Determination of Schistosomiasis Environmental Contamination and Microbial Source Tracking

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Abstract

Schistosomiasis and soil-transmitted helminths (STHs), cause major morbidity globally, predominantly in the world's poorest populations, and exacerbate the cycle of poverty. Poor sanitation facilitates the transmission of the parasitic worms which cause these diseases. The transmission of the *Schistosoma* species, *S. mansoni*, can be interrupted by water, sanitation, and hygiene (WASH) interventions if they prevent the faecally deposited schistosome eggs from reaching water and infecting the parasite's intermediate snail host. Adequate sanitation can also prevent STH ova being deposited into soils, and their ongoing transmission. The monitoring of schistosomiasis and the effect of WASH interventions has predominantly relied on epidemiological data, rather than direct examination of the parasite in the wider environment. In the last few years, environmental DNA (eDNA) techniques have been used to detect *S. mansoni* in water samples collected from known water contact sites in endemic areas. The sampling of the terrestrial environment has been neglected regarding environmental monitoring of schistosomiasis, despite the soil environment interfacing with sanitation practices such as open defecation and pit latrine usage. The overall aim of this thesis was to characterise the soil environment using eDNA techniques on soil samples that interface with sanitation facilities: sites of open defection and pit latrines in a community highly endemic for *S. mansoni* and co-endemic for STH.

As the soil environment has not been previously investigated using eDNA-based methods to detect *S. mansoni*, Chapter Two outlines the laboratory work carried out to apply existing eDNA-based techniques used on water samples, to the detection of *S. mansoni* in soil samples. An assay detecting the cytochrome oxidase I gene of *S. mansoni* was chosen and tested on soils spiked with varying numbers of *S. mansoni* eggs. It was tested to a lower limit of detection of a single *Schistosoma* egg extracted from 500 mg of soil. Although the qPCR assay could not reliably estimate the number of eggs in a sample, the assay was found to be highly sensitive. Therefore, this highly sensitive assay was then taken forward to be trialled in an absence/presence capacity on soil samples collected from a *S. mansoni* endemic area in Uganda in Chapter Three. Soil samples were collected from areas interfacing with sanitation (areas of open defecation and

pit latrines) as well as a predicted human defecation free area (community football field). The collected soils were then tested for the detection of *S. mansoni* using the DNA extraction methods and qPCR-assay from Chapter Two. Although the qPCR assay performed as expected from the standard curve data generated, none of the soils collected had detectable amounts of *S. mansoni* DNA.

Whilst this qPCR was not sensitive enough to detect *S. mansoni* eggs, or no eggs were present in these sanitation facilities-associated soils, that does not necessarily mean that faecal contamination of the soil environment was not occurring. Therefore, in Chapter Four, bacterial faecal markers were employed as a proxy for the presence of *S. mansoni* eggs, as they are transmitted into the environment through an infected individual's faeces. To gain a broader insight into the soil environment four qPCR assays were used to detect and quantify all eukaryotic DNA (universal 18S), prokaryotic DNA (universal 16S), as well as indicators of faecal contamination using a general *Bacteroides*, a bacterial indicator of homeothermic (bird and mammal) faeces and a human specific *Bacteroides* marker. Faecal contamination was observed across all samples which could have been caused by the free roaming animals observed in this community. However, fewer samples from the predicted negative control site (the community football pitch) had human faecal markers detected, than soils from the sanitation-associated sites.

As schistosomiasis is often co-endemic with STHs, including hookworm, additional studies were performed on these soil samples to assess if *Chryseobacterium nematophagum* (a nematode-eating bacteria) is found in areas endemic for hookworm. If found, this could indicate the potential for using these bacteria as a form of biological control for hookworm larvae in such an area. In the final experimental data chapter, Chapter Five, the soil samples collected from Uganda were therefore additionally tested for the presence of both hookworm and *C. nematophagum*. A single soil sample (out of 31) was positive for *C. nematophagum*. This soil sample was also positive for a hookworm species but as the technical replicate for this sample had C_T values more than 0.5 cycles apart, they were considered too disparate from one another to be considered reliable data. Further work is therefore needed before any potential biological

control intervention could be investigated further. The possible reasons for the variable results presented throughout the thesis, and recommendations for how to improve the assays, are discussed in the final chapter of the thesis, the General Discussion.

Table of contents

List of figures

List of tables

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Secondly, I would not have been able to complete this project without borrowing lab equipment! I would like to acknowledge Dr Gilbert Kasangaki from the Department of Civil and Environmental Engineering at Makerere University who was very accommodating in allowing me to use his soil lab. Closer to home, thank you to Jo Forde-Thomas at Aberystwyth University, who was very helpful in providing the infected mouse livers I required from Professor Karl Hoffman's lab. And thanks to Dr Alice Garrett and Professor Julia Edgar for letting me borrow their microscopes for counting the *S. mansoni* eggs.

Thirdly, thank you to members of the Lamberton Lab group and Water and Infrastructure group at Glasgow who helped in and out of the lab. Thank you to Dr Marta Vignola, Julie Russel, Dr Billy Sands and Chantal Potrafke for their help in the lab. The writing workshop for Stef's "Top Team", was also a great support during the initial Covid-19 lockdowns. Within the Lamberton Lab, special thanks to Dr Christina Faust for her help and guidance in organising the fieldtrip to Uganda whilst Poppy was on maternity leave and for Suzan and Rachel's company on the fieldtrip. I am also grateful for my colleagues who became friends over the four years based in IBAHCM, including, Lydia, Suzan, Agnes, Diana, and Maria. Danke Hans fürs Teilen deines Hausbüros mit mir während meines Schreibens und für deine Unterstützung.

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Authors declaration

The material presented in this thesis is the result of research conducted between October 2017 and May 2021. During this time, I was under the supervision of Dr Poppy Lamberton, Dr Stephanie Connelly and Professor Cindy Smith. This work has not been submitted as part of any other degree and is, for the most part, based on individual research carried out by me.

The field work was carried out with the help of technicians (Moses Adriko, Fred Besigye and the late Diana Ajambo) from Vector Control Division of the Ugandan Ministry of Health. Together we collected soil samples, and I was responsible for the planning, organisation, and execution of the work. In Chapter Five, the rerunning of the *Chryseobacterium nematophagum* PCR assay and visualisation by agarose gel were performed by Chantal Potrafke.

Teteh Champion

List of abbreviations

16S	Genes coding for the RNA component of the 30S subunit of prokaryotic ribosomes
18S	Genes coding for the 18S rRNA of eukaryotic organisms' ribosomes
ANOVA	Analysis of variance
bp	Base Pair
CCA	Circulating Cathodic Antigen
CFU	Colony-forming units
CI	Confidence Interval (in statistics) of an estimation
COI(COX1)	Mitochondrial cytochrome oxidase subunit 1 gene
C_T	Cycle threshold
CV	Coefficient of variation
DNA	Deoxyribonucleic Acid
dsDNA	Double stranded DNA
eDNA	Environmental DNA
FIB	Faecal indicator bacteria
JMP	WHO/UNICEF Joint Monitoring Programme for Water Supply and Sanitation
L1, L2, L3	Molts of nematode larvae: L1 first stage larvae, L2 second stage larvae and L3 are third stage larvae
LAMP	Loop-mediated isothermal amplification
MDA	Mass Drug Administration
MDG	Millennium Development Goals
MST	Microbial source tracking
ND5	NADH dehydrogenase subunit 5
NTC	No-template control

NTD	Neglected Tropical Disease
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction, also known as real-time PCR
R^2	Correlation coefficient
rDNA	Ribosomal DNA: rDNA is the chromosomal DNA that encodes for the 16s rRNA sequence
RNA	Ribonucleic acid
SD	Standard deviation
SDG	Sustainable development goals
SSU	Small subunit
STH	Soil-transmitted helminths
TO	Tuck Ordinary (TO) mice
UN	United Nations
WASH	Contraction of Water, Sanitation and Hygiene as a discipline or sector
WHA	World health assembly
WHO	United Nation World Health Organisation
WBE	Wastewater- based epidemiology

Chapter One: General introduction

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Schistosomiasis, is a chronic neglected tropical disease (NTD), caused by trematode helminths called *Schistosoma*. It is estimated that 236 million people worldwide are infected with these parasitic worms: with over 90% of cases occurring in underserved communities within sub-Saharan African countries (Figure 1.1) (WHO, 2021). Two species cause the majority of infection and disease on the African continent: *Schistosoma mansoni*, causing intestinal and hepatic schistosomiasis, and *S. haematobium* which causes urogenital schistosomiasis. In Asia, *S. japonicum* also causes intestinal and hepatic schistosomiasis. *Schistosoma* parasites require a mammalian and aquatic snail host to complete their lifecycle (Figure 1.2) and humans are the main mammalian hosts for *S. mansoni* (Colley *et al.*, 2014). This thesis focuses on *S. mansoni,* but examples within this literature review are used from the across species infecting humans.

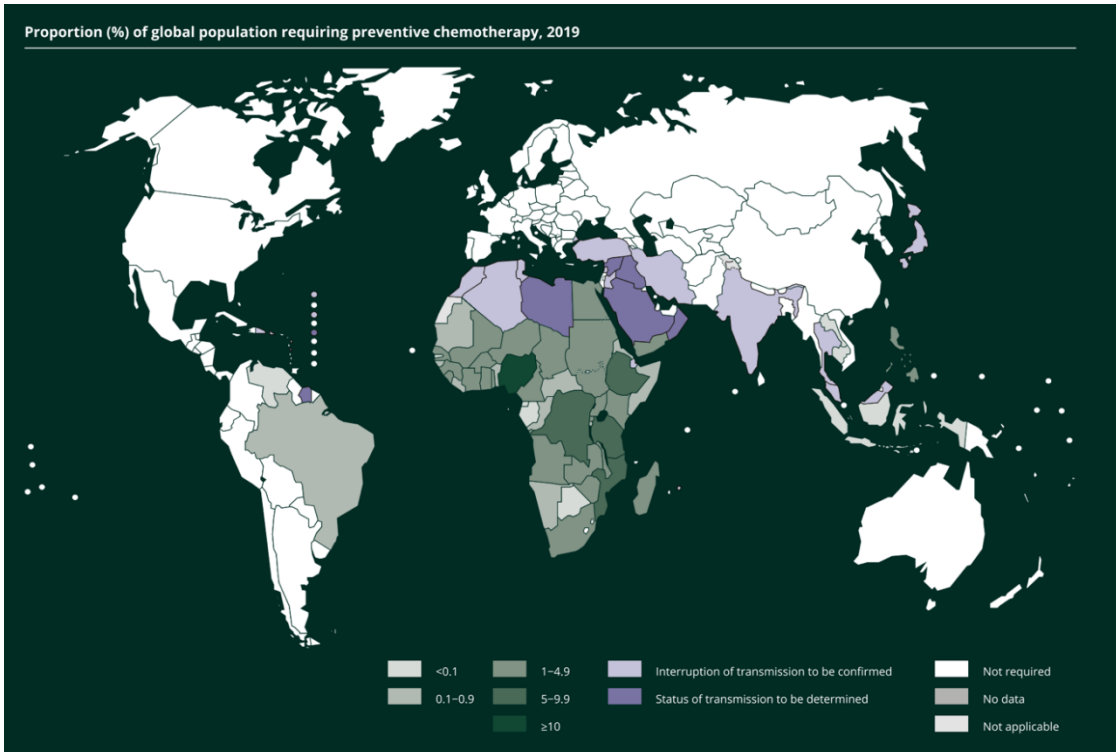


Figure 1. 1. Schistosomiasis is currently endemic in 78 countries across the globe, however more than 90% of the disease burden and therefore those requiring treatment, live in sub-Saharan Africa. Approximately 236 million people in 2019 required preventative chemotherapy with praziquantel. Figure from World Health Organization (WHO, 2020a).

S. mansoni has a complex lifecycle, their eggs are excreted from humans, their definitive host, hatch in freshwater and infect snails of the *Biomphalaria* genus, their intermediate host (Figure 1.2). When access to safe water is limited, a person can become infected through contact with cercariae-infested water: these cercariae are shed from infected aquatic snails. Once humans are infected, the immature stage of the schistosome (schistosomula) develop within the human host and mature into adult parasitic worms which then sexually reproduce. Their eggs are released into the environment through their host's excreta, faeces in the case of *S. mansoni* and *S. japonicum* and urine in the case of *S. haematobium*. Therefore, transmission of human schistosomiasis is intrinsically linked with inadequate water, sanitation, and hygiene (WASH) (Grimes *et al.*, 2015).

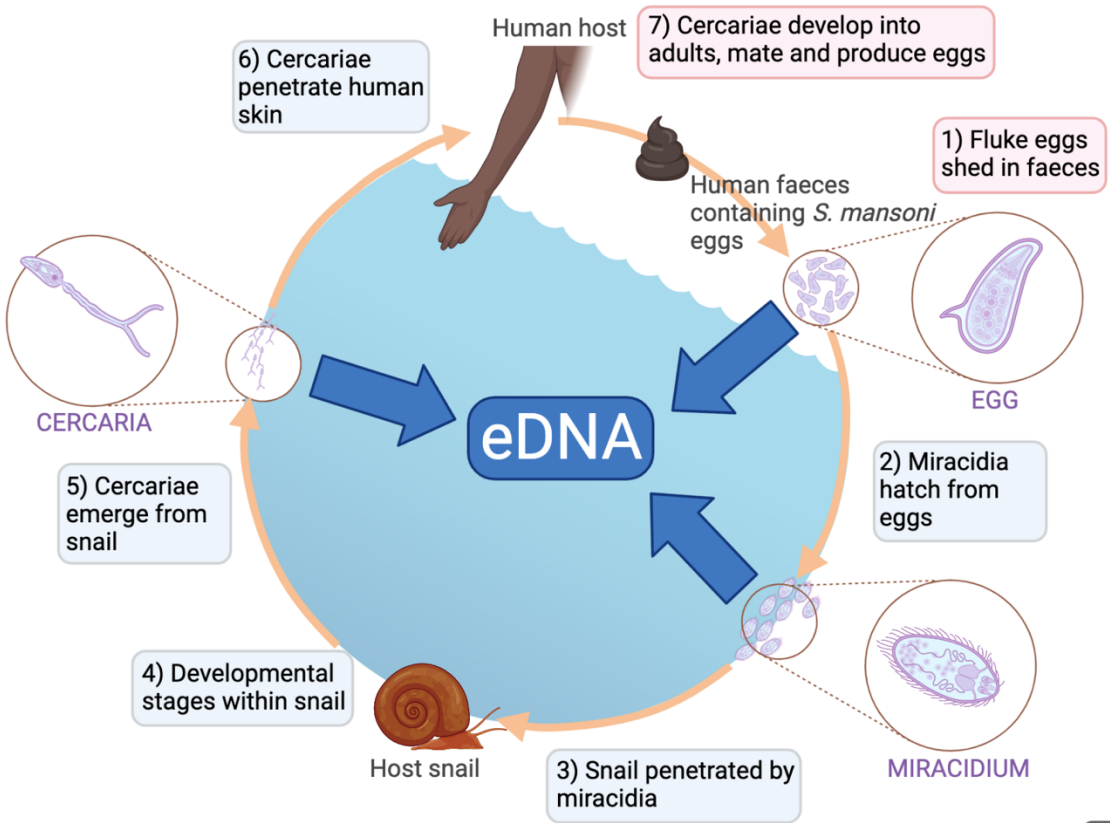


Figure 1. 2. Lifecycle of *Schistosoma mansoni*. The parasitic fluke has obligatory lifecycle stages in both a human host and snail host. An infected human sheds *S. mansoni* eggs in their faeces (1). If an egg reaches fresh water, it hatches, releasing the snail-infective miracidia (2). The miracidia then penetrate a susceptible snail (species of *Biomphalaria*) host (3) and undergo asexual developmental stages with the snail tissue (4). The human-infective cercariae are then released into the water (5). Without the provision of safe water, a human will encounter water containing cercariae and risk infection. The cercariae actively seek out their host and penetrate a person's skin (6). Within the human host the parasite migrates through the body and matures into an adult before pairing and mating to produce eggs (7), which are then released into the environment via the human host's faeces (1). If no, or inadequate, sanitation is used by the infected individual(s), the risk of the *S. mansoni* eggs being deposited in the aquatic habitat of the snails is increased. Lifecycle created using Biorender.com.

The World Health Organization's (WHO) recommended main control strategy is mass drug administration (MDA) with praziquantel, a schistosomicidal drug (WHO, 2021). Current control efforts therefore focus on large-scale preventative chemotherapy treatment using MDA of praziquantel, to at risk human populations and in 2017, 98.7 million people were administered praziquantel (WHO, 2021). The WHO had earmarked schistosomiasis for elimination as a "public health problem" by 2020 (Toor, Alsallaq, *et al.*, 2018) however, this target was missed (WHO, 2020a). The WHO NTD 2030 roadmap now aims for elimination of schistosomiasis as a public health problem by 2030 and the control strategy for reaching this new goal, is again, mainly through MDA (WHO, 2020a).

1.1. Limitations of mass drug administration

Successful treatment of an infected individual can stop the production of eggs if schistosome adults are killed by the praziquantel treatment. If a person is no longer shedding eggs into the environment, via their faeces in the case of *S. mansoni* (Figure 1.2: Step 1), this would prevent susceptible aquatic snails from being infected by the hatched eggs (Figure 1.2: Steps 2 and 3). However, praziquantel is not as effective in killing juvenile worms within the human host and if these juveniles survive praziquantel treatment, they can go on to mature into adults and produce eggs (Figure 1.2: Step 7). Additionally, praziquantel is not a prophylaxis and reinfection post-treatment can occur rapidly if no safe, cercariae-free water provisions are available (Figure 1.2: Step 6) (Grimes *et al.*, 2015). Transmission of schistosomiasis is multi-layered, and poverty ensures the continuing transmission of this parasite despite nearly two decades of MDA. Inadequate WASH plays a fundamental role in maintaining the transmission of the parasite, providing the opportunities for snail to human and human to snail transmission via the environment (Grimes *et al.*, 2015).

There have been successes in controlling and eliminating the parasite in low-transmission settings (Rollinson *et al.*, 2013). However, in highly endemic areas without an integrated approach to control, the transmission continues (Freeman *et al.*, 2013) and models predict that treatment alone is not sufficient to break the cycle of transmission (Toor, Alsallaq, *et al.*, 2018). Uganda, where the fieldwork for this thesis took place, was the first sub-Saharan African country to implement MDA for schistosomiasis (Adriko *et al.*, 2018). After 18 years of MDA,

Uganda still has an estimated national prevalence, estimated by CCA (Circulating Cathodic Antigen) testing, of 25.6%, which is over 10 million people (Exum *et al.*, 2019). Additionally, 55% of Uganda's population are thought to be at risk of infection by schistosomes (Loewenberg, 2014). There remain persistent hotspots for schistosomiasis that coincide with communities living with low sanitation coverage (Kabatereine *et al.*, 2011). Within the Ugandan district of Mayuge, where this project's fieldwork was carried out, the prevalence of schistosomiasis in school-aged children is often more than 80% (Trienekens *et al.*, 2020). An integrated strategy is needed to control transmission in moderate and high endemicity settings, and should encompass improved effectiveness of, access to, and use of, WASH facilities, alongside community-led behaviour change campaigns and where viable, snail control in the environment (Rollinson *et al.*, 2013). This thesis aimed to investigate molecular-based tools that could be used to monitor the improvements and effectiveness of such sanitation facilities.

1.2. Integrated control: WASH

As well as extending praziquantel treatment to all populations, the renewed WHO targets include targets for cross-sectoral collaboration (WHO, 2020a). This cross-sectorial partnerships could include NTD and WASH specialised stakeholders working together, as well as the mainstreaming of schistosomiasis and other NTDs into the more general efforts towards universal health coverage (WHO, 2020a). Improved sanitation could also have a positive effect on health outcomes beyond schistosomiasis, such as diarrhoea and stunting in children (Alzua *et al.*, 2015).

Without improved WASH, individuals within schistosomiasis endemic communities are continually exposed to high-risk environments. The risk of reinfection post-MDA remains without the provision of safe water: at least 250 - 600 *S. mansoni* cercariae per day can be shed from infected snails (Ayad, 1974). Malacological controls (chemical: molluscicides, biological: habitat alteration and introduction of predators) can be used to reduce the snail population but as snails are hermaphrodites, only one snail is required to survive to repopulate a habitat (Sokolow *et al.*, 2018). Without safe disposal of infective human waste, the snail habitats will continue to be contaminated, enabling the lifecycle of the schistosome to continue. The sustainable development goal

(SDG) 6 to provide sanitation for all by 2030 is not on track to be met, in 2020 3.6 billion people lacked safely managed sanitation services, which includes an estimated 494 million people practicing open air defecation (WHO UNICEF JMP, 2021a).

The primary risk factor in Uganda for *S. mansoni* infection is an individual's open defecation behaviours (Exum *et al.*, 2019). Prevalence of schistosomiasis is lower when individuals report that they exclusively use sanitation facilities compared with those who report open defecation: 22.3% population positive for schistosomiasis versus 29.0% positive respectively (Schisto, 2017). One of the research agendas for the WHO and Neglected Tropical Disease NGO Network is to investigate WASH interventions' effectiveness on reducing infection with NTDs (Boisson *et al.*, 2021). This requires monitoring tools for the environmental impact of WASH interventions, which could provide information on the risk to humans, through measuring snail output into the environment, and additionally monitoring the human output into the environment (Grimes *et al.*, 2014). Access to, and use of, WASH may be monitored via observations, surveys, and recall (Grimes *et al.*, 2014). Yet, translating these qualitative, and potentially biased, results into quantitative values to monitor the actual effectiveness of WASH facilities in limiting environmental transmission of schistosomiasis, remains challenging (Campbell *et al.*, 2018).

1.2.1. Sanitation's role in schistosomiasis transmission.

In the resource-poor settings where schistosomiasis is endemic, sanitation is inadequate, or sometimes non-existent (Campbell *et al.*, 2014). In 2013, it was estimated that for 1.77 billion people worldwide (~25% of the global population at that time), their primary means of sanitation was some form of pit latrine, a basic and often poorly managed sanitation solution (Graham and Polizzotto, 2013).

1.2.1.1. Successful sanitation interventions

The WHO/UNICEF Joint Monitoring Programme (JMP) for Water Supply, Sanitation and Hygiene have ranked sanitation on a scale from safely managed

(the best) to open defecation (the poorest sanitation service) (WHO UNICEF JMP, 2021b). Unimproved or basic sanitation services, as defined by the WHO/UNICEF JMP for Water Supply, Sanitation and Hygiene, fail to hygienically separate excreta from subsequent human contact (WHO UNICEF JMP, 2021b).

In sub-Saharan African countries, pit latrines are commonly employed, as they are often the cheapest and most logistically simple form of sanitation (WHO, 2017). A basic pit latrine consists of a dug-out pit in the ground, a squathole for the disposal of excreta, and a superstructure for privacy (WHO, 2017). An improved pit latrine deploys a slab or platform covering the ground in which the squathole is dug to serve as a physical barrier between the user and soil. If there is no lining to the dug-out pit, excreta within have the potential to leach into the groundwater. This has significant negative implications for human health, as it can result in the contamination of drinking water with faecal enteric pathogens (Graham and Polizzotto, 2013). To be a safely managed facility, a pit latrine must not be shared with other households and the excreta must be treated and disposed of safely, either *in situ* or stored temporarily before off-site treatment (WHO UNICEF, 2020). Many existing pit latrines will need to be improved to meet the UN's target for universal access to safely managed sanitation by 2030 (SDG 6) (WHO UNICEF, 2020).

In schistosomiasis endemic areas, if faecally contaminated ground water flows into the freshwater habitats of susceptible snails, the perpetuation of the schistosome's lifecycle can also be facilitated. The transportation of schistosome eggs and microbes into the aquatic habitat of the snails will be affected by environmental conditions. Hydroecological factors such as rock strata, soil characteristics and water table depth can affect the functionality of pit latrines, resulting in containment failure and water flowing freely in and out of the latrines (Graham and Polizzotto, 2013). Thin, rocky soils and rocky areas with shallow water tables could pose the highest risk of pit latrine effects on groundwater, although to date, there have been no studies investigating the transportation of helminth eggs into the unsaturated (vadose) zone of groundwater (Graham and Polizzotto, 2013). Arguably, some soil may also have protective properties, as they often have a role in the human waste purification,

including the removal of faecal microorganisms as water filters through the soil layer within the unsaturated zone (Lewis, Foster and Drasar, 1980).

In areas where groundwater and freshwater do not interact, as is likely during the wet/rainy season, heavy rain can cause the flooding of pit latrines: resulting in the transportation of schistosome eggs into the freshwater habitats of the snails. These seasons of high rains can coincide with the peak of snail numbers (Ernould, Ba and Sellin, 1999; Perez-Saez *et al.*, 2016). Schistosome eggs have been shown to survive for up to three weeks within drying pit latrine sludge (Newton *et al.*, 1948) and surviving eggs could hatch when the environmental conditions became more favourable (Jones *et al.*, 1947; Newton *et al.*, 1948; Maldonado, Acosta-Matienzo and Thillet, 1949; Kawata and Krusé, 1966). Thus, if the pit latrine does not effectively contain, or inactivate, the schistosome eggs, they have the potential to progress to their next lifecycle stages and contribute to onward transmission to humans. Unlike the transmission of bacteria, viruses (Graham and Polizzotto, 2013) and STH egg/ova (Baker and Ensink, 2012), the extent of schistosome egg containment by pit latrines is still an unknown.

1.3. Role of environmental monitoring tools in control efforts

The effects of schistosomiasis control measures are currently assessed by monitoring human infection (Colley *et al.*, 2014). These epidemiological data have been used to investigate the relationship between sanitation and schistosomiasis: however sensitive techniques to monitor the environment directly have been unavailable (Sengupta *et al.*, 2019). As control efforts become multifaceted, including, but not limited to, the improvement of sanitation facilities, direct environmental monitoring tools will be useful to assess the efficacy of these control measures. Snails can be collected and monitored to determine their infection status, but this form of environmental monitoring is labour-intensive, lacks sensitivity, and does not provide information on how the eggs are being transported into the snail habitat or the effect of sanitation facilities in this transmission (Sengupta *et al.*, 2019) and these survey methodologies remain unstandardised (Maes *et al.*, 2021).

1.3.1. Conventional environmental monitoring techniques

In the context of schistosomiasis, methods for determining what is occurring in the environments interfacing (in direct contact) with the sanitation system, remain underdeveloped and primarily focus on non-nucleic acid-based methods. Endemicity of *S. mansoni* within a community is used to inform the extent and frequency of praziquantel administration (WHO, 2021). Human infection status is determined by the presence of eggs in stool and enumerated by a standardised method called a Kato-Katz thick smear Katz, Chaves and Pellegrino, 1972). This gives indispensable information on individual parasite loads and community prevalence but provides limited information on the effectiveness of new sanitation interventions, or safe-water provisions in the wider context of WASH. Studies reporting the impact of sanitation on the infection rates in people, show adequate sanitation is associated with lower odds of infection with *S. mansoni* (systematically reviewed by Grimes *et al*. (2014)).

Historically, the impact of sanitation interventions has been measured by changes in human infection: not by direct evaluation of schistosome or faecal contamination related to sanitation interventions. These studies have relied on observational data (as opposed to cluster randomized controlled trials), often with weak statistical power (Grimes *et al.*, 2014). As such there is a requirement for an evidence-based argument to enable and monitor an integrated approach to schistosomiasis control programmes (Campbell *et al.*, 2018). This requires robust and direct investigations into the impact of sanitation on *S. mansoni* transmission, alongside the current monitoring of human schistosomiasis cases. Currently, there is no link-up between the monitoring of WASH access with epidemiological data (Campbell *et al.*, 2018) and despite the WHO 2030 roadmap calling for an investigation into environmental transmission, there are still no guidelines to enable robust and reproducible monitoring (WHO, 2020a). Furthermore, there is currently a lack of standardised tools fit for this purpose. Guidelines are also needed to inform the construction, maintenance and evaluation of improved sanitation facilities specifically relating to schistosomiasis and other human-related helminths (Campbell *et al.*, 2018). The current environmental monitoring options are summarised in Figure 1.3.

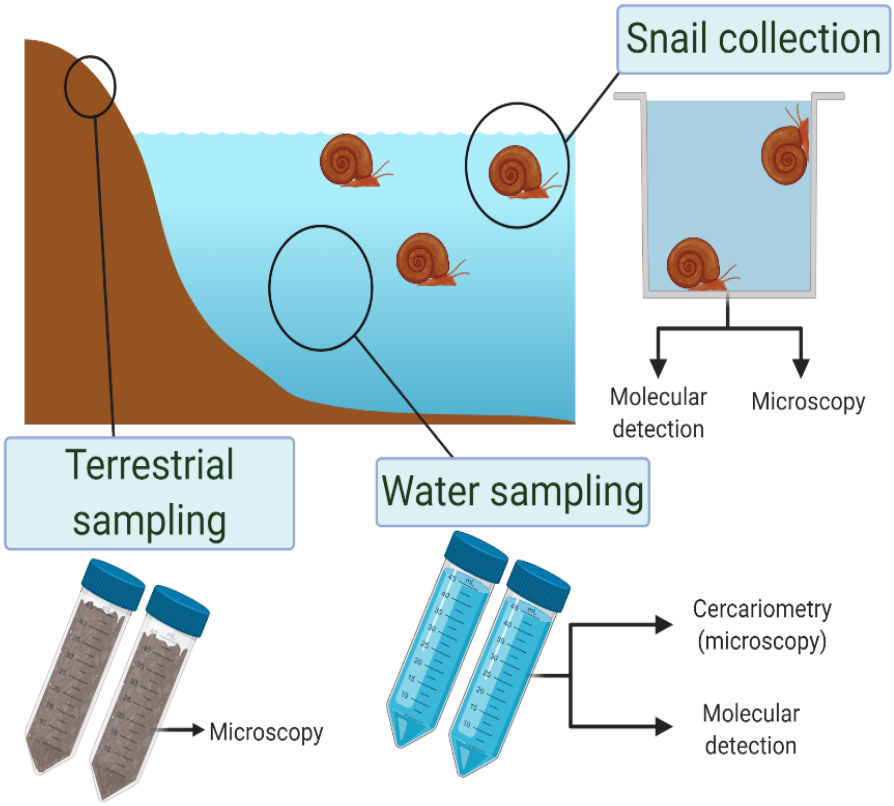


Figure 1. 3. Summary of schistosomiasis detection techniques available to sample freshwater and environments interfacing with sanitation. Sampling of the aquatic environment can consist of collecting snails, collecting water from the snail habitat, and collecting soli matrices associated with sanitation such as wastewater sludge and soil. Traditional methods to identify and quantify the parasite in environmental samples have relied upon microscopic techniques. Created using BioRender.com

Water-based epidemiology (WBE) has been used in contexts outside of schistosomiasis and STHs, to use wastewater as a matrix for analysis by molecular-based monitoring techniques (Sims and Kasprzyk-Hordern, 2020). These techniques use the same general sample processing procedures: extraction, detection and subsequent analysis and interpretation of biological/chemical compounds (i.e., biomarkers). In countries with centralised wastewater treatment, WBE has been used to monitor drug residues on a population-level (Sims and Kasprzyk-Hordern, 2020), these techniques can also be expanded to include biomarkers linked to other aspects of public health, including diet, stress, and communicable diseases' DNA and RNA. WBE can be utilised as a surveillance system at a community-level (Sims and Kasprzyk-Hordern, 2020) for infectious diseases, and its methodology could be adapted and optimised to de-centralised sanitation systems which are commonplace in rural and resource-poor communities where waterborne sanitation is not available.

1.3.1.1. Monitoring of the soil environment

Investigations into freshwater environments and water treatment have been at the forefront of research (e.g., Secor, 2014; Braun, Grimes and Templeton, 2018). Investigations quantifying eggs in environmental matrices such as wastewater, sludge and soils are infrequently used and were adapted from centrifuge-based separation methods of helminth eggs/ova from faeces (Faust *et al.*, 1938). The centrifugal flotation techniques developed in the early 20th century enable egg separation from the bulk of an environmental sample, by the specific gravity of the helminth eggs/ova. However, techniques used to detect STH in wastewater, such as centrifugal sedimentation or flotation, are less effective for recovering *Schistosoma* eggs (Smith, 1999). *Schistosoma* eggs can become shrivelled in flotation fluids, distorting them beyond the scope of microscopic identification and the sedimentation process can induce egg hatching. In the context of STH detection in such environmental matrices, molecular approaches have by contrast proven to be sensitive, specific, and rapid alternatives to detection by microscopy (Amoah *et al.*, 2017). However, as with schistosomiasis monitoring more broadly, the uptake of molecular methods for STH monitoring has been higher in clinical settings than for community level monitoring (Amoah *et al.*, 2017). The scientific literature is more limited to water-based investigations of the schistosomes regarding both microscopy- and nucleic acid- based methods to investigate the transmission of *Schistosoma* species in the environment.

1.3.1.2. Monitoring of the aquatic environment

1.3.1.2.1. Cercariometry & sentinel mice

Monitoring approaches for aquatic environments can be broadly categorised into collection of water samples, collection of the intermediate snail host or sentinel rodent infections (Sokolow *et al.*, 2018). Cercariometry, which requires filtering water from endemic areas, to collect and then count cercariae, can be used to infer the density of cercariae and transmission risk to humans in the environment (Aoki *et al.*, 2003). These data can be analysed with metadata such as hydrodynamic conditions, temporal variations related to seasonality and pre-existing knowledge of the peak cercarial emissions from the snails to estimate

cercarial densities (Théron, 1986). Counting is often performed using microscopy. As there are cercariae emerging from snails from other species of trematode, skilled personnel are required to identify the species based on their furcocercous tail morphologies, as first developed by Frandsen & Christensen (1984). As human and non-human schistosomes are often co-endemic, species identification is key to accurate xenomonitoring: the screening of intermediate hosts for the pathogen they can transmit.

An alternative to identification by microscopy techniques is the sentinel rodent technique in which mice are exposed to waterbodies containing cercariae, then maintained for six weeks whilst the parasites migrate and mature, before dissection to isolate and count adult worms in their bloodstream by perfusion. This technique is useful for directly assessing infection risk to humans from water contact and was developed in China where *S. japonicum* is endemic, this species' cercariae are stickier and more difficult to filter by methods used for *S. mansoni*. This technique, however, raises ethical issues and is logistically challenging and costly due to both the technical staff, laboratory time and resources required (Hung, Remais and Webster, 2008).

1.3.1.2.1. Snail surveys

Xenomonitoring, the surveillance of intermediate hosts (in this instance infected snails), can provide information on the dynamics of transmission between humans and snail vectors and are the mainstay of monitoring efforts after human infection surveys. Due to transmission focality and the resources available for surveillance, targeted sampling at transmission sites is favoured over systematic sampling (Sokolow *et al.*, 2018). The snails from these surveys are processed to determine their infection status, by the traditional method of cercarial shedding, crushing or by molecular methods as described below. To standardise snail collection a timed sampling effort should be carried out: the snails are commonly scooped with a sieve (mesh size of ~2 mm) for a set amount of time in each sampling location (Hairston *et al.*, 1958).

To determine the infection status of the collected snails, they are either placed in fresh water and exposed to light to test for cercarial shedding, or the snails can be crushed and dissected to identify the sporocyst (the lifecycle stage prior

to the cercarial stage) and immature cercariae. Cercarial shedding first occurs 28-42 days after the snail is exposed to miracidia (Sokolow *et al.*, 2018). Ideally snails are maintained and observed daily for up to six weeks but due to limitations associated with working in the field, a single time point can be chosen to observe the snails' infection status (Sokolow *et al.*, 2018). Large numbers of snails are needed as there can be high mortality of snails during this observation period (Hanelt *et al.*, 1997) and it is common to detect only a few infected snails amongst thousands of uninfected snails surveyed (Hamburger *et al.*, 1998). Identification of the shed cercariae by microscopy is most commonly used, alternatively PCR assays from DNA extracts from snail tissue can be used (Table 1.1).

