Detection, Identification and Quantitation of *Cryptosporidium parvum* in Water Samples and *Ascaris lumbricoides* in Sludge Samples using Real-Time Polymerase Chain Reaction Coupled with the High-Resolution Melt Curve Assay

By

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Summary

The intestinal parasites *Ascaris lumbricoides* and *Cryptosporidium parvum* are highly resistant to traditional water treatment processes and adverse health effects in individuals that come into contact with these parasites through contaminated water, have been reported. The research conducted in the current study was contracted by the East Rand Water Care Company (ERWAT) and the primary aim was to optimise a quantitative real-time PCR (qPCR) protocol for the detection and quantitation of *Cryptosporidium* oocysts in drinking water and environmental surface waters and develop a qPCR assay for the detection and quantitation of *Ascaris* eggs in sludge samples.

The first phase of the study focussed on developing and optimising a quantitative real-time PCR coupled with high-resolution melt curve (qPCR-HRM) assay for the detection and quantitation of *Ascaris lumbricoides* eggs in sewage sludge samples. Various DNA extraction protocols were compared and based on the reproducibility of the results, the QIAamp Fast DNA Stool Mini kit in conjunction with bead beating and freeze-boil cycles, was the most effective for the extraction of DNA from *A. lumbricoides* eggs. The use of the SensiFAST HRM kit with the Asc1-F/Asc2-R primer set (amplifying the cytochrome b fragment of *A. lumbricoides* mtDNA) also proved successful for the detection of the intestinal parasite in sludge samples. Standard curves for the quantitation of *A. lumbricoides* in the samples were also constructed using synthetic gene fragments (gBlocks Gene Fragments). However, a standard curve with dilutions up to $1x10^{-5}$ ng/µl could only be constructed and as the concentration detected in the sludge samples was lower, further quantitation was limited. The optimised method was then applied to sludge collected from six different wastewater treatment plants for the detection (presence/absence) of *A. lumbricoides*. The qPCR-HRM assay was also compared to a commercial primer-probe based qPCR detection kit (Genesig kit) and the results indicated that the molecular based methods have the potential to replace the currently employed microscopy method for the detection of *Ascaris* sp. The Genesig kit had the added advantage of quantifying the number of eggs present in the samples. The cost- and time-effectiveness of each method was calculated and compared. The qPCR-HRM assay and Genesig kits yielded similar turnaround times of ±7 hours compared to the microscopy method that yielded a turnaround time of ±50 hours in total. In addition, cost comparisons showed that the qPCR-HRM assay was the most cost-effective (R684.75) method for the detection of *A. lumbricoides* in sludge samples, followed by the ERWAT microscopy method (R706.86) and then the Genesig kit (R900.36).

In the second phase of the study a qPCR-HRM assay for the detection and quantitation of *Cryptosporidium parvum* oocysts in water samples was optimised. Various DNA extraction

protocols were compared and results showed that the QIAamp Fast DNA Stool Mini kit (no modifications) was the most effective for the extraction of DNA from *C. parvum* oocysts. The use of the SensiFAST HRM kit with the COWP-F/COWP-R primer set (amplifying the *C. parvum* oocysts wall protein) proved successful for the detection of the intestinal parasite. Standard curves intended for the quantitation of *C. parvum* in the samples were constructed using synthetic gBlocks Gene Fragments based on the *C. parvum* oocyst wall protein. Once again the standard curves could only quantify the *C. parvum* DNA up to $1x10^{-5}$ ng/µl, which was below the concentration of *C. parvum* DNA in the faecal samples and the standard curves could not be utilised for further quantitation. The optimised method was then applied for the detection (presence/absence) of *C. parvum* oocysts in drinking water spiked with faecal samples and it was determined that the sample limit of detection of the qPCR-HRM assay was less than 1 oocyst/ml. The qPCR-HRM assay was also applied to surface water samples collected up and downstream of a wastewater treatment plant for the detection of *Cryptosporidium* sp. and was compared to a commercial primer-probe based qPCR detection kit (Genesig kit). The qPCR-HRM assay was more sensitive in detecting *C. parvum* DNA, although the commercial kit was able to quantify the number of *Cryptosporidium* sp. oocysts in one of the samples. While similar turnaround times of ±7 hours were obtained for both methods, cost comparisons showed that the qPCR-HRM assay was more cost effective (R578.96) than the Genesig kit (R794.57) for sample analysis.

Opsomming

Die inwendige parasiete *Ascaris lumbricoides* en *Cryptosporidium parvum* is hoogs weerstandbiedig teen tradisionele waterbehandelings prosesse en nadelige gesondheids gevolge in individue wat in aanraking kom met die parasiete deur gekontamineerde water, is al aangemeld. Die navorsing uitgevoer in die huidige studie is gekontrakteer deur die East Rand Water Care Company (ERWAT) met die primêre doel om 'n kwantitatiewe werklike-tyd PKR (qPKR) protokol te optimiseer vir die deteksie en kwantifisering van *Cryptosporidium parvum* oösiste in drinkwater en omgewingswater, asook die ontwikkeling van 'n qPKR toets vir die deteksie en kwantifisering van *Ascaris* eiers in slykmonsters.

Die eerste fase van die studie het gefokus op die ontwikkeling van 'n kwantitatiewe werklike-tyd PKR gekoppel met hoë resolusie smelt kurwe (qPKR-HRM) analise vir die deteksie en kwantifisering van *Ascaris lumbricoides* eiers in slykmonsters. Verskeie DNS ekstraksie protokolle is met mekaar vergelyk. Na aanleiding van die herhaalbaarheid van die resultate, is die QIAamp Fast DNA Stool Mini kit in samewerking met kraal klop en vries-kook siklusse die mees effektiefste gevind vir die ekstraksie van DNS van *A. lumbricoides* eiers. Die gebruik van die SensiFAST HRM kit met die Asc1-F/Asc2-R inleier stel (amplifiseer die sitochroom b fragment van *A. lumbricoides* mtDNS) was suksesvol vir die deteksie van die inwendige parasiet in slykmonsters. Standaard kurwes vir die kwantifisering van *A. lumbricoides* in die slykmonsters is opgestel deur gebruik te maak van sintetiese geen fragmente (gBlocks Gene Fragments). 'n Minimum van $1x10^{-5}$ ng/µl *A. lumbricoides* eiers is egter benodig, wat hoër was as die konsentrasie gevind in die slykmonsters en het dus verdere kwantifisering ingeperk. Die ge-optimiseerde metode is daarna toegepas op slykmonsters van ses verskillende rioolwater behandelings werke vir die deteksie (teenwoordigheid/afwesigheid) van *A. lumbricoides*. Die qPKR-HRM toets is ook met 'n kommersiële inleier-peiler gebaseerde qPKR protokol (Genesig kit) vergelyk en die resultate het aangedui dat die molekulêre gebaseerde metodes die potensiaal het om die mikroskopie metode wat tans deur ERWAT gebruik word vir die deteksie van *Ascaris* sp. te vervang. Die Genesig protokol het ook die bygevoegde voordeel gehad dat die hoeveelheid eiers in die monsters gekwantifiseer kon word met die standaardkurwe wat deel uit maak van die protokol reagense. Die koste- en tyd-effektiwiteit van elke metode is bepaal en vergelyk. Die qPKR-HRM toets en die Genesig protokol het soortgelyke omkeertye opgelewer van ±7 ure in vergelyking met die ERWAT mikroskopie metode van ±50 ure. Daarby het koste vergelykings gewys dat die qPKR-HRM toets die mees koste-effektiewe (R684.75) metode was vir die deteksie van *A. lumbricoides* in slykmonsters, gevolg deur die ERWAT mikroskopie metode (R706.86) en dan die Genesig protokol (R900.36).

In die tweede fase van die studie is die qPKR-HRM toets geoptimiseer vir die deteksie en kwantifisering van *Cryptosporidium parvum* oösiste in watermonsters. Verskeie DNS ekstarksie protokolle is met mekaar vergelyk en resultate het gewys dat die QIAamp Fast DNA Stool Mini kit (geen modifikasies) die mees effektiefste was vir die ekstraksie van DNS van *C. parvum* oösiste vanuit stoelgangmonsters. Die gebruik van die SensiFAST HRM kit met die COWP-F/COWP-R inleier stel (amplifiseer die *C. parvum* oösist wand proteïen) was suksesvol vir die deteksie van die inwendige parasiet. Standaard kurwes vir die kwantifisering van *C. parvum* in die monsters is opgestel deur gebruik te maak van sintetiese geen fragmente gebaseer op die *C. parvum* oösist wand proteïen. Weereens kon die standaard kurwe slegs *C. parvum* DNS kwantifiseer tot by $1x10^{-5}$ ng/µl wat hoër was as die konsentrasie van *C. parvum* DNS in die stoelgangmonsters en die standaard kurwes kon nie verder gebruik word vir kwantifisering nie. Die ge-optimiseerde metode is daarna toegepas vir die deteksie (teenwoordigheid/afwesigheid) van *C. parvum* oösiste in drinkwater met bygevoegde stoelgangmonsters en daar is bevind dat die monster limiet van deteksie van die qPKR-HRM toets minder as 1 oösist/ml was. Die qPKR-HRM toets is ook toegepas op oppervlakwater monsters geneem op en afstroom van ‘n rioolwater behandelings werke vir die deteksie van *Cryptosporidium* sp. en is vergelyk met ‘n kommersiële inleier-peiler gebaseerde qPKR protokol (Genesig kit). Die qPKR-HRM toets was meer sensitief vir die deteksie van *C. parvum* DNS, alhoewel die kommersiële protokol die kwantifisering van die hoeveelheid oösiste in een van die monsters bewerkstellig het. Terwyl soortgelyke omkeer tye van ±7 ure vir beide metodes gevind is, het koste vergelykings gewys dat die qPKR-HRM toets meer koste effektief was (R578.96) as die Genesig kit (R794.57) vir monster analise.

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Chapter 1: Introduction and Literature Review

1.1 Introduction

Traditional water quality assessment methods have proven to be ineffective in isolating and accurately quantifying the wide spectrum of different pathogens present in water sources (Keserue *et al*., 2012; Elsafi *et al*., 2013). In addition routine water analysis relies mainly on standard plating techniques for the enumeration of indicator microorganisms, such as *Escherichia coli* (*E. coli*) and total and faecal coliforms (Ashbolt *et al*., 2001; WHO, 2011; Keserue *et al*., 2012), with incubation times ranging from 18 hours to several days (Allen *et al*., 2004; Reasoner, 2004). Traditional plating methods are also not applicable for the detection of intestinal parasites such as protozoa or helminth species and the methods employed to identify these pathogens are often labour intensive, time-consuming and requires trained personnel (Carey *et al*., 2003; Raynal *et al*., 2012).

Intestinal parasites have been detected in various water sources such as surface- and rainwater (Guy *et al*., 2003; Feng *et al*., 2011; Ayaz *et al*., 2013) as well as sludge generated during wastewater treatment (Maya *et al*., 2006; Yoder and Beach, 2010; Feng *et al*., 2011). Some of the most prevalent pathogenic intestinal parasites include *Cryptosporidium parvum* and *Ascaris lumbricoides* (WHO, 2011). Their zoonotic nature and ability to withstand traditional treatment processes as well as survive for extended periods in the environment, poses a significant threat to humans that come into contact with contaminated water. Since the waterborne transmission of these parasitic organisms has become more prevalent in recent years, regulatory agencies are also stipulating that source and finished water be screened for these organisms (WHO, 2011). Water authorities thus need to ensure that water sources adhere to regulatory standards [Department of Water Affairs and Forestry (DWAF) 1996, 2006; South African National Standards 214 (SANS) 2015] and methods, for the routine monitoring of drinking water, environmental water and wastewater, for the detection of these intestinal pathogens, need to be optimised and implemented. A major problem associated with the routine testing of these parasites however, is the lack of reliable methodologies. In addition, the methods employed to evaluate for intestinal parasites in water sources are often time-consuming and require the collection of large volumes of water samples, concentration/filtration methods and finally detection. Water analysis laboratories, such as the East Rand Water Care Company (ERWAT), thus have a vested interest in developing and optimising rapid and reproducible methods for the detection and quantitation of particularly *Ascaris* sp. and *Cryptosporidium* sp. in order to provide a service to municipalities and private clients seeking to adhere to regulatory standards and ensure safe water sources.

In order to accurately evaluate the removal of particularly *Cryptosporidium* sp. and *Ascaris* sp. in water treatment processes, the development of rapid, accurate and reproducible methods for analysing the concentrations of these pathogens are required. The primary aim of the current study was thus to optimise a quantitative real-time PCR (qPCR) assay for the detection and quantitation of *Cryptosporidium* oocysts in drinking water and environmental surface waters (rivers and dams) as well as develop a qPCR assay for the detection and quantitation of *Ascaris* eggs in sludge samples. This study was conducted in collaboration with the East Rand Water Care Company (ERWAT). Formed in 1992, this municipal entity is custodian of 19 wastewater treatment plants (WWTPs) located throughout the Ekurhuleni Metropolitan (East Rand) area of Gauteng. The current combined treatment capacity of the 19 WWTPs stands at 696 mega litres (million litres) of wastewater per day. The WWTPs operated by ERWAT extends geographically across three drainage districts, which include the Jukskei River (DD3), the Blesbokspruit (DD5) and the Klip River (DD6). ERWAT Laboratory Services provide analytical support to ERWAT Operations and routinely test samples from the water care works. In addition, the Ekurhuleni Metropolitan Council and a variety of private clients utilise the services offered by the ERWAT laboratory for routine chemical and microbiological water analyses, with the scope of analyses ranging from drinking water (tap water), boreholes, rivers, dams, storm water to industrial wastewater.

1.2 Water sources

Only 3% of the world's water is available as freshwater, with the remaining 97% comprised of saltwater. Agricultural activities are responsible for 70% of the global water consumption, while the industrial sector utilises 22% and the residential sector 8% of the global water supply (Alois, 2007). In South Africa 52% of available fresh water is used for agricultural purposes with most of these sources being utilised for irrigation and to a lesser extent as a source of drinking water for livestock. Irrigation for agricultural purposes is critical for food security and a large percentage of the water usage assigned to agricultural purposes can therefore be seen as water allocated to sustain the South African population. The industrial sector is responsible for 11% of South African water consumption, 8% is used for forestry purposes and 19% for the environment, whereas the residential sector utilises 10% for drinking and domestic purposes (Blignaut & van Heerden, 2009).

The two most important water sources generally used for drinking water and agricultural purposes are groundwater and surface water. Groundwater is water that saturates the tiny underground

spaces between the alluvial material (sand, gravel, silt, clay) or the crevices or fractures in rocks. Surface water on the other hand includes lakes, rivers and those waters stored as snow or ice. Water sources in South Africa, such as surface water and groundwater, are particularly susceptible to various contaminants and it is essential that these water sources are routinely monitored in order to determine the suitability thereof as a source of irrigation water or for domestic and drinking purposes.

1.2.1 Municipal Drinking Water

Rand Water provides bulk potable water to the Ekurhuleni Metropolitan Council, who in turn distributes this water to the resident population of approximately 3.2 million people. Potable water needs to adhere to certain standards as stipulated by the Department of Water Affairs and Forestry (DWAF, 1996) (currently known as the Department of Water and Sanitation) in order to be regarded as safe for human consumption. Similarly, the South African National Standards (SANS 241) for drinking water specifies the quality of potable water (defined in terms of microbiological, physical, aesthetic and chemical determinants) at the point of delivery. Water meeting this standard is considered to be acceptable for lifetime consumption (implying an average consumption of 2 L of water per day for 70 years by a person weighing 60 kg) (SANS 241, 2015). However, certain pathogens may persist in drinking water if inadequate treatment processes are utilised. **Table 1** outlines information on various pathogens, which may be transmitted through drinking water (WHO, 2011).

Worldwide, chlorination is frequently utilised by drinking water treatment plants for disinfection purposes. However, as can be seen from **Table 1**, while many pathogenic bacteria exhibit a low resistance to chlorine, viruses and helminths are generally classified as more resilient and exhibit a moderate resistance to chlorine treatment. In addition, pathogenic protozoan species such as *Cryptosporidium parvum* demonstrate a high resistance towards chlorination, highlighting the importance of rapid detection methods for these intestinal parasites in order to accurately evaluate the risk to the community.

Table 1. Pathogens transmitted through drinking water[a] (WHO, 2011)

Pathogen	Health Significance[b]	Persistence in Water Supplies[c]	Resistance to Chlorine[d]	Relative Infectivity[e]	Important Animal Source
Bacteria					
Burkholderia pseudomallei	High	May multiply	Low	Low	No
Campylobacter jejuni, C. coli	High	Moderate	Low	Moderate	Yes
Escherichia coli – Pathogenic[f]	High	Moderate	Low	Low	Yes
E. coli – Enterohaemorrhagic	High	Moderate	Low	High	Yes
Francisella tularensis	High	Long	Moderate	High	Yes
Legionella spp.	High	May multiply	Low	Moderate	No
Leptospira	High	Long	Low	High	Yes
Mycobacterium spp. (non-tuberculous)	Low	May multiply	High	Low	No
Salmonella typhi	High	Moderate	Low	Low	No
Other Salmonellae	High	May multiply	Low	Low	Yes
Shigella spp.	High	Short	Low	High	No
Vibrio cholerae	High	Short to long[g]	Low	Low	No
Viruses					
Adenoviruses	Moderate	Long	Moderate	High	No
Astrovirus	Moderate	Long	Moderate	High	No
Enteroviruses	High	Long	Moderate	High	No
Hepatitis A	High	Long	Moderate	High	No
Hepatitis E	High	Long	Moderate	High	Potentially
Noroviruses	High	Long	Moderate	High	Potentially
Rotaviruses	High	Long	Moderate	High	No
Sapoviruses	High	Long	Moderate	High	Potentially
Protozoa					
Acanthamoeba spp.	High	May multiply	High	High	No
Cryptosporidium hominis/parvum	High	Long	High	High	Yes
Cyclospora cayetanensis	High	Long	High	High	No
Entamoeba histolytica	High	Moderate	High	High	No
Giardia intestinalis	High	Moderate	High	High	Yes
Naelgleria fowlerei	High	May multiply[h]	Low	Moderate	No
Helminths					
Dracunculus medinensis	High	Moderate	Moderate	High	No
Schistosoma spp.	High	Short	Moderate	High	Yes

a This table contains pathogens for which there is some evidence of health significance related to their occurrence in drinking-water supplies.
b Health significance relates to the incidence and severity of disease, including association with outbreaks.
c Detection period for infective stage in water at 20°C: short, up to 1 week; moderate, 1 week to 1 month; long, over 1 month.
d When the infective stage is freely suspended in water treated at conventional doses and contact times and pH between 7 and 8. Low means 99% inactivation at 20 °C generally in < 1 min, moderate 1–30 min and high > 30 min. It should be noted that organisms that survive and grow in biofilms, such as *Legionella* and mycobacteria, will be protected from chlorination.
e From experiments with human volunteers, from epidemiological evidence and from experimental animal studies. High means infective doses can be 1–102 organisms or particles, moderate 102–104 and low > 104.
f Includes enteropathogenic, enterotoxigenic, enteroinvasive, diffusely adherent and enteroaggregative.
g *Vibrio cholerae* may persist for long periods in association with copepods and other aquatic organisms.
h In warm water.

The SANS 241 regulations state that drinking water must comply with the numerical standard limits for the microbiological indicator determinants as specified in **Table 2**. When a microbiological value exceeds the standard permissible limit (**Table 2)**, an unacceptable risk to human health is implied (SANS 241, 2015). As can be seen in **Table 2** and **Table 3**, SANS 241 regulations (SANS 241, 2015) as well as the Department of Water Affairs and Forestry (DWAF, 1996), respectively, stipulates that no protozoan parasites may be present in water sources intended for drinking purposes or domestic uses.

Table 2. South African National Standard regulations for drinking water compliance (SANS 214, 2015)

Determinant	Risk	Unit	Standard Limits
E. coli[a] or faecal coliforms[b]	Acute health – 1	Count per 100 ml	Not detected
Cytopathogenic viruses[c]	Acute health – 1	Count per 10 L	Not detected
Protozoan parasites[d]			
Cryptosporidium spp.	Acute health – 2	Count per 10 L	Not detected
Giardia spp.	Acute health – 2	Count per 10 L	Not detected
Total coliforms[e]	Operational	Count per 100 ml	≤ 10
Heterotrophic plate count[f]	Operational	Count per ml	≤ 1 000
Somatic coliphages[g]	Operational	Count per 10 ml	Not detected

a Definitive, preferred indicator of faecal pollution.
b Indicator of unacceptable microbial water quality, could be tested instead of *E. coli*, but is not the preferred indicator of faecal pollution. Also provides information on treatment efficiency and after growth in distribution networks.
c Confirms a risk of human infection and faecal pollution, and also provides information on treatment efficiency. The detection of selected viruses confirms faecal pollution of human origin.
d Confirms a risk of infection and faecal pollution, and also provides information treatment efficiency. The detection of selected protozoan parasites confirms a human health risk.
e Indicates potential faecal pollution and provides information on treatment efficiency and after growth.
f Process indicator that provides information on treatment efficiency, after growth in distribution networks and adequacy of disinfectant residuals.
g Process indicator that provides information on treatment efficiency.

Cryptosporidium is of great significance as it causes cryptosporidiosis, an emerging disease which occurs worldwide and greatly affects immuno-compromised individuals (Current, 1994; Meinhardt *et al*., 1996). In addition, several studies have found *Cryptosporidium parvum* present not only in environmental water sources, but in tap water as well (Ayaz *et al*., 2011; Feng *et al*., 2011; Johnson *et al*., 2011; Ayaz *et al*., 2013). This finding again stresses the importance of developing rapid and accurate methods for the routine monitoring of intestinal parasites in municipal water supplies in order to provide safe water to end users.

Table 3. Water quality standards for microorganisms for domestic purposes (DWAF, 1996)

Organism	Target Range for Water Quality
Coliphages	1 CFU/100 mL
Enteric viruses	0
Escherichia coli	0 CFU/100 mL
Faecal coliforms	0 CFU/100 mL
Heterotrophic plate count	<100 CFU/mL
Protozoan parasites	0
Total coliforms	≤5 CFU/100 mL

1.2.2 Surface Water

Surface water sources, such as river water, are utilised by many rural communities around the world for their daily water needs, which primarily includes irrigation of gardens, cleaning and cooking and as a source of drinking water (Nevondo & Cloete, 1991). However, these water sources may be severely contaminated and research has shown that it is often not fit for human consumption (Schulz, 2001; Ouyang, 2005; Mwenge Kahinda *et al*., 2007; Zhang *et al*., 2008).

The major sources of surface water contamination can be divided into two categories, namely point- and non-point source pollution. Point source pollution originates from a single, confined source i.e. a pipe from a factory that deposits contaminated waste into the river, while non-point source pollution originates from rainwater, agricultural, urban or other runoff sources as well as the daily activities from the community around the river system (United States Environmental Protection Agency, 2014). While it is speculated that rivers located near urban areas are more prone to point source pollution, and rivers located in rural areas are more subject to non-point source pollution, research has shown that the primary rivers located in the Boland area, such as the Lourens-, Plankenburg-, Berg- and Diep Rivers, contain chemical and microbial contaminants originating from both point- and non-point source pollution (Jackson *et al*., 2007; Paulse *et al*., 2009; Bollmohr and Schulz, 2009). The primary contaminants of river water may include chemical pollutants such as endocrine disrupting chemicals (Zhang *et al*., 2008) and heavy metals (Ouyang, 2005; Jackson *et al*., 2007) as well as a wide range of microbial pathogens (Lightfoot, 2003).

A study by Zhang *et al*. (2008) showed that pollutants such as endocrine disrupting chemicals, pharmaceuticals and personal care products are being introduced into river water through anthropogenic activities. These chemicals included 17-α-ethynylestradiol, the main component of oral contraceptives as well as carbamazepine and diclofenac, which are anti-epileptic and anti-

inflammatory drugs, respectively. Endocrine disrupting chemicals can also be found in the environment, excreted as female hormones such as estrone and 17-β-estradiol. These pollutants are not removed by normal sewage treatment methods but are rather reactivated during these processes (Zhang *et al*., 2008). A study performed on the lower St. Johns River [Florida, United States (U.S)] also revealed the presence of contaminants such as hydrocarbons and heavy metals. The sources of these contaminants were surface runoff generated by urban, rural and agricultural settings, including discharges from ditches and creeks, groundwater spillage from septic tanks, aquatic weed control and atmospheric deposition (Ouyang, 2005). The Berg River (South Africa) was also shown to be heavily contaminated with metals such as aluminium and iron, mostly at sites within agricultural areas (Jackson *et al*., 2007). Excessive ingestion of iron can cause haemochromatosis, which is tissue damage caused by iron accumulation in the body (DWAF, 1996). In addition the Lourens River (Western Cape, South Africa) has also been found to contain high levels of pesticide during rainfall events. It was hypothesised that the pesticides were being introduced into the river by runoff and spray drift carrying the contaminants from surrounding orchards (Schulz, 2001).

Microbial contaminants mainly originate from faecal matter introduced into rivers by rainwater runoff (Nnane *et al*., 2011). Improperly treated effluents from wastewater treatment plants routinely discharged into rivers are also a major source of microbial contamination (Dungeni *et al.* 2010). Studies conducted on various sites along river systems such as the Berg- and the Plankenberg Rivers, showed that microbial contamination was the highest at sites closest to informal settlements (Paulse *et al*., 2009; Paulse *et al*., 2012). The major sources of these contaminants are inadequate sanitation and waste removal services, disposal of sewage into the rivers and storm water drainage pipes that lead directly to the rivers to dispose of human and animal waste (Paulse *et al*., 2012). In contrast, studies conducted on the Diep River concluded that microbial contamination was the highest at sites close to industrial facilities such as paint and machine manufacturers, storage and maintenance facilities for steel containers, a wastewater treatment plant and an oil-refinery (Paulse *et al*., 2009).

The primary microbial contaminants of river water often include pathogens such as *E. coli* (certain strains only), *Vibrio cholerae*, *Campylobacter* spp., *Salmonella typhimurium*, *Shigella* spp. and *Aeromonas hydrophila* (Lightfoot, 2003). In addition, microorganisms frequently become attached to surfaces and each other to create a complex, slime-encased community known as a biofilm. Biofilms can grow on virtually any surface such as rocks or other objects in water (Willey *et al*., 2008). Biofilms protect microorganisms from numerous harmful agents such as UV light,

antibiotics and other antimicrobial agents. In addition, these microbial communities can also contain a number of microorganisms such as *Pseudomonas* spp., *Mycobacter* spp., *Klebsiella* spp., *Legionella* spp. and *Helicobacter pylori* (Gião *et al*., 2008). Several studies have also discovered enteric viruses such as adenovirus and rotavirus in groundwater sources such as river water (Jiang *et al*., 2001; Jothikumar, 2005; Van Heerden *et al*., 2005; Pickering *et al*., 2006; Verheyen *et al*., 2009). In addition, some of the most prevalent pathogenic protozoa found in surface water include *Acanthamoeba* spp., *Cryptosporidium hominis/parvum, Cyclospora cayetanensis, Entamoeba histolytica, Giardia intestinalis* and *Naelgleria fowlerei* (Marshall *et al*., 1997; Thurman *et al*., 1998; Guy *et al*., 2003; Feng *et al*., 2011; WHO, 2011; Ayaz, *et al*., 2013).

1.2.3 Wastewater

Wastewater treatment plants remove biological and chemical waste products from contaminated water allowing the treated effluent to be safely discharged back into the aquatic environment. Water treated by wastewater treatment plants can originate from agricultural wastewater (liquid animal waste and pesticide residues), sewage (human and other household waste) and industrial wastewater (waste from manufacturing industries and mining). **Figures 1** and **2** illustrates two examples of wastewater treatment plants generating different types of sludge as well as their relative stability throughout the process. Water care companies may use either one or a combination of wastewater treatment plant configurations in order to effectively treat wastewater. However, it should be noted that many other processes and configurations for the treatment of sludge also exist (DWAF, 2006). **Figure 1** illustrates the stabilisation of sludge through an activated sludge wastewater treatment process. The essential features of an activated sludge process are the solid–liquid separation stage followed by aeration, and a sludge recycling system. After primary treatment, wastewater enters an aeration tank and organic matter is brought into contact with sludge returned from the secondary clarifier. This sludge contains high numbers of microorganisms that are in an active state of growth (hence termed activated sludge). Air is introduced into the tank either in the form of bubbles through diffusers or by surface aerators. Microorganisms in the activated sludge utilise the oxygen introduced and convert organic matter into stabilised, low-energy compounds such as nitrate (NO_3), sulphate (SO_4), and carbon dioxide (CO_2), while new bacterial cells (biomass) is generated (Ellis *et al*., 2002). Effluent flowing from the aeration tank contains the flocculent microbial mass (sludge), which is separated from the liquid phase in a secondary settling tank (clarifier). A portion of the activated sludge is recycled back to the aeration tank as a continuous source of microorganisms, while the remainder of the sludge (waste sludge)

undergoes stabilisation, disinfection, dewatering and disposal. It is important to note that if all the activated sludge were recycled, then the bacterial mass would keep increasing to the stage where t he system gets clogged with solids (DWAF, 2006; Sanin *et al*., 2006).

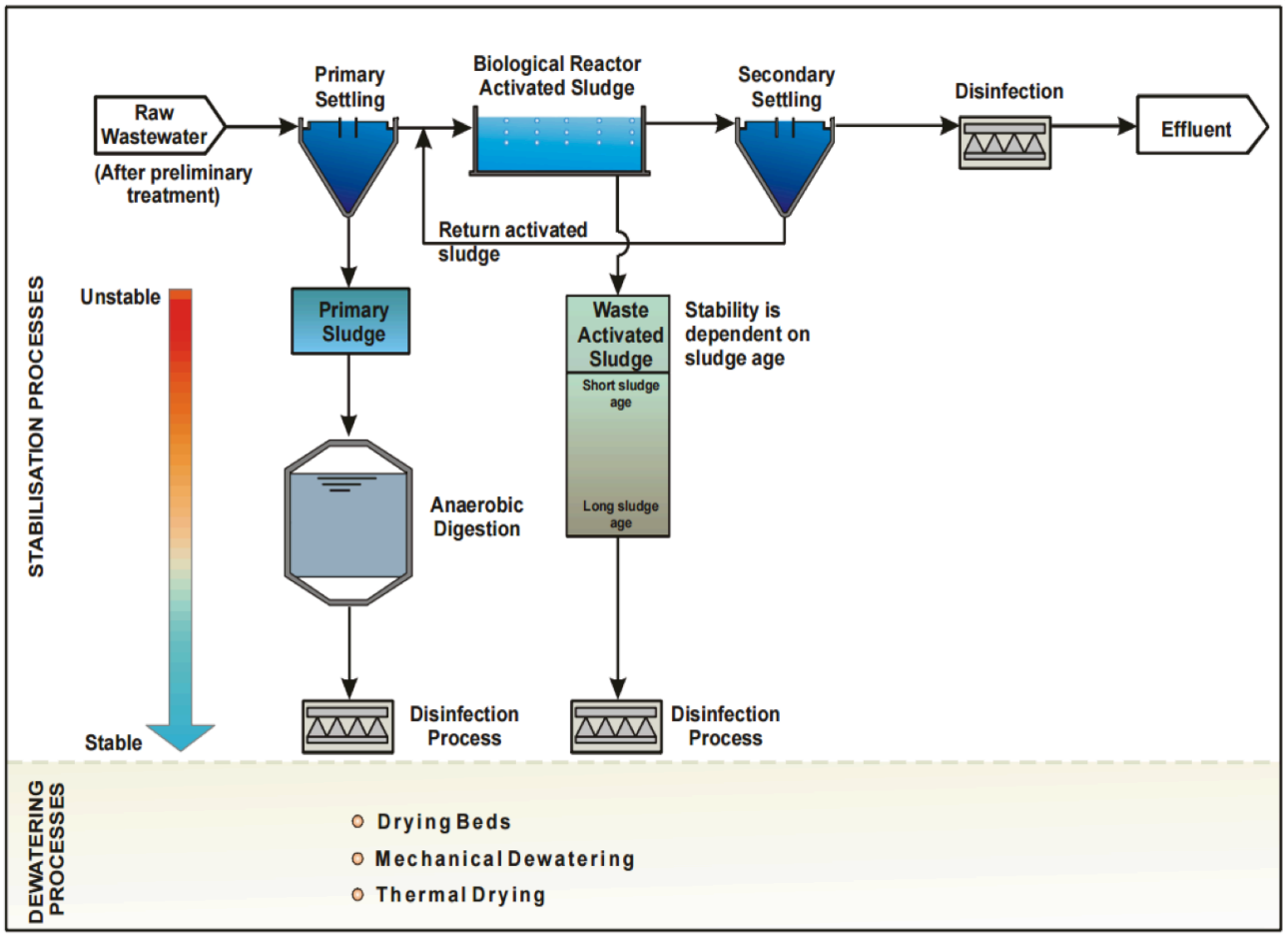

Figure 1. An example of an activated sludge wastewater treatment plant showing sludge stabilisation and dewatering options (DWAF, 2006)

Figure 2 illustrates the incorporation of trickling filters in the wastewater treatment process. Trickling filters are also called percolating filters and include a three-phase system. The first phase consists of a distribution system that introduces wastewater into the bioreactor by trickling the influent over the second phase, the fixed biofilm carriers. The biofilm carriers can consist of either rock or plastic media layered 1 to 3 metres deep with sufficient space between the materials to allow air to easily circulate. As the wastewater trickles over the packing material, air moves either upwards or downwards in the system (third phase) allowing oxygen and the dissolved organic matter to diffuse into the biofilm and be metabolised by the resident microorganisms. End products such as NO_3 and CO_2 diffuse back out of the biofilm and appear in the filter effluent. As microorganisms utilise the organic matter, the thickness of the biofilm increases to a point where it

can no longer be supported on the solid media and gets detached from the surface. This process is known as sloughing. A settling tank (clarifier) inserted after the trickling filter removes the detached bacteria film and some suspended matter. These treatment systems have a good adaptability to handle peak shock loads and the ability to function satisfactorily after a short period of time (Daigger and Boltz, 2011; DWAF, 2006).

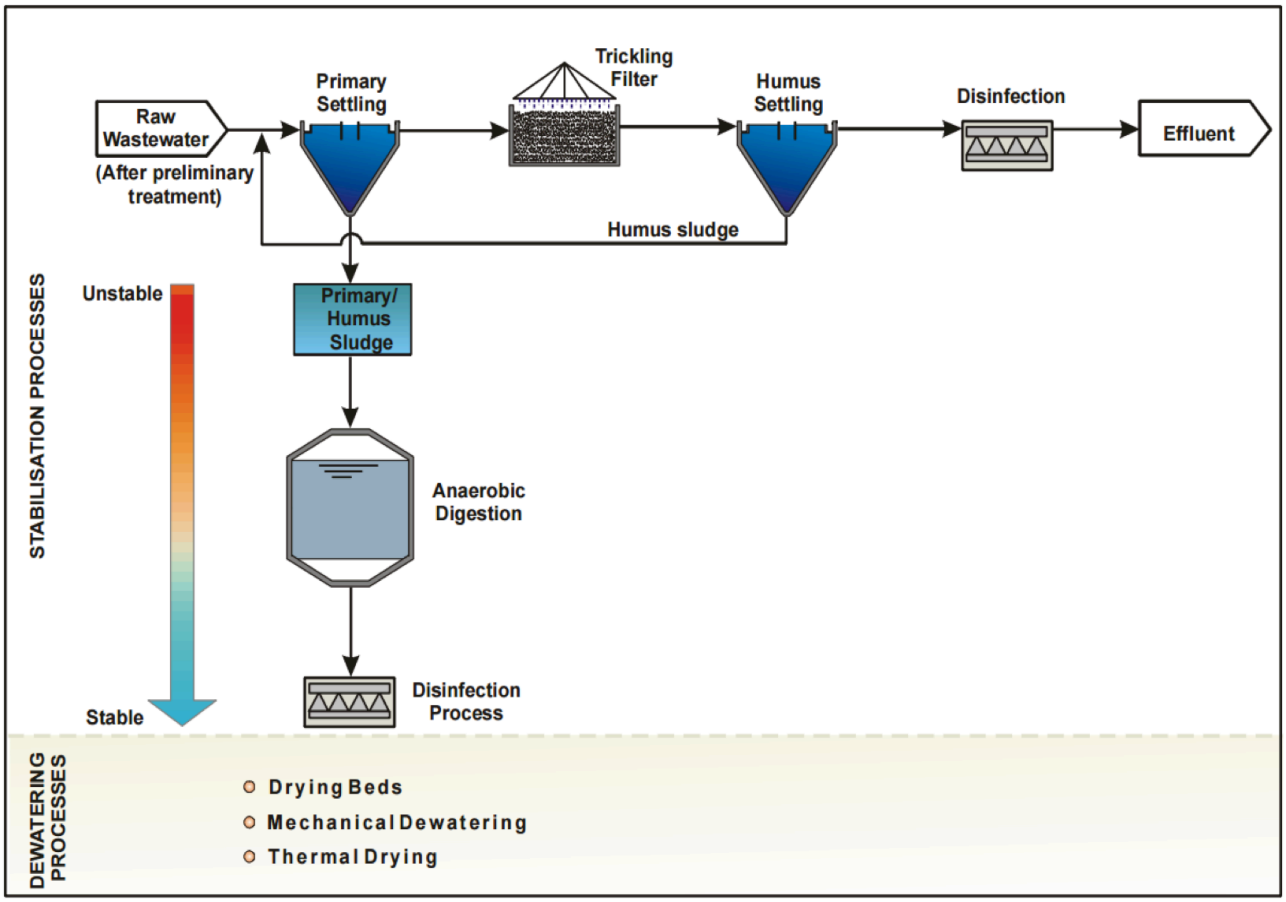

Figure 2. An example of a trickling filter wastewater treatment plant showing sludge stabilisation and dewatering options (DWAF, 2006)

Treated sewage effluent has become a popular alternative to other natural water sources such as river water as a source of irrigation water in the agricultural sector, due to the fact that it contains organic matter, nitrogen and phosphorous, promoting fertilising effects and increased crop yields (Maya *et al*., 2006; WHO, 2006a; Jimenez, 2007; Bastos *et al*., 2013). However, treated wastewater may contain large numbers of excreted organisms, including pathogens, with species and numbers that vary depending on the background levels of infection in the population (WHO, 2006a; Jimenez, 2007; Bastos *et al*., 2013). **Table 4** summarises the ranges of concentrations for various excreted organisms, which are commonly associated with wastewater (WHO, 2006a). As indicated, depending on the source water, microorganisms detected at highest levels in wastewater

are often thermotolerant coliforms with numbers ranging from 10^8 – 10^{10} per litre. *Ascaris lumbricoides* is the most prevalent helminth species found in wastewater, whereas the protozoan species *Cryptosporidium parvum* can be found at numbers as high as 10^4. In addition, enteric viruses associated with wastewater are found in numbers ranging from 10^2 – 10^6.

