherichia coli, Giardia lamblia cysts and Ascaris lumbricoides eggs in agricultural soils irrigated by untreated wastewater. J Environ Manage. 2013; 128:22–29 doi: 10.1016/j.jenvman.2013.04.049 PMID: 23722173
32. Strauss M, Blumenthal UJ. Human Waste Use in Agriculture and Aquaculture- Utilization Practices and health perspectives. International Reference Centre for Waste Disposal (now SANDEC), Duebendorf, Switzerland. Report no. 08/90 (main report). 1990.
33. Olsen A, Samuelsen H, Onyango-Ouma W. A study of risk factors for intestinal helminths infections using epidemiological and anthropological approaches. J Biosoc Sci. 2001; 33:569–584. PMID: 11683225

34. Peasey A. Human exposure to Ascaris infection through wastewater reuse in irrigation and its public health significance. PhD thesis, University of London, UK. 2000.

35. Fuhrimann S, Winkler MS, Kabatereine NB, Tukahebwa EM, Halage AA, Rutebemberwa E, Medlicott K, Schindler C, Utzinger J, Cissé G. Risk of Intestinal Parasitic Infections in People with Different Exposures to Wastewater and Fecal Sludge in Kampala, Uganda: A Cross-Sectional Study. PLoS Negl Trop Dis. 2016; 10(3): e0004469. doi: 10.1371/journal.pntd.0004469 PMID: 26938060

4. 0 CRITICAL OVERVIEW

4.1 Development of appropriate methods for the accurate detection and quantification of STH eggs.

4.1.1 What is the current state-of-art in the detection and quantification of STH eggs in the environment?

Accurate detection and quantification of STH eggs in environmental samples is critical for the determination of infection risks from exposure and the adoption of risk reduction strategies. The heterogeneous nature of environmental samples poses a challenge for researchers (Collender et al., 2015). Despite the occurrence of different methods, a general consensus has not been reached and there is no internationally accepted method for efficient detection and quantification of STH eggs in the environment (Bowman et al., 2003). The lack of a uniform method creates a challenge for comparative assessment of STH egg concentrations, both in different sample matrices as well as between locations. In the absence of a standardized method, a review of the existing methods, with a critical assessment of the various steps may help in the selection of the best procedure for accurate detection of STH eggs. **Paper I** was based on a literature search in PubMed and Science Direct and considered publications from different geographical regions. Methods reported in original research articles were grouped into conventional, molecular and emerging techniques and the main steps and challenges associated with each sample matrix discussed and compared.

The major steps in the methods reported in literature are filtration/sieving, egg recovery or separation from other particles, sedimentation, flotation and microscopy. These will result in the main variations in and between the methods. Concentration of STH eggs in the different sample matrices differed between studies (refer to Tables 1-4 in **Paper I**), which reflect differences in STH incidence within and between the populations. The difference in STH egg concentrations may also be attributed to the variation in egg recovery of the different methods. However, egg

concentrations in studies from developing countries generally are higher than from developed countries (Tables 1-4 of **Paper I**).

High solid content in samples has the potential to result in lower egg recovery, partly due to attachment of eggs to these particles. Additionally, particles on the microscope slide during the microscopy stage will interfere with the correct identification and quantification of the eggs (Bowman et al., 2003; Katakam et al., 2014; Engohang-Ndong et al., 2015). Most of the conventional methods employ a differential flotation step to achieve a further separation of the STH eggs from other particles. The loss of eggs in this step relates to the specific gravity of the flotation solution versus the relative density of the eggs. Section 3.3.2 (**Paper I**) discusses the various flotation solutions used and the reported recovery efficiencies, whilst also considering the challenges involved with each choice of flotation solution. **Paper I** established that the use of flotation solutions with specific gravities lower than 1.3 may result in the loss of STH eggs such as *Taenia* spp eggs (David and Lindquist, 1982). Therefore, the choice of solution and the specific gravity should be dependent on the type of STH eggs in the samples to be analyzed.

Some methods also incorporate a filtration step, using sieves of different pore size. The pore size of the sieve was found to be an important factor in ensuring that a high egg recovery is achieved whiles removing particles. The pore sizes are mainly influenced by the type of STH prevalent in the study area. Additionally, the use of detergents and other related solutions, that aid in the separation of the eggs from other particles result in enhanced egg recovery

In addition to the conventional methods described above, **Paper I** also presents some of the more advanced molecular methods (e.g. PCR, qPCR, LAMP etc.) used for the detection and in some cases quantification of STH eggs in environmental samples. These have the major advantage over the conventional microscopy methods in that they enable a differentiation of STH eggs to the

species level (Valero et al., 2009; Ai et al., 2010; Basuni et al., 2011; Verweij et al., 2007; Verweij et al., 2009). This is a major challenge with microscopy, due to similarities in egg morphology. The molecular techniques are also faster than the conventional methods, but conventional microscopy rapidly gives an overview of different parasites present. The molecular methods are further not fully optimized for routine use and there are major challenges hindering their widespread application. Extraction of DNA of good quantity and quality is a major bottleneck, mainly due to the tough egg shells. These shells serve as a protective shield for the STH eggs, impeding the extraction of the DNA. Several DNA extraction methods or protocols have been used with varying results but not systematically assessed. Table 6 of **Paper I** presents some of these protocols/methods and the STHs reported. Environmental samples may also contain substances, such as lipids, proteins etc. that may act as PCR inhibitors (Josefsen et al., 2015), which creates an additional challenge for the detection of the eggs with molecular methods. The high cost of molecular techniques has also hindered their adoption for routine laboratory analysis. For the future several other methodological candidates could be used for the detection and quantification of STH eggs but most of these are yet to be tested on environmental samples. In instances where the uses of these are reported in environmental samples, there is the lack of adequate data to confirm their applicability. Table 7 of **Paper I** present some of the advantages and disadvantages in the use of conventional, molecular and new/emerging techniques in the detection and quantification of STH eggs in environmental samples.

In addition to the identification and discussion of the critical steps in the analysis of environmental samples for STH eggs, **Paper I** also identified the knowledge gaps in the quest for an internationally accepted standardized method. For instance, it was determined that there is the need for quantitative assessment of the effect of the various chemicals/reagents that are used in these

methods on egg viability. As discussed in Section 3.3.3 of **Paper I**, ethyl acetate, diethyl ether, acetoacetic buffer etc. may result in the loss of over 95% of viable eggs (Satchwell, 1986).

4.1.2 Effect of reagent on STH egg viability

Since the environment, especially soil, plays a critical role in the transmission of STH infections (discussed in the literature **Chapter 2,** Section 2.1) viability assessments are essential. Therefore, to assess the infection risks or the efficiency of treatment barriers, it is necessary to accurately quantify the viable STH eggs in the environment. In the current variety of methods different reagents/chemicals are used that may both affect the efficient recovery and partly the viability of eggs. **Paper I** showed that steps such as the separation of eggs from other particles (Section 3.2.1), phase extraction (Section 3.3.3) and viability determination through incubation (Section 3.4), are major steps that may be affected and result in method variations. These variations are due to the use of different reagents based on personal preference of researchers and their reported improvement of viable egg recovery. Some researchers have reported that some of these reagents might affect viable egg recovery due to their inactivation of the eggs (Oksanen et al., 1990; Gaspard et al., 1995; Nelson and Darby, 2001). Using *Ascaris suum* eggs as a surrogate for STH eggs, **Paper II** provides an assessment of the impact of selected reagents (commonly used) on the viability of these eggs. The duration of exposure and the resulting impact was assessed. Prior to this viability assessment, the use of distilled water, 0.1N sulphuric acid and 0.5 % formalin as incubation solutions was determined and compared. Incubation with these three solutions for at least 28 days is commonly used to determine the viability of recovered STH eggs (see Section 3.4 of **Paper I**).

The use of 0.1N sulphuric acid for incubation gave the highest percentage of viability, followed by distilled water. The latter gives an option for laboratories that cannot afford the use of sulphuric acid. Additionally, distilled water does not have the same toxic effect, as reported for sulphuric

acid or formalin. The use of distilled water for incubation may however result in the growth of fungi and bacteria (Ciarmela et al., 2002) during the incubation stage, whereby it (distilled water) may be used with caution. **Paper II** and the works of others (Oksanen et al., 1990; Nelson and Darby, 2001; Pecson and Nelson, 2005; Karkashan et al., 2015) helps in the selection of the best incubation solution for viability determination. This, in itself, will not guarantee accurate quantification of viable eggs in environmental samples. There are a number of reagents used prior to incubation that have been reported to result in the inactivation of STH eggs. The loss in viability may be attributed to the degradation of the lipid layer of the eggs (which is the last defense for the eggs). As shown in Table 1 of **Paper II**, exposure of *Ascaris suum* eggs to acetoacetic acid, ethyl acetate and a combination of these resulted in the highest loss of viability as compared to Tween 80 and ammonium bicarbonate. These detergents (Tween 80 and ammonium bicarbonate) have been associated with improved recovery of STH eggs from environmental samples, such as sludge and UD toilet waste (Zenner et al., 2002; Trönnberg et al., 2010). Comparison of the inactivating impact of the reagents on eggs in distilled water, wastewater and sludge, showed higher inactivation in eggs suspended in distilled water than in other sample matrices. This was attributed to the antagonistic effect of substances/materials in the wastewater and sludge, or the absorption of these reagents by particulate matter (Lajeunesse et al., 2008; Gracia-Lor et al., 2010). As discussed in **Paper I**, reagents such as ethyl acetate, diethyl ether and acetoacetic buffer are only added to the samples after the removal of particles (through sieving/filtration, sedimentation and flotation). Therefore, the protective impact of the wastewater and sludge will be lower than reported in Table 4 and more in line with results in Table 1 (**Paper II**).

In circumstances where there is no other option, exposure of the eggs to ethyl acetate, diethyl ether and acetoacetic buffer should not exceed 5 minutes as was concluded in **Paper II**. Duration of

exposure may be reduced by thoroughly rinsing the eggs after steps involving these reagents so as to completely remove any residual concentration of the reagents on the eggs before incubation.

4.1.3 A revised method for STH egg detection in different environmental matrices.

A review of existing methods as presented in **Paper I** and discussed in Section 4.1.1 above, showed that two common methods have been used with several variations in different steps. These vary in relation to both reagents used as well as the sample matrices. **Paper I** discussed a number of critical steps that result in major variations and therefore have the potential to affect the recovery of viable eggs. **Paper II** (discussed in Section 4.1.2) addressed some of these contentious steps, such as the best incubation (among the three common solutions) and effect of selected reagents on egg viability. Based on the results from these two papers, a revised method for the detection and quantification of STH eggs was developed and a Standard Operation Procedure (SOP) (see **Appendix I** for full SOP) was prepared.

In brief, this method (and the accompanying SOP) presents an approach for the detection and quantification of STH eggs in different environmental matrices (wastewater, sludge, soil etc).

The various steps involved in the pre-processing are dependent on the type of sample and is largely influenced by the solid contents. Samples with high solid contents, such as untreated wastewater, sludge UD waste etc are initially mixed with Tween 80 or ammonium bicarbonate to aid in the separation of the STH eggs from other solids (see Figures 5-8). Dried sludge and other solid samples are soaked in deionized water for 12-24 hours to enable to the breaking of clumps of solids, this is essential in the release of eggs (see Step 2 in Figure 8). After these soaking steps (either with detergents or deionized water) the samples are treated through the same process as liquid samples with low solid contents (e.g treated wastewater and surface water). Samples are

poured through a 100µm unto a 20 µm sieve, this ensures the elimination of particles that may interfere with microscopy (Step 2 of Figure 5 and Step 3 for Figures 6-8). The filtrand on the 20 sieve is then carefully collected into Falcon tubes and processed.

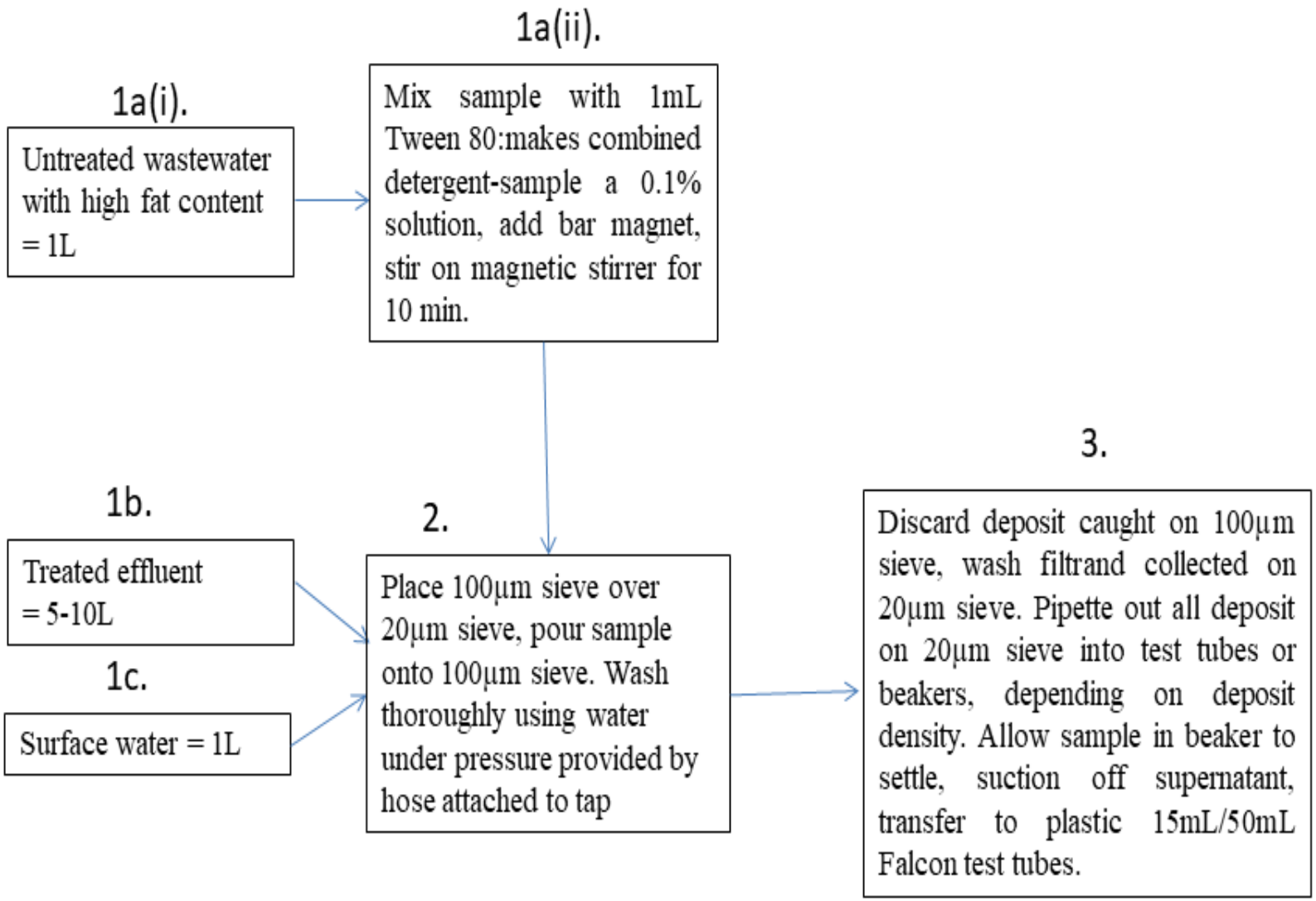


Figure 5: Flow chart for pre-processing of water and wastewater samples for the detection and quantification of STH eggs

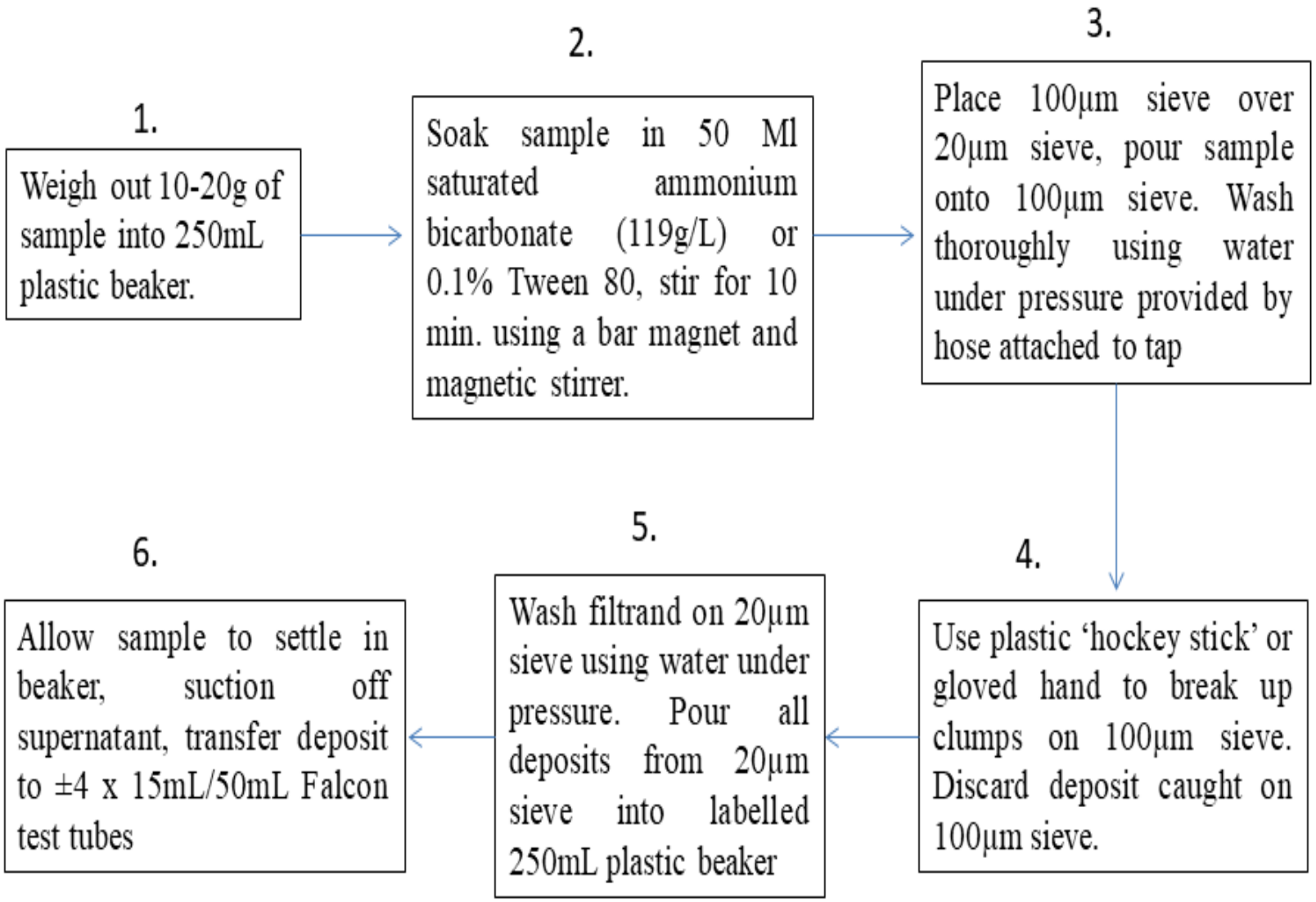


Figure 6: Flow chart for pre-processing of wet or moist sludge samples for the detection and quantification of STH eggs