PCRs were initially performed on DNA extracted directly from snail tissue and pooling of samples can increase the scale of snail processing and reduce costs. An alternative high throughput and sensitive method uses DNA extracts from the tank water incubating the snails. PCR has been the most widely applied and published molecular detection approach, however loop-mediated isothermal amplification (LAMP)-based methods using extracts from snail tissue have also been successfully employed more recently (Hamburger *et al.*, 2013; Gandasegui *et al.*, 2016; Caldeira, Jannotti-Passos and dos Santos Carvalho, 2017). Advantages of LAMP over PCR, include 1) simpler equipment requirements, such as a water bath or heat block, due to simpler reaction conditions required (only a single temperature, instead of precise cycling in PCR), and 2) visual readouts, where a positive reaction can be determined with the naked eye (Gandasegui *et al.*, 2016). PCR, by contrast, requires a thermocycler and fluorescence detection. It is noted that both LAMP and conventional PCR are presently limited in field use by the DNA extraction stage, which is often reliant on laboratory equipment, and is a pre-requisite for application of either method.

1.3.1.3. Indirect monitoring

In addition to traditional methods of estimating transmission risk from snails, mapping tools using remotely sensed environmental and climatic data are used to identify snail habitat niches and predict schistosomiasis "hotspots" (Wood *et al.*, 2019). Hotspots are areas with persistent transmission sites despite multiple years of well-implemented mass drug administration coverage (Kittur *et al.*,

2017). For monitoring sanitation interventions, a proxy for schistosomiasis "hotspots" in the environment could be microbial source tracking: the determination of faecal pollution sources. Faecal indicator bacteria, such as *Escherichia coli* and faecal coliforms can be used as a proxy for enteric pathogens. Environmental samples can be investigated by culturing samples and counting colony forming unit by eye or extracting the DNA from the samples for PCR. Research on the impact of sanitation, on enteric pathogens, have used faecal contamination to directly investigate transmission pathways (these techniques have been systematically reviewed by (Sclar *et al.*, 2016). In low-resource settings, where pit latrines are commonplace, investigations into the presence of faecal makers in households (e.g., Pickering et al., 2012; Boehm et al., 2016; Fuhrmeister et al., 2020) as well as the contamination of groundwater in relation to pit latrines (Graham and Polizzotto, 2013) have used microbial source tracking. In the context of schistosomiasis, faecal indicator bacteria along with human prevalence data were used by Ponce-Terashima *et al.* (2014) to explore the transmission dynamics of *S. mansoni* in water contact sites.

Table 1. 1. Summary of nucleic-acid detection of *Schistosoma mansoni* in environmental samples. Studies have successfully used polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP) assays to detect *S. mansoni* in the environment. The studies presented in this table have been selected arbitrarily to illustrate the different nucleic-acid-based methods used to detect *S. mansoni* from the non-human stages of its lifecycle. DNA was extracted from samples collected by malacological surveys, or more recently directly from water collected at transmission sites using environmental DNA (eDNA) methods. The lower limits of detection (pg= picogram, fg= fentogram) by each molecular method are stated as published.

Source of DNA extracts			Primer target	Method	Detection limit	Reference
Snail survey		eDNA (from water)				
Incubated tank water	Snail tissue					
	•		Mitochondrial DNA	Multiplex PCR	1 pg	(Jannotti-Passos *et al.*, 1997)
	•		18S rRNA	Nested PCR	10 fg	(Hanelt *et al.*, 1997)
•	•		Sm1-7 (M61098) (repetitive sequence)	PCR	1 fg	(Hamburger, Yu-Xin, *et al.*, 1998)
•			Sm1-7 (M61098) (repetitive sequence)	PCR	1 fg	(Hertel *et al.*, 2004)
	•		Small subunit (SSU) rRNA	two-step nested PCR, single- tube nested PCR & PCR	10 pg, 0.1 fg, 1 fg respectively	(Melo *et al.*, 2006)
	•		Mitochondrial DNA	Multiplex PCR	-	(Jannotti-Passos *et al.*, 2006)
	•		Sm1-7 (M61098) (repetitive sequence)	LAMP	0.1 fg	(Abbasi *et al.*, 2010)
	•		Sm1-7 (M61098) (repetitive sequence)	LAMP	-	(Hamburger *et al.*, 2013)
	•		Ribosomal intergenic spacer 28S-18S rRNA	LAMP	0.1 fg	(Gandasegui *et al.*, 2016)
	•		Mitochondrial gene ND5	PCR	> 0.1 fg	(Lu *et al.*, 2016)
	•		Internal transcript spacer (ITS) of rDNA gene	Low stringency-PCR & LAMP	70 fg	(Caldeira et al, 2017)
		•	Mitochondrial gene cytochrome oxidase I (COI)	Real-time PCR	-	(Sato *et al.*, 2018)
		•	Mitochondrial gene cytochrome oxidase I (COI)	Real-time PCR	1 DNA copy/ qPCR reaction	(Sengupta *et al.*, 2019)
•			Mitochondrial 16s rRNA gene	Real-time PCR	100 copies/µl	(Alzaylaee *et al.*, 2020)

1.4. Environmental DNA

Environmental DNA (eDNA) can be defined as the DNA retained by, or released, from organisms interacting in their surroundings, which is then extracted from environmental samples (Thomsen and Willerslev, 2015). In the context of schistosomiasis this eDNA could be extracted directly from an intermediate host organism (infected snail), samples from an infected organism (human excrement) and environmental matrices (soil, water, sewage) (Bass *et al.*, 2015). The downstream analysis of eDNA can be contentious (Bohmann *et al.*, 2014) and applications vary between aiming to capture the entire diversity of organisms within an environmental sample and those targeting a specific species or even strain (Thomsen and Willerslev, 2015). In the context of using eDNA as a monitoring tool to measure the successful uptake and impact of improved sanitation as a supplement to drug-based control strategies, detecting *Schistosoma* species specifically is likely of more value than a metagenomic survey of the environmental sample.

As discussed, current risk models and targeting of control measures rely upon ecological surveillance of snails, which have their drawbacks. Due to the parasite's asexual reproduction in snails, a single miracidium from an egg can result in 1000's of cercariae shed daily for the lifespan of the infected snail (Colley et al., 2014). Consequently, uptake of current and/or improved sanitation is unlikely to be linearly associated with reduced prevalence in humans, or in tank water containing snails sampled through xenomonitoring. Sampling the environment interfacing with a sanitation intervention, for example soil surrounding an improved pit latrine or the reusable products from sanitation interventions (composted sludge), would provide an indication of how the sanitation is functioning and its effect on transmission.

1.4.1. Application of environmental DNA in *Schistosoma mansoni* monitoring: water

Environmental DNA detection and sequencing from environmental samples, is an emerging environmental surveillance tool within the parasitological field (Bass *et al.*, 2015). In the last decade, eDNA has been successfully employed as a tool to specifically detect *S. mansoni* (Sato *et al.*, 2018; Sengupta *et al.*, 2019), *S.*

haematobium (Akande *et al.*, 2012) and *S. japonicum* (Fornillos *et al.*, 2019) in water samples. These studies sampled water from known water contact-based transmission sites in endemic settings to compare the arising data with that from traditional malacological surveys. Figure 1.4 illustrates the lifecycle stages that could contribute to schistosomal DNA detection in the wider environment.

In each of the eDNA studies, water samples were filtered and retained material fixed with 70% ethanol or RNAlater to preserve DNA for extraction and analysis by qPCR (quantitative PCR). The PCR techniques demonstrated specificity to single *Schistosoma* (and/or snail) species, being able to differentiate between *S. mansoni* and *S. haematobium* in co-endemic areas (Sato *et al.*, 2018). Both *S. mansoni* qPCR primers were designed to target a fragment of the mitochondrial cytochrome c oxidase gene (COI or COX1) and generate a short amplicon (Table 1.1). A short PCR product targeting a high copy number gene can enhance the sensitivity of the assay and still detect fragments of the DNA that result from environmental degradation. The sensitivity of the eDNA methods for *S. mansoni* were interrogated in a lab setting. Sengupta *et al.* (2019) used water samples taken from tanks containing different densities of infected host snails to determine the lower limit of detection: one DNA copy per qPCR reaction.

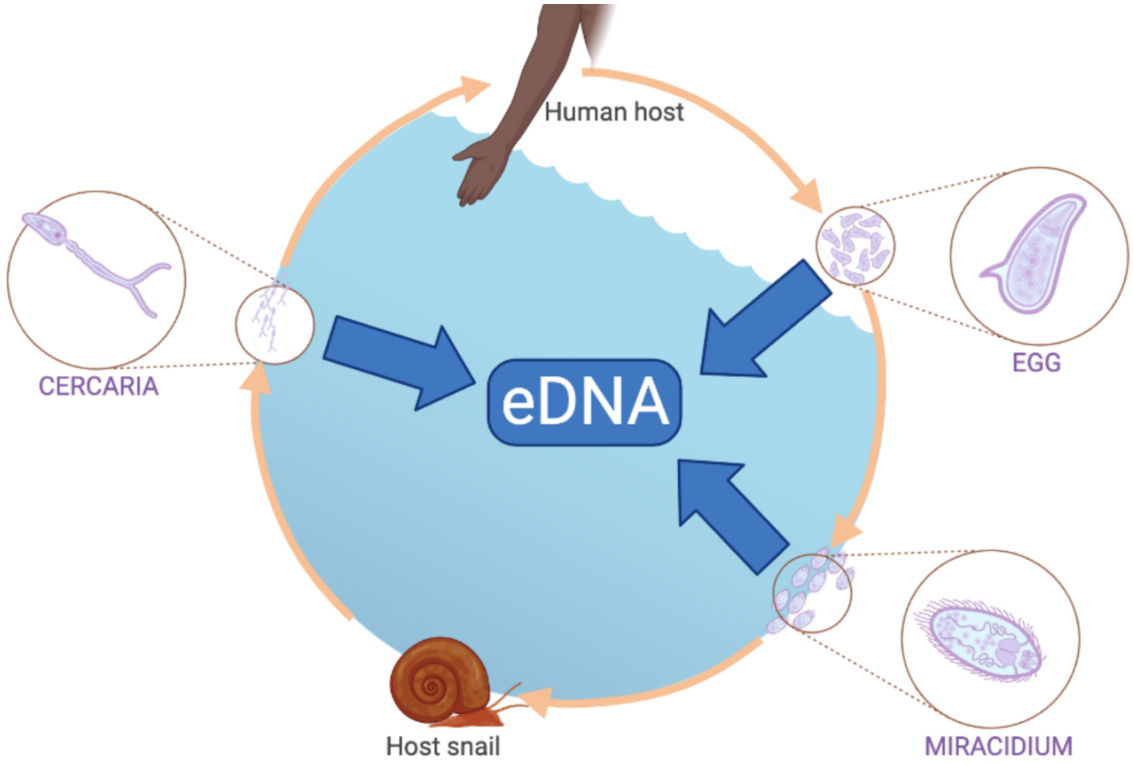


Figure 1. 4. Parts of the lifecycle of *Schistosoma mansoni* where environmental monitoring can be used. The free-living life stages of the parasite release DNA into the environment (eDNA). The transmission of *S. mansoni* can be disrupted with adequate water, sanitation, and hygiene (WASH) measures, that break the parasite's lifecycle. Figure created using BioRender.com.

Traditional snail surveys were used as a comparison of the molecular assay's sensitivity by both *S. mansoni* studies (Sato *et al.*, 2018; Sengupta *et al.*, 2019). The detection of *S. mansoni* by eDNA carried out by Sato et al. (2018) coincided with water contact sites where intermediate host snail species were found. Sengupta *et al.* (2019) maintained the surveyed snails to observe cercariae shedding and found the eDNA method more sensitive than the traditional methods of schistosomiasis detection. eDNA sampling has the potential to be an important, sensitive, and high throughput method of environmental surveillance.

Although the eDNA studies detected specific *Schistosoma* species by qPCR, they determined presence/absence rather than relative or absolute quantification. Estimating parasite numbers or densities in an environmental sample has its caveats. Aquatic stages of *Schistosoma* species, miracidia and cercariae, differ in size and cannot be distinguished by published eDNA assays, as the qPCR DNA targets are present across the parasite's lifecycle. Therefore, copy number determined by qPCR cannot currently be used to accurately infer the life-cycle stage or number of parasites from the amount of eDNA molecules detected in a water sample despite the potential of quantitative detection of DNA molecules by qPCR (Sengupta *et al.*, 2019).

1.4.2. Application of environmental DNA in *Schistosoma mansoni* monitoring: soil

There is potential for quantification of schistosome eggs in matrices associated with sanitation, as the eggs require specific environmental conditions to hatch (Standen, 1951). DNA could be extracted from samples collected directly from the sanitation facilities themselves (sludge, wastewater) or from soil interfacing with sanitation interventions. As soil is the main reservoir of STH eggs/ova, eDNA techniques have been used to detect and enumerate these parasites, for example hookworm ovum from soil and wastewater samples (Gyawali *et al.*, 2016, 2017). Copy number of a gene can vary for eggs (due to their maturity for example), therefore a range of gene copy numbers was estimated for a single hookworm ovum, this was then used to estimate the total number of ova in a wastewater sample (Gyawali *et al.*, 2017).

Whilst I have focused this introduction and literature review on *S. mansoni*, the techniques are highly transferable across *Schistosoma* spp. However, the biology of each species should be considered to tailor eDNA monitoring approaches beyond aquatic sampling that detect the parasite's obligatory water stages. The potential application to monitor sanitation has been discussed in the context of faecally transmitted *S. mansoni*, however for *S. haematobium* urination sites would need to be considered and sampling sanitation facilities may not provide a sufficient snapshot of the environmental transmission. For monitoring *S. japonicum*, where the majority of transmission does not occur from human to human, but from zoonotic hosts to humans, any soil eDNA detection could include monitoring of animal faecal contamination to improve understanding of transmission dynamics. The biology of the snails should also be considered to tailor eDNA detection of the snail host, for example the intermediate snail hosts of *S. japonicum* are amphibious, therefore sampling the soil environment as well as freshwater habitats can be informative in monitoring interventions (Fornillos *et al.*, 2019).

This chapter, and subsequent thesis, therefore, build on epidemiological and public-health focused reviews, by discussing the methods available for monitoring the various lifecycle stages of *S. mansoni* in soil and aquatic environments, and the potential for using eDNA as a tool to monitor the success of different sanitation interventions, far earlier than any subsequential reductions in human infection levels could be observed. This would enable uptake and success of interventions to be monitored in a more linear fashion, as well as identifying early on if interventions are not working as expected. In addition, combining eDNA data with mathematical models (such as Toor et al., 2018) may help to understand if there is a critical point of sanitation intervention uptake, where transmission to humans is significantly reduced. If sanitation intervention uptake has not reached this, monitoring only human infection levels might indicate that the sanitation intervention has not been successful at all, leading to premature cessation or lack of support of such interventions, when small additional increases in uptake may be all that is required to reap large rewards in reducing human infection levels. eDNA monitoring techniques developed for monitoring soil transmitted helminths (STH), other disease-causing parasites transmitted in the faeces which are often

co-endemic with schistosomiasis, are also reported and an analogy is drawn with the schistosomiasis field for which such tools are presently lacking.

1.4.3. Limitations of eDNA

As previously mentioned, eDNA cannot distinguish live and potentially infectious parasites from dead or dying organisms. RNA-based PCR assays have been used for molecular diagnostics of sampled snails (Table 1.1) and environmental RNA (eRNA) could enable detection of viability. Gyawali *et al.*, (2016) used propidium monoazide qPCR (PMA-qPCR) to successfully differentiate between viable and non-viable helminth ova of a species of hookworm (*Ancylostoma caninum*). Investigations into the decay of schistosome RNA will be key to assessing the usefulness of an eRNA tool for schistosomiasis. For both eDNA and eRNA, the environmental processes that will affect their movement and decay within the environment should also be noted. Studies into the spatial and temporal fate of eDNA in aquatic environments (Harrison, Sunday and Rogers, 2019) and soil (Prosser and Hedgpeth, 2018) in relation to biodiversity have been carried out but there are still knowledge gaps of the fate of eDNA in tropical ecosystems (reviewed in the context of faecal indicator bacteria by Rochelle-Newall et al., 2015).

The tools which are used to monitor schistosomiasis are ultimately determined by the rationale behind the type of intervention they are aiming to monitor, cost and local laboratory capacity. Snail surveys are imperfect but are field applicable: they require skilled personnel, but the equipment needed for both the snail surveying (scoop, container for collection) and shedding (beakers, microscope) are reusable and widely available. Sengupta *et al.* (2019) included a cost analysis of their eDNA study. The cost of eDNA based surveys is comparable with traditional methods if the number of samples taken from each site is reduced or pooled and therefore laboratory consumable costs lowered (PCR reagents, filters). If monitoring sanitation interventions, eDNA techniques cannot distinguish where the schistosome eggs have originated from: for example, if from an inadequate sanitation facility or from open defecation. Local information and observations during sample collection would complement the molecular data captured from the environment. Quantitative and qualitative information on community uptake of an intervention would also help to

understand if detection of the parasite in the wider environment is due to failure of sanitation to contain or treat the parasite or direct open defecation. Community engagement is also a key component to sustained adoption of any WASH intervention (Galvin, 2015; Schmidt, 2015).

1.5. Other WASH-linked parasites: soil transmitted helminths

As discussed, schistosomiasis won't be controlled and consequently eradicated by praziquantel alone in moderate and high endemicity areas. The sanitation-based interventions relevant for schistosomiasis are also effective against NTDs such as STHs (Vaz Nery *et al.*, 2019). If control moves forward in a more integrated fashion, the detection of STH eggs/ova and larvae will also be important; and the potential of eDNA in the surveillance of STH has already been noted by the WHO in their 2021-2030 roadmap (WHO, 2020a). Some species of STH have eggs that persist for long periods: *Schistosoma* eggs remain viable for up to six days whereas *Ascaris* eggs can survive within soil for several years in the right environmental conditions (Pitchford and Visser, 1972; Smith, 1999). eDNA could be used to inform quantitative microbrial risk assessment in the context of STH, specifically with insights into what is occurring within sanitation technologies.

In the context of this thesis, eDNA from soil samples will be used to form a picture of the environmental contamination related to current sanitation practices and infrastructure in Mayuge District, Uganda. By applying eDNA techniques to the soil, it is hoped to gain an understanding of *S. mansoni* and hookworm in the environment. As DNA extracts from environmental samples will be available for helminth detection, there is an associated opportunity to use eDNA to detect where human faecal contamination occurs in the environment, by means of bacterial faecal markers. Detecting both protozoan and eukaryotic eDNA together can provide a fuller picture of the soil environment.

1.6. Conclusion

Quantifying transmission risk of *S. mansoni*, by eDNA techniques to sample the aquatic environment, can provide valuable information on transmission risk

related to water access and use (Sato *et al.*, 2018; Sengupta *et al.*, 2019). However, data on the soil environment may be more beneficial for the evaluation of sanitation interventions including their uptake and effectiveness. eDNA could provide timely information on the success (or failure) of interventions in containing the faecal material and locate any leakage or general containment failure. The installation of safely managed sanitation interventions will have a delayed effect on a reduction of parasite burden for the local community, and environmental surveillance could be used as an early indicator. Uptake of interventions is as important as their functioning and information for local communities regarding sanitation upgrades could increase acceptability of interventions.

Monitoring the function of a sanitation technology, and water contact sites, by eDNA and human infection levels combined, could provide valuable information on local transmission pathways and the schistosomiasis force of infection within a given environment and how it could be reduced by different intervention types. This in turn would provide accurate data relating directly to the efficacy of sanitation and other WASH interventions which are currently difficult to monitor, and therefore to model their potential impact (Toor, Turner, *et al.*, 2018). eDNA has the potential to be a highly sensitive and high throughput tool, needed for enrolment and maintenance of sanitation for the long-term and sustainable control for schistosomiasis. Helping to fill key knowledge gaps on the potential for qPCR eDNA methods for detecting *S. mansoni*, hookworm and faecal pollution in the soil environment was the overall aim of this thesis.

1.7. Aims of the thesis

The first two aims of this PhD were to test the potential for, and trial the use of, eDNA-based techniques to detect *S. mansoni* in the soil environment and how the detection of this parasite relates spatially to sanitation facilities (pit latrines) (Chapters Two and Three). Building on previously published work on environmental monitoring of schistosomiasis in water, Chapter Two outlines a lab-based study to assess if eDNA-based qPCR methods could be used to quantify known numbers of *S. mansoni* eggs in soil. The specific aims of Chapter Two were:

- to select a qPCR assay for the detection of *S. mansoni*;

- to determine the relationship between known numbers of S. mansoni eggs in a controlled environment (phosphate-buffered saline) and corresponding qPCR C_T values from the extracted DNA;
- and to determine the relationship between known numbers of *S. mansoni* eggs in soil samples (by manual spiking with eggs) and qPCR C_T values of the extracted DNA.

Chapter Three is an investigation, using the optimised *S. mansoni* qPCR assay from Chapter Two, on soils collected proximal to sanitation sites (pit latrines and open air defecation) within a *S. mansoni* endemic village. The characterisation of the soil environment in this *S. mansoni* endemic village was expanded to the detection of faecal-associated bacteria (Chapter Four) and hookworm and a nematode-eating bacterium (Chapter Five).

Chapter Four's aims were

- to determine appropriate faecal-associated DNA targets for this S. mansoni high endemicity community in Uganda;
- to quantify the target DNA using 18S ribosomal RNA and 16S ribosomal RNA bacterial targets detected in the soil samples;
- and to determine the distribution of the faecal-associated bacterial concentrations in relation to increasing distances from three in-use pit latrines.

Chapter Five's two objectives were

- to apply a *N. americanus* qPCR assay to soils collected from soil-transmitted helminth endemic area;
- and to apply *C. nematophagum* PCR assay to soils collected from soil-transmitted helminth endemic area.

Chapter Six is the general discussion of the results from the previous data chapters and broader discussion of environmental monitoring of *S. mansoni* and hookworm using eDNA.

Chapter Two: Detection or quantification: verifying application of qPCR to assay *Schistosoma mansoni* DNA from known egg counts.

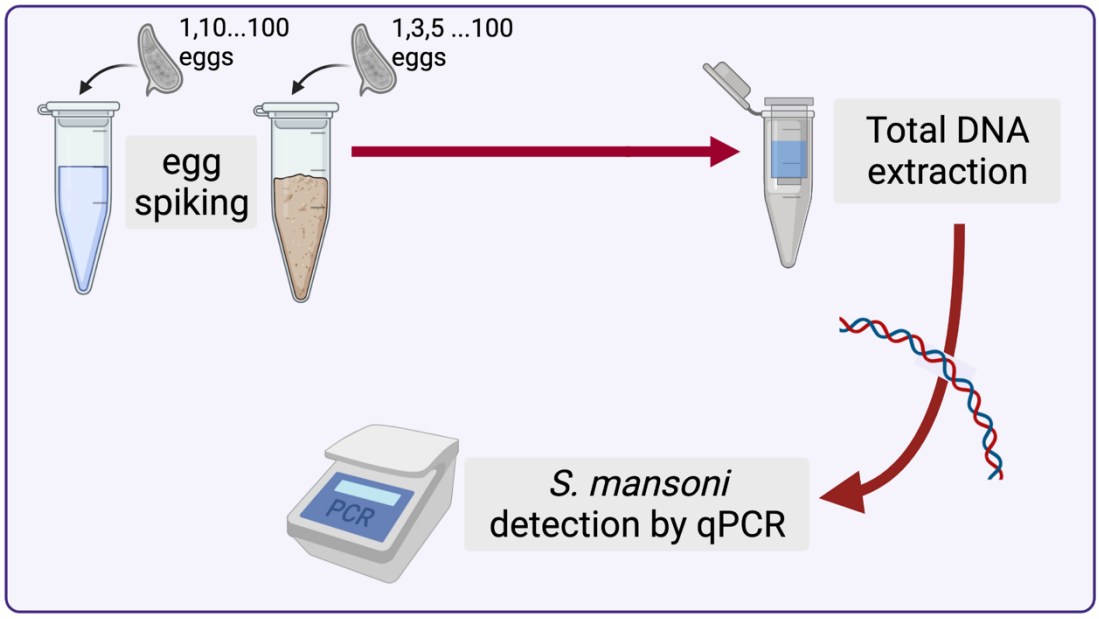


Figure 2. 1. Visual summary of Chapter 2's experiments: applying a *Schistosoma mansoni* qPCR assay to detect *S. mansoni* DNA extracted from egg-spiked matrices.

2.1. Abstract

The main aim of this chapter is to report on the application of eDNA-based techniques to the detection of *Schistosoma mansoni* in soil samples. The chosen qPCR assay, designed to specifically detect the cytochrome oxidase I gene of *S. mansoni*, was able to detect DNA from a single *Schistosoma* egg extracted in 500 mg of soil. The assay is highly sensitive but no relationship between egg number and C_T value was found. Therefore, the qPCR assay cannot reliably estimate the number of eggs in a sample but can be used in an absence/presence manner. This sensitive assay could be used as a surveillance tool for *S. mansoni* detection in the soil environment in the context of sanitation monitoring.

2.2. Introduction

Schistosomiasis is a human parasitic disease, which infects an estimated 236.6 million across the globe (WHO, 2019). In 2018, people in 52 countries required preventative chemotherapy for schistosomiasis, of which 41 were within Africa (WHO, 2018). In 2018 only 41.6% of people requiring praziquantel globally (40.9% in Africa), received treatment with this schistosomicidal drug (WHO, 2018a).

Treatment targets are consistently missed, and for those who are treated, praziquantel does not offer protection against reinfection (Wilson, 2020). Due to the obligatory aquatic snail stage of the parasite, a community's poor water, sanitation and hygiene (WASH) services can reduce the effectiveness of mass drug administration efforts in controlling transmission (Campbell *et al.*, 2018). Firstly, human sanitation practices and facilities affect the transmission of the schistosome from infected humans into the habitats of potential intermediate snail hosts (Grimes *et al.*, 2015). Secondly, in the absence of access to safe, cercariae-free water, routine activities that use water can result in schistosomiasis (re)infection.

2.2.1. Techniques for *Schistosoma mansoni* detection in the environment

WASH improvements are a key supplement to the current drug-based approaches to schistosomiasis control with the 2030 WHO roadmap highlighting their importance for reaching the 2030 goals (WHO, 2020a). These WASH improvements also require reliable, cost-effective monitoring methods to evaluate intervention uptake and success (Campbell *et al.*, 2018). Snail surveys are the current gold-standard for direct environmental surveillance of schistosomiasis (Sokolow *et al.*, 2018). The method involves collecting snails from transmission sites (human water contact sites) and determining if there are human-infecting species of schistosomiasis present in the surveyed snails. A trained individual can determine collected snails' infection statuses using histological examination of sporocysts or by induced cercarial shedding and morphological identification of the resulting cercariae (reviewed by Colley et al., 2014). There are also molecular-based methods to specifically detect the species of schistosome (Table 2.1), however the traditional microscopy-based methods are more commonplace. Snail surveys are labour-intensive, and have low sensitivity, due in part, to only 1-2% of the collected snail population being infected even in high endemicity areas (Lardans and Dissous, 1998).

Data on the presence of schistosomes at water contact sites, from directly detecting cercariae in water samples or indirectly by sampling snails, can provide information on the infection risk to humans. Methods to determine the infection risk from humans to snails, by investigating egg presence and

intensities in the environment, are less well developed. Contamination of the environment with schistosomes excreted from infected humans relies on the lack of adequate sanitation and/or safe defecation practices (Grimes et al., 2015) Open air defecation results in schistosome eggs being deposited directly into the soil environment and failing pit latrines can cause indirect contamination. Direct examination of the soil is uncommon to date, but methods to isolate schistosome eggs from this environmental matrix using centrifugal force, have been described (Smith, 1999). The flotation and centrifugal sedimentation methods used the specific gravity of *Schistosoma* eggs to separate the eggs from the bulk of environmental samples (Smith, 1999). However, the methods and reagents used to isolate the *Schistosoma* eggs can distort the eggs' shape rendering them beyond identification by microscopy and the flotation fluids can also induce egg hatching (Smith, 1999). Environmental DNA (eDNA) techniques to isolate, and specifically and sensitively detect, schistosomes in soil matrices could present a more accurate alternative to these current flotation-based methods.

Environmental DNA itself can be defined as DNA shed into the environment by an organism (Taberlet *et al.*, 2012). In the context of this thesis, eDNA is defined as all schistosomal DNA detectable from environmental samples: this includes the DNA shed by schistosome eggs into the environment and the parasites themselves which are free-living in the environment. Techniques used to characterise eDNA use nucleic acid extraction to capture DNA shed into the environment, these DNA extracts can then be characterised by molecular methods (Taberlet *et al.*, 2012). Depending on the aims of a research project, the molecular characterisation of eDNA extracts can employ metabarcoding methodologies to gain a broader insight into the taxa diversity within a sample, or take a more targeted approach, using predetermined PCR targets at a species or genus level (Taberlet *et al.*, 2012). eDNA is an emerging tool for biodiversity monitoring (Thomsen and Willerslev, 2015) and for environmental surveillance within the parasitology field (Bass *et al.*, 2015).

In the context of schistosomiasis, water samples have been collected to determine if species-specific PCR-based methods could provide an alternative to traditional snail surveys (Table 2.1). Studies have been published detecting the main three human-infecting *Schistosoma* species in water samples from endemic

areas, *S. mansoni* (Sato *et al.*, 2018; Sengupta *et al.*, 2019), *S. haematobium* (Akande *et al.*, 2012) and *S. japonicum* (Fornillos *et al.*, 2019). The *S. haematobium* PCR assay was based on the Dra 1 retrotransposon, a tandemly repeated DNA sequence (Akande *et al.*, 2012). The other studies report qPCR designed to target the mitochondrial cytochrome oxidase subunit 1 gene. This protein coding gene is commonly abbreviated as both Cox1 and COI, COI will be used throughout this thesis. COI is a popular target for phylogeographic studies of schistosomiasis due to the high copy number of this gene and the species specificity of the gene: resulting in a highly sensitive and specific PCR target (Zarowiecki, Huyse and Littlewood, 2007).

The two published *S. mansoni* specific eDNA studies both collected water samples from known *S. mansoni* transmission sites and were able to detect schistosome eDNA using the qPCR assays they developed to target the *S. mansoni* specific COI gene (Sato *et al.*, 2018; Sengupta *et al.*, 2019). The *S. mansoni* primers designed by Sato *et al.* (2018) and Sengupta *et al.* (2019) were both tested *in silico* for cross-reactivity with other *Schistosoma* species and the results indicated that they would both be species specific. Sato *et al.* (2018) additionally tested their *S. mansoni* COI qPCR assay using DNA extracted from adult *S. japonicum* and *S. mekongi* worms and they observed no amplification of these related *Schistosoma* species, confirming the species-specific amplification by their *S. mansoni* COI assay. The *S. mansoni* specific qPCR designed by Sengupta *et al.* (2019) was also tested for species specificity experimentally by using DNA extracts from *S. rhodaini, S. haematobium* and *S. bovis*. No amplification using their *S. mansoni* COI qPCR assay with these closely related *Schistosoma* species was detected either (Sengupta *et al.*, 2019). Species specificity within the *Schistosoma* genus is important as many areas of sub-Saharan Africa are co-endemic with human- and animal-infecting species and sanitation interventions will have different impacts on each (Leger and Webster, 2017).

Table 2. 1. Summary of a selection of molecular targets used to specifically detect *Schistosoma mansoni*. Monitoring of *S. mansoni* can be broadly categorised into two groups: determining the human input into the environment (infection risk from humans to snails) and measuring the output from snails into the aquatic environment (infection risk from snails to humans). The publications were selected arbitrarily to illustrate molecular assays that have been developed to specifically detect *S. mansoni* input into the environment from humans (faecal samples) or infected snail input into the environment (sporocyst samples, shed cercariae or water samples from known transmission sites). Three different molecular techniques have been employed for the specific detection of *S. mansoni* DNA: polymerase chain reaction (PCR), quantitative PCR (qPCR) and loop-mediated isothermal amplification (LAMP).

	Sample type	Molecular technique	Selected examples of *S. mansoni* specific molecular targets	Reference
Human input into the environment	Human faeces	PCR	Sm1-7 (M61098) (repetitive sequence)	(André Pontes, Dias-Neto and Rabello, 2002)
		qPCR	Small subunit rRNA	(ten Hove *et al.*, 2008)
		LAMP	Small subunit rRNA	(Gandasegui *et al.*, 2018)
Snail input into the environment	Snails (sporocyst examination)	PCR	Mitochondrial gene ND5	(Lu *et al.*, 2016)
		LAMP	Internal transcript spacer (ITS) of rDNA gene	(Caldeira, Jannotti-Passos and dos Santos Carvalho, 2017)
	Cercariae collected from snail shedding	PCR	Sm1-7 (M61098) (repetitive sequence)	(Hertel *et al.*, 2004)
		qPCR	Mitochondrial 16s rRNA gene	(Alzaylaee *et al.*, 2020)
	Water from transmission site	qPCR	Mitochondrial gene cytochrome oxidase I (COI)	(Sato *et al.*, 2018)
			Mitochondrial gene cytochrome oxidase I (COI)	(Sengupta *et al.*, 2019)

In addition to species specificity, these two *S. mansoni* qPCR assays were also shown to be highly sensitive. Sengupta *et al.* (2019) carried out two laboratory-based mesocosm experiments to investigate the lower limits of detection and decay of schistosome eDNA in water before carrying out a field-based trial of their qPCR eDNA assay. Sengupta *et al.* (2019) calculated the lower limit of detection for their qPCR assay to be one DNA copy per qPCR reaction. By using the qPCR assay to determine absence verses presence of the parasite, the eDNA method was shown to be more sensitive than traditional snail surveys: scooping snails for 20 minutes (Sengupta *et al.*, 2019). This was determined by snail surveys that were carried out in tandem with the eDNA-based sampling of water contact sites. The infection rates estimated by each method, snail survey and eDNA, were in the range of 0.0004-0.02 and 0.33-0.66 respectively (Sengupta *et al.*, 2019).

The qPCR assay developed by Sengupta *et al.* (2019) was used to determine the absence or presence of *S. mansoni* in water samples, the lower limit of quantification was determined to be 10 DNA copies per qPCR reaction. The qPCR outputs (C_T: cycle threshold) can be used to determine the sensitivity of an assay (lower limit of detection) and can also be used to quantify the copies of the specific gene that is being targeted by the assay within a sample. The qPCR reaction simultaneously amplifies the target DNA and monitors the real-time florescence output against a baseline which produces C_T values (Kubista *et al.*, 2007). These C_T values can then be used to calculate the copy number of the gene targeted by the qPCR assay if using a standard dilution series (standard curve) method for quantification (Kubista *et al.*, 2007). For single-celled organisms, copy number of the gene is a direct indicator of the number of individual organisms (such as bacteria) in the sample. However multicellular organisms, such as schistosomes, will have multiple copies of the gene as they are formed from more than one cell: therefore, estimating the number of organisms is challenging with regards to multicellular organisms such as schistosomes and soil-transmitted helminths (Papaiakovou, Gasser and Littlewood, 2019).