Table 4. Excreted organism concentrations in wastewater (WHO, 2006a)

Organism	Numbers in wastewater (per litre)
Bacteria	
Thermotolerant coliforms	$10^8 – 10^{10}$
Campylobacter jejuni	$10 – 10^4$
Salmonella spp.	$1 – 10^5$
Shigella spp.	$10 – 10^4$
Vibrio cholerae	$10^2 – 10^5$
Helminths	
Ascaris lumbricoides	$1 – 10^3$
Ancylostoma duodenale/Necator americanus	$1 – 10^3$
Trichuris trichiura	$1 – 10^2$
Protozoa	
Cryptosporidium parvum	$1 – 10^4$
Entamoeba hystolitica	$1 – 10^2$
Giardia intestinalis/lamblia	$10^2 – 10^5$
Viruses	
Enteric viruses	$10^5 – 10^6$
Rotavirus	$10^2 – 10^5$

Indicator organisms such as *E. coli* and thermotolerant coliforms are traditionally used to assess the risk associated with the use of wastewater in agriculture, however they are not suitable for routine monitoring as their concentrations often do not correspond with concentrations of viruses, protozoa or helminths (Ashbolt *et al*., 2001; Maya *et al*., 2006; WHO, 2006a). As can be seen from **Table 5**, helminth and protozoan species survive for long periods of time in water, on crops as well as in soil. It is therefore of great importance to assess their presence and viability in wastewater to be used for irrigation purposes, as they can be harmful to farmers and farmworkers, consumers of produce and surrounding communities (Sengupta *et al*., 2011).

Comprehensive characterisation and classification of sludge generated by a particular wastewater treatment plant needs to be carried out in order to determine which management options are suitable and what applications the sludge can be utilised for. The Department of Water Affairs and Forestry (2006) stipulates specific parameters and guidelines in order to evaluate the quality of sewage

sludge. These parameters address the physical characteristics, chemical quality and microbiological quality of the produced sludge.

Table 5. Survival of various organisms in selected environmental media at 20 – 30°C (WHO, 2006a)

Organism	Survival time (days)		
	Fresh Water and Sewage	**Crops**	**Soil**
Bacteria			
Thermotolerent coliforms	<60, usually <30	<30, usually <15	<70, usually <20
Salmonella spp.	<60, usually <30	<30, usually <15	<70, usually <20
Shigella spp.	<30, usually <10	<10, usually <5	ND
V. cholerae	ND	<5, usually <2	<20, usually <10
Viruses			
Enteroviruses[a]	<120, usually <50	<60, usually <15	<100, usually <20
Protozoa			
E. histolytica cysts	<30, usually <15	<10, usually <2	<20, usually <10
Cryptosporidium oocysts	<180, usually <70	<3, usually <2	<150, usually <75
Helminths			
Ascaris eggs	Years	<60, usually <30	Years
Tapeworm eggs	Many months	<60, usually <30	Many months

ND, no data
[a]Poliovirus, echovirus and coxsackievirus

Following physical characterisation, the chemical and microbiological quality is determined based on the South African sludge classification system, as stipulated by the Department of Water Affairs and Forestry (2006). A second classification system then classifies the sludge in terms of the microbiological content, stability as well as organic and inorganic pollutants. The second classification of the sludge can be used to select appropriate use and disposal methods or assess if the current practices are in line with the sludge guidelines.

It is however, evident from the prevalence and health risk associated with the protozoa species *Cryptosporidium parvum* and helminth species *Ascaris lumbricoides*, that accurate screening methods should be implemented as these organisms can persist for extended time periods in the environment.

1.3 *Cryptosporidium parvum*

Cryptosporidium parvum is an obligate, enteric, protozoan parasite that infects the gastrointestinal tract of both animals and humans leading to the acute diarrhoeal disease, cryptosporidiosis (Carey *et al*., 2003). The oocysts of this pathogen are extremely resistant to traditional water treatment methods such as chlorination and very low infectious doses have been reported (Xiao and Fayer, 2008).

1.3.1 Biology and life cycle of *Cryptosporidium parvum*

Cryptosporidium belongs to the phylum Apicomplexa and family Cryptosporidiidae and is an intracellular, coccidian parasite that reproduces both sexually and asexually (Carreno *et al.*, 2001). Currently, there are eight described *Cryptosporidium* species (*C. andersoni, C. canis, C. felis, C. hominis, C. meleagridis, C. muris, C. parvum* and *C. suis*) and five undescribed species (cervine, chipmunk, monkey, rabbit and skunk genotypes), which can infect both immuno-competent and immuno-compromised individuals (Xiao *et al*., 2004; Cacciò *et al*., 2005; Feltus *et al*., 2006; Nichols *et al*., 2006). Of these species, *C. parvum* and *C. hominis* are most commonly detected (Cacciò *et al*., 2005) and while both of these species infect humans, *C. parvum* also infects cattle and livestock, creating a higher risk of contamination in environmental waters and thus putting human health at greater risk (Smith and Nichols, 2009). Mature *Cryptosporidium* oocysts are 4 – 6 µm in size, have a thick wall and can survive in food and water for up to six months (Carreno *et al*., 2001). The nature of the robust oocysts make them highly resistant to oxidising disinfectants such as chlorine, however oocysts have been shown to be susceptible to UV light radiation (WHO, 2011). The oocysts of *Cryptosporidium* have been found in raw sewage, surface water, drinking water sources and recreational water (WHO, 2011). Water then becomes contaminated with the robust oocysts, which is excreted in the faeces of infected hosts.

The typical route of transmission follows the faecal-oral route, where ingestion of contaminated food and water leads to infection. However, the primary route of transmission is person-to-person contact (WHO, 2011). **Figure 3** illustrates the route of transmission and infection of a *Cryptosporidium* oocyst (CDC, 2014). After the oocysts are ingested by the host, excystation occurs within the small intestine, after which the released sporozoites attach to the epithelial cells of the gastrointestinal tract. Excystation of the oocysts requires reducing conditions, pancreatic enzymes,

and bile salts, however it has been shown that oocysts can excyst in warm aqueous environments. The sporozoites penetrate enterocytes and then develop into trophozoites. This stage occurs intracellularly beneath the host cell membrane but is extracytoplasmic. The trophozoites divide by asexual multiplication into forms with six to eight nuclei. They will each mature into a merozoite of type I meronts. The merozoite can then either develop into a type II meront and begin sexual multiplication and oocyst development, or the merozoite can reinitiate asexual multiplication. Environmentally resistant thick walled oocysts can develop from zygotes formed after fertilisation. They will then undergo sporogony to form sporulated oocysts containing four sporozoites. The sporulated oocysts are excreted through the faeces and can transmit infection from one host to another. If the zygotes develop into thin-walled oocysts, they enter the autoinfective life cycle and can maintain the parasite in the host. Only 20% of zygotes enter this stage. The autoinfective stage and the persistent meronts (the stage in the life cycle of sporozoans in which multiple asexual fission occurs, resulting in production of merozoites) are believed to be the cause for the life-threatening disease in children and immuno-deficient individuals (Current and Garcia, 1991; Sterling and Arrowood, 1993).

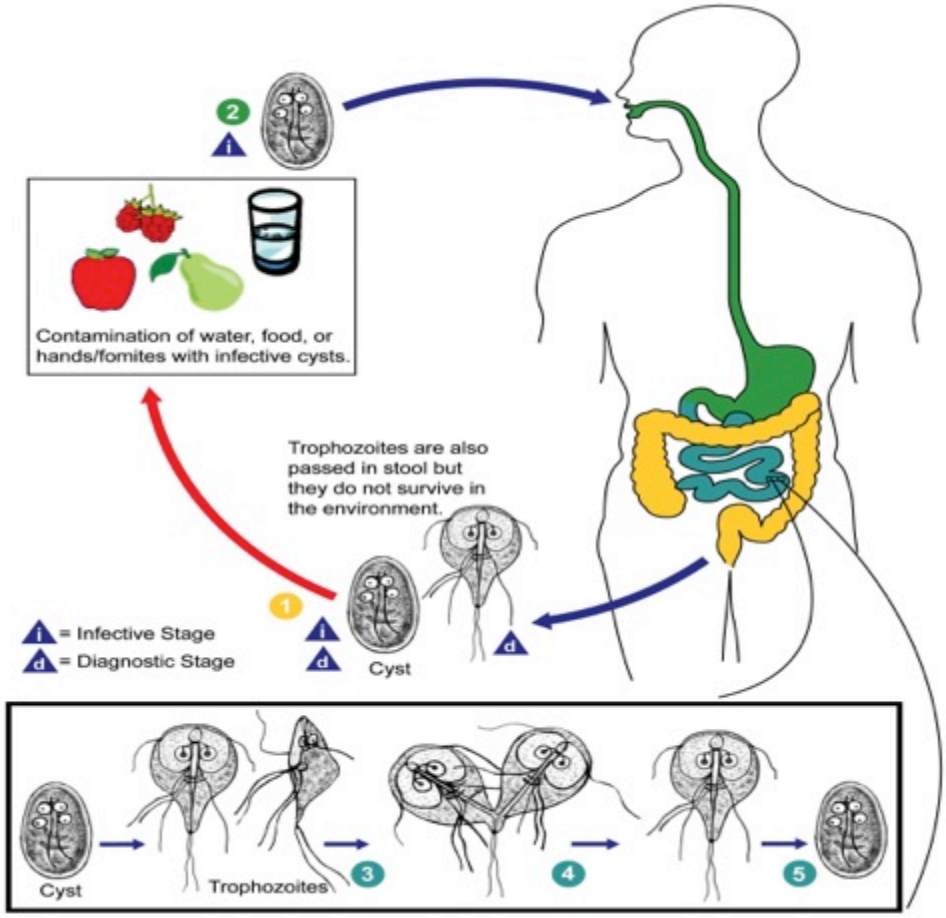

Figure 3. Route of transmission and infection by *Cryptosporidium* (CDC, 2014)

Cryptosporidiosis is recognised as an emerging disease and Karanis *et al*. (2007) stated that *Cryptosporidium* is responsible for 50.8% of water associated outbreaks of parasitic protozoan diseases documented worldwide. They also reported that 23.7% of these outbreaks were caused by *Cryptosporidium* that passed through filtered and unfiltered drinking water systems. In addition contaminated recreational waters were responsible for 50.3% of the cryptosporidiosis outbreaks (Karanis *et al*., 2007). The largest outbreak of cryptosporidiosis to date occurred in Milwaukee, United States, in 1993. An estimated 403 000 individuals were infected with the intestinal parasite (MacKenzie *et al*., 1994). In addition, outbreaks of cryptosporidiosis in West Hertfordshire and north London, UK (1997) included an incidence where an estimated 345 cases of infection were recorded (Smith, 1999). Moreover, during 2001 approximately 5 800 to 7 100 individuals were infected with *Cryptosporidium* in North Battleford, Canada (CCDR, 2001), 230 people were infected in Belfast, Ireland (Glaberman *et al*., 2002) and 563 individuals were infected in France (Dalle *et al*., 2003).

1.3.2 Detection of *Cryptosporidium parvum*

In order to evaluate for the presence of *Cryptosporidium* oocysts in water samples, the collected samples have to undergo three main processing steps, namely concentration, separation and detection. Various methods for the detection and quantitation of *Cryptosporidium* oocysts have been developed and compared. However, there is currently no internationally accepted molecular based method available (Smith and Grimason, 2003; Smith and Nichols, 2009; Sigudu *et al*., 2014).

1.3.2.1 Sampling methods

Low concentrations of the oocysts are generally present in the environment and two primary methods of sampling exist to obtain the maximum number of oocysts. The first method involves the collection of large volumes of water (100 – 1 000 L) over a period of hours at a defined flow rate. The second method consists of collecting several smaller volumes of water (10 – 20 L) over a certain sampling period and this is defined as grab samples. Significantly higher concentrations of oocysts are detected by grab samples than with the large volume sampling techniques tested, which requires greater manipulation of the sample. In addition, grab samples generate occurrence data in smaller volumes, can provide higher recovery efficiencies than large volume sampling and are easily collected (Smith, 1999).

As indicated, contamination of water sources can arise from both point and non-point source pollution and knowledge of the likely levels of contamination can influence whether larger volumes or grab samples are collected. It is generally advisable to make use of large volume samples if non-point source pollution occurs and little is known of the occurrence of oocysts in the matrix tested. When point source pollution occurs, such as with a sewage treatment plant, higher densities of oocysts are expected at the influent point. Thus, grab samples, which require fewer manipulations and minimal organism loss are preferred for sewage influent samples. In sewage effluents, where *Cryptosporidium* oocyst densities can be low (<10–60 oocysts l^{-4}, Bukhari *et al*. 1997), larger volume samples are preferred. In contrast, when oocysts numbers in both sewage influent and effluent are relatively high, grab samples, that result in fewer manipulations of the sample, are preferred (Robertson *et al*. 2000).

1.3.2.2 Concentration

Cartridge filtration is generally used to concentrate the protozoa in large volumes of water. The filters used are yarn-wound, 25.4 cm long, cartridge filters composed of polypropylene or other media and have a pore size of 1 mm. Large volumes of water are pumped through these filters at a set rate where after the filters are cut open manually and the trapped protozoa and particles are eluted by washing with an aqueous detergent solution (Musial *et al*., 1987; LeChevallier and Norton, 1995; Shepard and Wyn-Jones, 1996; Grimason *et al*., 1997; Sobsey, 1999). The oocysts are then further concentrated and recovered by centrifugation (WHO, 1992). A disadvantage of this method is that these filters have to be removed from the filter housing. In addition, filters only have normal pore size ratings and the cartridges are held in place by pressure in the plastic housings using flexible O-rings or gasket seals (Carey *et al*., 2003). This can result in the loss of oocysts through penetration or bypassing of the filter. Large volumes of water also contain higher loads of other particles, which can interfere with the purification and detection steps (Sobsey, 1999). The biggest obstacle regarding the detection and quantitation of *Cryptosporidium* oocysts from environmental samples is the ability to effectively concentrate and purify oocysts from water samples whilst limiting inhibiting factors (Carey *et al*., 2003).

Membrane filtration is used for smaller volumes of water and the filters have absolute pore sizes, smaller than the size of oocysts, which increases the expected retention of the oocysts (Ali *et al*., 2004; Castro-Hermida *et al*., 2008; Castro-Hermida *et al*., 2010; Feng *et al*., 2011; Keserue *et al*., 2012). The recovery efficiency of membrane filtration is much higher and the elution is much simpler than that of the cartridge filtration method (Carey *et al*., 2003). Different types of

membrane filters can be used, for example polycarbonate filters, cellulose-acetate filters and pleated capsule filters (1 µm pore size polyether-sulphone filters in polycarbonate housing) (Cacciò *et al*., 2003; Guy *et al*., 2003; Abo-Shehada *et al*., 2004; Castro-Hermida *et al*., 2009). The United States Environmental Protection Agency (USEPA) recommends the sampling of smaller volumes of water and the use of the membrane filtration technique as specified in the widely accepted Method 1622 and 1623 for the detection of *Cryptosporidium* and *Giardia* (oo)cysts.

The concentration of protozoa from water samples can also be achieved by flocculation, a chemical precipitation method (Smith 1999; Sobsey, 1999; Feng *et al*., 2011). Vesey *et al*. (1993) developed a calcium carbonate ($CaCO_3$) flocculation method. However, $CaCO_3$ affects the viability of (oo)cysts. Flocculation with aluminium sulphate ($Al[SO_4]_3$) does not affect viability and the recovery efficiency is comparable to that achieved with $CaCO_3$ flocculation (Sobsey, 1999; WHO, 1992; Karanis and Kimura, 2002). Ferric sulphate has also been shown to be effective in the recovery of *Cryptosporidium* oocysts (Karanis and Kimura, 2002).

In a study conducted by Shepard and Wyn-Jones (1996) these three methods of concentration were evaluated. The recovery efficiency of *Cryptosporidium* and *Giardia* from tap and river water was studied. Cartridge filtration showed a mean (oo)cyst recovery of 11.2% and 9.4% from tap water (n=8) and river water (n=6), respectively. For the membrane filtration technique evaluated it was found that a 1.2 µm cellulose acetate membrane and a 3.0 µm cellulose nitrate membrane was optimal for *Cryptosporidium* and *Giardia* recovery, respectively. Chemical flocculation using calcium carbonate resulted in mean recovery efficiencies for *Cryptosporidium* in river water (n=6) and tap water (n=8) of 71.3% and 73.6%, respectively, while *Giardia* recovery efficiencies were 72.5% in river water and 77.1% in tap water. In addition, LeChevallier and Norton (1995) found that the use of cotton, nylon or rayon filters instead of polypropylene filters during cartridge filtration resulted in a 10% higher recovery. Karanis and Kimura (2002) also analysed the recovery efficiency of *Cryptosporidium parvum* oocysts from tap water. The results obtained then indicated that ferric sulphate flocculation yielded a higher recovery efficiency of 61.5%, in comparison to calcium carbonate flocculation where a recovery efficiency of 58.1% was recorded.

1.3.2.3 Separation

Flow cytometry can be used to separate *Cryptosporidium* oocysts from other particles and solutes in water samples (Vesey *et al*., 1993). The method consists of labelling the oocysts with a fluorescein isothiocyanate-labelled monoclonal antibody (FITC-mAb) reactive with surface epitopes on oocysts, after which water concentrates are passed through a fluorescent activated cell sorter

(FACS) and the resulting droplets are separated according to a pre-determined size, shape and fluorescence characteristics (WHO, 1992; Smith, 1999; Abo-Shehada *et al*., 2004; Keserue *et al*., 2012; Ware *et al*., 2013). The instrument design must however first be modified to the electrical charge of the specific particle to be detected. This is achieved by vibrating the sheath streams droplets which flow by two charged plates where particles of interest are electrically charged with a voltage pulse (Mcpherson *et al.*, 2007). A drawback of this method is that organisms with similar sizes to *Cryptosporidium* may cross-react with the anti-*Cryptosporidium* monoclonal antibody and show similar fluorescence characteristic and false-positive results can occur. To prevent this, epifluorescent microscopy may rather be used to confirm the presence of oocysts (Carey *et al*., 2003). This method has been shown to be superior to direct microscopy on non-sorted samples with a success rate of 92% (Vesey *et al*., 1994).

Immuno-magnetic separation (IMS) selects, separates and purifies specific target organisms from other organisms and particles of similar size and shape as well as solutes. This method is a powerful approach for the recovery, enrichment, purification and concentration of oocysts from water as it is based on the specificity of an antigen-antibody reaction (Sobsey, 1999; Castro-Hermida *et al*., 2010). Immuno-magnetic separation consists of the binding of monoclonal antibody-coated magnetisable beads to the epitope of a specific antigen in the cell wall of the oocysts. This then causes an antigen-antibody complex to form. The sample is continuously mixed to enhance binding. The oocysts bind to the magnetisable particles and are concentrated from the debris by placing a magnet on the outside of the tube to attract the magnetisable particles. Debris is then aspirated from the beaded oocyst complex (Smith, 1999; Sobsey, 1999; Khouja *et al*., 2010).

A study conducted by Vesey *et al*. (1994) showed that the microscopic detection of both *Giardia* and *Cryptosporidium* (oo)cysts was easier to perform when flow cytometry was implemented. Recovery efficiencies from 179 oocysts and 77 cysts seeded into river and reservoir samples (100 ml) were all in excess of 92% showing flow cytometry to be a superior technique when compared to direct microscopy and non-sorted samples. Keserue *et al*. (2012) also concluded that flow cytometry was a very fast and convenient method, which was comparable to other separation techniques. Flow cytometry can thus be used as a rapid method for detecting *Cryptosporidium* oocysts in water samples; however a relatively large number of oocysts need to be present in the original sample (Carey *et al*., 2003), similar to the number (179 oocysts and 77 cysts) seeded into the water samples previously mentioned in the Vesey *et al*., (1994) study. In a study conducted by McCuin *et al*. (2001), IMS showed recovery efficiencies of *Cryptosporidium parvum* oocysts

between 55.9% and 83.1%. This study also showed that by utilising IMS, a small number of oocysts could be recovered from environmental samples (9 – 11 oocysts/ml). In addition, LeChevallier *et al*. (2000) showed that IMS was superior to other flotation methods for the separation of oocysts from raw and filtered samples, however the difference was not statistically significant ($p > 0.05$).

1.3.2.4 Detection

A widely accepted method for the detection of *Cryptosporidium* oocysts in water samples is method 1622 developed by the USEPA, which was validated in 1999. Method 1623 was also developed for the simultaneous detection of *Cryptosporidium* and *Giardia*. This method involves the concentration of water samples by means of filtration, immuno-magnetic separation (IMS) of the (oo)cysts and subsequently determining the concentration using an immuno-fluorescence assay (IFA). The presence of potential (oo)cysts are confirmed by staining with the vital dyes 4'-6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI), followed by differential interference contrast (DIC) microscopy (Carey *et al*., 2003). In a study conducted by Keserue *et al*. (2012), immuno-fluorescent microscopy was employed as an in-field method for the successful identification of both *Giardia* and *Cryptosporidium* (oo)cysts in water samples. However, due to the fact that the portable microscope used did not include a violet or UV light source, distinct nuclei could not be visualised with the addition of DAPI-staining as suggested by the USEPA method 1623. Method 1623 has also been assessed and optimised in numerous other studies (Simmons *et* al., 2001; McCuin and Clancy, 2003; Feng *et al*., 2003; Feng *et al*., 2011) and although no specialised equipment is required, it is apparent that this method is labour-intensive, time-consuming and requires extensive laboratory experience for the interpretation of results. It is also unable to distinguish between viable and non-viable (oo)cysts and between specific species, as different (oo)cyst sizes overlap (Carey *et al*., 2003; Feng *et al*., 2011; Smith and Nichols, 2009).

Fluorescent in situ hybridisation (FISH) is used to detect *Cryptosporidium parvum* oocysts in water samples by hybridising a fluorescently labelled oligonucleotide probe, which targets the 18S rRNA molecule (Carey *et al*., 2003). This molecule is present in high copy numbers in viable oocysts but has a short half-life and a rapid decline in copy numbers of non-viable oocysts (Carey *et al*., 2003). Although FISH can identify organisms to species level, distinguish between viable and non-viable oocysts and detect multiple organisms when using defined probes labelled with different fluorescent markers, the method includes lengthy fixation procedures and is more labour intensive than direct immuno-fluorescence (Carey *et al*., 2003).

Molecular based techniques, such as the polymerase chain reaction (PCR), have several advantages over standard techniques. These methods have been shown to be more reproducible and sensitive in the detection of *Cryptosporidium parvum* and can distinguish between different species (Carey *et al*., 2003; Mcpherson *et al*., 2007; Ayaz *et al*., 2013). Polymerase chain reactions allow for the detection and, in the case of certain PCR techniques, the quantitation of multiple samples in a relatively short period of time. Environmental samples may however, contain various inhibiting factors such as phenolic compounds, humic acids as well as heavy metals (Tsai and Rochelle, 2001). These inhibitors can influence DNA/DNA interactions and thus interfere with amplification (Carey *et al*., 2003; Keserue *et al*., 2012). Amplification may also be hindered by constituents of bacterial cells, non-target DNA and other contaminants that inhibit DNA polymerase activity. Immuno-magnetic separation (IMS) as well as fluorescent activated cell sorting may then be employed to reduce the level of inhibitors in water samples before PCR is performed (Smith, 1999). Commercial DNA extraction kits have also been shown to remove PCR inhibitors in environmental water samples (Behets *et al*., 2007). However, if the oocysts do not completely lyse then the extraction of DNA may be limited. To prevent this, enzymatic degradation of the cellular wall components is required. Another potential challenge is the degradation of nucleic acids by DNase activity (Carey *et al*., 2003). However, comparative studies have shown that PCR is the most effective and sensitive method for the detection of *Cryptosporidium parvum* oocysts (Leng *et al*., 1996; Mayer and Palmer, 1996; Elsafi *et al*., 2013). Several PCR techniques have thus been implemented for the detection of *Cryptosporidium parvum* oocysts and these methods include conventional PCR, quantitative real-time PCR (qPCR), nested PCR, reverse transcriptase-PCR (RT-PCR) as well as immuno-magnetic separation-PCR (IMS-PCR).

Quantitative real-time PCR (qPCR) coupled with high-resolution melt (HRM) curve analyses eliminates the need for post-PCR analysis, such as agarose gel analysis and sequencing. It also reduces variation, enhances sensitivity and has a higher throughput and reduced turnaround time with minimal risk of amplicon contamination due to the closed-vessel system (Carey *et al*., 2003; Guy *et al*., 2003). Fluorescent probes can be used to quantify the starting concentration of nucleic acids in the water samples where after the amount of amplified DNA can be calculated during the exponential phase (Carey *et al*., 2003). Limor *et al*. (2002) differentiated between *Cryptosporidium* species by analysing the melting curves and calculating melting temperatures (T_m) from qPCR data. Melting temperatures for the different species were as follows: *C. hominis* (66.5°C), *C. parvum* (59.3°C), *C. meleagridis* (62.4°C), *C. felis* (54.23°C) and *C. canis* (58.04°C). This method was found to have a detection limit of 5 oocysts, has similar sensitivity to conventional PCR and has an

increased specificity due to hybridisation of two probes during the detection and melting curve analysis phase (Limor *et al*., 2002). Guy *et al*. (2003) also employed qPCR for the simultaneous detection of both *Cryptosporidium* and *Giardia* (oo)cysts from environmental samples and sewage. The authors could then successfully detect DNA equivalent to one cyst of *Giardia lamblia* and one oocyst of *C. parvum*. They could also detect both assemblages A and B gene sequences of *G. lamblia* in the sewage samples.

The biggest disadvantage of qPCR still remains the inability to differentiate between viable and non-viable microorganisms. Studies have however been conducted with the DNA-intercalating dyes, ethidium monoazide (EMA) and propidium monoazide (PMA), in conjunction with qPCR for the selective detection of viable bacterial cells (Rudi *et al*., 2005; Inoue *et al*., 2008). These studies effectively detected viable bacteria cells by treating water samples with EMA prior to qPCR analysis. Ethidium monoazide qPCR (EMA-qPCR) has also been used to effectively detect bacteria in food (Rudi *et al*., 2005), biofilms (Chen and Chang, 2010) and water samples (Inoue *et al*., 2008). In a study conducted by Qin *et al.* (2012) EMA-qPCR was used to detect *Legionella* in environmental samples. Findings suggested that the percentage positive rate obtained by EMA-qPCR was significantly higher than with conventional PCR coupled with culturing methods and only slightly lower than qPCR. In another study conducted by Alonso *et al*. (2014), it was possible to distinguish between viable and non-viable *Cryptosporidium* and *Giardia* (oo)cysts spiked into phosphate-buffered saline and tertiary effluent wastewater using a PMA-qPCR method. Quantitative real-time PCR (qPCR) could thus be combined with DNA-intercalating dyes in order to evaluate the viability of parasites such as *Cryptosporidium* and *Ascaris* in wastewater and sludge samples.

1.4 *Ascaris lumbricoides*

Helminth species infects approximately 1.2 billion people worldwide with *Ascaris* being the most common helminth detected in wastewater (WHO, 2006b). **Figure 4** illustrates the prevalence of the five helminth species most commonly found in raw wastewater worldwide (Maya *et al*., 2006).

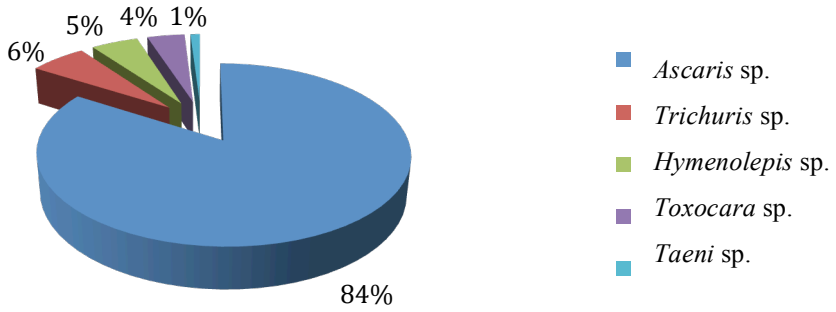

Figure 4. Average worldwide distribution of helminth ova in raw wastewater (Maya *et al*., 2006)

1.4.1 Biology and life cycle

Ascaris lumbricoides is a roundworm belonging to the phylum Nematoda. It is the largest roundworm and predominantly infects humans living in developing countries causing the disease ascariasis (Harhay *et al*., 2010). Male worms are 2 to 4 mm in diameter and are 15 to 31 cm long. In contrast, females are 3 to 6 mm wide and are 20 to 49 cm long. The female worm may contain up to 27 million eggs at a time with 200 000 being laid per day. Fertilised eggs are oval to round in shape and are 45 to 75 µm long and 35 to 50 µm wide with a thick outer shell (**Figure 5.A**). Unfertilised eggs measure 88 to 94 µm long and are 44 µm wide (**Figure 5.B**) (Roberts and Janovy, 2009).

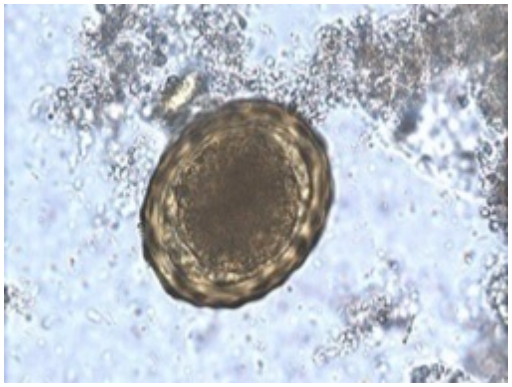

Figure 5.(A). Unstained wet mount of a fertilised *Ascaris lumbricoides* egg (CDC, 2014)

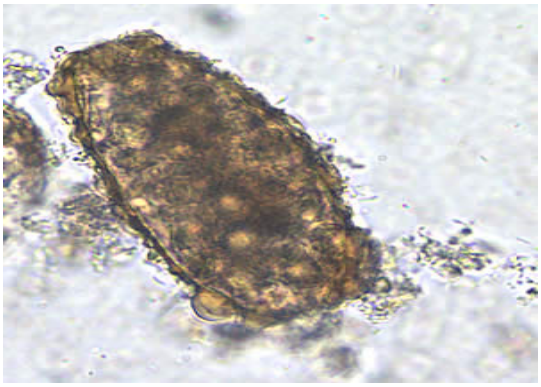

Figure 5.(B). Unstained wet mount of an unfertilised *Ascaris lumbricoides* egg (CDC, 2014)

Humans become infected when the fertilised egg is ingested. The egg becomes a larval worm inside the small intestine of the infected host, penetrating the wall of the duodenum and thus entering the blood stream. The larvated worm can spread throughout the body to the liver and heart, entering the pulmonary circulation to break free in the alveoli where it will grow and molt, shedding from the surface it is attached to in order to spread through the body. The larvae will pass from the respiratory system where it can be coughed up, swallowed and returned to the small intestine after three weeks. In the small intestine the larvae can mature to adult female or male worms. Fertilisation can then occur, with the female producing up to 200 000 eggs per day (Murray *et al*., 2005). The eggs can enter water sources through direct faecal contact, discharge from treated or untreated sewage water as well as surface runoff water (Kirby *et al*., 2003). The eggs of *Ascaris lumbricoides* have a resilient lipid layer which results in their increased resistance to acids and alkalis (Piper, 2007). In addition, agricultural crops can become contaminated with the eggs of *Ascaris lumbricoides* when they are irrigated with contaminated wastewater. Human infection can in turn occur when contaminated crops are ingested. According to the WHO (2006a) the biggest risk occurs when crops are not properly cooked before being consumed.

1.4.2 Detection of *Ascaris lumbricoides*

1.4.2.1 Detection of *Ascaris* sp. in water sources

Currently there is no widely accepted standard international method for the detection of helminth ova in water sources (Maya *et al*., 2006). Four main techniques can however be utilised for the detection of helminth ova in water and they include the USEPA method (Yanko, 1987), membrane filtration (Galván *et al*., 1996), Leeds I (Ayres, 1989) and Faust techniques (Faust *et al*., 1939). All four detection methods consist of two main steps; firstly separation, recovery and concentration of the helminth ova from the sample sediment and secondly the visual enumeration of helminth ova by means of microscopy (Maya *et al*., 2006). These methods are categorised based on the means of separation, recovery and concentration of the ova, whether microscopic analysis is carried out on all or only part of the concentrated sample and the volume of the initial sample (Maya *et al*., 2006).

To identify and detect viable *Ascaris* eggs in environmental water samples the USEPA 1994 method can be used, which consists of a laborious multi-step protocol to clean and concentrate the eggs from the samples, followed by a 4 week incubation period and a final step of counting larvated eggs under a light microscope. This method recommends the collection of a 5 L initial water

sample, after which flotation with zinc sulphate ($ZnSO_4$) with a specific gravity of 1.3 is combined with biphasic as well as sedimentation steps in order to separate the ova from the water. Microscope analysis to enumerate helminth ova is performed on the entire concentrated sample. The membrane filtration technique utilises flotation with $ZnSO_4$ (specific gravity of 1.2) and the indirect concentration of an initial 1 L sample. Helminth ova are retained on a cellulose acetate membrane (10 µm pore size), which is dehydrated, treated with ethanol and rendered transparent by treatment with glycerol. Enumeration of helminth ova is done by microscopy using the concentrated sample. Although no specialised or expensive equipment is needed, the incubation period is long, the method is extremely laborious and experienced technicians are required to correctly identify *Ascaris* eggs. The Leeds I technique consists of several consecutive centrifugation and flotation steps. The initial sample size is 1 L for wastewater with a high solid content and 40 L for wastewater with low solid content. If a 40 L initial sample is collected, the water is first filtered through a micro-wynd device to recover the ova before further flotation takes place (Maya *et al*., 2006). Flotation is achieved with a $ZnSO_4$ saturated solution with a specific gravity of 1.18. Final enumeration is carried out on only part of the flotation aliquot. *Ascaris* ova are recovered by removing the upper portion of the solution, from six 15 ml tubes, with a slide cover slip (four per tube). The slide cover slips are placed over glass slides and the ova are quantified under the microscope. Individual results from the six tubes are combined to provide the final concentration. The Faust technique relies on the same principles as the Leeds I technique where an initial sample of 1 L or 40 L is collected however, additional flotation, centrifugation and sedimentation steps are incorporated. The final concentration is determined by collecting five aliquots of 50 µl each from the final sediment and analysing these samples under the microscope. Results are averaged and extrapolated to yield the final concentration of helminth ova (Maya *et al*., 2006).

Maya *et al*. (2006) compared these four methods for the detection of helminth ova in drinking water, wastewater and synthetic wastewater samples and found that the USEPA technique demonstrated the greatest recovery and may be used on samples with both a high and a low solid content. They found that the total costs involved with the USEPA method were also the lowest and it requires the shortest time period for the training of personnel. Large numbers of samples can also be analysed simultaneously. In a study conducted by Sengupta *et al*. (2012) the re-suspension characteristic and settling characteristics of helminth eggs as well as cohesive sediments in natural freshwater were investigated. A modified McMaster technique developed by Sengupta *et al*. (2011) was applied to detect helminth eggs. The method consisted of concentrating the eggs by centrifugation followed by flotation and counting, using the McMaster slides.

1.4.2.2 Detection of viable *Ascaris* spp. in sludge

The detection, enumeration and determination of viable *Ascaris* ova in sludge samples can be carried out using the USEPA 2003 method. This method consists of blending the initial sample with buffered water containing a surfactant after which the blend is screened for large particles. Solids are then allowed to settle out and the supernatant is decanted. Magnesium sulphate with a specific gravity of 1.2 is added and the sediment is subjected to density gradient centrifugation. This flotation step yields a layer that will contain the *Ascaris* ova present in the sample. Small particles are removed in a second screening step and placed on a small mesh size screen. The concentrate is incubated at 26°C until control *Ascaris* eggs are fully embryonated. A Sedgwick-Rafter counting chamber is used to microscopically examine the concentrate for *Ascaris* ova (USEPA, 2003). In a study conducted by Bastos *et al*. (2013), a slightly modified version of the USEPA 2003 method was used to quantify *Ascaris* sp. and other helminth ova in sewage sludge collected from five wastewater treatment plants in Brazil. The modified version used 50 grams of the initial sample, instead of 300 grams as suggested by Bowman *et al*. (2003), as this reduced volume can favour the hydration of the sample and the quality of the sediment resulting in better microscope visualisation. The authors also found *Ascaris* sp. to be the most prevalent of all the helminths, which indicates that the use of the wastewater for irrigation purposes poses a significant public health concern.

Molecular methods for the detection of helminths, such as *Ascaris* spp. have not been extensively studied. However, Pecson *et al*. (2006) developed a quantitative PCR method, which targets the ITS-1 rRNA gene that is specific to *Ascaris*, for the detection of viable eggs. The method was able to differentiate between viable and non-viable eggs if a 10 day incubation step was introduced prior to DNA extraction. The method is based on the principle that a single-celled viable egg develops into the infective larval stage consisting of 600 cells when incubated (Rousell *et al*., 1994). Subsequently, the non-viable single-celled eggs will not develop into larvae and remain at the single-cell stage. The qPCR signal therefore increased by a factor of ~600 for viable eggs after incubation. In a study conducted by Raynal *et al*. (2012) further optimisation of the method described by Pecson *et al*. (2006) was conducted by determining the quantitative nature between the ITS-1 copy number and the egg number for single-celled and larvated eggs. They further determined the specificity of the qPCR method as well as whether viable and inactivated larvated eggs could be distinguished. The qPCR method was not tested on an actual sample matrix. Ngui *et al*. (2012) then evaluated a real-time polymerase chain reaction (PCR) coupled with high-resolution melting-curve (HRM) assay, as a rapid and sensitive tool for the identification of the five human

hookworm species *Necator americanus*, *Ancylostoma duodenale*, *Ancylostoma ceylanicum*, *Ancylostoma caninum* and *Ancylostoma braziliense*. The study demonstrated that the real-time PCR coupled with HRM assay can serve as a rapid screening protocol for large sample numbers and was more sensitive and specific than a semi-nested PCR for the detection of the hookworm species.

1.5 Rationale and aims

Evaluation of *Cryptosporidium* oocysts in water samples are currently performed using a series of steps which include the collection of a 10 to 1 000 L water sample, concentration by means of filtration, separation of oocysts from environmental debris and subsequent detection and identification. Methods most commonly used for final detection and identification of *Cryptosporidium* oocysts include immuno-fluoresence assays (IFA), flow cytometry, fluorescent *in situ* hybridisation and various polymerase chain reaction (PCR) methods such as conventional PCR, nested PCR, RT-PCR, qPCR, cell culture PCR and IMS-PCR. In addition, the current methods used for the enumeration of helminth ova such as *Ascaris* spp. are extremely time-consuming. These methods are also highly laborious requiring an extensive amount of work to prepare samples and perform the microscopic evaluation, which requires experienced laboratory personnel to perform the analysis.

Molecular biology based methods such as quantitative real-time PCR coupled with HRM curve analyses, offers a powerful alternative to standard methods, as targeting specific genetic markers implies that the detection assays are highly selective, sensitive and rapid. The use of a qPCR method coupled with HRM curve analyses as opposed to a primer-probe based qPCR method was selected in the current study due to the lower cost of a DNA binding dye such as EvaGreen. EvaGreen is a saturated dye which intercalates with all the nucleotides of a double-stranded DNA sequence. The effectiveness of EvaGreen in qPCR and post-PCR DNA melt curve analysis has also been proven in several studies (Ihrig *et al*., 2006; Sang and Ren, 2006; Wang *et al*., 2006; White *et al*., 2007; Akiyama *et al*., 2009; Dagar *et al*., 2009; Li *et al*., 2010; Sun *et al*., 2010; Ujino-Ihara *et al*., 2010). In addition, EvaGreen remains stable and effective as a qPCR reagent for up to 6 months even when exposed to temperatures as high as 65°C (Nowadly *et al*., 2014). Another advantage of a qPCR-HRM approach as opposed to a primer-probe based qPCR analyses, is the elimination of the difficulties that can arise in designing the probe for the sensitive detection of organisms. Primer-probe based qPCR methods thus require sensitivity experiments to ensure that

primer-probe sequences do not amplify more than one species. Quantitative real-time PCR coupled with HRM curve analyses will thus enable the sensitive detection and quantitation of *Cryptosporidium parvum* oocysts in water samples and *Ascaris lumbricoides* eggs in sludge samples. However, due to the fairly novel approach of using a qPCR-HRM assay for the detection of these organisms in the respective matrixes, thorough optimisation of the method will be required before implementation in a routine analysis laboratory.