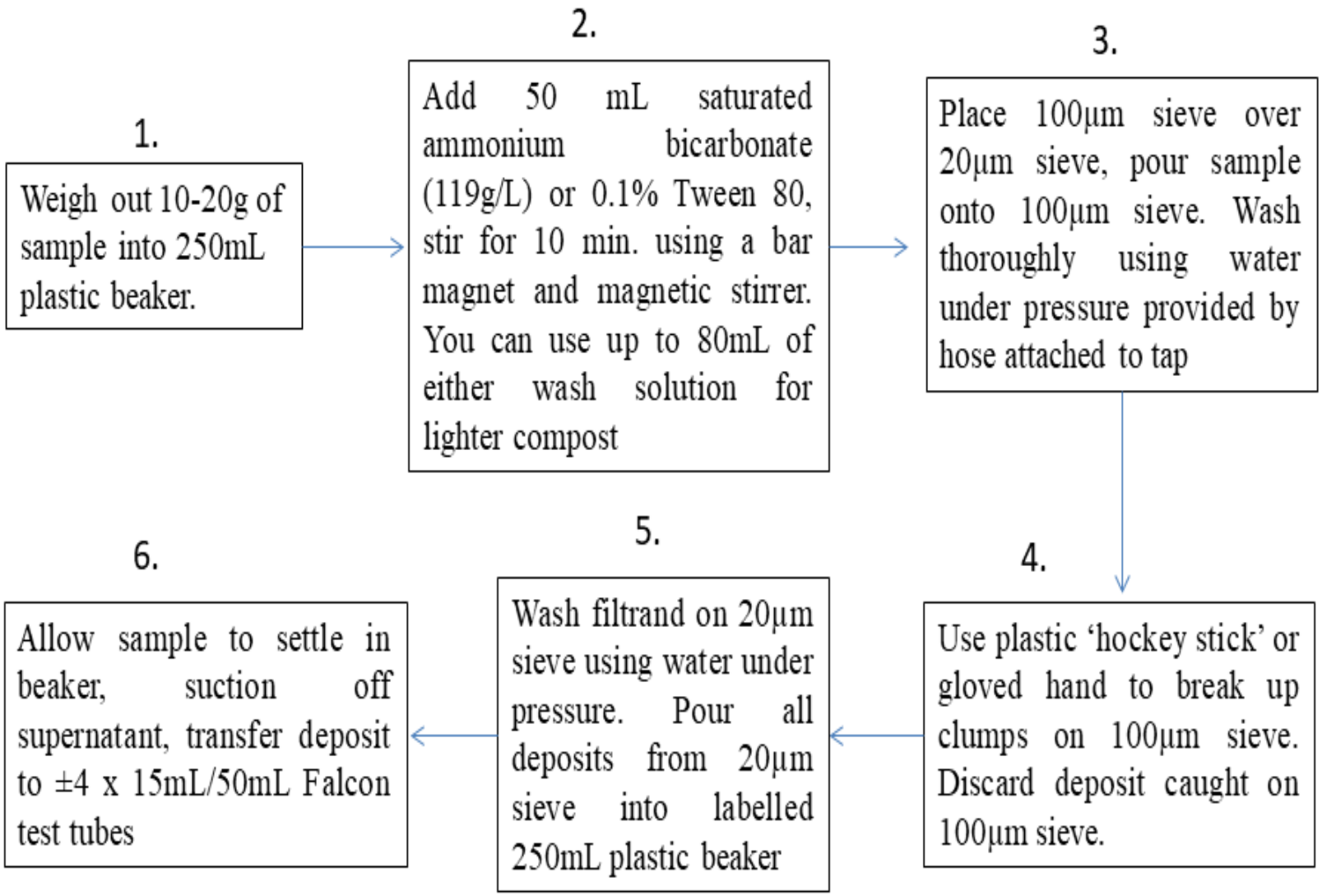


Figure 7: Flow chart for pre-processing of composted sludge and UD waste samples for the detection and quantification of STH eggs

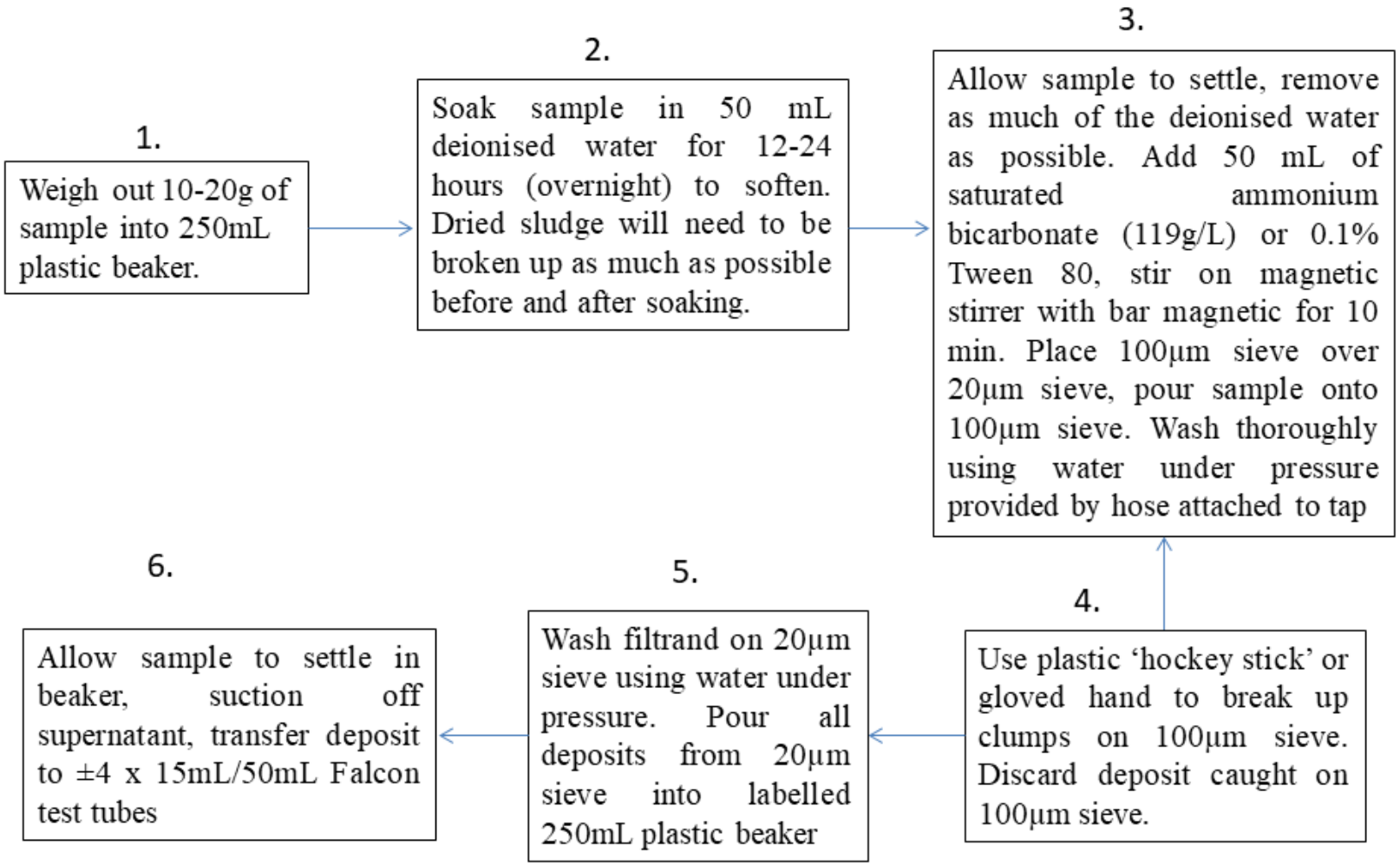


Figure 8: Flow chart for pre-processing of dry or hard sludge samples for the detection and quantification of STH eggs

The different approaches help to account for the heterogeneity and variability of the various environmental samples. The use of 'one-fit-all' method for analyzing different environmental samples, has the potential to adversely affect egg recovery. Environmental samples have high variability therefore development of an internationally accepted method must account for this sample variability as shown in the approaches presented by Figures 5-8. Although there is variability in the processing of samples depending on the solid contents in this method, the use of same steps after sieving ensures consistency in the subsequent steps. With this revised method, the introduction of specific steps for different sample matrices ensures a consistent approach providing a uniform method for the detection of STH eggs in these samples.

Spiking of sludge and wastewater samples gave recovery percentages of 80.25% to 97.63% respectively. These recovery percentages were calculated using the "split-spike" technique reported in Bowman et al., (2003). In this technique, each sample was divided into three sub-samples, the first two examined for precision and the third was spiked with a known concentration of *Ascaris suum* eggs and then analyzed for accuracy. These spiking experiments were done six (6) times in different sample matrices to provide the need precision and accuracy data. Sensitivity analysis indicated that the method could detect eggs as low as 2-3 eggs per liter in wastewater samples, however in sludge sensitivity was between 5-7 eggs per 20 grams. The reduction in sensitivity for sludge analysis could be due to the attachment of eggs to the solids in the samples, which might result in the loss of eggs during the sieving step (see Figures 2-4). Method sensitivity is very important in determining the quality of wastewater and sludge in relation to international and national wastewater/sludge treatment and reuse standards or guidelines. The recovery percentages and sensitivity results reported determine during the method validation stage were used to accurately determine the STH egg concentration in actual samples. This method has been

successfully used to consistently detect and quantify different types of STH eggs from a variety of sample matrices. The results of which were published and will be discussed in the subsequent sections of this chapter.

The consistent recovery percentage of this method could be attributed to several factors. The use of sieves for filtration reduces the amount of particulate matter in the final pellets, whereby there is less interference in the microscopy counting step. Additionally, the removal of the sedimentation step considerably reduces the time required for the separation of the eggs from other particles, both for sludge, sewage, soil and other sample matrices with high solid contents. Passive sedimentation (**Section 3.3.1** of **Paper I)** is time consuming and susceptible to error, resulting in lower recovery percentages. The increased recovery of eggs could also be attributed to the use of zinc sulphate with a specific gravity of 1.3. The use of flotation solutions with lower specific gravity has the potential to result in the loss of some STH eggs, e.g. *Taenia* spp eggs (**Section 3.3.2** of **Paper I)**. The revised method does not involve the use of ethyl acetate and acetoacetic acid, based on their impact on egg viability as concluded in **Paper II.** These reagents are mainly used to remove lipids and proteins from samples (Verbyla et al., 2016; Satchwell, 1986), their exclusion may result in high lipid content in the samples after processing and may interfere with microscopy. However, the use of the detergents in the pre-processing stage eliminates these lipids and related materials/substances. The removal of this phase extraction step also reduces the number of steps involved in sample processing. Reduction of steps reduces time needed to process samples and gives less room for errors.

Selectivity in the use of conventional methods for the detection and quantification of STH eggs is subject to the experience of the microscopists. Differentiating between STH eggs and debris and between different types of eggs is solely dependent on the experience of the individual. However,

with considerable reduction in the amount of debris on the microscope slide, due to the sieving step, the influence of debris on the method selectivity is reduced. The selectivity of the method could also be enhanced by using zinc sulphate of different specific gravity, since different eggs have varying relative densities.

The method and accompanying SOP, presented in **Appendix I,** were developed as part of a project funded by the Bill and Melinda Gates Foundation (BMGF) with the collaboration of researchers and laboratories from South Africa, Mexico, India, Senegal, Brazil and Ghana. In addition to the method validation in relation to recovery percentages, a full quality assurance and quality control (QA/QC) assessment is currently ongoing in these partner laboratories. The outcome of this would provide QA/QC data to back the method which is very important for its adoption as an internationally accepted method. The aim of this process is to use this method as a basis for the development of an ISO certified method for the detection and quantification of STH eggs in the environment.

4.2 Reduction of STH eggs through wastewater and sludge treatment

Wastewater and sludge are known to contain a variety of contaminants including STH eggs (**Section 2.5.1 of Chapter 2**). These may adversely affect the health of exposed populations. Exposure to wastewater or sludge either intentionally or unintentionally has been linked to an increase in STH infections (Seidu et al., 2008b; Blumenthal et al., 2001; Pham-Duc et al., 2013; Rutkowski et al., 2007; Trang et al., 2006; Habbari et al., 1999). This exposure may be through occupational (e.g waster irrigation or sludge application in agriculture) or recreational use (such as swimming in wastewater contaminated rivers, streams or lakes). To protect public health there

is the need for treatment of wastewater and sludge before discharge to the environment and the determination of treatment efficiency is therefore important.

4.2.1 Comparison of the STH removal efficiency by centralized and decentralized wastewater treatment

Different wastewater treatment technologies have been developed and implemented over the years. Conventional centralized wastewater treatment plants (WWTPs) are the main treatment option but conventional WWTPs are expensive to construct and maintain (Massoud et al., 2009). Low cost decentralized wastewater treatment options have been researched and implemented. Anaerobic baffled reactors (ABRs) and planted gravel filters (PGFs) are some of these decentralized treatment technologies that have seen an increased use in the last decade due to simple, inexpensive construction and stable operational conditions (Tilley et al., 2014; Reynaud and Buckley, 2016).

Pathogen reduction by ABRs and PGFs has been studied (Foxon et al., 2004; Nasr et al., 2009) but these studies have focused on indicator microorganisms such as *E. coli* and total coliforms with limited information on the removal of STH eggs (Johansen et al., 2013; Hailu, 2006). The removal of bacteria varies significantly from the removal of STH eggs due to difference in their survivability, size and the mode of removal. STH eggs are known to form part of the solid component of wastewater and are therefore removed largely by sedimentation and flocculation processes (Mara and Horan, 2003). Despite the increase in the use of DEWATS for wastewater treatment, there is no comparative assessment of their performance in removing STH eggs as against the widely used conventional WWTPs. This knowledge is important in validating these (DEWATS) treatment approaches and will provide an indication of the public safety issues surrounding wastewater treatment.

Paper III presents a comparative study on the removal of STH eggs in decentralized ABRs and PGFs (referred to as DEWATS here) and centralized WWTPs in Durban, South Africa and Maseru, Lesotho. Lesotho was chosen for the assessment of the ability of the DEWATS in removing these eggs due to the large number of operational plants. These were therefore chosen based on availability and accessibility. Using the method discussed above (Section 4.1.3) and presented in the **Appendix I**, the STH egg concentration in wastewater from different stages of treatment (see Section 2.1.1 of **Paper III**) at the three (3) centralized and one DEWATS plants in Durban was determined. The conventional WWTPs have different treatment capacities and serve communities with varying socio-economic status. A total of ten DEWATS plants were studied in Maseru. These were chosen to represent individual households and institutional plants.

STH egg concentrations differed between the populations in the city of Durban as was evident by the variation in egg concentrations in the untreated wastewater at the WWTPs. STH egg concentration gives an indication of the level of infection within different populations, with temporal and spatial variations depending on the source of the wastewater (Chan, 1997; Mara and Horan, 2003). In Maseru, the concentration of STH eggs in raw wastewater differed between the different DEWATS plants. The ones located near orphanages and schools had a higher egg concentration. This also adds to the knowledge on the high incidence of STH infections among children of school age as reported in literature. However, it must be noted that the concentration of STH eggs in the wastewater and sludge in this study was different from reports from clinical studies. This could be attributed to several factors, such as, prevalence of infections in the study populations, survivability of the STH eggs in the environment and possible eggs from other species of STH that infect animals.

The DEWATS showed a much higher removal of STH eggs than the centralized WWTPs which could be attributed to the differences in the treatment process (Mara and Horan, 2003; Rose et al., 1996; Mayer and Palmer, 1996; Dowd et al., 1998). The high removal efficiencies seen in the DEWATS plants could be attributed to the process of forcing the influent wastewater through the sludge bed/blanket of the upflow baffles in the DEWATS. This process enhances egg removal through filtration and aggregation (Mara and Horan, 2003). DEWATS plants with biogas accumulation had lower removal efficiencies, attributed to lower hydraulic retention time (HRT). Additionally, the PGFs increases the STH removal efficiencies (Stott et al., 2002), in Lesotho it was observed that in DEWATS plants with functioning PGFs, the removal efficiencies were much higher.

By applying the developed method (Appendix I), it was possible to show in **Paper III** that DEWATS plants are efficient in removing STH eggs from wastewater, comparatively higher than the centralized WWTPs.

4.2.2 Concentration of STH eggs in sludge samples after drying

Reduction or elimination of STH eggs through wastewater treatment is achieved by removing the eggs from the wastewater into the sludge through processes such as filtration, sedimentation, flocculation etc (Jimenez-Cisneros, 2006; Mara and Horan, 2003). STH egg concentration in sludge is mostly higher than in the wastewater as confirmed by results in **Table 4** of **Paper III** and **Table 2** of **Paper IV**. Contamination of wastewater and sludge in this case with animal faeces could lead to high counts of STH eggs, especially from STHs that infect animals, such as *Toxocara* spp or *Ancylostoma* spp (type of hookworm). Therefore, sludge application as a fertilizer has the potential to greatly increase the risk of STH infections. Application of sludge as a fertilizer or soil

conditioners has so many benefits for soil fertility resulting in high crop yield (Navarro-Pedreño et al. 2003; Almendro-Candel et al. 2014). However, with the high concentrations of STH eggs, this practice must be regulated to ensure public safety.

Sludge can be treated by a variety of processes as elaborated in **Table 7 of Chapter 2** with varying efficiencies. In South Africa and many other parts of Africa, including Senegal, sludge is treated by drying. The effectiveness of this process in inactivating viable STH eggs is dependent on the ambient temperature and solar irradiation under which the drying takes place (Berendes et al., 2015; McKinley et al., 2012; Szabova et al., 2010). Duration of drying under these conditions is very important. Sludge is most commonly dried for 60 days in Durban, due to high demand for drying space/beds. Determination of the number of viable STH eggs in the sludge at the end of the drying period is important in determining the health implications associated with the use of this sludge as a fertilizer or soil conditioner.

Paper IV gives an assessment of STH egg concentration in sludge from Durban, South Africa and Dakar, Senegal. Using the method developed and discussed already, the viable egg concentrations were assessed after the 60-day drying period. The rate of decay of STH eggs under the drying conditions described was also calculated by determining the concentration of viable STH eggs on a weekly basis for a period of 90 days.