In the context of quantifying schistosome eggs in a sample using qPCR, the size of the eggs, and number of cells, will vary depending on their age/maturity

(Papaiakovou, Gasser and Littlewood, 2019). A study researching the quantitative capacity of a hookworm qPCR assays to enumerate eggs in wastewater samples circumvented the issue of an individual egg's potential copy number by instead determining a range of gene copy numbers for hookworm ova (Gyawali *et al.*, 2017). The potential to enumerate *Schistosoma* eggs in a terrestrial environment would add an extra level of detail when calculating the force of infection from humans to snails (Gurarie *et al.*, 2018).

Quantitative PCRs on eDNA extracts could have the potential to be highly sensitive, and in the context of sample processing within the laboratory, a high throughput tool for monitoring water contact sites. However, eDNA-based methods are yet to be trialled in the context of sanitation and schistosomiasis at the life-cycle stage of transmission from humans to snails (Champion et al., 2021). If an eDNA-based qPCR assay were able to detect schistosome DNA in the soil as sensitively as the assays used in the context of water samples, it could be possible to monitor the transmission of schistosomiasis from the terrestrial environment, where defecation occurs, to the aquatic environment where the intermediate hosts live (Stothard *et al.*, 2017; Campbell *et al.*, 2018). The occurrence of schistosomiasis in the soil environment could also provide a tool to monitor the effectiveness (or failure) of sanitation facilities such as the commonplace pit latrines, and new improvements and interventions, in containing the faeces and eggs within them. Due to the asexual reproductive stage occurring in the snail hosts, a single miracidium infection of a snail can result in 250–600 cercariae being released per day into water (Ayad, 1974). Therefore, evaluating sanitation interventions through snail surveys or infection prevalence in the human community may underestimate the uptake of and the impact of an improved sanitation facility.

2.2.2. Aims and objectives

The overarching aim was to assess if previously developed eDNA-based qPCR methods can be used to quantify known numbers of *S. mansoni* eggs in soil. eDNA has been successfully employed to detect *S. mansoni* in water samples from an endemic area (Sato *et al.*, 2018; Sengupta *et al.*, 2019) but how these techniques can be adapted for use in the soil environment is currently unknown.

Objectives:

1. To select a qPCR assay for the detection of *S. mansoni* from the range of published primer sets.
2. To determine the relationship between known numbers of *S. mansoni* eggs in a controlled environment (phosphate-buffered saline) and corresponding qPCR C_T values from the extracted DNA.
3. To determine the relationship between known numbers of *S. mansoni* eggs in soil samples (by manual spiking with eggs) and qPCR C_T values of the extracted DNA.

2.3. Methods

2.3.1. Source of *Schistosoma mansoni* eggs

Frozen mouse liver samples containing *S. mansoni* eggs were provided by Professor Karl Hoffman's group at Aberystwyth University. The livers were from Tuck Ordinary (TO) mice which had undergone a percutaneous infection with 180 *S. mansoni* cercariae 48 days previous. All procedures performed on mice adhered to the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986 (project licenses PPL 40/3700 and P3B8C46FD) as well as the European Union Animals Directive 2010/63/EU and were approved by Aberystwyth University's Animal Welfare and Ethical Review Body. Eggs were isolated from the liver tissue by sieving through 250 µm and 150 µm sieves into 1% PBS and stored at -20˚C. The eggs were counted out using a light dissecting microscope at 10x magnification. Repeats of counts of eggs in increasing numbers (1-100) were stored in PBS for DNA extraction (n=1) or counted and spiked into soil samples prior to DNA extraction (n=3), described in more detail below. The soils were collected from a *S. mansoni* endemic village in Uganda. The collection of soils is described more in depth in Chapters Three and Four which report the field testing of the *S. mansoni* and bacterial marker assays respectively. Briefly, the soils used in this study are surface soils (maximum depth of 2 cm) from the village's football field: an area assumed free of defecation and sanitation facilities. Approximately 1 g of soil was aliquoted into a 2 ml cryovials (Corning®), and flash frozen on-site using a Taylor Wharton CXR500 liquid

nitrogen dry shipper. The samples were transported in the charged dry shipper to the University of Glasgow where they were stored at -20ºC until use.

2.3.2. DNA extraction methods

DNA was extracted using two different DNA spin kits according to the sample type. The DNA from *S. mansoni* eggs spiked into 125 µl PBS was extracted using the QIAamp® DNA Mini Kit according to the manufacturer's guidelines (Qiagen). The Mini Kit was also used to extract DNA from eggs from mouse liver tissue which was used to trial a collection of *S. mansoni* PCR primer assays. This kit uses a proteinase K step to lyse the cells for DNA extraction.

The MPBio Soil Spin Kit was used to extract DNA from soil samples spiked with *S. mansoni* eggs. 500 mg of Ugandan soil were weighed into the first set of extraction tubes of the MPBio Soil Spin Kit, these tubes had previously been spiked with known counts of *S. mansoni* eggs. The extraction was carried out according to the manufacturer's protocol and the samples were homogenised using the FastPrep-24™ Classic Instrument (MPBio) at speed 5.5 for two 30 second runs. The quality of the DNA extracted was assessed by running the extraction product on a 2% agarose gel. All DNA extracts were stored at -20˚C before further use.

2.3.3. Selection of quantitative PCR assay

Before choosing a qPCR assay to take forward, three published *S. mansoni* specific PCR primer sets were trialled targeting: the small subunit (SSU) rRNA gene (do Vale Gomes *et al.*, 2006); the NADH dehydrogenase subunit 5 (ND5) sequence (Lu *et al.*, 2016); and the mitochondrial cytochrome oxidase I (COI) gene (Sato *et al.*, 2018) (Table 2.2). DNA extracted from *S. mansoni* infected mice livers were used as the positive DNA template. The PCR amplification was performed using Applied Biosystems SimpliAmp Thermal Cycler and 2% agarose gels were used to visualise the PCR products from these three assays.

The assay targeting the COI gene, designed by Sato *et al* (2018), was chosen to take forward (see results for full findings of the three PCR assays). The COI qPCR cycling parameters were as follows: 2 mins at 50˚C and 10 mins at 95˚C as initial

steps, followed by 40 cycles of 15 secs at 95˚C and 60 secs at 60˚C (Sato *et al.*, 2018). The qPCR amplification was performed in 20 µl reactions using 10 µl TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems), 0.9 µl 10 µM primer mixtures, 1.25 µl 10 µM probe mixture, 4.95 µl water and 2 µl of DNA. The qPCR assays were performed using the StepOne™ Real-Time PCR System.

For absolute quantification using this qPCR assay, a standard curve was used. A 162-base pair (bp) target segment of the mitochondrial COI gene of *S. mansoni* DNA sequence, (CAGGGGTTTCAAGTCTAATTGGATCTGTCAATTTCATTTCTACGATT-TTTAGTCGTTTAAGATTCAAATGTTCGATAATAGTATGGGCTTATCTATTTACGTCTGTC TTACTATTGCTTTCATTACCTGTATTAGCCAGAGGAATAACGATGTTATTATTTG) was purchased from Integrated DNA Technologies (IDT Technology, USA). Gene copy numbers were calculated by multiplying the DNA concentration by Avogadro's number and dividing by the size of the COI gene product (bp) and an average weight of a base pair (Yun *et al.*, 2006):

$$\text{number of copies (molecules)} = \frac{X\text{ng} \times 6.0221 \times 10^{23}\text{molecules / mole}}{(\text{N} \times 660\text{g/mole}) \times 1 \times 10^{9}\text{ng / g}}$$

$X = \text{amount of amplicon (ng)}$
$\text{N} = \text{lengh of amplicon (bp)}$
$660\ \text{g/mole} = \text{average mass of 1 bp double stranded DNA (dsDNA)}$

The efficiencies of the qPCR assays were determined by assembling a linear regression. Serial dilutions were prepared ranging from $10x10^{12}$ to $10x10^{4}$ gene copies per µl which served as a standard for qPCR amplification. The dilution series' C_T values were plotted against the log gene copy number to produce a standard curve. The equation of this line was then used to determine qPCR efficacies (*E%*) using the following equation:

$$E\% = \left(10^{\left(\frac{-1}{slope}\right)} - 1\right) \times 100\%$$

2.3.4. Determination of quantitative relationship between known number of *Schistosoma mansoni* eggs and their corresponding C_T values

The COI qPCR assay was also tested to establish if the gene copy quantification correlated with the number of *S. mansoni* eggs from which the DNA was extracted. The DNA templates used in the assay were DNA extracts from known counts of eggs: 1, 10, 25, 50 and 100. The eggs were isolated from experimentally infected mouse livers and individually counted by microscope into 125 µl of PBS buffer (n = 1). DNA was extracted by the QIAamp® DNA Mini Kit as described previously. Similarly, the COI qPCR assay was used to determine whether the gene copy number estimated by the COI qPCR assay correlated with the number of *S. mansoni* eggs extracted from a soil matrix. Microscopy-determined counts of *S. mansoni* eggs (1, 3, 5, 10, 20, 50 and 100 eggs) were spiked into 500 mg of Ugandan soils (n = 3). The DNA from each egg spiked soil sample was then extracted using the MPBio Soil Spin as described above.

For both these experiments, a negative template control (NTC) was run on the qPCR plate and the COI standard curve was used as a positive control. When analysing the results, if a reaction had a C_T within three C_T values of the water control (NTC) and therefore within 90% difference of the water control or had an undetermined C_T, the sample was categorised as negative. The quantification threshold was set to within three C_T of the NTC as samples with a high C_T value could be the result of an amplification artefacts (Ruiz-Villalba, Ruijter and van den Hoff, 2021) .The correlation coefficient statistic was calculated to compare the strength of the relationship between the independent and dependent variables of the three qPCR assay studies: manufactured COI dsDNA; eggs spiked into PBS and eggs spiked into soil.

Additionally, the DNA recovery of the soil extraction process was investigated by spiking three different concentrations (10 $x10^{10}$, 10 $x10^{7}$ and 10 $x10^{4}$ copy numbers) of the manufactured *S. mansoni* COI standard, into 500 mg of soil before DNA extraction (repeats n=1). The spiked DNA extracts were consequently run using the COI qPCR assay and C_T values compared with their corresponding standard dilutions generated from the “clean” standard curve: standards prepared in nuclease free water.

Table 2. 2. *Schistosoma mansoni* PCR and qPCR assays trailed on DNA extracts from *S. mansoni* eggs. Three assays were trialled that were designed to detect the small subunit rRNA gene (Schfo111 and Shre111), the reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 5 (Nd5-2) and the mitochondrial cytochrome oxidase I gene (Sma-COI). Tm is the melting temperature.

Name of Primers	Target	PCR assay type	Primer/probe sequence (5' to 3')	Product size (bp)	Tm (°C)	Reference
Schfo111	small subunit (SSU) rRNA gene	SYBR Green real-time (qPCR) assay	5'- CGATCAGGACCAGTGTTCAGC -3'	106	83	(do Vale Gomes *et al.*, 2006)
Shre111			5'- GACAGGTCAACAAGACGAACTCG -3'			
Nd5-2	NADH dehydrogenase subunit 5 (ND5) sequence	Endpoint PCR	5'-ATT AGA GGC AAT GCG TGC TC-3'	302	96	(Lu *et al.*, 2016)
			5'-ATT GAA CCA ACC CCA AAT CA-3'			
Sma-COI	mitochondrial cytochrome oxidase I (COI) gene	TaqMan real-time (qPCR) assay	5'-CAGGGGTTTCAAGTCTAATTGGAT-3'	162	95	(Sato *et al.*, 2018)
			5'-CAAATAATAACATCGTTATTCCTCTGG-3'			
			5'-FAM-TTCAAATGTTCGATAATA-NFQ-MGB-3'			

2.4. Results

2.4.1. *Schistosoma mansoni* PCR assays

The first objective of the study was to select a *S. mansoni* specific qPCR assay. Three PCR primer pairs (Table 2.2) were trailed before taking one assay forward for use as a qPCR assay. The results from the three PCRs, (SSU rRNA gene, ND5 sequence and COI gene) were visualised by a 2% agarose gel (Figure 2.2).

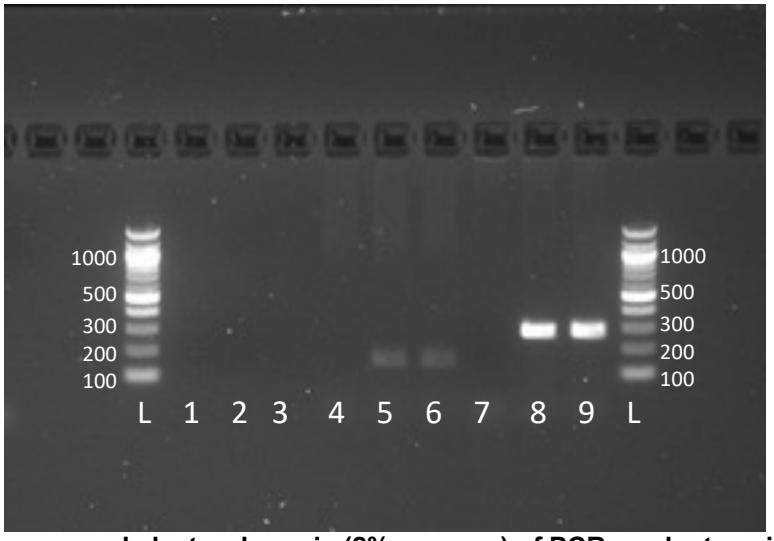


Figure 2. 2. Agarose gel electrophoresis (2% agarose) of PCR products using *Schistosoma mansoni* specific PCR primer sets. Lane 1-3 small subunit (SSU) rRNA gene. Lane 4-6 mitochondrial cytochrome oxidase I (COI) gene. Lane 7-9 NADH dehydrogenase subunit 5 (ND5) sequence. For each primer set a negative template control (no DNA) was used (Lanes 1, 4 and 7) and DNA extracted from *S. mansoni* eggs were used to test each primer pair, Lanes 2+3, 5+6 and 8+9. Lanes L, 1 kb DNA size marker, values are in basepairs (bp).

The COI-based qPCR assay was chosen as the PCR assay using the SSU primers did not amplify the *S. mansoni* egg DNA extracts (Figure 2.2: Lanes 2+3). While amplification was achieved with the ND5-based primers producing the brightest band (Figure 2.2: Lanes 8+9) the PCR product was larger in length than the 162bp product amplified by the COI-based assay, which also produced specific amplification (Figure 2.2: Lanes 5+6).

A small PCR product size is beneficial to eDNA studies and can help to increase the detection level of the assay, as DNA degradation will occur naturally in the environment (Harrison, Sunday and Rogers, 2019). The *S. mansoni* assay based on the COI gene (Sato *et al.*, 2018) was therefore chosen to be the qPCR assay applied to DNA extracted from soil samples. All NTCs (water) for the three PCR assays were negative, Lanes 1, 4 and 7 of Figure 2.2.

2.4.2. Quantitative PCR assay efficiency and reproducibility

A manufactured standard designed for the *S. mansoni* COI qPCR assay was used to determine the reaction efficiency (Figure 2.3A). The manufactured standards had a linear range of quantification from 10 x 10^5 to 10 x 10^{10} gene copies per 1µl of the standard. There was amplification for 10 x 10^4 gene copies but as the C_T value of 10 x 10^4 copies (37.84) was within three C_T values of the NTC (39.44) it was excluded from the dynamic range of the assay as this was the quantification threshold cut-off. The efficiency, slope of the standards, correlation coefficient (r^2) and *y*-intercept were 89%, -3.6, 0.978 and 52 respectively. The concentration of the COI standards and their corresponding average C_T values measured by the qPCR assay, were also used to calculate a coefficient of correlation to measure the strength and direction of the linear relationship and compare it with the subsequent tests of this qPCR assay with counts of *S. mansoni* eggs. It was calculated as -0.989, suggesting a strong negative correlation.

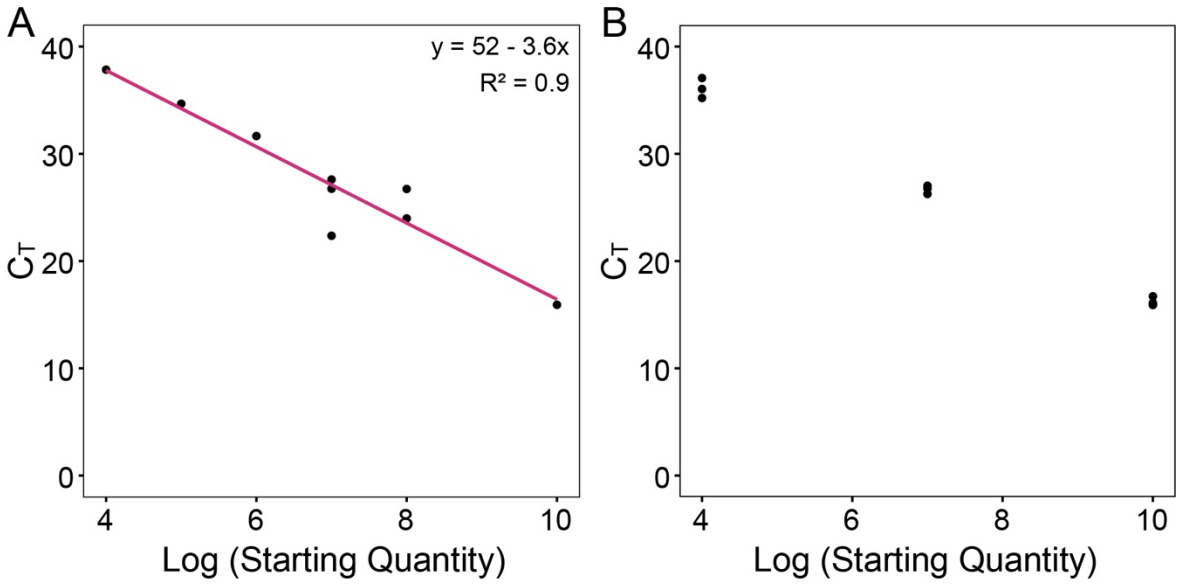


Figure 2. 3. A standard curve generated using the *Schistosoma mansoni* COI manufactured standard and plot of the of the cycle threshold (C_T) values detected by qPCR of DNA extracts from the *S. mansoni* COI standard spiked into from 500 mg Ugandan soil before DNA extraction. A) The logged concentrations of the gene copies are plotted against the C_T values. Each concentration of the COI standard was run in triplicate on the qPCR plate. The C_T is the cycle number at which the fluorescence signal increased above the defined threshold. This threshold was calculated by the real-time PCR software. R^2 is the correlation coefficient. B) The three concentrations of the *S. mansoni* standard (10 x10^{10}, 10 x10^7 and 10 x10^4) were spiked into 500 mg of Ugandan soil (n=3) and the variation of each of the three soil extract's C_T value is plotted. Each DNA extract was used neat in the qPCR assay.

The COI assay was also used to test the recovery of DNA using the bead beating method of DNA extraction. To measure DNA recovery of the DNA extraction process, three concentrations of the *S. mansoni* COI standard was spiked into soils from Uganda before the DNA extraction process, subsequently the DNA recovery was determined by qPCR (Figure 2.3A).

The DNA extractions from soil samples spiked with 10 $x10^{10}$, 10 $x10^{7}$ and 10 $x10^{4}$ *S. mansoni* standard had average C_T values of: 16.2, 26.6 and 35.6. The corresponding average C_T values from the standards prepared in water (Figure 2.3A), at concentrations 10 $x10^{10}$, 10 $x10^{7}$ and 10 $x10^{4}$ were: 15.9, 25.5 and 37.8. The C_T values for the soil-spiked and "pure" standards measured suggest the DNA extraction process was efficient. Additionally, the qPCR assay was not greatly affected by any inhibitors within the soil that would be expected to raise the C_T values dues to reduced efficiency of the qPCR reaction. The NTC was undetermined in this specific qPCR run.

2.4.3. Egg counts and quantification by qPCR

The second objective of this study was to determine if there was a relationship between the number of eggs spiked into PBS, a controlled matrix, and the quantification of the *S. mansoni* COI gene by qPCR using the DNA extracted from the counts of *S. mansoni* eggs. Using known counts of *S. mansoni* eggs spiked into PBS, the correlation coefficient, calculated by comparing the average C_T values for each egg count with the original number of eggs spiked into each sample (Table 2.3), was -0.75, signifying there is a strong negative correlation between the two variables. The negative correlation fits with the hypothesis that an increased number of eggs/*S. mansoni* DNA in a sample would result in a lower C_T value, indicating the sample has a higher quantity of the COI gene.

Table 2. 3. Summary of cycle threshold (C_T) measured for each count of *S. mansoni* eggs spiked into PBS buffer and total DNA concentration (µg/ml) of the DNA extract used in the qPCR assay. DNA concentration was calculated by broad range qubit. NTC = negative template control. SD = standard deviation.

Number of eggs	C_T value (SD)	*Total DNA concentration (µg/ml)*
1	29.9 (± 1.5)	3.1
10	26.7 (± 1.4)	4.3
25	27.4 (± 1.6)	2.7
50	26.6 (± (0.4)	3.7
100	25.7 (± 0.7)	Too low
NTC	34.2 (± 0.3)	-

In addition to calculating the correlation coefficient, the qPCR efficiency was determined using the C_T values measured for number of eggs, rather than gene copy number used to measure the efficiency of the manufactured standard (Figure 2.3.A). A 1 in 10 factor was used (1, 10 and 100 eggs) to generate the curve (Figure 2.4). The efficiency, slope of the standards, correlation coefficient (r^2) and *y*-intercept were 694%, -0.9, 0.67 and 30 respectively. Each DNA extract was run in triplicate, the standard deviation of the C_T measured for each sample is shown on Figure 2.4. The NTC had a C_T of 34.2.

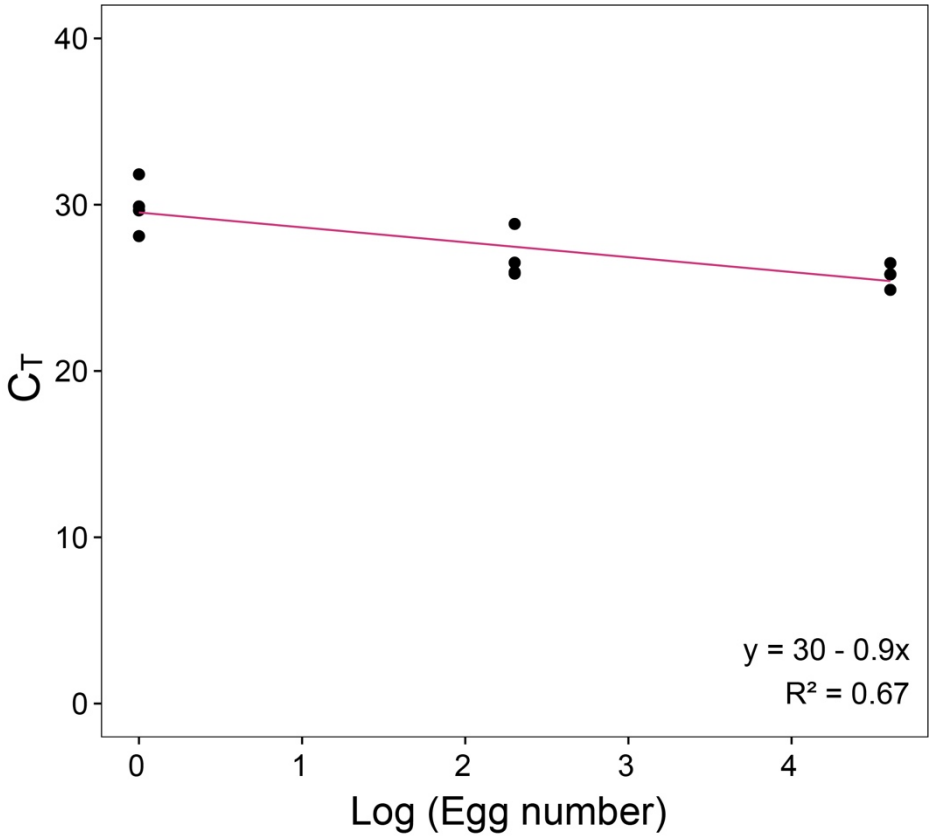


Figure 2. 4. A standard curve generated using known counts of *Schistosoma mansoni* eggs in phosphate buffered saline (PBS). Gene copies plotted against the cycle threshold (C_T) values. The C_T is the cycle number at which the fluorescence signal increased above the defined threshold. This threshold was calculated by the real-time PCR software. R^2 is the correlation coefficient.

To fulfil Objective Three, to determine if there was a relationship between the number of eggs spiked into soil and their corresponding C_T values, *S. mansoni* eggs were counted by microscopy and spiked into soil samples prior to DNA extraction. Table 2.4 summarises the results from the DNA extraction and qPCR assay for each count of eggs carried out (1, 3, 5, 10, 25, 50 and 100 eggs per extraction). Three biological replicates were made for DNA extraction and each DNA sample was run in triplicate in the qPCR assay. The relationship between the egg counts and the C_T value is not monotonous. As a log plot, the R^2 value of linear regression is 0.1.

Table 2. 4. Summary of cycle threshold (C_T) measured for each count of *Schistosoma mansoni* eggs spiked into soil and total DNA concentration (µg/ml) of the DNA extract used in the qPCR assay. NTC = negative template control. SD = standard deviation.

Number of eggs	C_T (SD)	*Total DNA concentration (µg/ml)*
1	26.7 (±1.9)	64.6
3	19.7 (±1.6)	42.8
5	26.1 (±1.7)	77.1
10	25.2 (±0.1)	92.8
25	25.7 (±3.1)	154.0
50	25.4 (±1.0)	100
100	19.5 (±1.9)	65.3
NTC	37.7	-
Extraction control	Undetermined	Too low

The efficiency of the qPCR assay was determined using the C_T values for 1, 10 and 100 eggs (Figure 2.5). For the 1 in 10 dilution factor of eggs spiked into soil, the efficiency, slope of the standards, correlation coefficient (r^2) and *y*-intercept were 38%, -0.14, 0.903 and 26 respectively (Figure 2.4).

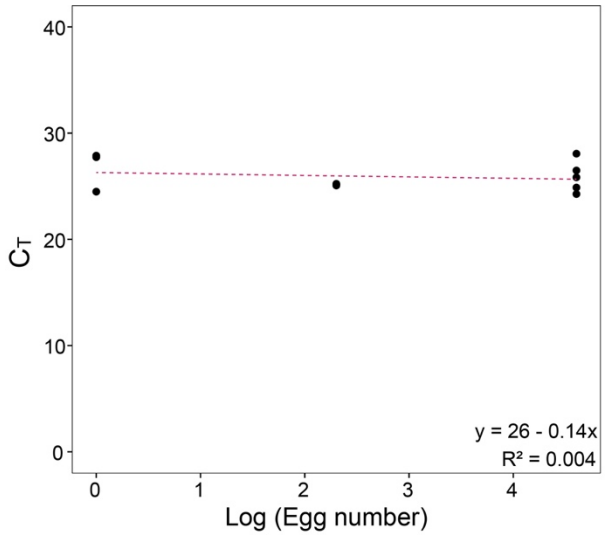


Figure 2. 5. A standard curve generated using known counts of *Schistosoma mansoni* eggs spiked into soil. The concentrations of the gene copies are plotted against the cycle threshold (C_T). The C_T is the cycle number at which the fluorescence signal increased above a defined threshold. This threshold was calculated by the real-time PCR software. R^2 is the correlation coefficient.

The efficiency of the standard curve (Figure 2.5) was 38%, and the standard curve only had three 10-fold concentrations (1,10 and 100) and comparing the average C_T values from the full range of egg counts (Table 2.4). Comparing the three spiking experiments, the correlation between the concentration of *S. mansoni* DNA in the sample and the C_T values measured became weaker as the starting materials changed from manufactured standard in NF water, to DNA extracted from egg in PBS to eggs in soil . When comparing the C_T values between the DNA extracts from eggs spiked into the PBS and eggs spiked into soil, the C_T values for the eggs in PBS were higher for each of the corresponding counts of *S. mansoni* eggs.

2.5. Discussion

2.5.1. Potential for environmental monitoring of schistosomiasis using quantitative PCR

Due to the obligatory development stage of the schistosome in a snail host, the interaction of humans (and human excrement) with the snail's aquatic habitats is key to schistosomiasis' continued transmission. Both human exposure to the schistosomes' cercariae and human transmission of schistosome eggs into the environment containing (susceptible) snail habitats require an absence, or

failure, of WASH services. Molecular methods have been adapted to detect schistosomiasis species in water samples of the three most common human-infecting species (Akande *et al.*, 2012; Sato *et al.*, 2018; Fornillos *et al.*, 2019; Sengupta *et al.*, 2019). Here I adapted these water-based eDNA methods to another environmental matrix, soil, to offer new methods which can potentially offer new insights into the role of sanitation interventions.

Objective One of this chapter was to choose a *S. mansoni* PCR assay to use on environmental samples and the COI-based qPCR assay chosen was shown to be highly sensitive. Objectives Two and Three were to determine if the assay was quantitative with respect to the DNA extracted from a given number of *S. mansoni* eggs in PBS and soil samples. The detection of the COI gene using the qPCR assay was semi quantitative with respect to the DNA extracted from a given number of *S. mansoni* eggs in PBS buffer. However, once the eggs were added to a soil matrix prior to DNA extraction, this quantitative relationship was lost. The qPCR was however sensitive enough to detect a single egg in either matrix (soil or PBS) and whilst not quantitative, the assay was demonstrated to be an effective absence/ presence measure with at least a lower limit of detection of 1 egg/ 500mg soil. An individual with a light infection of *S. mansoni* would have faeces containing 1-99 eggs/ gram (WHO, 2002), this assay was sensitive to 2 eggs/ gram, at the lowest limit tested.

2.5.2. Quantitative PCR assay for *Schistosoma mansoni* in soils

To fulfil Objective One, out of the three *S. mansoni* PCR assays tested the COI-based *S. mansoni* assay, designed by Sato *et al.* (2018) was taken forward, primarily due to its small PCR product size (Sato *et al.*, 2018), which is beneficial for DNA which might have undergone extensive physical and UV degradation in an external environment. Next, a synthesised DNA sequence, designed to replicate the specific PCR product of the *S. mansoni* COI assay, was used to determine the efficiency of the assay and check the range of gene copy numbers that the assay can quantify the COI gene across. The lower limit of detection was shown to be 10×10^5 gene copies per 1 µl. The qPCR assay had an amplification efficiency of 89%; a figure between 90% and 110% is considered an acceptable efficiency (Taylor *et al.*, 2019).

Efficiency is important to consider as it is an indicator of what proportion of the target molecules in the PCR reaction are copied in one PCR cycle, which can vary across assays (Ruiz-Villalba, Ruijter and van den Hoff, 2021). Any comparisons made between different qPCR assays require similar reaction efficiencies to ensure a valid comparison of the data. Poor efficiency can be caused by a range of factors such as inhibitors within the DNA extract. For example, soil samples contain humic and fulvic acids from the soils' organic matter which copurify with nucleic acids during the DNA extraction process (Thomsen and Willerslev, 2015). The investigation into the DNA extraction efficiency using the manufactured DNA standards spiked into soils showed little evidence of inhibitors to the qPCR assay as the C_T values were only slightly higher (less than 2 cycles) than their corresponding concentrations measured for the "pure" standards (those prepared in nuclease free water). The higher C_T values could be a result of both the proportion of DNA recovered from the extraction from soils and/or any soil-derived inhibitors present in the DNA extracts. As the C_T values of the soil-spiked standards were only slightly higher, both these factors have only a negligible effect on the COI-qPCR assay, but these differences could become more important at the lower limits of detection and give false negative results in some soil samples. The presence of PCR inhibitors from soil-derived DNA extracts was further tested in Chapter Three.

The test to determine DNA extraction recovery was not a perfect proxy for egg recovery from the DNA extraction process, as the DNA extracted from eggs would be lysed from the cell, rather than already being free dsDNA in the soil sample and the *Schistosoma* eggshells consists of three layers that must be ruptured (Neill *et al.*, 1988). However, as the COI-based *S. mansoni* assay showed high sensitivity and a good efficiency for quantification, *S. mansoni* eggs were then used to fulfil the second and third aims of this study: determining the relationship between known numbers of *S. mansoni* eggs and qPCR C_T values from the eggs' extracted DNA.

As *S. mansoni* eggs are multicellular, estimating egg counts from gene copy number can be challenging. The C_T values of COI genes measured by the COI assay and the DNA extracted from eggs spiked into PBS and soil had correlations of -0.75 and -0.50 respectively. The DNA extracts from counts of eggs in soil

successfully amplified but did not have the strong negative corelation that the manufactured standards had. Therefore, the C_T values did not reflect the starting number of eggs and would not be an accurate proxy for enumerating *S. mansoni* eggs from environmental samples where a similarly low number of eggs would be predicted to be found.

2.5.3. Challenges and limitations of quantification by qPCR

To test the quantitative potential of the *S. mansoni* COI qPCR assay, absolute quantification can by employed (Pabinger *et al.*, 2014). This relies on comparing the C_T of a sample with a standard curve generated from a serial dilution of a standard. The reliability of this standard curve method is dependent on the amplification efficiency of the qPCR target, in this case *S. mansoni* COI gene. In the context of using manufactured standards which were prepared and diluted in nuclease-free water, the qPCR assay for *S. mansoni* was quantitative. To apply this technique to a real-world environmental monitoring of *S. mansoni*, it would be useful to be able to calculate, using the gene copy number measured by the qPCR, how many eggs are in each sample collected from the environment. The gene copy number of COI in *S. mansoni* eggs was unknown but it was reasoned that a positive relationship between the egg numbers and the COI genes would be seen; fewer eggs would result in a lower gene copy number (higher C_T value). Unfortunately, the assay was not successful for enumeration of eggs as the assay was adapted from measuring genes to quantifying eggs in PBS and then quantifying eggs in soil. Possible reasons for this are discussed below.

2.5.3.1. DNA extraction processes and DNA recovery

The loss of the strong, negative correlation between amount of *S. mansoni* DNA within a sample and measured C_T values could be due to several reasons, for example, human error in preparing the samples, as the eggs were counted manually by microscopy before the DNA extractions were carried out. The results from the comparison of eggs spiked into PBS and into soil samples could also be due to the DNA extraction process and extraction efficiency. Due to the matrices that the eggs were extracted from (PBS vs soil), the DNA extraction process was different between the two experiments and the bead beating kit

(MPBio) resulted in high total DNA yields and lower C_T values for the *S. mansoni* egg extracts. A proteinase K-based kit was used to extract the DNA from the eggs within PBS buffer whereas mechanical lysis by a bead beating step was used on the soil samples. Therefore, differences in the DNA recovery by the two extraction techniques (lower total DNA from the proteinase K kit and higher from the bead beating lysis method) could be a contributing factor to this variation in the C_T values.

As the Qubit measures total DNA, it is not possible to know before the qPCR assay what the starting quantities of *S. mansoni* were in each of the DNA extracts. The soil would have DNA from many other organisms present and the extracts from the eggs in PBS would have contained organic material from the mouse liver the eggs were isolated from. The DNA extracts from the eggs in soil could have had a lower proportion of *S. mansoni* DNA compared with non-schistosomal DNA extracted from the soil, however the qPCR data suggest that more *S. mansoni* DNA was present in these DNA extracts compared with the egg counts extracted from PBS by the proteinase K method.

The DNA extracts from the soil samples would also have contained PCR inhibitors and DNA not originating from the *S. mansoni* eggs, however the qPCR assay still specifically detected the *S. mansoni* COI gene from these extracts and resulted in higher C_T values, another result suggesting the bead-beating method resulted in better DNA recovery compared with the proteinase K method. Even with the differences in DNA recovery from the extraction processes, the downstream qPCR assay was shown to be sensitive to the lowest limit tested: it could detect as little as one egg in 125 µl PBS (C_T 30) and in 500 mg soil (C_T 27).