The primary aims of this study were to develop a quantitative real-time polymerase chain reaction (qPCR) coupled with high-resolution melt curve (HRM) assay for the detection and quantitation of *Ascaris lumbricoides* eggs in sludge samples and to optimise a qPCR-HRM assay for the detection and quantitation of *Cryptosporidium parvum* oocysts in drinking water and environmental surface water. These aims were achieved as follow:

- Chapter two:
    - Optimisation of qPCR-HRM assay for the detection and quantitation of *Ascaris lumbricoides* eggs in sewage sludge samples.
    - Application of the optimised qPCR-HRM assay for the detection of *Ascaris lumbricoides* eggs in sludge collected from wastewater treatment plants.
    - Cost and efficiency comparison of the optimised qPCR-HRM assay with traditional microscopy and a primer-probe based qPCR detection kit for the detection of *Ascaris lumbricoides* eggs in sludge collected from wastewater treatment plants.
- Chapter three:
    - Optimisation of qPCR-HRM assay for the detection and quantitation of *Cryptosporidium parvum* oocysts in water samples.
    - Application of the optimised qPCR-HRM assay for the detection of *Cryptosporidium parvum* oocysts in drinking water.
    - Application of the optimised qPCR-HRM assay for the detection of *Cryptosporidium parvum* oocysts in environmental surface water.
    - Cost and efficiency comparison of the optimised qPCR-HRM assay with a primer-probe based qPCR detection kit for the detection of *Cryptosporidium parvum* oocysts in environmental surface water.

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Chapter 2:

Optimisation of Real-Time Polymerase Chain Reaction Coupled with High-Resolution Melt Curve Assay for the Detection, Identification and Quantitation of *Ascaris lumbricoides* in Sludge

(Chapter 2 is compiled in the format of Science of The Total Environment Journal and UK spelling is employed)

Optimisation of a Real-Time Polymerase Chain Reaction (qPCR) Coupled with High-Resolution Melt (HRM) Curve Assay for the Detection, Identification and Quantitation of *Ascaris lumbricoides* in Sludge

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Abstract

Molecular biology based methods such as quantitative real-time polymerase chain reaction (qPCR), are a powerful alternative to standard methods for the routine monitoring of environmental samples. These assays are highly specific and can offer rapid detection and quantitation of intestinal parasites such as *Ascaris lumbricoides*. The primary aim of the current study was to optimise a real-time PCR coupled with high-resolution melt (HRM) curve assay for the detection and quantitation of *A. lumbricoides* eggs in sludge. The DNA extraction efficiency of three different kits coupled with bead beating and freeze-boil cycles as well as with liquid nitrogen and sonication were compared. For quantitation purposes, standard curves were set up using synthetic gBlocks Gene Fragments, with the detection limits calculated in terms of ng/µl. However, a standard curve with dilutions up to $1x10^{-5}$ ng/µl could only be constructed, which was lower than concentration detected in the sludge samples and limited further quantitation. The optimised qPCR-HRM assay was then applied to sludge samples collected from six different wastewater treatment plants and compared to traditional microscopy analysis as well as the commercially available Genesig kit for *Ascaris* detection (presence/absence). The QIAamp Fast DNA Stool Mini kit coupled with bead beating and freeze-boil cycles proved to be the most effective method for DNA extraction and qPCR-HRM assay of *A. lumbricoides* eggs. High-resolution melt (HRM) profiles obtained from target amplicons were characterised by a peak of 78.10 ± 0.26 °C for *A. lumbricoides* and the qPCR procedure was successful in detection of *A. lumbricoides* eggs in sludge samples. Application and comparative studies between the qPCR-HRM assay, Genesig kit and traditional microscopy method indicated that the qPCR-HRM assay was the most cost- and time-effective method for the evaluation of the presence/absence of *Ascaris lumbricoides*, however the Genesig kit had the added advantage of quantifying the *Ascaris lumbricoides* copy numbers (2 copies/µl to $2x10^{5}$ copies/µl) in the sludge samples.

Key words: Quantitative real-time PCR; High-resolution melt curve assay; *Ascaris lumbricoides*; gBlocks Gene Fragments; Microscopy

2.1 Introduction

Wastewater has become a popular alternative as a source of irrigation water in the agricultural sector, as it contains organic matter, nitrogen and phosphorous, leading to fertilising effects and increased crop yields (Maya *et al*., 2006; WHO, 2006a; Jimenez, 2007; Bastos *et al*., 2013). Indicator organisms such as *Escherichia coli (E. coli)* and thermotolerant coliforms are traditionally used to assess the risk associated with the use of wastewater in agriculture, however they are not suitable for routine monitoring as their concentrations often do not correspond with the concentration of viruses, protozoa or helminths (Ashbolt *et al*., 2001; Maya *et al*., 2006; WHO, 2006a). Helminth species infects approximately 1.2 billion people worldwide (WHO, 2006b), with *Ascaris lumbricoides* (*A. lumbricoides*) being the most common helminth detected in wastewater (Maya *et al*., 2006).

Ascaris lumbricoides is a roundworm belonging to the phylum Nematoda. It is the largest roundworm that infects humans (especially those living in developing countries) causing the disease ascariasis (Harhay *et al*., 2010). The eggs of *A. lumbricoides* can enter water sources through direct faecal contact, discharge from treated or untreated sewage water as well as surface runoff water (Kirby *et al*., 2003). In addition, the eggs are surrounded by a resilient lipid layer, which results in their increased resistance to the effects of acids and alkalis (Piper, 2007). Helminth species such as *Ascaris* spp. also survive for extremely long periods of time in water, on crops as well as in soil and it is therefore of great importance to assess their presence and viability in wastewater to be used for irrigation purposes, as they can be harmful to farmers, consumers and surrounding communities (Sengupta *et al*., 2011).

Currently there is no internationally accepted standard method for the detection of helminth ova in wastewater sources (Maya *et al*., 2006). Four main techniques can however be utilised for the detection of helminth ova in water and they include the USEPA method (Yanko, 1987), membrane filtration (Galván *et al*., 1996), Leeds I (Ayres, 1989) and Faust techniques (Faust *et al*., 1939). All four detection methods consist of two main steps; firstly separation, recovery and the concentration of the helminth ova from the sample sediment and secondly the visual enumeration of the helminth ova by means of microscopy (Maya *et al*., 2006). These methods are categorised based on the means of separation, recovery and concentration of the ova, whether microscopic analysis is carried out on all or only part of the concentrated sample, and the volume of the initial sample (Maya *et al*.,

2006). Comparison of these four methods for the analysis of drinking water, wastewater and synthetic wastewater samples showed that the USEPA technique demonstrated the greatest recovery and may be used on samples with both a high and a low solid content. It was also found that the USEPA method was the most cost-effective of the four methods analysed and large numbers of samples could be analysed simultaneously. Moreover, an advantage of utilising these four methods for the enumeration of helminth ova is that specialised or expensive equipment is not required. However, these methods are highly laborious and time-consuming, often requiring approximately 16 hours to complete (Maya *et al*., 2006). In addition, trained personnel are required to perform the analysis and correctly identify and enumerate the helminth through microscopic analysis (Raynal *et al*., 2012).

In order to accurately evaluate the removal of *Ascaris* sp. in wastewater treatment processes, the development of rapid, accurate and reproducible methods for the detection and quantitation of these pathogens in sludge is required. Molecular biology based methods are a powerful alternative to standard methods and the polymerase chain reaction (PCR) technique in particular is a highly specific and rapid detection method which targets specific genetic markers. In addition, the PCR technique has been shown to be more reproducible and sensitive in the detection of different parasites and is capable of distinguishing between different species (Carey *et al*., 2003; McPherson *et al*., 2007; Ayaz *et al*., 2013). This method also allows for the detection and, in the case of certain PCR techniques, the quantitation of multiple samples in a relatively short period of time. Quantitative real-time PCR (qPCR) has thus emerged as a promising method for the detection and quantitation of target gene sequences in a variety of matrices. The entire assay can be completed within a few hours, making qPCR an extremely rapid method of detecting pathogens of all types, including bacteria, viruses, protozoa, and helminths. In a study conducted by Pecson *et al*. (2006), a primer-probe based quantitative real-time PCR (qPCR) method was developed, which targets the ITS-1 rRNA gene that is specific to *Ascaris*. The method was able to differentiate between viable and non-viable eggs if a 10 day incubation step was introduced prior to DNA extraction. The method was based on the principle that a single-celled viable egg develops into the infective larval stage consisting of 600 cells when incubated (Rousell *et al*., 1994). Subsequently, the non-viable single-celled eggs will not develop into larvae and remain at the single-cell stage. The qPCR signal therefore increased by a factor of ~600 for viable eggs after incubation.

Thus while a primer-probe based qPCR method allows for the sensitive detection and quantitation of a specific organism, a qPCR high-resolution melt (HRM) curve assay approach eliminates the

need for costly probes that needs to be synthesised and the complex process of designing the sequence specific probes. Typically for HRM assays a fluorescent intercalating dye is added to the PCR reaction. This dye binds to the double stranded amplicon DNA, which is then gradually heated from approximately 50˚C to 95˚C. At some point during this heating process, the melting temperature of the double stranded DNA is attained and the two strands of DNA dissociates into single strands. Thus, qPCR-HRM assay reduces variation and turnaround time, enhances sensitivity and has a higher throughput with minimal amplicon contamination due to the closed-vessel system utilised (Pangasa *et al*., 2009; Al-Mohammed, 2011; Zhang *et al*., 2012). Ngui *et al*. (2012) evaluated a real-time polymerase chain reaction (qPCR) coupled with HRM analysis, as a rapid and sensitive tool for the identification of the five human hookworm species *Necator americanus*, *Ancylostoma duodenale*, *Ancylostoma ceylanicum*, *Ancylostoma caninum* and *Ancylostoma braziliense*. The study demonstrated that the real-time PCR coupled with HRM assay can serve as a rapid screening protocol for large sample numbers and was more sensitive and specific than a semi-nested PCR for the detection of the hookworm species.

The primary aim of the current study was to develop and optimise a novel, quantitative real-time PCR coupled with high-resolution melt curve (qPCR-HRM) assay for the routine detection, identification and quantitation of *Ascaris lumbricoides* eggs in sludge samples. The efficiency of the optimised method was investigated utilising wet and dry sludge samples obtained from six wastewater treatment plants operated by the East Rand Water Care Company (ERWAT), South Africa. This study was contracted by ERWAT in order to replace the current microscopy method employed for the routine detection of *Ascaris lumbricoides* in sludge samples. The commercially available Genesig kit for *Ascaris lumbricoides/Ascaris suum* genomes was also utilised for the detection of *Ascaris* species in the sludge samples collected from the respective waste water treatment plants (WWTP) in order to compare the use of a primer-probe detection system with a DNA intercalating dye coupled with the HRM assay approach.

2.2 Materials and Methods

2.2.1 Sources of Ascaris lumbricoides *for optimisation experiments*

Isolates of *Ascaris lumbricoides* were required for use in DNA extraction and PCR optimisation experiments. For this, sewage sludge samples containing viable *Ascaris lumbricoides* eggs were obtained from a private client of ERWAT for the optimisation of the qPCR-HRM assay. The eggs were concentrated from the sludge samples (section 2.2.2) and their presence was confirmed using

phase contrast microscopy (section 2.2.2). Various DNA extraction protocols were then compared (section 2.2.3) followed by qPCR-HRM assay optimisation (section 2.2.4).

2.2.2 Concentration and confirmation of Ascaris lumbricoides eggs from sludge

Ascaris lumbricoides eggs were separated from the sludge sample using the zinc sulphate ($ZnSO_4$, specific gravity 1.3) flotation method coupled with membrane filtration (as developed by ERWAT). Briefly, the sludge sample (1 L) was mixed by swirling and stirring with a plastic rod. From the total sample, 15 ml sub-samples* were decanted into 50 ml test tubes. Ten millilitres of ammonium bicarbonate (AmBic) solution was added to the samples. The samples were vortexed for 30 seconds and another 10 ml wash solution (AmBic) was added. This procedure was repeated until the tubes were filled to approximately 1 cm from the top. A 150 µm sieve was placed over a plastic beaker and the well-mixed solutions were filtered through consecutively, rinsing the tube with tap water in between filtering. The filtrate was poured into test tubes and centrifuged at 2 900 rpm for three minutes. The supernatant was carefully removed with a plastic pipette and discarded. The remaining deposits were combined into a suitable number of tubes so that a maximum of 5 ml was placed into each 50 ml tube. Each of the deposits were then re-suspended in 10 ml $ZnSO_4$ and mixed well by vortexing. This procedure was repeated until the tubes were filled to approximately 1 cm from the top. The tubes were then centrifuged at 1 900 rpm for three minutes. The supernatant was filtered through a 10 µm nitrocellulose filter using a vacuum pump at a flow rate of approximately ≥ 65 mL/min/cm^2 at 0.7 bar (70 kPa). The filters were then placed into 50 ml tubes and washed with 5 ml dH_2O. The tubes were centrifuged at 4 000 rpm for one minute and the filters were removed. The 5 ml dH_2O was concentrated in 2 ml microcentrifuge tubes to obtain a pellet. Each pellet was then re-suspended in 200 µl dH_2O and subsequent experiments were performed.

When dry sludge samples were used for the application of the qPCR-HRM assay, 3 g sub-samples* were weighed out from the total sample into 50 ml test tubes. Ten millilitres of AmBic solution was then added to the samples. The samples were vortexed and another 10 ml wash solution was added. This procedure was repeated until the tubes were filled to approximately 1 cm from the top of the tube. Dry sludge samples were vortexed for an extended period of 30 minutes in total (dry sludge samples need to be vortexed longer than wet sludge samples in order to ensure complete release of eggs from solid materials).

*Note: It was preferable to work with small sub-samples as eggs may not be as easily released from a large sample to float out of the sludge when performing the $ZnSO_2$ flotation technique. The number of sub-samples was increased to prevent overloading of the tubes.

After the eggs were concentrated from the sludge samples using the $ZnSO_4$ flotation method, all samples were analysed using the BX43 phase contrast microscope (Olympus Life Science Solutions, Japan) under 10X magnification in order to confirm the presence of *Ascaris lumbricoides* eggs in the sample before subsequent DNA extractions continued.

2.2.3 Optimisation of DNA extractions from concentrated sewage sludge samples

To optimise the qPCR-HRM assay, DNA extracted from *A. lumbricoides* was required. Three different protocols (modified as outlined below) were used to extract DNA from the concentrated sewage sludge samples for use in the optimisation of the qPCR-HRM assay. After DNA extraction utilising the respective methods, representative samples were sent for DNA concentration analysis by means of spectrophotometry (NanoDrop, Inqaba Biotec, South Africa) in order to evaluate the efficiency of each kit. It should however, be noted, that as general DNA extraction kits and extraction methods were utilised in the study, the DNA concentrations were representative of all the DNA present in the original concentrated sludge samples.

2.2.3.1 Instagene Matrix kit

This DNA extraction kit was applied to the concentrated sewage sludge samples using a slightly modified protocol. Briefly, 200 µl of Instagene Matrix solution was added to the sample pellet and incubated at 56°C for 20 minutes. The suspensions were boiled at 98°C for 15 minutes, mixed and then frozen at -22°C for 30 minutes and again boiled at 98°C for 15 minutes. Hereafter the solution was centrifuged at 10 000 rpm for 2 minutes and the supernatant, containing purified DNA, was used for further qPCR analyses. All extracted DNA was stored at -22°C until further use.

2.2.3.2 QIAamp DNAeasy Blood and Tissue kit

DNA was extracted from 200 µl of concentrated sludge sample containing *Ascaris lumbricoides* eggs using the QIAamp DNAeasy Blood and Tissue kit (Qiagen, Germany) according to the manufacturer's instructions. The protocol for the purification of total DNA from animal tissue was used and the lysis step was carried out for a total of three hours. Extracted DNA was stored at -22°C until further use.

The QIAamp DNAeasy Blood and Tissue kit (Qiagen, Germany) was also used in conjunction with bead beating and freeze-boil cycles to extract DNA from the *A. lumbricoides* eggs. Briefly, the 200 µl sample was transferred to a Beadbug tube containing 0.5 mm acid-washed silica beads (Sigma-Aldrich, USA). The Beadbug tube was vortexed for 5 minutes and then subjected to three

consecutive freeze-boil cycles (freeze at -22°C, boil at 100°C). The QIAamp DNAeasy Blood and Tissue kit protocol was then followed according to the manufacturer's instructions with a lyses step of three hours. Extracted DNA was stored at -22°C until further use.

2.2.3.3 QIAamp Fast DNA Stool DNA Mini kit

DNA was extracted from concentrated sludge samples containing *A. lumbricoides* eggs using the QIAamp Fast DNA Stool Mini kit (Qiagen, Germany) with minor modifications as outlined in Dobrowsky *et al*. (2014). Briefly, 1 ml InhibitEX buffer was added to the 200 µl sample and vortexed for 1 minute. The sample was incubated at 95°C for 5 minutes. In order to remove the inhibitors bound to InhibitEX, the sample was centrifuged at 14 000 rpm for 1 minute. The supernatant (400 µl) was transferred to a microcentrifuge tube containing 15 µl of proteinase K. Before incubating the sample at 70°C for 10 minutes, 400 µl of buffer AL was added, and after incubation, 400 µl of ethanol (96 to 100%) was added to each sample. In order to filter the complete lysate through the QIAamp spin column, approximately 600 µl of the lysate was added to the QIAamp spin column and centrifuged at 14 000 rpm for 1 minute. This process was repeated until the full lysate had been filtered through the QIAamp spin column. The manufacturer's protocol was then followed. Extracted DNA was stored at -22°C until further use.

The QIAamp Fast DNA Stool Mini kit was also used in conjunction with bead beating coupled with freeze-boil cycles to extract DNA from the *A. lumbricoides* eggs. Briefly, the 200 µl sample was transferred to a Beadbug tube containing 0.5 mm acid-washed silica beads (Sigma-Aldrich, USA). The Beadbug tube was vortexed for 5 minutes and then subjected to three freeze-boil cycles (freeze at -22°C, boil at 100°C). The 200 µl sample was then transferred to a 2 ml microcentrifuge tube and the QIAamp Fast DNA Stool Mini kit protocol was then followed as previously described. Extracted DNA was stored at -22°C until further use.

The QIAamp Fast DNA Stool Mini kit was lastly used in conjunction with freeze-boil cycles using liquid nitrogen and boiling, followed by sonication as outlined in Guy *et al*. (2003) to extract DNA from concentrated sludge samples containing *A. lumbricoides* eggs. Briefly, 1 ml InhibitEX buffer was added to the 200 µl sample and vortexed for 1 minute. The sample was heated at 95°C for 5 minutes. Fifteen microliters Proteinase K was pipetted into a separate microcentrifuge tube. The sample was then spun down at 14 000 rpm for 1 minute and 400 µl supernatant added to the Proteinase K along with 400 µl buffer AL and vortexed briefly. The sample was incubated at 55 °C for 1 hr. This was followed by three freeze-boil cycles at 2 minute intervals using liquid nitrogen

and boiling (100°C) followed by three 20 second bursts of sonication (Branson Ultrasonic Corp., CT, USA). The QIAamp Fast DNA Stool Mini kit protocol was then followed as previously described. Extracted DNA was stored at -22°C until further use.

2.2.4 Optimisation of the real-time polymerase chain reaction coupled with high-resolution melt curve analysis (qPCR-HRM) assay

Ascaris lumbricoides DNA isolated from sewage sludge samples were used for the optimisation of the qPCR-HRM assay. Reactions were prepared using the SensiFAST HRM kit (Bioloine, UK) containing the reaction buffer and all reaction components. Three different pairs of suitable primers (IDT, USA) were compared for the amplification of *Ascaris lumbricoides* gene targets (**Table 1**). Reactions were initially set up as outlined in Pecson *et al*. (2006). Each PCR reaction was made up to a final volume of 25 µl and the mixture contained 5 µl DNA as template, a final concentration of 1x SensiFAST Mix and 0.7 µM of each respective primer. As outlined by Pecson *et al*. (2006), the amplification was performed with an initial incubation of 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of alternating temperatures of 95°C for 15 seconds and 60°C for 1 minute. All samples were analysed using a RotorGene 6000, 2-plex PCR thermal cycler with HRM capability (Qiagen, Germany). Fluorescence data was collected at the end of each cycle. A negative control was included in every assay. High-resolution melt curve assay was performed after each run with programming consisting of an initial 90 second pre-melt conditioning step followed by ramping from 70°C to 90°C with ramping rate 0.05°C/sec. Melting profiles of the amplification products were then established.

Table 1. Primer sequences utilised for the detection of *Ascaris lumbricoides* by HRM qPCR

Primer name	Sequence 5' – 3'	Target	Reference
ITS1-F	TGCACATAAGTACTATTTGCGCGTAT	82 bp of ITS-1 region of rDNA	Pecson *et al*. 2006
ITS1-R	TGATGTAATAGCAGTCGGCGG		
Asc1-F	GTTAGGTTACCGTCTAGTAAGG	142 bp of cytochrome b fragment	Loreille *et al*. 2001
Asc2-R	CACTCAAAAAGGCCAAAGCACC		
NC5-F	GTAGGTGAACCTGCGGAAGGATCAT	1000 bp of entire ITS nuclear region (ITS1, 5.8S, ITS2)	Cavallero *et al*. 2013
NC2-R	TTAGTTTCTTCCTCCGCT		

Based on the results obtained, the PCR reaction was consequently optimised by increasing stringency in order to eliminate extensive primer dimer formation and non-specific binding. The optimised PCR reaction for *A. lumbricoides* was made up to a final volume of 20 µl (SensiFAST Mix manufacturer's protocol), rather than the volumes previously outlined in Pecson *et al*. (2006).

The mixture contained 4 µl template DNA, a final concentration of 1x SensiFAST Mix and 0.2 µM of each respective primer. The optimised amplification was performed with an initial incubation step of 95˚C for 10 minutes, after which 45 cycles of denaturation at 95˚C for 30 seconds, annealing at 62˚C for 30 seconds and elongation at 72˚C for 30 seconds was completed with a final elongation step at 72˚C for 5 minutes. Fluorescence data was collected at the end of each cycle and all samples were analysed using a RotorGene 6000, 2-plex PCR thermal cycler with HRM capability (Qiagen, Germany). A negative control was included in every assay. High-resolution melt curve assay was performed after each run and the Tm for amplification products was established.

Representative qPCR amplification products that produced a single peak during HRM analysis were submitted for nucleotide sequencing (Inqaba Biotec, South Africa). Sequence identification was completed using the National Centre for Biotechnology Information (NCBI) and the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the closest match of local similarity between samples and the international database in GenBank, EMBL, DDBJ and PDB sequence data (Altschul *et al*., 1997). The sequences of representative samples that showed >97% similarity (<3% diversity) to organisms was recorded.

2.2.5 Construction of standard curves intended for quantitation

After numerous attempts, DNA extracted from the sludge samples failed to deliver usable standard curves and the gBlocks gene fragments were then utilised to construct the standard curve for the quantitation of *Ascaris lumbricoides* in samples.

2.2.5.1 Preparation of gBlock Gene Fragments and construction of standard curve

Standard curves intended for the quantitation of *A. lumbricoides* in samples were constructed using gBlocks Gene Fragments (IDT, USA). gBlock fragments are double stranded DNA that is sequence verified and have a high sequence fidelity. They are linear fragments of double stranded DNA and the gBlocks gene fragment used in this study was constructed according to the sequences of the Asc1-F/Asc2-R primers (**Table 1**) that amplify the cytochrome b fragment of *Ascaris lumbricoides*. Solutions of each fragment were prepared according to the manufacturer's instructions. Briefly, each tube containing a lyophilised pellet was centrifuged at 14 000 rpm for 10 seconds after which 20 µl of 1X TE Buffer was added to yield a final concentration of 10 ng/µl. The tubes were vortexed and incubated at 50°C for 20 minutes. Each tube was again vortexed and centrifuged at 14 000 rpm for 10 seconds. A 10 fold dilution series was prepared for each gBlock Gene Fragment for use in setting up standard curves.

The standard curve was constructed by using the serially diluted gBlock Gene Fragment preparation as template in the optimised qPCR-HRM assay. A negative control was included in very run and HRM curve assay was performed after amplification to confirm the Tm of the amplification product. Upon completion of the run, the RotorGene software was used to construct the standard curve. The auto-find threshold function of the RotorGene software scans the selected region of the graph to find a threshold setting which delivers optimal estimates of given concentrations. The range of threshold levels is scanned to obtain the best fit of the standard curve based on the samples that have been defined as standards (i.e. where the R value is closest to 1.0). The dynamic tube normalisation option is selected by default in the software and is used to determine the average background of each sample just before amplification commences. The R and R^2 values indicate the correlation coefficient and the coefficient of determination, respectively and should be as close to 1 as possible indicating a low variance between samples. The slope of the standard curve indicates the efficiency of the qPCR and should be as close to -3.32 as possible indicating 100% efficiency (Efficiency = $10^{(-1/slope)}$-1). The efficiency of the experiment should be 90% or higher in order for the standard curve to be seen as valid and usable for quantitation (Bustin *et al*., 2009).

Detection of the *A. lumbricoides* gBlocks Gene Fragments were carried out using the SensiFAST HRM kit as per manufacturer's instructions. The Asc1-F/Asc2-R primers used for the assay are indicated in **Table 1** and each PCR reaction was made up to a final volume of 20 µl. The mixture contained 1 µl gBlocks Gene Fragment, a final concentration of 1X SensiFAST mix and 0.2 µM of both the respective forward and the reverse primers. Amplification was performed with an initial incubation at 95°C for 10 minutes, after which 45 cycles of denaturation at 95°C for 30 seconds, annealing at 62°C for 30 seconds and elongation at 72°C for 30 seconds was completed with a final elongation step at 72°C for 5 minutes. Fluorescence data was collected at the end of each cycle. All samples were analysed using a RotorGene 6000, 2-plex PCR thermal cycler with HRM capability (Qiagen, Germany). A negative control was included in every assay. High-resolution melt curve assay was performed after each run and the Tm for the amplification product was established. The software of the RotorGene 6000, 2-plex PCR machine with HRM capability was used to extrapolate a standard curve.

2.2.6 Application of optimised qPCR-HRM assay for the detection of Ascaris *in wastewater sludge samples*

As indicated, the current study was contracted by ERWAT in order to determine whether the qPCR-HRM assay could replace the current microscopy method used. To directly compare these two methods, sludge samples were collected from six different wastewater treatment plants (WWTPs) operated by ERWAT. The six different WWTPs utilise either an activated sludge process or a combination of an activated sludge and bio-filter process to treat wastewater. Two samples (total of 1 L wet sludge and total of 500 g dried compost) were collected from the Heidelberg WWTP (**Figure 1**). In addition, a total of 1 L wet sludge was collected from the Welgedacht WWTP (**Figure 2**) and a total of 500 g dried compost was collected from the Olifantsfontein (**Figure 3**), Ratanda (**Figure 4**), Jan Smuts (**Figure 5**) and Waterval (**Figure 6**) WWTPs, respectively. An aerial view of each WWTP, indicating sampling points and their respective methods of wastewater treatment is indicated in **Figures 1** to **6**.

Figure 1. Map of the Heidelberg WWTP (26°32'23.75"S 28°19'46.52"E) indicating the sampling site for the wet sludge as well as the compost sample. Heidelberg WWTP makes use of an activated sludge process only.

Figure 2. Map of the Olifantsfontein WWTP (25°56'29.77"S 28°12'46.98"E) indicating the sampling site for the compost sample. Olifantsfontein WWTP makes use of a combination of activated sludge and bio-filter process.

Figure 3. Map of the Ratanda WWTP (26°34'57.39"S 28°18'6.23"E) indicating the sampling site for the compost sample. Ratanda WWTP makes use of an activated sludge process only.

Figure 4. Map of the Welgedacht WWTP (26°11'37.04"S 28°28'22.04"E) indicating the sampling site for the wet sludge sample. Welgedacht WWTP makes use of an activated sludge process only.

Figure 5. Map of the Jan Smuts WWTP (26°13'24.09"S 28°22'30.59"E) indicating the sampling site for the compost sample collection. Jan Smuts WWTP makes use of a combination of activated sludge and bio-filter process.

Figure 6. Map of the Waterval WWTP (26°26'13.81"S 28° 5'53.78"E) indicating the sampling site for the compost sample. Waterval WWTP makes use of an activated sludge process only.

The current ERWAT microscopy method was firstly used to enumerate *A. lumbricoides* eggs in seven sludge samples (60 ml each of the two wet sludge samples and 12 g each of the 5 compost samples) collected from the different wastewater treatment plants as described in section 2.2.2. The seven sludge samples were concentrated (section 2.2.2), followed by subsequent DNA extractions with the QIAamp Fast DNA Stool Mini kit in conjunction with bead beating coupled with freeze-boil cycles (section 2.2.3.3). The qPCR coupled with HRM assay was carried out as described in section 2.2.4 using the extracted DNA.

2.2.6.1 Application of the commercial Genesig kit for the detection of Ascaris *in wastewater sludge samples*

The commercial Genesig kit for the detection of *Ascaris* (PrimerDesign, UK) was utilised for the detection of *Ascaris* species in the sludge samples collected from the respective WWTP (section 2.2.6) in order to compare the use of a primer-probe detection system with a DNA intercalating dye coupled with HRM analysis approach. Each Genesig kit PCR reaction was made up to a final volume of 20 µl. The mixture contained 5 µl DNA as template, 10 µl of Oasig 2 x qPCR Mastermix, 1 µl *A. lumbricoides/A. suum* primer-probe mix, 1 µl internal extraction control primer-probe mix and 3 µl RNAse/DNAse free water. In addition, a positive control of *A. lumbricoides/A. suum* DNA, an internal extraction control and a negative control, provided with

the kit, were also included in the qPCR assay. The positive control *Ascaris lumbricoides/Ascaris suum* DNA was also used in order to set up a standard curve with the copy numbers ranging from 2 X 10^5 copies/µl to 2 copies/ µl. The amplification was performed with an initial incubation at 95°C for 15 minutes, followed by 50 cycles of alternating temperatures of 95°C for 10 seconds and 60°C for 1 minute. All samples were analysed using a RotorGene 6000, 2-plex PCR machine. The *A. lumbricoides/A. suum* primer-probe mix that binds to the *Ascaris lumbricoides* DNA was detected through the FAM channel whereas the internal extraction control primer-probe mix was detected through the VIC channel.

Finally the costs and time required for the completion of the ERWAT microscopy method, the qPCR-HRM assay and the Genesig kit (sections 2.2.6 and 2.2.6.1) were calculated and compared.

2.3 Results and Discussion

2.3.1 Ascaris lumbricoides *samples obtained for optimisation of qPCR-HRM assay*

Sewage sludge samples containing high concentrations of *A. lumbricoides* eggs were obtained and concentrated using the $ZnSO_4$ (specific gravity 1.3) flotation method coupled with membrane filtration. The presence of the eggs in the concentrated samples was confirmed using phase contrast microscopy. The DNA extraction methods were optimised using these sewage sludge samples and the subsequent DNA was utilised for the optimisation of the qPCR-HRM detection assay.

2.3.2 Concentration and confirmation of Ascaris lumbricoides *eggs from sewage sludge*

A significant challenge that arises with the detection of *A. lumbricoides* is the initial concentration and recovery of the eggs from the sewage sludge. The $ZnSO_4$ flotation method coupled with membrane filtration was used in this study to concentrate and recover the eggs from the sewage sludge (optimisation of qPCR-HRM assay) as this is the standard method utilised by ERWAT. In addition, this method is based on the USEPA 1994 protocol, where flotation with $ZnSO_4$ with a specific gravity of 1.3 is combined with biphasic as well as sedimentation steps in order to separate the ova from the water source. In a study conducted by Gyawali *et al*. (2015), the $MgSO_4$ (specific gravity 1.2) flotation method was also successful in the concentration and recovery of *Ancylostoma caninum* eggs from raw wastewater and sludge prior to DNA extraction and qPCR assays. However, it was found that the *Ancylostoma caninum* eggs were better recovered from treated wastewater than from the raw wastewater and sludge. This was attributed to the low suspended solid content of treated wastewater as this water source could be directly passed through the 8 µm

polycarbonate filters utilised and the ova could be washed from the membranes. Previous studies also found better retention of ova on membranes during filtering of water samples compared to a potential loss of ova during flotation methods (Nieminski *et al*., 1995; Ferguson *et al*., 2004). Flotation techniques involve multiple steps of centrifugation, flotation and concentration with potential loss of eggs in each step compared to membrane filtration, which involves a single step for the direct recovery of the eggs from the filters (Gyawali *et al*., 2015). However, due to the high solid content and turbidity of raw wastewater and sludge, membrane filtration is not suitable for processing of these matrices. In the current study the $ZnSO_4$ flotation method coupled with membrane filtration was thus used to recover *Ascaris* eggs from sludge. The flotation method was first applied due to the high solid content and turbidity of the sludge, followed by the membrane filtration membrane in order to retain a maximum amount of *Ascaris* eggs for further assay.

After the eggs were concentrated and recovered from the sewage sludge using the $ZnSO_4$ flotation method, all samples were analysed using the BX43 phase contrast microscope under 10X magnification in order to confirm the presence of *Ascaris lumbricoides* eggs. As shown in **Figure 7**, the red arrow indicates a fertilised *Ascaris lumbricoides* egg, oval to round in shape, 45-75 µm long and 35-50 µm wide with a thick outer shell. Following confirmation of the fertilised *A. lumbricoides* eggs, the various DNA extraction protocols were applied and optimised using the concentrated sludge samples.

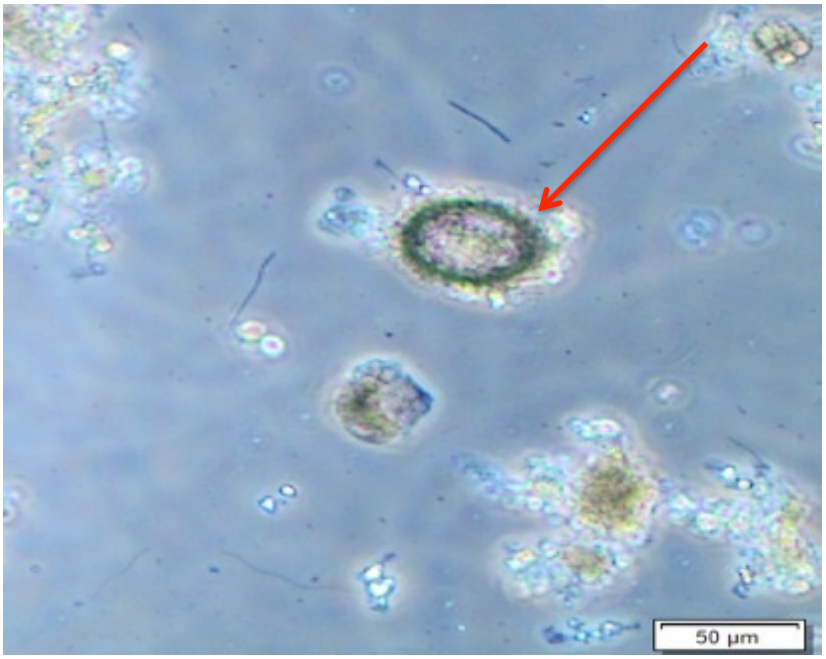

Figure 7. Fertilised *Ascaris lumbricoides* egg concentrated from a sewage sludge sample viewed with phase contrast microscopy at 10X maginification.

2.3.3 DNA extraction methods

The Instagene Matrix, DNAeasy Blood and Tissue kit (utilised with and without acid-washed silica beads and freeze-boil cycles) as well as the QIAamp Fast DNA Stool Mini kit (utilised with and without acid-washed silica beads and freeze-boil cycles as well as in conjunction with liquid nitrogen and sonication) were utilised for the extraction of DNA from the concentrated sludge samples. The eggs of *A. lumbricoides* have a strong lipid layer that is responsible for their increased resistance to both chemical and physical stresses and it is therefore more difficult to extract DNA from *Ascaris* spp. than from bacteria and viruses (Taniuchi *et al*., 2011). The extraction of DNA for molecular analysis is directly dependant on whether the strong lipid layer of the *Ascaris* eggs is adequately lysed during the DNA extraction process. Incomplete lyses of the eggs will result in a reduced concentration of DNA in the eluate causing false-negative results when conducting qPCR assays and thus three different DNA extraction protocols were used and evaluated in this study.

The efficiency of each DNA extraction method was initially evaluated using spectrophotometry however, DNA concentration results obtained proved to be highly inconsistent, even when analysing the exact same DNA extracts. The efficiency of the DNA extraction methods were then evaluated in terms of the amplification and cycle threshold (Ct) or crossing point values, obtained when the qPCR assay is performed using each DNA sample, provided the expected single peak was produced during HRM analysis (**Table 2**). The Ct value of the qPCR outcome reflects the cycle at which the fluorescence signal exceeded a defined background threshold. At the end of each amplification cycle, the fluorescence signal is measured and the obtained Ct value results from the interpolation of the two signal measurements between which the threshold was crossed. Cycle threshold values, therefore, are positive and continuous up to the last cycle of the reaction. Real-time PCR results differ from the conventional continuous outcome of other diagnostic assays (e.g., enzyme-linked immuno-sorbent assay) in that negative specimens do not yield Ct values [also referred to as quantitation cycle (Cq)] because the fluorescent signal stays below the specified threshold (Caraguel *et al*., 2011).

As indicated in **Table 2**, the Instagene Matrix, the DNAeasy Blood and Tissue kit with no modifications and the QIAamp Fast DNA Stool Mini kit coupled with liquid nitrogen and sonication did not result in any qPCR amplification of DNA extracted from the sewage sludge samples (optimisation experiments) utilising the primer sets indicated in **Table 1**. However, the DNAeasy Blood and Tissue kit in conjunction with bead beating and freeze-boil and the QIAamp Fast DNA Stool Mini kit with no modifications resulted in mean Ct values of 30.03 and 33.55,

respectively. The lowest mean Ct value (29.53) was obtained for the QIAamp Fast DNA Stool Mini kit in conjunction with the bead beating and freeze-boil cycles.

Table 2. Efficiency of DNA extraction kits as determined by positive or negative amplification and Ct values obtained from qPCR-HRM assay of representative samples

DNA extraction kit	qPCR amplification	Number of samples (n)	Mean Ct value
Instagene Matrix	Negative	2	N/A*
DNAeasy Blood and Tissue kit, Animal Protocol	Negative	3	N/A*
DNAeasy Blood and Tissue kit, Animal Protocol in conjunction with bead beating and freeze-boil	Positive	5	30.03
QIAamp Fast DNA Stool Mini kit	Positive	7	33.55
QIAamp Fast DNA Stool Mini kit in conjunction with bead beating and freeze-boil	Positive	7	29.53
QIAamp Fast DNA Stool Mini kit coupled with liquid nitrogen and sonication	Negative	2	N/A*

*N/A = Not applicable

The Instagene Matrix is a low cost DNA extraction kit, can extract DNA from up to 10 samples in less than 45 minutes and requires less ancillary equipment and reagents than many other DNA extraction methods. It has also been shown to be an effective method for DNA extraction from intestinal protozoa such as *Cryptosporidium parvum* oocysts yielding satisfactory templates for PCR purposes (Higgins *et al*., 2001). *Cryptosporidium parvum* oocysts and *A. lumbricoides* eggs have a similar resistance to chemical and physical stresses. The Instagene Matrix was therefore compared for the extraction of DNA from *A. lumbricoides* eggs during this study, however subsequent qPCR-HRM assay resulted in no amplification of DNA (**Table 2**) indicating that the Instagene Matrix did not enable extraction of DNA from the *A. lumbricoides* eggs utilising the conditions employed in the current study.