STH egg concentrations were higher in sludge from Dakar than Durban, with variation in the type of STH detected in the two study areas (Table 2 of **Paper IV**). The variation in the egg concentrations may be attributed to the difference in the prevalence and intensity of STH infections. Over the ten-month study period, the concentration of viable STH egg in the sludge from Durban showed significant variations, probably due to changes in the environmental

conditions. This change in environmental conditions has the potential to affect the rate of decay of the eggs under the conditions mentioned (Berendes et al., 2015; Belcher et al., 2015). The rate of decay therefore needs to be determined for different locations where this information is important to determine the best treatment process and duration of treatment needed to produce sludge that is safe for reuse.

The concentration of STH eggs reported in **Paper IV** and the decay rate calculated implies the sludge needs further treatment with processes such as composting, thermophilic digestion, lime stabilization etc. (see **Table 7 of Chapter 2**) to produce sludge meeting the various guidelines presented in **Table 5 of Chapter 2**.

4.3 Risk of STH infection from exposure to eggs in the environment

Exposure to wastewater/sludge either treated or untreated could result in an increase in STH infections. As shown in **Papers III** and **IV**, wastewater and sludge treatment does not always result in the total removal or inactivation of these eggs, which may result in intentional or unintentional exposures. The risks of infections differ depending on the amount of viable STH eggs ingested by exposed populations where these risks will also differ based on the populations exposed. Risks of infection could be estimated either directly or indirectly as presented in **Section 2.6**.

4.3.1 Probabilistic estimation of STH infection risks from direct and indirect exposure to effluents from centralized and decentralized WWTPs.

The QMRA approach, as described in **Section 2.6.2 of Chapter 2** was used to estimate risks of STH infections for different populations exposed, either directly or indirectly, to effluents from the WWTPs studied and reported in **Paper III.** *Ascaris* spp was used as a surrogate for STHs. QMRA played an important role in developing the WHO and Australian wastewater reuse guidelines (NRMMC, 2004; NRMMC et al., 2006; WHO, 2006). Several researchers have effectively used this approach to estimate risks of STH infections for different populations exposed

to wastewater directly or indirectly (Cutolo et al., 2012; Navarro and Jiménez, 2011; Navarro et al., 2009; O'Connor et al., 2017; Kundu et al., 2014). The estimated risks from these studies vary, due to the choice of dose response models and most importantly the ingested dose. Dose of pathogens used in QMRA is either assumed or measured. The accurate detection of the STH eggs in environmental samples is therefore important for estimation of risks from environmental exposure.

The QMRA framework used for the determination of probability of infection with exposure to the effluents from the WWTPs, presented in **Paper III,** was developed based on observed and assumed exposure scenarios. This framework takes into account farmer and consumer risks associated with wastewater irrigation, risks of infection from immersion in the maturation ponds (centralized WWTPs) and playing new the PGFs of the DEWATS were also considered (**Section 2.5** of **Paper III**).

Ascaris spp egg concentration, in the effluents from all the WWTPs (**Tables 4 and 5** of **Paper III**), was beyond the WHO guideline value of 1 egg per liter for irrigation water. Risks of infection for farmers were therefore found to be higher than the WHO tolerable risks figure (10^{-2} pppy) (WHO, 2006). Reuse of the effluents from the DEWATS plants posed the least risk, as compared to effluents from the centralized WWTPs. This was expected as the concentration of viable *Ascaris* spp eggs in the effluents from the centralized WWTPs was higher than the DEWATS plants. In Maseru, Lesotho, reuse of the effluents from the DEWATS plants is common, driven by dry conditions. Consumption of farm produce, especially vegetables from these farms may result in infections, due to possible contamination. **Paper III** presents risks estimate for this exposure (See **Table 8** of **Paper III**), which were also higher than the tolerable risks index. One-time consumption of these vegetables may not result in higher risks of infection, however considering

the fact that multiple consumption would increase risk as shown in the table (**Table 8** of **Paper III**). Although risks of infection for farmers using the effluents of the DEWATS plants is low, it is imperative to incorporate further risk reduction measures as suggested by the WHO multi-barrier approach. The multi-barrier approach to risk reduction is important especially for the consumers in situations where further wastewater treatment is not feasible. This approach incorporates a series of steps aimed at reducing the concentration of microbial pathogens. This could be achieved by a further treatment step, use of different irrigation approaches to reduce contamination of edible parts of crops, cessation of irrigation before harvest, washing or disinfection of produce with either bleach, vinegar etc. (Amoah et al., 2011). Farmer risks could be reduced by simple on-farm interventions, such as the use of protective clothing, effluent storage for some days and adoption of irrigation methods that reduce their exposure to the water (Keraita et al., 2008; Qadir et al., 2010).

Risks of infection from the other exposure scenarios considered in **Paper III**, such as immersion/swimming in the maturation ponds (centralized WWTPs) or playing near the PGFs of the DEWATS plants could potentially lead to higher infections. It was observed in Maseru that most of these plants were not fenced and in some instances, there is overflow of the PGFs exposing households to the wastewater. Although lower viable STH eggs were recorded in the effluents from the DEWATS plants, poor maintenance of the plants resulting in the observed overflows may defeat the purpose of wastewater treatment.

4.3.2 Estimation of STH infection risks from agricultural application of sludge

Sludge from Durban and Dakar contained higher concentrations of STH eggs, than the South African national and WHO guideline values for sludge reuse (**Paper IV)**. Therefore, application of sludge on these farms could lead to STH infections for both farmers and consumers of farm

produce. Probabilistic assessment of the STH infection risks showed that farmers applying this sludge without adequate protective measures have high risks of infection (**Table 4** of **Paper IV**). These risks were higher than the WHO tolerable risks figure (10^{-2} pppy) for both study areas but risks were marginally lower in Durban than Dakar, as expected, due to a lower viable egg concentration.

Agriculture in developing countries is labor-intensive and accidental ingestion of small amounts of soil during land tilling is a very likely event (Seidu et al., 2008a). Ingestion of sludge amended soil is another major route of possible infections. Estimation of STH infection risks from this scenario must take into account the decay of the eggs on the field. Most risk assessment studies use decay rates calculated from different locations where the environmental conditions contributing to the decay of the eggs are different. The use of these rates has the potential to result in the wrong estimation of risks. **Paper IV** presents a decay rate from the drying of sludge in Durban, giving a rate that is applicable to this area and therefore reduces the uncertainty in the risks estimates. Taking this decay into consideration, the risks of infections reduced over time as shown in Figure 6 (**Paper IV**). Based on the estimated risks of infection after decay, exposure to farm soil after 40-50 days of sludge application may reduce the risks of infection to levels lower the WHO tolerable risks figure. However, this may not be practical due to the farmers needs to attend to their crops frequently.

At the time of harvest, produce (e.g lettuce) could be contaminated with sludge with a direct transmission route to the consumer (Westrell et al., 2004; Schönning et al., 2007). Since produce are harvested days or months after the application of the sludge, the concentration of viable eggs is expected to reduce. **Paper IV** presents a QMRA framework that was effectively used to estimate the risks of STH infection following sludge application taken into account the number of days after

application to harvesting. For instance, using lettuce as a representative vegetable, harvesting of vegetables in Dakar could be done after 40 days of sludge application to reduce the risks of infection to the WHO tolerable value but in Durban harvesting after 30 days ensures that consumers are protected.

Accurate estimation of viable STH eggs after sludge treatment is a key component in ensuring public health where the findings of **Paper IV** indicates that drying of sludge in Durban and Dakar does not produce sludge meeting both local and international standards and guidelines for sludge reuse whereby there is the need further treatment.

4.3.3 Direct estimation of contribution of wastewater irrigation to STH infection among farmers.

Estimation of risks of STH infection using the QMRA approach is indirect though the use of mathematical models. This approach has a number of limitations and uncertainties but risks of infection or actual infections could also be accurately determined through epidemiological studies. The advantages and disadvantages of these two risk estimation approaches have been discussed in **Section 2.6 of Chapter 2**. Different epidemiological approaches have been used to successfully determine the link between wastewater/sludge reuse and STH infections (see **Table 3 of Chapter 2**).

STH egg concentration in wastewater used for vegetable irrigation and in the farm soil was determined and the contribution of exposure to these by farmers on infections determined through stool analysis. Wastewater used for irrigation of urban and peri-urban farms in the Ghanaian city of Kumasi was found to contain high *Ascaris* spp and hookworm egg concentrations exceeding the WHO guidelines (See **Table 1** of **Paper V**). As suspected, egg concentrations in the soil were significantly higher than the wastewater, which might be due to accumulation of STHs eggs in the

soil. Additionally, *Ascaris* spp eggs have been found to attach to soil particles (especially clay) thereby contributing to their high concentrations (Landa-Cansigno et al., 2013). Egg concentrations in both irrigation water and soil were higher in the wet season (**Figures 2** and **3** of **Paper V**) than the dry season, which may be attributed to rainfall and reduced temperatures extending the survival period of the eggs (Gaasenbeek et al., 1998; Feachem et al., 1983).

Farmers were found to have a higher prevalence of STH infections than a non-farmer control group. Due to frequent exposure to the wastewater and farm soil with high STH egg concentrations, farmers had higher risk of infections with *Ascaris* spp (OR = 3.9, 95% CI, 1.15±13.86) and hookworm (OR = 3.07, 95% CI, 0.87±10.82). These odds were higher due to soil than wastewater ingestion, understandably so due to a higher egg concentration in the soil than the irrigation water.

The prevalence and intensity of infections among the farmers were higher in the wet season than the dry season. A seasonal variation in infection consequently occurred. This could be attributed to the increase in STH egg concentrations in both the irrigation water and soil. An increased infection prevalence and intensity could also be responsible for the higher egg concentrations in the wastewater since the source of contamination in the wastewater or soil is faecal matter from infected individuals. This variation in infection prevalence and intensity could therefore indicate a dose response relationship, which plays an important role in understanding the role the environment plays in the transmission of infections (such as STHs). Deworming of infected individuals prior to the start of the studies and after the first round of stool sampling showed the re-infection rate, which is a function of continuous exposure. Chemotherapy has been used as the first intervention in reducing or eliminating STH infections, however the rate of re-infection determined in **Paper V** shows that this approach alone would not achieve the desired results.

Continuous exposure to the infective eggs/larvae in the environment as seen in this **Paper V** requires the adoption of a combination of both chemotherapy, behavioral and occupational interventions.

Direct (epidemiological) and indirect (QMRA) estimation of STH infection risks from exposure to wastewater, sludge, consumption of crops etc. play an important role in reducing STH infections. These approaches vary in their limitations and the inputs necessary for accurate risk estimation. However, they both require accurate quantification of STH egg concentrations in the exposure medium (e.g. wastewater, sludge, soil etc.). For instance, inaccurate estimation of STH egg concentrations in wastewater, sludge etc. will result in the use of wrong dose for QMRA and this may not provide a clear link between the exposure and infection and therefore result in the wrong estimation of the contribution of the exposure to the infection.

Therefore, the revised/amended method for the detection and quantification of STH eggs created an avenue to accurately quantify these eggs. The quantification of the eggs was instrumental in determining the STH egg removal efficiencies in different WWTPs and sludge treatment. The data also provided inputs for the estimation of STH infection risks for different populations using both QMRA and epidemiological approaches.

4.4 STANDING OF JOURNALS AND RECEPTION OF PUBLICATIONS.

Paper I: This was published in Acta Tropica, a journal with a 5-year impact factor of 2.414. This paper is currently ranked number 5 on the most downloaded articles in Acta Tropica and has received 9 citations as at 25th March, 2018.

Paper II: Published in Water Science and Technology, an International Water Association journal with an impact factor of 1.197. It currently has 283 downloads as at 25th March, 2018 with 192 abstract reads and one citation.

Paper III: This paper was published in Environmental Science and Pollution Research with an impact factor of 2.741. From 24 February, 2018 (date it was published online) to 25th March, 2018 it had 140 downloads.

Paper IV: Published in Journal of Environmental Management, with a 5-year impact factor of 4.712. As at 25th March, 2018 it had one citation.

Paper V: This paper is published in PLOS Neglected Tropical Diseases, a journal with impact factor of 3.948. It has over 1,723 reads and 5 citations as at 25th March, 2018.

5.0 CONCLUSION

The major conclusions based on the objectives of the thesis are;

There are a number of methods used for the detection and quantification of STH eggs in environmental samples. These methods can be categorized into conventional (based on the main principles of sedimentation, flotation and microscopy), molecular (PCR, qPCR) and emerging techniques (LAMP, Flow cytometry).

- Conventional methods are the most commonly used techniques, due to their ease of use and low cost.
- There are a number of these conventional methods, with similar steps and reagents.
- Some of the reagents used in the conventional methods may result in the loss of egg viability, this affects the accurate quantification of viable eggs which is important for determination of treatment efficiency or calculation of infection risks.
- Detection and quantification of STH eggs during the microscopy stage may result in inaccurate data due to subjectivity of the microscopists.
- Molecular techniques have been used to effectively detect, and in some cases quantify (qPCR) STH eggs, in environmental samples.
- These molecular techniques address some of the major challenges with conventional techniques, such as the long duration of sample analysis and possible errors during microscopy.
- Differentiation of eggs at the species level is possible with the molecular methods, a feature which is difficult (if not impossible) with the conventional methods.
- A major challenge with these molecular techniques is the high costs, this limits their routine use. In addition, extraction of DNA of good quality and quantity is a challenge with STH eggs, making the application of molecular methods difficult.
- There are a number of emerging techniques (flow cytometry, LAMP etc.) that could also be applied for the detection and quantification of STH eggs in the environment, however there is the need to further validate these methods.

The use of reagents such as ethyl acetate and acetoacetic acid may result in the inactivation of viable STH eggs during sample analysis. This effect is enhanced with prolonged exposure and also in combination with other reagents such as formalin. Therefore, methods used for the detection and quantification of viable STH eggs must avoid the use of these reagents.

- Using 0.1N sulphuric acid gives the best assessment of egg viability after incubation, distilled water may be used but caution must be taken to avoid growth of fungi and bacteria.
- Ethyl acetate and acetoacetic acid should not be used in the detection and quantification of viable eggs, these reagents result in the loss of egg viability.

- Duration of exposure to reagents such as tween 80 should also be limited to the shortest time possible (below 5 minutes)
- Prolonged exposure of the eggs to reagents used during sample processing may be reduced with thorough washing of the samples with distilled water after each step.

The review of methods and laboratory tests were used to develop a new method for the detection and quantification of STH eggs in environmental samples. This new method provides procedures for analyzing different environmental matrices (wastewater, sludge etc.) and improves the recovery of eggs in these samples. The method is faster than the commonly used conventional methods and has less impact on egg viability resulting in accurate quantification of viable STH eggs.

- The use of sieves of different pore sizes for sample filtration reduces the time need for sedimentation and also reduces the amount of debris on the slides during the microscopy stage.
- Zinc sulphate solution of specific gravity 1.3 ensured the detection of all STH eggs, lower specific gravities may lead to lower recovery of eggs, such as *Taenia* spp eggs.
- With lesser steps than the common methods, it reduces possibility for error as well as improving the consistency and reliability of results.
- This new method can consistently recover between 80.25% to 97.63% of eggs in sludge and wastewater.

The concentration of STH eggs in wastewater from Durban, South Africa and Maseru, Lesotho varied between and within the cities. The removal of these STH eggs by centralized and decentralized (DEWATS) wastewater treatment plants differed, with higher removal percentage achieved with DEWATS treatment. The concentration of STH eggs in the effluents from these plants was above the WHO, USEPA and local guidelines for wastewater reuse in agriculture. Risks of infections were therefore higher than the WHO tolerable risk figure (1×10^{-2}).

- *Ascaris* spp, hookworm, *Trichuris* spp, *Taenia* spp and *Toxocara* spp were the common STH eggs in untreated wastewater in Durban.
- The concentration of the STH eggs varied over the ten-month study period, indicating a change in infection prevalence or intensity in the populations.
- All the STH eggs mentioned above were also found in untreated wastewater in Maseru except for *Toxocara* spp. DEWATS plants in orphanages and schools had higher STH concentrations than individual house plants.
- STH egg removal was higher in the DEWATS plants (between 95-100% removals) than the centralized plants (67-100% removal).

- *Ascaris* spp egg removal was significantly higher than the other STH eggs irrespective of the wastewater treatment plant and type of treatment.
- Exposure to the effluents from these plants during irrigation resulted in high risks of infections for farmers, above the WHO tolerable risks figure.
- Consumers of vegetables irrigated with these effluents are also at risk of STH infection, therefore implementation of the WHO multi-barrier risk reduction strategy will be essential to protect public health.
- Risks of infections were lower due to exposure to effluents from the DEWATS than the centralized plants, due to lower concentrations of STH eggs in the effluents from these DEWATS plants.

Drying of sludge under ambient environment conditions for 60 days in Durban and Dakar is inadequate to inactivate STH eggs. The rate of inactivation of the eggs is too low to produce sludge meeting local and international guidelines and standards. Reuse of this sludge as fertilizer therefore would result in high risks of STH infections among farmers and consumers of the farm produce. Using the decay rate, harvesting of produce after 30 and 40 days, after sludge application, in Durban and Dakar respectively could considerably reduce the risks of infections, due to egg die-off.

- Eggs of *Ascaris* spp, hookworm, *Trichuris* spp, *Taenia* spp and *Toxocara* spp were detected in the sludge, except for Dakar where *Taenia* spp and *Toxocara* spp were not detected.
- STH egg concentrations were higher in Dakar than in Durban, with viable STH egg concentrations from both study areas exceeding the WHO guideline value and USEPA regulatory value after the 60 days drying.
- Concentration of viable STH eggs in the sludge in Durban varied over the ten-month study period, probably due to changes in environmental conditions or variation in infection levels.
- The decay rate determined over a 90 day period was 0.0056, indicating a need for further treatment to produce sludge for acceptable reuse. .
- Risk of STH infections for farmers exposed during application of sludge dried for 60 days was higher than the WHO tolerable risk figure.
- The concentration viable STH eggs in the sludge after drying and the risk of infections estimated with QMRA indicated that 60 days drying of sludge was inadequate to protect public health.

Direct exposure to wastewater during irrigation was associated with a higher risk of STH infection for farmers as compared to an unexposed population. Clinical examination of the stool of the

farmers showed higher prevalence and intensity of STH infection, this was higher in the wet season compared to the dry season.

- Concentration of STH eggs in wastewater used for irrigation in Kumasi, Ghana was found to be higher than the WHO guideline value for unrestricted irrigation.
- Wastewater and farm soil in the wet season had higher egg concentrations than the dry season and is associated with higher STH infection prevalence and intensity among the farmers.
- The observed higher STH egg concentration in the wastewater and soil and the increase in infection prevalence indicate a dose response relationship between environmental exposure and infections.
- The odds ratio for infection for farmers vs non-famers indicate that farmers had a higher risk of infections compared to the non-farmers, especially in the wet season.