As the C_T values were lower for the counts of eggs extracted from soil samples compared with their responding count from PBS, this is suggestive that the extraction efficiency was greater in the bead beating method. It would have been interesting to visualise the effects of the lysis process under the microscope: to have compared the *S. mansoni* eggs structure of the samples post-bead beating or post proteinase K-treatment.

2.5.3.2. Variable gene copy numbers in multicellular eggs

Within this study it was not possible to precisely determine the yield of *S. mansoni* DNA from egg counts or estimate the COI gene copy number for one egg. The genome of *S. mansoni* contains approximately 580 fentograms (do Vale Gomes *et al.*, 2006) but as the eggs are multicellular, they will therefore have a range of gene copy numbers, determined by their size and maturity. The variation in copy number of the COI gene in a given egg calculated by qPCR will also be compounded by variable gene copy numbers estimated by the C_T value qPCR assay itself (Taylor *et al.*, 2019).

As an alternative to absolute quantification method used in this chapter with a standard curve, relative quantification could have been used. The C_T values could be normalised with a house-keeping gene, such as PSMD Smp_000740 which has been used in previous studies (Anderson *et al.*, 2017). Despite the quantitative limitations, the qPCR assay was successful in detecting *S. mansoni* eggs in spiked soil samples as positive, even with just one egg present. This highly sensitive assay was consequently used on soil samples collected in a *S. mansoni* endemic area to investigate the presence of *S. mansoni* DNA in soil samples in relation to sanitation facilities in the next chapter.

2.6. Conclusion

The primary aim of this study was to determine the quantitative nature of the *S. mansoni* COI qPCR assay in relation to egg number in PBS and soil matrices. It was possible to use the *S. mansoni* eDNA-based qPCR to investigate the presence and absence of *S. mansoni* in soil samples spiked in a lab-based setting. However, the quantitative aspect of this qPCR assay was unsuccessful: when tested with *S. mansoni* spiked soil samples, there was no correlation between copy number based on the qPCR assay and original number of eggs spiked into each sample: the relationship between concentration of *S. mansoni* DNA and C_T value became weaker as the matrix changed from DNA standard in NF water, eggs in PBS and eggs in soil.

In a field setting, complex samples such as soil would always be used, rather than purer sample matrices (e.g., PBS) to detect *Schistosoma* contamination.

Despite being unable to quantify *S. mansoni* egg intensity in soil samples, using this qPCR for measuring *S. mansoni* presence or absence could help to measure the success of sanitation interventions in an endemic setting and to see how far from open defecation sites, or potentially leaky latrines, *S. mansoni* eggs could be detected. In fact, the qPCR assay could detect as little as one egg, in 0.5 g of soil. A study to explore the validity of using this eDNA qPCR assay in a field-based setting was carried out in the next chapter, Chapter Three.

Chapter 3: Field detection of *Schistosoma mansoni* in soil samples using environmental DNA

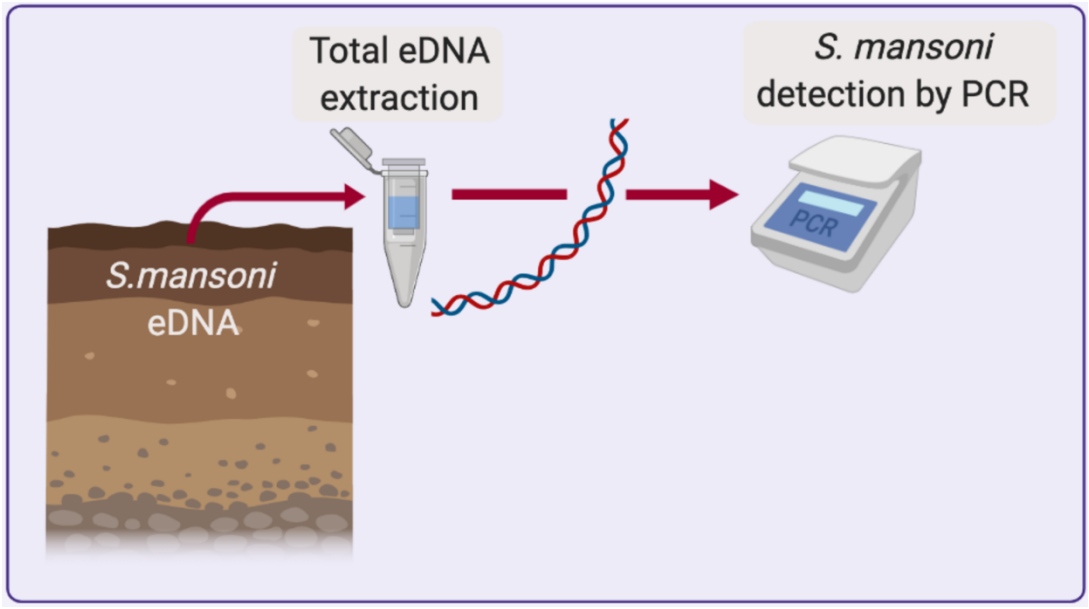


Figure 3. 1. Visual summary of Chapter Three: the field testing of the *Schistosoma mansoni* eDNA-based qPCR assay on soils from a *S. mansoni* endemic village.

3.1. Abstract

Current methods to monitor interventions for schistosomiasis focus mainly on human based diagnostics, or occasionally the intermediate snail host stage of the life cycle. Both of these are likely to have a non-linear association with the uptake and/or success of sanitation-based interventions. qPCR has been shown to be able to detect the presence or absence of a single *Schistosoma mansoni* egg in buffer or spiked soil (Chapter Two). The aim of this chapter is therefore to report on the field applicability of the *S. mansoni* qPCR assay for detecting the presence of environmental contamination in soil samples collected from a high *S. mansoni* endemicity community and to test the hypothesis that there was more contamination detected in open defecation sites, or soils surrounding pit latrines, than negative control samples from the community football pitch. Surface soils were collected from three distinct areas: an area predicted to be free from human defection (the community football field), sites of open defecation and samples collected proximal to in-use pit latrines. Soil samples were frozen in liquid nitrogen at time of collection and for shipment and then stored at -20°C until processing in the lab. DNA extracted from these soils were tested for the presence/absence of *S. mansoni*, using a *S. mansoni* specific qPCR

assay targeting the mitochondrial cytochrome oxidase (COI) gene. The results indicate that *S. mansoni* could not be detected in soil samples taken around pit latrines and even in soils taken directly from open defecation sites, despite using a highly sensitive qPCR assay that could detect just one egg in 500 mg of soil. As no positive samples were detected, it was not possible to test the hypothesis that *S. mansoni* would be more frequent in samples collected from the area of open defecation and soils proximal to the pit latrines compared to the soils sampled at the football field. This eDNA-based technique needs further optimising to enable it to be used as a monitoring technique for schistosomiasis in the soil and sanitation-linked environment.

3.2. Introduction

The relationship between poor sanitation and unsafe water and infection with schistosomiasis is well established but the link remains rarely directly and quantitatively investigated (Grimes *et al.*, 2014). Studies exploring the relationship between schistosomiasis and water, sanitation, and hygiene (WASH) have been limited to comparing WASH facilities', presence, and use, with schistosome infection rates in people (Grimes *et al.*, 2015). Due to the complex lifecycle of the schistosome, a non-linear humans-to-snail force of infection, an amplification step in the snails, and a non-linear snails-to-humans force of infection, and the relationship between the level of WASH and *Schistosoma* prevalence in the human population will therefore also be non-linear (Gurarie *et al.*, 2018). The information on WASH facilities, commonly collected through household surveys, can provide insights to sanitation use and household behaviour. However, these surveys cannot provide accurate, or quantitative information on how the pit latrine is functioning or insights about any environmental contamination caused by an inadequately functioning sanitation facility (Grimes *et al.*, 2014).

3.2.1. Basic sanitation services and schistosomiasis

Although the link between WASH and its role in the transmission of schistosomiasis is well established (Grimes *et al.*, 2014), provision of adequate WASH is not a standard adjunct to schistosomiasis control campaigns, which remain largely confined to MDA (Campbell *et al.*, 2018; WHO, 2021). Where

there are sanitation provisions, predominantly pit latrines, local guidelines on their design specifications are lacking and ways to monitor or assess their effectiveness to contain (and/or destroy helminth eggs) do not yet exist (Campbell *et al.*, 2018).

Pit latrines consist of a dug-out hole in the ground, a squathole that enables the disposal of excreta into the larger dug-out pit and a superstructure providing privacy (Baker and Ensink, 2012). Pit latrines are a cheap form of sanitation and one of the most common forms of sanitation (WHO, 2017; WHO UNICEF JMP, 2021b). Their construction varies depending on the resources and equipment available to the owner. The most basic pit latrines will have an unlined pit and built without a ground slab, and therefore do not protect the user from exposed soil around the drop hole. Consequently, basic pit latrines are classed as unimproved: a facility that does not effectively sperate faecal matter from human contact (WHO UNICEF JMP, 2021b).

The WHO recommends, where possible, that pit latrines are constructed at least two metres above the water table, at least 30 metres from water sources, and downhill rather than uphill of any water sources (WHO, 2017). In practice these guidelines are not always met, for example in the village of Bugoto, Uganda, where the fieldwork for this study was carried out which is a persistent hotspot for *S. mansoni* (Crellen *et al.*, 2016; Prada *et al.*, 2018; Clark *et al.*, 2021). The photo from the village (Figure 3.2), shows the household dwellings and pit latrines uphill, and in close proximity to the shoreline. Like in all endemic areas, the role of sanitation on the prevalence of schistosomiasis in this community is difficult to monitor directly.

Access to safely managed sanitation is only one part of WASH: the use and maintenance of sanitation facilities also impact the effectiveness of any sanitation intervention (WHO UNICEF JMP, 2021a). Although the 2030 SDG goal is for ‘access’ to sanitation, sanitation facilities must be acceptable for their users to ensure uptake and maintenance: an unused safely managed sanitation facility will have little effect on schistosomiasis transmission (Campbell *et al.*, 2018) or any other faecally transmitted infection. Any monitoring of the environment in relation to schistosomiasis and sanitation would be well complimented by parasitological infection data and quantitative and qualitative data on access

and use from sanitation-users and community members to gain an insight into the overall use of sanitation and sanitation practices and their impact on environmental contamination (Sacolo, Chimbari and Kalinda, 2018).

3.2.3. Methods for investigating the soil environment in relation to *Schistosoma mansoni*

The majority of evaluation and monitoring of *S. mansoni* control programmes is through measuring prevalence and intensity in humans (Stothard *et al.*, 2017). However, this is only a proxy of environmental transmission in each community, in part due to the mobility of the human hosts between endemic areas, as well as their individual treatment histories and biological and behavioural risk factors (Arinaitwe *et al.*, 2021). Detection of transmission levels in a specific area requires the monitoring of life-cycle stages outside of the human hosts (McManus, Gordon and GAD Weerakoon, 2018). Methods to detect the free-living stages of the schistosome (eggs, miracidia and cercariae) have historically been focused on water contact sites and on the collection of aquatic snails.

Figure 3. 2. Photo of proximity of some of Bugoto's residential dwellings to the shore of Lake Victoria. Pit latrines in Bugoto have been built uphill and within 30 metres of the shoreline. Photographed by T. Champion November 2017.

Environmental DNA (eDNA) techniques have been used to detect specific species of human infecting schistosomes from waters samples collected in endemic areas as reviewed by Champion *et al.*, (2021). These eDNA techniques, tested using water samples from endemic areas, have also been used to detect the snail species *Bulinus truncatus* (Mulero *et al.*, 2019) and *Oncomelania hupensis* (Calata *et al.*, 2019), intermediate hosts of *S. haematobium* and *S. japonicum* respectively. In the previous chapter, an eDNA-based technique was developed to detect *S. mansoni* in soil samples, which whilst not quantitative was shown to be a highly sensitive presence/absence assay. eDNA detection of the parasite is a promising new tool to supplement current monitoring efforts. Due to the low percent of infected snails, even in areas with high human infection rates, the sensitivity of snail surveys is low (Standley *et al.*, 2010; Angelo *et al.*, 2014; Odero *et al.*, 2019). Sampling of water from endemic areas, for use in eDNA-based qPCR detection, has been shown to be a sensitive method of *S. mansoni* monitoring (Sato *et al.*, 2018; Sengupta *et al.*, 2019). eDNA-based detection of the parasite from soil samples could be useful if it can successfully be adapted and applied for use on field collected soil samples. The previous chapter found the *S. mansoni* COI qPCR assay to be highly sensitive and able to detect a single *S. mansoni* egg spiked into a 500 mg soil sample (detectable with a C_T value 27 (NTC 38)) but the assay remains untested on soils from a *S. mansoni* endemic area.

The proportion of *Schistosoma* infected snails in an endemic area is dependent on 1) eggs contaminating the environment, 2) eggs contacting fresh water to hatch, and 3) the presence of susceptible snail hosts in that aquatic environment. Therefore, snail infection levels are not linearly associated with inadequate containment of infected faeces (Gurarie *et al.*, 2018). Additionally, the exponential reproduction of the parasite within the snail hosts, makes quantifying sanitation intervention uptake, and success, using snail and human survey data alone even more challenging (Dietz, 1979). The terrestrial environment in a *S. mansoni* endemic area could provide information on human to snail transmission facilitated by inadequate sanitation infrastructure or by unsafe sanitation practices. It is currently unknown how far eggs are transported if they are deposited into the soil environment by open defecation for example, and to what degree terrestrial-based open defecation contributes to the

continuation of the schistosomes' lifecycle. A tool to sensitively and specifically detect the parasite in the terrestrial environment is missing from the schistosomiasis toolbox and eDNA methods could fulfil this. Centrifuge-based separation methods of helminth eggs/ova from soil, wastewater and sludge remains the only published technique for these environmental matrices (Smith, 1999). The eDNA methods developed in the previous chapter to detect *S. mansoni* in soil samples were therefore trailed on samples collected from a *S. mansoni* hotspot in Uganda.

3.2.4. Aims and objectives

The aim of this chapter was to gain an understanding of the presence/absence of *S. mansoni* in the soil environment in a *S. mansoni* endemic area. With specific regard to areas interfacing with sanitation facilities such as the area surrounding pit latrines, as well as a site of open defecation. It was expected that more soils collected from the high contamination sites (areas proximal to the pit latrines and sites of open defecation) would have more soils samples positive for *S. mansoni* than an area assumed free from open defecation (the community football field).

Objectives:

1. Apply DNA extraction and detection by qPCR methods for *S. mansoni* eggs developed in Chapter Two on soils from a *S. mansoni* endemic area.

2. Determine if there is a difference between the presence/absence of *S. mansoni* COI gene detected in soil samples from three different sampling areas: an area free from defection, sites of open defecation and samples collected proximal to in-use pit latrines.

3. Determine if is there a difference in the absence/presence of *S. mansoni* detected with the *S. mansoni* COI assay, with increasing distance from pit latrines using a sampling transect approach.

3.3. Methods

3.3.1. Ethics

This research study was nested within Dr Poppy Lamberton's research group's ongoing work on schistosomiasis in Uganda under research ethics permit (UNCST-HS 2193) in collaboration with Vector Control Division, Ministry of Health Research Ethics Committee (VCDREC/062) and University of Glasgow Medical, Veterinary and Life Sciences Research Ethics Committee (200160068).

3.3.2. Study site description

The Ugandan village of Bugoto, Mayuge District (Figure 3.3) was selected for this study because of its persistently high prevalence of *S. mansoni* and proximity of pit latrines to surface waters. Bugoto is inhabited by approximately 3500 people and split into two villages, Bugoto A and Bugoto B. Bugoto A is situated on a spit of land on the shores of Lake Victoria (Figure 3.3C). The majority of the adult population make their living from Lake Victoria's fish stocks, being either fisherfolk or fishmongers (Adriko *et al.*, 2018).

The area is a *S. mansoni* hotspot, with prevalence >90% in primary-school children (Lamberton *et al.*, 2014; Crellen *et al.*, 2016; Clark *et al.*, 2021). Bugoto started annual community-wide MDA in 2003, however coverage has been low, with only less than half of eligible people being treated in 2016 and nearly one third of eligible residents never having been treated up until 2016, despite 14 years of annual MDA by that time point (Adriko *et al.*, 2018). It was believed that MDA had not recently occurred before soil sample collection, however even if it had, as MDA coverage is low and drug efficacy is not 100%, it was predicted that *S. mansoni* eggs would be in the soil at open defecation sites at the time of the soil sampling. The lake borders the south of the village with marshland and rice paddy fields on its southwest edge (Figure 3.3C). The houses of Bugoto's residents, and associated pit latrines, were constructed near to the shoreline (Figure 3.2). Open defecation is also practised in the village, with high levels of open defecation observed (evidence of open defecation was highly concentrated in the West part of the village, highlighted in Figure 3.3.C.).

Soil sampling

Soils were sampled from multiple sites in the village of Bugoto the week of 27th October 2019. To increase the likelihood of collecting soil contaminated with *S. mansoni* eggs, soil was collected from areas adjacent to three in-use pit latrines and from six sites of open defecation. Figure 3.4 shows the sampling plan for the soil samples collected from transects of three pit latrines, an area of open defecation and an area free of human defecation (football field). The samples from each sampling site were collected over the course of a single day. The transects for the pit latrines started behind each pit latrine and ran down the slope towards Lake Victoria (Figure 3.2), with the aim of gaining a spatial insight into *S. mansoni* presence with increasing distance from the pit latrines. Soils were collected horizontally along the transect 1 metre behind the in-use pit latrines, at the midpoint between the pit latrine and shore of Lake Victoria, 2 metres from the shore and sediment samples (Figure 3.4). The soils collected from the negative site were collected across five straight transect lines randomly placed on the football field. The soils collected from the open defecation sites were taken from directly under six sites of open defecation, increasing the likelihood of collecting soil.

The soil at each sampling point was collected by scraping the surface layer within a 400 cm^2 (20 cm x 20 cm) square area using a metal hoe (Pickering *et al.*, 2012). The soils were then scooped into a new zip lock bag and homogenised by hand. Approximately 1 g of soil was aliquoted from the homogenised soil sample into each of the five 2 ml cryovials (Corning®) before flash freezing (Tatti *et al.*, 2016). All tubes were flash frozen in liquid nitrogen on the day of collection using a Taylor Wharton CXR500 dry shipper. This provided five technical replicates to each biological replicate. The football field had five biological replicates; each transect line was categorised as an individual biological replicate (Figure 3.4d).

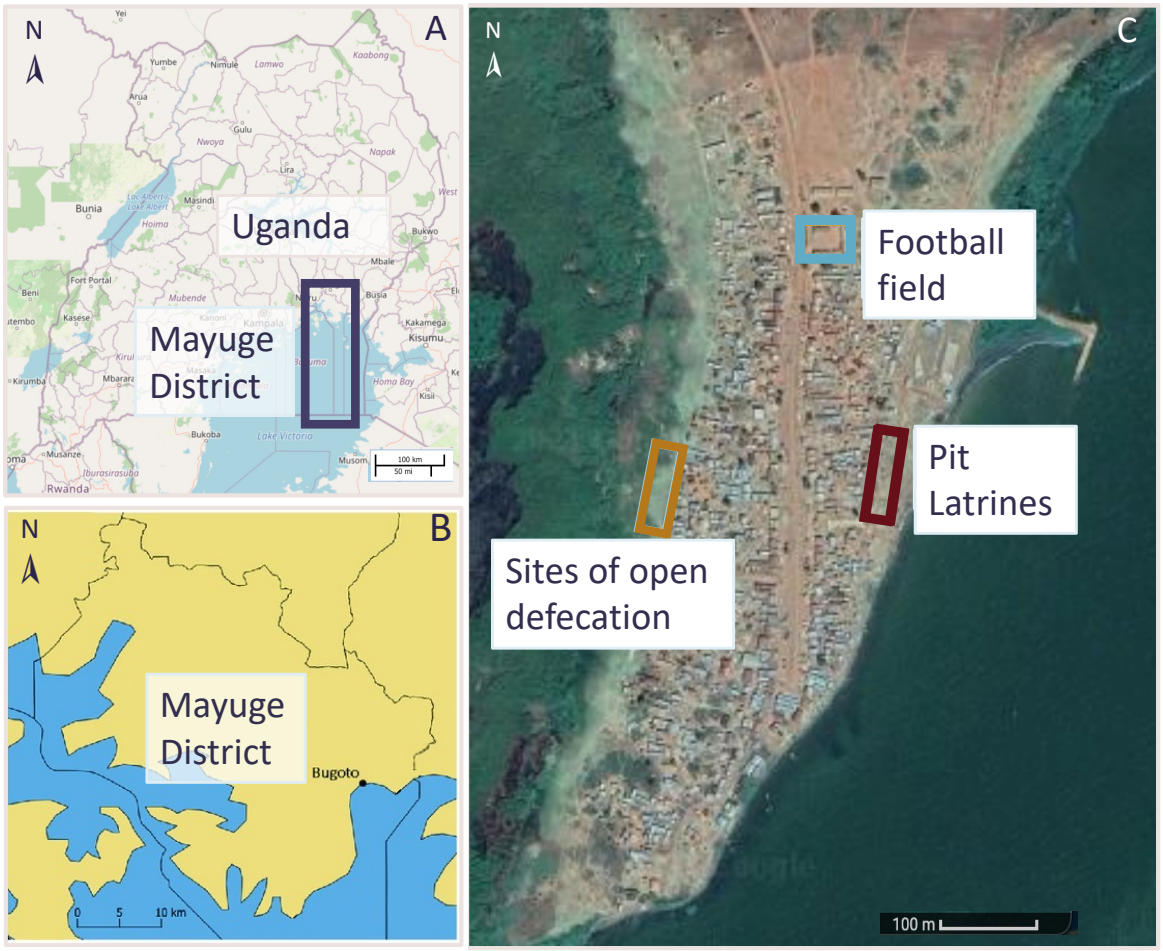


Figure 3.3. Map of study site. A) Map of Uganda with districts outlined and Mayuge District highlighted (Open Street Map). B) Mayuge district with location of study site, the village of Bugoto (adapted from Ssali *et al* (2021)). C) Bugoto A, the study site, is shown with sampling points labelled: football field, sites of open defecation and the pit latrines (satellite imagery from Google Maps).

The open defecation site had samples from six biological replicated, each from a distinct area of defecation and therefore had six biological replicates (Figure 3.4e). For the three pit latrines, Pit Latrine 1, Pit Latrine 2, and Pit Latrine 3 there were n= 11, n=14, n=13 biological replicates respectively. The different numbers of samples collected from each pit latrine transect were due to the presence of fishing boats moored on the shoreline: it was not possible to collect sediment samples from each planned sampling point. The samples were then transported to the University of Glasgow within the shipper and upon arrival stored at -20°C.

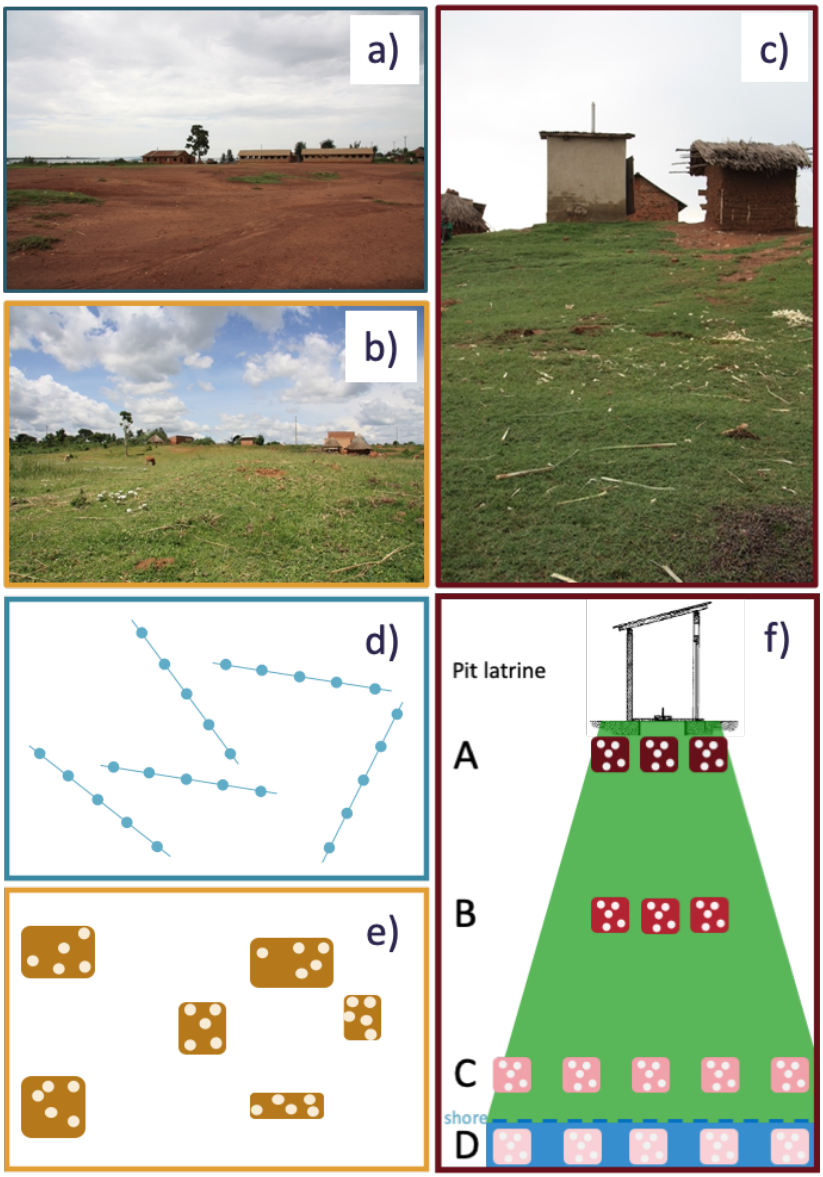


Figure 3. 3. Photo and schematic representation of sampling areas. a) Photograph of the negative control flat sampling site, the football field, chosen as a sanitation-free control site. b) Photograph of the sampling site where open defecation was present. c) Photograph of a pit latrine that was sampled and the slope down towards Lake Victoria. d) Schematic of sampling transect technique used to collect soil samples from the football field. e) Schematic of sampling technique used to sample soil from sites of open defecation, each brown square represents an individual faecal sample, of which the soil was sampled beneath. f) Schematic of sampling plan used to sample soils proximal to each pit latrine. Each circle (●) represents a sampling spot within a biological replicate soil sample: the five sampling spots within each transect shown in d), e) and f) were combined and homogenised to make each sample used for subsequent DNA extraction.

3.3.3. Application of quantitative PCR method

3.3.3.1. DNA extraction

The COI-based qPCR eDNA assay developed in Chapter Two, was used on these soil samples to screen for absence or presence of *S. mansoni*. Soil DNA was extracted using the MPBio Soil Spin Kit, using 500 mg of soil according to manufacturer's protocol and outlined previously in more detail in Chapter Two.

Briefly, the samples were homogenised using the FastPrep-24™ Classic Instrument (MPBio) at speed 5.5 for two 30 second runs. A blank DNA extraction containing 500 µl nuclease free water was performed with each extraction run as an extraction negative control. DNA was eluted in nuclease free water in a final volume of 50 µl. The DNA concentration was measured using a broad range Qubit assay (Invitrogen) (see Table 3.1).

3.3.3.2. *Schistosoma mansoni* detection by quantitative PCR

The mitochondrial cytochrome oxidase (COI) qPCR assay was carried out as described in Chapter Two. A standard curve was run on each qPCR plate: a five-point dilution of the standard from 10^{12} to 10^{4} gene copies. The logged starting quantity of each standard was plotted against its corresponding C_T to produce an equation of the linear regression and determine the efficiency of each qPCR run. Each soil DNA extract was run neat, in triplicate, on the qPCR plate. No-template controls (NTCs) were also included in triplicate within each plate and the standard curve acted as a positive control. If a reaction had an average C_T value within three C_T values of the water control (NTC) or all C_T values were undetermined, the sample was categorised as negative.

To determine the presence of PCR inhibitors in the DNA extracted from the soils, a serial dilution was carried out on two of the DNA extracts: P3A3 a sample from pit latrine three and PFD11 a sample from the site of open defecation. These soil samples were chosen as they had been extracted in duplicate. The DNA extracts were run in a series of three dilutions: 1 in 10, 1 in 100 and 1 in 1000.As the C_T values for the soil samples were all undetermined, apart from one sample which had a C_T value of 40, the two soil samples extracts were spiked with the COI manufactured standard and then serially diluted. The qPCR assay was also run on these DNA extracts spiked with the COI standard: 10^{7} copies of the reference standard DNA. The DNA extracts were again then diluted to 1:10, 1:100 and 1:1000 and run, in triplicate, on the qPCR plate.

3.3.3.3. Assay variation and statistical analysis

Due to the number of soil samples processed, ten qPCR runs were made to characterise all the samples. The samples were randomly allocated to a plate

and well position within the plate to minimise plate biases. As a serial dilution of the COI standard curve was carried out on each qPCR plate, the inter-assay variation was assessed by calculating the coefficient of variation for each of the five dilutions. The equation for percentage coefficient of variation was as follows:

$$Coefficient\ Variation\ (CV)\% = \left(\frac{standard\ deviation}{mean}\right) \times 100$$

The variation of the slope and elevations of the ten standard curves were also analysed to determine the reproducibility of the assay. An ANOVA was carried out to determine if there was a difference between the means of the slope and elevations of the standard curves using the R packages ggplot2 (Wickham, 2016), gridExtra (Baptiste, 2017), Lme4 (Bates *et al.*, 2015) and LmerTest (Kuznetsova, Brockhoff and Christensen, 2017) (R version 4.1.0).

From observation, it was theorised that any pit latrines built proximal to the water's edge could be seeding the lake with *S. mansoni* eggs. As well as using the *S. mansoni* qPCR assay, to compare the absence/presence of *S. mansoni* DNA in the soils collected from high contamination environments (site of open defecation and pit latrines), it was also hoped to gain a spatial insight into the presence of *S. mansoni* within soils collected between the pit latrines and lakeshore to fulfil Objective 3, however due to the finding described below this objective could not be investigated further.

3.4. Results

3.4.1. Quality assurance of *Schistosoma mansoni* COI quantitative PCR assay

3.4.1.1. Quality assurance of soil sample processing

Following DNA extraction from the soil samples, total DNA concentration was estimated using a Qubit Fluorometer (Tables 3.1). Total DNA extracted from the soil samples ranged from 0.21ng/µl to 780ng/µl across all the sampling sites. The median and mean amounts were 140.50ng/µl and 193.71 ng/µl respectively. All DNA extraction water negative controls had DNA concentrations undetectable by Qubit Fluorometer.

Table 3. 1. Summary of the ranges of the total DNA estimated by Qubit for the football field (negative control), open defecation (positive control) and three pit latrines. The area of open defecation and football field had five and six biological replicates collected respectively. Each pit latrine transect had four distances from the structure (as illustrated in Figure 3.4), these were annotated as levels A-D. Pit Latrine 1 could not have the furthest sample collected. Each biological replicate had five technical replicates collected, resulting in the range of total DNA. DNA was extracted from 500mg of each Ugandan soil samples.

Sampling area	**Biological replicate**	**Range of total DNA (ng/µl)**
Area of open defecation	1	273-279
	2	157-227
	3	98-197
	4	306-396
	5	111-190
	6	90-239
Football Field	1	8-28
	2	42-56
	3	21-42
	4	5-20
	5	37-47

Sampling area	Sample level	Range of total DNA (ng/µl)
Pit Latrine 1	A	9-401
	B	70-780
	C	75-540
Pit Latrine 2	A	28-261
	B	243-438
	C	48-297
	D	0.2-429
Pit Latrine 3	A	77-670
	B	274-381
	C	43-374
	D	49-323

3.4.1.2. Inhibitors

For the two soil sample DNA extracts that were spiked with the manufactured COI standard and diluted by factors 10, 100 and 1000, the C_T values ranged between 27.97-28.13 and 28.08 and 28.41 (Table 3.2). There was less than half a cycle between the C_T values measured.

Table 3.2. CT values for soil extract dilutions used to determine the presence of PCR inhibitors in the extracted DNA samples. The samples were soils collected from Pit Latrine 3 (P3A3) and a site of open defecation (PFD11). The samples were diluted and run without spiking and with spiking of the *S. mansoni* standard. The spiking was performed after DNA extraction but before dilution. Each sample was run in triplicate and C_T presented in the table are the averaged C_T values. UD: undetermined C_T value.

Soil sample ID and dilution factor	C_T value (average)	Spiked soil sample ID and dilution factor	C_T (average)
P3A3 1:10	UD	Spiked P3A3 1:10	28.0
P3A3 1:100	39.6	Spiked P3A3 1:100	28.1
P3A3 1:1000	UD	Spiked P3A3 1:1000	28.1
PFD11 1:10	UD	Spiked PFD11 1:10	28.1
PFD11 1:100	UD	Spiked PFD11 1:100	28.1
PFD11 1:1000	UD	Spiked PFD11 1:1000	28.4

3.4.1.3. Quantitative PCR assay reproducibility

It was determined in Chapter Two that this qPCR assay lacked the sensitivity to quantify the number of *S. mansoni* eggs in a soil sample, although it was sensitive enough to detect a single *S. mansoni* egg DNA extracted from 500 mg of soil. The standard curves, using the manufactured COI standard, were used to assess the performance of each qPCR run: its efficiency and lower C_T value cut-off. The correlation coefficient (r^2), efficiency (*E*) and *y*-intercept generated from the ten standard curves are summarised in Table 3.3. The serial dilution ranged from 10^{12} to 10^4 per 1µl of standard DNA and inter-assay variation ranged from 3.6% to 7.5% (Appendix 1: Table 2).

Table 3. 2. Summary of the logistic regressions from 10 separate qPCR runs of the *Schistosoma mansoni* COI standards. r^2 is the correlation coefficient, E is the efficiency of the qPCR assay and y-int is the intercept of the slope. The standard curves were obtained using dilutions of DNA ranging from 10^{12} to 10^4 copies per reaction.

	q-PCR assay run									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
r^2	**0.948**	**0.993**	**0.999**	**0.978**	**0.998**	**0.972**	**0.969**	**0.810**	**0.997**	**0.979**
E (%)	**56**	**89**	**85**	**80**	**95**	**76**	**80**	**88**	**69**	**76**
y-int	**73**	**58**	**59**	**58**	**56**	**60**	**58**	**59**	**63**	**60**

The first qPCR run's standard curve was an outlier from the other nine runs (Appendix 1: Figure 1). Both the slope of the line and *y*-intercept for this qPCR run were outside the interquartile range: 0.27 (2dp) and 2.03 (2dp) for the slope and *y*-intercept respectively. There was no significant difference between the slope of the line (F= 0.589, Df= 1, Pr(>F) = 0.468) or for the *y*-intercept (F= 3.512, Df= 1, Pr(>F) = 0.103) between the different runs of the qPCR assay. The C_T values were negatively associated with increasing COI standard concentration (Figure 3.5). The standard deviation across the five standards varied from 0.3 to 2.2, as illustrated by the error bars in Figure 3.5 (full results reported in Appendix 1: Table 2).