The DNAeasy Blood and Tissue kit and the QIAamp Fast DNA Stool Mini kit have been proven effective in the lyses of highly resistant organisms such as *Cryptosporidium* oocysts and *Giardia* cysts from environmental water samples and sewage (Guy *et al*., 2003; Dobrowsky *et al*., 2014). The QIAamp Fast DNA Stool Mini kit has the added advantage of the InhibitEX buffer that reduces

qPCR inhibition caused by environmental factors present in the sludge samples. A study conducted by Raynal *et al*. (2012) used the Mobio Ultraclean Faecal DNA isolation kit (Carlsbad, CA) that makes use of bead-beating in order to extract DNA from *Ascaris* eggs. The DNA was extracted from a pure egg solution and not an environmental sample. In the current study bead-beating as well as freeze-boil cycles were added to the respective DNA extraction kits' protocols to evaluate its effect on the lyses of the eggs. The DNAeasy Blood and Tissue kit, Animal protocol, however resulted in no amplification of DNA (**Table 2**) in the current study. The addition of the bead beating and freeze-boil cycles to the DNAeasy Blood and Tissue kit protocol resulted in positive amplification of *Ascaris lumbricoides* DNA with a mean Ct value of 30.03. The addition of the bead beating and freeze-boil cycles to the QIAamp Fast DNA Stool Mini kit also resulted in improved amplification based on the lower mean Ct values (29.53) (**Table 2**) when conducting the qPCR-HRM assay. It can thus be concluded that these physical stresses aided in lysing the strong lipid layer of the *A. lumbricoides* eggs. When conducting qPCR assays, the quantity as well as the quality of the template DNA must be taken into consideration. In the current study it was concluded that the template DNA obtained from the QIAamp Fast DNA Stool Mini kit in conjunction with bead beating and freeze-boil cycles produced the highest quality of DNA resulting in the most consistent results.

In a study conducted by Guy *et al.* (2003) liquid nitrogen and sonication were coupled with the DNAeasy Blood and Tissue kit in order to effectively lyse highly resistant *Cryptosporidium* oocysts and *Giardia* cysts from environmental water samples and sewage. In the current study the QIAamp Fast DNA Stool Mini kit containing the InhibitEX buffer was then also coupled with liquid nitrogen and sonication in order to evaluate its effect on the lyses of the *Ascaris lumbricoides* eggs. This approach however did not result in amplification of DNA as determined by qPCR-HRM assay (**Table 2**). Since it is unlikely that this method did not lyse the eggs, it was hypothesised that the template DNA obtained through this extraction method was not of an acceptable quality in order to conduct qPCR-HRM assay. The DNA would be considered of an acceptable quality if the dsDNA is still intact and not degraded by the physical or chemical lyses and purification methods.

2.3.4 Real-time polymerase chain reaction coupled with high-resolution melt curve (qPCR-HRM) assay

In the current study, the qPCR-HRM assay was carried out with the DNA of each sewage sludge sample extracted with the various DNA extraction protocols as outlined in sections 2.2.3.1 to 2.2.3.3. The three primer pairs compared for the amplification of *Ascaris lumbricoides* during the

current study were the ITS1-F/ITS-R primers, the Asc1-F/Asc2-R primers and the NC5-F/NC2-R primers (**Table 1**). The qPCR-HRM assay was performed with each primer pair for the DNA extracted from the sewage sludge utilising the respective extraction methods as outlined in sections 2.2.3.1 to 2.2.3.3. Results indicated that the Asc1-F/Asc2-R primers (Loreille *et al*., 2001) were the only primer pair that produced positive amplification of *A. lumbricoides* DNA and was therefore used for the optimisation of the qPCR-HRM assay. The Asc1-F/Asc2-R primers amplify a mitochondrial marker instead of genomic DNA. Mitochondrial DNA is present in higher copy numbers than genomic DNA, which makes amplification of mtDNA more likely, especially if the number of eggs in the sample is limited. The specific Tm categorisation of the samples (**Table 3**) was determined by sending representative samples amplified with the Asc1-F/Asc2-R primers and that produced a single peak during HRM analysis for nucleotide sequencing. In the present study, the melting profile was characterised by a peak of 78.10°C ± 0.26°C (*A. lumbricoides*) (**Table 3**). The lowest Tm that was found to be positive for *A. lumbricoides* DNA was 77.63°C, while the highest peak was obtained at 78.69°C. Environmental samples often impact the Tm of a specific qPCR product and it is therefore necessary to consider a wider Tm range than for single organisms. Thus qPCR products with a Tm between the range of 77.63°C and 78.69°C were considered positive for *A. lumbricoides* DNA. Real-time PCR products within this Tm range were sent for sequencing and BLAST analysis confirmed the presence of the *Ascaris lumbricoides* mitochondrion complete genome (accession number HQ704900.1) with an identity of 97% or higher.

Table 3. Results achieved by qPCR-HRM assay of the specific amplicons from mitochondrial DNA extracted from *Ascaris lumbricoides* eggs.

Melting curve assay	Number of samples	Mean melting temperature ± standard deviation (SD)
Ascaris lumbricoides	31	78.17°C ± 0.28°C

As previously discussed (section 2.3.3), DNA extracted with the Instagene Matrix, the DNAeasy Blood and Tissue kit (with no modifications) and the QIAamp Fast DNA Stool Mini kit coupled with liquid nitrogen and sonication did not result in any qPCR amplification. Therefore, only qPCR-HRM results obtained with DNA from the DNAeasy Blood and Tissue kit in conjunction with bead beating and freeze-boil, the QIAamp Fast DNA Stool Mini kit with no modifications and the QIAamp Fast DNA Stool Mini kit in conjunction with bead beating and freeze-boil are discussed below. High-resolution melt curve assay of representative samples are indicated, with the

cycling green fluorescence data indicating positive qPCR amplification from representative samples included in **Appendix 2A**.

2.3.4.1 DNAeasy Blood and Tissue kit (Animal Protocol) in conjunction with bead beating and freeze-boil for the detection of Ascaris lumbricoides *using qPCR- HRM*

Ascaris lumbricoides qPCR-HRM was successfully carried out with the Asc1-F/Asc2-R primers using DNA extracted from the sewage sludge obtained from a private client of ERWAT by means of the DNAeasy Blood and Tissue kit in conjunction with bead beating and freeze-boil method (**Appendix 2A**). As indicated in **Figure 8**, two peaks formed during the HRM analysis. The qPCR product was submitted for sequencing and the BLAST analysis results confirmed that the product with a Tm of 78.07°C (blue curve, **Figure 8**) was positive for *Ascaris lumbricoides* mitochondrion complete genome (accession number HQ704900.1) with an identity of 99%. BLAST analysis results confirmed that the second peak with a Tm of 81.14°C (blue curve, **Figure 8**) showed no significant similarity. An increase in fluorescence was observed for the negative control (red curve, **Figure 8**), however this was attributed to the formation of primer dimers. Primer dimers can form when the stringency of the qPCR conditions are not high enough, they have a lower GC content and will melt at lower temperatures as observed in **Figure 8**.

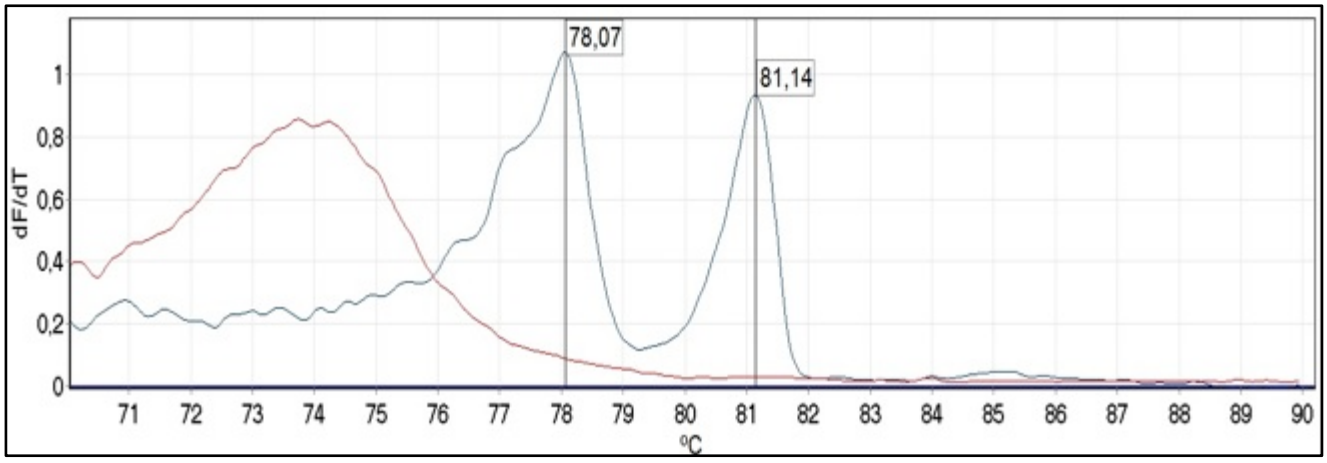

Figure 8. High-resolution melt-curve assay of representative *Ascaris lumbricoides* sewage sludge DNA extracted using the DNAeasy Blood and Tissue kit in conjunction with bead beating and freeze-boil cycles.

The qPCR method was optimised by increasing the stringency of the conditions in order to ensure that the primers only bind to the intended mtDNA marker and produce a single product. As indicated in **Figure 9**, the optimised qPCR-HRM conditions resulted in the production of a single

peak (blue curve) during the HRM analysis with a Tm of 78.16°C. The formation of less primer dimers can also be observed in the negative control (red curve, **Figure 9**).

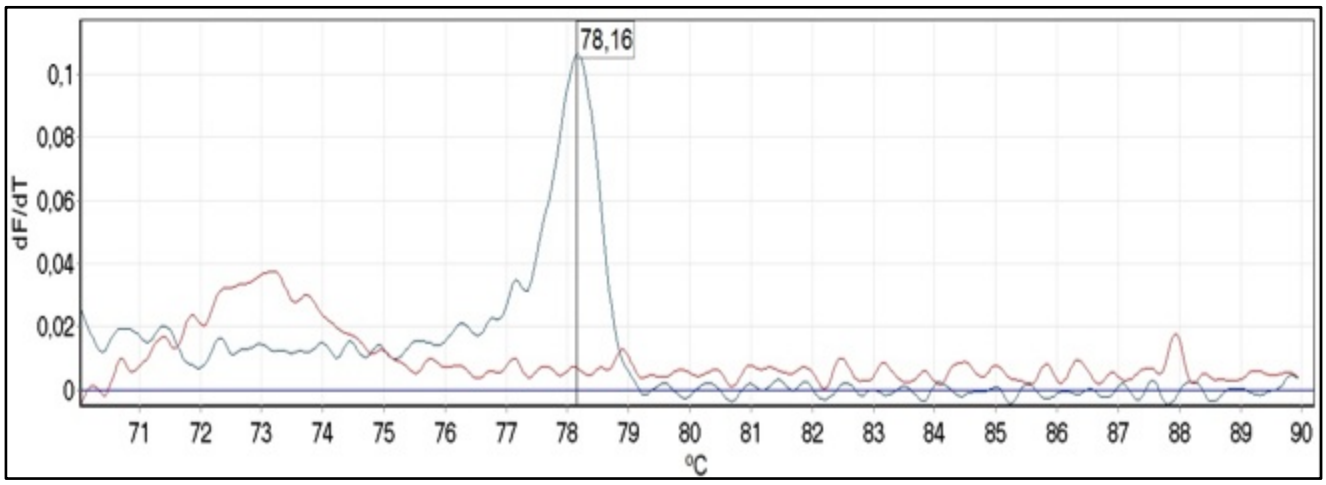

Figure 9. High-resolution melt-curve assay of representative *Ascaris lumbricoides* sewage sludge DNA extracted using the DNAeasy Blood and Tissue kit in conjunction with bead beating and freeze-boil cycles and optimised protocol.

2.3.4.2 QIAamp Fast DNA Stool Mini kit for the detection of Ascaris lumbricoides *using qPCR-HRM*

Ascaris lumbricoides qPCR-HRM was successfully performed using the Asc1-F/Asc2-R primers and DNA extracted from the sewage sludge obtained from a private client of ERWAT by means of the QIAamp Fast DNA Stool Mini kit (**Appendix 2A**). As can be seen in **Figure 10**, this method resulted in the production of a single peak during the HRM analysis with a Tm of 78.00°C and no formation of primer dimers.

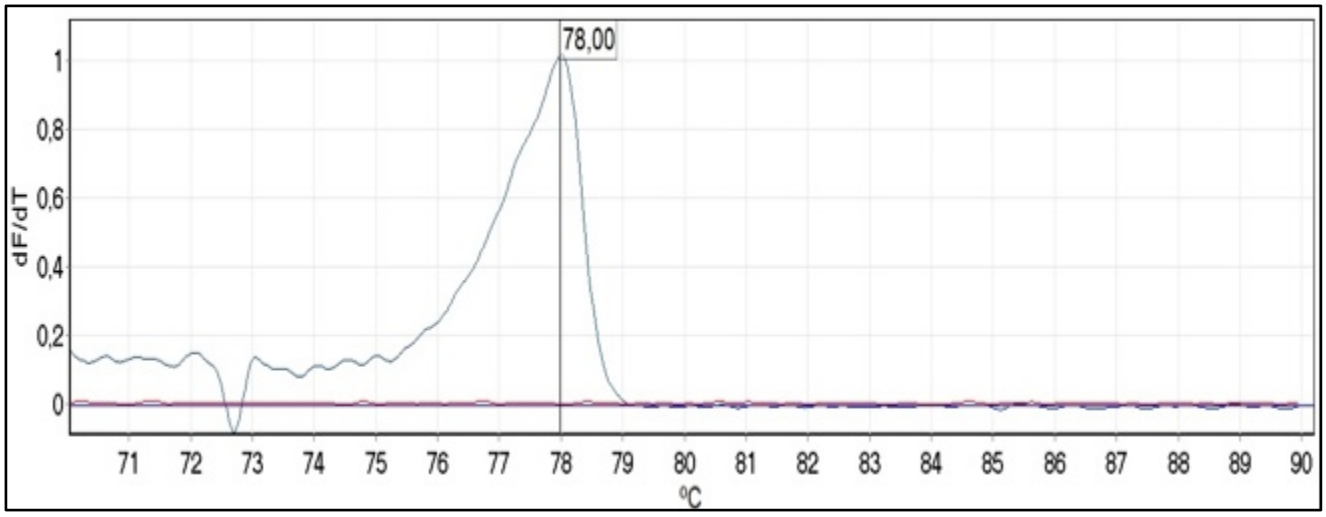

Figure 10. High-resolution melt-curve assay of representative *Ascaris lumbricoides* sewage sludge DNA extracted using the QIAamp Fast DNA Stool Mini kit and optimised protocol.

The qPCR-HRM assay was also successfully carried out on *Ascaris lumbricoides* with the Asc1-F/Asc2-R primers using DNA extracted from the sewage sludge by means of the QIAamp Fast DNA Stool Mini kit in conjunction with bead beating and freeze-boil cycles (**Appendix 2A**). As can be seen in **Figure 11**, this method resulted in the production of a single peak during the high-resolution melt-curve assay with a Tm of 77.63°C and no formation of primer-dimers.

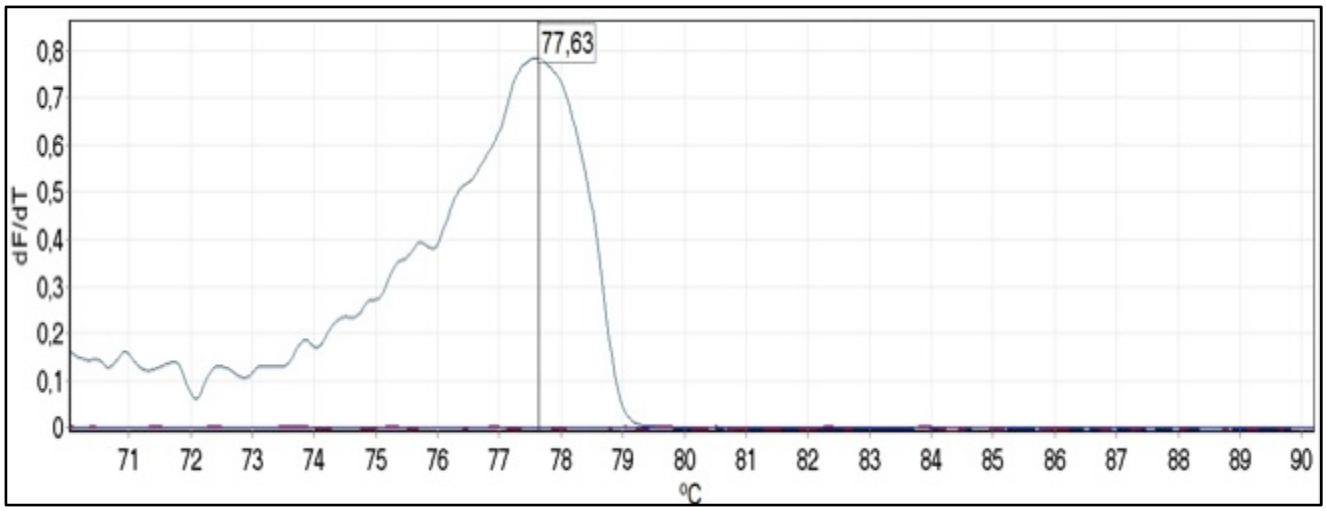

Figure 11. High-resolution melt-curve assay of representative *Ascaris lumbricoides* sewage sludge DNA extracted using the QIAamp Fast DNA Stool Mini kit in conjunction with bead beating and freeze-boil cycles and optimised protocol.

Comparison of the different DNA extraction methods showed that the QIAamp Stool DNA Mini kit in conjunction with the bead beating and freeze-boil cycles was the most succesful method for lyses of the *A. lumbricoides* eggs and produced the highest quality of DNA based on the subsequent Ct values resulting in the most consistent qPCR-HRM results. Adjusting the concentrations of the primers and the DNA, implementing a three step cycling qPCR rather than two step as well as increasing the anealing temperature of the reaction also improved the results of the amplification. The optimised qPCR conditions resulted in only one product forming and thus only one peak during HRM analysis. These results proved to be consistent and reproducible throughout the study.

In the current study, the SensiFAST HRM kit was used for the amplification and detection of the qPCR-HRM products. This kit makes use of EvaGreen, a double strand DNA binding dye. EvaGreen is a saturating dye which intercalates with all the nucleotides of a double-stranded DNA molecule. As the qPCR-amplified DNA fragments are heated and disassociate, the fluorescence of the saturating intercalating dye is measured generating sequence-defined melt curves with single-nucleotide resolution. EvaGreen has a lower cost than other fluorescent dyes and its effectiveness

in quantitative PCR and post-PCR DNA melt curve assay has been proven in several studies (Ihrig *et al*., 2006; Sang and Ren, 2006; Wang *et al*., 2006; White *et al*., 2007; Akiyama *et al*., 2009; Dagar *et al*., 2009; Li *et al*., 2010; Sun *et al*., 2010; Ujino-Ihara *et al*., 2010). EvaGreen has also been shown to be stable and remains effective as a real-time PCR reagent for up to 6 months even when exposed to temperatures as high as 65°C (Nowadly *et al*., 2014).

One of the major limitations when conducting PCR experiments using wastewater matrices is the potential presence of PCR inhibitors (organic and inorganic compounds) (Toze, 1999; Shannon *et al*., 2007). In a study conducted by Gyawali *et al*. (2015) a primer-probe based qPCR method was developed for the detection of *Ancylostoma caninum* ova in wastewater matrices. The presence of qPCR inhibitors in the DNA samples extracted from tap water, secondary treated wastewater, raw wastewater and sludge samples collected from two WWTPs were determined through spiking the DNA samples with 10 pg of *Oncorhynchus keta* (*O. keta*) DNA. The mean Ct values and standard deviation of the *O. keta* contaminated DNA samples were compared to equivalent quantities of *O. keta* DNA suspended in pure water. Results showed that no PCR amplification took place in secondary treated wastewater from one WWTP and sludge from both WWTPs, however after dilution of the DNA samples to reduce any PCR inhibitors, the DNA samples had similar mean Ct values and standard deviation values to that of the *O. keta* DNA spiked in pure water (27.8 ± 0.36). During the current study the presence of PCR inhibitors in the sewage sludge DNA samples were not determined and as a result could be a possible reason for late amplification during qPCR-HRM assay. Future studies should focus on the potential pressence and effects PCR inhibitors may have on the qPCR-HRM detection assay.

2.3.5 Construction of standard curves for quantitation of Ascaris lumbricoides

Obtaining pure samples of RNA or DNA for the use as a positive control or the construction of a standard curve of pathogenic organisms can often be a significant challenge. Alternatively, the construction of synthetically produced DNA may be used in this regard. In the current study synthetic gene fragments [gBlocks Gene Fragments (IDT, USA)] were utilised as positive controls and templates for standard curves intended for the quantitation of an organism. The qPCR-HRM conditions and reagents used for the DNA extracted from the sewage sludge samples were also effective in amplification of the gBlocks gene fragment dilutions (**Appendix 2A**). High-resolution melt curve assay of the amplified products showed that the fragments had the expected Tm of 78.43°C (**Figure 12**) that falls within the Tm range (77.63°C to 78.69°C) determined for the cytochrome b gene (Asc1-F/Asc2-R primer set) through qPCR-HRM results presented in section

2.3.4. The formation of limited primer dimers can be observed in the negative control. The gBlock gene fragments can therefore be used in order to compare and quantitate the DNA concentration of actual samples amplified using the same primer set.

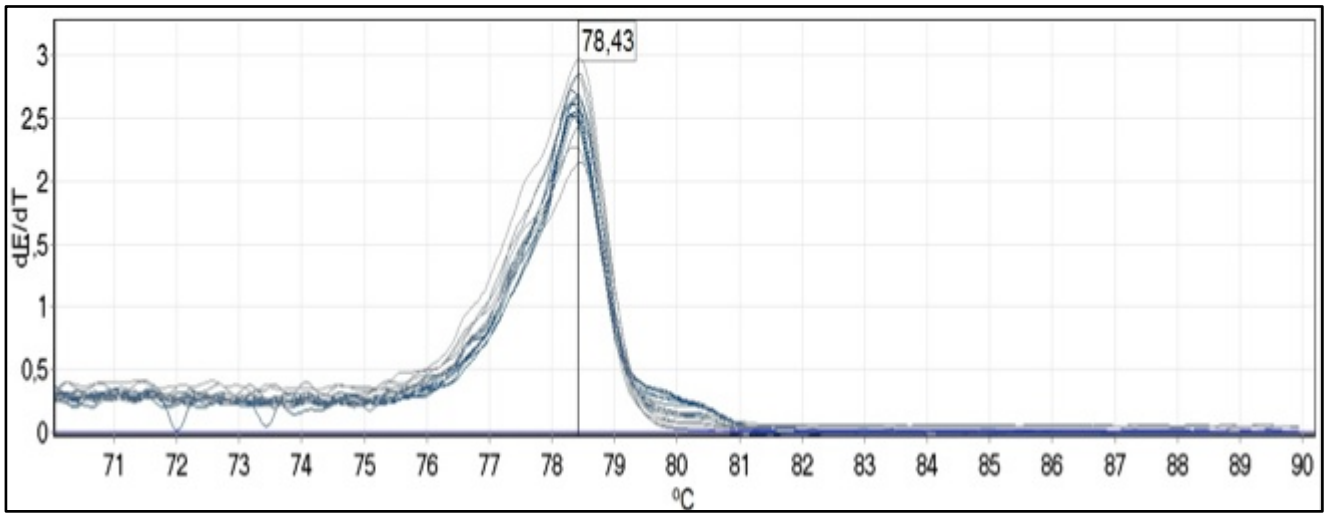

Figure 12. High-resolution melt-curve assay of the tenfold serial dilution of the *Ascaris lumbricoides* cytochrome b gBlocks Gene Fragment ranging from $1x10^{-1}$ ng/µl to $1x10^{-5}$ ng/µl.

The results of the standard curve ($1x10^{-1}$ ng/µl to $1x10^{-5}$ ng/µl) are presented in **Figures 13** and **14**. A strong positive correlation coefficient was observed for the data (R = 0.991) as well as a coefficient of determination very close to 1 indicating low variance between the samples (R^2 = 0.983). The slope of the standard curve indicates the efficiency of the qPCR and should be as close to -3.32 as possible indicating 100% efficiency (Efficiency = $10^{(-1/slope)}$-1). In **Figure 14** the slope (M) was shown to be –3.366 indicating high efficiency of the qPCR experiment (98%). The standard curve (**Figure 14**) corresponds to the amplification plot (**Figure 13**).

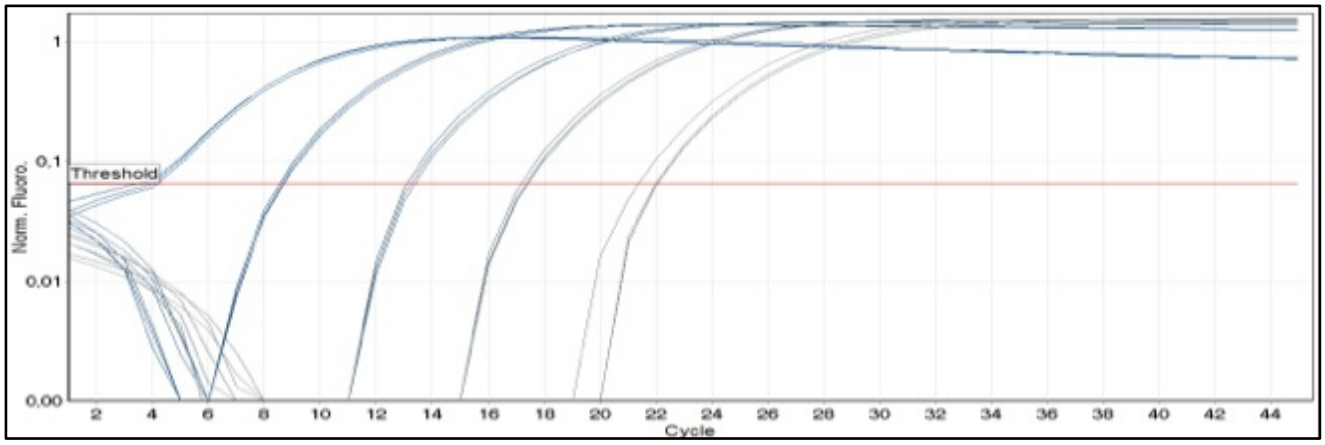

Figure 13. Logarithmic view of the tenfold serial dilution of the *Ascaris lumbricoides* cytochrome b gBlocks Gene Fragment ranging from $1x10^{-1}$ ng/µl to $1x10^{-5}$ ng/µl. Fluorescent detection through the FAM channel. The horizontal line indicates the threshold.

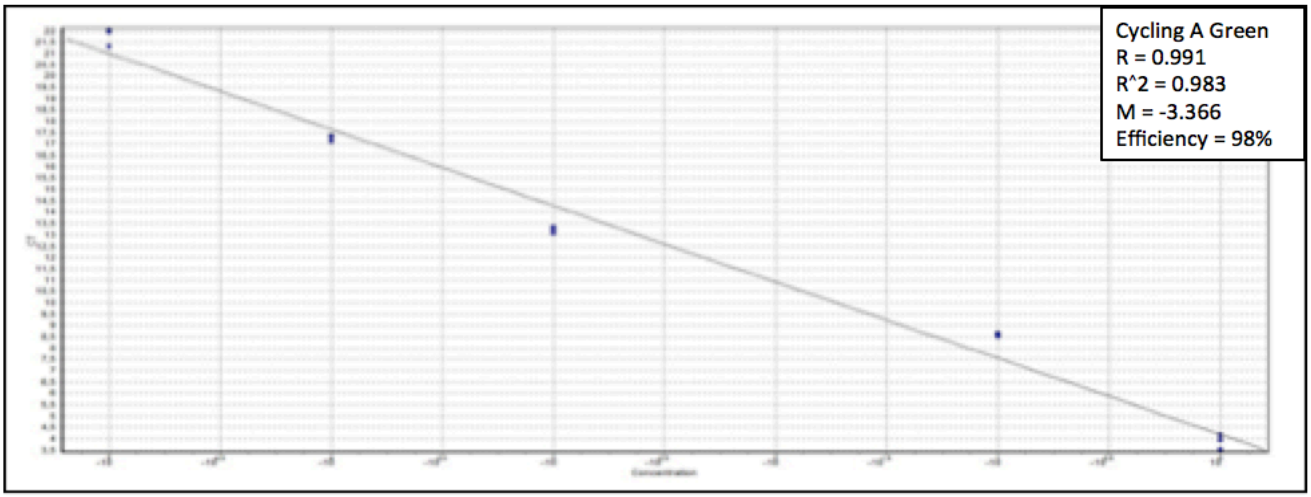

Figure 14. Real-time PCR detection of the tenfold serial dilution of the *Ascaris lumbricoides* cytochrome b gBlocks Gene Fragment ranging from $1x10^{-1}$ ng/µl to $1x10^{-5}$ ng/µl.

The Rotorgene 6000, 2-plex PCR machine software allows for the standard curve to be imported into each separate *Ascaris* qPCR run. This in turn then allows for quantitation of the DNA concentration in the unknown samples. A triplicate sample of one of the gBlocks dilutions used to set up the standard curve must also be included in the run containing the unknown samples in order for the software to recognise it as a standard sample. For quantitation however, the Ct value of the unknown sample must fall within the dilution range of the standard curve. During the current study it was not possible to construct an effective standard curve with dilutions up to $1x10^{-8}$ ng/µl due to the low efficiency observed when working with such low concentrations. The results of the standard curve with dilutions up to $1x10^{-8}$ ng/µl are represented in **Figures 15** and **16**.

Although a strong positive correlation coefficient was observed for the data (R = 0.997) and a coefficient of determination very close to 1 (R^2 = 0.994) was obtained, the slope (M) was shown to be –3.736 indicating a low efficiency (85%) of the experiment (**Figure 16**). As can be seen from the amplification plot (**Figure 15**), triplicates of the lower concentrations do not have similar Ct values or slopes. These discrepancies in turn cause the lower efficiency of the standard curve. In order for a standard curve to be effective in the quantitation of unknown samples, efficiencies of 90% and higher are required (Bustin *et* al., 2009). The most important shortcoming of the qPCR-HRM assay used in this study was thus the inability of the assay to quantify the number of *Ascaris lumbricoides* eggs present (up to $1x10^{-8}$ ng/µl) in the sewage sludge samples. Future studies should aim at optimising the construction of a standard curve which can be successfully used for quantitation purposes.

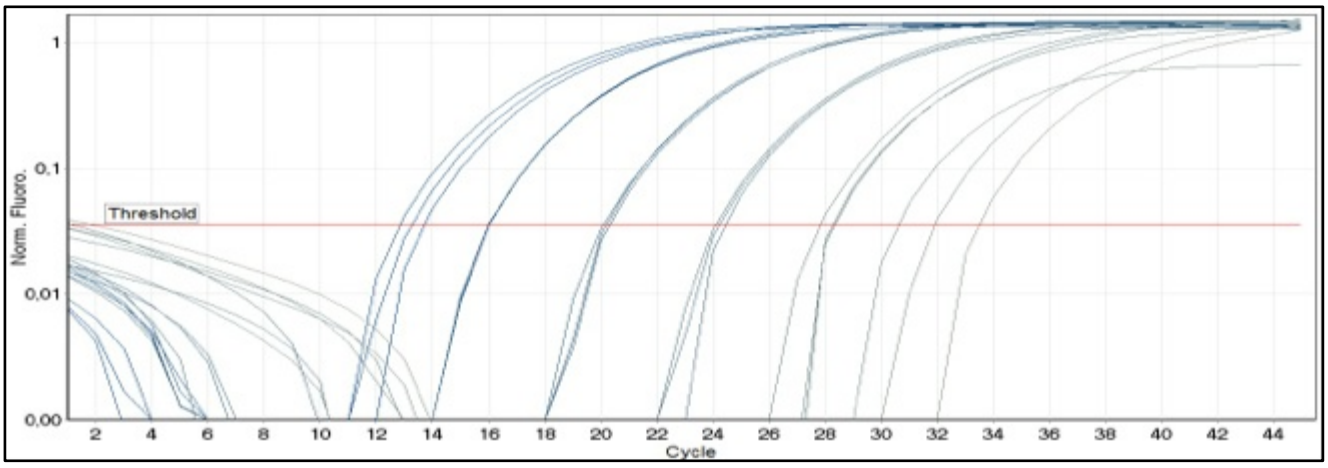

Figure 15. Logarithmic view of the tenfold serial dilution of the *Ascaris lumbricoides* cytochrome b gBlocks Gene Fragment ranging from $1x10^{-3}$ ng/µl to $1x10^{-8}$ ng/µl. Fluorescent detection through the FAM channel. The horizontal line shows the threshold.

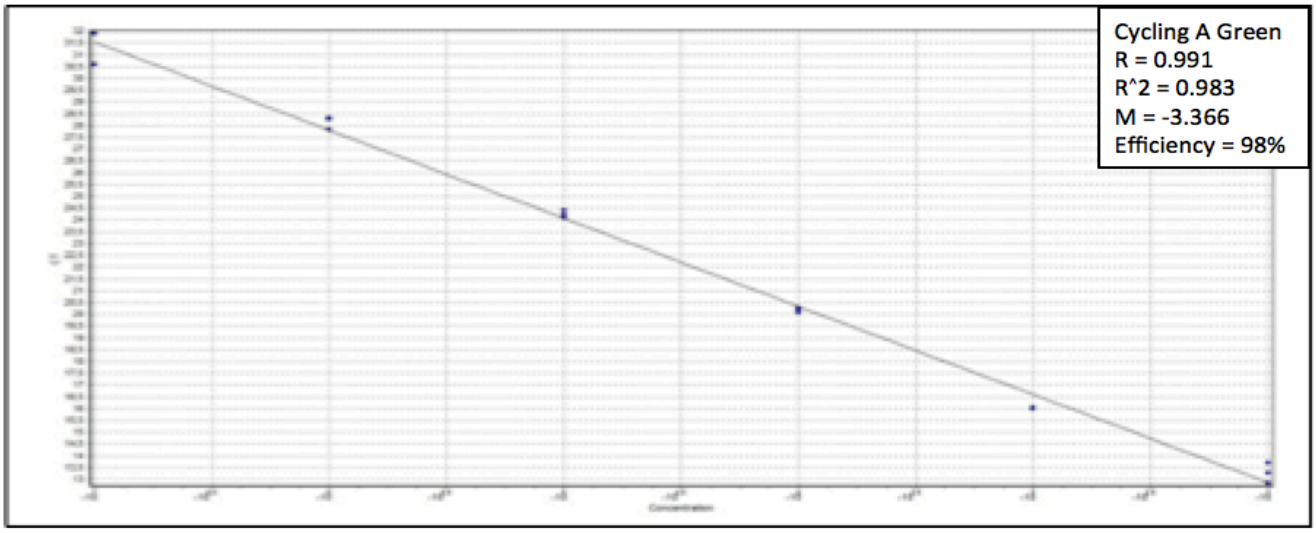

Figure 16. Real-time PCR detection of the tenfold serial dilution of the *Ascaris lumbricoides* cytochrome b gBlocks Gene Fragment ranging from $1x10^{-3}$ ng/µl to $1x10^{-8}$ ng/µl.

2.3.6 Comparison of qPCR assay coupled with HRM assay, Genesig kit and ERWAT microscopy method

To directly compare the qPCR-HRM assay with the current ERWAT microscopy method, seven sludge samples were collected from six different wastewater treatment plants (WWTPs) operated by ERWAT. Two samples were collected from the Heidelberg WWTP and one sample was collected from the Olifantsfontein, Ratanda, Welgedacht, Jan Smuts and Waterval WWTPs, respectively. In addition, the Genesig kit for *Ascaris lumbricoides/Ascaris suum* genomes was also utilised for the detection of *Ascaris* spp. in the sludge samples collected from the respective WWTP

(section 2.2.6) in order to compare the use of a primer-probe detection system with a DNA intercalating dye coupled with HRM analysis approach.

2.3.6.1 Application of the ERWAT microscopy method for the detection of Ascaris *in wastewater sludge samples*

The seven sludge samples collected from the different WWTPs were concentrated using the $ZNSO_4$ flotation method coupled with membrane filtration (section 2.2.2). The ERWAT microscopy method was hereafter used in order to enumerate the *Ascaris lumbricoides* eggs. No eggs could be enumerated in the Heidelberg WWTP wet sludge sample, the Welgedacht WWTP wet sludge sample and the Jan Smuts WWTP compost sample. The Heidelberg WWTP compost sample contained 3 eggs/12 g, the Olifantsfontein WWTP compost sample contained 9 eggs/12 g, the Ratanda WWTP compost sample contained 3 eggs/12 g and the Waterval WWTP compost sample contained 6 eggs/ 12g (**Table 4**).

Table 4. ERWAT microscopy results of seven sludge samples collected from the six different WWTPs

Wastewater treatment plant	Sample type	Number of eggs enumerated per 60 ml (wet sludge) or 12 g (compost)
Heidelberg WWTP	Wet sludge	0 eggs/60 ml
Heidelberg WWTP	Compost	3 eggs/12 g
Olifantsfontein WWTP	Compost	9 eggs/12 g
Ratanda WWTP	Compost	3 eggs/12 g
Welgedacht WWTP	Wet sludge	0 eggs/60 ml
Jan Smuts WWTP	Compost	0 eggs/12 g
Waterval WWTP	Compost	6 eggs/12 g

2.3.6.2 Application of the optimised qPCR-HRM assay for the detection of Ascaris *in wastewater sludge samples*

After the concentration of the seven sludge samples collected from the different WWTPs, DNA extractions were performed using the QIAamp Fast DNA Stool Mini kit in conjunction with bead beating coupled with freeze-boil cycles (section 2.2.3.3). The qPCR-HRM assay was carried out as described in section 2.2.4 using the extracted DNA. Previously extracted *Ascaris lumbricoides* DNA (sewage sludge sample), used in the optimisation of the qPCR-HRM assay, was included as a positive control. The positive control had a Ct value of 26.72 and a melting temperature of 77.90°C. All Ct values and Tm results of the seven sludge sample qPCR-HRM assay are presented in **Table 5**.