6.0 REFERENCES

Abreu-Acosta, N. and Vera, L. 2011. Occurrence and removal of parasites enteric bacteria and faecal contamination indicators in wastewater natural reclamation systems in Tenerife-Canary Islands, Spain. *Ecological Engineering,* 37(3):496–503.

Adams, V. J., Markus, M. B., Adams, J.F.A., Jordaan, E., Curtis, B., Dhansay, M.A., Obihara, C.C. and Fincham, J.E. 2005. Paradoxical helminthiasis and giardiasis in Cape Town, South Africa: epidemiology and control. African Health Sciences, 5(3):276–280.

Adams, V.J., Lombard, C.J., Dhansay, M.A., Markus, M.B. and Fincham, J.E. 2004. Efficacy of albendazole against the whipworm *Trichuris trichiura* - a randomised, controlled trial. South African Medical Journal, 94(12):972-976.

Adamu, N.B., Adamu, J.Y. and Mohammed, D. 2012. Prevalence of helminth parasites found on vegetables sold in Maiduguri, Northeastern Nigeria. Food Control, 25(1):23-26.

Adanir, R. and Tasci, F. 2013. Prevalence of helminth eggs in raw vegetables consumed in Burdur, Turkey. Food Control, 31(2):482–484.

Ai, L., Dong, S.J., Zhang, W.Y., Elsheikha, H.M., Mahmmod, Y.S. and Lin, R.Q. 2010. Specific PCR-based assays for the identification of *Fasciola* species: their development, evaluation and potential usefulness in prevalence surveys. Annals of *Tropical Medicine* and *Parasitology,* 104(1):65–72.

Alemu, A., Atnafu, A., Addis, Z., Shiferaw, Y., Teklu, T., Mathewos, B., Birhan, W., Gebretsadik, S. and Gelaw, B. 2011. Soil transmitted helminths and *Schistosoma mansoni* infections among school children in Zarima town, northwest Ethiopia. BMC Infectious Disease, 11(189):1-7.

Alli, J., Abolade, G., Kolade, A., Salako, A., Mgbakor, C., Ogundele, M., Oyewo, A. and Agboola, M. 2011. Prevalence of intestinal parasites on fruits available in Ibadan Markets, Oyo State, Nigeria. *Acta Parasitologica* Globalis, 2(1):6-10.

Al-Megrin, W.A.I. 2010. Prevalence of intestinal parasites in leafy vegetables in Riyadh, Saudi Arabia. International Journal of Tropical Medicine, 5(2):20-23.

Almendro-Candel, M.B., Navarro-Pedreño, J., Jordán-Vidal, M.M., Gómez, I. and Meléndez-Pastor, I. 2014. Use of municipal waste compost to reclaim limestone quarries mine spoils as soil amendments: effects on cd and Ni. Journal of Geochemical Exploration, 144:263–366.

Almendro-Candel, M.B., Navarro-Pedreño, J., Gómez Lucas, I., Jordán-Vidal, M.M., García Sánchez, E. and Mataix Solera, J. 2002. Movement of Fe, Mn, Cu and Zn in a sewage sludge-treated soil. Waste Management and the Environment, ed. D. Almorza, C.A. Brebbia, D. Sales and V. Popov, WIT Press, pp. 311-320.

Amahmid, O. and Bouhoum, K. 2005. Assessment of the health hazards associated with wastewater reuse: transmission of geohelminthic infections (Marrakech, Morocco). International Journal of Environmental Health Research, 15(2):127–133.

Amoah, I. D., Seidu, R., Reddy, P. and Stenström, T. A. 2018. Removal of soil-transmitted helminth egg in selected centralized and decentralized wastewater treatment plants in South Africa and Lesotho: Health implications for direct and indirect exposure to the effluents. Environmental Science and Pollution Research, 17:1-13. https://doi.org/10.1007/s11356-018-1503-7

Amoah, I.D., Abubakari, A., Stenström, T.A., Abaidoo, R.C. and Seidu, R. 2016. Contribution of Wastewater Irrigation to Soil Transmitted Helminths Infection among Vegetable Farmers in Kumasi, Ghana. PLoS Neglected Tropical Diseases, 10(12):e0005161. https://doi.org/10.1371/journal.pntd.000516.

Amoah, P., Keraita, B., Akple, M., Drechsel, P., Abaidoo, R.C. and Konradsen, F. 2011. Low-cost Options for Reducing Consumer Health Risks from Farm to Fork where Crops Are Irrigated with Polluted Water in West Africa. International Water Management Institute. Research Report 141, pp. 4

Andreoli, C., Ferreira, A., Teles, C., Cherubini, C., Bernet, P., Favarin, F. and Castro, L.2000.Operation of different options to disinfect and dry sludge anaerobically digested. I Seminário Nacional De Microbiologia Aplicada Ao Saneamento. Anais.Vitória. in Portuguese.

Angelakis, A.N., Marecos do Monte, M.H., Bontoux, L. and Asano, T. 1999. The Status of Wastewater Reuse Practice in the Mediterranean Basin. Water Research, 33(10):2201-2217.

Appleton, C.C., Mosala, T.I., Levin, J. and Olsen, A. 2009. Geohelminth infection and reinfection after chemotherapy among slum-dwelling children in Durban, South Africa. South Africa. Annals of *Tropical Medicine* and *Parasitology,* 103(3):249–261.

Appleton, C.C., Maurihungirire, M. and Gouws, E.1999. The distribution of helminth infections along the coastal plain of KwaZulu-Natal province, South Africa. Annals of Tropical Medicine and Parasitology, 93:859–868. doi: 10.1080/00034989957862

Appleton, C.C. and Gouws, E. 1996. The distribution of common intestinal nematodes along an altitudinal transect in KwaZulu-Natal, South Africa. Annals of Tropical Medicine and Parasitology, 90:181–188.

Arvaniti, O. S. and Stasinakis, A. S. 2015. Review on the occurrence, fate and removal of perfluorinated compounds during wastewater treatment. Science of the Total Environment, 524–525:81–92.

Asano, T. 2002. Water from (Waste)Water -- The Dependable Water Resource. Paper delivered on August 13, 2001 at the 11th Stockholm Water Symposium, August 12-18, 2001, Stockholm, Sweden.

Ayres, R.M. and Mara, D.D. 1996. Analysis of Wastewater for Use in Agriculture: a Laboratory Manual of Parasitological and Bacteriological Techniques. World Health Organization, Geneva, Switzerland.

Baker, S.M. and Ensink, J.H.J. 2012. Helminth transmission in simple pit latrines. Transactions of the Royal Society of Tropical Medicine and Hygiene, 106(11):709–710.

Barrios, J., Jiménez, B. and Maya, C. 2004. Treatment of sludge with peracetic acid to reduce the microbial content. Journal of Residuals Science and Technology, 1(1):69-74.

Bartram, J. and Cairncross, S. 2010. Hygiene, Sanitation, and Water: Forgotten Foundations of Health. PLoS Medicine, 7(11): e1000367. doi:10.1371/journal.pmed.1000367.

Bastos, V.K., Cutolo, S.A., Doria, M.C.O. and Razzolini, M.T.P. 2013. Detection and quantification of viable *Ascaris* sp. and other helminth eggs in sewage sludge. *International Journal of Environmental Health Research*, 23(4):352-362.

Basuni, M., Muhi, J., Othman, N., Verweij, J.J., Ahmad, M., Miswan, N., Rahumatullah, A., Aziz, F.A., Zainudin, N.S. and Noordin, R. 2011. A pentaplex real-time polymerase chain reaction assay for detection of four species of soil-transmitted helminths. American Journal of Tropical Medicine and Hygiene, 84(2):338–343.

Battilani, A., Steiner, M., Andersen, M., Back, S.N., Lorenzen, J., Schweitzer, A., Dalsgaard, A., Forslund, A., Gola, S., Klopmann, W., Plauborg, F. and Andersen, M.N. 2010. Decentralised water and wastewater treatment technologies to produce functional water for irrigation. Agricultural Water Management, 98(3):385-402.

Beaudequin, D., Harden, F., Roiko, A., Stratton, H., Lemckert, C. and Mengersen, K. 2015. Modelling microbial health risk of wastewater reuse: A systems perspective. Environment International, 84:131–141.

Belcher, D., Foutch, G.L., Smay, J., Archer, C. and Buckley, C.A. 2015. Viscous heating effect on deactivation of helminth eggs in ventilated improved pit sludge. Water Science and Technology, 72(7):1119-1126.

Belyhun, Y., Medhin, G., Amberbir, A., Erko, B., Hanlon, C., Alem, A., Venn, A., Britton, J. and Davey, G. 2010. Prevalence and risk factors for soil-transmitted helminth infection in mothers and their infants in Butajira, Ethiopia: a population based study. BMC Public Health, 10(21):DOI: 10.1186/1471-2458-10-21.

Berendes, D., Levy, K., Knee, J., Handzel, T. and Hill, V.R. 2015. Ascaris and Escherichia coli Inactivation in an Ecological Sanitation System in Port-au-Prince, Haiti. PLoS ONE, 10(5):1-14.

Bethony, J., Brooker, S., Albonico, M., Geiger, S. M., Loukas, A., Diemert, D., Hotez, P. J. 2006. Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet, 367(9521):1521–1532.

Blaszkowska, J., Kurnatowski, P. and Damiecka, P. 2011. Contamination of the soil by eggs of geohelminths in rural areas of Lodz district (Poland). Helminthologia, 48(2):67–76.

Blumenthal, U.J., Cifuentes, E., Bennett, S., Quigley, M. and Ruiz-Palacios, G. 2001. The risk of enteric infections associated with wastewater reuse; the effect of season and degree of storage of wastewater. Transactions of the Royal Society for Tropical Medicine and Hygiene, 95(2):131–137.

Bogitsh, B.J. and Cheng, T.C. 1990. Human Parasitology. WB Saunders, Philadelphia. USA.

Borecka, A., Gawor, J, Niedworok, M. and Sordyl, B. 2010. Occurrence of *Toxoccara* spp. eggs in household environment of children with diagnosed toxocarosis in Lodz voivodeship. Wiadomości Parazytologiczne, 56(2):141–144.

Bouhoum, K. and Schwartzbrod, J. 1998. Epidemiological study of intestinal helminthiasis in a Marrakech raw sewage spreading zone. Zentralblatt für Hygiene und Umweltmedizin. 200:553–561.

Bowman, D.D., Little, M.D. and Reimers, R.S. 2003. Precision and accuracy of an assay for detecting *Ascaris* eggs in various biosolid matrices. Water Research, 37(9):2063-2072.

Brooker, S., Clements, A. and Bundy, D. 2006. Global epidemiology, ecology and control of soil-transmitted helminth infections. Advances in *Parasitology*, 62: 221–261. doi: 10.1016/S0065-308X(05)62007-6 PMID: 16647972.

Brooker, S., Rowlands, M., Haller, L., Savioli, L. and Bundy, D.A.P. 2000. Towards an atlas of human helminth infection in sub-Saharan Africa: the use of geographical information systems (GIS). Parasitology Today, 16(7):303–307.

Brooker, S., Peshu, N., Warn, P.A., Mosobo, M., Guyatt, H.L., Marsh, K. and Snow, R.W. 1999. The epidemiology of hookworm infection and its contribution to anaemia among preschool children on the Kenyan coast. Transaction of the Royal Society for Tropical Medicine and Hygiene, 93(3):240–246.

Bundy, D.A.P., Chan, M.S., Medley, G.F., Jamison, D. and Savioli, L. 2004. Intestinal nematode infections. In: Murray, C.J.L., Lopez, A.D., Mathers, C.D. eds. The global epidemiology of infectious diseases. World Health Organization, Geneva, Switzerland.

Burkholder, J., Libra, B., Weyer, P., Heathcote, S., Kolpin, D., Thorne, P.S. and Wichman, M. 2007. Impacts of waste from concentrated animal feeding operations on water quality. Environmental Health Perspectives, 115(2):308–312.

Campbell, S.J., Savage, G.B., Gray, D.J., Atkinson, J.A., Soares Magalhães, R.J., Nery, S.V., McCarthy, J.S., Velleman, Y., Wicken, J.H., Traub, R.J., Williams, G.M., Andrews, R.M. and Clements, A.C. 2014. Water, Sanitation, and Hygiene (WASH): a critical component for sustainable soil-transmitted helminth and schistosomiasis control. PLoS Neglected Tropical Diseases, 8(4):e2651. doi: 10.1371/journal.pntd.0002651.

CAMRA-Center for Advancing Microbial Risk Assessment. 2013. Michigan State University, East Lansing, Michigan. http:// http://qmrawiki.canr.msu.edu/index.php/Quantitative_Microbial_Risk_Assessment_(QMRA)_Wiki. Accessed on 12th July, 2017.

Capizzi, S. and Schwartzbrod, J. 2001. Irradiation of *Ascaris* ova in sludge using an electron beam accelerator. Water Research, 35(9): 2256-2260.

Capizzi, S., Chevallier, A. and Schwartzbrod, J. 1999. Destruction of *Ascaris* ova by accelerated electron. Radiation Physics and Chemistry, 56(5-6):591-595.

Capizzi-Banas, S., Deloge, M., Remy, M. and Schartzbrod, J. 2004. Liming as an advanced treatment for sludge sanitisation: helminth eggs elimination--*Ascaris* eggs as model. Water Research, 38(14-15):3251-3258.

Casmo, V., Augusto, G., Nala, R., Sabonete, A. and Carvalho-Costa, F.A. 2014. The effect of hookworm infection and urinary schistosomiasis on blood hemoglobin concentration of schoolchildren living in northern Mozambique. Revista do Instituto de Medicina Tropical de *São Paulo*, 56(3):219-224.

Castells, X.E. 2012. Vías de tratamiento y valorización de fangos de depuradora: Reciclaje de residuos industriales. Ed. Díaz de Santos, Madrid.

Castro, G.A. 1996. Helminths: Structure, Classification, Growth and Development. Chapter 86. Baron S., Ed. Medical Microbiology 4th Edition. NCBI Bookshelf. Galveston (TX). USA.

CDC-Centers for Disease Control and Prevention. 2013. Soil-transmitted helminths.Global Health-Division of Parasitic Diseases. Accessed on 18th September, 2017.

Chan, M.S. 1997. The global burden of intestinal nematode infections-Fifty years on. Parasitology Today, 13(11):438-443.

Ciarmela, M. L., Minvielle, M. C., Lori, G. and Basualdo, J. A. 2002. Biological interaction between soil fungi and *Toxocara canis* eggs. Veterinary Parasitology, 103 (3):251–257.

Cifuentes, E., Blumenthal, U., Ruiz-Palacios, G., Bennett, S. and Peasey, A. 1994. Epidemiologic Setting of the Agricultural Use of Sewage: Valle Del Mezquital, Mexico. Salud Publica de Mexico, 36 (1):3-9.

Collender, P.A., Kirby, A.E., Addiss, D.G., Freeman, M.C. and Remais, J.V. 2015. Methods for quantification of soil-transmitted helminths in environmental media: current techniques and recent advances. *Trends* in *Parasitology,* 31(12):623–639.

Crompton, D.W. and Stephenson, L.S. 1990. Hookworm infection, nutritional status, and productivity. In: Schad GA, Warren KS, eds. Hookworm disease: current status and new directions. Taylor and Frances, London, UK. Pp. 231–264.

Crompton, D.W.T. and Nesheim, M.C. 2002. Nutritional Impact of Intestinal Helminthiasis during the Human Life cycle. Annual Review of Nutrition, 22:35-59.

Crook, J. 2003. Water reuse—an overview. Keynote Speech Presented at MED-REUNET 1 Project, International Seminar on Wastewater Reclamation and Reuse, Izmir, Turkey, 25–26 September 2003. Website: Mediterranean network on wastewater reclamation and reuse. www.med-reunet.com

Cutolo, S.A., Piveli, R.P., Santos, J.G., Montes, C. R., Sundefeld, G., Campos, F., Gomes, T.M. and Melfi, A. J. 2012. Parasitological risk assessment from wastewater reuse for disposal in soil in developing countries. Water Science and Technology, 65(8):1357-1367.

Dabrowaska, J., Zdybel, J., Karamon, J., Kochanowski, M., Stojecki, K., Cencek, T. and Klapec, T. 2014. Assessment of viability of the nematode eggs (*Ascaris*, *Toxocara* and *Trichuris*) in sewage sludge with the use of LIVE/DEAD Bacterial Viability Kit. *Annals* of Agricultural and *Environmental Medicine*, 21(1):35–41.

Dalsgaard, A. 2007. Special issue: Wastewater use—Food safety and health aspects. *Tropical Medicine* and *Health*, 12(2):1–90.

David, E. D. and Lindquist, W. D. 1982. Determination of the Specific Gravity of Certain Helminth Eggs Using Sucrose Density Gradient Centrifugation. Journal of Parasitology, 68(5):916-919.

de Silva, N.R., Brooker, S., Hotez, P.J., Montresor, A., Engels, D. and Savioli, L. 2003. Soil-transmitted helminth infections: updating the global picture. *Trends* in *Parasitology,* 19(12):547–551.

De Victorica, J. and Galvan, M. 2003. Preliminary testing of a rapid coupled methodology for quantitation/viability determination of helminth eggs in raw and treated wastewater. Water Research, 37(6):1278-1287.

de Ybanez, M. R. R., Garijo, M. M. and Alonso, F. D. 2001. Prevalence and viability of eggs of *Toxocara* spp. and *Toxascaris leonina* in public parks in eastern Spain. Journal of Helminthology, 75(2):169 – 173.

Dickin, S.K., Schuster-Wallace, C.J., Qadir, M. and Pizzacalla, K. 2016. A Review of Health Risks and Pathways for Exposure to Wastewater Use in Agriculture. Environmental Health Perspectives, 124(7):900-909.

Dowd, S.E., Gerba, C.P. and Pepper, I.L. 1998. Confirmation of the human-pathogenic microsporidia *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis* and *Vittaforma corneae* in water. Applied and Environmental Microbiology, 64(9):3332-3335.

Drechsel, P. and Evans, A. E. V. 2010. Wastewater use in irrigated agriculture. Irrigated and Drainage Systems, 24(1):1–3.

Drechsel, P., Scott, A.C., Raschid-Sally, L., Redwood, M., Bahri, A. 2010. Wastewater Irrigation and Health : Assessing and Mitigating Risk in Low-Income Countries; Drechsel, P.; Scott, A.C.; Raschid-Sally, L.; Redwood, M.; Bahri, A., Eds.; Earthscan, IWMI, IDRC: London, UK, 2010.

Drechsel, P., Graefe, S., Sonou, M. and Cofie, O. 2006. Informal irrigation in urban West Africa: An overview, Research Report 102, International Water Management Institute, Colombo, Sri Lanka.

Dubná, S., Langrová, I., Jankovská, I., Vadlejch, J.,Pekár, S., Nápravník, J. and Fechtner, J. 2007. Contamination with *Toxocara* eggs in urban (Prague) and rural areas in the Czech Republic. Veterinary Parasitology, 144(1-2):81–86.