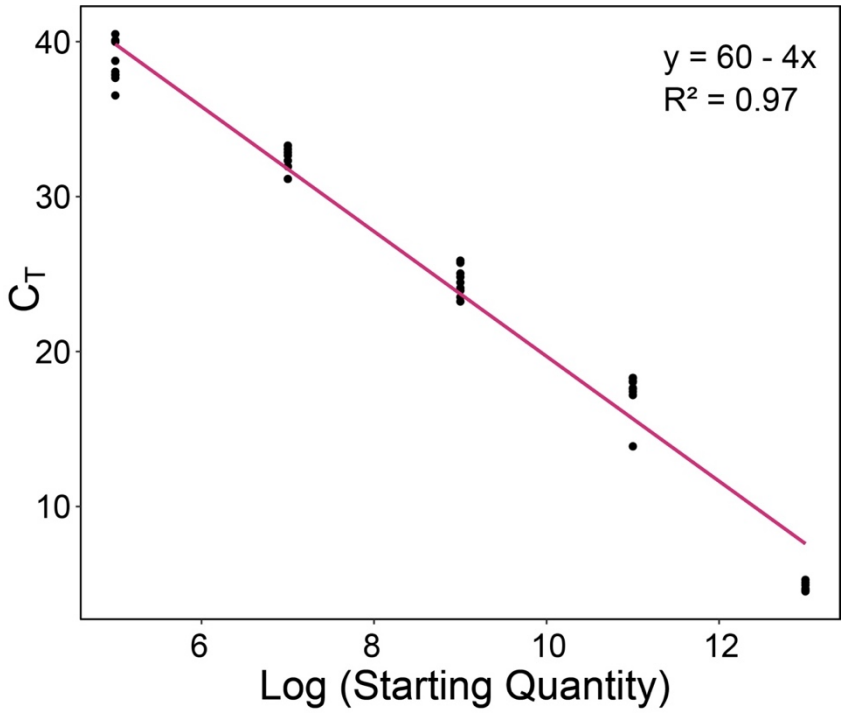


Figure 3. 5. Dynamic range of the real-time PCR assay for *Schistosoma mansoni* detection. The logistic regression was performed on mean data from ten, separate qPCR runs. Standard curve obtained using dilutions of DNA ranging from 10x 10^{12} to 10x10^4 copies per reaction. The concentrations of the gene copies are plotted against the logged C_T values. The C_T is the cycle number at which the fluorescence signal increased above a defined threshold. This threshold was calculated by the real-time PCR software. R^2 is the correlation coefficient.

3.4.2. Quantitative PCR estimated presence of *Schistosoma mansoni* in soil samples.

The qPCR assay did not detect the *S. mansoni* COI gene in any of the soil samples collected from the endemic Ugandan field sites (Table 3.4) with all C_T values within three C_T of the NTC. The NTC C_T values ranged from 36.5 to 42.0 C_T or were measured as undetermined by the software (Full results in Appendix 1: Table 1). The extraction negative controls (DNA extracted from nuclease free water alongside the soil samples) also showed no specific amplification. The assay's positive controls (standard curves prepared in nuclease free water) successfully amplified in all the qPCR runs (Table 3.3.), indicating the assay worked, and the results from the soil extracts are true negatives.

Table 3. 3. Summary table of the soil collection points and amplification of *Schistosoma mansoni* COI gene. No specific amplification was detected in the soil samples.

Site	No. of positive (No. of samples tested)
Area of open defecation	0 (6)
Football Field	0 (6)
Pit latrine 1	0 (11)
Pit latrine 2	0 (13)
Pit Latrine 3	0 (12)

3.5. Discussion

3.5.1. Monitoring of *Schistosoma mansoni* in relation to sanitation

S. mansoni's survival and proliferation is dependent on completing each one of its lifecycle stages. This lifecycle is reliant on the transmission of viable eggs, excreted within human faeces, to the habitat of susceptible aquatic snail hosts, where these eggs hatch, releasing the snail-infective miracidia (Roach, 2018). This human-to-snail transmission is facilitated by a lack of, or inadequate, sanitation facilities and/or sanitation practices (Grimes *et al.*, 2015). Water

contact sites can be directly monitored using snail surveys and newer eDNA methods, but sanitation use, and effectiveness in retaining the parasites, remains more challenging to monitor. This study used eDNA-based methods to investigate the presence of *S. mansoni* DNA in soils from an endemic area. The results indicate that soil samples taken from around pit latrines and even those taken directly from open defecation sites were not positive for *S. mansoni,* using a highly sensitive qPCR assay optimised in Chapter Two.

3.5.1.1. Quantitative PCR reproducibility and lower limits of detection

This study applied eDNA techniques to soils collected from a *S. mansoni* endemic area, from locations specifically chosen due to their likelihood of being contaminated, such as open defecation sites, or sites surrounding pit latrines, but the COI qPCR assay did not detect any *S. mansoni*. The positive controls successfully amplified and the NTC ranged between 36.5 and 42.0 or were undetermined, indicting the PCRs were working and that there was either, no *S. mansoni* in the soil samples, or the amount of *S. mansoni* DNA in these was below the low limit of detection (one egg in 500 mg soil) of this qPCR assay.

The C_T values' coefficient of variation results, a measurement of variation around the mean for each COI standard concentration, suggest that one assay was not carried out in a reproducible manner. This could have been due to, for example, inaccurate pipetting and subsampling error of the standards (Taylor et al., 2019). This qPCR run had outlier slope and *y*-intercept values that would indicate this qPCR run had a lower sensitivity than the other qPCR runs, potentially resulting in false-negatives. This qPCR assay should have been re-run but due to laboratory closures, time constraints and the remaining volume of the DNA extracts available, this was not performed. However, all other assays did appear to be reproducible, and given the samples were distributed between qPCR runs to minimise plate effects, and the high sensitivity of the assay demonstrated in Chapter Two, it is likely that our negative findings overall indicate that *S. mansoni* was not present in the soil samples that were used for the DNA extraction.

3.5.1.2. PCR inhibition test

A factor that could affect the qPCR assay used in this chapter is the presence of inhibitors in the DNA extracts carried over from the soil. There are DNA purification steps within the commercial DNA extraction kit used but it is not 100% effective (Opel, Chung and McCord, 2010). Inhibitors can reduce the sensitivity of qPCR assays, leading to an underestimation of the PCR target and potentially false-negative results. There are a variety of inhibition mechanisms that can affect PCR reactions, such as humic acid (a component of soil) which has been shown to bind to the template DNA, resulting increased C_T values measured (Opel, Chung and McCord, 2010). Inhibitors can also interact with the Taq polymerase, resulting in reduced assay efficiency (Opel, Chung and McCord, 2010).

There are different ways to minimize the effect of inhibitors, such as diluting the DNA extracts (and therefore reducing the concentration of inhibitors present in the sample) or changing the PCR conditions to be more resistant to inhibitors. To rule out the possibility of organic and inorganic inhibitors within the soils altering the qPCR results, a serial dilution of two different soil sample extracts was performed but there was still no amplification of *S. mansoni* COI gene when diluted. The soils were then diluted and spiked with the COI standard to detect any differences in the C_T values measured, but there was no evidence from this that the soil samples contained inhibitors.

The qPCR master mix used was designed by its manufacturers to be a robust reaction mixture and specially formulated for qPCR reactions containing inhibitors and has been shown to be robust against inhibition when tested on environmental water samples (Cao *et al.*, 2012). Therefore, as 1) the qPCR master mix was robust was against inhibitors, 2) there was no evidence of inhibitors from the diluting experiment, and 3) diluting the DNA extracts would reduce the concentration of any *S. mansoni* DNA present in the DNA extracts, reducing the sensitivity of the assay, the neat soil DNA extracts were used. Using the DNA extract neat is the equivalent of running DNA extracted from 20 mg of soil sample in each qPCR assay as 500 mg soil was extracted into to 50 µl and 2 µl was run in each qPCR well. By using a 1 in 10 dilution this would be the equivalent of running 200 µg in each qPCR reaction well which may have led to

false negatives and was therefore avoided where possible to maximise sensitivity.

3.5.2. Possible reasons for the lack of detection of *Schistosoma mansoni* in DNA extracts

3.5.2.1. No *Schistosoma mansoni* present in soil sampling areas

The sampling of soil in Bugoto was designed to cover a range of *S. mansoni* environmental contamination by collecting soils from an area of no/low likelihood of human faecal contamination (football field) and areas highly likely to have faecal pollution (sites of open defecation and pit latrines). As *S. mansoni* is endemic within the community of Bugoto, *S. mansoni* eggs would be present in an infected person's stool. The sanitation facilities available in Bugoto are pit latrines and open air defecation. For the area of open defecation sampled, it was hypothesised that the soil interfacing with the stool deposited on the ground, would also contain *S. mansoni* eggs. The soil proximal to the pit latrines was also predicted to be an area of high contamination for *S. mansoni*. These predictions of the presence of *S. mansoni* eggs (or *S. mansoni* eDNA) may have been incorrect, resulting in the absence of *S. mansoni* detected by qPCR on the soil samples.

Additionally, only surface soil was chosen for sampling and collected. The movement of eggs within the soil environment after being deposited onto the soil via defecation, is still unknown. At the open defecation sample sites, it was expected that any eggs that were excreted in faeces would be detectable in the soil adjacent to the faecal matter. It is known that MDA coverage is low in this communities (Adriko *et al.*, 2018), and if sampling had happened just after a MDA effort, it is still likely that people within the community, and their faeces, would still be infected with schistosomiasis. It could be that the eggs remain in the faeces or that eggs which transported out of the faeces had moved deeper into the soil or washed away and where not in the sampled layer.

It is most likely that eggs were missed by the sampling methods chosen for this study, and further optimisation is required to increase the volume of soil being processed. For example, this optimisation could include power calculations into

the prevalence of the infections across whole communities, to make sure we have sampled enough open defecation sites to have at least one site from an infected person. These calculations could use infection intensity data of *S. mansoni* in the local population to inform a sample size calculation: the lower the intensity of infection, the greater sampling of soils would be needed to detect *S. mansoni* eggs deposited in the soil.

The pit latrine transects were chosen as another area of high faecal contamination risk. From observation it was theorised that the pit latrines could be leaching faeces (carrying *S. mansoni* eggs) into Lake Victoria as the lake is proximal to where the latrines were built, the latrines are uphill from the lakeshore and the water table is high. However, none of the soils collected from the pit latrine transects had detectable *S. mansoni* DNA. It could have been useful to have additionally sampled directly from inside the pit latrine, both the ground surrounding the drop hole and sludge within the pit latrine, to see if *S. mansoni* is detectable in these samples using the qPCR assay. This would help to understand if there was no detectable *S. mansoni* in the latrine to leak out, or if the latrine appeared to be successfully containing the *S. mansoni* eggs. As it is known that *S. mansoni* transmission is maintained at a high rate in this community, it was expected to detect *S. mansoni* DNA in the environmental samples. Therefore, it is more likely that the lack of detection of *S. mansoni* in the soils was due to the low sensitivity of the sampling methods and/or sample size, rather than due to the eggs/DNA not being in the soil environment.

3.5.2.2. Low sensitivity of assay due to small soil sample size

If there were *S. mansoni* eggs and associated eDNA in the areas of Bugoto that were sampled, the other reason *S. mansoni* was undetected could be due to the sample volume of the soils. As described before, surface soils were collected, and DNA was extracted from 500 mg of the soil sample. As there were five technical replicates extracted for each soil sample, there were five chances for an egg to be present in the sampling spot and have its DNA extracted. However, the small volume (2 µl/50 µl) of DNA extract used in each qPCR reaction well is the equivalent of 20 mg of the 500 mg soil sample. Therefore, only a 100 mg subset of each sampling spot along the transect was, in actual terms, analysed by qPCR. Concentrating the sample, either before or after DNA extraction, or

increasing the volume of DNA used in the qPCR assays could have increased the probability of detecting *S. mansoni* DNA if it was present in the soil sample.

It may be that to capture *S. mansoni* for detection by qPCR, either the amount of soil processed per extraction would need to be increased, or the number of technical replicates, or both, to increase the chance of extracting DNA from a sample containing one or more eggs.

It was not possible to estimate how many eggs could be expected in the soil and what minimum amount of soil would need to be sampled to detect schistosome eDNA if it was present. The number of eggs excreted by a *S. mansoni* infected individual can be estimated, for example a person would be classified as having a light infection intensity if they were estimated to have 1-99 eggs per gram of stool, as determined by Kato-Katz (WHO, 2002). This increases to more than 1000 eggs per gram in individuals with a high infection intensity. However, how this translates into soil samples interfacing with egg-containing stool is unknown, although it can be hypothesised that the concentration of eggs will be lower compared with the number of eggs contained within a faecal sample openly defecated.

As the lower limit of quantification for the number of eggs was determined to be 1 egg/ 500mg. Therefore, each 1g aliquot of the soil sample flash frozen in Uganda would have to contain a minimum of two *S. mansoni* eggs for this test to detect their presence. However, the probability of detecting an egg was increased due to the five technical replicates for each sample, therefore only one of the five 1g aliquots of soil would need to contain an egg to detect *S. mansoni* eDNA.

There are data from previous studies on the range of soil-transmitted helminths (STHs) eggs found in different soil environments (For example: Mandarino-Pereira et al., 2010; Blaszkowska et al., 2013; Nooraldeen, 2015; Rocha, Eboly Barés and Braga, 2016). For the STHs, the number of helminth eggs enumerated from soil varies between species. For example, it can range between 5-53 eggs in 100g for *Ascaris lumbricoides* (Blaszkowska *et al.*, 2013; Nooraldeen, 2015; Rocha, Eboly Barés and Braga, 2016), 1-17 *Trichuris tricuria* eggs measured in

100g (Mandarino-Pereira *et al.*, 2010; Blaszkowska *et al.*, 2013; Nooraldeen, 2015) and 5-20 eggs in 100g for hookworm species (Nooraldeen, 2015).

STH eggs are not necessarily a good proxy for *S. mansoni* eggs as different STH species eggs, or larvae, remain viable in the environment for variable amounts of time, and all of them likely to remain viable longer than Schistosome eggs. For example, *Trichuris* eggs can remain viable for up to 1.5 years and *Ascaris* for several years (Smith, 1999). Their resistance to degradation in the environment may result in higher counts of these eggs being enumerated from soil samples. Hookworm eggs persist for a shorter time in the soil environment and hatch within 24-48 hours of reaching a suitable soil environment, but the hatched larvae can survive for weeks to months (Udonsi and Atata, 1987) and would also be detectable by DNA techniques. *Schistosoma* eggs typically only remain viable (able to hatch) in the soil environment for up to six days (Pitchford and Visser, 1972). Therefore, the overall number of eggs in an environment will be low, transient, and highly dependent on the infection intensities of any open defecators, as well as environmental factors.

To increase the likelihood of capturing parasite DNA in a sample, a concentration method could be employed before DNA extraction. Gyawali *et al.* (2015), used a centrifugation and flotation approach to concentrate hookworm ova before DNA extraction for qPCR detection (Gyawali *et al.*, 2015). These researchers used wastewater and sludge matrices, but their sequential centrifugation and flotation steps could be adapted for environmental samples with a lower water content. The flotation solutions can cause the distortion of *Schistosoma* eggs, rendering them beyond identification by microscopy (Smith, 1999), however this problem would be circumvented by DNA-based species identification. If hatching was unintentionally induced, this COI qPCR assay would also remain a species-specific tool to determine presence or absence of *S. mansoni*, as the mitochondrial gene is expressed throughout the parasite's lifecycle stages (Jolly *et al.*, 2007). Therefore, further studies, utilising concentration steps and larger samples sizes would help elucidate the presence of *S. mansoni* in soil samples or other environmental samples, as transmission is occurring at a very high level in the Bugoto community, eggs must be reaching water bodies inhabited by *Biomphalaria* snails.

3.5.3. *Schistosoma mansoni* eggs in the environment and *S. mansoni* environmental DNA

The soils collected for this Chapter were theorised to contain *S. mansoni* eggs and/or fragments of *S. mansoni* DNA either from degraded eggs or fragments excreted in the faeces. The DNA extraction technique should have extracted DNA from both sources: eggs and/or *S. mansoni* DNA in the wider environment. As the COI gene fragment targeted by the qPCR assay is small (162 bp) this should have reduced the chances of the assay failing to detect *S. mansoni* DNA because of the DNA fragments extracted being too damaged or degraded.

The surface soils collected during this study will have been subjected to environmental processes resulting in egg and eDNA degradation. As discussed above, *Schistosoma* eggs in faeces deposited into the wider environment can remain viable for up to six days (Pitchford and Visser, 1972) but the persistence of its eDNA beyond this remains unknown. Any *S. mansoni* eDNA within the soil environment, would be subjected to both protective and destructive processes: the mechanics of this degradation require further investigation to inform soil-based eDNA studies (Harrison, Sunday and Rogers, 2019). An interesting additional step of this study would have been to investigate the degradation of *S. mansoni* eggs and eDNA, as although tropical environments have more rapid DNA degradation, eDNA bound to clay soil, such as the red ferralsol soils found in Bugoto, are protective against degradation (Taberlet *et al.*, 2018). Further work into UV- and temperature -based degradation and protection from degradation by soil type is warranted.

3.5.4. Case for environmental DNA as tool for monitoring

This study was not able to successfully detect *S. mansoni* in the soil environment by using an eDNA qPCR assay. However, eDNA from samples such as these could possibly still provide a rapid, sensitive, and specific tool to assess the functioning of sanitation facilities and monitoring of environmental contamination. This could either be performed using concentrated soil samples or increasing the amount of the soil DNA is extracted from. An alternative to directly detecting *S. mansoni* eDNA could be to utilise bacterial faecal markers

to detect faecal contamination. Ponce-Terashima *et al.* (2014) investigated the prevalence of *S. mansoni* in communities living along a specific river and the levels of faecal contamination of this river's surface waters. Bacterial markers were used as a proxy for *S. mansoni* to assess the spatial distribution of human faecal bacteria in surface water. Using faecal indicator bacteria detection assays on the Ugandan soil samples could help understand the level of containment of faeces in pit latrines and the level of contamination in open defecation sites and is the focus of Chapter Four.

The affordability of environmental surveys is an important factor for both neglected tropical diseases and the strengthening of WASH services (WHO UNICEF, UN-Water Global Analysis and Assessment of Sanitation and Drinking-Water and Expert Group on WASH Affordability, 2021). The cost of carrying out eDNA surveys to detect either the snail host or the parasite itself, has been found to be more effective than traditional snail surveys (Mulero *et al.*, 2019; Sengupta *et al.*, 2019). Although this cost effectiveness was dependent on the scale of the monitoring, with the price per assay reducing as the scale of the survey increased. If absence and presence is sufficient data output of eDNA-based assays, end-point PCR could be used instead of the more expensive qPCR methods used in this study.

The limitations of snail surveys and using data from human epidemiology studies to infer WASH provisions on schistosomiasis have been discussed in this chapter and in review papers (Grimes *et al.*, 2014, 2015). If eDNA techniques can be harnessed to detect schistosomiasis in terrestrial samples, control programmes would have a supplementary tool to assess WASH intervention use, impact and subsequent schistosomiasis transmission reductions in communities at different life-cycle stages.

3.6. Conclusion

From the literature, eDNA techniques offered promise as a rapid, sensitive, and specific tool to monitor schistosomes in the wider environment. However, further research is needed, in particular to improve sensitivity, by increasing the amount of soil that DNA is extracted from and/or using concentration techniques. For the control of schistosomiasis, the sanitation infrastructures are

needed to confine faeces away from snail habitats, but in endemic areas the standards of pit latrines are often insufficient. This research study indicates that if using these eDNA methods, it is currently only possible to detect the presence of *Schistosoma* eDNA in water samples, rather than directly at the potential source of schistosome DNA in the soils proximal to sanitation faculties: further research is required. As the eDNA field continues to develop, eDNA techniques could aid public health assessments associated with sanitation and schistosomiasis, complementing behavioural surveys. One possible method to monitor sanitation interventions might be to use faecal biomarkers, rather than schistosome specific markers, this is addressed in the next chapter, Chapter Four.

Chapter Four: Occurrence of bacterial markers in surface soils proximal to pit latrines in a Ugandan village.

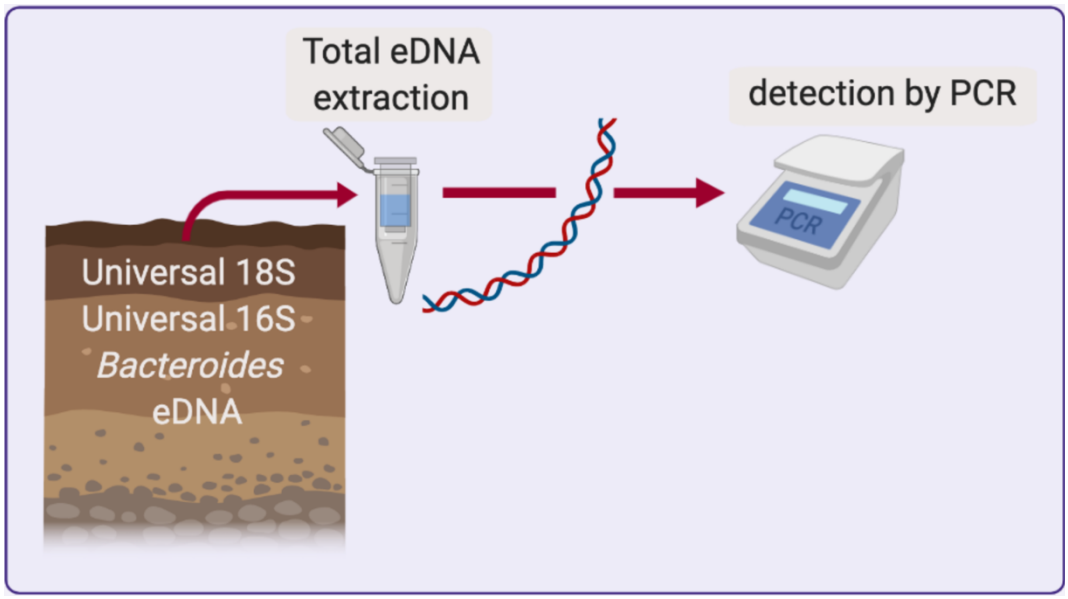


Figure 4. 1. Visual summary for Chapter Four: the detection of universal 18S and bacterial markers (universal 16S and *Bacteroides*) in soils sampled from a *S. mansoni* endemic village.

4.1. Abstract

The relationship between the human parasite, *Schistosoma mansoni*, and poor sanitation is well established. However, it is rarely directly quantified and the link between sanitation and schistosomiasis has been mainly evidenced by epidemiological data. Faecal bacterial biomarkers have been used to identify faecal contamination of a range of substrates, but not for detecting potential schistosomiasis contamination in a soil environment. The aim of this chapter was to investigate the soils proximal to sanitation facilities (pit latrines and areas of open defecation) using bacterial indicators of faecal contamination, as a proxy for the faecally transmitted *S. mansoni*. It was predicted that soils collected from sanitation facilities would have higher concentrations of faecal biomarkers than soils from predicted negative control sites on a football pitch. It was also predicted that faecal contamination would decrease as physical distance from a pit latrine increased. Four qPCR assays were chosen to characterise the soil environment of a *S. mansoni* endemic community in Uganda: universal 18S, universal 16S, general *Bacteroides* and a human specific *Bacteroides*. The four qPCR assays were successfully employed to detect their targets in the soil samples. The faecal bacterial markers (general *Bacteroides* and human specific

Bacteroides qPCRs) were detected within all sampling sites. The human specific *Bacteroides* marker however had lower bacterial counts in the football field samples compared with samples from the other sampling areas, whilst the general *Bacteroides* marker had no significant variation between the different sites. No spatial relationship between the concentrations of the *Bacteroides* faecal markers, universal 18S or universal 16S was observed along the pit latrine transects. The results indicate that faecal contamination is relatively ubiquitous in this community, but that there is more contamination at open defecation sites and surrounding pit latrines than at the football field. The higher detection at the latrines may be due to either unknown open defecation near them or faecal leaking from the latrines warranting further research. Applying molecular-based assays to environmental samples could expand the capabilities of monitoring the environment with respect to human health. However, the application of these methods must be carefully approached to avoid, or help explain, some of the results observed in this study.

4.2. Introduction

Monitoring the presence of schistosomiasis in the environment can provide insights into the use of and effectiveness (or failure) of water and sanitation services. The survival and proliferation of *Schistosoma mansoni* is dependent both on the infection of humans with the water-bound cercariae and the deposit of eggs, transported within an infected humans' faeces, into the aquatic habitat of *Biomphalaria* snails (Campbell *et al.*, 2014). The previous chapter, field tested an eDNA assay to specifically detect *S. mansoni* in soil samples proximal to sanitation facilities (pit latrines and areas of open defecation). This assay was unsuccessful, potentially due to sensitivity issues in the sample volume and the number of eggs within any faecally contaminated soils. Detecting the faeces themselves, using faecal specific bacterial markers, could provide an alternative method to monitor the soil environment for human faecal pollution, as an indirect marker for schistosomiasis contamination. If there is contamination of the soil environment due to poor sanitation, faecal bacteria will be present in greater number compared with *S. mansoni* eggs in the soil. Hence, faecal indicator bacteria (FIB) could be a more sensitive monitoring tool, despite not being designed to specifically detect *Schistosoma* species. Regarding sanitation

and *S. mansoni*, the monitoring of faecal markers in the environment, including the aquatic snail habitats could provide an indication of how sanitation services are working to contain faecal matter, and by proxy parasitic eggs.

4.2.1. Techniques for human faeces detection

Bacterial indicators that track human sources of faecal contamination in soil can contribute to our understanding of the transmission dynamics of faecal-associated parasites. Microbial source tracking (MST), which aims to distinguish the source organism of faecal contamination, is commonly used in studies investigating diarrhoeal diseases and drinking water contamination (Ferguson and Signoretto, 2011). For example, there are numerous published studies that researched the presence of faecal bacterial markers in the household environment with regards to sanitation improvements (Fuhrmeister *et al.*, 2020; Holcomb *et al.*, 2020; Contreras *et al.*, 2021).

MST is based on detecting faecal indicator bacteria (FIB): a range of bacteria that inhabit the gastrointestinal tract of birds and mammals (homeotherms) and these bacteria are consequently excreted in these animals' faecal material (Ferguson and Signoretto, 2011). The principal method for detecting and enumerating faecal indicator bacteria are culture based: whereby colony-forming units (CFU) are identified and counted (Harwood *et al.*, 2014). Molecular techniques, including PCR-based methods, also enable the targeting of specific faecal-associated bacteria, such as species-specific microbial biomarkers, and can be detected directly from environmental samples without prior culture (Harwood *et al.*, 2014). Methods for the PCR-based amplification of bacterial ribosomal DNA (rDNA) extracted directly from a faecally contaminated environment without prior culture, were chosen for this study.

In addition to choosing between culture-based and molecular-based methods for MST, there are also various faecal-associated bacteria shown to accurately and specifically infer the presence of human faecal matter (Wuertz *et al.*, 2011). *Escherichia coli* has been employed as a FIB due to it being an easily cultured microorganism. *E. coli* can be a useful faecal indicator but lacks the host-specificity to be used as a faecal source indicator: a bacteria indicative of which organism the faeces derived from (Mclellan and Murat Eren, 2014). Faecal

anaerobic bacteria are some of the most promising alternative indicators to *E. coli* (Ponce-Terashima *et al.*, 2014). Some genera of these bacteria have sufficient specificity to a host species' gut that they can be employed to identify individual host source species (e.g., human, dog, cow) (Boehm *et al.*, 2013). Organisms of the *Bacteroidale* genus for example have a high host specificity and encompass a significant proportion of the animal faecal bacteria population, which means they are highly specific and sensitive MST targets (Layton *et al.*, 2006).

Surface water contamination by human and animal faeces in schistosomiasis transmission spots has been previously investigated with the primary aim of correlating the spatial concentration of faecal contamination in river water with the prevalence of *S. mansoni* in the nearby communities in Brazil (Ponce-Terashima *et al.*, 2014). Ponce-Teraschima *et al.* (2014) found that human-originating faecal bacteria accumulated as the river they sampled ran downstream. This spatial association of faecal contamination also correlated with the proportion of people with schistosomiasis living along the river: the risk for schistosomiasis was higher as faecal contamination of surface water increased (Ponce-Terashima *et al.*, 2014).

The main aim of this chapter was to apply this eDNA methodology to an area with active *S. mansoni* transmission and gain a spatial and quantitative understanding of human faecal contamination in the soil environment. This study was carried out to determine if MST could be an additional tool for the monitoring of the uptake and effectiveness of sanitation in schistosomiasis endemic areas.

4.2.2. Aim and objectives

The main aim of this chapter was to investigate the presence and abundance of human faecal bacterial markers in soil in relation to pit latrines in a *S. mansoni* endemic community. The soil sampling was designed to facilitate the detection and enumeration of faecal contamination along a transect leading away from a pit latrine and down a slope towards the shoreline of Lake Victoria. Two control sites were also sampled for comparison with the pit latrine transects: an area

predicted to be free from defecation (village football field) and an area of active open defecation.

Objectives:

1. Determine appropriate faecal-associated DNA targets for this *S. mansoni* high endemicity community in Uganda.

2. Quantify the target DNA using 18S ribosomal RNA and 16S ribosomal RNA bacterial targets detected in the soil samples.

3. Determine the distribution of the faecal-associated bacterial concentrations in relation to increasing distances from three in-use pit latrines.

4.3. Methods

4.3.1. Ethics

This research study in Bugoto, Uganda was nested within Dr Poppy Lamberton's research group's ongoing work on schistosomiasis in Uganda under research permit (UNCST-HS 2193) in collaboration with Vector Control Division, Ministry of Health (VCDREC/062) and University of Glasgow Medical, Veterinary and Life Sciences Research Ethics Committee (200160068).

4.3.2. Field site description

Soil samples were collected from the village of Bugoto A, Uganda in September 2019, as outlined in Chapter Three. In brief, the village of Bugoto A is a *S. mansoni* hotspot whose residents are predominately fisherfolk of Lake Victoria (Lamberton *et al.*, 2014; Crellen *et al.*, 2016). The soil sampling sites within the village are shown in Figure 3.4, as well as the location of Bugoto A in relation to Lake Victoria. Stool samples were collected, as part of Lauren Carruthers's PhD, from primary school children in September 2017 and frozen on dry ice before being shipped to Glasgow for DNA extraction (Carruthers, 2021). The DNA from these faecal samples were used for use in the PCR primer validation of the universal 18S and bacterial markers.

4.3.3. Soil sampling

Soil samples were collected from transects of three in-use pit latrines, an area of open defecation and an area presumed free of defecation (football field), as outlined in Chapter Three. The same soils collected for the detection of *S. mansoni* were also used for the qPCRs for faecal bacteria detection performed in this chapter. To recap, the soils collected from the negative control site were collected across six straight transects randomly placed on the village football field (Figure 3.4.). The soils collected from the site of open defecation were taken from directly under six points of open defecation (Figure 3.4.). For the three pit latrines, a transect was designed to start behind a pit latrine and continued down towards the shore of Lake Victoria (Figure3.4.). Soils were collected along the transect, 1 metre behind an in-use pit latrine, at the midpoint between the pit latrine and shore of Lake Victoria, and 2 metres from the shore and sediment samples from the shoreline.

The surface soil at each site was collected by scraping the surface layer within a 400 cm^2 area using a metal hoe. The soils were then scooped into a new Ziplock bag and approximately 1 g of soil was aliquoted from the hand-homogenised soil sample into five 2 ml cryovials (Corning®). The tubes were flash frozen in liquid nitrogen on the day of collection using a Taylor Wharton CXR500 dry shipper. The samples were then transported to the University of Glasgow within the shipper and upon arrival stored at -20°C. Soils were imported under Licence no. IMP/SOIL/6/2016/2.

4.3.4. DNA extraction

The DNA of the soil samples were extracted using the MPBio Soil Spin Kit using 500 mg of soil according to manufacturer's protocol as outlined in Chapters Two and Three. Briefly, samples were homogenised using the FastPrep-24™ Classic Instrument (MPBio) at speed 5.5 for two 30 second runs and a tube containing nuclease free water was added to each extraction run as an extraction control. DNA extracts were stored at -20°C until use.

4.3.5. Validation of proxy bacteria quantitative PCR on faecal samples.

A validation study to choose sensitive and specific markers to infer the presence of human faecal matter in the soil environment was conducted. Local testing for microbial markers is recommended before use on samples from a study site/population (Reischer *et al.*, 2013), consequently, a screening of potential bacterial targets was carried out. Seven qPCR primers targeting bacterial faecal indicators (Table 4.1) were tested using DNA extracts from human faecal samples of residents of Bugoto. To gain a broad insight into the bacteria present in the soil environment PCR assays specific to both kingdom and genus were chosen. Prokaryotic verses eukaryotic DNA were targeted using universal 16S and universal 18S assays respectively. At the genus level a general *Bacteroides* target was chosen as a faecal bacterial indicator as *Bacteroides* species are estimated to comprise 30 to 40% of the amount of total faecal bacteria in homeotherms (Layton *et al.*, 2006). For comparison with an estimate of general animal faecal bacteria in the soil environment, a human specific *Bacteroides* assay was used to estimate the amount of human faeces present in the soil.

The DNA used to test the seven potential PCR primers (Table 4.1) was previously extracted by Lauren Carruthers using the extended bead beating method (Alcon-Giner *et al.*, 2017) with adaptations (Carruthers *et al.*, 2019). The DNA extraction process for these stool samples used the MP Biomedical Soil Spin Kit as described previously for the soil samples but with three variations: firstly 200 mg of stool was used as the starting material; secondly, the stool samples were homogenised using a TissueLyser II (Qiagen, Hilden, Germany) at a speed setting of 25; thirdly, there was also an additional 2-minute centrifugation step used after the addition of the binding matrix.

The seven primer pairs (Table 4.1) were used in endpoint PCR reactions. Two of the PCR reactions did not result in amplification: human *Bacteroidale* target (Hf183) and *Enterococcus* target (ESP). For the five primer pairs that did result in specific amplification there were two non-species specific *Bacteroides* primers, AllBac and GenBac3. As the AllBac primers are specific for only *Bacteroides*, compared with GenBac3 which targets both *Bacteroides* and *Prevotella* bacteria, the AllBac primers were chosen to take forward and used to

compare the amplification of human specific *Bacteroides* based on the results from this general *Bacteroides* assay.

The four primers chosen (universal 18S rRNA, universal 16S rRNA, general *Bacteroides* and human-specific *Bacteroides*) from the end point PCR experiment were used in qPCR assays on the soils. The universal 18S assay is a SYBR assay and were 20 μl reactions using 10 μl Applied Biosystems® SYBR® Green PCR Master Mix, full conditions are outlined in Table 4.2. The other four assays were TaqMan probe-based and were amplified in 20 μl reactions using 10μl TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems) (Table 4.2). An Applied Biosystems™ 7500 Real-Time PCR System was used for all qPCR assays with MicroAmp Fast Optical 96-well Reaction Plates (Applied Biosystems™). MicroAmp™ Optical Adhesive Film were used to seal the plates (Applied Biosystems™).

Table 4. 1. Bacterial and 18S qPCR assays tested on faecal samples from the field site.