Table 5. qPCR-HRM assay of seven sludge samples collected from the six different WWTPs

Wastewater treatment plant	Sample type	Cycle threshold (Ct) value	Melting temperature (Tm) of peaks
Heidelberg WWTP	Wet sludge	28.63	80. 21°C
Heidelberg WWTP	Compost	27.21	73.17°C
Olifantsfontein WWTP[1]	Compost	26.47	78.31°C; 80.91°C
Olifantsfontein WWTP[2]	Compost	29.86	81.16°C
Ratanda WWTP	Compost	26.44	78.31°C; 80.77°C
Welgedacht WWTP	Wet sludge	24.54	80.79°C
Jan Smuts WWTP	Compost	28.60	77.97°C
Waterval WWTP	Compost	26.59	77.89°C

[1] = First qPCR-HRM results

[2] = Second qPCR-HRM results

The DNA from the seven sludge samples amplified during qPCR had Ct values corresponding to the positive control Ct value (26.72). However, when the HRM analysis was conducted, it was found that some of the samples did not produce HRM peaks within the melting temperature profile range (77.63°C to 78.69°C), as determined during the qPCR-HRM optimisation results for *Ascaris lumbricoides* DNA amplified using the Asc1-F/Asc2-R primers. The high Tm (80. 21°C) of the product from the Heidelberg WWTP wet sludge sample could be attributed to non-specific binding of the primers. The low Tm (73.17°C) of the product from the Heidelberg WWTP compost sample could be attributed to the formation of primer dimers. Primer dimers can form when the stringency of the qPCR conditions are not high enough, they have lower GC content and will thus melt at lower temperatures. The compost samples from both the Olifantsfontein and the Ratanda WWTP produced two melt peaks during HRM analysis. The first melt peak had the desired Tm (78.31°C) which fell within the melting temperature profile range of the confirmed *Ascaris lumbricoides,* thus indicating the presence of *Ascaris lumbricoides* DNA, however the Tm of the second peak was much higher (80.91°C and 80.77°C, respectively) which could be attributed to non-specific binding. The high Tm (80. 21°C) of the product formed from the Welgedacht WWTP wet sludge sample could also be attributed to non-specific binding of the primers and was therefore considered negative for the presence of *Ascaris lumbricoides* DNA. The compost samples from both the Jan Smuts and the Waterval WWTPs, also formed products with the desired Tm (77.97°C and 77.89°C respectively), which fell within the melting profile range (77.63°C to 78.69°C) of the confirmed *Ascaris lumbricoides*.

It should be noted that after the seven sludge samples were analysed using the Genesig kit (section 2.3.6.3), it was found that no *Ascaris lumbricoides* DNA could be detected in the Olifantsfontein

WWTP sample (**Table 6**) which was contradictory to the results of the qPCR-HRM results that formed two peaks, 78.31°C confirming the presence of *Ascaris lumbricoides* DNA and 80.91°C indicating non-specific binding. The qPCR-HRM assay was then repeated for all the samples. Results from the second qPCR-HRM assay produced similar results to that of the first qPCR-HRM assay for all the samples (results not shown), with the exception of the Olifantsfontein WWTP compost sample (**Table 5**), which produced only one peak (81.16°C) for the Olifantsfontein WWTP compost sample indicating non-specific binding as opposed to the first qPCR-HRM assay that produced two peaks (78.31°C; 80.91°C), the first of which indicated the presence of *Ascaris lumbricoides* DNA. Results of the second qPCR-HRM assay for the Olifantsfontein sample are also represented in **Table 5**. The qPCR products were sent for sequencing and the BLAST analysis confirmed the presence of *Ascaris lumbricoides* DNA in the positive control, Ratanda WWTP sample, Jan Smuts WWTP sample, Waterval WWTP sample and the first qPCR-HRM assay product of the Olifantsfontein WWTP sample. In addition, *Ascaris lumbricoides* DNA was not detected in the Heidelberg WWTP sample, Welgedacht WWTP sample and the second qPCR-HRM assay product of the Olifantsfontein WWTP (**Appendix 2B**).

2.3.6.3 Application of the commercial Genesig kit for the detection of Ascaris *in wastewater sludge samples*

The DNA extracted from the seven sludge samples obtained from the six WWTP's was used for the qPCR assay utilising the the commercial Genesig kit for the detection of *Ascaris* (PrimerDesign, UK) to compare the use of a primer-probe detection system with a DNA intercalating dye coupled with HRM analysis approach. The standard curve included in the kit quantifies the unknown samples in terms of copy number/µl. A strong positive correlation coefficient was observed for the Genesig kit standard curve data (R = 0.999), with a coefficient of determination very close to 1 indicating low variance between the samples (R^2 = 0.998) and a slope (M) of –3.376 indicating high efficiency (accuracy and consistency) of the qPCR experiment (98%) (**Appendix 2B**).

Due to the high efficiency of the data, the standard curve constructed using the Genesig kit could thus be used in order to quantify the amount of *Ascaris lumbricoides* DNA copy numbers per µl. Amplification of products with Ct values lower than 35 and detected through the FAM channel is considered as positive for the presence of *Ascaris lumbricoides* DNA according to the manufacturer. The internal extraction control DNA added to each sample before the DNA extraction protocol is followed and amplified by the internal extraction control primer-probe mix was detected through the VIC channel and showed positive results for all the samples except the

Olifantsfontein WWTP sludge sample. This indicates that there could have been qPCR inhibitors present in the DNA sample. Results of the Genesig kit assay are represented in **Table 6**. In turn, amplification of the Olifantsfontein WWTP sludge sample was very late (Ct = 44.46) resulting in no *A. lumbricoides/A. suum* DNA quantified by the standard curve (**Table 6**). Very late amplification also took place for the Heidelberg WWTP wet sludge sample and thus the concentrations were too low for detection of copies/µl by the standard curve. This was not attributed to the presence of qPCR inhibitors as the internal extraction control DNA did amplify and was detected in this sample. No qPCR amplification took place for the Welgedacht WWTP sample indicating the absence of *A. lumbricoides/A. suum* DNA. As determined by the standard curve, the Heidelberg WWTP compost sample contained 2 *Ascaris lumbricoides* DNA copies/µl, the Ratanda WWTP compost sample contained 52 *Ascaris lumbricoides* DNA copies/µl, the Jan Smuts WWTP contained 36 *Ascaris lumbricoides* DNA copies/µl and the Waterval WWTP contained 97 *Ascaris lumbricoides* DNA copies/µl.

Table 6. Genesig kit assay of seven sludge samples collected from the six different WWTPs

Wastewater treatment plant	Sample type	Cycle threshold (Ct) value	Copies/µl
Heidelberg WWTP	Wet sludge	37.43	0
Heidelberg WWTP	Compost	33.37	2
Olifantsfontein WWTP	Compost	44.46	0
Ratanda WWTP	Compost	28.74	52
Welgedacht WWTP	Wet sludge	NPA*	N/A*
Jan Smuts WWTP	Compost	29.26	36
Waterval WWTP	Compost	27.81	97

*NPA = No qPCR amplification

*N/A = Not applicable

2.3.6.3.1 Comparison of the efficiency of the ERWAT microscopy method, the optimised qPCR-HRM assay and the Genesig kit for the detection of Ascaris *in wastewater sludge samples*

During the current study it was found that the three methods utilised for the detection of *Ascaris lumbricoides* from sludge samples were relatively comparable with minor differences noted as indicated in **Table 7**. Comparison of the three detection methods yielded negative results for the detection of *Ascaris lumbricoides* in the Heidelberg WWTP wet sludge sample. In addition, the three detection methods yielded positive results for the detection of *Ascaris lumbricoides* in the Ratanda WWTP compost sample and negative results for the detection of *Ascaris lumbricoides* in the Welgedacht WWTP wet sludge sample. The positive detection of *Ascaris lumbricoides* DNA in the Jan Smuts WWTP compost sample, using the qPCR-HRM assay and the Genesig kit, as

opposed to the negative detection in the same sample using the ERWAT microscopy method, could be attributed to human error when conducting microscopy analysis. The three detection methods also yielded positive results for the detection of *Ascaris lumbricoides* in the Waterval WWTP compost sample.

Table 7. Comparison of positive or negative detection of *Ascaris lumbricoides* DNA through qPCR-HRM, Genesig kit and the ERWAT microscopy method from seven samples obtained from six different WWTPs operated by ERWAT.

Wastewater treatment plant	Sample type	qPCR-HRM	Genesig kit	ERWAT microscopy
Heidelberg WWTP	Wet sludge	Negative	Negative (0 copies/µl)	Negative (0 eggs/60 ml)
Heidelberg WWTP	Compost	Negative	Positive (2 copies/µl)	Positive (3 eggs/12 g)
Olifantsfontein WWTP	Compost	Positive[1] Negative[2]	Negative (0 copies/µl)	Positive (9 eggs/12 g)
Ratanda WWTP	Compost	Positive	Positive (52 copies/µl)	Positive (3 eggs/12 g)
Welgedacht WWTP	Wet sludge	Negative	Negative (0 copies/µl)	Negative (0 eggs/60 ml)
Jan Smuts WWTP	Compost	Positive	Positive (36 copies/µl)	Negative (0 eggs/12 g)
Waterval WWTP	Compost	Positive	Positive (97 copies/µl)	Positive (6 eggs/12 g)

*NPA = No qPCR amplification

[1] = First qPCR-HRM results

[2] = Second qPCR-HRM results

The ability to detect *Ascaris lumbricoides* DNA in the Heidelberg WWTP compost sample using the Genesig kit and the ERWAT microscopy method, as opposed to the negative result in the same sample with qPCR-HRM assay, was attributed to the presence of extremely low copy numbers (2 copies/µl) and eggs (3 eggs/12 g) obtained by the two methods, respectively. In addition, *Ascaris lumbricoides* DNA was detected during the first qPCR-HRM assay followed by a negative result during the second qPCR-HRM assay in the Olifantsfontein WWTP compost sample. The internal extraction control is included in the Genesig kit in order to determine whether pure DNA added to the qPCR reaction would amplify and if no amplification takes place, qPCR inhibitors could be present. It was thus hypothesized that qPCR inhibitors were present in this sample due to the fact that the internal extraction control included in the Genesig kit for the Olifantsfontein WWTP compost sample did not amplify and there was no amplification of *Ascaris lumbricoides* DNA in

the sample. It should however, be noted that positive enumeration of *Ascaris lumbricoides* eggs (9 eggs/12 g) was recorded for the ERWAT microscopy method in the Olifantsfontein WWTP compost sample.

A major advantage of the qPCR-HRM assay is that it eliminates the need for post-PCR analysis, such as agarose gel analysis and sequencing. If the HRM product amplified during qPCR shows a resulting Tm of 78.17°C ± 0.28°C, it can be concluded that the product is in fact *Ascaris lumbricoides* DNA. An advantage of the Genesig kit is that it was able to quantify the *Ascaris lumbricoides* copy numbers present in the samples, whereas the qPCR-HRM assay could only detect the presence or absence of *Ascaris lumbricoides*. During the current study it was thus concluded that both the qPCR-HRM assay and the Genesig kit could be compared to the ERWAT microscopy method and applied to sludge samples, however based on the results obtained for certain samples (Olifantsfontein WWTP) further optimisation and monitoring studies will have to be conducted in order to accurately evaluate the efficiency and sensitivity of each kit compared to current microscopy methods.

The cost- and time- efficiency of the ERWAT microscopy method, the qPCR-HRM assay and the Genesig kit was then comprehensively calculated (**Appendix 2C**) and compared (**Table 8**). The ERWAT microscopy method currently used for the enumeration of *Ascaris lumbricoides* eggs in sludge samples has a very low cost for reagents, however due to the extremely long time period (± 50 hrs) and specialised knowledge required to accurately analyse a single concentrated sample under the microscope, the labour costs are relatively high.

Table 8. Comparison of total time required to process samples and costs involved for each method

Indicator	ERWAT Microscopy method	Real-Time PCR coupled with HRM assay	Genesig kit
Total Time	± 50hrs	± 7hrs	± 7hrs
Total Cost	R706.86	R684.75	R900.36

In contrast, the qPCR-HRM assay approach and the Genesig kit are extremely time-effective for the detection of *Ascaris lumbricoides* eggs, with the assay completed in approximately 7 hours. Overall, based on the labour, reagents and analysis costs, the qPCR-HRM assay approach was the most cost-effective method for the detection of *Ascaris lumbricoides* eggs in sludge samples (**Table 8**). The ERWAT microscopy method was marginally more expensive (labour costs), while the

Genesig kit was the least cost-effective. However, an advantage of the Genesig kit is that it is able to quantify the copy numbers of *A. lumbricoides* eggs.

2.4 Conclusions

The efficiency of various protocols for the extraction of *Ascaris lumbricoides* DNA from sewage sludge was evaluated in the current study. The results showed that the QIAamp Fast DNA Stool Mini kit in conjunction with the bead beating and freeze-boil cycles was the most efficient method for the lyses of the *Ascaris lumbricoides* eggs from environmental sludge samples. Template DNA extracted using the QIAamp Fast DNA Stool Mini kit in conjunction with the bead beating and freeze-boil cycles also produced the highest quality of *Ascaris lumbricoides* DNA resulting in the most consistent qPCR-HRM results. The Asc1-F/Asc2-R primers, amplifying the cytochrome b fragment of *Ascaris lumbricoides* mtDNA, also proved effective in detection of this helminth and a Tm was established at 78.17°C ± 0.27°C. A real-time PCR coupled with HRM assay utilising the DNA intercalating dye, EvaGreen, for the detection of *Ascaris lumbricoides* in sludge was thus successfully developed and partially optimised. This method has the potential to be implemented as a routine monitoring method for the detection of *Ascaris lumbricoides* eggs in sludge samples. It can therefore also be used as a valuable tool to aid in the characterisation and classification of sludge generated at wastewater treatment plants in order to determine which management options are suitable and what processes the characterised sludge can be utilised for as stipulated by the Department of Water Affairs and Forestry (2006). Further studies could include quantitative real-time PCR coupled with HRM assay of the *Ascaris lumbricoides* eggs through the optimisation of a standard curve using gBlock gene fragments or purified *Ascaris lumbricoides* DNA.

Comparative analysis of the qPCR-HRM assay, the Genesig kit and the ERWAT microscopy method indicated that the qPCR-HRM assay was the most cost-effective for the evaluation of the presence/absence of *Ascaris lumbricoides*, however the Genesig kit had the added advantage of quantifying the *Ascaris lumbricoides* copy numbers in the samples. These molecular based methods have the potential to replace the current ERWAT microscopy method, however further routine monitoring studies will have to be conducted in order to accurately evaluate and compare the efficiency and sensitivity of the qPCR-HRM assay and the Genesig kit with the current ERWAT microscopy method. In conclusion, implementation of the molecular based qPCR-HRM assay could potentially evaluate the presence or absence and thus, in turn, the removal efficiency of *Ascaris*

lumbricoides from wastewater treatment systems and shows great potential for further quantitation and monitoring studies for water analysis laboratories.

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Chapter 3:

Optimisation of Real-Time Polymerase Chain Reaction Coupled with High-Resolution Melt Curve Assay for the Detection, Identification and Quantitation of *Cryptosporidium parvum* in Water Samples

(Chapter 3 is compiled in the format of Science of the Total Environment Journal and UK spelling is employed)

Optimisation of a Real-Time Polymerase Chain Reaction (qPCR) Coupled with High-Resolution Melt (HRM) Curve Assay for the Detection, Identification and Quantitation of *Cryptosporidium parvum* in Water Samples

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Abstract

Routine water quality monitoring relies mainly on traditional plating methods for the enumeration of indicator microorganisms such as *Escherichia coli* and faecal coliforms. These methods are not applicable for the detection of protozoan parasites such as *Cryptosporidium parvum*. A major problem with the routine testing for these protozoa is the lack of reliable methodologies and currently no widely accepted international method is being employed. The primary aim of the current study was to optimise a real-time PCR analysis coupled with high-resolution melt (HRM) curve assay for the detection, identification and quantitation of *C. parvum* oocysts in water samples. The extraction of DNA from *Cryptosporidium* oocysts is challenging and the efficiency of three different commercially available kits (with and without modifications to the protocols) was compared. For quantitation purposes, standard curves were prepared using synthetic gBlocks Gene Fragments and the detection limits were calculated in terms of ng/µl. Once optimised, the qPCR-HRM assay was applied to environmental water samples collected upstream and downstream of a wastewater treatment plant. The success of the qPCR-HRM assay was further compared to the commercially available Genesig kit for *Cryptosporidium* detection. Of the three kits used, the QIAamp Fast DNA Stool Mini kit proved to be the most effective for DNA extraction from *C. parvum* oocysts. Constant HRM profiles for the target gene (COWP) could be obtained, characterised by an amplicon Tm of 79.38 ± 0.31°C. High-resolution melt profiles obtained for the gene marker targeted were characterised by peaks of 79.38°C ± 0.31°C and the qPCR-HRM assay was fully optimised for the detection of *C. parvum* oocysts. The qPCR-HRM assay showed a high sensitivity with the ability to detect less than one *C. parvum* oocysts per 1 ml/spiked tap water sample. Comparison of the qPCR-HRM assay and the Genesig kit indicated that the Genesig kit was able to quantify the *Cryptosporidium* copy numbers in one of the environmental samples tested however, compared to the commercial kit, the qPCR-HRM assay was found to be the more sensitive and cost effective method for the evaluation of the presence/absence of *Cryptosporidium parvum* oocysts in environmental samples.

Key words: Quantitative real-time PCR; high-resolution melt curve assay; *Cryptosporidium parvum* oocysts; drinking water; environmental water; gBlocks Gene Fragments; Genesig kit

3.1 Introduction

Intestinal parasites have been detected in various water sources such as surface water and roof harvested rainwater (Guy *et al*., 2003; Feng *et al*., 2011; Ayaz *et al*., 2013). Their zoonotic nature and ability to withstand traditional treatment processes as well as their ability to survive for extended periods of time in the environment, poses a significant threat to humans that come into contact with contaminated water. Since waterborne transmission of these parasitic organisms has become more prevalent in recent years, regulatory agencies are stipulating that source and finished water be screened for these organisms (WHO, 2011). A major problem associated with the routine testing of these parasites however, is the lack of reliable methodologies. In addition, the methods employed to evaluate for intestinal parasites in water sources are often time-consuming and include the collection of large volumes of water samples, followed by concentration and filtration methods and finally detection.

Cryptosporidium parvum is an obligate enteric protozoan parasite that infects the gastrointestinal tract of both animals and humans leading to the acute diarrhoeal disease, cryptosporidiosis (Carey *et al*., 2003). The oocysts of this pathogen are extremely resistant to traditional water treatment methods such as chlorination and have subsequently been found in both raw and treated sewage, surface water, drinking water sources and recreational water (WHO, 2011). Very low infectious doses have been reported (Xiao and Fayer, 2008) for this organism and it is thus recognised as one of the most prevalent pathogenic intestinal parasites (WHO, 2011). Currently there are eight described *Cryptosporidium* species (*C. andersoni, C. canis, C. felis, C. hominis, C. meleagridis, C. muris, C. parvum* and *C. suis*) and five undescribed species (cervine, chipmunk, monkey, rabbit and skunk genotypes), which can infect both immuno-competent and immuno-compromised individuals (Cacció *et al*., 2005; Feltus *et al*., 2006; Nichols *et al*., 2006; Xiao *et al*., 2004). Of these species, *C. parvum* and *C. hominis* are most commonly detected (Cacció *et al*., 2005) and while both of these species infect humans, *C. parvum* also infects cattle and livestock, creating a higher risk of contamination in environmental waters, placing human health at greater risk (Smith and Nichols, 2009).

Evaluation of *Cryptosporidium parvum* oocysts in water samples are currently performed using a series of steps, which include the collection of a 10 – 1 000 L water sample, concentration by means of a filtration step and separation of the oocysts from environmental debris, after which final

detection can be carried out. Methods most commonly used for the final detection of *Cryptosporidium parvum* oocyst concentrations include microscopic analysis, immuno-fluoresence assays (IFA), flow cytometric detection and fluorescent *in situ* hybridisation. A widely accepted method for the detection of *Cryptosporidium* oocysts (and *Giardia*) in water samples is method 1622 developed by the United States Environmental Protection Agency (USEPA, 2005), which was validated in 1999 and update in 2005. This method consists of concentrating the water sample through filtration, immuno-magnetic separation (IMS) of the (oo)cysts and subsequently determining the concentration through an immuno-fluorescence assay (IFA). The presence of potential (oo)cysts are then confirmed by staining with the vital dyes 4'-6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI), followed by differential interference contrast (DIC) microscopy (Carey *et al*., 2003). Method 1623 has also been assessed and optimised in numerous studies (Simmons *et* al., 2001; McCuin and Clancy, 2003; Feng *et al*., 2003; Feng *et al*., 2011) and while no specialised equipment is required, it is apparent that this method is labour-intensive, time-consuming and requires extensive laboratory experience for the interpretation of results. It is also unable to distinguish between viable and non-viable (oo)cysts and between specific species, as different (oo)cyst sizes overlap (Carey *et al*., 2003; Feng *et al*., 2011; Smith and Nichols, 2009).

Techniques based on molecular assays, such as the polymerase chain reaction (PCR), have been applied broadly due to their sensitivity that permits specific amplification of genetic loci from minimal amounts of intestinal parasite material (Widmer, 1998). Polymerase chain reactions overcome the limitations of specific diagnosis by traditional microscopic techniques, resulting in the reproducible and sensitive detection of *Cryptosporidium parvum* and distinguishing between different species (Carey *et al*., 2003; McPherson *et al*., 2007; Pangasa *et* al., 2009; Ayaz *et al*., 2013). In addition it allows for the detection and, in the case of certain PCR techniques, the quantitation of multiple samples in a relatively short period of time. Comparative studies have also shown that PCR is the most effective and sensitive method for the detection and quantitation of *Cryprosporidium parvum* oocysts (Leng *et al*., 1996; Mayer and Palmer, 1996; Elsafi *et al*., 2013). Several PCR techniques have thus been implemented for the detection of *Cryptosporidium parvum* oocysts and these methods include conventional PCR, quantitative real-time PCR (qPCR), nested PCR, reverse transcriptase-PCR (RT-PCR) as well as immuno-magnetic separation-PCR (IMS-PCR).

Quantitative PCR (qPCR) allows for the simultaneous detection and quantitation of the target organism by means of fluorescent dyes and fluorescently labelled genetic probes are a popular choice in qPCR assays. Studies conducted by Guy *et al*. (2003) successfully utilised qPCR and fluorescently labelled genetic probes to detect both *Giardia* and *Cryptosporidium* in environmental water samples and sewage. However, qPCR assays using high-resolution melt curve assay relies on intercalating dyes such as EvaGreen and the specific melt temperature (Tm) of the gene marker targeted. A qPCR coupled with HRM analyses approach could thus be utilised for the rapid screening of large numbers of *Cryptosporidium parvum* oocysts. Studies conducted by Al-Mohammed (2011) and Pangasa *et al*. (2009) successfully detected *Giardia* and *Cryptosporidium,* respectively, by coupling HRM analyses with qPCR.

While various methods for the detection and quantitation of *Cryptosporidium* oocysts have been developed and compared, no internationally accepted molecular based method is currently being implemented (Smith and Grimason, 2003; Smith and Nichols, 2009). The primary aim of the current study was to optimise a real-time PCR assay coupled with high-resolution melt curve (qPCR-HRM) assay for the routine detection, identification and quantitation of *Cryptosporidium parvum* oocysts in water samples. This study was contracted by the East Rand Water Care Company (ERWAT) in South Africa and the efficiency of the optimised method was then investigated utilising environmental water samples obtained upstream and downstream of a wastewater treatment plant operated by the ERWAT. To further evaluate the assay, the commercial Genesig kit for the detection of *Cryptosporidium* (PrimerDesign, UK) was utilised in order to compare the use of a primer-probe detection system with a DNA intercalating dye coupled with HRM approach.

3.2 Materials and Methods

3.2.1 Sources of Cryptosporidium parvum *for optimisation experiments*

Isolates of *Cryptosporidium parvum* were required for use in DNA extraction and PCR optimisation experiments. For this, EasySeed parasite suspensions (BioMérieux, France) containing inactivated *Cryptosporidium parvum* oocysts were obtained and prepared according to the manufacturer's instructions. Briefly, the complete solution (8 ml) was concentrated by centrifugation at 14 000 rpm for 5 min. The concentrate was transferred to a 1.5 ml microcentrifuge tube and stored at 4°C until used in subsequent optimisation experiments.

In addition, faecal samples positive for *Cryptosporidium parvum* oocysts (microscopic examination) were obtained from a local pathologist and were utilised in optimisation experiments. Each faecal sample (~4 g) was concentrated by centrifugation at 14 000 rpm for 5 min. The number of *C. parvum* oocysts in the faecal samples was determined using phase contrast microscopy (BX43, Olympus, Japan) and Sedgwick-Rafter counting chambers (SPI, USA). All faecal samples were stored at 4°C until further analysis was conducted.

3.2.2 Optimisation of DNA extractions from Cryptosporidium parvum *oocysts*

To optimise the qPCR-HRM assay, DNA extracted from *Cryptosporidium parvum* was required. Three different commercial DNA extraction kits (modified as outlined below) were utilised to extract DNA from the *Cryptosporidium parvum oocysts*. The extraction kits were applied to the sources of *Cryptosporidium parvum* as mentioned in section 3.2.1. All DNA extracted was stored at -22°C until further use. As general kits and DNA extraction methods were utilised in the current study, the DNA concentrations were representative of all DNA present in the original samples.

3.2.2.1 Instagene Matrix kit

This DNA extraction kit was applied to both the EasySeed and faecal samples using a slightly modified protocol. Briefly, 200 µl of Instagene Matrix solution was added to the sample pellet and incubated at 56°C for 20 minutes. The suspensions were boiled at 98°C for 15 minutes, mixed and then frozen at -22°C for 30 minutes and again boiled at 98°C for 15 minutes. Hereafter the solution was centrifuged at 10 000 rpm for 2 minutes and the supernatant, containing purified DNA, was used for further qPCR analyses. All extracted DNA was stored at -22°C until further use.

3.2.2.2 DNAeasy Blood and Tissue kit

The QIAamp DNAeasy Blood and Tissue kit (Qiagen, Germany) was applied to the EasySeed and faecal samples. The protocol for the purification of total DNA from animal tissue was used and the lysis step was carried out for a total of three hours. Extracted DNA was stored at -22°C until further use.

The QIAamp DNAeasy Blood and Tissue kit (Qiagen, Germany) was also used in conjunction with bead beating and freeze-boil cycles to extract DNA from the *Cryptosporidium parvum* EasySeed parasite suspension and the faecal samples (4 g each) containing *Cryptosporidium parvum* oocysts. Briefly, the sample pellet was resuspended in 200 µl nuclease-free water and transferred to a Beadbug tube containing 0.5 mm acid-washed silica beads (Sigma-Aldrich, USA). The Beadbug

tube was vortexed for 5 minutes and then subjected to three consecutive freeze-boil cycles (freeze at -22°C, boil at 100°C). Follwing this, the manufacturer's instructions were followed with the lysis step carried out for a total of three hours. Extracted DNA was stored at -22°C until further use.

3.2.2.3 QIAamp Fast DNA Stool Mini kit

DNA was extracted from the EasySeed parasite suspension and the faecal samples using the QIAamp Fast DNA Stool Mini kit (Qiagen, Germany) with minor modifications as outlined in Dobrowsky *et al*. (2014). Briefly, the sample pellet was resuspended in 1.4 ml buffer ASL and incubated at 95°C for 5 min. Once the sample had undergone subsequent centrifugation (14000 rpm for 1 min), the InhibitEX buffer was added to 1.2 ml of the supernatant and the mixture was vortexed for 1 min and left at room temperature for 1 min. In order to remove the inhibitors bound to InhibitEX, the sample was centrifuged at 14000 rpm for 3 min. The supernatant (400 µl) was added into a 1.5 ml microcentrifuge tube containing 30 µl of proteinase K. Before incubating the sample at 70°C for 15 min, 400 µl of buffer AL was added, and after incubation, 400 µl of ethanol (96%) was added to each sample. In order to filter the complete lysate through the QIAamp spin column, approximately 600 µl of the lysate was added to the QIAamp spin column and centrifuged at full speed for 1 min. This process was then repeated until the full lysate had been filtered through the QIAamp spin column. To further purify the DNA, the manufacturer's protocol was then followed. Extracted DNA was stored at -22°C until further use.

In addition, the QIAamp Fast DNA Stool Mini kit was also used in conjunction with bead beating and freeze-boil cycles. Briefly, the sample pellet was resuspended in 200 µl nuclease-free water and transferred to a Beadbug tube containing 0.5 mm acid-washed silica beads (Sigma-Aldrich, USA). The Beadbug tube was vortexed for 5 minutes and then subjected to three freeze-boil cycles (freeze at -22°C, boil at 100°C). Following this, DNA was further purified using the protocol as previously outlined. Extracted DNA was stored at -22°C until further use.

Finally the QIAamp Fast DNA Stool Mini kit was used in conjunction with freeze-boil cycles using liquid nitrogen and boiling, followed by sonication as outlined in Guy *et al*. (2003). This method was only applied to the faecal samples (4 g each) containing *Cryptosporidium parvum* oocysts. Briefly, 1 ml of InhibitEX buffer was added to the 200 µl of faecal sample and vortexed for 1 minute. The sample was heated at 95°C for 5 minutes. The sample was then centrifuged at 14 000 rpm for 1 minute and 400 µl supernatant added to a separate microfuge tube containing 15 µl of Proteinase K along with 400 µl buffer AL and vortexed briefly. The sample was incubated

at 55 °C for 1 hr and was then subjected to three freeze-boil cycles at 2 minute intervals using liquid nitrogen and boiling. Each freeze-boil cycle was followed by a 20 second burst of sonication (Bransonic 5510E, Branson Ultrasonic Corp., CT, USA). From here the protocol was followed as previously outlined. Extracted DNA was stored at -22°C until further use.

3.2.3 Optimisation of the real-time polymerase chain reaction coupled with high-resolution melt curve analysis (qPCR-HRM) assay

Cryptosporidium parvum DNA isolated from the EasySeed parasite suspension and the faecal samples were used for the optimisation of the qPCR-HRM assay. Reactions were prepared using the SensiFAST HRM kit (Bioline, UK) containing the reaction buffer and all reaction components.

The gene coding for a *Cryptosporidium* oocyst wall protein (COWP) was selected as target where a 151 bp region from position 165 to 316 of the coding sequence of the precursor COWP gene was amplified (**Table 1**) using suitable primers (IDT, USA). Reactions were initially set up as outlined in Guy *et al*. (2003). Each PCR reaction was made up to a final volume of 25 µl. The mixture contained 5 µl DNA as template, a final concentration of 1x SensiFAST mix and 0.3 µM COWP-F and COWP-R primer. Amplification was performed starting with an initial incubation step at 95°C for 10 minutes followed by 40 cycles of alternating temperatures at 95°C for 15 seconds and at 60°C for 1 minute. All samples were analysed using a RotorGene 6000, 2-plex PCR thermal cycler with HRM capability (Qiagen, Germany). Fluorescence data was collected at the end of each cycle and a negative control was included in every PCR reaction. High-resolution melt curve analysis was performed after target amplification with programming consisting of an initial 90 second pre-melt conditioning step followed by ramping from 70°C to 90°C with ramping rate 0.05°C/sec. Melting profiles of the amplification product was established.

Table 1. *Cryptosporidium parvum* oocysts outer wall protein (COWP) primer sequences

Primer name	Sequence 5' – 3'	Nucleotide position	Reference
COWP-F	CATCCGCGAGGAGGTCAA	165	Guy *et al*. 2003
COWP-R	GCAGCCATGGTGTCGATCT	316	

Poor results obtained using this protocol necessitated further optimisation of the qPCR-HRM assay to improve target amplification. The optimised PCR reaction was made up to a final volume of 20 µl as outlined in the SensiFAST Mix manufacturer's protocol in contrast to the volumes outlined in Guy *et al*. (2003). The mixture contained 4 µl DNA as template, a final concentration of 1x

SensiFAST Mix and 0.4 µM COWP-F and COWP-R primers. Reaction conditions were optimised to include an initial incubation step at 95˚C for 10 minutes, followed by 45 cycles of denaturation at 95˚C for 30 seconds, annealing at 65˚C for 30 seconds and elongation at 72˚C for 30 seconds. The run was completed with a final elongation step at 72˚C for 5 minutes. Fluorescence data was collected at the end of each cycle. All samples were analysed using a RotorGene 6000, 2-plex PCR thermal cycler with HRM capability (Qiagen, Germany). A negative control was included in every PCR reaction. High-resolution melt curve analysis was performed after target amplification with programming consisting of an initial 90 second pre-melt conditioning step followed by ramping from 70°C to 90°C with ramping rate 0.05°C/sec. Melting profiles of the amplification product was established.

Representative qPCR products that produced a single melt peak during HRM analysis were submitted for nucleotide sequencing (Inqaba Biotec, South Africa). Sequence identification was completed using the National Centre for Biotechnology Information (NCBI) and The Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the closest match of local similarity between the amplicon sequence and the international database in GenBank, EMBL, DDBJ and PDB sequence data (Altschul *et al*., 1997). The sequences of representative samples that showed >97% similarity (<3% diversity) to the gene marker targeted were recored..

3.2. 4 Construction of standard curves intended for quantitation

After numerous attempts, DNA extracted from the EasySeed parasite suspension and the faecal samples failed to deliver usable standard curves and the gBlocks gene fragments were then utilised to construct the standard curve for the quantitation of *Crytposporidium* spp. in samples.

3.2.4.1 Preparation of gBlock Gene Fragments and construction of standard curve

Standard curves intended for optimising the quantitation of *Cryptosporidium* in samples were constructed using synthetic gBlock Gene Fragments (IDT, USA). gBlock fragments are synthetic, linear, double stranded DNA that are sequence verified and have a high sequence fidelity. The gBlock gene fragment used in this study was constructed to contain the binding sequences for the COWP-F/COWP-R primers (**Table 1**) used for amplification of the *Cryptosporidium* oocyst COWP gene. Solutions of each fragment were prepared according to the manufacturer's instructions. Briefly, each tube containing a lyophilised pellet was centrifuged at 14 000 rpm for 10 seconds after which 20 µl of 1X TE Buffer was added to yield a final concentration of 10 ng/µl. The tubes were vortexed and incubated at 50°C for 20 minutes. Each tube was again vortexed and centrifuged at

14 000 rpm for 10 seconds. A 10 fold dilution series was prepared for each gBlock Gene Fragment for use in setting up standard curves.

The standard curve was constructed by using the serially diluted gBlock Gene Fragment preparation as template in the optimised qPCR-HRM assay (as described in section 3.2.3). A negative control was included in every run and HRM curve assay was performed after amplification to confirm the Tm of the amplification product. Upon completion of the run, the RotorGene software was used to construct the standard curve. The auto-find threshold function of the RotorGene software scans the selected region of the graph to find a threshold setting which delivers optimal estimates of given concentrations. The range of threshold levels is scanned to obtain the best fit of the standard curve based on the samples that have been defined as standards (i.e. where the R value is closest to 1.0). The dynamic tube normalisation option is selected by default in the software and is used to determine the average background of each sample just before amplification commences. The R and R^2 values indicate the correlation coefficient and the coefficient of determination, respectively and should be as close to 1 as possible indicating a low variance between samples. The slope of the standard curve indicates the efficiency of the qPCR and should be as close to -3.32 as possible indicating 100% efficiency (Efficiency = $10^{(-1/slope)}$-1). The efficiency of the experiment should be 90% or higher in order for the standard curve to be seen as valid and usable for quantitation (Bustin *et al*., 2009).

Detection of the *Cryptosporidium parvum* COWP gBlock Gene Fragments were carried out using the SensiFAST HRM kit as per manufacturer's instructions. The primers used were mentioned previously (**Table 1**). Each PCR reaction was made up to a final volume of 20 µl. The mixture contained 1 µl gBlock Gene Fragment, a final concentration of 1X SensiFAST mix and 0.4 µM of both the forward and the reverse primers. Amplification was performed with an initial incubation at 95°C for 10 minutes after which 45 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C for 30 seconds and elongation at 72°C for 30 seconds, was completed with a final elongation step at 72°C for 5 minutes. Fluorescence data was collected at the end of each cycle. All samples were analysed using a RotorGene 6000, 2-plex PCR thermal cycler with HRM capability. A negative control was included in every assay. High-resolution melt curve analysis was performed after each run and the Tm for the amplification product was established. The software of the RotorGene 6000, 2-plex PCR machine with HRM capability was used to extrapolate a standard curve.

3.2.5 Determining the lower limit of detection (LLOD) of the qPCR-HRM assay

The LLOD of the qPCR-HRM assay was determined using DNA extracted from *Cryptosporidium parvum* oocysts. The DNA extracted was quantified using spectrophotometry (Inqaba Biotec, South Africa). Ten-fold serial dilutions (10^{-1} to 10^{-5}) of 4.9 ng/µl of DNA were prepared in replicates (n=9) and were used as templates in the qPCR-HRM assay. The lowest quantity of DNA resulting in the successful amplification of the target gene was considered as the LLOD.

3.2.6 Determining the sample limit of detection (SLOD) of the qPCR-HRM assay

The SLOD determines the lowest amount of *C. parvum* oocysts detectable by the qPCR-HRM assay. To determine the SLOD, 1 L drinking water (tap water) samples were collected and artificially contaminated (spiked) using *C. parvum* positive faecal samples. A ten-fold serial diltution (10^{-1} to 10^{-5}) was prepared using the faecal sample and bacteriological saline. Of this series, 1 ml of each dilution was used to spike a 1 L tap water sample. Spiked tap water samples were then subjected to DNA extraction and the qPCR-HRM assay. The SLOD experiment was conducted in triplicate (n=3). A blank consisting of 1 L unspiked tap water was included for each batch to check for cross contamination during sample processing. All water samples were filtered through a 0.45 µm nitrocellulose filter at a flow rate of approximately ≥ 65 mL/min/cm^2 at 0.7 bar (70 kPa). The filters were placed in 50 ml tubes and washed with 5 ml dH_2O. Tubes were centrifuged at 14 000 rpm for 1 minute where after the filters were removed. The 5 ml dH_2O was concentrated in 2 ml microcentrifuge tubes to obtain a single pellet. Each pellet was then resuspended in 200 µl dH_2O and subsequent DNA extractions were performed using the QIAamp Fast DNA Stool Mini kit as previously described (section 3.2.2.3).

3.2.7 Application of the optimised qPCR-HRM assay for the detection of **Cryptosporidium** *in environmental samples*

Once optimised, the qPCR-HRM assay was applied to environmental water samples (i.e. river water). To determine the application of the qPCR-HRM method on environmental samples, 1 L river water samples were collected both upstream and downstream of the ERWAT Hartebeesfontein wastewater treatment plant (WWTP) at three different time intervals (**Figure 1**). The respective 1 L river water samples were then filtered through a 0.45 µm nitrocellulose filter at a flow rate of approximately ≥ 65 mL/min/cm^2 at 0.7 bar (70 kPa). The filters were placed in 50 ml tubes and washed with 5 ml sterile dH_2O. Tubes were centrifuged at 14 000 rpm for 1 minute where after the filters were removed. The 5 ml dH_2O was concentrated in 2 ml microcentrifuge tubes to obtain a single pellet. Each pellet was then resuspended in 200 µl dH_2O. The QIAamp Fast DNA Stool

Mini kit was then followed as previously described (section 3.2.2.3). An aerial view of the WWTP, indicating the upstream and downstream sampling points, is indicated in **Figure 1**.