Dunn, J. C., Turner, H. C., Tun, A. and Anderson, R. M. 2016. Epidemiological surveys of, and research on, soil-transmitted helminths in Southeast Asia: a systematic review. Parasites and Vectors, 9(31):1-13.

Echazú, A., Bonanno, D., Juarez, M., Cajal, S.P., Heredia, V., Caropresi, S., Cimino, R. O., Caro, N., Vargas, P.A., Paredes, G. and Krolewiecki, A. J. 2015. Effect of Poor Access to Water and Sanitation as Risk Factors for Soil-Transmitted Helminth Infection: Selectiveness by the Infective Route. PLoS Neglected Tropical Diseases, 9(9): e0004111. doi:10.1371/journal.pntd.0004111.

Eggimann, S., Truffer, B. and Maurer, M. 2015. To connect or not to connect? Modelling the optimal degree of centralisation for wastewater infrastructures. Water Research, 84:218-231.

Elmitwalli, T. A., Oahn, K.L.T., Zeeman, G. and Lettinga, G. 2002. Treatment of domestic sewage in a two-step anaerobic filter/anaerobic hybrid system at low temperature. Water Research, 36(9):2225–2232.

Engohang-Ndong, J., Uribe, R.M., Gregory, R., Gangoda, M., Nickelsen, M.G. and Loar, P. 2015. Effect of electron beam irradiation on bacterial and Ascaris ova loads and volatile organic compounds in municipal sewage sludge. Radiation Physics and Chemistry, 112:6–12.

Ensink, J.H., Blumenthal, U.J. and Brooker, S. 2008. Wastewater quality and the risk of intestinal nematode infection in sewage farming families in Hyderabad, India. American Journal of Tropical Medicine and Hygiene, 79(4):561–567.

Ensink, J.H.J., Mahmood, T. and Dalsgaard, A. 2007. Wastewater-irrigated vegetables: market handling versus irrigation water quality. Tropical Medicine and International Health, 12(Suppl 2): 2–7.

Ensink, J.H., van der Hoek, W., Mukhtar, M., Tahir, Z. and Amerasinghe, F. P. 2005. High risk of hookworm infection among wastewater farmers in Pakistan. Transactions of the Royal Society of Tropical Medicine and Hygiene, 99(11):809—818.

Fallah, A.A., Makhtumi, Y. and Pirali-Kheirabadi, K. 2016. Seasonal study of parasitic contamination in fresh salad vegetables marketed in Shahrekord, Iran. Food Control, 60(4):538–542.

Feachem, R.G., Bradley, D.J., Garelick, H. and Mara, D. 1983. Sanitation and Disease: Health Aspects of Excreta and Wastewater Management. Wiley, New York.

Ferguson, C., Kaucner, C., Krogh, M., Deere, D. and Warnecke, M. 2004. Comparison of methods for the concentration of *Cryptosporidium* oocysts and *Giardia* cysts from raw waters. Canadian Journal of Microbiology, 50(9):675-682.

Ferré, P. and Dorchies, P. 2000. Prevalence of *Toxocara* eggs in sand pits in eight public parks in Toulouse. Revue de Médecine Vétérinaire, 151(6): 501–506.

Fincham, J.E., Adams, V., Curtis, B., Jordaan, E. and Dhansay, M.A. 2001. Intestinal parasites, growth of children, sanitation and water quality at primary schools in the Boland/Overberg health region, Western Cape Province. South African Medical Research Council, Cape Town, ISBN 1-919809-21-X.

Fortin, N., Beaumier, D., Lee, K. and Greer, C.W. 2004. Soil washing improves the recovery of total community DNA from polluted and high organic content sediments. Journal of Microbiology Methods, 56(2):181-191.

Foxon, K.M., Pillay, S., Lalbahadur, T., Rodda, N., Holder, F. and Buckley, C. A. 2004. The anaerobic baffled reactor (ABR): An appropriate technology for on-site sanitation. Water SA, 30(5):44-50.

Fuhrimann, S., Winkler, M.S., Kabatereine, N.B., Tukahebwa, E.M., Halage, A.A., Rutebemberwa, E., Medlicott, K., Schindler, C., Utzinger, C. and Cissé, G. 2016a. Risk of Intestinal Parasitic Infections in People with Different Exposures to Wastewater and Fecal

Sludge in Kampala, Uganda: A Cross-Sectional Study. PLoS Neglected Tropical Disease,10(3): e0004469. https://doi.org/10.1371/journal.pntd.0004469

Fuhrimann, S., Winkler, M.S., Pham-Duc, P., Do-Trung, D., Schindler, C., Utzinger, J. and Cissé, G. 2016b. Intestinal parasite infections and associated risk factors in communities exposed to wastewater in urban and peri-urban transition zones in Hanoi, Vietnam. Parasites and Vectors, 9(1):537-552.

Fung, I.C. and Cairncross, S. 2009. Ascariasis and handwashing. Transactions of the Royal Society of Tropical Medicine and Hygiene, 103(3):215–222.

Gaasenbeek, C.P.H. and Borgsteede, F.H.M. 1998. Studies on the survival of *Ascaris suum* eggs under laboratory and simulated field conditions. Veterinary Parasitology, 75(2-3):227-234.

Gallizzi, K. 2003. Co-composting reduces helminth eggs in fecal sludge, a field study in Kumasi, Ghana, SANDEC Switzerland http://www.eawag.ch/fileadmin/Domain1/Abteilungen/sandec/publikationen/SWM/Co-composting/Gallizzi_2003.pdf (access: 2017.7.03).

Gantzer, C., Gaspard, P., Gálvez, L., Huyard, A., Dumouthier, N. and Schwartzbrod, J. 2002. Monitoring of bacterial and parasitological contamination during various öeatment of sludge. Water Research, 35(16):3763-3770.

Gaspard, P.G., Wiart, J. and Shwartzbrod, J. 1995. Urban sludge reuse in agriculture: waste treatment and parasitological risk. Bioresource Technology, 52(1):37–40.

GBD. 2015.-Global Burden of Disease Study 2013 Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet, 386(9995):743–800.

Gerba, C.P. and Smith Jr., J.E. 2005. Sources of pathogenic microorganisms and their fate during land application of wastes. Journal of Environmental Quality, 34(1):42–48.

Giacometti, A., Cirioni, O., Fortuna, M., Osimani, P. Antonicelli, L., Del Prete, M. S., Riva, A., D'Errico, M. M., Petrelli, E. and Scalise, G. 2000. Environmental and serological evidence for the presence of toxocariasis in the urban area of Ancona, Italy. European Journal of Epidemiology, 16(11):1023–1026.

Gordon, C.A., Gray, D.J., Gobert, G.N. and McManus, D.P. 2011. DNA amplification approaches for the diagnosis of key parasitic helminth infections of humans. Molecular Cell Probes, 25(4):143–152.

Gracia-Lor, E., Sancho, J. V. and Hernández, F. 2010. Simultaneous determination of acidic, neutral and basic pharmaceuticals in urban wastewater by ultra-high-pressure liquid

chromatography-tandem mass spectrometry. Journal of Chromatography A, 1217(5):622–632.

Guerrero, C., Gómez, I. and Mataix-Solera, J. 2007. El uso de enmiendas en la restauración de suelos quemados. In: Incendios forestales, Suelos y Erosión hídrica. Ed. Caja Mediterráneo CEMACAM, Alcoy, pp 119–154.

Gumbo, J.R., Malaka, E.M., Odiyo, J.O. and Nare, L. 2010. The health implications of wastewater reuse in vegetable irrigation: a case study from Malamulele, South Africa. International Journal of Environmental Health Research, 20(3):201–211.

Gwetu, T P, Chhagan, M, Craib, M, Taylor, M, and Kauchali, S. 2015. Persistent and new-onset anaemia in children aged 6 - 8 years from KwaZulu-Natal Province, South Africa. South African Journal of Child Health, 9(4):127-129.

Gyawali, P., Ahmed, W., Sidhu, J.P.S., Jagals, P. and Toze, S. 2015. Comparison of concentration methods for rapid detection of hookworm ova in wastewater matrices using quantitative PCR. Experimental Parasitology, 159(8):160–167.

Haas, C. N., Rose, J. B. and Gerba, C. P. 2014. Quantitative Microbial Risk Assessment (second ed.). John Wiley and Sons, New York, NY.

Habbari, K., Tifnouti, A., Bitton, G. and Mandil, A. 2000. Geohelminthic infections associated with raw wastewater reuse for agricultural purposes in Beni-Mellal, Morocco. Parasitology International, 48(3):249-254.

Habbari, K., Tifnouti, A., Bitton, G. and Mandil, A. 1999. Helminthic infections associated with the use of raw wastewater infections associated with the use of raw wastewater for agricultural purposes in Beni Mellal, Morocco. East Mediterranean Health Journal. 5:912–921.

Hailu T. 2006. Assessment of intestinal parasites in the effluent slurry of toilet-linked biogas digesters. M.Sc thesis. Addis Ababa University, Ethiopia.

Harrison, E. Z., Oakes, S. R., Hysell, M. and Hay, A. 2006. Organic chemicals in sewage sludges. Science of the Total Environment, 367(2-3):481–497.

Havelaar, A.H., 2012. QMRA — a framework for assessing microbiological public health risks. Dutch National Institute for Public Health and the Environment (RIVM) and Utrecht University: The European College of Veterinary Public Health Annual Scientific Conference, Maastricht, 23 August 2012.

Herselman, J.E. and Moodley, P. 2009. Guidelines for the Utilisation and Disposal of Wastewater Sludge Volume 4. WRC Project No. K5/1622. Water Research Commission, Pretoria, South Africa.

Hill, V.R., Kahler, A.M., Jothikumar, N., Johnson, T.B., Hahn, D. and Cromeans, T.L. 2007. Multistate evaluation of an ultrafiltration-based procedure for simultaneous recovery of enteric microbes in 100-liter tap water samples. Applied Environmental Microbiology, 73(13):4218-4225.

Hill, V.R., Polaczyk, A.L., Hahn, D., Narayanan, J., Cromeans, T.L., Roberts, J.M. and Amburgey, J.E. 2005. Development of a rapid method for simultaneous recovery of diverse microbes in drinking water by ultrafiltration with sodium polyphosphate and surfactants. Applied Environmental Microbiology, 71(11):6878-6884.

Holland, C. V., Olorcain, P., Taylor, M. R. H. and Kelly, A. 1995. Seroepidemiolgy of toxocariasis in school children. Parasitology, 110(5):535–545.

Hong, S.T., Chai, J.Y., Choi, M.H., Huh, S., Rim, H.J. and Lee, S.H. 2006. A successful experience of soil-transmitted helminth control in the Republic of Korea. Korean Journal of Parasitology, 44(3):177–185.

Horiuchi, S., Paller, V. and Uga, S. 2013. Soil contamination by parasite eggs in rural village in the Philippines. Tropical Biomedicine, 30(3):495–503.

Hotez, P.J. and Herricks, J.R. 2015. Helminth Elimination in the Pursuit of Sustainable Development Goals: A "Worm Index" for Human Development. PLoS Neglected Tropical Diseases, 9(4):e0003618. Doi: 10.1371/ journal.pntd.0003618.

Hotez, P. J., Brindley, P.J., Bethony, J. M., King, C.H., Pearce, E. J. and Jacobson, J. 2008. Helminth infections: the great neglected tropical diseases. The Journal of Clinical Investigation, 118(4):1311-1322.

Hughes, R.G., Sharp, D.S., Hughes, M.C., 'Akau'ola, S., Heinsbroek, P., Velayudhan, R., Schulz, D., Palmer, K., Cavalli-Sforza, T. and Galea, G. 2004. Environmental influences on helminthiasis and nutritional status among Pacific schoolchildren. International Journal of Environmental Health Research, 14(3):163-177.

Istenic, D., Bodík, I. and Bulc, T. 2014. Status of decentralised wastewater treatment systems and barriers for implementation of nature-based systems in central and eastern Europe. Environmental Science and Pollution Research, 22(7):12879-12884.

Jia, T-W., Melville, S., Utzinger, J., King, C.H. and Zhou, X-N. 2012. Soil-transmitted helminth reinfection after drug treatment: a systematic review and metaanalysis. PLoS Neglected Tropical Diseases, 6(5):e1621. doi: 10.1371/journal.pntd.000162

Jiménez, B., Maya, C., Barrios, J. A. and Navarro, I. 2017. Helminths and their Role in Environmental Engineering, Human Helminthiasis, Prof. Luis Rodrigo (Ed.), InTech, DOI: 10.5772/64878. Available from: https://www.intechopen.com/books/human-helminthiasis/helminths-and-their-role-in-environmental-engineering

Jiménez, B., Austin, A., Cloete, E., Plasha, C. and Beltran, N. 2007. Biological risks to foods crops fertilized with Ecosan sludge. Water Science and Technology, 55(7):S21–S29.

Jimenez, B. and Wang, L. 2006. Municipal Wastewater Management in Developing Countries: Principles and Engineering, Ujang Z. and Henze M. Editors. International Water Association Publishing. London, U.K. pp 237-292.

Jiménez, B. and Álvarez, A.M. 2005. Control de la degradación de suelos. Ed. Universidad Autónoma de Madrid, Madrid.

Jimenez-Cisneros, B.E. and Maya-Rendon, C. 2007. "Helminths and Sanitation". Communicating Current Research and Educational Topics and Trends in Applied Microbiology A. Méndez-Vilas (Ed.). Formatex Research Center, Badajoz, Spain. pp 60-71.

Jimenez-Cisneros, B. E. 2006. Helminth ova control in wastewater and sludge for agriculture reuse. Water and Health – Vol. II. ©Encyclopedia of Life Support Systems (EOLSS).

Johansen, A., Nielsen, H.B., Hansen, C.M., Andreasen, C., Carlsgart, J., Hauggard-Nielsen, H. and Roepstorff, A. 2013. Survival of weed seeds and animal parasites as affected by anaerobic digestion at meso- and thermophilic conditions. *Waste Management*, 33(4):807-812.

Josefsen, M.H., Andersen, S.C., Christensen, J. and Hoorfar, J. 2015. Microbial food safety: Potential of DNA extraction methods for use in diagnostic metagenomics. Journal of Microbiology Methods, 114:30–34.

Julca-Otiniano, A., Meneses-Florian, L., Blas-Sevillano, R. and Bello-Amez, S. 2006. Organic matter, importance and experience of its use in agriculture. IDESIA, 24(9):49-61

.

Kamizoulis, G. 2008. Setting health based targets for water reuse (in agriculture). Desalination, 218(1-3):154–163.

Karagiannis-Voules, D.-A., Biedermann, P., Ekpo, U. F., Garba, A., Langer, E., Mathieu, E., Midzi, N., Mwinzi, P., Polderman, A.M., Raso, G., Sacko, M., Talla, I., Tchuem Tchuenté, L.-A., Touré, S., Winkler, M. S., Utzinger, J. and Vounatsou, P. 2015. Spatial and temporal distribution of soil-transmitted helminth infection in sub-Saharan Africa: a systematic review and geostatistical meta-analysis. *Lancet* Infectious Diseases, 15(1):74–84.

Karkashan, A., Khallaf, B., Morris, J., Thurbon, N., Rouch, D., Smith, S.R. and Deighton, M. 2015. Comparison of methodologies for enumerating and detecting the viability of Ascaris eggs in sewage sludge by standard incubation-microscopy, the BacLight Live/Dead viability assay and other vital dyes. Water Research, 68:533–544.

Katakam, K.K., Mejer, H., Dalsgaard, A., Kyvsgaard, N.C. and Thamsborg, S.M. 2014. Survival of *Ascaris suum* and *Ascaridia galli* eggs in liquid manure at different ammonia concentrations and temperatures. Veterinary Parasitology, 204(3-4):249–257.

Keller, R., F. Passamani, S. Cassini and F. Goncalves, 2004. Disinfection of sludge using lime stabilization. Water Science and Technology, 50(1):13-17.

Keraita, B. and Amoah, P. 2011. Fecal Exposure Pathways in Accra: A Literature Review with Specific Focus on IWMI's Work on Wastewater Irrigated Agriculture (IWMI). Literature Review Report. Submitted to the Centre for Global Safe Water, Emory University.

Keraita, B., Drechsel, P. and Konradsen, F. 2008. Perceptions of farmers on health risks and risk reduction measures in wastewater-irrigated urban vegetable farming in Ghana, Journal of Risk Research, 11(8):1047-1061.

Khouja, L. B. A., Cama, V. and Xiao, L. 2010. Parasitic contamination in wastewater and sludge samples in Tunisia using three different detection techniques. Parasitology Research, 107(1):109–116.

Klapec, T. 2009. Contamination of soil with geohelminth eggs on vegetable organic farms in the Lublin voivodeship, Poland. Wiadomości Parazytologiczne, 55(4):405–409.

Kobayashi, A., Hara, T. and Kajima, J. 2006. Historical aspects for the control of Soil-transmitted helminthiasis. *Parasitology International*, 55:S289–S291.

Kone´, D., Cofie, O., Zurbrügg, C., Gallizzi, K., Moser, D., Drescher, S. and Strauss, M. 2007. Helminth eggs inactivation efficiency by faecal sludge dewatering and co-composting in tropical climates. Water Research, 41(19):4397–4402.

Krzyzanowski Jr, F., Lauretto, M.S., Nardocci, A. C., Sato, M. I. Z. and Razzolini M.T.P. 2016. Assessing the probability of infection by *Salmonella* due to sewage sludge use in agriculture under several exposure scenarios for crops and soil ingestion. Science of the Total Environment, 568(12):66–74.

Kundu, A., Poma, H.R., Jenkins, M. W., Rajal, V.B. and Wuertz, S. 2014. QMRA of intestinal nematode infection via multimedia exposure pathways. International Environmental Modelling and Software Society (iEMSs) 7th Intl. Congress on Env. Modelling and Software, San Diego, CA, USA, Daniel P. Ames, Nigel W.T. Quinn and Andrea E. Rizzoli (Eds.)

Kwitshana, Z. L., Tsoka, J. M. and Mabaso, M. L. H. 2008. Intestinal parasitic infections in adult patients in KwaZulu-Natal. South African Medical Journal, 98(9):709–711.

LaBeaud, A.D., Nayakwadi, S. M., McKibben, M., Mungai, P., Muchiri, E.M., McKibben, E., Gildengorin, G., Sutherland, L. J., King, C. H., King, C. L. and Malhotra, I. 2015. Parasitism in Children Aged Three Years and Under: Relationship between Infection and

Growth in Rural Coastal Kenya. PLoS Neglected Tropical Diseases, 9(5): e0003721. https://doi.org/10.1371/journal.pntd.0003721

Lajeunesse, A., Gagnon, C. and Sauvé, S. 2008. Determination of basic antidepressants and their N-desmethyl metabolites in raw sewage and wastewater using solid-phase extraction and liquid chromatography-tandem mass spectrometry. Analytical Chemistry, 80(14):5325–5333.