Primer name	Classification level	Target organism/group	Target host	Amplification	Reference
Universal 16S rRNA (Bact2)	Kingdom	16S SSU rDNA	Prokaryotes	Yes	(Suzuki, Taylor and Delong, 2000)
Universal 18S	Kingdom	18S rRNA gene of Eukarya	Eukaryotes	Yes	(Medlin *et al.*, 1988)
AllBac	Genus	*Bacteroides*	Non-specific	Yes	(Layton *et al.*, 2006)
GenBac3	Order	*Bacteroidale-Prevotella* group	Non-specific	Yes	(Shanks *et al.*, 2009)
HuBac	Genus	*Bacteroides*	Human-indicative	Yes	(Layton *et al.*, 2006)
Hf183	Order	*Bacteroidale* (HF8 cluster)	Human indicative	No	(Seurinck *et al.*, 2005)
ESP	Genus	Enterococcus	Human-indicative	No	(Ahmed *et al.*, 2008)

Table 4. 2. Quantitative PCR assays used in the study

Target	Primer/probe sequence (5' to 3')	Product size (bp)	Tm (°c)	Reaction conditions	Reference
Universal 16S SSU rDNA (BAC2)	1369-F (5'-CGGTGAATACGTTCYCGG-3') 1492-R (5'-GGTTACCTTGTTACGACTT-3') 1389-P (5' -CTTGTACACACCGCCCGTA-3')	123	56	1.8 µl 10µM primer mixtures, 0.4 µl of the 10µM probe mixtures, 5 µl nuclease free water and 2 µl of DNA. Cycling conditions: initial step 15 mins at 95ºC followed by 40 cycles of 30 sec 94 ºC, 30 sec 57 ºC and 30 sec 72 ºC.	(Suzuki *et al.,* 2000)
Universal 18S (V9 region)	Euk_1391 (5'- GTA CAC ACC GCC CGT C -3') EuKBr (5'-TGATCCTTCTGCAGGTTCACCTAC-3')	180	57	0.8 µl of each 10 µM primer mixtures, 7.4 µl water and 2 µl of DNA. Cycling conditions: initial steps of 10 mins at 95ºC, followed by 40 cycles of 30 sec 94ºC, 30 sec 57ºC and 30 sec 72ºC.	(Medlin *et al.,* 1988)
General *Bacteroides* 16S rRNA	AllBac-296-F (5'-GAGAGGAAGGTCCCCCAC-3') AllBac412-R (5'- GAGAGGAAGGTCCCCCAC-3') AllBac375-P (5'-FAM-CCATTGACCAATATTCCTCACTGCTGCCT-MGB-3')	106	60	0.9 µl 10µM primer mixtures, 1.25µl of the 10µM probe mixtures, 4.95 µl nuclease free water and 2 µl of DNA. Cycling conditions: initial steps of 2 mins at 50ºC and 10 mins at 95ºC, followed by 40 cycles of 30 sec 95ºC and 45 sec 60ºC	(Layton *et al.,* 2006)
Human *Bacteroides* 16S rRNA	HuBac-566-F (5'-GGGTTTAAAGGGAGCGTAGG-3') HuBac-692-R (5'-CTACACCACGAATTCCGCCT-3') HuBac-594-P (5'-FAM-TAAGTCAGTTGTGAAAGTTTGCGGCTC-MGB-3')	116	60	0.9 µl 10µM primer mixtures, 1.25µl of the 10µM probe mixtures, 4.95 µl nuclease free water and 2 µl of DNA. Cycling conditions: initial steps of 2 mins at 50ºC and 10 mins at 95ºC, followed by 40 cycles of 30 sec 95ºC and 45 sec 60ºC	(Layton *et al.,* 2006)

4.3.6. Application of quantitative PCR assays on environmental samples

The qPCR assays outlined in this chapter were used on the DNA extracts from the environmental samples collected in Bugoto, described, and used in the previous chapter. Each soil sample's DNA extract was run neat, in triplicate, in each of the four qPCR assays described above. In each of the qPCR assays, 2 µl starting volume was used in each assay. For absolute quantification, each qPCR plate included a standard curve. The specific PCR product sequence for each assay was purchased from Integrated DNA Technologies (IDT Technology, USA). Each dilution of the standard was run in triplicate on the plate. No-template controls (NTC) were also included in triplicate on each plate. A sample was recorded as positive if at least one of the three replicates amplified, and the cycle threshold (C_T) was not within three C_T values of the NTC.

4.3.6.1. Estimation of gene copy number

The copy number of each gene targeted by the qPCR assay was interpolated from each qPCR plate's standard dilution curve. The following equation was used for absolute quantification with data inputted from the equation of the standard curve's logistic regression (y= mx + c).

$$Quantity = 10^{\left(\frac{C_T - b}{m}\right)}$$

$b = y$-int
$m =$ slope of the line

4.3.6.2. Quantitative PCR reproducibility

The method to determine the coefficient of variation (CV) of each of the qPCR primer sets was identical to that used for the *S. mansoni* qPCR assay, described previously in Chapter Three. The equation used was:

$$Coefficient\ Variation\ (CV)\% = \left(\frac{standard\ deviation}{mean}\right) \times 100$$

4.3.7. Statistical Analysis

To compare the occurrence of the bacterial markers and universal 18S between sampling sites, a linear mixed effects model was used in R. The model was used to determine if there was a statistical difference between the site free of sanitation (football field), site of open defecation and the three pit latrine sites. The one-way ANOVA from this model has two outputs: *F* value (a ratio of two quantities that are expected to be equal under the null hypothesis) and significance code (calculated probability). To visualise the gene copy numbers estimated by the qPCR assays, box plots for the four sampling areas were also created in R Studio (R version 4.1.0). The following packages were used: ggplot2 (Wickham, 2016), gridExtra (Baptiste, 2017), Lme4 (Bates *et al.*, 2015) and LmerTest (Kuznetsova, Brockhoff and Christensen, 2017).

4.4. Results

4.4.1. Quantitative PCR assay reproducibility and lower limit of quantification

The qPCR standards for each of the four qPCR assays were analysed to determine the reaction efficiencies and dynamic range of the logistic regressions across the multiple qPCR runs carried out (Figure 4.3).

4.4.1.1. Universal 16S rRNA quantitative PCR assay standard curve

Due to the number of Ugandan soil samples, the samples were run over ten individual qPCR plates, consequently, there were results for the ten individual standard curves and the intra-assay variation of each qPCR assay could be interrogated. Figure 4.3A shows the dynamic range of the logistic regressions from the ten standard curves run using the universal 16S qPCR assay. The universal 16S qPCR assay's standards had a linear range of quantification from 10^9 to 10^1 gene copies per 1 µl of manufactured double stranded oligo (Figure 4.2A). The average C_T value for the NTC across the ten plates was 37.5. The efficiency, correlation coefficient (r^2), and the intercept ranged from 141% to 160%, 0.991 to 0.999 and 35 to 38 respectively (Appendix 2: Table 5). The inter-assay CV of the standards ranged from 3.8% to 16.3%.

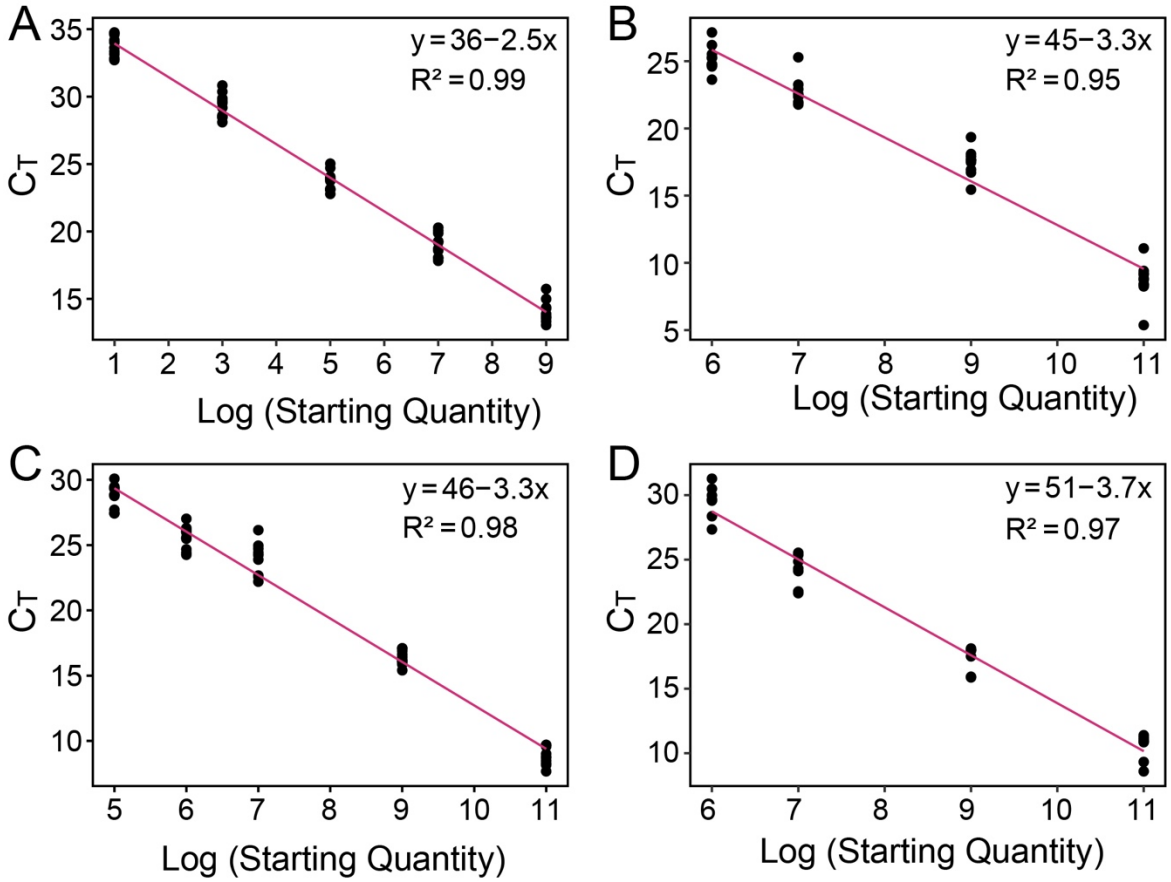


Figure 4. 2. Dynamic range of the four quantitative PCR (qPCR) assays. A) The universal 16S specific small-subunit (SSU) rDNA gene assay. The data were obtained over ten qPCR runs using dilutions of DNA ranging from 10^9 to 10^1 copies per reaction. B) The universal 18S standards, the mean data are from ten separate qPCR runs and dilutions of DNA ranged from 10^{11} to 10^5 copies per reaction. C) The general *Bacteroides* genus assay: the mean data are from ten separate qPCR runs and dilutions of DNA ranged from 10^{11} to 10^5 copies per reaction. D) The human specific *Bacteroides* assay: mean data are from seven separate qPCR runs and the dilutions of DNA ranged from 10^{11} to 10^4 copies per reaction. In all the graphs, the concentrations of the gene copies are plotted against the C_T values. The C_T is the cycle number at which the fluorescence signal increased above a defined threshold. This threshold was calculated by the real-time PCR software. R^2 is the correlation coefficient.

4.4.1.2. Universal 18S quantitative PCR assay standard curve

The dynamic range of the logistic regressions from the ten repeats of the universal 18S qPCR assay are summarised in Figure 4.2B. The universal 18S qPCR assay's standards had a linear range of quantification from 10^{11} to 10^5 gene copies per 1 µl of manufactured double stranded oligo. The average C_T value for the NTC across the ten plates was 33.1. The efficiency, correlation coefficient (r^2), and the intercept ranged from 87% to 129%, 0.903 to 0.988 and 41 to 47 respectively (Appendix 2: Table 6). The intra-assay CV of the standards ranged from 3.2% to 7.8%.

4.4.1.3. General *Bacteroides* quantitative PCR assay standard curve

The dynamic range of the logistic regressions from the ten repeats of the general *Bacteroides* qPCR assay are summarised in Figure 4.2C. The general *Bacteroides* qPCR assay's standards had a linear range of quantification from 10^{11} to 10^{5} gene copies per 1 μl of manufactured double stranded oligo (Figure 4.2C). The average C_T value for the NTC across the ten plates was 34.4. The efficiency, correlation coefficient (r^2), and the intercept ranged from 95% to 110%, 0.979 to 0.993 and 44 to 48 respectively (Appendix 2: Table 7). The intra-assay CV of the standards ranged from 3.2% to 7.8%.

4.4.1.4. Human *Bacteroides* quantitative PCR assay standard curve

The dynamic range of the logistic regressions from the seven repeats of the human specific *Bacteroides* qPCR assay are summarised in Figure 4.2D. The human *Bacteroides* qPCR assay's standards had a linear range of quantification from 10^{11} to 10^{6} gene copies per 1 μl of manufactured double stranded oligo Figure 4.2D. The average C_T value for the NTC across the seven plates was 30.5 . The efficiency, correlation coefficient (r^2), and the intercept ranged from 80% to 122%, 0.909 to 0.998 and 41 to 54 respectively (Appendix 2: Table 8). The intra-assay CV of the standards ranged from 4.5% to 10.3%.

4.4.2. Environmental faecal contamination

The universal 16S, general *Bacteroides* and human *Bacteroides* assays had statistically significant differences of their qPCR outputs (C_T values) when comparing C_T values of the negative control site (community football field) with those of the soils collected from the pit latrine transects and site of open defecation and (Table 4.3). The universal 18S did not show a difference in the C_T values means between the football field and other sites (*F* value of 1.37 (2dp) Pr(>(F)= 0.246, df= 4). None of DNA extraction controls or qPCR negative, water controls (NTCs) specifically amplified under any of the four qPCR assays. As it was determined that there was variation between the C_T values measured by the bacterial qPCR assays, the source and potential spatial aspect of this variation was investigated.

Table 4. 3. Summary of the percentage positive (number of positive) for the soil samples using the universal 16S, universal 18S, General *Bacteroides* and human *Bacteroides* qPCR assays from each sampling site: negative control site (football field), positive control site (area of open defecation) and pit latrine sites. The variation between the C_T values between the football field and the soils from the pit latrines and points of open defecation are summarised in the final row.

PCR target	Universal 16S rRNA (BAC2)	Universal 18S	General *Bacteroides* 16S rRNA	Human *Bacteroides* 16S rRNA
Percentage of samples from the football field positive (%)	100 (n= 5/5)	100 (n=5/5)	80 (n=4/5)	20 (n=1/5)
Percentage of samples from the site of open defecation positive (%)	100 (n= 6/6)	100 (n= 6/6)	100 (n= 6/6)	17 (n=1/6)
Percentage of samples collected from the pit latrine transects positive (%)	100 (n=35/35)	95 (n=35/36)	29 (n=10/36)	31 ((n=11/35)
F value (2dp) (P value for the *F* statistic and degrees of freedom (df)	18.61 P= 6.58e-13 df= 4	1.37 P= 0.246 df= 4	9.65 P = 3.83e-7 df= 4	9.12 P = 2.565 df= 4

4.4.2.1. Spatial distribution of faecal contamination: changes in bacterial communities across the pit latrine transect

Although bacteria were detected in all sites across the football field, pit latrines and open defecation (as defined by the presence of 16S), there were significantly more bacteria, as quantified by the universal 16S qPCR assay, in samples from the area of open defecation and soils proximal to pit latrines, compared with the soils sampled from the football field (F= 18.61 Pr(>(F)= 6.575e-13, df=4) (Figure 4.3). The universal 16S was detected at very low levels in the samples from the football fields compared with the other sites, the average across the five sampling sites was 60 copies per gram of soil. However, although there was variation of copy number of the 16 gene target within each soil sampling transect, the variation along each pit latrine transect was not significant: no spatial aspect to this variation of bacteria number was seen with increasing distance from the pit latrines (Full ANOVA results in Appendix 2: Table 9).

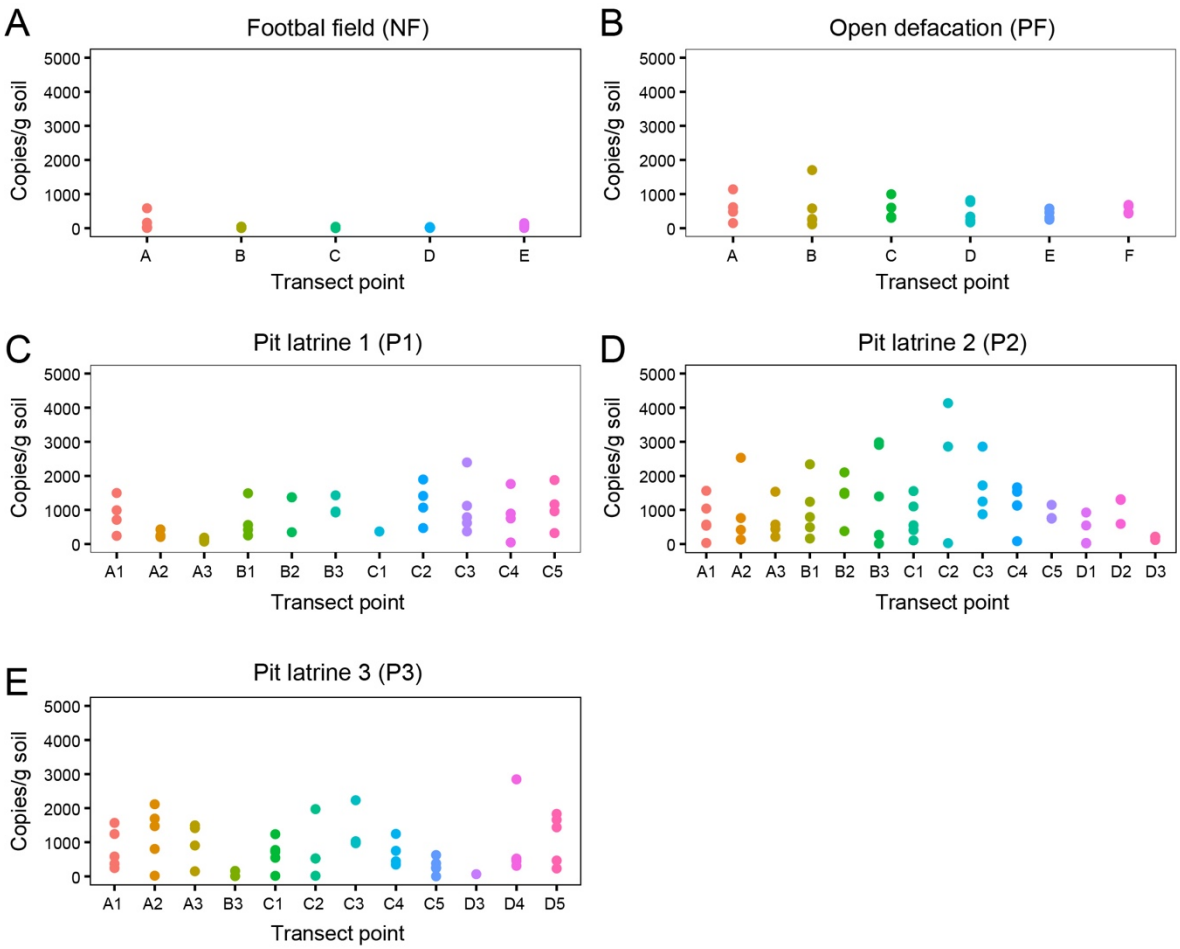


Figure 4. 3. Summary plot of the copy number per gram of soil interpolated from the 16S qPCR assays for the five transects. A) negative flat transect (NF), soils were collected from five different spots: NF1, NF2, NF3, NF4 and NF 5. B) site of open defecation positive flat (PF) where soils were collected from six different spots of open defecation, PF1- PF6. C), D) and E) are the three transects proximal to pit latrines, P1, P2 and P3 respectively. Levels A (red) were collected 1 metre behind the pit latrine, Levels B (green) at the midpoint between the pit latrine and the lake shoreline. Level C soils (blue) were collected at the shoreline. For Pit Latrine 2 and 3, D) and E), it was also possible to collect sediment samples: Level D. Upper outlier restriction was set at 5000 copies per gram of soil.

There was no significant difference in eukaryotic 18S genes measured by the universal 18S qPCR assay, across the different sampling sites (Figure 4.4). This finding was the same when analysing both the copy number values per gram of soil (*F* value = 1.28 (2dp), Pr(>(F)= 0.27, df= 4) and the C_T values (*F* value = 1.37 (2dp), Pr(>(F)= 0.25, df=4) calculated from this qPCR assay (Full ANOVA results in Appendix 2: Table 10).

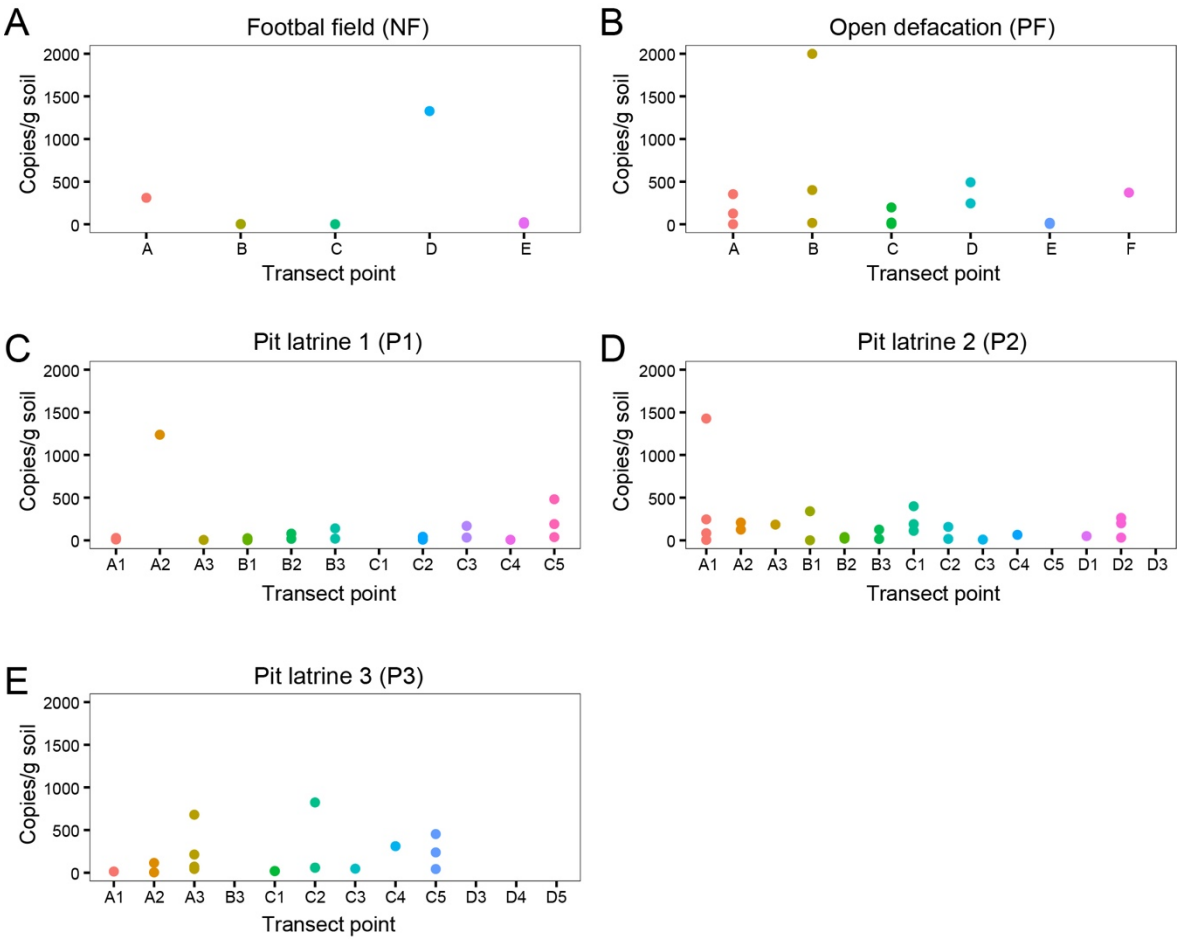


Figure 4. 4. Plots summarising the copy numbers per gram of soil interpolated from the 18S qPCR assays for the five transects. A) negative flat transect (NF), soils were collected from five different spots: N1- NF5. B) site of open defecation positive flat (PF) where soils were collected from six different spots of open defecation, PF1- PF6. C), D) and E) three transects proximal to pit latrines, P1, P2 and P3 respectively. Levels A (red) were collected 1 metre behind the pit latrine, Levels B (green) at the midpoint between the pit latrine and the lake shoreline. Level C soils (blue) were collected at the shoreline. For Pit Latrine 2 and 3, D) and E), it was also possible to collect sediment samples: Level D. Upper outlier restriction was set at 2000 copies per gram of soil on the y-axis.

Unlike the universal 16S assay, the general *Bacteroides* qPCR assay's results showed no significant variance in the copy number of *Bacteroides* gene between the negative flat soil samples (football field) and the other four sampling sites (Figure 4.5). However, when comparing the original C_T values for the soils, as opposed to interpolated gene copy numbers, the general *Bacteroides* gene was detected in greater number in soils from Pit Latrine 3 compared with the football field (*F* statistic 9.65 (2dp), Pr (>F) =3.828e-07, df= 4). Transect level B of the pit latrines, the mid-point between the pit latrine and lakeshore, showed lower C_T values compared with soils collected along the transect (*F* statistic 3.40 (2dp), Pr (>F) =0.01885, df=3).

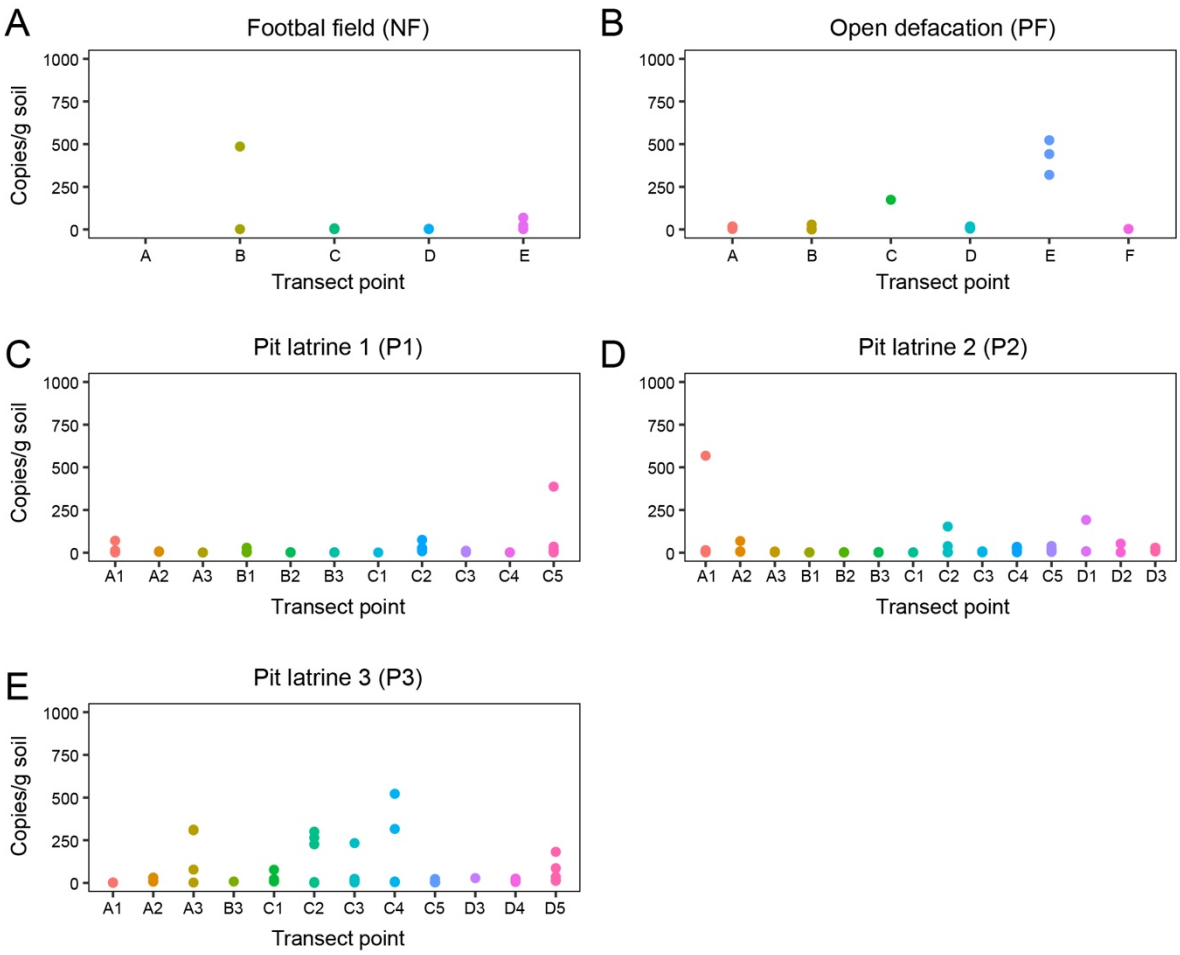


Figure 4. 5. Plots summarising the copy numbers per gram of soil interpolated from the general *Bacteroides* qPCR assays for the five transects. A) negative flat transect (NF), soils were collected from five different spots: NF1- NF5. B) site of open defecation positive flat (PF) where soils were collected from six different spots of open defecation, PF1- PF6. C), D) and E) three transects proximal to pit latrines, P1, P2 and P3 respectively. Levels A (red) were collected 1 metre behind the pit latrine, Levels B (green) at the midpoint between the pit latrine and the lake shoreline. Level C soils (blue) were collected at the shoreline. For Pit Latrine 2 and 3, D) and E), it was also possible to collect sediment samples: Level D. An upper restriction of the y axis of 1000 was set for these boxplots.

Like the universal 16S qPCR assay results, the human specific *Bacteroides* qPCR assay showed higher gene copy numbers from soils along the pit latrine transects and the open defecation site, compared with the football field (*F* value was 9.12 (2dp), Pr (>F) = 2.560e-07, df = 4) (Figure 4.6). Like the general *Bacteroides* assay, transect level B of the pit latrines (midpoint between the pit latrine and lake shore) had a significantly lower number of gene copies detected (P value between 0 and 0.001) compared with the other transect soils. However, the analyses using the C_T values instead of the estimated gene copy numbers showed no statistically significant differences along the distinct pit latrine transects.

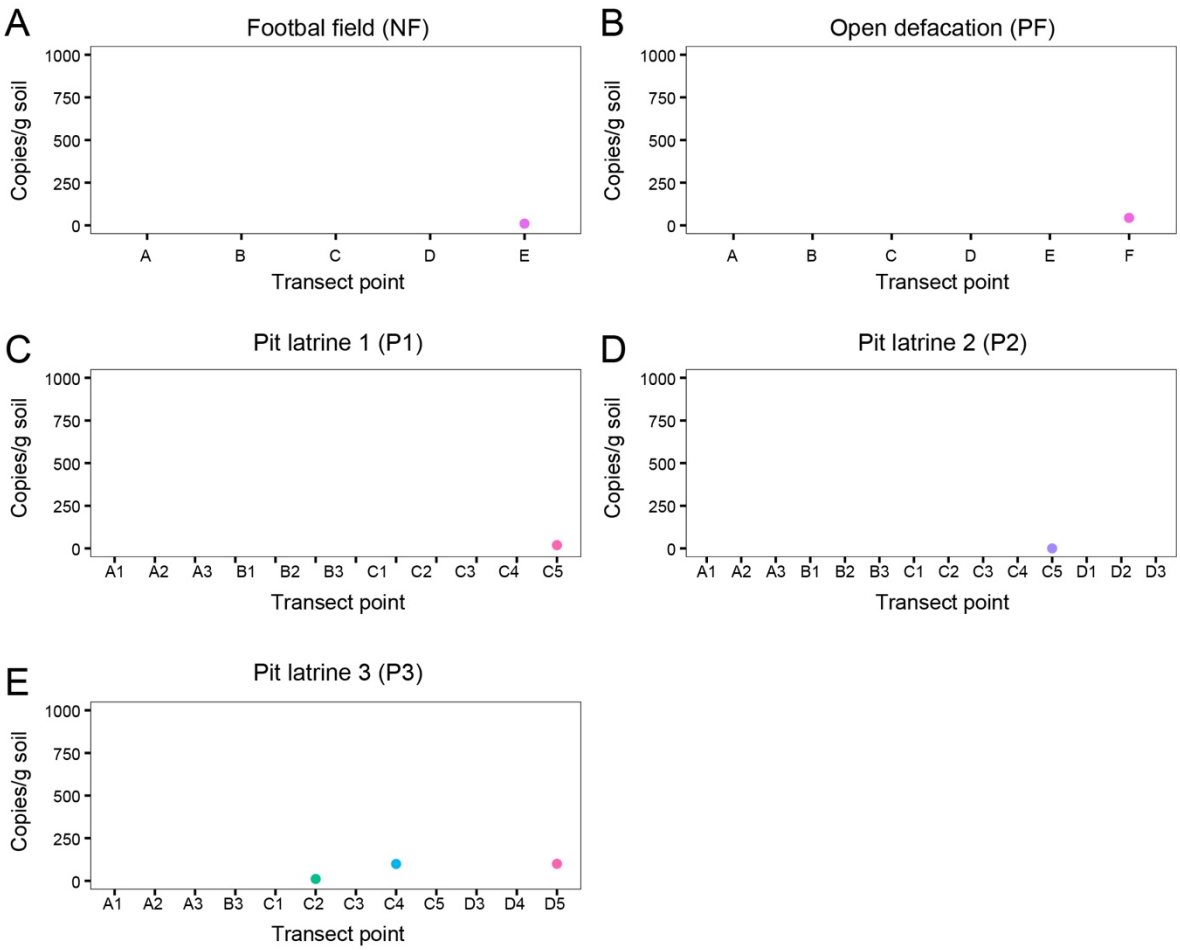


Figure 4. 6. Box plots summarising the copy numbers per gram soil interpolated from the human specific *Bacteroides* qPCR assays for the five transects. A) negative flat transect (NF), soils were collected from five different spots: NF1- NF5. B) site of open defecation positive flat (PF) where soils were collected from six different spots of open defecation, PF1- PF6. C), D) and E) three transects proximal to pit latrines, P1, P2 and P3 respectively. Levels A (red) were collected 1 metre behind the pit latrine, Levels B (green) at the midpoint between the pit latrine and the lake shoreline. Level C soils (blue) were collected at the shoreline. For Pit Latrine 2 and 3, D) and E), it was also possible to collect sediment samples: Level D. The y axis had an outlier restriction of 1000 copies.

4.5. Discussion

There is currently no gold-standard method for monitoring schistosomiasis in the soil environment related to sanitation, nor a method to assess the effectiveness of sanitation interventions to adequately contain human faeces. *S. mansoni* is seeded into the soil environment via the faeces of infected human host and the continuation of the parasite's lifecycle is dependent on a failure of sanitation services (Campbell *et al.*, 2014). As an alternative to the qPCR assay designed to specifically detect *S. mansoni* used in the previous chapter, a collection of bacterial markers was used as a measure of faecal contamination as a proxy for parasite presence. Four qPCR assays were used: universal 16S, universal 18S, general *Bacteroides* and human-specific *Bacteroides*. Three different sampling

areas of a *S. mansoni* endemic village were chosen to be characterised using the four qPCR assays: a site predicted to be free of human faeces, sites of open defecation and three transects designed to measure the soil proximal and distal to three pit latrines.

Amplification of DNA extracts from soils using the four qPCR assays was successful (Table 4.4), compared with the lack of any specific amplification in Chapter Three using the *S. mansoni* specific qPCR assay. The faecal bacterial markers (general *Bacteroides* and human specific *Bacteroides* qPCRs) were detected within all sampling sites: the area of open defecation, soils collected from the pit latrine transects and area presumed free of human faeces (football field). The human specific *Bacteroides* marker however had lower bacterial counts in the football field samples compared with samples from the other sampling areas, whilst the general *Bacteroides* marker had no significant variation between the different sites. No spatial relationship between the concentrations of the *Bacteroides* markers was however observed along the pit latrine transects.