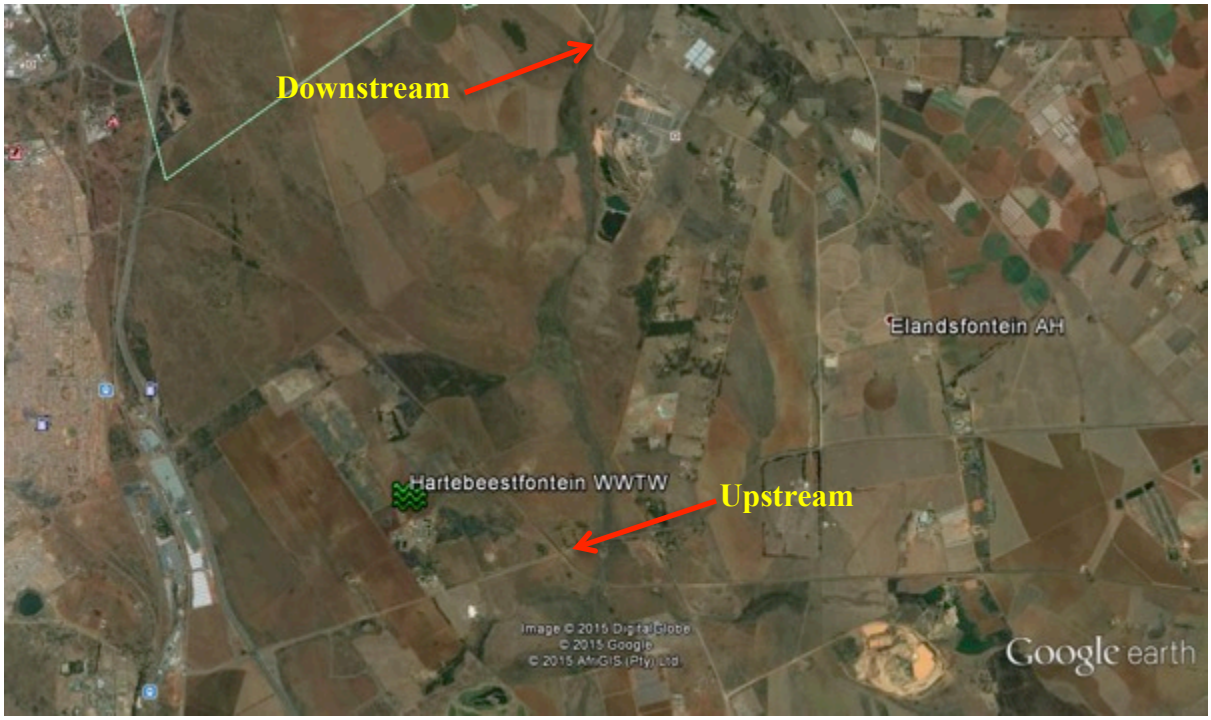

Figure 1. Map of the Hartebeesfontein WWTP indicating the site of the upstream (26° 1'8.26"S 28°18'15.22"E) and the downstream (25°58'8.09"S 28°18'3.70"E) sampling points.

3.2.8 Application of the commercial Genesig kit for the detection of Cryptosporidium *in environmental samples*

The commercial Genesig kit for the detection of *Cryptosporidium* (PrimerDesign, UK) was also utilised for the detection of *Cryptosporidium* species in the river water samples collected upstream and downstream of the ERWAT Hartebeesfontein wastewater treatment plant (WWTP) (section 3.2.7) in order to compare the use of a primer-probe detection system with a DNA intercalating dye coupled with HRM analysis approach. Each Genesig kit PCR reaction was made up to a final volume of 20 µl. The mixture contained 5 µl DNA as template, 10 µl of Oasig 2 x qPCR Mastermix, 1 µl *Cryptosporidium* primer-probe mix, 1 µl internal extraction control primer-probe mix and 3 µl RNAse/DNAse free water. In addition, a positive control of *Cryptosporidium* DNA, an internal extraction control and a negative control provided with the kit were also included in the qPCR assay. The positive control *Cryptosporidium* DNA was also used in order to set up a standard curve and included in the qPCR assay. The standard curve copy numbers ranged from 2 X 10^5 copies/µl to 20 copies/µl as stipulated by the manufacturers. The amplification was performed with an initial incubation at 95°C for 15 minutes, followed by 50 cycles of alternating temperatures of

95°C for 10 seconds and 60°C for 1 minute. All samples were analysed using a RotorGene 6000, 2-plex PCR thermal cycler with HRM capability (Qiagen, Germany). Fluorescence data was collected at the end of each cycle. The *Cryptosporidium* primer-probe mix that binds to the *Cryptosporidium* DNA was detected through the FAM channel whereas the internal extraction control primer-probe mix was detected through the VIC channel.

Finally the costs and time required for the completion of the qPCR-HRM assay and the Genesig kit (sections 3.2.7 and 3.2.8) were also calculated and compared.

3.3 Results and Discussions

3.3.1 Cryptosporidium parvum *samples obtained for optimisation of qPCR-HRM assay*

EasySeed parasite suspensions (BioMérieux, France) containing inactivated *Cryptosporidium parvum* oocysts and *Cryptosporidium parvum* oocysts obtained from faecal samples that tested positive by microscopic examination at a local pathologist were utilised as positive controls for the optimisation experiments. The EasySeed parasite suspensions proved ineffective as a reliable source of *Cryptosporidium parvum* DNA showing low cycle threshold values (Ct) and inconsistent qPCR amplification. It was hypothesised that the formalin, in which the oocysts are suspended, could have inhibited the qPCR-HRM assay. The EasySeed parasite suspension is intended for experiments such as microscopy and flow cytometry and not PCR assays. The suspension also contains 1 000 oocysts/ml. Faecal samples that tested positive for *Cryptosporidium parvum* oocysts by microscopic examination were thus selected as a suitable positive control for the optimisation of the methods due to the high number of oocysts present in the sample (6.5×10^6, varying slightly between samples). qPCR inhibitors may however be present in the faecal samples. The QIAamp Fast DNA Stool Mini kit however was designed specifically to extract DNA from faecal samples with the InhibitEx buffer included in the protocol, which eliminates qPCR inhibitors.

3.3.2 DNA extractions methods

As indicated in Chapter two (section 2.3) the efficiency of the DNA extraction methods were evaluated through the amplification and cycle threshold (Ct) or crossing point values obtained by the qPCR assay performed on each DNA sample that produced a single peak during HRM analysis (**Table 2**). The QIAamp Fast DNA Stool Mini kit, in combination with bead beating and freeze-boil cycles as well as in combination with liquid nitrogen and sonication, did not result in any qPCR amplification of the target gene when using DNA extracted from the EasySeed parasite suspension

(**Table 2**). However, the DNAeasy Blood and Tissue kit in conjuntion with bead beating and freeze-boil cycles resulted in the lowest mean Ct value (35.00) when using DNA extracted from the EasySeed parasite suspension.

Table 2. Efficiency of DNA extraction kits as determined by positive or negative amplification and mean Ct values obtained from qPCR-HRM assay of representative samples

DNA extraction kit	**EasySeed parasite suspension**			**Faecal samples**		
	qPCR amplification	*Number of samples (n)*	*Mean Ct value*	*qPCR amplification*	*Number of samples (n)*	*Mean Ct value*
Instagene Matrix	Positive	1	36.73	Negative	1	*N/A
DNAeasy Blood and Tissue kit, Animal Protocol	Positive	3	36.80	Negative	3	*N/A
DNAeasy Blood and Tissue kit, Animal Protocol in conjunction with bead beating and freeze-boil	Positive	2	35.00	Negative	2	*N/A
QIAamp Fast DNA Stool Mini kit	Negative	2	*N/A	Positive	14	26.65
QIAamp Fast DNA Stool Mini kit in conjunction with bead beating and freeze-boil	Negative	2	*N/A	Positive	5	33.31
QIAamp Fast DNA Stool Mini kit coupled with liquid nitrogen and sonication	*N/A	2	*N/A	Positive	2	28.21

*N/A = Not applicable – analysis not performed on this sample

The Instagene Matrix and the DNAeasy Blood and Tissue kit with no modifications also resulted in mean Ct values of 36.73 and 36.80, respectively. In contrast, the Instagene Matrix, the DNAeasy Blood and Tissue kit (without modifications) and in combination with bead beating and freeze-boil cycles did not result in any qPCR amplification of DNA extracted from the faecal samples. However, the lowest mean Ct value (26.65) for DNA extracted from the faecal samples was obtained for the QIAamp Fast DNA Stool Mini kit with no modifications. The QIAamp Fast DNA Stool Mini kit in combination with bead beating and freeze-boil and coupled with liquid nitrogen and sonication resulted in mean Ct values of 33.31 and 28.21, respectively.

The Instagene matrix has previously been shown to be an effective method for DNA extraction from the intestinal protozoa *Cryptosporidium parvum* oocysts, yielding satisfactory templates for PCR purposes (Higgins *et al*., 2001). Due to the low cost, high turnover time and simple protocol of the Instagene matrix it was compared for the extraction of DNA from both the EasySeed parasite suspension and the faecal samples during the current study. However, subsequent qPCR-HRM assay resulted in no amplification of DNA from the faecal samples indicating that the Instagene matrix was not successful in extracting DNA from the *Cryptosporidium parvum* oocysts present in these samples. It was hypothesised that the faecal samples contained inhibitors still present in the DNA sample after DNA extraction which in turn possibly hindered qPCR amplification. This matrix was however, successful in the extraction of DNA from the Easyseed parasite suspension as can be seen from the mean Ct value in **Table 2** (36.73), however amplification was very late possibly indicating lower numbers of *Cryptosporidium parvum* oocysts or incomplete lyses of the oocysts.

The DNAeasy Blood and Tissue kit has proven to be effective in the lyses of *Cryptosporidium* oocysts and *Giardia* cysts from environmental water samples and sewage (Guy *et al*., 2003). In the current study bead beating as well as freeze-boil cycles were utilised with DNAeasy Blood and Tissue kits' protocol to evaluate the effect on the lyses of *Cryptosporidium parvum* oocysts. However, while the DNAeasy Blood and Tissue kit, Animal protocol (in combination with bead beating and freeze-boil cycles or not) resulted in qPCR amplification of DNA from the EasySeed parasite suspension, no amplification was observed for the DNA extracted from the faecal samples. It was again hypothesised that the faecal samples either contained inhibitors hindering qPCR amplification or that the kit did not achieve complete lyses of the oocyts in the faecal samples. It should be noted that the addition of the bead beating and freeze-boil cycles to the DNAeasy Blood and Tissue kit resulted in improved amplification based on lower mean Ct values (**Table 2**) of the EasySeed parasite suspension DNA when conducting the qPCR-HRM assay. It was thus concluded that the physical stresses aided in breaking open the strong multi-layer of the *Cryptosporidium parvum* oocysts. The effieciency of the extraction method also depended on the source of the *Cryptosporidium parvum* oocysts.

In a previous study conducted by Dobrowsky *et al*. (2014) the QIAamp Fast DNA Stool Mini kit was effective in the lyses of *Cryptosporidium* oocysts and *Giardia* cysts isolated from roof-harvested rainwater. The QIAamp Fast DNA Stool Mini kit has the added advantage of the InhibitEX buffer that reduces the amount of qPCR inhibition caused by environmental inhibiting

factors present in samples. Thus, during the current study it was found that the QIAamp Fast DNA Stool Mini kit resulted in qPCR amplification of DNA extracted from the faecal samples but not the EasySeed parasite suspension. In addition, while the combination of the bead beating and freeze-boil cycles with the QIAamp Fast DNA Stool Mini kit also resulted in qPCR amplification of the DNA extracted from the faecal samples, lower mean Ct values (**Table 2**) were not obtained. Moreover, the addition of the bead beating and freeze-boil cycles to the the QIAamp Fast DNA Stool Mini kit did not result in amplification of DNA extracted from the EasySeed parasite suspension. The QIAamp Fast DNA Stool Mini kit containing the InhibitEX buffer was also coupled with liquid nitrogen and sonication in order to evaluate its effect on the lyses of the *Cryptosporidium parvum* oocysts from faecal samples. This approach resulted in qPCR amplification of DNA, however no decrease in the mean Ct value was observed as determined by qPCR-HRM assay.

When conducting qPCR assays, the quantity as well as the quality of the template DNA must be taken into consideration and in the current study the template DNA obtained from the faecal samples and extracted using the QIAamp Fast DNA Stool Mini kit produced the highest quality of DNA resulting in the best and most consistent results, as determined by the subsequent mean Ct value of the qPCR-HRM assay (**Table 2**).

3.3.3 Real-time polymerase chain reaction coupled with high-resolution melt curve analysis (qPCR-HRM) assay

The qPCR-HRM assay technique was carried out with the DNA extracted from each sample (EasySeed parasite suspension and faecal samples) utilising the respective DNA extraction methods as outlined in sections 3.2.2.1 to 3.2.2.3. The SensiFAST HRM kit was used for the amplification and detection of the qPCR-HRM products. The melting temperature of the targeted gene marker used in the current study was confirmed by nucleotide sequence analysis of the amplication products produced after qPCR. The HRM melt profile for the COWP gene were characterised by a peak of 79.48°C ± 0.24°C (*Cryptosporidium parvum*) (**Table 3**). For the COWP amplicon, the lowest Tm recorded was 78.76°C, while the highest Tm was recorded at 79.90°C. Variation of the Tm may be as a result of various factors including the presence of salts and other inhibiting compounds. Thus, qPCR products with a Tm between the range of 78.76°C and 79.90°C were considered positive for *Cryptosporidium parvum* DNA. Real-time PCR products obtained within this Tm range were confirmed by nucleotide sequencing and results showed homology to the

Cryptosporidium parvum oocysts wall protein gene (accession number AF481960.1), with an identity of 97% or higher.

Table 3. Mean melting temperature obtained for the COWP amplification product following qPCR.

Melting curve analysis	Number of samples	Mean melting temperature ± standard deviation (SD)
Cryptosporidium parvum	18	79.38°C ± 0.31°C

As previously discussed (section 3.3.2), DNA extracted with the QIAamp Fast DNA Stool Mini kit and the QIAamp Fast DNA Stool Mini kit in conjunction with bead beating and freeze-boil did not result in any qPCR amplification of DNA extracted from the EasySeed parasite suspension. In contrast the Instagene Matrix, the DNAeasy Blood and Tissue kit with no modifictions and the DNAeasy Blood and Tissue kit in conjunction with bead beating and freeze-boil did not result in any qPCR amplification of DNA extracted from the faecal samples. Therefore, only qPCR-HRM results obtained from the Easyseed DNA extracted with the Instagene Matrix, the DNAeasy Blood and Tissue kit with no modifications and the DNAeasy Blood and Tissue kit in conjunction with bead beating and freeze-boil are discussed below. Similarly, only qPCR-HRM results obtained from faecal sample DNA extracted with the QIAamp Fast DNA Stool Mini kit with no modifications, the QIAamp Fast DNA Stool Mini kit in conjunction with bead beating and freeze-boil and the QIAamp Fast DNA Stool Mini kit coupled with liquid nitrogen and sonication are discussed below. High-resolution melt curve analysis of representative samples are indicated, with the cycling green fluorescence data indicating positive qPCR amplification from representative samples included in **Appendix 3A**.

3.3.3.1 Cryptosporidium parvum *Easyseed Parasite Suspension*

3.3.3.1.1 Instagene Matrix

Cryptosporidium parvum qPCR-HRM was successfully carried out with the COWP-F/COWP-R primers using DNA extracted from the EasySeed parasite suspension by means of the Instagene Matrix method (**Appendix 3A**). The qPCR product was sent for sequencing and BLAST analysis confirmed that the product with Tm 78.76°C (blue curve, **Figure 2**) was positive for *Cryptosporidium parvum* oocysts wall protein 1 (COWP1) gene, with an identity of 99% and accession number JQ349359.1. The negative control (**Figure 2**) did not amplify thus no contamination took place.

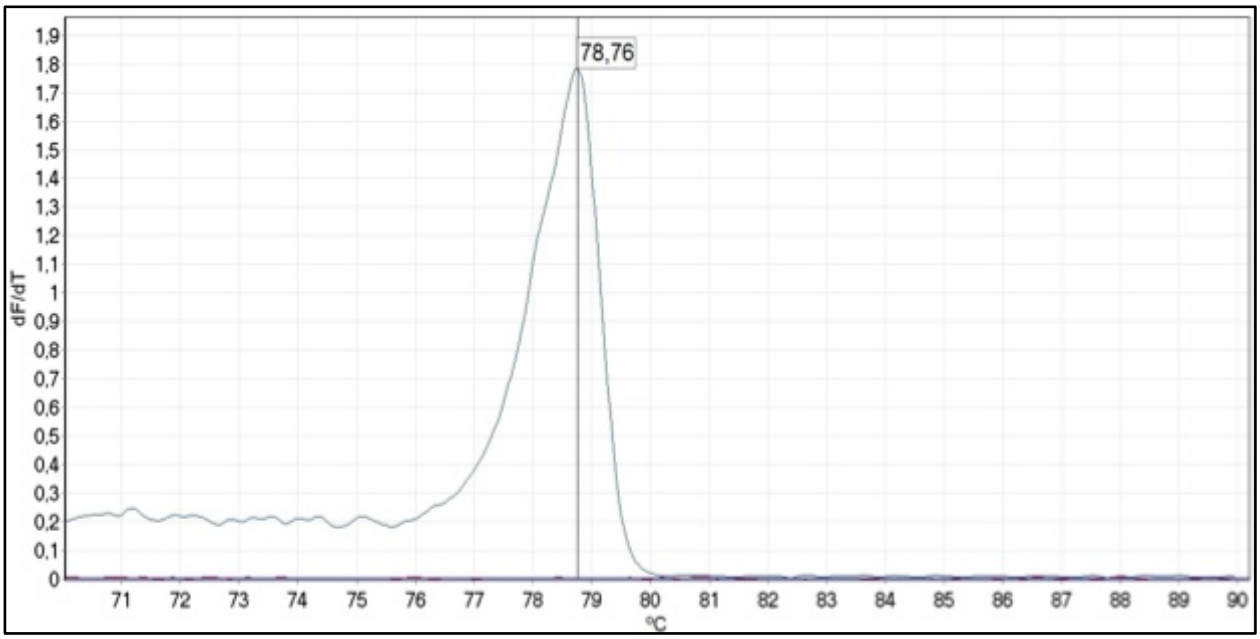

Figure 2. High-resolution melting-curve analysis of *Cryptosporidium parvum* EasySeed parasite suspension DNA extracted using the Instagene Matrix.

3.3.3.1.2 DNAeasy Blood and Tissue kit, Animal Protocol

Cryptosporidium parvum qPCR-HRM assay was successfully carried out with the COWP-F/COWP-R primers using DNA extracted from the EasySeed parasite suspension by means of the DNAeasy Blood and Tissue kit method (**Appendix 3A**). The qPCR product was sent for sequencing and BLAST analysis confirmed that the product with Tm 79.57°C (blue curve, **Figure 3**) was positive for *Cryptosporidium parvum* oocysts wall protein 1 (COWP1) gene, with an identity of 99% and accession number JQ349359.1. A slight increase in fluorescence was observed for the negative control (red curve, **Figure 3**), however this was attributed to the formation of primer dimers. Primer dimers can form when the stringency of the qPCR conditions are not high enough, they have lower GC content and will thus melt at lower temperatures (**Figure 3)**.

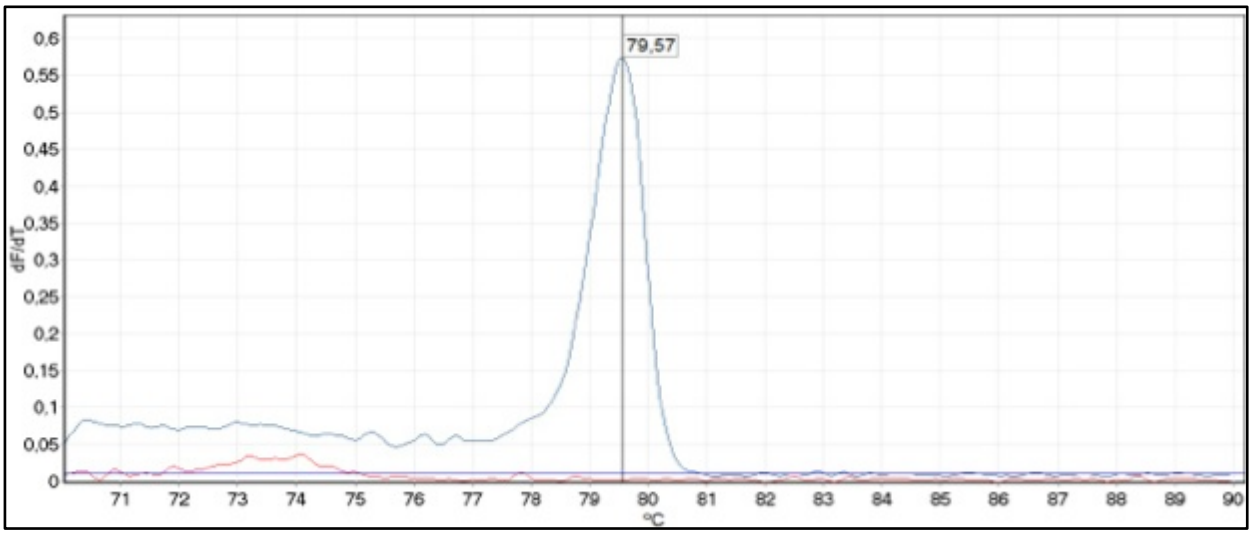

Figure 3. High-resolution melting-curve analysis of *Cryptosporidium parvum* Easyseed parasite suspension DNA extracted using the DNAeasy Blood and Tissue kit.

In order to obtain better amplification a higher starting concentration was needed to ensure a lower Ct value. The QIAamp DNAeasy Blood and Tissue kit was thus used in conjunction with bead beating and freeze-boil cycles to try and extract maximum amount of DNA, however the Ct value did not significantly decrease (**Appendix 3A**). No change was observed with the HRM curve analysis as can be seen from **Figure 4,** where a Tm of 78.89°C was recorded. An increase in fluorescence was again observed in the negative control (red curve, **Figure 4**), however this was attributed to the formation of primer dimers due to the low melting temperature.

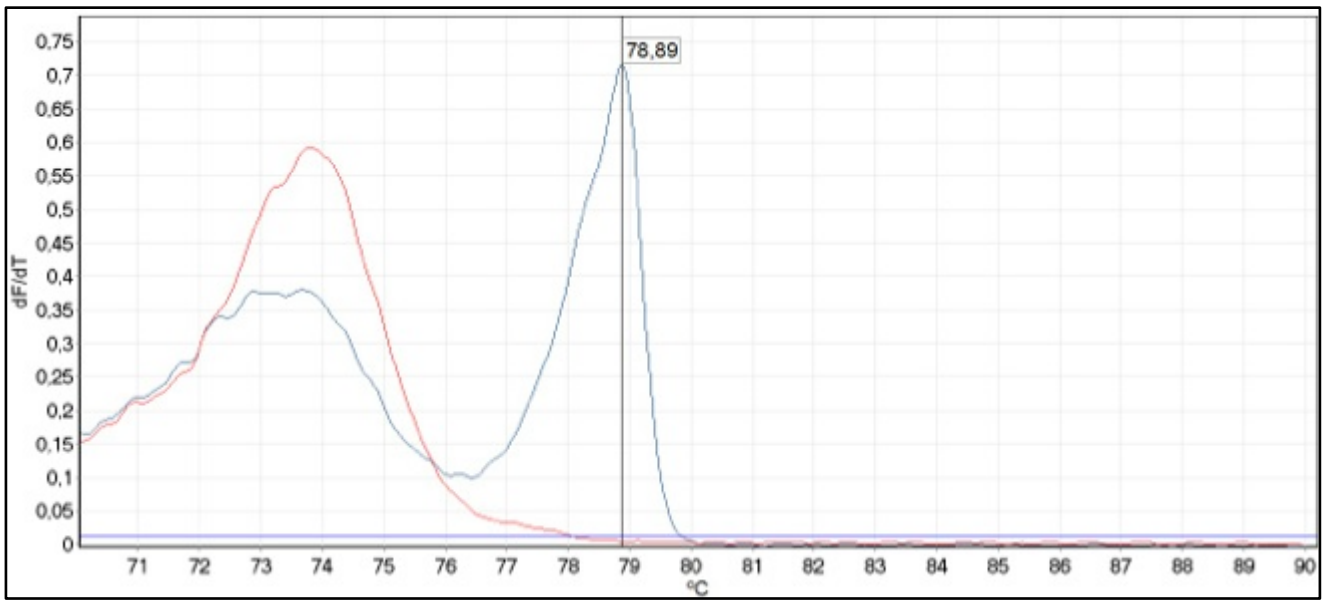

Figure 4. High-resolution melting-curve analysis of *Cryptosporidium parvum* Easyseed parasite suspension DNA extracted using the DNAeasy Blood and Tissue kit in conjunction with bead beating and freeze-boil cycles.

3.3.3.2 Cryptosporidium parvum *faecal sample*

3.3.3.2.1 QIAamp Fast DNA Stool Mini kit

Cryptosporidium parvum qPCR-HRM was successfully performed with the COWP-F/COWP-R primers using DNA extracted from the *Cryptosporidium parvum* faecal samples and the QIAamp Fast DNA Stool Mini kit (**Appendix 3A**). The qPCR product was sent for sequencing and BLAST analysis confirmed that the product with Tm 79.27°C (blue curve, **Figure 5**) was positive for *Cryptosporidium parvum* oocysts wall protein 1 (COWP1) gene, with an identity of 99% and accession number JQ349359.1. An increase in fluorescence was again observed in the negative control (red curve, **Figure 5**), however this was attributed to the formation of primer dimers due to the low melting temperature.

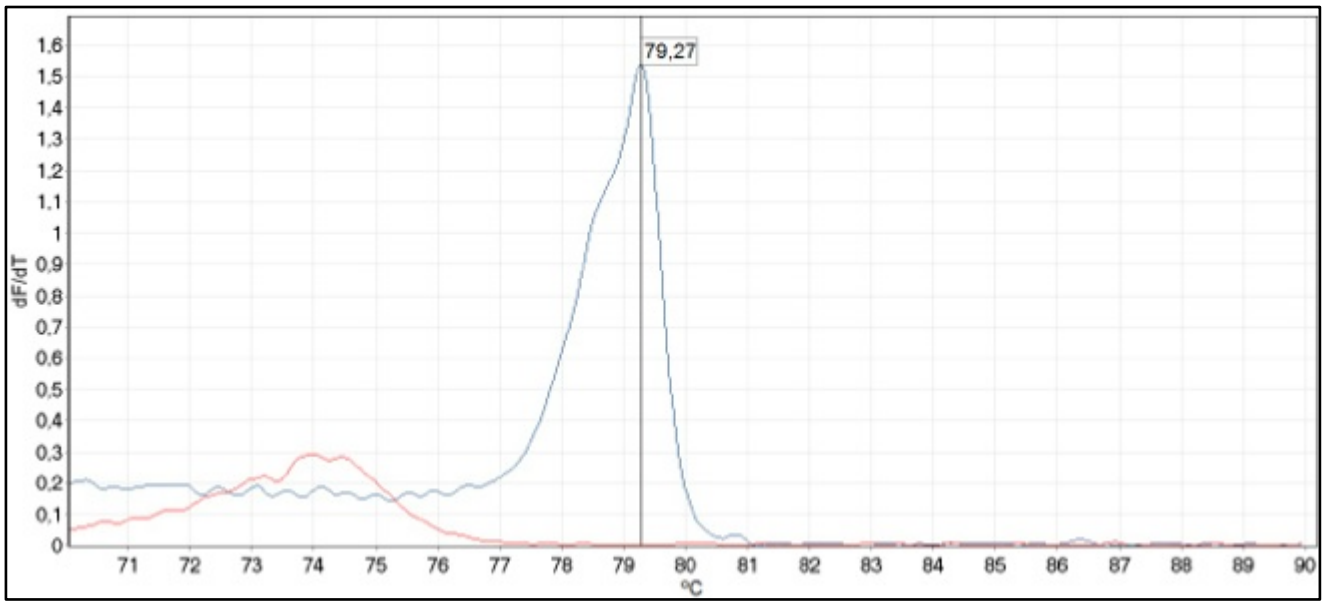

Figure 5. High-resolution melt-curve analysis of *Cryptosporidium parvum* faecal sample DNA extracted using the a QIAamp Fast DNA Stool Mini kit

In order to obtain better amplification a higher starting concentration was needed to ensure a lower Ct value. The QIAamp Fast DNA Sool Mini kit was thus used in conjunction with bead beating and freeze-boil cycles to try and extract maximum amount of DNA, however no significant decrease in the Ct value was observed (**Appendix 3A**). In addition, no change was observed with the HRM curve analysis as can be seen from **Figure 6** where the Tm was 79.56°C. A slight increase in fluorescence was again observed in the negative control (red curve, **Figure 6**), however this was attributed to the formation of primer dimers due to the low melting temperature.

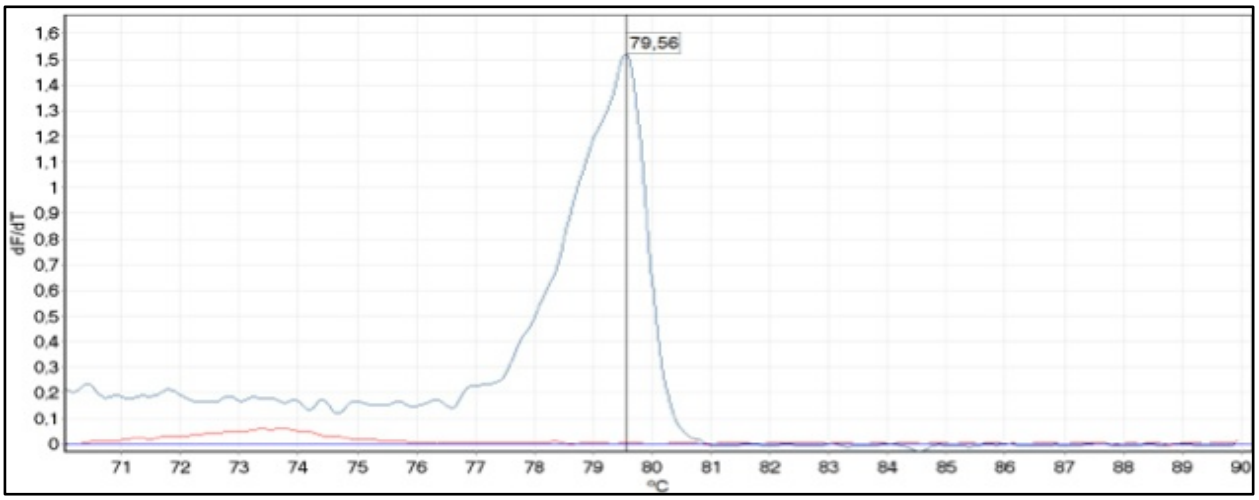

Figure 6. High-resolution melt-curve analysis of *Cryptosporidium parvum* faecal sample DNA extracted using the QIAmp Fast DNA Stool Mini kit in conjunction with bead beating and freeze-boil cycles.

The qPCR method was then changed from the protocol suggested by Guy *et al*. (2003) to the SensiFAST mix manufacturer's protocol. This was done to increase the stringency of the conditions in order to ensure that the primers only bind to the intended DNA marker and produce a single product (**Appendix 3A**). As indicated in **Figure 7**, the optimised qPCR-HRM conditions resulted in the production of a single peak (blue curve) during the high-resolution melt-curve analysis with a Tm of 79.35°C and no formation of primer dimers in the negative control (red curve, **Figure 7**).

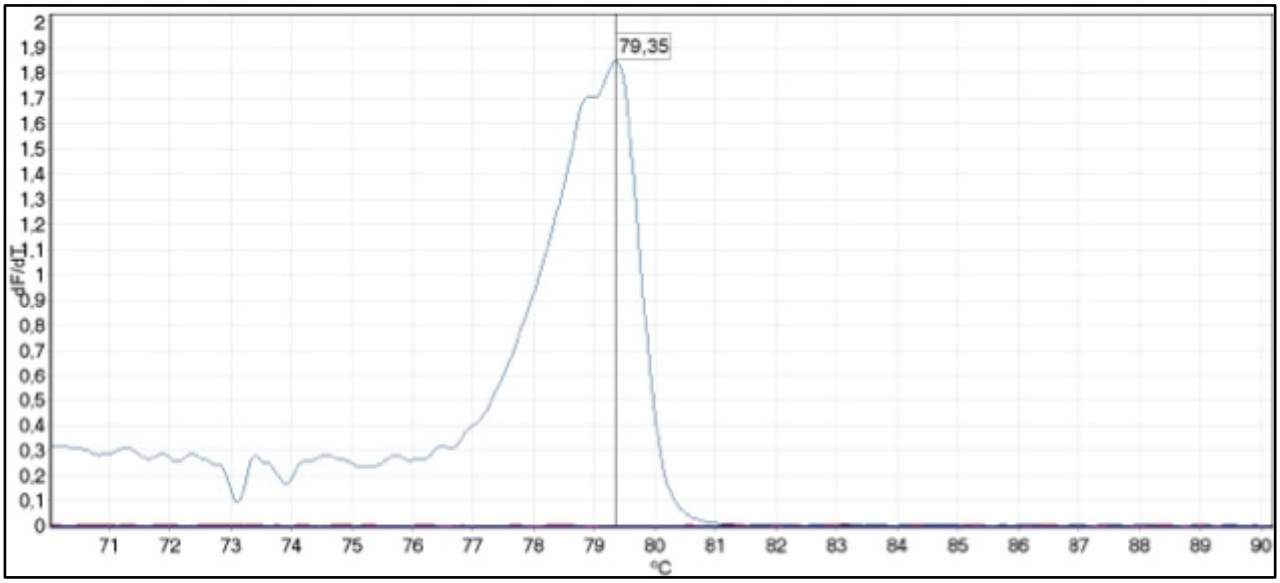

Figure 7. High-resolution melt-curve analysis of *Cryptosporidium parvum* faecal sample DNA extracted using the QIAmp Fast DNA Stool Mini kit and optimised qPCR conditions.

Comparison of the different DNA extraction methods showed that the QIAmp Fast DNA Stool Mini kit (no modifications) was the most succesful method for lyses of the *Cryptosporidium parvum* oocysts and produced the highest quality of DNA based on the subsequent Ct values resulting in the most consistent qPCR-HRM results. A lower Ct value indicates a higher starting concentration of DNA and is optimum when developing a quantitative qPCR-HRM method. Adjusting the concentrations of the primers and the DNA, implementing a three step cycling qPCR rather than two step as well as increasing the anealing temperature of the reaction, proved to increase the results of the amplification and eliminated the formation of primer dimers. These results also proved to be consistent and reproducible throughout the study.

Real-time PCR coupled with HRM analysis offers a high throughput diagnostic analysis of pathogens as well as improves data handling, analysis and storage. It substantially decreases the time needed for testing and can yield qualitative or quantitative results. A study conducted by Pangasa *et al*. (2009) evaluated the use of high-resolution melting-curve (HRM) analysis for the diagnosis of cryptosporidiosis in humans. The PCR-HRM assay approach was found to be suited for the rapid screening of large numbers of *Cryptosporidium* oocyst DNA samples. Although this approach was qualitative and not quantitative, advantages was found over electrophoretic techniques such as shorter analysis time, higher sample throughput and increased data storage and analysis capacities. Pouring, handling and/or scanning of electrophoretic gels was also excluded reducing the overall time and cost of the analysis.

In addition, the SensiFAST HRM kit used in the current study makes use of a double strand DNA binding dye called EvaGreen. This DNA intercalating dye is a saturated dye which intercalates with the nucleotides of all double-stranded DNA. Several studies have indicated that EvaGreen is more cost-effective than other fluorescent dyes and its effectiveness in quantitative PCR and post-PCR DNA melt curve analysis has been proven (Ihrig *et al*., 2006; Sang and Ren, 2006; Wang *et al*., 2006; White *et al*., 2007; Akiyama *et al*., 2009; Dagar *et al*., 2009; Li *et al*., 2010; Sun *et al*., 2010; Ujino-Ihara *et al*., 2010).

3.3.4 Construction of standard curves for quantitation

In the current study synthetic gene fragments [gBlocks Gene Fragments (IDT, USA)] were utilised as positive controls and templates for standard curves intended for the quantitation of an organism. The qPCR-HRM conditions and reagents used for the DNA extracted from the *Cryptosporidium parvum* oocyst samples were also effective in amplification of the gBlock gene fragment dilutions

(**Appendix 3A**). High-resolution melt curve analysis of the amplified products also indicated that the fragments had the expected Tm of 79.33°C (**Figure 8**), which was within the range (78.76°C to 79.90°C) as determined for the COWP gene (COWP-F/COWP-R pimer set) through qPCR-HRM results presented in section 3.3.3. The formation of limited primer dimers can be observed in the negative control. The gBlock gene fragment can therefore be used in order to compare and quantify the DNA concentration of actual samples amplified using the same primer set.

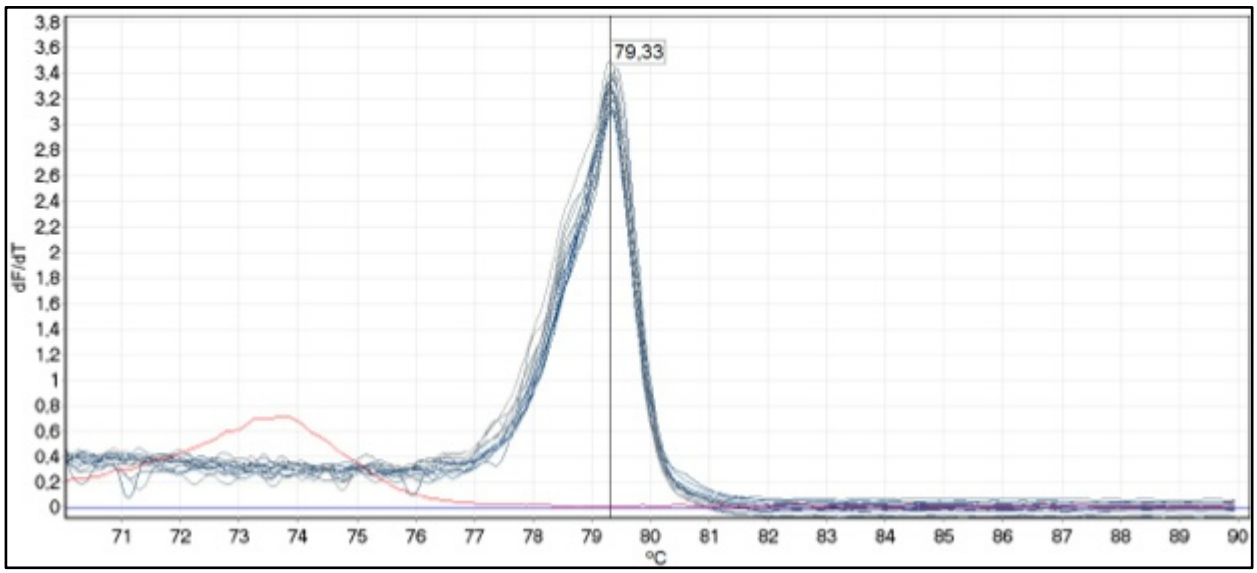

Figure 8. High-resolution melt-curve analysis of the tenfold serial dilution of the *Cryptosporidium parvum* COWP gBlock Gene Fragment ranging from $1x10^{-1}$ ng/µl to $1x10^{-5}$ ng/µl.

The results of the standard curve ($1x10^{-1}$ ng/µl to $1x10^{-5}$ ng/µl) are represented in Figures 9 and 10. A strong positive correlation coefficient was observed for the data (R = 0.993) as well as a coefficient of determination very close to 1 indicating low variance between the samples (R^2 = 0.986). The slope of the standard curve indicates the efficiency of the qPCR and should be as close to -3.32 as possible indicating 100% efficiency (Efficiency = $10^{(-1/slope)}$-1). In **Figure 10** the slope (M) was shown to be –3.598 indicating high efficiency of the qPCR experiment (90%). The standard curve (**Figure 10**) corresponds to the amplification plot (**Figure 9**).