Lallana, C., Krinner, W., Estrela, T., Nixon, S., Leonard, J. and Berland, J.M. 2001. Sustainable water use in Europe, Part 2: demand management. In: Environmental Issues. Report No. 19. European Environment Agency. Denmark.

Lam, S., Nguyen-Viet, H., Tuyet-Hanh, T.T., Nguyen-Mai, H. and Harper, S. 2015. Evidence for Public Health Risks of Wastewater and Excreta Management Practices in Southeast Asia: A Scoping Review. International Journal of Environmental Research and Public Health, 12(10):12863-12885.

Landa-Cansigno, O., Durán-Álvarez, J.C. and Jiménez-Cisneros, B. 2013. Retention of *Escherichia coli, Giardia lamblia* cysts and *Ascaris lumbricoides* eggs in agricultural soils irrigated by untreated wastewater. *Journal* of *Environmental Management,* 128:22-29.

Luo, Z. J., Wang, G. X., Yang, C. L., Luo, C. H., Cheng, S. W. and Liao, L. 1999. Detection of circulating antigens and antibodies in *Toxocara canis* infection among children in Chengdu, China. Journal of Parasitology, 85(2):252 – 256.

Mabaso, M.L.H., Appleton, C.C., Hughes, J.C. and Gouws, E. 2004. Hookworm (Necator americanus) transmission in inland areas of sandy soils in KwaZulu-Natal, South Africa. *Tropical Medicine* and *International Health*, 9(4):471–476.

Mabaso, M.L., Appleton, C.C., Hughes, J.C. and Gouws, E. 2003. The effect of soil type and climate on hookworm (Necator americanus) distribution in KwaZulu-Natal, South Africa. Tropical Medicine and International Health. 8:722-727. 10.1046/j.1365-3156.2003.01086.x

Mara, D. and Sleigh, A. 2010. Estimation of *Ascaris* infection risks in children under 15 from the consumption of wastewater irrigated carrots. Journal of Water and Health, 8(1):35–38.

Mara, D., Lane, J., Scott, B. and Trouba, D. 2010. Sanitation and health. PLoS Medicine, 7(11): e1000363. doi:10.1371/journal.pmed.1000363.

Mara, D. and Horan, N. 2003. Handbook of water and wastewater microbiology. Academic Press. London, ISBN 0-12-470100-0.

Masi, F., Rochereau, J., Troesch, S., Ruiz, I. and Soto, M. 2015. Wineries wastewater treatment by constructed wetlands: a review. Water Science and Technology, 71(8):1113–1127.

Massoud, M. A., Tarhini, A. and Nasr, J. A. 2009. Decentralized approaches to wastewater treatment and management: Applicability in developing countries. Journal of *Environmental Management,* 90(1):652-659.

Mateo-Sagasta, J., Raschid-sally, L. and Thebo, A. 2015. Global Wastewater and Sludge Production, Treatment and Use, in: Drechsel, P., Qadir M., Wichelns, D. (Ed.), Wastewater-Economic Asset in an Urbanizing World. Springer Dordrecht Heidelberg New York London. pp. 15-38.

Mateo-Sagasta, J. and Burke, J. 2010. Agriculture and Water Quality Interactions: *A Global Overview*. SOLAW Background Thematic Report-TR08. Rome, Food and Agricultural Organization of the United Nations (FAO).

Matsuo, J. and Nakashio, S. 2005. Prevalence of fecal contamination in sandpits in public parks in Sapporo City. Japan. Veterinary Parasitology, 128(1-2):115–119.

Matthys, B., Bobieva, M., Karimova, G., Mengliboeva, Z., Jean-Richard, V., Hoimnazarova, M., Kurbonova, M., Lohourignon, L. K., Utzinger, J. and Wyss, K. 2011. Prevalence and risk factors of helminths and intestinal protozoa infections among children from primary schools in western Tajikistan. Parasites and Vectors, 4(195):1-13.

Maya, C., Jimenez, B. and Schwartzbord, J. 2006. Comparison of techniques for the detection of helminth ova in drinking water and wastewater. *Water Environment Research,* 78(2): 118-124.

Mayer, C.L. and Palmer, C.J. 1996. Evaluation of PCR, Nested PCR and Fluorescent antibodies for detection of *Giardia* and *Cryptosporidium* species in wastewater. Applied and Environmental Microbiology, 62(6):2081-2085.

McKinley, J.W., Parzen, R.E. and Mercado, G. A. 2012. Ammonia inactivation of *Ascaris* ova in ecological compost by using urine and ash. Applied and Environmental Microbiology, 78(15):5133–5137.

Medema, G.J., Schets, F.F., Tuenis, P.F.M. and Havelaar, A.H. 1998. Sedimentation of free and attached Cryptosporidium oocysts and Giardia cysts in water. Applied and Environmental Microbiology, 64(11):4460-4466.

Mendez, J.M., Jimenez, B.E. and Barrios, J.A. 2002. Improved alkaline stabilization of municipal wastewater sludge. Water Science and Technology, *46*(10):*139*-146.

Mes, T.H.M. 2003. Technical variability and required sample size of helminth egg isolation procedures. Veterinary Parasitology, 115(4): 311–320.

Mizgajska, H. 1997. The role of some environmental factors in the contamination of soil with *Toxocara* spp. and other geohelminth eggs. Parasitology International, 46(1):67–72.

Mkhize, B. T., Mabaso, M., Mamba, T., Napier, C. E. and Mkhize-Kwitshana, Z. L. 2017. The Interaction between HIV and Intestinal Helminth Parasites Coinfection with Nutrition among Adults in KwaZulu-Natal, South Africa. BioMed Research International, 3(22):1-12.

Molleda, P., Blanco, I., Ansola, G. and de Luis, E. 2008. Removal of wastewater pathogen indicators in a constructed wetland in Leon, Spain. *Ecological Engineering,* 33:252–257.

Molvik, M., Heiland, E., Zulu, S. G., Kleppa, E., Lillebo, K., Gundersen, S. G., Kvalsvig, J.D., Taylor, M., Kjetland, E. F. and Vennervald, B.J. 2017. Co-infection with *Schistosoma haematobium* and soil-transmitted helminths in rural South Africa. South African Journal of Science, 113(3-4):1-6.

Moodley, P., Archer, C. and Hawksworth, D. 2008. Standard methods for the recovery and enumeration of helminth ova in wastewater, sludge, compost, and urine diversion waste in South Africa, Water Research Commission. In: WRC Report No. TT322/08.

Moubarrad, F.Z.L. and Assobhei, O. 2007. Health risks of raw sewage with particular reference to *Ascaris* in the discharge zone of El Jadida (Morocco). Desalination, 215(1-3):120–126.

Murray, C.J. and Acharya, A.K.1997. Understanding DALYs (disability-adjusted life years). Journal of Health Economics, 16(6):703-30.

Nasr, N.A., Al-Mekhlafi, H.M., Ahmed, A., Roslan, M.A. and Bulgiba, A. 2013. Towards an effective control programme of soil-transmitted helminth infections among Orang Asli in rural Malaysia. Part 1: prevalence and associated key factors. Parasites and Vectors, 6(27): doi.org/10.1186/1756-3305-6-27.

Nasr, F.A., Doma, H. S. and Nassar, H. F. 2009. Treatment of domestic wastewater using an anaerobic baffled reactor followed by a duckweed pond for agricultural purposes. Environmentalist, 29:270–279 DOI: 10.1007/s10669-008-9188-y.

Navarro, I. and Jiménez, B., 2011. Evaluation of the WHO helminth eggs criteria using a QMRA approach for the safe reuse of wastewater and sludge in developing countries. Water Science and Technology, 63:1499-1505

Navarro, I., Jiménez, B., Lucario, S. and Cifuentes, E. 2009. Application of helminth ova infection dose curve to estimate the risks associated with biosolid application on soil. Journal of Water and Health, 7(1):31–44.

Navarro,I., Jiménez, B., Cifuentes, E. and Lucario, S. 2008. A quantitative microbial risk assessment of helminth ova in reusing sludge for agricultural production in developing countries. Risk Analysis, 39(6):65-74.

Navarro-Pedreño, J., Almendro-Candel, M.B., Jordán-Vidal, M.M., Mataix-Solera, J. and García-Sánchez, E. 2003. Mobility of cadmium, chromium and nickel through the profile of a calcisol treated with sewage-sludge in the southeast of Spain. Environmental Geology, 44(8):545-553.

Navitsky, R.C., Dreyfuss, M.L., Shrestha, J., Khatry, S.K., Stoltzfus, R.J. and Albonico, M.1998. *Ancylostoma duodenale* is responsible for hookworm infections among pregnant women in the rural plains of Nepal. Journal of Parasitology, 84(3):647–651.

Nelson, K. L. and Darby, J. L. 2001. Inactivation of viable *Ascaris* eggs by reagents during enumeration. Applied Environmental Microbiology, 67(12):5453–5459.

Ngui, R., Lim, Y.A., Chong Kin, L., Chuen, C. S. and Jaffar, S. 2012. Association between anaemia, iron deficiency anaemia, neglected parasitic infections and socioeconomic factors in rural children of West Malaysia. PLoS Neglected Tropical Diseases, 6(3):e1550.doi: 10.1371/journal.pntd.0001550 PMID: 22413027.

Ngui, R., Ishak, S., Chuen, C.S., Mahmud, R. and Lim, Y.A.2011. Prevalence and risk factors of intestinal parasitism in rural and remote West Malaysia. PLoS Neglected Tropical Diseases, 5(3): e974. doi: 10.1371/journal.pntd. 0000974 PMID: 21390157.

Nguyen, P.H., Nguyen, K.C., Nguyen, T.D., Le, M.B., Bern, C., Flores, R. and Martorell, R. 2006. Intestinal helminth infections among reproductive age women in Vietnam: Prevalence, co-infection and risk factors. *Southeast Asian Journal* of *Tropical Medicine* and *Public Health*, 37(5):865–874.

NRMMC 2004 Guidelines for Sewage Systems, Biosolids Management. Natural Resource Management Ministerial Council (NRMMC), Australia.

NRMMC, EPHC & AHMC 2006.Australian Guidelines for Water Recycling: Managing Health and Environmental Risks (Phase 1). In Environment Protection and Heritage Council Australian Health Ministers' Conference. Natural Resource Management Ministerial Council, Canberra, Australia

O'Connor, N. A., Surapaneni, A., Smith, D. and Stevens, D. 2017. Occurrence and fate of *Ascaris lumbricoides* ova in biosolids in Victoria, Australia: a human health risk assessment of biosolids storage periods. Water Science and Technology, 76(6):1332-1346.

Oge, S. and Oge, H. 2000. Prevalence of *Toxocara* spp. eggs in the soil of public parks in Ankara. Turkey. *Dtsch Tierarztl Wochenschr*, 107(2):72–75.

Oksanen, A., Eriksen, L., Roepstorff, A., Ilsøe, B., Nansen, P. and Lind, P. 1990. Embryonation and infectivity of *Ascaris suum* eggs: a comparison of eggs collected from worm uteri with eggs isolated from pig faeces. Acta Veterinaria Scandinavica, 31(4):393–398.

Oswald, W. E., Stewart, A.E. P., Kramer, M. R., Endeshaw, T., Zerihun, M., Melak, B., Sata, E., Gessese, D., Teferi, T., Tadesse, Z., Guadie, B., King, J. D., Emerson, P.M., Callahan,

E. K., Freeman, M. C., Flanders, W. D., Clasen, T. F. and Moe, C. L. 2017. Association of community sanitation usage with soil-transmitted helminth infections among school-aged children in Amhara Region, Ethiopia. Parasites and Vectors, 10(1):91-104.

O'Toole, J., Sinclair, M., Malawaraarachchi, M., Hamilton, A., Barker, S.F. and Leder, K. 2012. Microbial quality assessment of household greywater. Water Research, 46(13):4301–4313.

Peasey, A. 2000. Human exposure to *Ascaris* infection through wastewater reuse in irrigation and its public health significance. PhD thesis, University of London, UK.

Pecson, B.M., Barrios, J.A., Jimenez, B.E. and Nelson, K.L. 2007. The effects of temperature, pH, and ammonia concentration on the inactivation of *Ascaris* eggs in sewage sludge. Water Research, 41(13):2893–2902.

Pecson, B. M. and Nelson, K. L. 2005. Inactivation of *Ascaris suum* eggs by ammonia. Environmental Science and Technology, 39(20):7909–7914.

Pepper, I.L., Zerzghi, H., Brooks, J.P. and Gerba, C.P. 2008. Sustainability of land application of Class B biosoloids. Journal of Environmental Quality, 37:S58–S67.

Pham-Duc, P., Nguyen-Viet, H., Hattendorf, J., Zinsstag, J., Phung-Dac, C., Zurbrügg, C. and Odermatt, P. 2013. *Ascaris lumbricoides* and *Trichuris trichiura* infections associated with wastewater and human excreta use in agriculture in Vietnam. Parasitology International, 62(2):172–180.

Pham-Duc, P., Nguyen-Viet, H., Hattendorf, J., Zinsstag, J., Phung-Dac, C. and Odermatt, P. 2011. Risk factors for *Entamoeba histolytica* infection in an agricultural community in Hanam province, Vietnam. Parasites and Vectors. 4:102-10.1186/1756-3305-4-102.

Pike, E.B. 1990. The removal of *Cryptosporidium* oocysts during sewage treatment. In: Cryptosporidium in water supplies. Dept. of Env and Dept. of Health Report of the Group of experts. Chair Sir John Badenoch. Part II Paper X, PP. 205-208. HMSO, London

Pillay, S., Foxon, K., Rodda, N., Smith, M., Buckley, C. 2005. The use of effluent from an anaerobic baffled reactor (ABR) for irrigation in a peri-urban community. In: Ecosan GTZ (ed.) 3rd International Ecological Sanitation Conference. Durban, South Africa: DFID, EcoSanRes, CSIR. pp. 445–449.

Pompeo, R., Andreoli, C., Alcântara de Castro, E. and Aisse, M. 2016. Influence of Long-Term Storage Operating Conditions on the Reduction of Viable *Ascaris* Eggs in Sewage Sludge for Agricultural Reuse. *Water*, *Air* and *Soil Pollution*, 227(144):1-14.

Pullan, R.L., Smith, J.L., Jasrasaria, R. and Brooker, S.J. 2014. Global numbers of infection and disease burden of soil transmitted helminth infections in 2010. Parasites and Vectors, 7(1):37–56.

Pullan, L. and Brooker, S.J. 2012. The global limits and population at risk of soil-transmitted helminth infections in 2010. Parasites and Vectors, 5(81):1-14.

Qadir, M., Wichelns, D., Raschid-Sally, L., McCornick, P.G., Drechsel, P., Bahri, A. and Minhas, P.S. 2010. The challenges of wastewater irrigation in developing countries. Agricultural Water Management, 97(4):561–568.

Quintero, K., Duran, C., Duri, D., Medina, F., Garcia, J., Hidalgo, G., Nakal, S., Echeverria-Ortega, M., Albano, C., Incani, R.N., Cortez, J., Jiménez, S., Díaz M, Maldonado, C., Matute, F. and Rodriguez-Morales, A.J. 2012. Household social determinants of ascariasis and trichuriasis in North Central Venezuela. International Health, 4(2):103–110.

Rai, S. K., Uga, S., Ono, K., Rai, G. and Matsumura, T. 2000. Contamination of Soil with Helminth Parasite Eggs in Nepal. The Southeast Asian Journal of Tropical Medicine and Public Health, 31(2):388-93.

Reinoso, R. and Becares, E. 2008. The occurrence of intestinal parasites in swine slurry and their removal in activated sludge plants. Bioresource Technology, 99(4):6661–6665.

Reinoso, R., Torres, L.A. and Bécares, E. 2008. Efficiency of natural systems for removal of bacteria and pathogenic parasites from wastewater. Science of the Total Environment, 395(2-3):80–86.

Reynaud, N. and Buckley, C.A. 2016. The anaerobic baffled reactor (ABR) treating communal wastewater under mesophilic conditions: a review. Water Science and Technology, 73(3):463-478.

Riahi, K., Mammou, A.B. and Thayer, B.B. 2009. Date-palm fibers media filters as a potential technology for tertiary domestic wastewater treatment. Journal of Hazardous Materials, 161(2-3):608–613.

Rojas-Oropeza, M., Castro, P., Ortega, S. and Cabirol, N. 2002. XII National Congress of the Mexican Federation of Sanitary Engineering and Environmental Science, 1, 789. Morelia, México.

Rose, J.B., Dickson, L.J., Farrah, S.R. and Carnahan, R.P. 1996. Removal of pathogenic and indicator microorganisms by a full-scale water reclamation facility. Water Research, 30(11):2785–2797.

Rostami, A., Ebrahimi, M., Mehravar, S., Omrani, V.F., Fallahi, S. and Behniafar, H. 2016. Contamination of commonly consumed raw vegetables with soil transmitted helminth eggs in Mazandaran province, Northern Iran. International Journal of Food Microbiology, 225(12):54–58.

Rutkowski, T., Raschid-Sally, L. and Buechler, S. 2007. Wastewater irrigation in the developing world—Two case studies from the Kathmandu Valley in Nepal. Agricultural Water Management, 88:83 – 91.

Saddoud, A., Ellouze, M., Dhouib, A. and Sayadi, S. 2007. Anaerobic membrane bioreactor treatment of domestic wastewater in Tunisia. Desalination, 207(78):205–215.

Said, D.S. 2012. Detection of parasites in commonly consumed raw vegetables. Alexandria Journal of Medicine, 48:345-352.

Salonen, A., Nikkilä, J., Jalanka-Tuovinen, J., Immonen, O., Rajilić-Stojanović,M., Kekkonen, R.A., Palva, A. and de Vos, W.M. 2010. Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: effective recovery of bacterial and archaeal DNA using mechanical cell lysis. Journal of Microbiology Methods, 81(2):127–134.

Sanchez-Ramos, D., Sánchez-Emeterio, G. and Beltrán, M. F. 2016. Changes in water quality of treated sewage effluents by their receiving environments in Tablas de Daimiel National Park, Spain. Environmental Science and Pollution Research, 23(7):6082–6090.

Satchwell, M.G. 1986. An adaptation of concentration technique for the enumeration of parasitic helminth eggs from sewage sludge. Water Research, 20(7):813–816.

Schoen, M. E. and Garland, J. 2017. Review of pathogen treatment reductions for onsite non-potable reuse of alternative source waters. Microbial Risk Analysis, 5:25–31.

Schönning, C., Westrell, T., Stenström, T.A., Arnbjerg-Nielsen, K., Hasling, A. B., Høibye, L. and Carlsen, A. 2007. Microbial risk assessment of local handling and use of human faeces. Journal of Water and Health, 5(1):117–128.