4.5.1. Main findings of quantitative PCRs

There was variation in gene copy number measured across the five different transects (Figures 4.3- 4.6). It was originally planned to compare the findings of each qPCR assay, however as the efficiencies varied across the four assays (Figure 4.2), any comparison would not be reliable (Taylor *et al.*, 2019). However, the results from within the individual assays were compared with consideration to their inter-assay coefficients of variation. It was predicted when designing the study that there would be a significantly higher proportion of soils sampled that were positive for faecal indicators measured in the soils from the site of open defecation and proximal to pit latrines, compared with the football field. However, this was not the case for all three of the bacterial qPCRs, indicating that all sites had some soils contaminated with faecal matter.

The universal 18S was employed to compare the ratio of eukaryote and prokaryote DNA present in the soil samples. There was no statistically significant variation in copy number estimated for the universal 18S assay, with regards to the sampling site (football field versus site of open defecation or pit latrine

sites) or the transect levels varying in proximity to the three pit latrines (Table 4.4). In contrast, there were however higher copy numbers of the universal 16S gene, an indication of total bacteria present in the soil samples, in soils from the site of open defecation and pit latrine transects compared with the football field. This lower detection of total 16S genes in the football field compared with all other sites was not replicated when measuring the presence of all *Bacteroides* bacteria. The general *Bacteroides* qPCR assay, an indicator of mammal and bird faeces, showed no difference between the copy number across the different sampling sites. However, the human specific *Bacteroides* qPCR assay, an bacterial target indicative of human faeces, did replicate the results seen with the universal 16S assay: a lower gene copy number in the football field compared with the other sites. No consistent pattern was observed using these three bacteria-based assays, but these results indicate that the football field is contaminated with animal or bird faecal matter, which could be explained by the free roaming livestock observed in the area during the sampling period. The presence of low levels of human faecal matter (using the human specific *Bacteroides* qPCR assay) in some samples collected from the football field does highlight the community level contamination in this area, despite the lack of high levels of the human specific *Bacteroides* gene detected at the pit latrine and open defecation sites.

It was also hypothesised that there would be a spatial aspect to the detection of the two faecal indicator bacteria across the pit latrine transects: concentration of faecal-linked bacteria decreasing with distance from the pit latrine. However, there was only amplification of the human specific *Bacteroides* assay in the lower transects, the sampling points closest to the lake shore. This could be due to leaching of faecal material from the pit latrines as the toilets are uphill from these sampling areas, but further investigation would be needed to confirm this theory. The only consistent result between the two faecal indicator assays was that the midpoint of the pit latrine transects had the lowest copies of both *Bacteroides* genes present.

4.5.2. Limitations of quantitative PCR findings: inter-assay variation

The reproducibility of qPCR assays is likely to impact the results of this chapter. The interpretation of the copy numbers estimated by standard curve-based absolute quantification, must take into consideration the efficiency of the assay itself. Efficiency is considered "good" if within the range of 90% and 110% (Taylor *et al.*, 2019). Across the different qPCR runs of all the soil samples, the General *Bacteroides* assay was the only assay to have efficiencies within the acceptable range: 95% to 110% (full results in Appendix 2: Table 5). The universal 16S assay was likely impacted by the poor efficiency of this assay as measured across the ten separate qPCR runs: 152% (± 5.8). The human specific *Bacteroides* assay also had a range of efficiency across the individual qPCR runs that exceeded the acceptable range of 90-110%: 100% (± 22.1). A confidence interval so large suggests the quantitative results of the human specific *Bacteroides* assay are likely to be incomparable across qPCR plates, it would be possible to compare the results from the qPCR runs that had similar efficiencies, however, the number of samples that specifically amplified this *Bacteroides* target were low, 4% of all soil samples run (Table 4.4).

Poor efficiency can be influenced by a range of factors such as: inhibitors within the DNA extracts, suboptimal primer/probe design and inaccurate pipetting (Taylor *et al.*, 2019). As the efficiency of a qPCR assay is defined as the fraction of target molecules that are copied in one PCR cycle, any comparisons between different primer assays require similar reaction efficiencies (Ruiz-Villalba, Ruijter and van den Hoff, 2021). An important caveat when interpreting this study's data. The subsampling of standards by pipetting the samples onto the qPCR plates could have caused the inter-assay variation due to Poisson variation (Ruiz-Villalba, Ruijter and van den Hoff, 2021). The variation of efficiencies could also be due to random effects in each run occurring within the cycler or batch of reagents (Svec *et al.*, 2015).

4.5.3. Microbial source tracking and *Schistosoma mansoni* monitoring

Using the data from the general *Bacteroides* assay, that were shown to be reliable across the qPCR runs, no difference was observed between the defecation free site and areas proximal to the pit latrines and open defecation. Unfortunately, due to the variability of the other three qPCR assays, no cohesive relationship along the pit latrine transects could be determined. If it had been possible without the time constraints due to Covid-19, the qPCR assay runs with efficiencies outside the acceptable range could have been repeated to generate more comparable data.

Gaps remain in the direct monitoring of schistosomiasis in the terrestrial environment or within sanitation facility outputs (wastewater, sludge, compost). This study collected outdoor surface soils, collecting ground samples from within the pit latrines could have added an extra layer of information when interpreting the results from the pit latrine transects. As would have categorising the latrines and comparing transects from, for example, lined and unlined pit latrines or improved and unimproved latrines. It would also have been interesting to have added a temporal aspect to the soil sampling. As discussed previously, it was hypothesised that *S. mansoni* eggs could be transported from sites of open defecation to the lakeshore by surface water during the rainy season. Additionally, pit latrines can flood and overflow during heavy rains and this surface water could also be carrying faecal matter, potentially containing parasitic eggs, into the lake. By employing the MST qPCR assays on soils collected from different time points (before, during and after the rainy season) evidence to investigate this theory could have been gained.

Improvements to sanitation services, as supplements to MDA-based schistosomiasis control should reduce immediate faecal input into the environment, which can be measured directly through MST. MST could be used to gain an understanding of *S. mansoni* transmission in the wider environment and to monitor the effectiveness of off-grid sanitation facilities such as pit latrines. Although unfortunately, this study was unable to determine a microbial difference between sites linked to sanitation and those free of sanitation using the chosen qPCR assays.

4.6. Conclusion

The chosen faecal indicator qPCR assays were successfully employed to detect *Bacteroides* genes in the soils from a *S. mansoni* endemic area, including detecting higher concentrations of human specific *Bacteroides* at the open defecation and pit latrine sites than the negative control site of the football field. However, no spatial relationship between the concentrations of these faecal markers was observed along the pit latrine transects. Despite the shortcomings of this study, MST remains a potential additional tool in WASH. Research in the field of eDNA continues to offer more tools to expand the capabilities of qPCR methods to characterise the environment with respect to human health. However, the application of these methods must be carefully approached to avoid the irregular results observed in this study.

Chapter Five: Prescence of *Chryseobacterium nematophagum* and *Necator americanus* in surface soils in a Ugandan village.

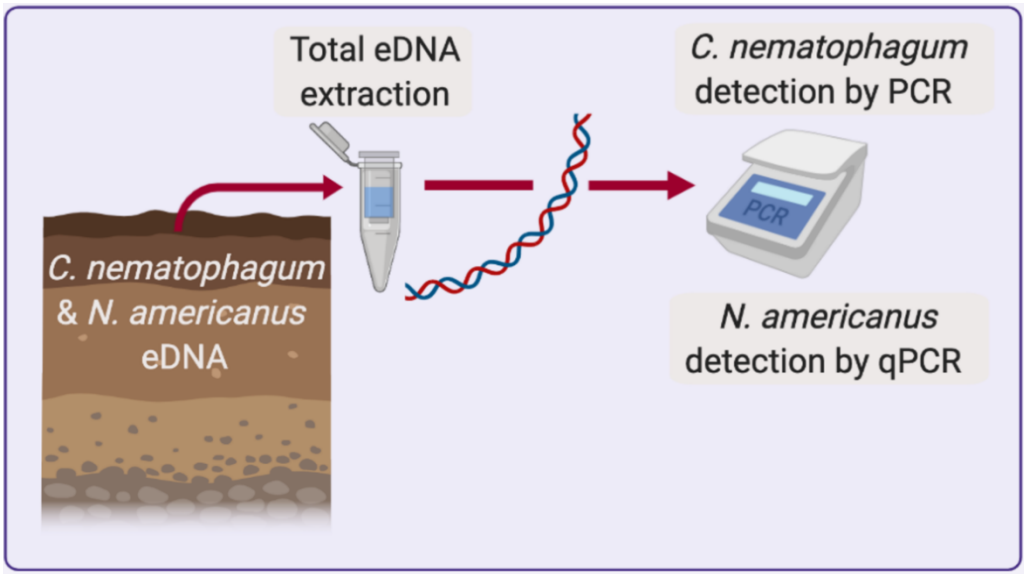


Figure 5. 1. Visual summary for Chapter Five: the detection of hookworm DNA (*Necator americanus*) and a nematode eating bacterium (*Chryseobacterium nematophagum*) in soils sampled from a *S. mansoni* endemic village.

5.1. Abstract

Human hookworm, a soil-transmitted helminth (STH), is a major cause of morbidity across the globe. Akin to other neglected tropical diseases (NTDs) the burden of disease is predominantly carried by the world's poorest populations. Mass drug administration (MDA) is the cornerstone of hookworm control and supplementary control measures such as improved sanitation and biological controls, e.g., natural pathogens of nematodes, could be useful interventions to prevent reinfection post-MDA. *Chryseobacterium nematophagum* has been found to be a bacterium pathogenic to nematodes and has been previously co-detected in rotten fruit with free-living nematode species. However, to date it has never been detected in sub-Saharan Africa, where it is estimated 472 million people live with hookworm infections. The aim of this chapter was to determine if the hookworm species *Necator americanus* and the nematode killing species bacteria *C. nematophagum* were detectable in soils proximal to sanitation in a hookworm endemic area. If *C. nematophagum* is found to be native to this area, then it opens doors for biological control intervention trials, without concern for the potential negative effects an invasive species might have. Soils were collected from an area predicted to be free of human defecation (community football

field) and two areas interfacing with sanitation: soils proximal to in-use pit latrines and an area of open defecation. A *N. americanus* qPCR and *C. nematophagum* PCR were used on the soil DNA extracts. One soil sample out of the 31 samples tested was positive for *C. nematophagum,* whilst one sample from each of the three pit latrine sampling transects and one sample out of the four football field samples (site free of sanitation facilities) were positive for *N. americanus,* totalling four positives out of 31 samples. The soil sample positive for hookworm from Pit Latrine 3 was also positive for *C. nematophagum*, however due to the variability in the qPCR results, the technical replicates for this sample had C_T values more than 0.5 cycles apart, they were considered too disparate from one another to be considered reliable data. Further optimisation of the assays is required but the initial findings presented in this chapter indicate that the human parasitic nematode (*N. americanus*) and a nematode-eating bacterium (*C. nematophagum*) could be present in the soil environment together, and that *C. nematophagum* may be native to Uganda.

5.2. Introduction

5.2.1. Nematodes and human health

Hookworm species *Necator americanus*, *Ancylostoma duodenale* and *An. Ceylanicum* are human-infecting hookworm species which, along with other intestinal nematode species, are commonly categorised as soil-transmitted helminths (STHs) (WHO, 2020b). Like schistosomiasis, STH infections are primarily controlled by mass drug administration (MDA). In 2018, 576.4 million children were treated for STHs, but this is only 59.9% of the NTD roadmap target for STH preventative chemotherapy (Hotez, 2013; WHO, 2019). The disparity between the WHO target, and actual number of people treated is analogous to the challenges faced in schistosomiasis control, and other neglected tropical diseases (NTDs) (Parker and Allen, 2011). Again, like schistosomiasis, the highest prevalence of hookworm is concentrated in sub-Saharan Africa, and burden of disease disproportionately carried by those living in the most resource-poor settings (Hotez, 2008).

The transmission of hookworm infections is facilitated by unimproved sanitation and unsafe excreta disposal (Haldeman, Nolan and Ng'habi, 2020). This is due to

the nematode's eggs being passed into the environment within an infected individual's stool (Figure 5.2). Hookworms, unlike schistosomes, are independent of an obligatory intermediate host and only require damp, warm and shady soil conditions for their larval stage to hatch from the eggs (Figure 5.2: Stage 2). To become the human infective filariform larva, the freshly hatched larva will undergo two molts, taking between five to ten days. Analogous to schistosome cercariae, the infective larvae can penetrate the skin on contact with a human host (Figure 5.2: Stage 4). As the larvae occur in the soil environment (surviving for three to four weeks) infection usually occurs due to contact of bare feet with contaminated soil.

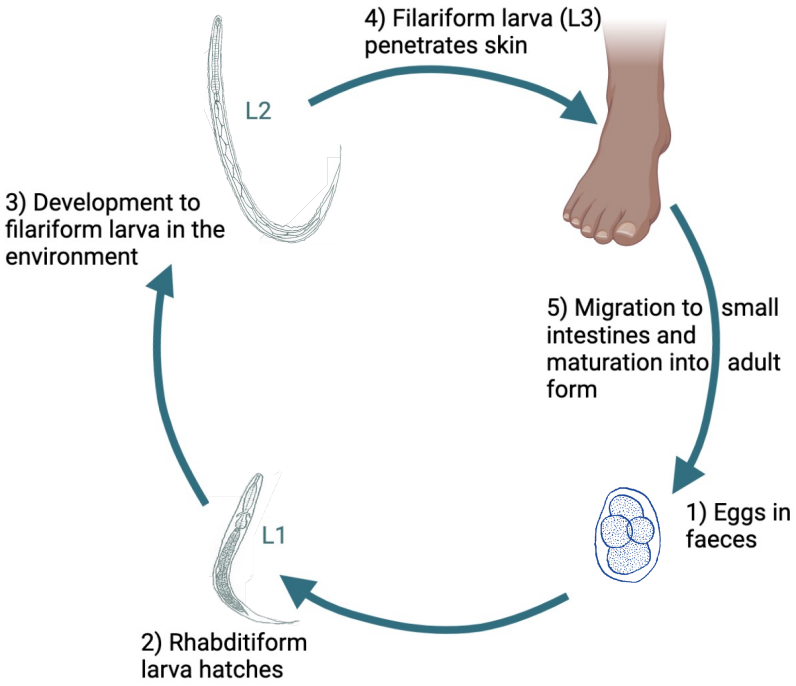


Figure 5. 2. Lifecycle of human hookworm species: *Necator americanus*, *Ancylostoma duodenale* and *An. Ceylanicum*. An infected individual sheds hookworm eggs in their faeces (1). If the soil conditions are met, the rhabditiform larvae (L1) will hatch from the egg (2). After two molts, to develop into its filariform (L3), the larva is now infective to a human host (3). Human infection occurs by contact, and consequential penetration, of the skin by the L3 larva (4). The larva is carried by the blood vessels to the heart and then lungs where the larvae actively penetrate the alveoli to reach the pharynx where they are swallowed, and then travel to the small intestine where they reside and mature into adults (5). Adults live in the lumen of the small intestine and produce eggs which are passed in the faeces of their host (1). Inadequate sanitation facilities and/or unsafe defecation practices, facilitate the transmission of eggs in faeces to the soil environment where they can advance to their infective phase and continue the endemicity of this parasitic disease. Lifecycle created using BioRender.com.

5.2.2. Natural pathogens for soil-transmitted helminth control

The beneficial role of improved water, sanitation, and hygiene (WASH) on the transmission of STH and schistosomiasis have been previously reviewed and

discussed in Chapter One. WASH can be used to support MDA efforts by preventing reinfection following successful treatment, resulting in more long-term effects on reduced endemicity (Coffeng *et al.*, 2018). Biological controls for helminthic diseases have so far not been discussed in this thesis. Biological controls can be broadly categorised as organisms which are predators (parasites/micropathogens) and competitors and are an attractive option compared with their more ecotoxic chemical control counterparts (Levine, 1969). Research on biological controls have been mainly focused on livestock parasites and natural predators to the larval stage of some parasitic nematodes have been discovered (Canhão-Dias, Paz-Silva and Madeira De Carvalho, 2020; Szewc, de Waal and Zintl, 2021). Biological controls for STH include nemophagous fungi, predatory invertebrates (beetles and earthworms) (Canhão-Dias, Paz-Silva and Madeira De Carvalho, 2020) and a pathogenic bacterium of nematodes *Chryseobacterium nematophagum* (Page *et al.*, 2019). These nemophagous fungi have been shown to kill the nematodes by trapping and digesting the parasite larvae, whereas the nemaphagous bacteria infect (and kill) the bacterivore STH larvae via ingestion (Page *et al.*, 2019). The focus of this chapter is the nemaphagous bacteria, *Chryseobacterium nematophagum*.

This species of nematocidal *Chryseobacterium* has been co-isolated, along with free-living nematodes from rotten fruit (Page *et al.*, 2019). The *C. nematophagum* isolated from these fruit samples were shown to have nematocidal properties (Page *et al.*, 2019). Isolates of this nematocidal bacterium were tested in a lab setting on free-living *Caenorhabditis elegans* (wild type strain N2) (Page *et al.*, 2019). Exposure to the *C. nematophagum* isolates resulted in the killing of free-living, bacterivorous *C. elegans* larvae (L1, L2, L3 and L4 larval stages) as the nematode larvae actively ingested the bacteria which then went on to digest the nematode internally (Page *et al.*, 2019).

The effect of *C. nematophagum* was also tested on various parasitic nematodes including *Ancylostoma caninum*, a hookworm species that infects dogs, wolves and foxes. The bacterivorous stages of the hookworm larvae (L1-L3) were tested and were all killed within 24 hours of exposure to *C. nematophagum* (Page *et al.*, 2019). If this bacterial species is also found to kill human hookworm species,

trials into its use in endemic areas would be warranted, to supplement ongoing MDA programmes. However, biological control studies are complex and have the particular concern that any biological control species being used do not cause harm to non-target organisms, and if they are introduced, the biological control species will not damage a fine ecosystem balance (Levine, 1969). One way to minimise the risk of adverse environmental events, and to enable a trial to go ahead, is if a species that is already known to be native to a given area is employed. At present, although *C. nematophagum* has been isolated in Asia and Europe, it has not yet been tested for its presence in sub-Saharan Africa (Page *et al.*, 2019). Given the public health problem of hookworm in this area (Chami *et al.*, 2015), and consequential potential of a biological control programme, this chapter investigated the possible occurrence of *C. nematophagum* and the free-living larval forms of *N. americanus* in soil samples from a hookworm endemic community.

5.2.3. Aims and objectives

Aim: Determine if a PCR-based assays can detect *C. nematophagum* and *N. americanus* in the soil environment in a STH endemic area in sub-Saharan Africa.

Objectives:

1. Apply *N. americanus* qPCR assay to soils collected from soil-transmitted helminth endemic area.

2. Apply *C. nematophagum* PCR assay to soils collected from soil-transmitted helminth endemic area.

5.3. Methods

5.3.1. Source of *Chryseobacterium nematophagum*

The positive control used in the PCR assay was a *C. nematophagum* isolate and the negative control was a *Escherichia coli* isolate, both provided by Professor Tony Page (University of Glasgow). For both controls, single colonies of freshly grown bacteria were picked into 100 µl of distilled water, boiled for 10 minutes

and then centrifuged for 10 minutes. The supernatant was then stored at -20ºC until use.

5.3.2. DNA extraction

The DNA extracts used in this chapter were the same extracts used in the previous chapters investigating the presence of *S. mansoni* and faecal bacterial indicators. Mayuge District, Uganda, where the village of Bugoto is situated, is also endemic for hookworm (Chami *et al.*, 2015). The soil sampling and DNA extraction methods are detailed in full in Chapter Three. To summarise, soils were collected from Bugoto, a village on the Ugandan shores of Lake Victoria. Soils were sampled at five sites: football field (sanitation-free site), area of open defecation and three areas proximal to in-use pit latrines (Figure 3.4.). Five technical replicates were collected for each sampling point, and for this study, the DNA extracts from these five replicates were pooled before use in the PCR and qPCR assays.

The DNA extracts were prepared from the sampled soils using the MPBio Soil Spin Kit (MPBio). Briefly, 500 mg of soil was homogenised using the FastPrep-24™ Classic Instrument (MPBio) at speed 5.5 for two 30 second runs for the initial cell lysis stage and the manufacturer's protocol was followed for all previous and consequential steps. The DNA was eluted into a final volume of 50 µl of nuclease free water.

5.3.3. PCR method: *Chryseobacterium nematophagum*

The previously published primers and PCR assay designed to detect *C. nematophagum* isolates were used (Page *et al.*, 2019). The forward and reverse primers used were: CnemF1 5'-TGA TTC TTT CGG GAA TCA GA-3' and CnemR2 5'- GCT TCC CAC ACG TGG AAA GG-3'. These primers amplify a 394 bp fragment (Appendix 3: Figure 2). The cycling parameters were 92°C for 1 min, 53°C for 1 min and then 72°C for 1 min for 30 cycles. The PCR amplification was performed using Applied Biosystems SimpliAmp Thermal Cycler.

Each PCR was carried out in a 25 µl reaction mixture containing 12.5 µL Hotstart G2 Go Taq Master Mix (Promega), 1 µl of each 10 µM primer, 1 µl dNTPs, 1 µl

$MgCl_2$, 2 µl of template DNA and nuclease-free water (Qiagen). Each soil DNA extract was diluted (1 in 20) before use in this PCR assay. A non-template control (NTC), positive control (*C. nematophagum* gDNA) and negative control (*Escherichia coli* gDNA) were used in each PCR plate. PCR products were visualised using a 2% gel, run at 90V for 30 minutes.

5.3.4. Quantitative PCR method: *Necator americanus*

Previously published primers, F: 5′-CCA GAA TCG CCA CAA ATT GTA T-3 ′ and R: 5'-GGG TTT GAG GCT TAT CAT AAA GAA-3' were used to detect *N. americanus* (Pilotte *et al.*, 2016). The cycling parameters, 95˚C for 5 mins, followed by 40 cycles of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 10 sec, followed by a final extension step of 72ºC for 10 sec, were used to amplify the target gene. The qPCR amplification was performed in 10 µl reaction mixtures containing 5 µl SYBR® Green PCR Master Mix (Applied Biosystems®), 0.1 µM of each primer, 2 µl of template DNA and made up to final volume with nuclease free water (Qiagen). Each soil DNA extract was used neat in the reaction. The qPCR assays were performed using the StepOne™ Real-Time PCR System with MicroAmp™ Optical 96-well plates (Applied Biosystems®). Each sample was run in duplicate. A NTC (water) was included in each plate run. All C_T values that were less than 3.3 cycles lower that the C_T values of the NTC were considered negative. Any technical duplicates whose C_T values differed more than 0.5 cycles were considered too variable and excluded from the analysis (Ruiz-Villalba, Ruijter and van den Hoff, 2021).

5.4. Results

5.4.1. *Chryseobacterium nematophagum* PCR

One soil sample (collected from Pit latrine Three) had clear *C. nematophagum* amplification (Figures 5.4). The top row of Figure 5.4 is the 2% agarose gel used to visualise the PCR products from pooled DNA extracts collected from the control sites: the defecation free site (Lanes 1-5) and site of open defecation (Lanes 6-9). The bottom row of Figure 5.4 shows the PCR products from the DNA extracts from soils collected proximal to the three pit latrines, including the soil DNA extract which produced a faint, but clear, specific band (Sample 30). The

gel's negative control, *E. coli* gDNA and NTC both showed no amplification compared with the specific 394 bp band visualized in Lane '+', containing *C. nematophagum* gDNA.

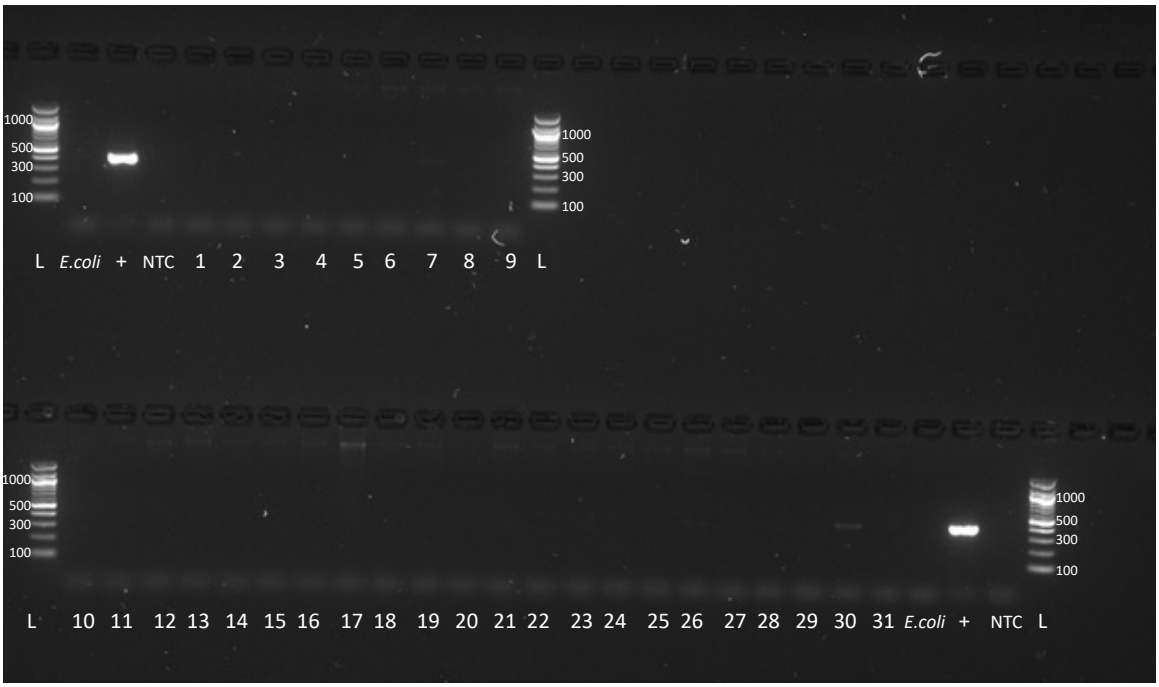


Figure 5. 3. Agarose gel electrophoresis (2% agarose) of PCR products using *C. nematophagum* specific PCR. The top gel shows the PCR products from soil DNA extracted from the football field (lanes 1-5) and area of open defection (lanes 6-9). The bottom gel shows the PCR products from pit latrine transects: pit latrine one (lanes 10- 14); pit latrine (lanes 15-24) and pit latrine three (lanes 25 -31). For the gel: lanes L is the 1 kb DNA size marker, values are in basepairs. Lanes *E. coli*, *Escherichia coli* gDNA; +, *C. nematophagum* gDNA (394 bp fragment); NTC, negative template control.

5.4.2. *Necator americanus* quantitative PCR

The results from the *N. americanus* qPCR assay carried out on pooled soil samples, run in duplicate on the qPCR plate, are shown in Table 5.1. The soils collected from the site of open defecation had no specific amplification of the specific hookworm gene. The other four sites (football field and three pit latrines) all had one sampling spot that was positive. These C_T values ranged from 4.46 to 17.11 (Table 5.1). The soil sample that was positive for *C. nematophagum* along the transect of Pit Latrine 3 was also positive for hookworm (C_T value mean of 6.36 and standard deviation 1.02).

Table 5. 1. Summary of the soil collection points and amplification of *Necator americanus* mRNA gene on soil samples. The C_T values reported are the cycle number at which the fluorescence signal increased above a defined threshold. The mean and standard deviation (SD) for the C_T values are shown.

Site	No. of positive/ No. of samples tested	Mean and SD of C_T of positive samples
Area of open defecation	0 (5)	-
Football Field	1 (4)	4.5 ± 0.4
Pit latrine 1	1 (12)	17.1 ± 0.2
Pit latrine 2	1 (13)	11.5 ± 1.3
Pit Latrine 3	1 (11)	6.4 ± 1.0

5.5. Discussion

The nematocidal bacteria, *C. nematophagum,* shows potential as a biological control for both human- and livestock- infecting roundworm parasites due to its nematode-killing properties (Page *et al.*, 2019). In this study, for the first time, the *C. nematophagum* species of bacteria was detected in a soil sample in sub-Saharan Africa. It was found in a soil sample collected adjacent to a pit latrine. As only 31 samples were tested, further studies into how frequently it is found and where are warranted.

5.5.1. Presence of *Chryseobacterium nematophagum* in Ugandan soils by PCR

As there was no concentration step before DNA extraction, and soil sample size used in each DNA extraction was small (0.02g), the likelihood of extracting DNA from *C. nematophagum* DNA would have been low, regardless of the sensitivity of the PCR assay. For the *S. mansoni* qPCR assay, it was determined that the assay was sensitive enough to detect a single egg (Chapter Two), however a similar experiment was not carried out to determine the lower limit of detection of the *C. nematophagum* assay. Adapting this PCR assay into a qPCR assay could have provided an extra layer of information and enabled the calculation of how

many *C. nematophagum* bacteria were present in the soil sample, as well as determining the lower limit of detection for this assay. There were also bands at the top of the gel with a high molecular weight, an indication of genomic DNA present at the termination of the PCR reaction. This may have been due to too much DNA being used as the starting material for the PCR reaction, a serial dilution of the starting DNA could be carried out to optimise this PCR assay further.

An additional factor that could have affected the performance of the *C. nematophagum* assay, was the DNA extraction method. It was not optimised for the extraction of this specific bacterium, although Chapter Four demonstrated that other bacterial markers were amplified from these same DNA extracts. Given these two limitations, it is exciting that *C. nematophagum* was detected and could potentially be present in soils in other hookworm endemic areas in Uganda or more widely across the African continent. This highlights the potential for this bacterium as a biological control method for hookworm in this area and future studies are needed.

5.5.2. Presence of *Necator americanus* in Ugandan soils by quantitative PCR

DNA from hookworm species has been successfully (specifically and sensitively) detected from wastewater using qPCR (Gyawali *et al.*, 2017) and propidium monoazide qPCR (PMA-qPCR) methods (Gyawali *et al.*, 2016). Detection from other sanitation-related matrices have relied on microscopy-based detection (Amoah *et al.*, 2017). A published qPCR assay for *Necator americanus* which reported a lower limit of detection of between 100 ag/µl and 10 ag/µl (Pilotte *et al.*, 2016) was used in this study to detect this specific hookworm species in the Bugoto soils.

A single sample from four of the five sampling sites produced a C_T value that could be considered positive. One of the soil samples positive for hookworm was the same sample from Pit Latrine 3 which was also positive for *C. nematophagum*. However, the reproducibility of these results is important to consider when interpreting these qPCR data. Each sample was run in duplicate and the standard deviation of the C_T of the positive samples ranged between 0.2

and 1.3. If the technical replicates are more than 0.5 cycles apart, they are considered too disparate from one another and should be removed from any analysis (Ruiz-Villalba, Ruijter and van den Hoff, 2021). These spurious results could be due to the high sensitivity of this assay and detection of any contamination whilst carrying out the loading of these qPCR plates in the lab, or due to very low hookworm DNA levels. If *C. nematophagum* had very strong anti-hookworm properties then it would also not be expected for them to be found in the same soil sample, but similarly it could be that the presence of *C. nematophagum* was in part causing the low reproducibility if this qPCR assay, by digesting any hookworm present. However, this is speculative at this stage and requires further research on associations between hookworm and *C. nematophagum* present in sanitation-related soil samples. To determine the accuracy of this hookworm qPCR assay and reliably interpret this data, it would have been beneficial to use another *N. americanus* qPCR to compare with the detection using the assay designed by Pilotte *et al* (2016) and determine if the positive results are truly reproducible.

Both PCR-based assays showed the potential as tools to detect hookworm and a nematocidal *Chryseobacterium* species but require further optimising for their application to soil samples collected from soils associated with sanitation facilities. However, the *C. nematophagum* PCR demonstrated that it is likely that this specific bacterial species is present in this village's soil environment and therefore opens the door for future research. For example, *C. nematophagum* could be used in a controlled way to treat the internal slab or floor of latrines, potentially reducing the risk of infection for barefooted latrine users.

5.6. Conclusion

C. nematophagum was detected in one of the 31 soil samples tested, warranting further investigation into this potential new biological control agent. The use of inadequate sanitation is a public health risk due to the faeces-based transmission of STHs, including the human-infecting hookworm species. If existing or newly implemented sanitation facilities do not destroy hookworm eggs/larvae, or if facilities do not sufficiently contain the larvae to prevent human contact, these interventions will have a limited effect on reducing

hookworm transmission. Within the pit latrine there are natural degradative processes, and the additional treatment of waste, or treatment of the latrine's floors, with the nematophagic bacteria *C. nematophagum* could be a useful biological additive to the composting and digestive processes used to treat waste and this bacterium has been found to be present in soils collected from a community in Uganda, highlighting its potential for use here and potentially across sub-Saharan Africa.

Chapter Six: General Discussion

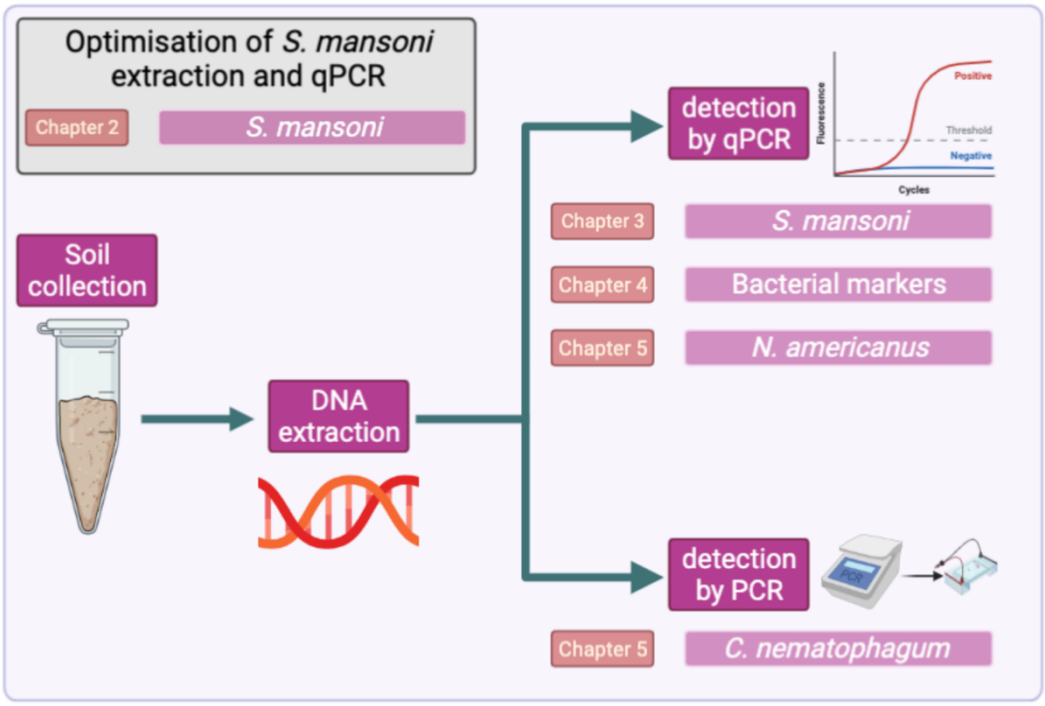


Figure 6. 1. Visual summary of Chapter Six, the general discussion of the four data chapters.

6.1. Abstract

This chapter summarises the key findings of this thesis and discusses the strengths and limitations of the research and the potential applications of these techniques. Firstly, the key findings from the previous chapters are summarised, followed by an evaluation of the strengths of the research, the issues that limit the validity and applicability of the data and suggestions for future improvements to the eDNA methods used in this thesis. To finish, the potential of eDNA-based methodologies is discussed in the context of monitoring sanitation.

6.2. Considering my chapters collectively

The primary aim of this PhD was to apply environmental DNA (eDNA)-based molecular techniques to soil samples in the context of schistosomiasis and sanitation. Aside from being a basic human right, adequate sanitation aims to prevent the transmission of faecal pathogens by sequestering human faeces away from the human contact. However, in 2020 more than 3.6 billion people lacked access to safely managed sanitation services (WHO UNICEF JMP, 2021b).