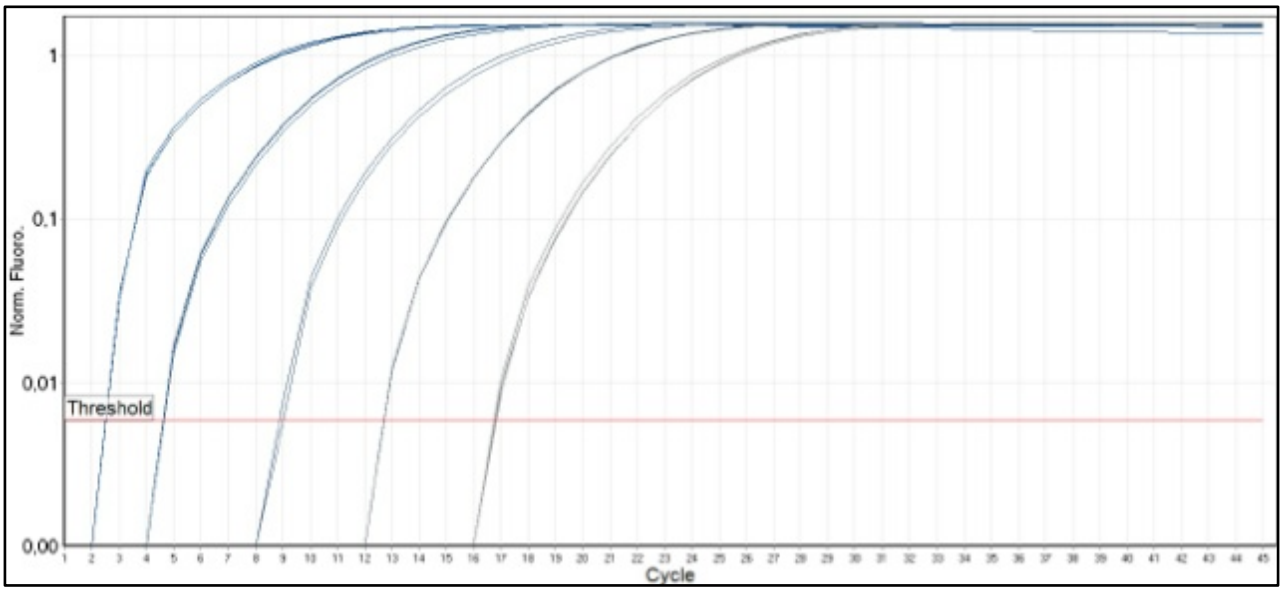

Figure 9. Logarithmic view of the tenfold serial dilution of the *Cryptosporidium parvum* COWP gBlock Gene Fragment ranging from $1x10^{-1}$ ng/µl to $1x10^{-5}$ ng/µl. Fluorescent detection through the FAM channel. The horizontal line shows the threshold.

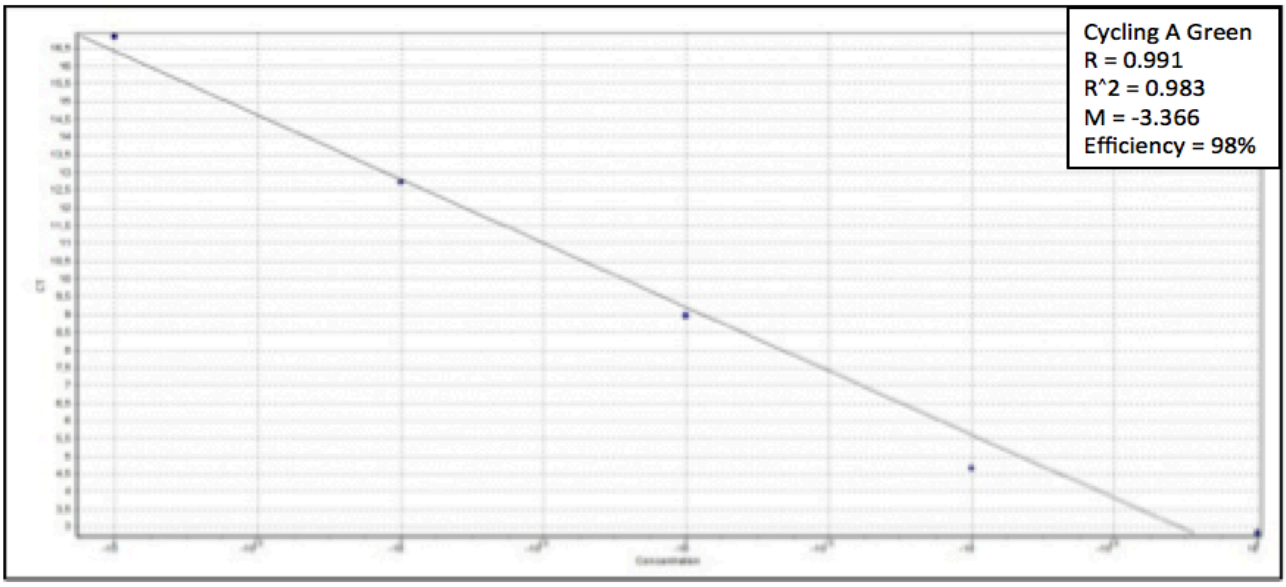

Figure 10. Real-time PCR detection of the tenfold serial dilution of the *Cryptosporidium parvum* COWP gBlock Gene Fragment ranging from $1x10^{-1}$ ng/µl to $1x10^{-5}$ ng/µl.

The Rotorgene 6000, 2-plex PCR machine software allows for the standard curve to be imported into each separate *Cryptosporidium* qPCR run. This in turn then allows for quantitation of the DNA concentration in the unknown samples. A triplicate sample of one of the gBlock dilutions used to set up the standard curve must also be included in the run containing the unknown samples in order for the software to recognise it as a standard sample. For quantitation however, the Ct value of the unknown sample must fall within the dilution range of the standard curve. During this

study it was however not possible to construct an effective standard curve with dilutions up to $1x10^{-8}$ ng/µl due to the low efficiency observed when working with such low concentrations. The results of the standard curve with dilutions up to $1x10^{-8}$ ng/µl are represented in Figures 11 and 12.

Although a strong positive correlation coefficient was observed for the data (R = 0.997) as well as a coefficient of determination very close to 1 (R^2 = 0.995), the slope (M) was shown to be –3.950 indicating a low efficiency (79%) of the experiment (**Figure 12**). As can be seen from the amplification plot (**Figure 11**), triplicates of the lower concentrations do not have similar Ct values or slopes. These discrepancies in turn cause the lower efficiency of the standard curve. In order for a standard curve to be effective in the quantitation of unknown samples, efficiencies of 90% and higher are required (Bustin *et al*., 2009). The biggest shortcoming of the qPCR-HRM assay in the current study was thus the inability of the assay to quantify the *Cryptosporidium parvum* oocysts in the samples at lower dilutions. Future studies could therefore optimise the construction of a standard curve by using either the gBlock Gene fragment or pure *Cryptosporidium parvum* DNA with known concentrations.

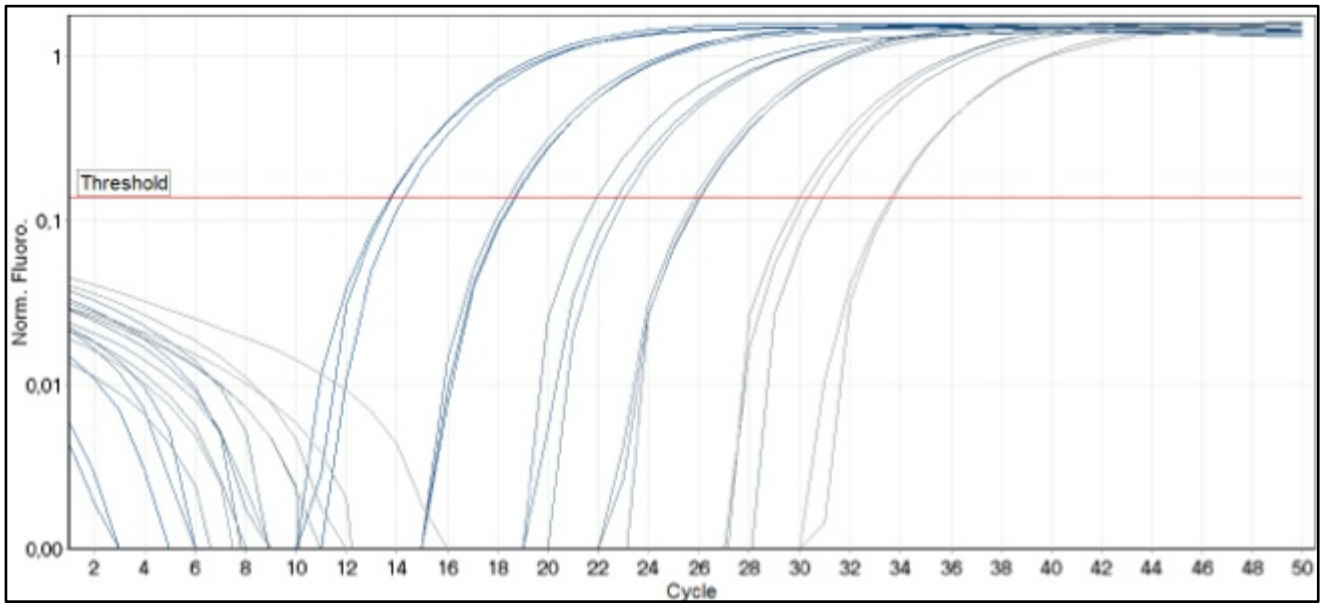

Figure 11. Logarithmic view of the tenfold serial dilution of the *Cryptosporidium parvum* COWP gBlock Gene Fragment ranging from $1x10^{-3}$ ng/µl to $1x10^{-8}$ ng/µl. Fluorescent detection through the FAM channel. The horizontal line shows the threshold.

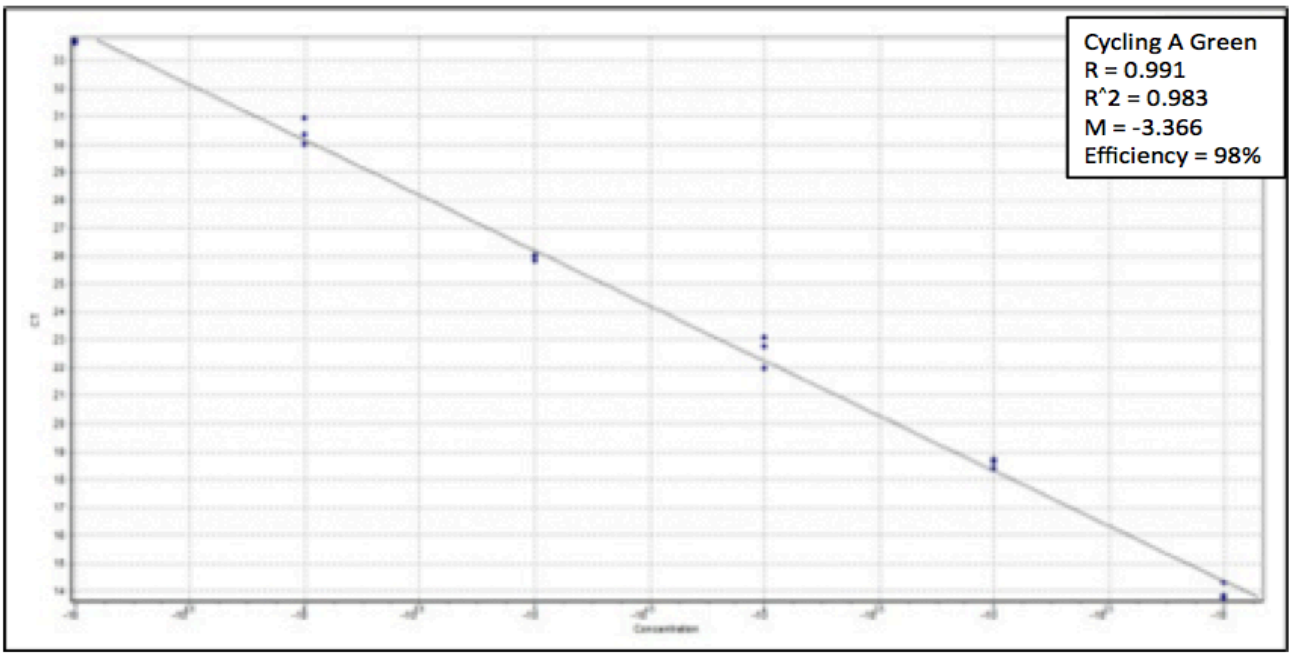

Figure 12. Real-time PCR detection of the tenfold serial dilution of the *Cryptosporidium parvum* COWP gBlock Gene Fragment ranging from $1x10^{-3}$ ng/µl to $1x10^{-8}$ ng/µl.

3.3.5 Real-Time Polymerase Chain Reaction coupled with HRM assay lower limit of detection (LLOD)

The LLOD or sensitivity of the qPCR-HRM assay was tested by amplifying genomic DNA from faecal samples positive for *Cryptosporidium parvum* oocysts with a known concentration of 4.9 ng/µl as determined through NanoDrop analysis. The qPCR-HRM assay was able to detect *Cryptosporidium parvum* DNA dilution 10^{-1} (equivalent to 0.49 ng/µl) consistently (**Table 4**). Dilutions up to 10^{-2}, 10^{-3} and 10^{-4} could also be detected however, results were not consistent between qPCR-HRM assay (**Table 4**), while dilutions of up to 10^{-5} could not be detected during qPCR-HRM assay. It was hypothesised that the inconsistent detection of *Cryptosporidium parvum* DNA dilutions from 10^{-2} – 10^{-4} was due to pipetting inacurracy that could arise when conducting experiments with such low DNA concentrations.

Table 4. Real-time PCR coupled with HRM assay lower limit of detection (LLOD) of serially diluted *Cryptosporidium parvum* genomic DNA (n=9)

Dilution series of DNA	Positive samples (%) (n=9)	Concentration of DNA (ng/µl)
10^{-1}	100%	0.49
10^{-2}	89%	0.049
10^{-3}	34%	4.9×10^{-3}
10^{-4}	45%	4.9×10^{-4}
10^{-5}	0	4.9×10^{-5}

3.3.6 Enumeration of Cryptosporidium parvum *oocysts*

The *Cryptosporidium parvum* oocysts used in the current study were obtained from faecal samples that tested positive for the protozoa with microscopic examination at a local pathologist. *Cryptosporidium parvum* oocyst numbers in faecal samples were determined by using the BX43 phase contrast microscope under 10X magnification. Sedgewick-Rafter Counting Chambers were used to enumerate the oocysts in each grid. The total amount of *Cryptosporidium parvum* oocyst per ml was calculated at approximately 6.5 x 10^6. The enumerated faecal samples were stored at 4°C and were used for subsequent spiking and sample limit of detection experiments. The *Cryptosporidium parvum* oocysts (red arrow) obtained from a faecal sample wet mount is indicated in **Figure 13**. The *Cryptosporidium parvum* oocyst is round in shape, 4 – 6 µm in diameter and the nuclei in the centre stains a pinkish colour when covered with immersion oil.

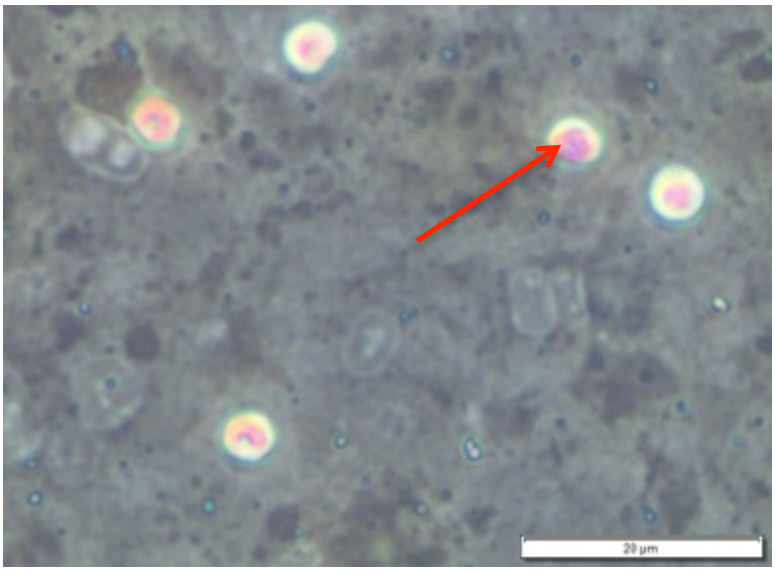

Figure 13. Magnification (10X) of *Cryptosporidium parvum* oocysts obtained from a faecal sample using the BX43 phase contrast microscope.

3.3.7 Sample limit of detection (SLOD)

The qPCR-HRM assay developed in the current study was able to detect *Cryptosporidium parvum* oocysts up to the dilution of 10^{-4} (<1 oocyst/ml) consistently for spiked tap water samples. No *Cryptosporidium parvum* oocysts were detected in the unspiked samples indicating that no cross contamination took place (**Table 5**). The recovery results reported for the present study are similar to results reported in comparable studies. In a study conducted by Hill *et al*. (2009), the recovery efficiency of parasites such as *Giardia intestinalis* and *Cryptosporidium parvum* through hollow-fiber ultrafiltration was compared to the recovery efficiency of these parasites through the

established USEPA method 1623. Results obtained from low-seeding experiments, in which 150 (oo)cysts were seeded into dechlorinated 100 L tap water samples, indicated that the ultrafiltration method yielded higher mean recovery efficiency (51 ± 18%) than Method 1623 (3.9 ± 1.7%). In a study conducted by Morales-Morales *et al*. (2003), recovery efficiencies of 54 ± 1.5% were also reported for 100 *Cryptosporidium parvum* oocysts in 10 L of surface water.

Studies have shown that *Cryptosporidium* is the etiological agent responsible for 60.3% of water associated outbreaks from parasitic protozoan diseases documented worldwide between 2004 and 2010 (Karanis *et al*., 2007; Baldursson and Karanis, 2011). It has also been reported that 23.7% of these outbreaks were caused by *Cryptosporidium* that passed through filtered and unfiltered drinking water systems. In addition contaminated recreational waters were responsible for 50.3% of the cryptosporidiosis outbreaks (Karanis *et al*., 2007). The oocysts of this pathogen are extremely resistant to traditional water treatment methods such as chlorination and very low infectious doses (10 – 100 oocysts) have been reported (Xiao and Fayer, 2008; Yang *et al*., 2014). The detection of <1 oocyst/ml in the current study thus indicates that the qPCR-HRM assay developed could be sensitive enough to be used as a routine monitoring tool for the detection of *Cryptosporidium parvum* oocysts in drinking water samples, however further monitoring studies would have to be conducted in order to evaluate the efficiency over a specific time period.

Table 5. Sample limit of detection (SLOD) of real-time PCR assay coupled with high-resolution melt curve assay for the detection of *Cryptosporidium parvum* oocysts in spiked tap water (n=3)

Dilution series of spiked tap water	Positive samples (%) (n=3)	Number of oocysts/ml
10^{-1}	100%	650
10^{-2}	100%	65
10^{-3}	100%	6.5
10^{-4}	100%	0.65
10^{-5}	0	0
Unspiked tap water	0	0

3.3.8 Application of the optimised qPCR-HRM assay for the detection of Cryptosporidium *in environmental samples*

After the 1 L river water samples collected upstream and downstream of the ERWAT Hartebeesfontein WWTP at three different time intervals were concentrated through membrane filtration (section 3.2.8) and DNA extractions were performed with the QIAamp Fast DNA Stool Mini kit (section 3.2.2.3), the qPCR-HRM assay was then carried out as described in section 3.2.3

using the extracted DNA. Previously extracted *Cryptosporidium parvum* DNA from a faecal sample, used in the optimisation of the qPCR-HRM assay, was also included as a positive control. The positive control had a Ct value of 28.28 and a melting temperature of 79.59°C. All Ct values and Tm results of the six environmental samples qPCR-HRM analyses are presented in **Table 6**. The DNA from the six environmental samples amplified during qPCR and produced HRM analysis peaks that fell within the melting temperature profile range (78.76°C to 79.90°C) as determined during the qPCR-HRM optimisation results for *Cryptosporidium parvum* DNA amplified using the COWP-F/COWP-R primers (**Table 6**). The qPCR products were sequenced and the BLAST analysis confirmed the presence of *Cryptosporidium parvum* oocysts wall protein gene, partial cds (accession number AF481960.1) with an identity of 97% or higher (**Appendix 3B**).

Table 6. Triplicate qPCR-HRM assay of six environmental samples collected upstream and downstream of the ERWAT Hartebeesfontein WWTP

Sampling Date	**Sampling point relative to ERWAT Hartebeesfontein WWTP**	**Cycle threshold (Ct) value**	**Melting temperature (Tm) of peaks**
First Sampling (03/09/2015)	Upstream Downstream	31.13 35.40	79.48°C 79.64°C
Second sampling (07/09/2015)	Upstream Downstream	32.62 31.89	79.01°C 78.93°C
Third sampling (16/10/2015)	Upstream Downstream	31.07 31.25	79.24°C 78.96°C

3.3.9 Application of the commercial Genesig kit for the detection of Cryptosporidium *in environmental samples*

The DNA extracted from the six environmental samples collected upstream and downstream of the ERWAT Hartebeesfontein WWTP were also analysed by qPCR using the Genesig kit for the *Cryptosporidium* genome to compare the use of a primer-probe detection system with a DNA intercalating dye coupled with HRM analysis approach. The standard curve included in the kit quantifies the unknown samples in terms of copy number/µl. A strong positive correlation coefficient was observed for the Genesig kit standard curve data (R = 0.995), with a coefficient of determination very close to 1 indicating low variance between the samples (R^2 = 0.990) and a slope (M) of –3.464 indicating high efficiency of the qPCR experiment (94%) (**Appendix 3B**).

Due to the high efficiency of the data, the standard curve constructed using the Genesig kit could thus be used in order to quantify the number of *Cryptosporidium* DNA copies per µl. Amplification of products with Ct values lower than 35 and detected through the FAM channel are considered as positive for the presence of *Cryptosporidium* DNA. Results of the Genesig kit assay are represented in **Table 7**. The internal extraction control DNA, added to each sample before the DNA extraction protocol is followed and amplified by the internal extraction control primer-probe mix, was detected through the VIC channel and showed positive results for both the up- and downstream samples collected during the first sampling date (03/09/2015) and the downstream sample collected during the third sampling date (16/10/2015) indicating no qPCR inhibitors were present in these samples. The only sample however that showed positive detection of *Cryptosporidium* DNA was the upstream sample collected during the first sampling date (**Table 7**). As determined by the standard curve, this sample contained 3 084 *Cryptosporidium* DNA copies/µl. Although the internal extraction control DNA added to the downstream sample collected during the first sampling date and the downstream sample collected during the third sampling date amplified indicating that no qPCR inhibitors were present in the reaction, the Genesig kit did not detect any *Cryptosporidium* DNA. In addition, the Genesig kit did not detect any *Cryptosporidium* DNA in the up- and downstream samples collected during the second sampling date as well as the upstream sample collected during the third sampling date (**Table 7**). This could be attributed to qPCR inhibitors that are present in the samples due to no amplification observed of the internal extraction control DNA added to these samples.

Table 7. Genesig kit assay of six environmental samples collected upstream and downstream of the ERWAT Hartebeesfontein WWTP

Sampling Date	**Sampling point relative to ERWAT Hartebeesfontein WWTP**	**Cycle threshold (Ct) value**	**Copies/µl**
First Sampling (03/09/2015)	Upstream Downstream	31.10 NPA*	3 084 N/A*
Second sampling (07/09/2015)	Upstream Downstream	NPA* NPA*	N/A* N/A*
Third sampling (16/10/2015)	Upstream Downstream	NPA* NPA*	N/A* N/A*

*NPA = No qPCR amplification

*N/A = Not applicable

3.3.9.1 Comparison of the efficiency of the optimised qPCR-HRM assay and the Genesig kit for the detection of **Cryptosporidium** *in environmental samples*

During the current study it was found that the qPCR-HRM assay was more sensitive in comparison to the Genesig kit for the detection of *Cryptosporidium* DNA (**Table 8)**. Comparison of the two detection methods only yielded similar positive results for the detection of *Cryptosporidium* in the upstream sample collected during the first sampling date (**Table 8**). The positive detection of *Cryptosporidium parvum* DNA in all the samples collected using the qPCR-HRM assay as opposed to *Cryptosporidium* DNA not being detected in the same samples using the Genesig kit, could be attributed to the presence of very low concentrations of *Cryptosporidium* oocysts. Although the Genesig kit is designed to detect all *Cryptosporidium* species and not only *Cryptosporidium parvum* specifically, in the current study *Cryptosporidium* DNA was not detected in any of the five environmental samples using the Genesig kit.

Table 8. Comparison of positive or negative detection of *Cryptosporidium* DNA through qPCR-HRM assay and the Genesig kit

Sampling Date	**Sampling point relative to ERWAT Hartebeesfontein WWTP**	**qPCR-HRM**	**Genesig kit**
First Sampling (03/09/2015)	Upstream Downstream	Positive Positive	Positive Negative
Second sampling (07/09/2015)	Upstream Downstream	Positive Positive	Negative Negative
Third sampling (16/10/2015)	Upstream Downstream	Positive Positive	Negative Negative

Quantitation of *Cryptosporidium* DNA through a primer-probe based qPCR assay, such as the Genesig kit, is indirect and not absolute, as it is based on the concentrations of the standards, which are defined by the Genesig kit manufacturer. Thus, a major limitation of the Genesig kit is that the quantitative data generated is only as accurate as the standards used. The DNA standards are usually obtained using dilutions of cloned *Cryptosporidium* DNA in recombinant plasmids, or dilutions of genomic DNA extracted from known numbers of oocysts (Yang *et al*., 2014). The quantitation of nucleic acids is thus achieved using either spectrophotometry to measure UV absorbance, which can be directly related to the amount of DNA or RNA present, or by measuring

the fluorescence intensity of nucleic acids in the presence of nucleic acid-binding dyes such as EvaGreen. Spectrophotometric quantitation are simple, rapid and inexpensive, but recommended for relatively highly purified and concentrated DNA preparations (5 – 90 ng/µL), as several contaminating compounds show specific absorption coefficients overlapping with that of pure double-stranded DNA (Sambrook and Russel, 2001), which in turn can lead to low selectivity of the method and thus overestimation of the amount of template DNA in a sample or standard (Anon, 2010). Fluorescent-based methods, on the other hand, potentially allow estimation of single stranded DNA (ssDNA), dsDNA or RNA, which is more sensitive. However, this approach requires the use of standards and the generation of standard curves to convert the fluorescent measurement into a DNA concentration. Thus, quantitation of DNA in reference standards for qPCR could be improved by using dyes such as SYTO9, Picogreen or EvaGreen, which excite preferentially when bound to dsDNA and not primer-probe sequences that have to bind to the specific region on the DNA sequence in the sample. For example, the Qubit1.0 fluorometer (Life Technologies, Mulgrave Victoria, Australia) is highly selective for dsDNA through the use of a DNA-specific dye and has been shown to be more accurate than other systems at lower DNA concentrations (Hoffmann and Griebeler, 2013). Similarly, manual enumeration of oocysts using a haemocytometer is relatively inaccurate (due to manual counting, pipetting and dilution errors), whereas counting by a flow cytometer has been shown to be more reliable (Reynolds *et al*., 1999).

A major advantage of the qPCR-HRM assay is that it eliminates the need for post-PCR analysis, such as agarose gel analysis and sequencing. If the HRM product amplified during qPCR falls within the melting temperature profile range (78.76°C to 79.90°C) as determined during the qPCR-HRM optimisation results for *Cryptosporidium parvum* DNA amplified using the COWP-F/COWP-R primers, it can be concluded that the product is in fact *Cryptosporidium parvum*. An advantage of the Genesig kit is that it was able to quantify the *Cryptosporidium* copy numbers present in one of the samples, whereas the qPCR-HRM assay could only detect the presence or absence of *Cryptosporidium parvum*. During the current study it was thus concluded that the qPCR-HRM assay could be applied to environmental water samples for the detection of *Cryptosporidium parvum*, however further optimisation and monitoring studies will have to be conducted in order to accurately evaluate the efficiency and sensitivity of the method.

The costs-and time-effectiveness of each method was comprehensively calculated (**Appendix 3C**) and compared in **Table 9**. In the current study it was found that the qPCR-HRM assay approach was the more cost-effective method for the detection of *Cryptosporidium parvum* oocysts in

environmental samples. The Genesig kit however, has the potential to quantify the amount of *Cryptosporidium* oocysts in the environmental samples. It should also be noted, that analysis of environmental samples through both methods can be completed in approximately 7 hours. One of the standard methods used internationally for the detection of *Cryptosporidium* in water is the U.S. Environmental Protection Agency Method 1623.1 (USEPA, 2012). This method detects *Cryptosporidium* and *Giardia* in water samples by concentration, immunomagnetic separation (IMS), and immunofluorescence assay (FA) microscopy. *Cryptosporidium* and *Giardia* are further characterized using 4',6-diamidino-2-phenylindole (DAPI) staining and differential interference contrast (DIC) microscopy (USEPA, 2012). Method 1623.1 identifies the genera, *Cryptosporidium* or *Giardia*, but not the species and cannot be utilised to determine the host species of origin, nor can it determine the viability or infectivity of detected oocysts and cysts (USEPA, 2012). In addition, this method can only be completed by analysts highly experienced in water filtration techniques, IMS, fluorescent antibody staining with monoclonal antibodies, and microscopic examination of biological particulates using bright-field and DIC microscopy, which can take up to two weeks to complete. Specialised laboratory equipment is also needed for the detection of *Cryptosporidium* and *Giardia* through method 1623.1 (USEPA, 2012). Thus when comparing molecular analysis methods such as the qPCR-HRM assay and the Genesig kit with the U.S. Environmental Protection Agency Method 1623.1 (USEPA, 2012) it is evident that molecular based methods such as these used in the current study are a more cost- and time-effective option for the routine detection of *Cryptosporidium* in water samples.

Table 9. Comparison of total time required to process samples and costs involved for each molecular based method

	Real-Time PCR coupled with HRM assay	**Genesig kit**
Total Time	± 7hrs	± 7hrs
Total Cost	R578.96	R794.57

3.4 Conclusions

The efficiency of various protocols for the extraction of *Cryptosporidium parvum* DNA from parasite suspensions and faecal samples containing *Cryptosporidium parvum* oocysts was evaluated in the current study. Subsequent results showed that DNA obtained using the QIAamp Fast DNA Stool Mini kit was the most efficient method for the lyses of the *Cryptosporidium parvum* oocysts. Template DNA extracted using the QIAamp Fast DNA Stool Mini kit also produced the highest

quality of *Cryptosporidium parvum* DNA resulting in the most consistent qPCR-HRM results. The COWP-F/COWP-R primers, amplifying the *Cryptosporidium parvum* oocysts wall protein genomic DNA, also proved effective in detection of this intestinal parasite and a Tm was established at 79.38°C ± 0.31°C. The qPCR-HRM assay also showed high detection sensitivity with the ability to detect less than one *C. parvum* oocysts per ml from spiked tap water samples. A real-time PCR coupled with HRM assay utilising the DNA intercalating dye, EvaGreen, for the detection of *Cryptosporidium parvum* was thus successfully developed and partially optimised. This method has the potential to be implemented for the routine detection of *Cryptosporidium parvum* oocysts in drinking water and environmental water samples. It can therefore also be used as a valuable tool to aid in the characterisation and classification of water intended for drinking purposes as stipulated by the Department of Water Affairs and Forestry (1996). Further studies should however, include quantitative real-time PCR coupled with HRM analysis of the *Cryptosporidium parvum* oocysts through the optimisation of a standard curve using gBlock gene fragments or purified *Cryptosporidium parvum* DNA.

While there is an increased focus by municipalities throughout South Africa to detect *Cryptosporidium* and *Giardia* (oo)cysts in water supplies, current methods employed are often time-consuming and laborious. These organisms are also highly resistant to numerous chemical and physical treatments and can in turn be released in effluent water. It is therefore important to develop methods with lower costs and turnaround time as a routine monitoring method. Comparative studies between the qPCR-HRM assay and the Genesig kit then indicated that the qPCR-HRM assay was the most cost-effective method for the evaluation of the presence/absence of *Cryptosporidium parvum*, however the Genesig kit has the added advantage of potentially quantifying the *Cryptosporidium* copy numbers in the samples. Further studies should thus include routine monitoring in order to accurately evaluate the efficiency and sensitivity of the qPCR-HRM assay and the Genesig kit on drinking water and environmental water samples. However, implementation of the molecular based qPCR-HRM assay could potentially evaluate the presence or absence and thus in turn the removal efficiency of *Cryptosporidium parvum* from drinking water and environmental water and shows great potential for implementation in routine water analysis laboratories such as ERWAT.

3.5 References

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Chapter 4:

General Conclusions and Recommendations

4.1 General Conclusions and Recommendations

The routine monitoring of drinking water, environmental water and wastewater for intestinal pathogens is essential as water authorities need to ensure that the different water sources adheres to regulatory standards as stipulated by the South African National Standards (SANS 214, 2015) and the Department of Water Affairs and Forestry (DWAF, 1996; DWAF, 2006) and can in turn be used for its intended purposes. In order to accurately evaluate the removal of particularly *Ascaris* sp. and *Cryptosporidium* sp. in water treatment processes, rapid, accurate and reproducible methods, such as molecular based methods, are required for the routine detection and quantitation of these pathogens.

4.2 *Ascaris lumbricoides*

The first aim of the current study was to develop and optimise a qPCR-HRM protocol for the detection and quantitation of *Ascaris lumbricoides* eggs in sludge samples. The $ZnSO_4$ flotation method coupled with membrane filtration was found to be effective for the separation and concentration of *Ascaris lumbricoides* eggs from sludge samples with a high solid content and turbidity. The efficiency of various protocols for the extraction of *Ascaris lumbricoides* DNA from sewage sludge was evaluated and subsequent results showed that DNA obtained using the QIAamp Fast DNA Stool Mini kit, in conjunction with the bead beating and freeze-boil cycles, was the most efficient method for the lyses of the *Ascaris lumbricoides* eggs in sewage sludge samples. Because of recurring inconsistent results obtained when using spectrophotometry, this evaluation was based on the lowest Ct value (29.53) obtained when utilising the qPCR-HRM assay.

Template DNA extracted using the QIAamp Fast DNA Stool Mini kit in conjunction with the bead beating and freeze-boil cycles also produced the highest quality of *Ascaris lumbricoides* DNA resulting in the most consistent qPCR-HRM results. A real-time PCR coupled with HRM analysis utilising the DNA intercalating dye, EvaGreen, for the detection of *Ascaris lumbricoides* in sludge was thus successfully developed and partially optimised. The Asc1-F/Asc2-R primers, amplifying the cytochrome b fragment of *Ascaris lumbricoides* mtDNA, proved effective in the successful detection of this helminth species and a consistent amplicon Tm was established at 78.17°C ± 0.27°C. Resulting real-time PCR products were submitted for DNA sequencing and BLAST

analysis confirmed homology to the *Ascaris lumbricoides* mitochondrion complete genome (accession number HQ704900.1) with an identity similarity of 97% or higher.

After numerous attempts, DNA extracted from the sludge samples failed to deliver usable standard curves, possibly due to the low concentration of DNA present and synthetic gBlock Gene Fragments were then utilised to construct the standard curve for the quantitation of *Ascaris lumbricoides*. Construction of a standard curve for quantitation of *Ascaris lumbricoides* using synthetic gBlock Gene Fragments was successfully carried out for dilutions up to $1x10^{-5}$ ng/µl. However, it was not possible to construct an effective standard curve with dilutions up to $1x10^{-8}$ ng/µl due to the low PCR efficiency observed when working with such low concentrations. A standard curve spanning the complete Ct range when performing the qPCR-HRM assay was crucial for accurate quantification purposes and quantitation of *Ascaris* eggs could thus not be achieved using the qPCR-HRM assay in combination with the constructed standard curve.

Comparative analysis of the optimised qPCR-HRM assay, the Genesig kit and the ERWAT microscopy method (Chapter 2) indicated that the qPCR-HRM analysis was the most cost-effective. It also allowed for the rapid detection and identification of *Ascaris lumbricoides* in sewage sludge samples requiring much less labour and staff experience. Although the microscopy method allowed for the quantification of *Ascaris* in sewage sludge samples, the time and labour required to perform this method is substantial. Laboratory analysts performing this method also require experience to correctly identify *Ascaris* eggs. The Genesig kit was successful in detecting and identifying *Ascaris* in the sewage samples and had the added advantage of quantifying the *Ascaris lumbricoides* copy numbers in the samples analysed.

Extracting genomic DNA of a high quality and yield from *Ascaris lumbricoides* eggs remains a big challenge. Though fairly successful, the current method to isolate and purify *Ascaris* eggs from sewage sludge samples needs further optimisation. The efficiency of the DNA extraction methods used in this study also require further evaluation and possible optimisation to ensure high DNA extraction rates from *Ascaris* eggs. Further studies to extensively evaluate and compare the sensitivity and selectivity of the optimised qPCR-HRM assay are thus recommended. Comparing the performance of the qPCR-HRM assay to commercial kits such as the Genesig kit and extending the use of the qPCR-HRM assay to analyse a larger sample set and compare it with concurring microscopy results, is also recommended. Once fully optimised, it is recommended that the qPCR-HRM assay undergoes validation in accordance with the requirements as stipulated for ISO/IEC

17025 accreditation purposes. Having the qPCR-HRM assay ISO/IEC 17025 accredited will add credibility and value. This will allow water analysis laboratories to offer a niche service to municipalities and other water analysis agencies for the evaluation of the presence/absence of *Ascaris lumbricoides* in wastewater and sludge. These molecular methods could therefore also be used by ERWAT for further quantitation and monitoring studies and could be used as a valuable tool to aid in the characterisation and classification of sludge generated at wastewater treatment plants in order to determine which management options are suitable and what processes the characterised sludge can be utilised for (DWAF, 2006).

4.3 *Cryptosporidium parvum*

The second aim of the study then focussed on optimising a qPCR-HRM assay for the detection and quantitation of *Cryptosporidium parvum* oocysts in faecal samples. The efficiency of various protocols for the extraction of *Cryptosporidium parvum* DNA from parasite suspensions and faecal samples containing *Cryptosporidium parvum* oocysts was also evaluated. Subsequent results showed that the QIAamp Fast DNA Stool Mini Kit was the most efficient method for the lyses of the *Cryptosporidium parvum* oocysts and extraction of DNA based on the lowest Ct value (26.65) obtained when utilising this method on faecal samples.

Template DNA extracted from faecal samples using the QIAamp Fast DNA Stool Mini Kit also produced the highest quality of *Cryptosporidium parvum* DNA resulting in the most consistent qPCR-HRM results. The COWP-F/COWP-R primers, amplifying the *Cryptosporidium parvum* oocysts wall protein genomic DNA, also proved effective in detection of this intestinal parasite and a Tm was established at 79.38°C ± 0.31°C. Real-time PCR products within this Tm range were sent for sequencing and BLAST analysis confirmed the presence of *Cryptosporidium parvum* oocysts wall protein 1 (COWP1) gene (accession number JQ349359.1) with an identity of 97% or higher. The qPCR-HRM assay showed high detection sensitivity with the ability to detect less than one *C. parvum* oocysts per ml from spiked tap water samples. A real-time PCR coupled with HRM assay utilising the DNA intercalating dye, EvaGreen, for the detection of *Cryptosporidium parvum* was thus successfully developed and partially optimised.