Seidu, R., Drechsel, P., Amoah, P., Löfman, O., Heistad, R., Fodge, M., Jenssen, P. Stenström, T.A. 2008b. Quantitative microbial risk assessment of wastewater and faecal sludge reuse in Ghana. 33rd WEDC International Conference, Accra, Ghana, 2008a.

Seidu, R., Heistad, A., Amoah, P., Drechsel, P., Jenssen, P. D. and Stenström, T.A., 2008b. Quantification of the health risk associated with wastewater reuse in Accra, Ghana: a contribution toward local guidelines. Journal of Water and Health, 6(4):461-471.

Semblante, G.U., Hai, F. I., Huang, X., Ball, A. S., Price, W. E. and Nghiem, L. D. 2015. Trace organic contaminants in biosolids: Impact of conventional wastewater and sludge processing technologies and emerging alternatives. Journal of Hazardous Materials, 300(14):1–17.

Sengupta, M.E., Keraita, B., Olsen, A., Boateng, O.K., Thamsborg, S.M., Pálsdóttir, G.R. and Dalsgaard, A. 2011. Use of *Moringa oleifera* seed extracts to reduce helminth egg numbers and turbidity in irrigation water. Water Research, 46(11):3646–3656.

Shuval, H.I., Adin, A., Fattal, B., Rawitz, E. and Yekutiel, P. 1986. Wastewater Irrigation in Developing Countries: Health Effects and Technical Solutions. World Bank, Washington DC.

Simmons, O.D., Sobsey, M.D., Heaney, C.D., Schaefer, F.W. and Francy, D.S. 2001. Concentration and detection of *Cryptosporidium* oocysts in surface water samples by method 1622 using ultrafiltration and capsule filtration. Applied Environmental Microbiology, 67(3):1123-1127.

Sinclair, M., O'Toole, J., Forbes, A., Carr, D. and Leder, K. 2010. Health status of residents of an urban dual reticulation system. International Journal of Epidemiology, 39(6):1667–1675.

Smith, J.L. and Brooker, S. 2010. Impact of hookworm infection and deworming on anaemia in non-pregnant populations: a systematic review. Tropical Medicine and International Health, 15(7):776–795.

Sørensen, S.J., Müller, A.K., Hansen, L.H., Rasmussen, L.D., Lipthay, J.R. and Barkay, T. 2002. Molecular methods for assessing and manipulating the diversity of microbiological populations and processes. In: Burns RG, Dick RP, Eds., Enzymes in the Environment, Marcel Dekker, New York.

Starr, M.C. and Montgomery, S.P. 2011. Soil-transmitted helminthiasis in the United States: a systematic review–1940–2010. *American Journal* of *Tropical Medicine* and *Hygiene*, 85(4):680–684.

Stats SA. 2014. Census 2011 Statistical release – P0301.4 Statistics South Africa. Pretoria

Steinbaum, L., Njenga, S.M., Kihara, J., Boehm, A.B., Davis, J., Null, C. and Pickering, A. J. 2016. Soil-Transmitted Helminth Eggs Are Present in Soil at Multiple Locations within Households in Rural Kenya. PLoS ONE 11(6):e0157780. doi:10.1371/journal. pone.0157780.

Stephenson, L.S., Latham, M.C. and Ottesen, E.A. 2000. Malnutrition and parasitic helminth infections. Parasitology, 121(Suppl):S23–S38.

Stolk, W.A., Kulik, M.C., le Rutte, E.A., Jacobson, J., Richardus, J.H. and de Vlas, S.J. 2016. Between-country inequalities in the neglected tropical disease burden in 1990 and 2010, with projections for 2020. PLoS Neglected Tropical Diseases, 10 (5), e0004560, http://dx.doi.org/10.1371/journal.pntd.0004560.

Stoltzfus, R.J., Chwaya, H.M., Montresor, A., Albonico, M., Savioli, L. and Tielsch, J.M. 2000. Malaria, hookworms and recent fever are related to anemia and iron status indicators in 0- to 5-y old Zanzibari children and these relationships change with age. Journal of Nutrition, 130(7):1724–1733.

Stoltzfus, R.J., Dreyfuss, M.L., Chwaya, H.M. and Albonico, M.1997. Hookworm control as a strategy to prevent iron deficiency. Nutrition Review, 55(6):223–232.

Stott, R., May, E. and Mara, D.D. 2002. Parasite removal by natural wastewater treatment systems: Performance of waste stabilization ponds and constructed wetlands. Proceedings of the 5th IWA Waste Stabilization Ponds, 2-5 April. Auckland, New Zealand

Stott, R., Jenkins, T., Shabana, M. and May, E.1997. A survey of the microbial quality of wastewaters in Ismailia, Egypt and the implications for wastewater reuse. Water Science and Technology, 35(11-12):211-217.

Strunz, E.C., Addiss, D.G., Stocks, M.E., Ogden, S., Utzinger, J. and Freeman, M.C. 2014. Water, sanitation, hygiene and soil-transmitted helminth infection: a systematic review and meta-analysis. PLoS Medicine, 11(3):e1001620. doi.org/10.1371/journal.pmed.1001620

Szabova, E., Juris, P. and Papajova, I. 2010. Sanitation composting process in different seasons *Ascaris suum* as model. Waste Management, 30(2):426–432.

Tavassoli, M., Hadian, M., Charesaz, S. and Javadi, S. 2008. *Toxocara* spp. Eggs in Public Parks of Urmia City, West Azerbaijan Province Iran. *Iranian Journal* of *Parasitology*, 3(3):24–29.

Tebbe, C.C. and Vahjen, W. 1993. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant-DNA from bacteria and a yeast. Applied and Environmental Microbiology, 59(8):2657-2665.

Teh, X.Y., Poh, P.E.., Gouwanda, D. and Chong, M.N.2015. Decentralized light greywater treatment using aerobic digestion and hydrogen peroxide disinfection for non-potable reuse. Journal of Cleaner Production.99:305-31.1

Tikasingh, E.S., Chadee, D.D. and Rawlins, S.C. 2011. The control of hookworm in the Commonwealth Caribbean countries. Acta Tropica, 120(1-2):24–30.

Tilley, E., Ulrich, L., Luthi, C., Reymond, P. and Zurbrüg, C. 2014. Compendium of sanitation systems and technologies. 2nd Edition. Swiss Federal Institute of Aquatic Sciences and Technology (EAWAG). Dübendorf, Switzerland.

Toze, S. 2006. Reuse of effluent water—benefits and risks. Agricultural Water Management, 80(57):147– 159.

Trang, D.T., Mølbak, K., Cam, P.D. and Dalsgaard, A. 2007. Helminth infections among people using wastewater and human excreta in peri-urban agriculture and aquaculture in Hanoi, Vietnam. Tropical Medicine and International Health, 12(Suppl 2):82–90.

Trang, D.T., van der Hoek, W., Cam, P.D., Vinh, K.T., Hoa, V.N. and Dalsgaard, A. 2006. Low risk for helminth infection in wastewater-fed rice cultivation in Vietnam. Journal of Water and Health, 4(3):321–331.

Trönnberg, L., Hawksworth, D., Hansen, A., Archer, C. and Stenström, T. A. 2010. Household-based prevalence of helminths and parasitic protozoa in rural KwaZulu-Natal, South Africa, assessed from faecal vault sampling. Transactions of the Royal Society of Tropical Medicine and Hygiene, 104(10):646–652.

Uga, S.,Hoa, N.T.V., Noda, S., Moji, K., Cong, L., Aoki, Y., Rai, S.K. and Fujimaki, Y. 2009. Parasite egg contamination of vegetables from a suburban market in Hanoi, Vietnam. *Nepal Medical College Journal*, 11(2):75–78.

Uhlikova, M. and Hubner, J. 1998. Seroprevalence of *Toxocara canis* infection in Czech Republic. Central European Journal of Public Health, 6(3):195–198.

USEPA-United States Environmental Protection Agency. 1999. Control of Pathogens and Vector Attraction in Sewage Sludge EPA/625/R-92/013. Revised Edition. U.S. Environmental Protection Agency, Washington, D.C.

USEPA- United States Environmental Protection Agency. 1993. Part 503—Standards for the Use and Disposal of Sewage Sludge. Rules and Regulations. Federal Register, 58(32):9248–9415.

Valero, M.A., Perez-Crespo, I., Periago, M.V., Khoubbane, M. and Mas-Coma, S. 2009. Fluke egg characteristics for the diagnosis of human and animal fascioliasis by *Fasciola hepatica* and *F gigantica*. Acta Tropica, 111(2):150–159.

van der Hoek, W., De, N.V., Konradsen, F., Cam, P.D., Hoa, N.T.V., Toan, N.D. and Cong, L.D. 2003. Current status of soil-transmitted helminths in Vietnam. *Southeast Asian Journal* of *Tropical Medicine* and *Public Health*, 34(Suppl I):1–11.

van der Hoek, W., Hassan, M.U.l., Ensink, J.H.J., Feenstra, S., Raschid-Sally, L. and Munir, S. 2002. Urban Wastewater: A Valuable Resource for Agriculture: a Case Study from Haroonabad, Pakistan'. Research Report 63, Colombo, Sri Lanka. International Water Management Institute. pp 2-5.

Vandemark, L.M., Jia, T.W. and Zhou, X.N. 2010. Social science implications for control of helminth infections in Southeast Asia. Advances in Parasitology, 73:137–170.

Verbyla, M.E., Symonds, E.M., Kafle, R.C., Cairns, M.R., Iriarte, *M.M.*, Mercado Guzmán, *A.*, Coronado, O., Breitbart, M., Ledo, C., Mihelcic, J.R. 2016. Managing microbial risks *from* indirect wastewater reuse for irrigation in *urbanizing watersheds*. *Environmental Science* and Technology, 50(13):6803–6813.

Verweij, J.J., Canales, M., Polman, K., Ziem J., Brienen, E.A., Polderman, A.M. and van Lieshout, L. 2009. Molecular diagnosis of *Strongyloides stercoralis* in fecal samples using real-time PCR. Transactions of the Royal Society of Tropical Medicine and *Hygiene,* 103(4):342 – 346.

Verweij, J.J., Brienen, E.A., Ziem, J., Yelifari, L., Polderman, A.M. and Van Lieshout, L. 2007. Simultaneous detection and quantification of *Ancylostoma duodenale*, *Necator americanus*, and *Oesophagostomum bifurcum* in fecal samples using multiplex real-time PCR . American Journal of Tropical Medicine and Hygiene, 77(4):685 – 690.

Westrell, T., Schönning, C., Stenström, T.A. and Ashbolt, N.J. 2004. QMRA (quantitative microbial risk assessment) and HACCP (hazard analysis and critical control points) for management of pathogens in wastewater and sewage sludge treatment and reuse. Water Science and Technology, 50(2):23-30.

WHO-World Health Organization. 2016. Quantitative Microbial Risk Assessment: Application for Water Safety Management. WHO Press, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

WHO-World Health Organization. 2015. Investing To Overcome The Global Impact Of Neglected Tropical Diseases Third Who Report On Neglected Tropical Diseases. WHO Document Production Services, Geneva, Switzerland (WHO/HTM/NTD/2015.1).

WHO-World Health Organization. 2013. Neglected tropical diseases - prevention, control, elimination and eradication. WHA 66.20. In: Sixty-Sixth World Health Assembly. Geneva: World Health Organization.

WHO-World Health Organization. 2012. Eliminating soil-transmitted helminthiases as a public health problem in children: progress report 2001–2010 and strategic plan 2011–2020. Geneva: World Health Organization.

WHO-World Health Organization. 2006. Guidelines for the Safe Use of Wastewater, Excreta and Greywater—Volume 2: Wastewater Use in Agriculture. Geneva, Switzerland.

Worrell, C.M., Wiegand, R.E., Davis, S.M., Odero, K.O., Blackstock, A., Cuéllar, V.M., Njenga, S. M., Montgomery, J.M., Roy, S.L. and Fox, L. M. 2016. A Cross Sectional Study of Water, Sanitation, and Hygiene Related Risk Factors for Soil-Transmitted Helminth Infection in Urban School- and Preschool-Aged Children in Kibera, Nairobi. PLoS ONE 11(3): e0150744. doi:10.1371/journal.pone.0150744.

Worrell, C., Davis, S., Wiegand, R., Lopez, G., Odero, K. 2013. Water, sanitation, and hygiene-related risk factors for soil-transmitted helminth infection in urban school- and pre-school-aged children in Kibera, Nairobi 2013; ASTMH 61st Annual Meeting; 11–15 November 2013, Atlanta.

WWAP (United Nations World Water Assessment Programme). 2017. The United Nations World Water Development Report 2017. Wastewater: The untapped resource. Paris, UNESCO.

Yajima, A., Jouquet, P., Trung, D.D., Cam, T.D.T., Cong, D.T., Orange, D. and Montresor, A. 2009. High latrine coverage is not reducing the prevalence of soil-transmitted helminthiasis in Hoa Binh province, Vietnam. Transactions of the Royal Society for Tropical Medicine and Hygiene, 103(3):237–241.

Yang, S., Hai, F. I., Price, W. E., McDonald, J., Khan, S. J. and Nghiem, L. D. 2016. Occurrence of trace organic contaminants in wastewater sludge and their removals by anaerobic digestion. Bioresource Technology, 210(8):153–159.

Yaya-Beas, R., Cadillo-La-Torre, E., Kujawa-Roeleveld, K., van Lier, J.B. and Zeeman, G. 2016. Presence of helminth eggs in domestic wastewater and its removal at low temperature UASB reactors in Peruvian highlands. Water Research, 90:286–293.

Yen-Phi, V.T., Rechenburg, A., Vinneras, B., Clemens, J. and Kistemann, T. 2010. Pathogens in septage in Vietnam. Science of the Total Environment, 408(9):2050–2053.

Zdybel, J., Cencek, T., Karamon, J. and Kłapéc, T. 2015. Effectiveness of selected stages of waste water treatment in elimination of eggs of intestinal parasites. Bulletin of the Veterinary Institute in Pulawy, 59(1): 51-57.

Zenner, L., Gounel, J. M. and Chauve, C. M. 2002. A standardized method for detecting parasite eggs and oocysts in soils. Revue de Médecine Vétérinaire, 153(11):729–734.

Zhou, J.Z. and Thompson, D.K. 2002. Challenges in applying microarrays to environmental studies. Current Opinion in Biotechnology, 13(3):204-207.

Ziegelbauer, K., Speich, B., Mäusezahl, D., Bos, R., Keiser, J. and Utzinger, J. 2012. Effect of Sanitation on Soil-Transmitted Helminth Infection: Systematic Review and Meta-Analysis. PLoS Medicine, 9(1):e1001162. https://doi.org/10.1371/journal.pmed.1001162.

7.0 APPENDIX I

Standard Operating Procedure for Detecting, Enumerating and viability determination of Soil-Transmitted Helminth eggs in environmental samples

1. Scope and Field of Application

This method defines the procedural protocol for the detection, enumeration and determination of viability of soil-transmitted helminths (using *Ascaris* spp. ova as a surrogate) in water, wastewater, sludge and urine diversion (UD) toilet waste, and compost matrices. All modifications in this version are done based on analytical testing as well as consensus reached with partners. Further quality assurance and statistical data will be used to validate this method which will be revised accordingly. The procedure in the method refers to water, sludge and UD wastes, other matrices will be added as and when necessary.

2. Principle

The main aim of this method is to concentrate helminth ova from wastewater, sludge and UD waste. There are three main principles to achieve this: (1) washing and sieving, (2) sedimenting, and (3) floating to obtain helminth ova. However the incorporation of these principles in sample analysis depends on the type of sample and solids content. The flotation step is achieved through density gradient centrifugation achieved by the use of zinc sulphate of specific gravity (SG) 1.30. This allows helminth ova to float based on their relative densities (RD), e.g. *Ascaris* spp. ova have a RD of 1.13 and *Taenia* spp. ova, 1.27. The viability of ova is determined on microscopy, either after processing is complete, or after incubation in 0.1N sulfuric acid for 18 – 28 days at 26^{o}C.

3. Interferences

Freezing of the samples interferes with the buoyant density of helminth ova and thereby decreases their recovery. During the verification and validation of this method further investigations will be undertaken to identify additional interferences that may be caused due to holding times, sampling methods, chemicals used, etc.

4. Sampling

A sampling methodology will be created indicating the various sampling protocols as per the sample matrix. As a starting point the following sampling techniques are advised. For liquid matrices, 1L - 10L samples may be taken and for solid matrices, 10 - 50g. Adjustments can be made depending on sample matrices and the prevalence of infection in areas sampled. A suitable number of sub-samples should be tested to allow for egg recovery differences due to the heterogeneous nature of samples.

- The sampling container should be placed on wet ice or chemical ice packs and sent back to the laboratory. Analysis should be conducted within 24 hours of sampling.

- If analysis cannot be completed within a 24hr period, samples must be stored in a laboratory refrigerator set to temperatures between 2 and 5°C. Samples should not freeze during transportation or storage.

5. Safety Precautions

When handling samples that may contain parasite ova, personal protective measures must be employed to prevent infection, i.e. by implementing good personal hygiene and good laboratory practice.

- Personal protective equipment, such as safety glasses, latex/nitrile gloves and laboratory coats, should always be worn in the laboratory.
- After analysis, all laboratory utensils used should be washed thoroughly in a bleach-soap solution, rinsed and allowed to dry.
- Gloves must be disposed-off after completion of analysis, and hands must be washed with antiseptic soap.
- Avoid spillages and contact with skin, work should be performed on a paper towel or similar surface and 70% ethanol used as a disinfected should any spillages occur.
- All waste must be disposed of in a bio-hazardous waste bin and NOT in the general waste.

6. Apparatus

Capital Equipment:

- Light microscope (with 100 and/or 400 x magnification AND if possible, a camera)
- Top pan balance (weight range: 0 – 1 000gm, accurate to 2 decimal places)
- Magnetic stirrer and magnetic stirring bars
- Vortex mixer
- Centrifuge with swing-out rotor and buckets to hold 15ml (and, if possible 50ml) Falcon tubes
- Stainless steel sieves (1 x 200mm diam., mesh size 100µm; 1 x 200mm diam., mesh size 20µm; and 1 x 100mm diam., mesh size 20µm)
- Incubator (Temp. range 20 - 40°C – If Viability Testing is to be conducted - optional)
- Blender (optional)
- Hydrometer (specific gravity range between 1.2 to 1.3)
- MUST HAVE A DEEP SINK TO WORK IN

Reusable Items:

- Microscope slides
- 1 x 2lt Plastic & 1 x 3lt Plastic Beakers with spout and handle

- 1 x 1 000ml measuring cylinder
- Wash bottles x 2
- 250ml Plastic beakers for sample weighing and preparation (+/- 20)
- Test tube racks (2 large to accommodate 50mL Falcon centrifuge tubes and 4 small to accommodate 15mL Falcon centrifuge tubes)
- Centrifuge tubes: 50mL and 15mL Falcon tubes
- Pasteur pipettes – 3ml plastic disposable
- China markers for labelling plastic ware (washes off better than marking pens)

Single-use & discard items:

- 22 x 40mm coverslips (optional, also 22 x 22mm coverslips)
- Gloves, non-sterile – must be correct size and a good quality and thickness

7. Reagents

Reagent grade chemicals should be used throughout the analysis. If other grades of reagent are used, it should first be ascertained that the purity of the chemical is sufficient without decreasing the recovery and accuracy of the determination.