The continued spread of faecally-associated diseases, such as schistosomiasis and STHs, is dependent on the absence or failure of sanitation services (Freeman *et al.*, 2013). In the case of *Schistosoma mansoni*, universal access to and use of adequate sanitation would prevent the transmission of egg-infected faeces into the aquatic habitat of snails.

The installation of adequate sanitation is not necessarily a silver-bullet for disease control as a common failure of sanitation implementations is their maintenance and monitoring (Martin *et al.*, 2017). Having a tool to assess the extent of environmental contamination of helminth eggs in the environment before a sanitation intervention is implemented and a method to monitor the soil environment post-installation would be useful. The effect of sanitation on schistosomiasis has historically been investigated indirectly using epidemiological human data rather than direct monitoring of the soil environment which interfaces with sanitation (Grimes *et al.*, 2014). Any uptake and effectiveness of sanitation interventions are unlikely to have a linear impact on human infection levels and therefore a method to monitor the actual contamination of the environment is needed. Chapters Two and Three aimed to optimise and then use qPCR assays to detect *S. mansoni* eDNA in the soil environment of a *S. mansoni* endemic village. Microbial source tracking (MST) can provide data on the source of faecal contamination and four qPCR assays were employed to characterise the soil environment in this regard in Chapter Four. The effects of poor sanitation are not purely limited to schistosomiasis endemicity and in Chapter Five the presence of hookworm species *Necator americanus* and a nematode-eating bacteria *Chryseobacterium nematophagum* in the soils of the Ugandan village were also investigated with two PCR-based assays, to assess the potential for using this bacterium as a biological control for hookworm.

6.2.1. Key findings

The chosen COI-based qPCR assay that had previously been used to detect *S. mansoni* in water samples (Sato *et al.*, 2018) was shown to be a highly sensitive assay: it could detect a single egg spiked into 500 mg of soil. However, when this assay was applied to soil samples collected from soils proximal to sanitation in a village known to be a *S. mansoni* hotspot, the assay did not detect any *S. mansoni* eDNA in these soils. Further research is needed, particularly to improve

the accuracy of the assay by increasing the amount of soil the DNA is extracted from and/or using concentration techniques to adapt existing eDNA-based methods to monitor *S. mansoni* in the soil environment.

Following the attempt to specifically detect *S. mansoni* in the soils, a universal 18S, universal 16S, general *Bacteroides* and human specific *Bacteroides* qPCR assay were used to characterise the soils with relation to open defecation and sites proximal to pit latrines. The *Bacteroides* targets acted as a proxy for *S. mansoni* as they are faecally-associated bacteria. Although it was possible to detect and enumerate these qPCR targets, no spatial relationship between the concentrations of these faecal markers was observed along the pit latrine transects.

As discussed, poor sanitation is also connected to STH transmission, the penultimate chapter of this thesis set out to investigate the presence of the hookworm species *N. americanus* and the nematode eating bacterium, *C. nematophagum,* which had previously been co-isolated with *Caenorhabditis* nematodes from a rotten fruit sample(Page *et al.*, 2019). In this study *C. nematophagum* was detected for the first time in soils from sub-Saharan Africa, opening up the opportunity for use of this bacteria as a biological control for nematode larvae as the results of Chapter Five show it to naturally occur within the soil in a Ugandan village.

From epidemiological data of this village, it is known that there are active *S. mansoni* and hookworm infections in this population. 22% of rural population in Uganda practice open defection (Government of Uganda Ministry of Water and Environment, 2020) and open-air defecation was observed in this village whilst soil sampling, but the eDNA-based qPCR assays employed in this thesis were unable to detect the *S. mansoni* or consistently differentiate between areas of open defecation and a site free of sanitation (community football field). The sensitivities of the assays were determined in a laboratory setting to be adequate for detecting each target: if the target organism/gene was present in the soil DNA extracts, it should have been specifically detected. The lack of detection could therefore have been, for example, due to the soil sampling technique or other factors, such as recent weather events, temperature, and UV

level prior to sample collection. For further research into sanitation and human helminths, it could be useful to learn from the limitations of this project.

6.2.2. Strengths and limitations of the research

6.2.2.1. Soil sampling

The starting material for the DNA extracts was 500 mg of soil, but due to the volume of the qPCR reaction, the equivalent of 20 mg of soil were tested for the presence of target organism. To increase the probability that genetic material will be present in a soil sample, the initial sampling method must be improved. As suggested in Chapter Three an extra step of concentrating the helminth eggs before the DNA extraction process by centrifugation or flotation could be used before the downstream qPCR to identify the parasites present (Smith, 1999).

Although this still leaves the question of how many *S. mansoni* eggs would be expected in soils in contact with open-air faecal deposits or poorly functioning pit latrines? If someone has a low intensity of *S. mansoni* infection, the concentration of eggs in their stool would be between 1-99 eggs per gram of stool (WHO, 2002). If egg-carrying stool is deposited onto soil by open air defecation how the eggs move or are transported into the interfacing soil is unknown. An informative study could be to carry out a mesocosm experiment: spiking an artificial soil environment with *S. mansoni* eggs and collecting soil samples over different spatial transects and over time to investigate how focal *S. mansoni* detection can be expected when collecting soils in contact with openly deposited stool. The temporal aspect to this experiment would also help to determine the decay of *S. mansoni* eDNA in the soil environment and what length of PCR primers are the best to target the degraded eDNA of remnants of the eggs themselves. It is known that *S. mansoni* eggs are viable in the soil environment for up to six days (Pitchford and Visser, 1972) but how long their DNA persists in the soil environment, and therefore potentially remaining detectable using eDNA techniques, is unknown. Further studies using RNA would also provide extra information on egg viability but given the difficulty in detecting DNA this would require extensive optimisation of sampling strategy and likely large sample sizes.

Surface soils were collected for investigating in this study at a single time point. It was theorised that one of the ways *S. mansoni* transmission is facilitated in Bugoto was that during the rainy season, *S. mansoni* eggs deposited uphill on bare soil from open defecation, would be transported by surface water run-off into Lake Victoria. The soil sampling took place at the end of September 2019, at the beginning of the rainy season. It could be interesting to observe if the detection of *S. mansoni* and MST bacteria would change over the course of a rainy season, to see if this hypothesis was correct: that surface run-off would contaminate the soil environment and distribution of the schistosome eggs from their initial deposit into soil environment would increase. As the rains had already begun, it could also be hypothesised that our negative findings for *S. mansoni* could be because any soil-deposited eggs had already been washed away, but at this stage this is speculative and would require further testing. The effect of the season (rainy vs dry) could therefore impact the success of sampling soils using eDNA methods and edaphic factors (e.g. soil structure, temperature, pH, and salinity) will therefore likely affect the longevity of *Schistosoma* eggs deposited into soil and the longevity of the eDNA shed by the eggs (Furtak and Gałazka, 2019).

Soil type was important metadata that would have complemented this research. Any future studies using eDNA extracts from soil would be enriched by characterising the soil. The qualities of the soil (soil structure and type, temperature, moisture, pH and acidity and salinity) will impact eDNA longevity. As briefly mentioned in Chapter 3, the ferralsol found in Bugoto is high in metal oxides and this soil type is likely to have a positive effect on the longevity of any *S. mansoni* eDNA present (Taberlet *et al.*, 2018).

6.2.2.2. Panel of PCR assays

To ensure the accuracy of the data generated from this study, all primers were tested before their use on the soils collected in Bugoto. The *S. mansoni* qPCR was tested in the lab and lower limit of detection determined. The 18S, 16S and two *Bacteroides* were also screened for their detectability in faecal samples donated by Bugoto residents. The hookworm assay had been successfully used to detect *N. americanus* by Lauren Carruthers on faecal samples from Bugoto for her PhD research (Carruthers, 2021).

The *Bacteroides* provided the most promising qPCR data out of the three chapters characterising the Ugandan soils. Without the specific detection of parasites, a faecal indicator, such as a *Bacteroides*-based target, can provide an indication of the presence of faeces in an environment. The presence or absence of faecal indicator bacteria (FIB) cannot however confirm the presence or absence of enteric pathogens in the environment or identify the diversity of enteric pathogens present or the number of these pathogens in the wider environment (Goddard *et al.*, 2020). FIB can provide information on the source of faecal matter (humans vs livestock animals for example) which could be useful in a one health context (Penakalapati *et al.*, 2017). The importance of NTD and WASH sectors collaborating has been previously discussed, and although outside the scope of this PhD, animal faeces and zoonotic NTDs also negatively affect human health (Campbell *et al.*, 2016) indicating a wider scope for this type of faecal monitoring research.

With the push for universal access to sanitation (WHO, 2020a), there are also growing concerns over the effectiveness of pit latrines to prevent faecal contamination and their failure to contain faeces (Contreras *et al.*, 2021). This PhD has been focused on schistosomiasis and hookworm, but the presence of FIB can also be used in the context of enteric pathogens (Goddard *et al.*, 2020). Metagenomic sequencing would have been an interesting addition to this PhD research, as one of the pitfalls of the targeted eDNA approach used in this thesis is that only specific targets are detected. Although taking a broad stroke approach may not be appropriate outside of a research context, it could be informative to understand the effects of sanitation on environmental contamination and gain a more complete picture of the range of enteric pathogens present in different soil environments where poor sanitation is in use. For example, it may be predicted that viruses and bacteria can travel further from 'leaky' latrines than larger macroparasites such as *S. mansoni* and other helminths.

6.2.3. Potential of environmental DNA and quantitative PCR for environmental monitoring

Utilising new technologies for the surveillance of STH with eDNA was one of the recommendations of the WHO's 2030 roadmap to renew efforts of NTD

eradication over the next decade (WHO, 2020a). Implementation of improved sanitation facilities in low-income households is challenging and universal coverage of sanitation has many barriers (cost, community investment, behaviour change, facility maintenance to name a few) but an integrated approaches to NTD control could result in an improvement of the effectiveness of control initiatives and their health outcomes (Villacorta Linaza et al., 2021).

A tool that could be used to monitor different diarrhoeal agents (parasitic, viral, and bacterial in cause) could help to facilitate the move to increase multilateral partnerships of those working in parasitology, child health and WASH fields (Freeman *et al.*, 2013). However, the use of any molecular-generated data should be interpreted with the caveat that they are unable to capture the complex picture of all sanitation practices and their effect on environmental contamination, for example the disposal of child faeces (Majorin *et al.*, 2019) or the extent of hygienic bathing (Sow *et al.*, 2008). In this research project, information about sanitation coverage and usage was not collected but are valuable data to provide a full picture of the WASH situation in communities endemic with schistosomiasis. An extra layer of information when monitoring the presence of schistosomiasis, such as the type of sanitation facilities and practices can explain the 'how' of schistosomiasis detection within the wider environment.

As research on monitoring expands and increases its focus on molecular-based methods it should be remembered that NTD efforts remain underfunded, and the cost-effectiveness of these methods could also be improved by multiplexing detection assays, for example using TaqMan array cards (Lappan *et al.*, 2021). If the context and cost-effectiveness is appropriate, environmental monitoring could be integrated across NTD and WASH sectors.

6.3. Conclusion: importance of integrated control and monitoring

Good sanitation matters for human dignity, public health, and environmental protection from human faecal pollution. The soil environment in relation to sanitation was investigated in this thesis, as the impact of poor sanitation is one of the factors that has entrenched the lifecycles of diseases such as

schistosomiasis. In this thesis, a *S. mansoni* specific qPCR assay, microbial source bacterial markers and *C. nematophagum* and *N. americanus* PCRs and qPCRs were used to characterise soils collected from sites associated with sanitation in a *S. mansoni* and hookworm endemic area in rural Uganda. A robust tool to monitor schistosomiasis in the soil environment using eDNA-based techniques was not fully realised during this PhD but eDNA still remains a promising tool for the environmental monitoring of parasitic helminths with targeted future studies required.

Appendices

Appendix 1: Chapter Two manufactured COI standard extended dynamic range

Table 1: Cycle threshold (C_T) values for the extended dilution curve of the COI standard. [b] Mean ± standard deviation (SD), each C_T values is to 2 dp.

Dilution of standard	*Cycle threshold (C_T)* [b]
10^{12}	8.5
10^{11}	11.2 ± 0.7
10^{10}	18.3 ±1.2
10^{9}	23.6 ± 0.2
10^{8}	24.0 ±0.3
10^{7}	8.0 ± 0.1
10^{6}	31.7 ± 0.3
10^{5}	35.9 ± 1.7
10^{4}	38.7 ± 0.6
10^{3}	UD
NTC	40.6

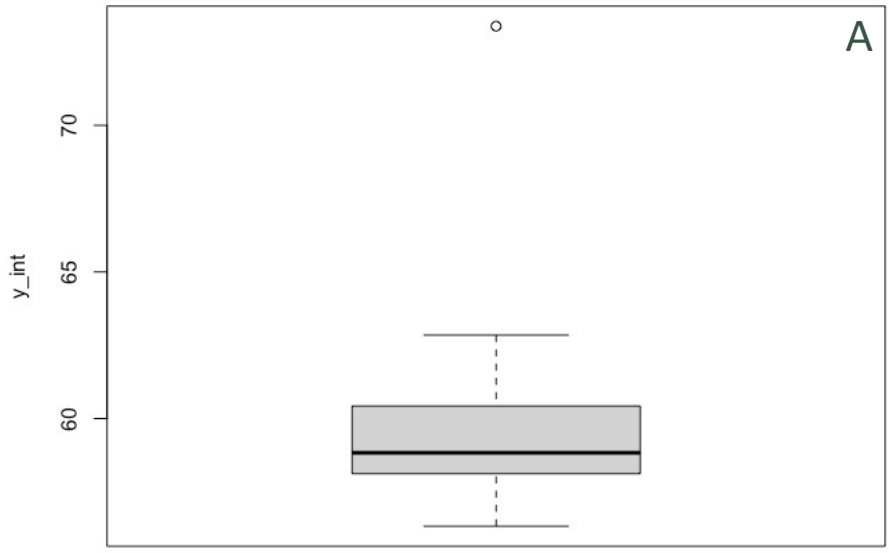
Appendix 2: Chapter Three quantitative PCR results

Table 2: C_T values for the no template controls measured from the ten runs of the *Schistosoma mansoni* COI gene qPCR assay. The average NTC C_T value was 38.39 across the ten runs. UD: undetermined.

PCR run	C_T values
1	**37.1**
2	**40.2**
3	**42.0**
4	**38.4**
5	**38.8**
6	**UD**
7	**36.5**
8	**UD**
9	**UD**
10	**37.7**

Table 3: The inter-assay coefficient of variation (CV) for the qPCR assay of *Schistosoma mansoni* COI gene. [a]Inter-assay variation was determined using the values obtained in 10 separate PCR runs. [b] Mean ± standard deviation (SD), *CV* coefficient of variation.

Dilution series of standard	Inter-assay variation [a]	
	Cycle threshold (C_T) [b]	CV (%)
10^{12}	4.9 ± 0.3	6.1
10^{10}	17.5 ± 1.3	7.6
10^{8}	24.5 ± 0.9	3.6
10^{6}	33.1 ± 2.2	6.6
10^{4}	38.0 ± 2.2	5.8



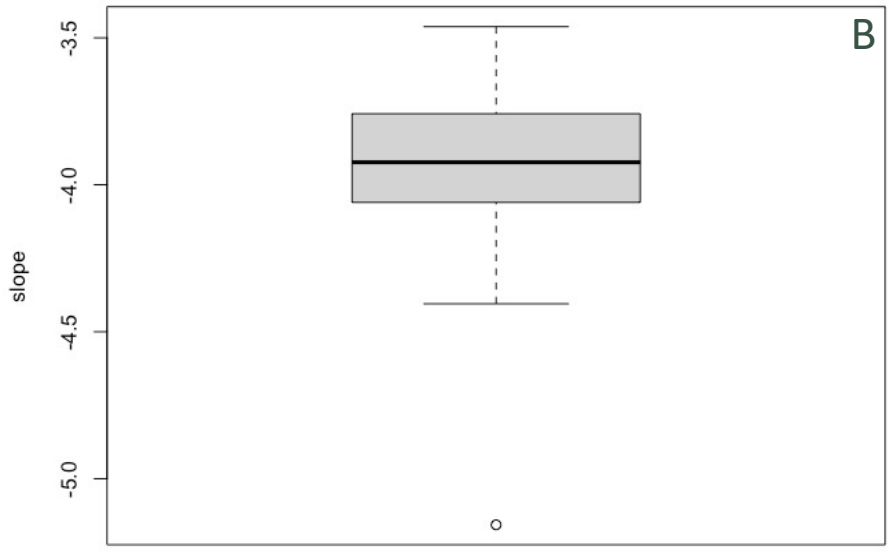


Figure 1: Box and whiskers plot for *y*-intercept and slope of the ten *Schistosoma mansoni* COI qPCR runs. A) Box plot for the *y*-intercept values. Interquartile range of 2.0 (2dp). B) Box plot for the slopes of the standard curves where interquartile range was 0.2 (2dp). The box plots show the *y*-intercept (A) and slope (B) between 25th and 75th data quartiles; whiskers extend to the outermost data point within ±1.5 x this interquartile range. There is an outlier (º) in each of the plots.

Appendix 2: Chapter Four quantitative PCR results

Table 4: C_T values for the no template controls measured from the ten runs of the universal 16S, universal 18S, and *Bacteroides* qPCR assay runs. Each C_T values reported to 2dp.

C_T values			
universal 16S	**universal 18S**	**general *Bacteroides***	**human *Bacteroides***
36.8	**36.5**	**33.2**	**27.6**
36.1	**33.6**	**33.4**	**31.9**
37.5	**35.0**	**35.1**	**32.3**
37.1	**32.8**	**35.3**	**32.5**
39.4	**32.6**	**35.7**	**28.6**
37.5	**31.0**	**34.7**	**30.9**
38.6	**35.5**	**35.9**	**29.8**
38.3	**31.0**	**35.6**	**-**
36.4	**30.2**	**34.2**	**-**
36.8	**32.9**	**31.5**	**-**

Table 5: Summary of the logistic regressions from 10 separate qPCR runs of the universal 16S standards: the efficiency of the qPCR assay *(E)*, correlation coefficient (r^2) and *y*-int is the intercept of the slope. The standard curves were obtained using dilutions of DNA ranging from 10^9 to 10^1 copies per reaction.

	Q-PCR assay									
	1	2	3	4	5	6	7	8	9	10
r^2	0.9984	0.9986	0.9989	0.9993	0.9975	0.9982	0.9996	0.9910	0.9983	0.9996
E (%)	151.1	160.1	152.7	154.4	149.2	150.5	147.9	140.8	160.2	155.7
y-intercept	36.41	35.365	35.75	35.31	37.19	36.77	35.85	37.71	34.80	37.10

Table 6: Summary of the logistic regressions from 10 separate qPCR runs of the universal 18S standards: the efficiency of the qPCR assay (*E)*, correlation coefficient (r^2) and y-int is the intercept of the slope. The standard curves were obtained using dilutions of DNA ranging from 10^{10} to 10^4 copies per reaction.

	Q-PCR assay									
	1	2	3	4	5	6	7	8	9	10
r^2	0.9673	0.9819	0.9883	0.9853	0.9712	0.973	0.9828	0.981	0.9029	0.9812
E (%)	105.8	108.7	103.2	103.4	86.96	112.2	103.8	113.4	128.8	106.9
y-intercept	44.62	44.44	45.04	44.65	46.96	44.01	47.40	43.56	41.37	44.77

Table 7: Summary of the logistic regressions from 10 separate qPCR runs of the general *Bacteroides* standards: the efficiency of the qPCR assay (*E*), correlation coefficient (r^2) and y-int is the intercept of the slope. The standard curves were obtained using dilutions of DNA ranging from 10^{10} to 10^4 copies per reaction.

	Q-PCR assay									
	1	2	3	4	5	6	7	8	9	10
r^2	0.9906	0.9876	0.9934	0.9898	0.9902	0.9868	0.9929	0.9882	0.9799	0.9832
E (%)	109.9	106.6	101.6	95.9	95.0	100.0	102.3	99.4	94.5	94.9
y-intercept	43.67	43.90	46.31	46.54	47.21	46.67	44.22	46.06	48.32	47.06

Table 8: Summary of the logistic regressions from 10 separate qPCR runs of the human-specific *Bacteroides* standards: the efficiency of the qPCR assay (*E*), correlation coefficient (r^2) and y-int is the intercept of the slope. The standard curves were obtained using dilutions of DNA ranging from 10^{10} to 10^2 copies per reaction.

	Q-PCR assay						
	1	2	3	4	5	6	7
r^2	0.9713	0.9823	0.9621	0.9653	0.9093	0.9947	0.998
E (%)	121.9	79.8	94.2	97.1	139.8	83.7	87.9
y-intercept	43.54	53.55	47.26	47.88	41.38	52.54	51.28

Test statistics from the linear mixed effects models for the four qPCR assays used in Chapter Four.

Table 9: Universal 16S: test statistic from the Type III ANOVA with Satterthwaite's method. Both estimated copy number data and original cycle threshold data were analysed by this statistical test.

Variable	Copy Number							Cycle Threshold (C_T)						
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	Signif. codes	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	Signif. codes
Sampling Site	3.7843e+26	9.4606e+25	4	86371	1.3187	0.2602		172.576	43.144	4	188.68	18.608	6.575e-13	*** (0)
Transect Level	2.9736e+26	9.9121e+25	3	143621	1.3817	0.2462		6.274	2.091	3	188.60	0.902	0.4413	

Table 10: Universal 18S: test statistic from the Type III ANOVA with Satterthwaite's method. Both estimated copy number data and original cycle threshold data were analysed by this statistical test.

Variable	Copy Number						Cycle Threshold (C_T)					
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Sampling Site	1.0190e+28	2.5474e+27	4	Inf	1.2822	0.2743	255.957	63.989	4	171.49	1.3723	0.2456
Transect Level	6.6643e+27	2.2214e+27	3	1.3511e+16	1.1181	0.3401	66.133	22.044	3	170.97	0.4727	0.7017

Table 11: General *Bacteroides*: test statistic from the Type III ANOVA with Satterthwaite's method. Both estimated copy number data and original cycle threshold data were analysed by this statistical test.

Variable	Copy Number							Cycle Threshold (C_T)						
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	Signif. codes	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	Signif. codes
Sampling Site	7.3213e+18	1.8303e+18	4	477225	0.6247	0.64483		332.2	83.050	4	197	9.6452	3.828e-07	*** (0.001)
Transect Level	1.8358e+19	6.1193e+18	3	800153	2.0886	0.09937	. (0.05)	87.8	29.266	3	197	3.3989	0.01885	* (0.01)

Table 12: Human specific *Bacteroides*: test statistic from the Type III ANOVA with Satterthwaite's method. Both estimated copy number data and original cycle threshold data were analysed by this statistical test.

Variable	Copy Number							Cycle Threshold (C_T)						
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	Signif. codes	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	Signif. codes
Sampling Site	8.3912e+26	2.0978e+26	4	2584.49	9.1249	2.56e-07	*** (0)	3.8738	0.9685	4	140.50	0.2966	0.8798	
Transect Level	3.8200e+26	1.2733e+26	3	6.68	5.5387	0.0311	* (0.01)	13.1187	4.3729	3	141.19	1.3390	0.2642	

Appendix 3: Chapter Five

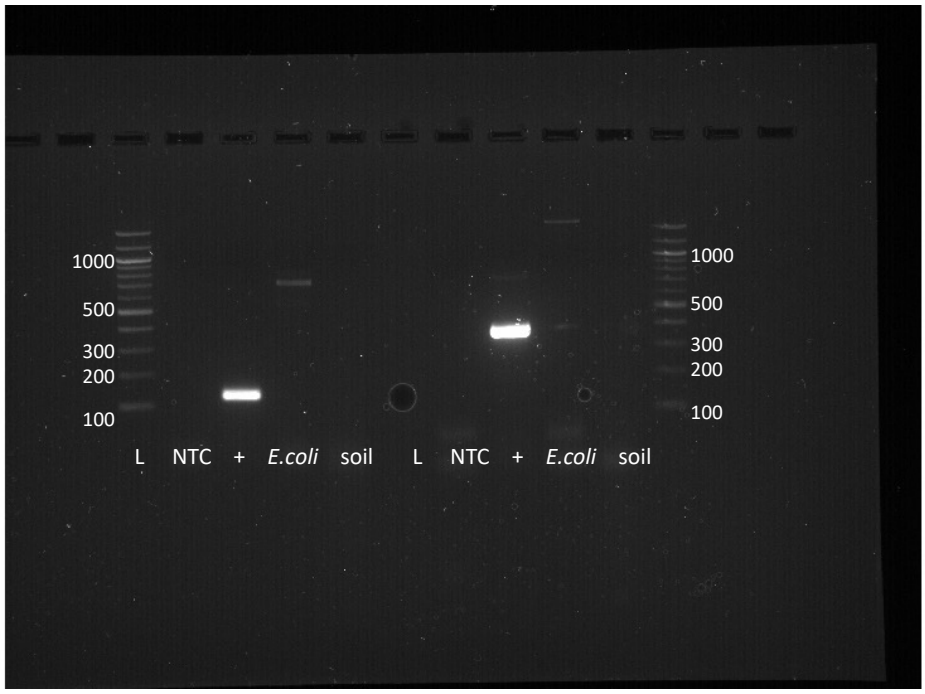


Figure 2: Agarose gel electrophoresis (2% agarose) of PCR products using two *C. nematophagum* specific PCR. Two primer sets were used (CnemF1 and CnemR1 and CnemF1 and CnemR2) producing a 129 bp and 394 bp fragment respectively. Lanes L, 1 kb DNA size marker, values are in basepairs. NTC, negative template control. +, *C. nematophagum* gDNA isolated from freshly grow bacteria. *Escherichia coli* gDNA isolated from freshly grow bacteria. Soil, DNA extracted from a Ugandan soil sample.

Appendix 4: MVLS ethical approval and soil importation agreement

The methods used in this study were reviewed and approved by the Vector Control Division (VCD) Research Ethics Committee (REC) (VCDREC/062), the Uganda National Council of Science and Technology (UNCST) (UNCST-HS 2193) and the University of Glasgow Medical, Veterinary and Life Sciences (MVLS) Research Ethics Committee (200160068).

Appendix 4.1: MVLS ethical approval

7th February 2017

Dear Dr Lamberton

MVLS College Ethics Committee

Project Title: New approaches to characterise Schistosoma mansoni infections persisting despite mass drug administration
Project No: 200160068

The College Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study. It is happy therefore to approve the project, subject to the following conditions:

- Project end date: 30 April 2021
- The data should be held securely for a period of ten years after the completion of the research project, or for longer if specified by the research funder or sponsor, in accordance with the University's Code of Good Practice in Research: (http://www.gla.ac.uk/media/media_227599_en.pdf)
- The research should be carried out only on the sites, and/or with the groups defined in the application.
- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.
- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely

Appendix 4.2: Vector Control Division ethical approval

TEL General: 256-414-251927
Direct: 256-414-253407
Email: vcdmohrec@gmail.com
IN ANY CORRESPONDENCE ON
THIS SUBJECT PLEASE QUOTE
VCD-REC/078

VECTOR CONTROL DIVISION (MOH)
RESEARCH & ETHICS COMMITTEE
15 BOMBO ROAD
P O BOX 1661
KAMPALA, UGANDA

Your Ref: **VCDREC/078** *Our Ref:* **UG-REC-018** Date: 28th March 2017

VECTOR CONTROL DIVISION-RESEARCH & ETHICS COMMITTEE APPROVAL NOTICE

To: Dr. Poppy Lamberton, Principal Investigator

Re: **(HS 2193) New approaches to characterise Schistosoma mansoni infections persisting despite mass drug administration (SCHISTO_PERSIST)**

Type: [ ] Initial Review
[ ] Protocol Amendment
[ ] Letter of Amendment (LOA)
[ ] Continuing Review
[ ] Material Transfer Agreement
[√] Other. Specify: Informed Assent and Sample storage Consent form

I am pleased to inform you that at the [**VCDREC Meeting/08**] convened meeting on [**22/03/2017**], the [VCDREC, committee meeting] voted to approve the above referenced application.

Approval of the research is for the period of **28/03/2017** to **28/12/2017**.

This research is considered a risk high level for pediatric risk category. As Principal Investigator of the research, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the protocol or the consent form must be submitted to the REC for re-review and approval **prior** to the activation of the changes. The REC application number assigned to the research should be cited in any correspondence.
3. Reports of unanticipated problems involving risks to participants or other must be submitted to the REC. New information that becomes available which could change the risk: benefit ratio must be submitted promptly for REC review.
4. Only approved consent forms are to be used in the enrollment of participants. All consent forms signed by subjects and/or witnesses should be retained on file. The REC may conduct audits of all study records, and consent documentation may be part of such audits.
5. Regulations require review of an approved study not less than once per 12-month period. **Therefore, a continuing review application must be submitted to the REC eight weeks prior to the above expiration date of [28/12/2017] in order to continue the study beyond the approved period.** Failure to submit a continuing review application in a timely fashion may result in suspension or termination of the study, at which point new participants may not be enrolled and currently enrolled participants must be taken off the study.
6. You are required to register the research protocol with the Uganda National Council for Science and Technology (UNCST) for final clearance to undertake the study in Uganda.

The following is the list of all documents approved in this application by [**VCDREC/078**]:

	Document Title	Language	Version	Version Date
1.	A cover letter	English	Revised	27/03/2017
2.	Informed Assent form for mature minor	English, Lusoga & Adhola	Revised	27/03/2017
3.	Consent form for sample storage	English, Lusoga & Adhola	Revised	27/03/2017

Signed,

VECTOR CONTROL DIVISION (MoH)
APPROVED
VALID UNTIL
28 DEC 2017
RESEARCH & ETHICS COMMITTEE
P. O. BOX 1661, KAMPALA

Dr. Abbas Kakembo

Chair, VCDREC

[Chair, 28/03/2017]

cc: The Assistant Commissioner/NTD National Coordinator, Vector Control Division

Appendix 4.3: Ugandan National Council for Science and Technology (UNCST) ethical approval

Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

UNCST

Our Ref: HS 2193 **29th August 2017**

Dr. Poppy Hellen Louise Lamberton
Principal Investigator
C/o Vector Control Division, Ministry of Health (MoH)
Kampala

Dear Dr. Lamberton,

Re: Research Approval: **New Approaches to Characteristics Schistosoma Mansoni Infections Persisting Despite Mass Drug Administration (SCHISTO _ PERSIST)**

I am pleased to inform you that on **08/05/2017**, the Uganda National Council for Science and Technology (UNCST) approved the above referenced research project. The Approval of the research project is for the period **08/05/2017** to **08/05/2022.**

Your research registration number with the UNCST is **HS 2193**. Please, cite this number in all your future correspondences with UNCST in respect of the above research project.

As Principal Investigator of the research project, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the research protocol or the consent form (where applicable) must be submitted to the designated Research Ethics Committee (REC) or Lead Agency for re-review and approval **prior** to the activation of the changes. UNCST must be notified of the approved changes within five working days.
3. For clinical trials, all serious adverse events must be reported promptly to the designated local REC for review with copies to the National Drug Authority.
4. Unexpected events involving risks to research subjects/participants must be reported promptly to the UNCST. New information that becomes available which alters the risk/benefit ratio must be submitted promptly for UNCST review.
5. Only approved study procedures are to be implemented. The UNCST may conduct impromptu audits of all study records.
6. A progress report must be submitted electronically to UNCST within four weeks after every 12 months. Failure to do so may result in termination of the research project.

Below is a list of documents approved with this application:

	Document Title	Language	Version	Version Date
1.	Research proposal	English	N/A	N/A
2.	Informed assent form for stool, urine and finger prick blood sample collection	English, Adhola and Lusoga	N/A	N/A
3.	Participant information sheet for stool, urine and finger prick blood sample collection	English, Adhola and Lusoga	N/A	N/A
4.	Side effect questionnaire	English, Adhola and Lusoga	N/A	N/A

Yours sincerely,

Isaac Makhuwa
for: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

Copied to: Chair, Vector Control Division (MoH), Research Ethics Committee

LOCATION/CORRESPONDENCE

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Appendix 4.4: Scottish Government Soil importation license

LICENCE NO. IMP/SOIL/6/2016-2
(Renewal of Licence No. IMP/SOIL/30/2015)

Scottish Government
Riaghaltas na h-Alba
gov.scot

PLANT HEALTH (SCOTLAND) ORDER 2005

Scottish Ministers, by virtue of the provisions of Part 8 of the Plant Health (Scotland) Order 2005, hereby authorise:

Professor Chris Pearce, Dr Cindy Smith, Dr Caroline Gauchotte-Lindsay,
Dr Stephanie Connelly

School of Engineering
Rankine Building
Oakfield Avenue
University of Glasgow
Glasgow
G12 8QQ

(hereinafter referred to as "the licensee") to import and retain samples of soil and sediment from any country for research purposes only.

This licence is subject to all other provisions of the Order and to the following conditions:

1. The samples must be transported securely packaged, double-wrapped in sealed polythene bags, accompanied by this licence and Letter of Authority or copies thereof.

2. Prior to importation, the consignment shall be carefully examined and found to be free from invertebrate pests.

3. Upon landing the samples must be conveyed directly to the containment facilities at the above address where they shall be stored, separate from UK material, clearly labelled with respective licence number and handled by authorised personnel only in such a way as to avoid contamination of any other similar material.

4. All redundant packing material must be destroyed by incineration or autoclaving following arrival of the material at the above given address.

5. The licensee must take all necessary precautions to prevent dissemination of the material kept under the authority of this licence from the containment facilities.

6. The material must be kept within the facilities at the above address which have been authorised for this purpose and handled in accordance with the Standard Operating Procedures developed for this work.

7. Access to the licensed material shall be restricted to those directly involved with the work. This licence or a copy thereof must be shown or given to all authorised personnel to read prior to handling the licensed material.

8. The discovery in the imported material of any suspected non-indigenous plant pest or pathogen must be reported immediately to the Scottish Government.

9. The licensee shall permit an authorised officer of the Scottish Government to inspect the conditions under which the material is kept and handled for investigation.

LICENCE NO. IMP/SOIL/6/2016-2
(Renewal of Licence No. IMP/SOIL/30/2015)

10. All equipment used in association with the licensed material must be sterilised by autoclaving or proven chemical methods as they become redundant. All work surfaces used while working with the licensed material must be disinfected after use with an approved disinfectant.

11. The material must not be removed or distributed from the containment facilities at the above address, except with written permission from the Scottish Government.

12. The licensee must notify the Scottish Government immediately in the event of an unplanned/accidental release of licensed material from the containment facilities.

13. On completion of the investigations for the purposes of which this licence is issued, the licensed material and any associated waste must be destroyed by incineration or autoclaving at 15 psi until a temperature of 121°C has been reached and held for one hour.

14. A record must be kept of all the material imported under the authority of this licence for the period from the 1st of July of the preceding year to the 30th of June of the current year, and a list submitted to the Scottish Government by the 7th of July each year.

15. The licensee must notify the Scottish Government when the work to which this licence relates is completed and the material has been destroyed.

16. Acknowledgement of the authority of this licence for holding the imported material in the UK shall be made and the licence number quoted in all publications relating to work with the material.

17. This licence may be amended or revoked by the Scottish Government at any time.

UK Plant Health Service
The Scottish Government

Dated: 10th July 2019

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For and on behalf of the Scottish Ministers

The Scottish Government
Science and Advice for Scottish Agriculture (SASA)
Roddinglaw Road
Edinburgh
EH12 9FJ

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