- 0.1% Tween80 (for washing solution)
- Ammonium bicarbonate (for washing solution)
- Zinc Sulphate (for floatation solution, SG 1.3)
- O.1N H_2SO_4 (for incubation of eggs)
- 70% ETOH (for cleaning up spills)

8. Calibration

- Centrifuge should be calibrated and verified using a tachometer to ensure that the revolutions per minute correlate with the speed gauge.
- Incubator temperature must be calibrated with a traceable thermometer.
- Microscope optics must be cleaned and the condenser adjusted, so the Köhler illumination is established.
- Refrigerators for storage of samples must be verified on a daily basis to ensure the temperature setting.
- Further quality control protocols will be discussed in an additional quality assurance document outlining the procedural requirements for quality control and in particular to inter and intra-laboratory quality control.

9. Reagent Preparation

- **0.1%Tween 80**
  To prepare 1L solution use 999mL distilled water and 1 mL Tween80.

- **Zinc Sulphate ($ZnSO_4$)**
  Use 500g Zinc Sulphate in +/- 800ml de-ionised or distilled water. Use a hydrometer to check and adjust the SG to 1.3 - if the specific gravity of the solutions is less than 1.3 more chemical should be added. If the specific gravity is higher than 1.3 then more water should be added to the solution.

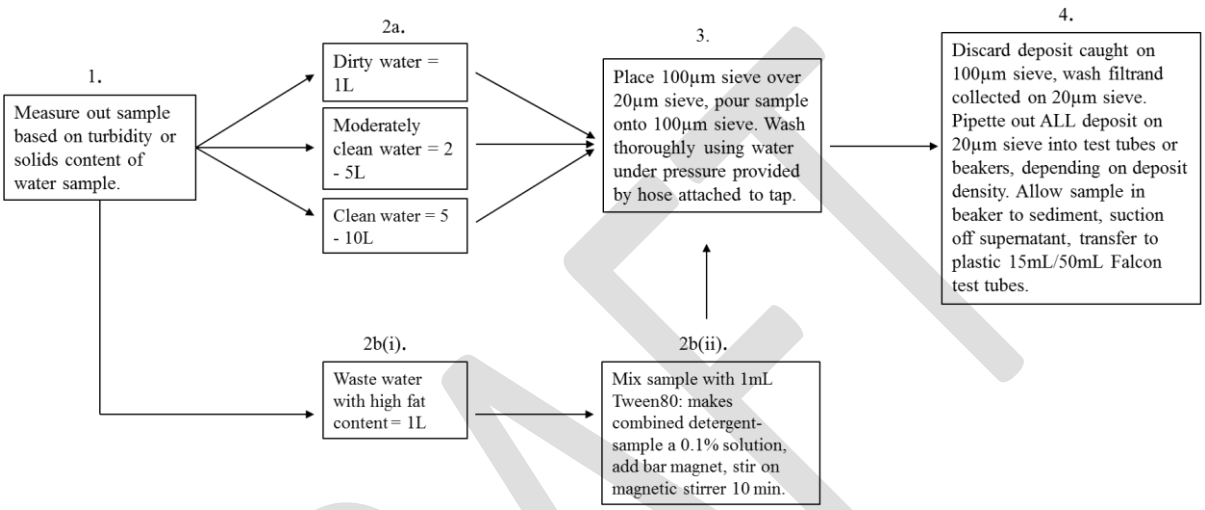
- **Ammonium bicarbonate**
  To prepare, weigh 119g and add to 1000ml of de-ionised water, dissolve completely using a magnetic stirring bar and stirrer.

- **0.1N Sulphuric acid (H_2SO_4)**
  Add 500ml of de-ionised water to a 1 litre plastic bottle, pour 3ml of concentrated H_2SO_4 into a 10ml graduated cylinder, then pour the H_2SO_4 into the plastic bottle containing the water, re-cap and shake. Un-cap, add 497ml of de-ionised water to the plastic bottle, re-cap and shake.

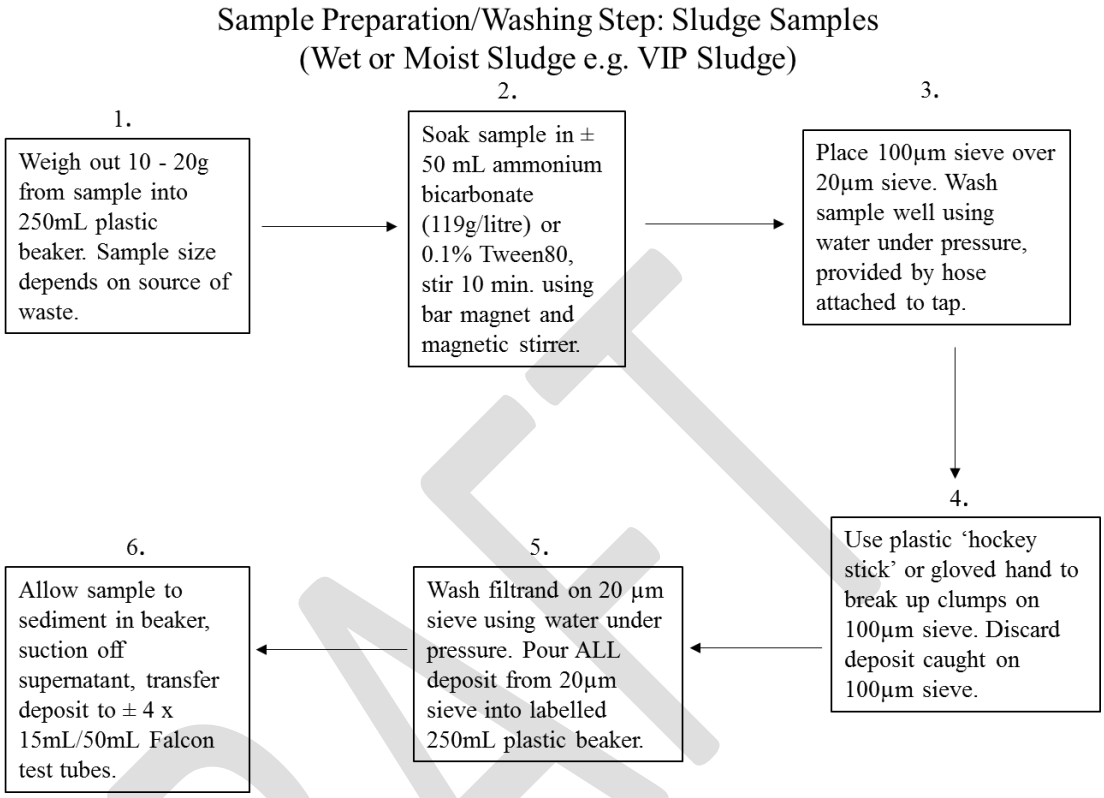
10. Sample Preparation

Wastewater / Water

Sample Preparation/Washing Step: Water or Wastewater Samples

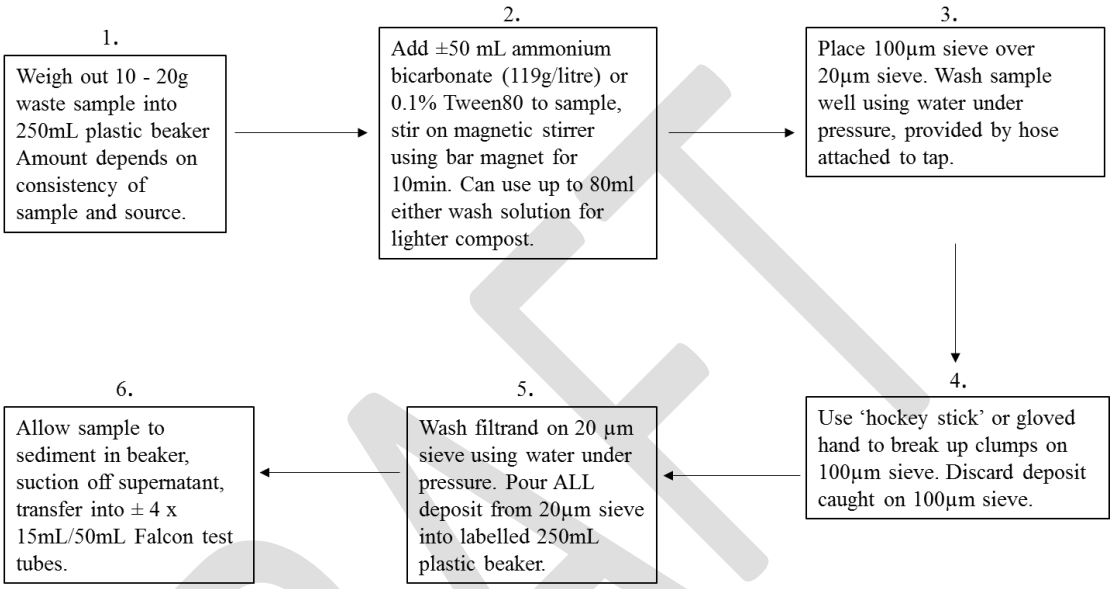


Wet Sludge

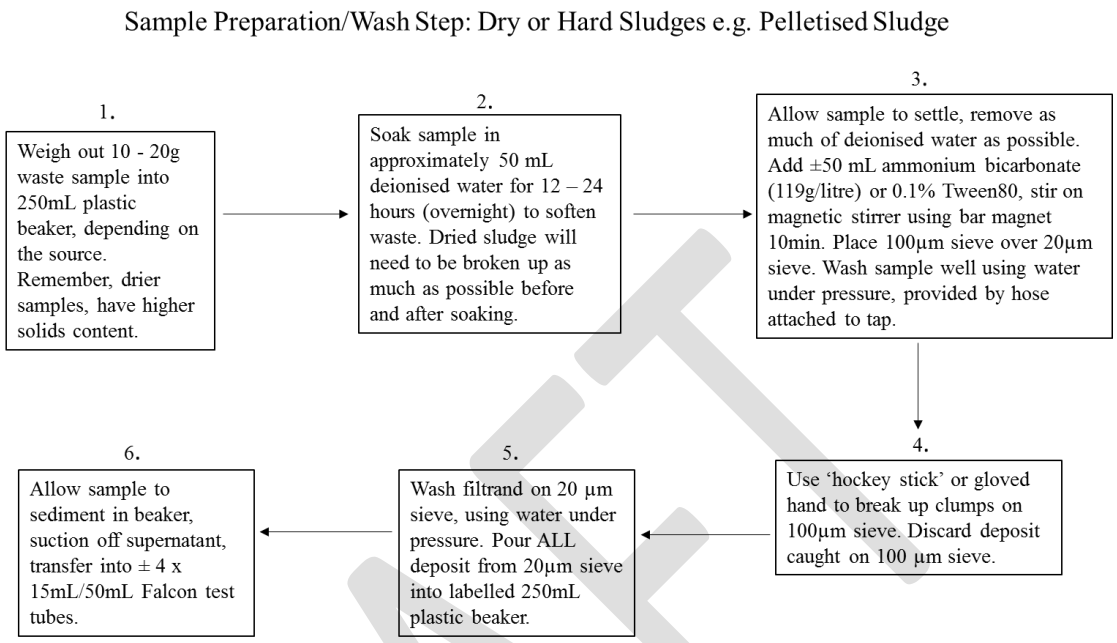


Composted Sludge/UD Wastes/Soil Samples

Sample Preparation/Wash Step: Composted Sludge and UD Waste Samples



Hard Sludges/Pelletised Waste



11. Egg Recovery Procedure

- Centrifuge the sample (using 15 or 50mL centrifuge tubes) for 10 minutes at 3000rpm. Remove all the supernatant from each tube so only the sediment remains. (The packed sediment in each tube should not exceed 0.5mL in a 15mL tube or 5mL in a 50mL tube. If any deposits are larger than this, add water and distribute the sediment evenly among 2 or more tubes and repeat this step.
- Add 10 to 15mL of $ZnSO_4$ solution (SG 1.3) to each tube and mix for 15 to 20 seconds on a vortex mixer.
- Add additional $ZnSO_4$ solution to each tube to make the volumes equal (+/- 14mL in 15mL tubes and +/- 45mL in 50mL tubes). Centrifuge samples for 5-10 minutes at 2000rpm, with the centrifuge brake off.
- Allow the centrifuge to stop and pour all the supernatant from each tube through the 100mm diameter 20µm sieve.
- Using a hose on a tap, carefully wash the deposit on the sieve to remove the $ZnSO_4$ and return the sample to a normal SG...
- Rinse sediment collected on the sieve into a 15 or 50mL centrifuge tube using the hose and/or water from a wash bottle.
- After thoroughly washing the sediment from the sieve into the required number of centrifuge tubes, centrifuge the tubes for 10 minutes at 3000rpm, and then discard the

supernatant. If more than one tube has been utilised for one sample, transfer the sediment to one tube, fill with water and repeat the centrifugation. Discard all the supernatant taking care not to lose the deposit.

- ***Quantification:*** Add a drop or 2 of water to the deposit and pipette it onto one microscope slide at a time (if there is too much for one slide) and place a coverslip on top. Examine the sample thoroughly from one side of the coverslip to the other under 100x magnification, using 400x to determine the stage of development of the ova. Count every helminth egg, classifying them as viable, potentially viable or dead. The final concentration of ova should be reported per litre for wastewater/water and per gram for sludges, compost and soil samples. The calculation for the final concentration should take into account the initial volume/weight of sample analysed.
- ***Viability:*** Re-suspend the solids in 4mL 0.1 N H_2SO_4 and pour into a 50mL test tube and cap loosely.
- Mark the liquid level on each tube before incubation. Incubate the tubes at 26°C for three to four weeks. Check the liquid level in each tube every day, swirl for about 30 seconds to oxygenate the solution, then top it up to the initial liquid level as marked, to compensate for evaporation.
- After 18 days, suspend by vortexing, remove a small aliquot to a microscope slide and examine to check viability status.
- Classify the ova as unembryonated, embryonated to the first or second stage larva, and non-viable for ova that have not developed larvae.

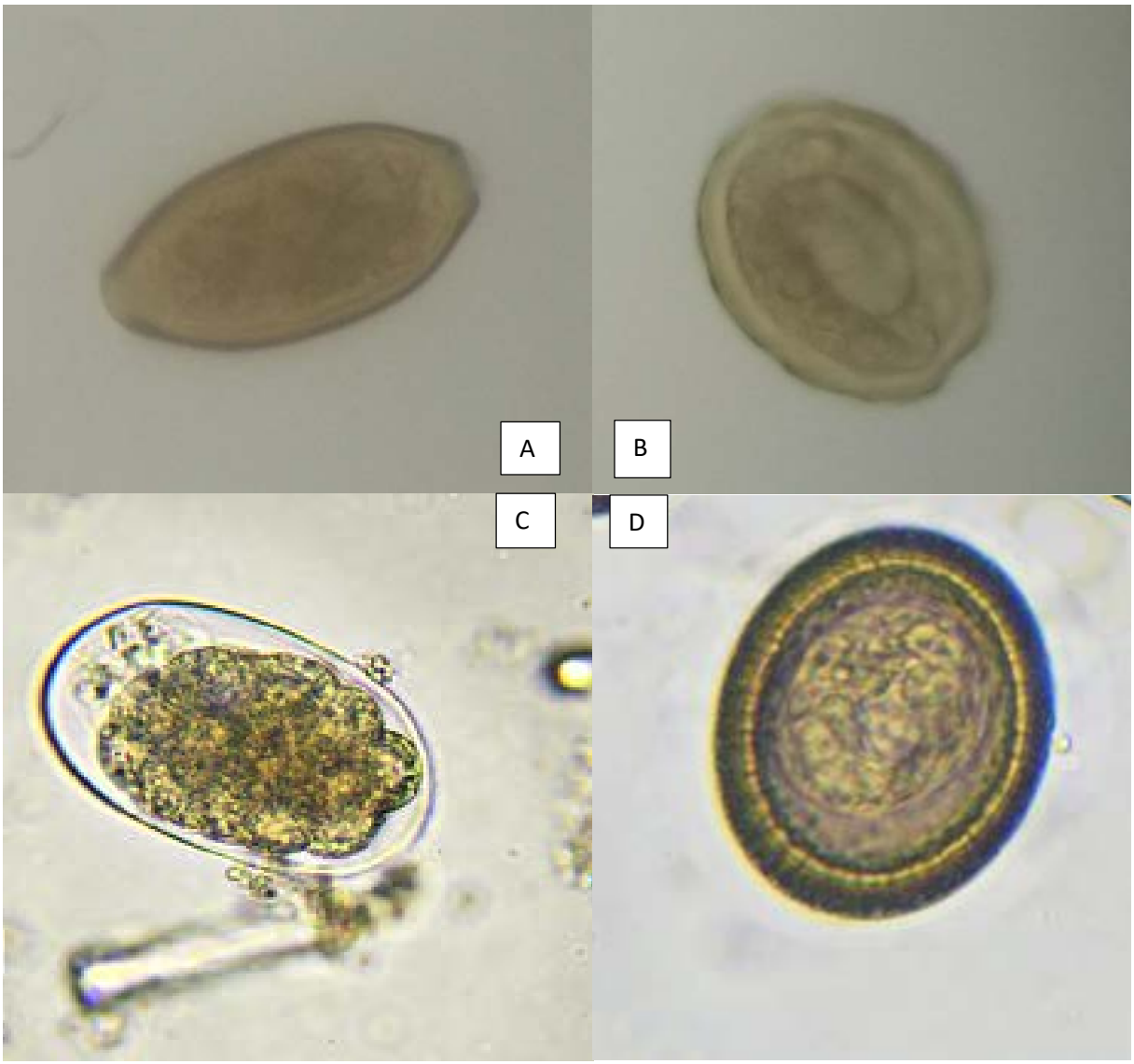
12. Additional Notes

Substitution of $ZnSO_4$ with $MgSO_4$ as the flotation solution is still to be determined so as to allow laboratories to use either of these based on availability.

In addition distilled water may be used as the incubation solution based on availability and preference of laboratories. However, the preferred medium is 0.1 N H_2SO_4

APPENDIX II

Common soil-transmitted helminth eggs detected and reported on in this study.



Common soil-transmitted helminth eggs detected in this study; A: *Trichuris* spp; B: *Ascaris* spp with larvae; C: hookworm and D: *Taenia* spp.