Similar to the results obtained for *Ascaris*, DNA extracted from the EasySeed suspension and faecal samples failed to deliver usable standard curves and synthetic gBlock Gene Fragments were then

utilised to construct the standard curve for the quantitation of *Cryptospordium parvum*. Construction of a standard curve for quantitation of *Cryptosporidium parvum* using synthetic gene fragments was successfully carried out for dilutions up to $1x10^{-5}$ ng/µl however, it was not possible to construct an effective standard curve with dilutions up to $1x10^{-8}$ ng/µl due to the low PCR efficiency observed when working with such low concentrations. Comparative studies between the qPCR-HRM assay and the Genesig kit (Chapter 3) then indicated that the qPCR-HRM assay was the most cost-effective method for the evaluation of the presence/absence of *Cryptosporidium parvum*, however the Genesig kit had the added advantage of quantifying the *Cryptosporidium parvum* copy numbers in one of the samples.

There is an increased focus by municipalities to detect intestinal parasites such as *Cryptosporidium parvum* in various water sources, due to the possible adverse health implications on the surrounding communities. It is therefore important to explore alternative methods for the detection of *Cryptosporidium parvum* that are rapid, reliable and cost-effective. The methods described in the current study have the potential to be implemented as a routine monitoring method but will also have to undergo validation and evaluation in accordance with the requirements as stipulated for ISO/IEC 17025 accreditation purposes. Companies such as ERWAT could therefore offer an alternative method for the evaluation of the presence/absence of *Cryptosporidium parvum,* in different water sources, to municipalities and other water analysis agencies. Newly developed methods for the detection of *Cryptosporidium parvum* oocysts in drinking water and environmental water samples can therefore be used by ERWAT as a valuable tool to aid in the characterisation and classification of water intended for drinking purposes as stipulated by the South African National Standards (SANS 214, 2015) and the Department of Water Affairs and Forestry (DWAF, 1996). However, due to the fairly novel approach of using qPCR-HRM curve assay for the detection of these protozoan species in the respective matrixes, thorough optimisation of the method will be required before implementation in a routine analysis laboratory.

The biggest disadvantage of qPCR still remains the inability to differentiate between viable and non-viable microorganisms and future studies could thus include qPCR assay coupled with DNA-intercalating dyes such as ethidium monoazide (EMA) and propidium monoazide (PMA) (Delgado-Viscogliosi *et al*., 2009). Ethidium monoazide and PMA are membrane impermeable, and do not pass through the membranes of intact cells. The dye will thus covalently bind to deoxyribonucleic acid (DNA) originating from dead or damaged cells after photoactivation and this DNA cannot be amplified during quantitative PCR. Thus, only the DNA of cells with intact membranes can be

amplified and quantified. Previous studies have been conducted with EMA and PMA, in conjunction with qPCR for the selective detection of viable bacterial cells (Rudi *et al*., 2005; Inoue *et al*., 2008). These studies effectively detected viable bacteria cells by treating water samples with EMA prior to qPCR assay. Ethidium monoazide qPCR (EMA-qPCR) has also been used to effectively detect bacteria in food (Rudi *et al*., 2005), biofilms (Chen and Chang, 2010) and water samples (Inoue *et al*., 2008). In a study conducted by Qin *et al.* (2012), EMA-qPCR was used to detect *Legionella* in environmental samples. Findings suggested that the percentage positive rate obtained by EMA-qPCR was significantly higher than with conventional PCR coupled with culturing methods and only slightly lower than qPCR. In a study conducted by Alonso *et al*. (2014), there authors were able to distinguish between viable and non-viable *Cryptosporidium* and *Giardia* (oo)cysts spiked into phosphate-buffered saline and tertiary effluent wastewater using a PMA-qPCR method. Quantitative real-time PCR (qPCR) could thus be combined with DNA-intercalating dyes in order to evaluate the viability of parasites such as *Ascaris* sp. and *Cryptosporidium* sp. in different water sources.

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Appendix 2A

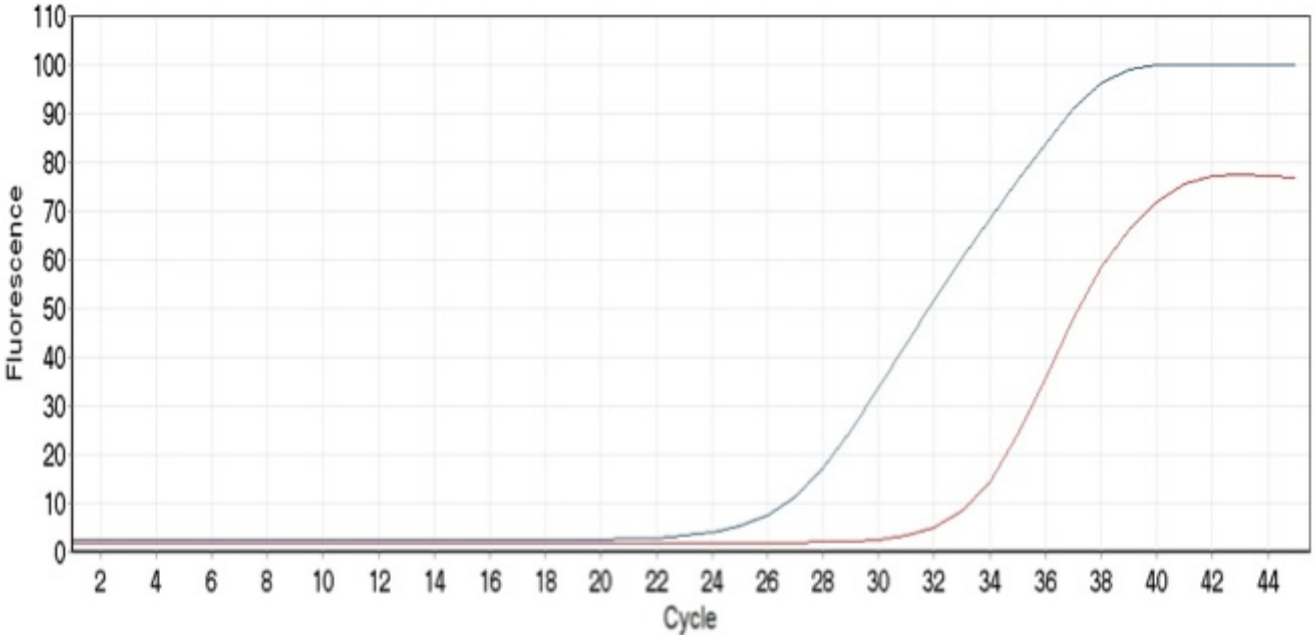

Figure 1. Cycling green fluorescence data from representative *Ascaris lumbricoides* sewage sludge DNA extracted using the DNAeasy Blood and Tissue kit in conjunction with bead beating and freeze-boil cycles.

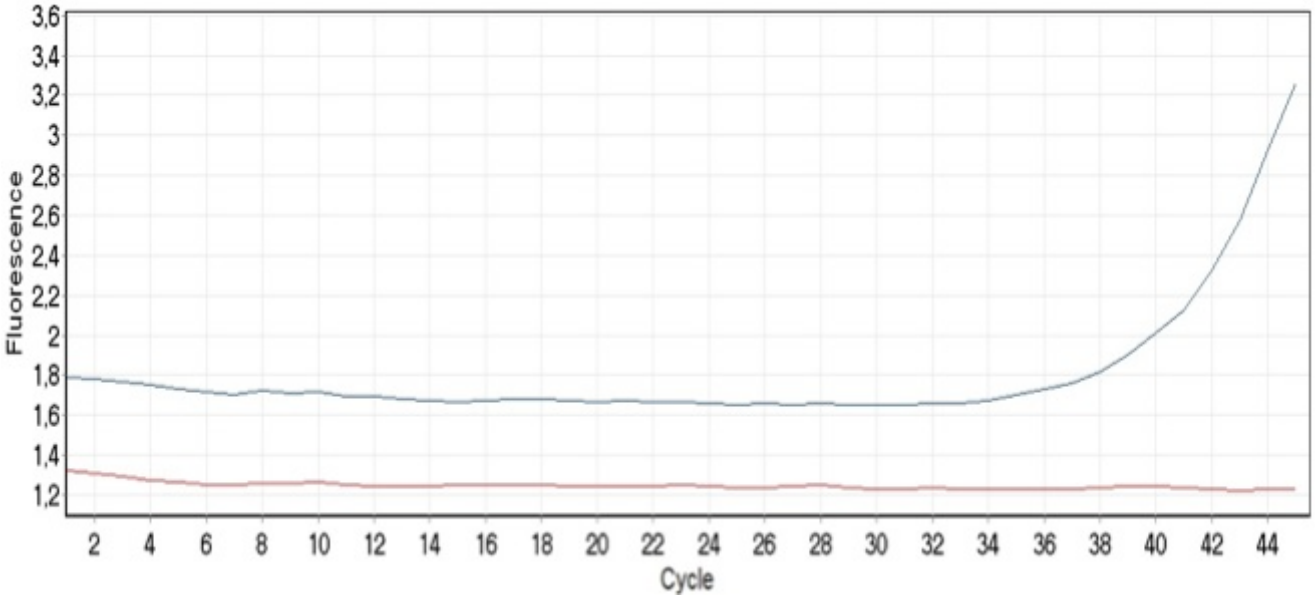

Figure 2. Cycling green fluorescence data from representative *Ascaris lumbricoides* sewage sludge DNA extracted using the DNAeasy Blood and Tissue kit in conjunction with bead beating and freeze-boil cycles and optimised qPCR conditions.

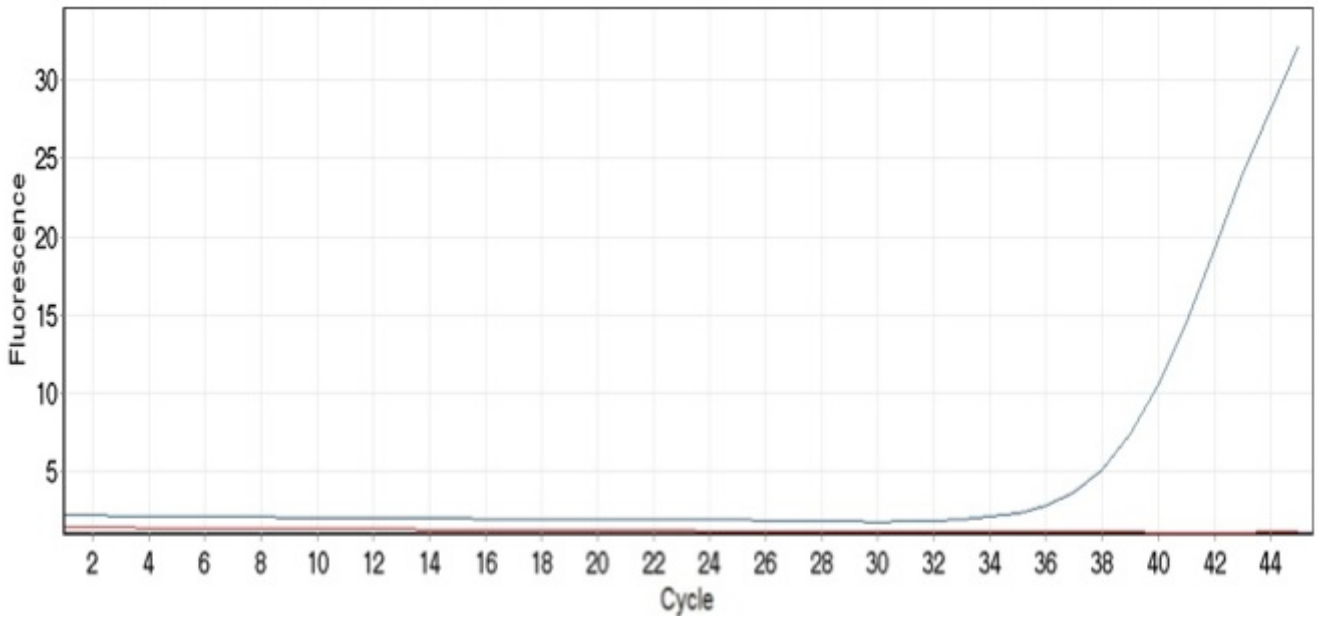

Figure 3. Cycling green fluorescence data from representative *Ascaris lumbricoides* sewage sludge DNA extracted using the QIAamp Fast DNA Stool Minikit and optimised qPCR assay.

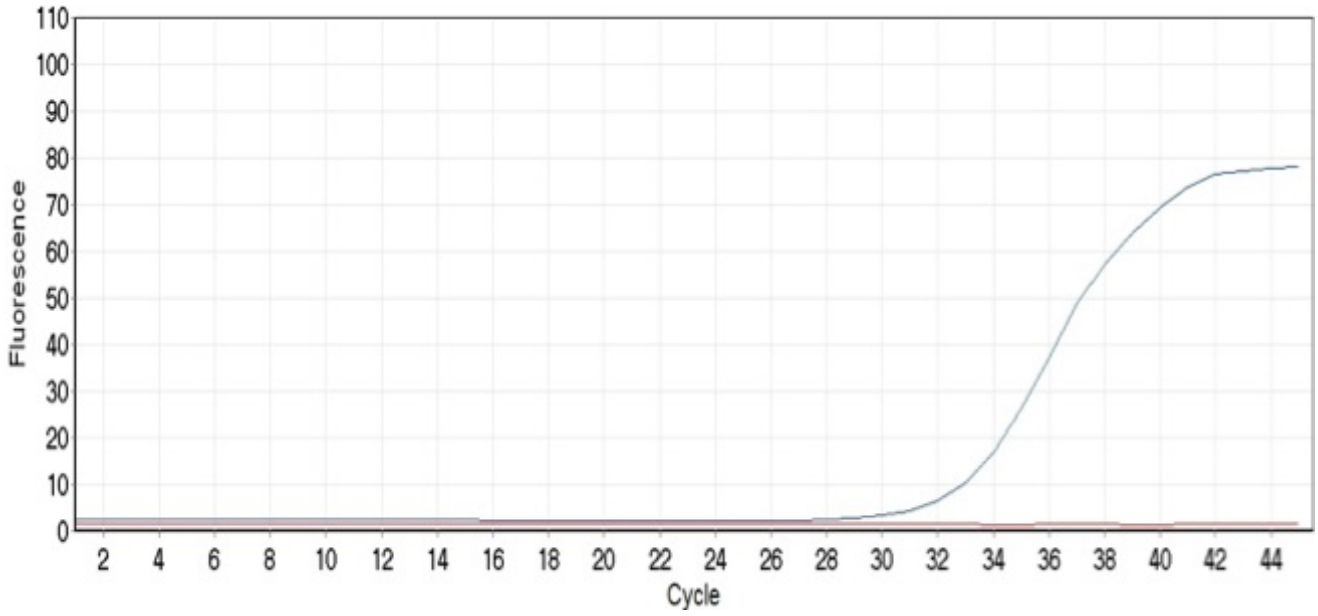

Figure 4. Cycling green fluorescence data from *Ascaris lumbricoides* wet sludge sample DNA extracted using the QIAamp Fast DNA Stool Minikit in conjunction with bead beating and freeze-boil cycles and optimised protocol.

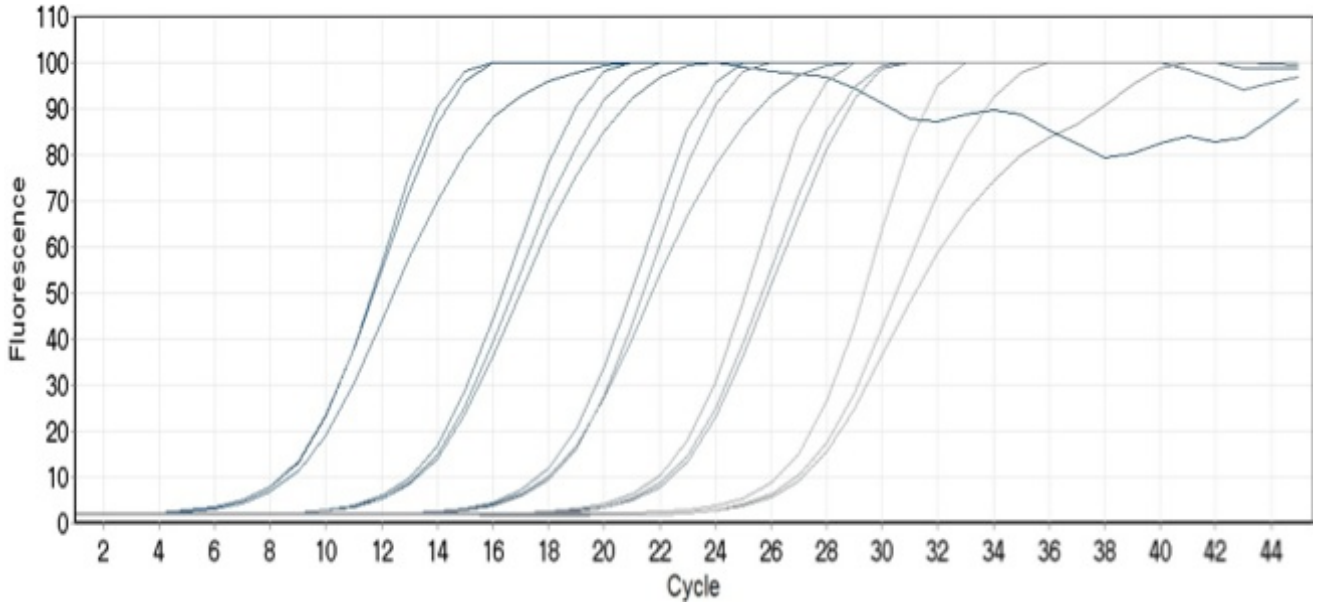

Figure 5. Cycling green fluorescence data from the tenfold serial dilution of the *Ascaris lumbricoides* cytochrome b gBlocks Gene Fragment ranging from $1x10^{-1}$ ng/µl to $1x10^{-5}$ ng/µl.

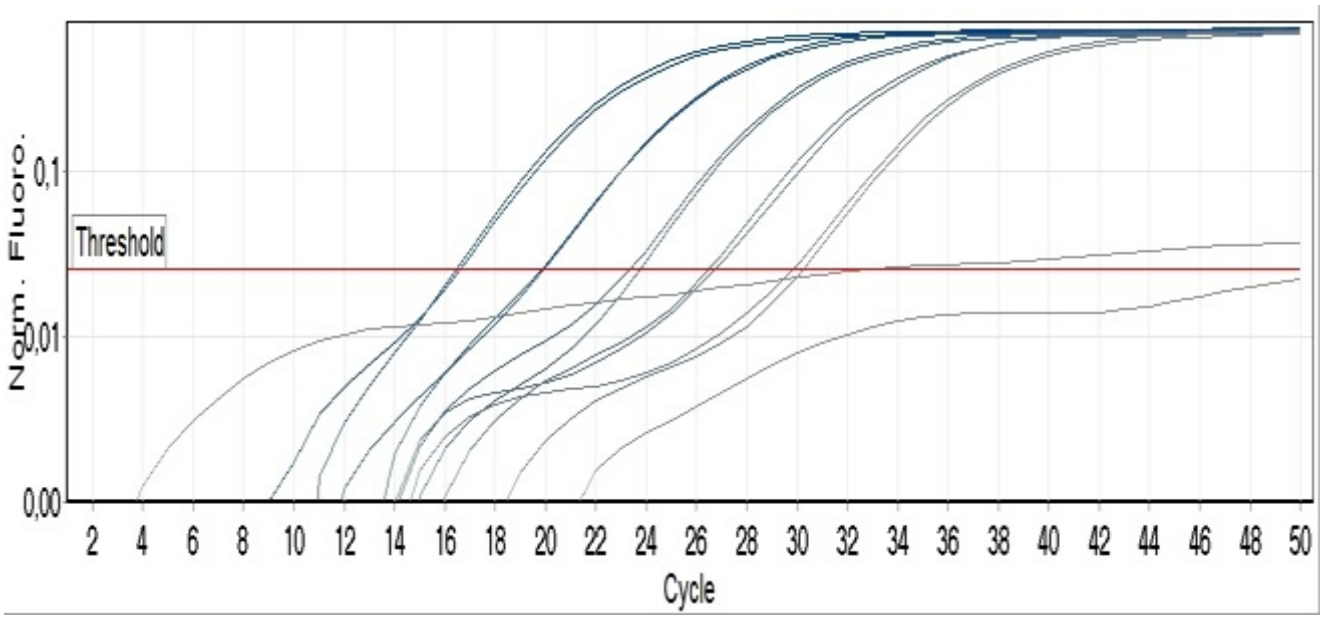

Figure 6. Logarithmic view of the tenfold serial dilution of the *Ascaris lumbricoides/Ascaris suum* Genesig Kit DNA ranging from 2 copies/µl to $2x10^{5}$ copies/µl. Fluorescent detection through the FAM channel. The horizontal line shows the threshold.

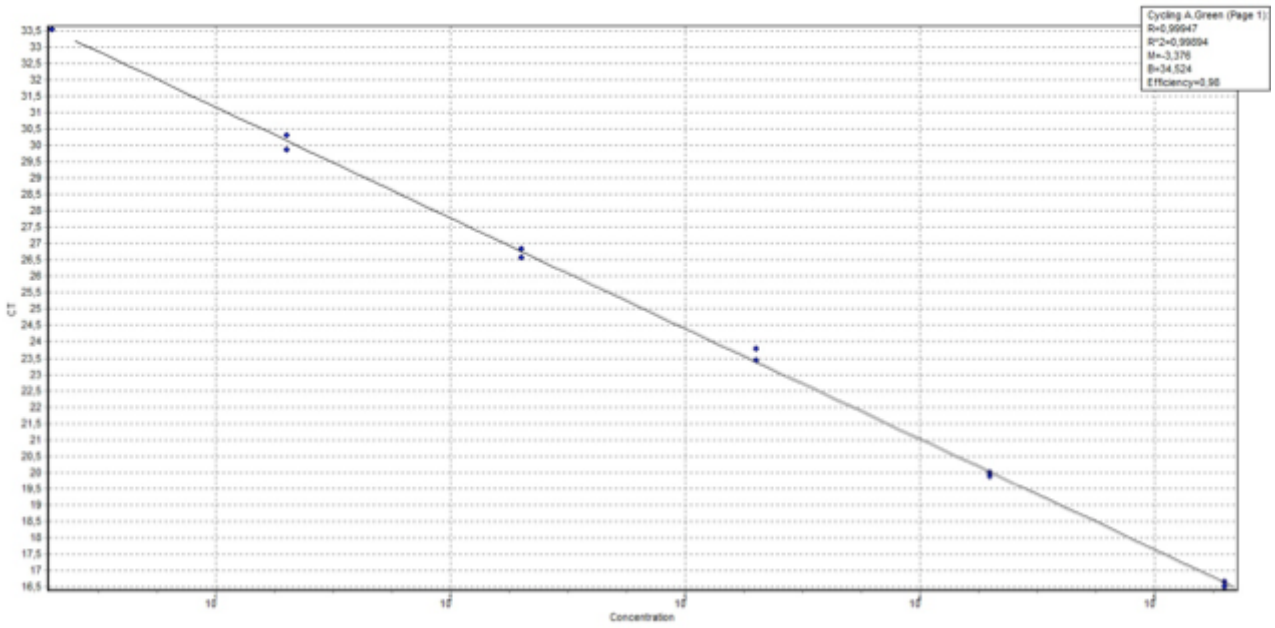

Figure 7. Real-time PCR detection of the tenfold serial dilution of the *Ascaris lumbricoides/Ascaris suum* Genesig Kit DNA ranging from 2 copies/µl to $2x10^5$ copies/µl.

Appendix 2B

Table 1. Evaluation of qPCR-HRM assay of seven sludge samples collected from the six different WWTPs

Sample source	**Melting temperature (Tm) of peaks**	**Description**	**Identity**	**Accession number**
Ascaris lumbricoides Positive Control	77,90°C	*Ascaris lumbricoides*, mitochondrion, complete genome	97%	HQ704900.1
Heidelberg WWTP	80, 21°C	No significant similarity found	N/A	N/A
Heidelberg WWTP	73,17°C	No significant similarity found	N/A	N/A
Olifantsfontein WWTP[1]	78,31°C	*Ascaris lumbricoides*, mitochondrion, complete genome	97%	HQ704900.1
Olifantsfontein WWTP[2]	81,16°C	No significant similarity found	N/A	N/A
Ratanda WWTP	78,31°C	*Ascaris lumbricoides*, mitochondrion, complete genome	98%	HQ704900.1
Welgedacht WWTP	80,79°C	No significant similarity found	N/A	N/A
Jan Smuts WWTP	77,97°C	*Ascaris lumbricoides*, mitochondrion, complete genome	98%	HQ704900.1
Waterval WWTP	77,89°C	*Ascaris lumbricoides*, mitochondrion, complete genome	98%	HQ704900.1

*NPA = No qPCR amplification

[1] = First qPCR-HRM results

[2] = Second qPCR-HRM results

Appendix 2C

Table 1. ERWAT microcsopy method cost breakdown

Reagent or Consumable	Cost per unit (excl. VAT)*	Required	Total Cost (excl. VAT)*
Falcon tube	R0.80	5	R4.00
Ammonium bicorbonate	R2.856 per 1000 ml	140 ml	R0.43
Zinc sulphate	R95.45 per 1000 ml	160 ml	R15.27
20 µm nitrocellulose membrane	R10.62	2	R21.24
2 ml microcentrifuge tube	R0.25	1	R0.25
1000 µl filter tip	R0.67	8	R5.36
Microscope slide	R0.24	1	R0.24
Cover slip	R0.07	1	R0.07
Total cost for reagents or consumables			R46.86
Total cost of labour			R660.00
Time required			50 hours
Total cost of ERWAT microscopy method			R706.86

*VAT = Value added tax at 14 %

Table 2. Sludge sample preparation costs

Reagent or Consumable	Cost per unit (excl. VAT)*	Required	Total Cost (excl. VAT)*
Falcon tube	R0.80	5	R4.00
Ammonium bicorbonate	R2.856 per 1000 ml	140 ml	R0.43
Zinc sulphate	R95.45 per 1000 ml	160 ml	R15.27
20 µm nitrocellulose membrane	R10.62	2	R21.24
2 ml microcentrifuge tube	R0.25	1	R0.25
1000 µl filter tip	R0.67	8	R5.36
Total cost for reagents or consumables			R46.55
Total cost of labour			R110.00
Time required			60 minutes
Total cost for sample preparation			R156.55

*VAT = Value added tax at 14 %

Table 3. DNA Extraction costs

Reagent or Consumable	**Cost per unit (excl. VAT)***	**Required**	**Total Cost (excl. VAT)***
Beadbug Tube	R27.20	1	R27.20
2 ml microcentrifuge tube	R0.25	1	R0.25
1,5 ml microcentrifuge tube	R0.25	2	R0.50
1000 µl filter tip	R0.67	10	R6.70
200 µl filter tip	R0.67	1	R0.67
10 µl filter tip	R0.67	2	R1.34
QIAamp Fast DNA Stool Minikit	R95.99	1 reaction	R95.99
Total cost for reagents or consumables			R132.65
Total cost of labour			R275.00
Time required			150 minutes
Total cost for DNA extraction			R407.65

*VAT = Value added tax at 14 %

Table 4. Real-Time PCR assay coupled with HRM assay costs

Reagent or Consumable	**Cost per unit (excl. VAT)***	**Required**	**Total Cost (excl. VAT)***
1.5ml microfuge tube	R0.25	1	R0.25
100µl filter tip	R0.67	2	R1.34
200µl filter tip	R0.67	4	R2.68
10µl filter tip	R0.67	4	R2.68
Nuclease free water	R0.004 per µl	100µl	R0.40
SensiFast HRM kit	R13.50 per reaction	1 reaction	R13.50
Oligo nucleotides	R2.50 per µM	0.4µM	R1.00
Quality control:	**Negative control (NTC)**		
Reagent or consumable:	**Cost per unit (excl. VAT)*:**	**Required:**	**Total cost (excl. VAT)*:**
1.5ml microfuge tube	R0.40	1	R0.25
100µl filter tip	R0.67	2	R1.34
200µl filter tip	R0.67	4	R2.68

10µl filter tip	R0.67	4	R2.68
Nuclease free water	R0.004 per µl	100µl	R0.40
SensiFast HRM kit	R13.50 per reaction	1 reaction	R13.50
Oligo nucleotides	R2.50 per µM	0.4µM	R1.00
Quality control:	**Positive control (PC)**		
Reagent or consumable:	**Cost per unit (excl. VAT)*:**	**Required:**	**Total cost (excl. VAT)*:**
1.5ml microfuge tube	R0.40	1	R0.25
100µl filter tip	R0.67	2	R1.34
200µl filter tip	R0.67	4	R2.68
10µl filter tip	R0.67	4	R2.68
Nuclease free water	R0.004 per µl	100µl	R0.40
SensiFast HRM kit	R13.50 per reaction	1 reaction	R13.50
Oligo nucleotides	R2.50 per µM	0.4µM	R1.00
Total cost for reagents or consumables			R65.55
Total cost of labour			R55.00
Time required for set up			30 minutes
Time required for qPCR run			152 minutes
Total cost of qPCR-HRM assay			R120.55

*VAT = Value added tax at 14 %

Table 5. Genesig Kit costs

Reagent or Consumable	**Cost per unit (excl. VAT)***	**Required**	**Total Cost (excl. VAT)***
1.5ml microfuge tube	R0.25	1	R0.25
100µl filter tip	R0.67	2	R1.34
200µl filter tip	R0.67	4	R2.68
10µl filter tip	R0.67	4	R2.68
Nuclease free water	R0.004 per µl	100µl	R0.40
Genesig Kit	R67.76 per reaction	1 reaction	R67.76
Oasig 2 x qPCR Mastermix	R18.61 per reaction	1 reaction	R18.61
Quality control:	**Negative control (NTC)**		
Reagent or consumable:	**Cost per unit (excl.**	**Required:**	**Total cost (excl.**

	VAT)*:		VAT)*:
1.5ml microfuge tube	R0.40	1	R0.25
100µl filter tip	R0.67	2	R1.34
200µl filter tip	R0.67	4	R2.68
10µl filter tip	R0.67	4	R2.68
Nuclease free water	R0.004 per µl	100µl	R0.40
Genesig Kit	R67.76 per reaction	1 reaction	R67.76
Oasig 2 x qPCR Mastermix	R18.61 per reaction	1 reaction	R18.61
Quality control:	**Positive control (PC)**		
Reagent or consumable:	**Cost per unit (excl. VAT)*:**	**Required:**	**Total cost (excl. VAT)*:**
1.5ml microfuge tube	R0.40	1	R0.25
100µl filter tip	R0.67	2	R1.34
200µl filter tip	R0.67	4	R2.68
10µl filter tip	R0.67	4	R2.68
Nuclease free water	R0.004 per µl	100µl	R0.40
Genesig Kit	R67.76 per reaction	1 reaction	R67.76
Oasig 2 x qPCR Mastermix	R18.61 per reaction	1 reaction	R18.61
Total cost for reagents or consumables			R281.16
Total cost of labour			R55.00
Time required for set up			30 minutes
Time required for qPCR run			152 minutes
Total cost of qPCR-HRM assay			R336.16

*VAT = Value added tax at 14 %

Appendix 3A

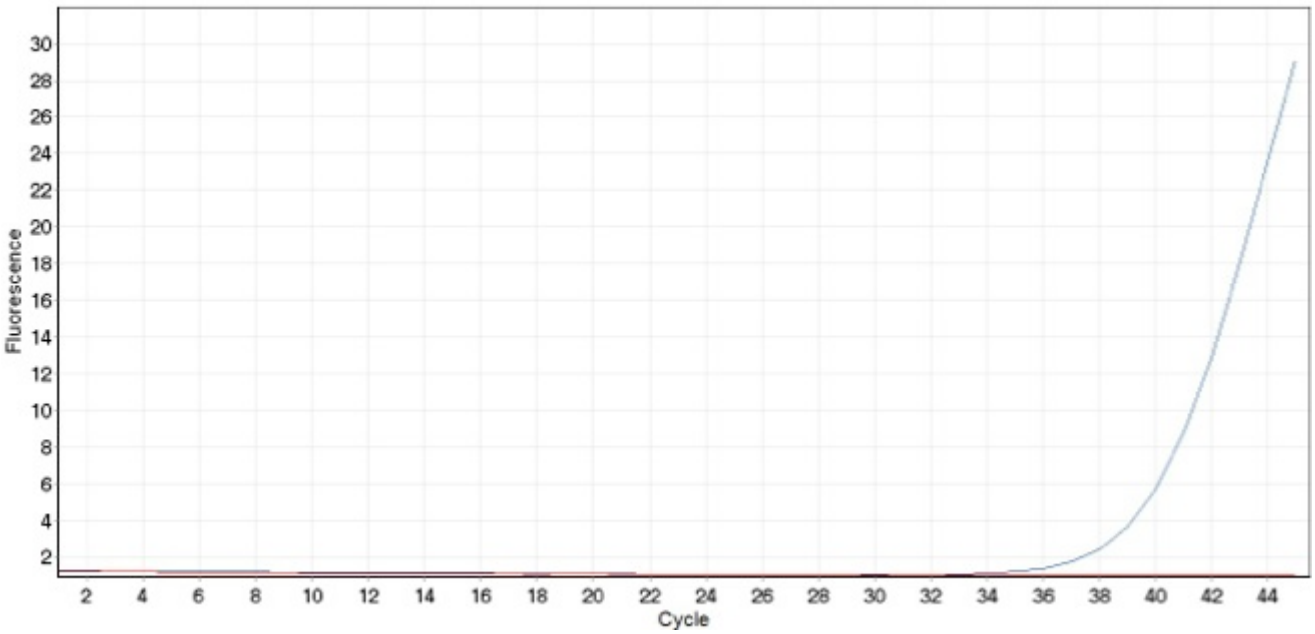

Figure 1. Cycling green fluorescence data from *Cryptosporidium parvum* EasySeed parasite suspension DNA extracted using the Instagene Matrix.

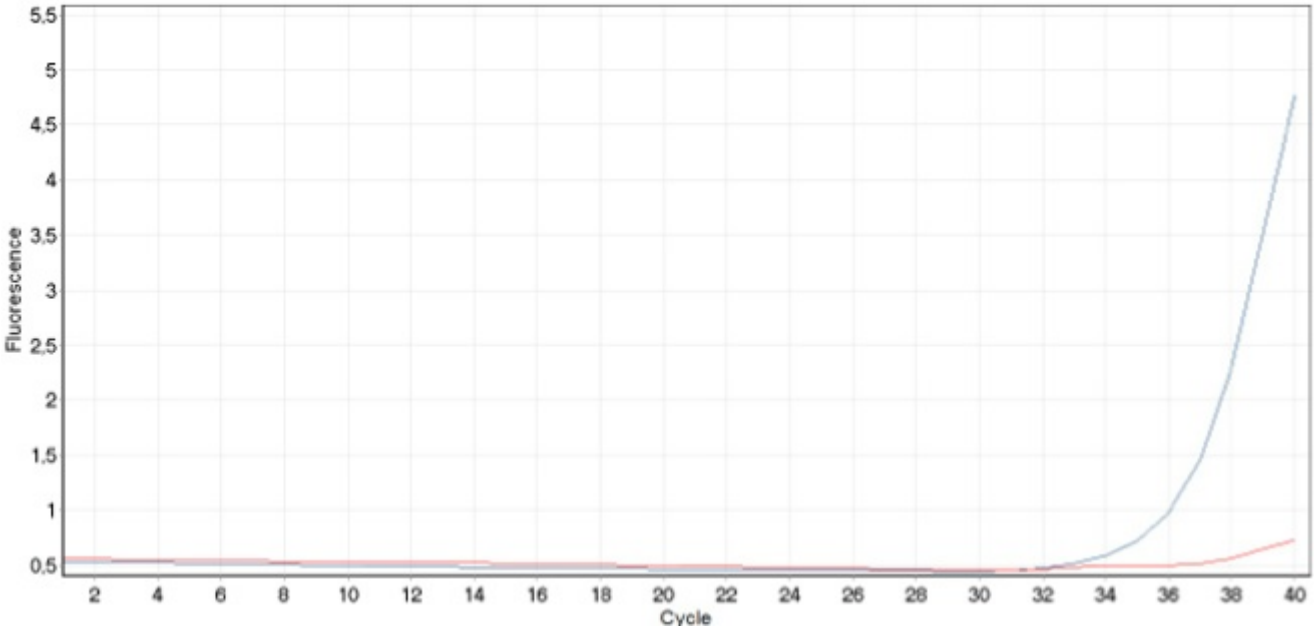

Figure 2. Cycling green fluorescence data from *Cryptosporidium parvum* Easyseed parasite suspension DNA extracted using the DNAeasy Blood and Tissue Kit.

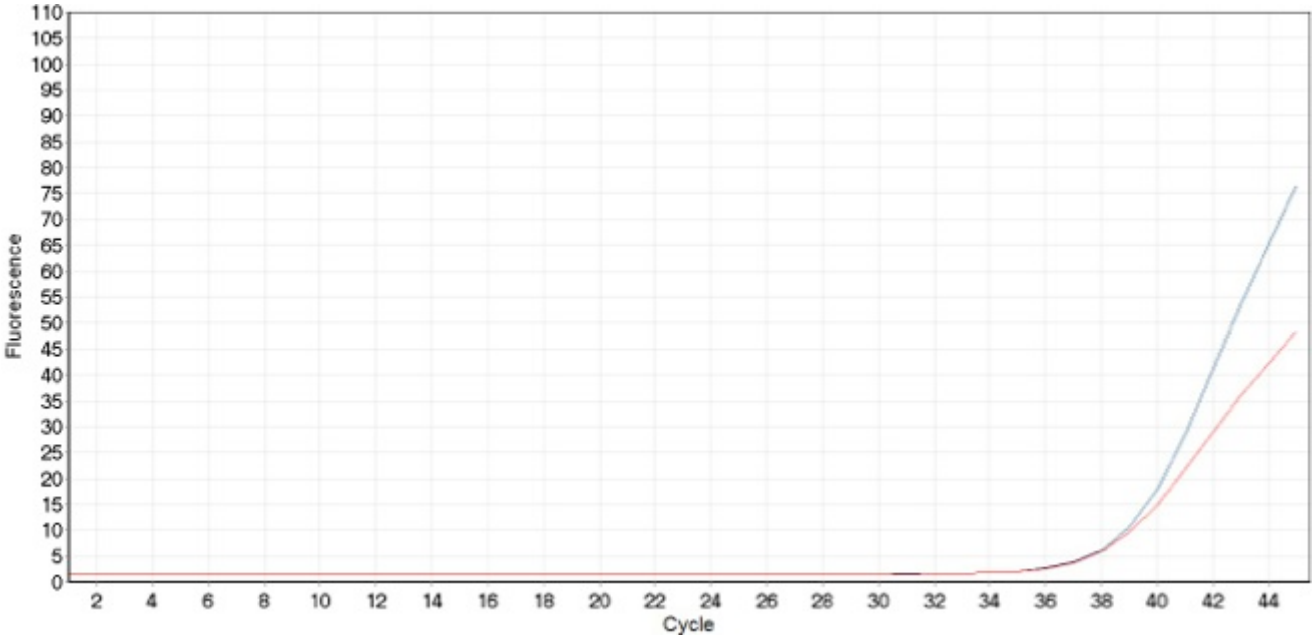

Figure 3. Cycling green fluorescence data from *Cryptosporidium parvum* Easyseed parasite suspension DNA extracted using the DNAeasy Blood and Tissue Kit in conjunction with bead beating and freeze-boil cycles.

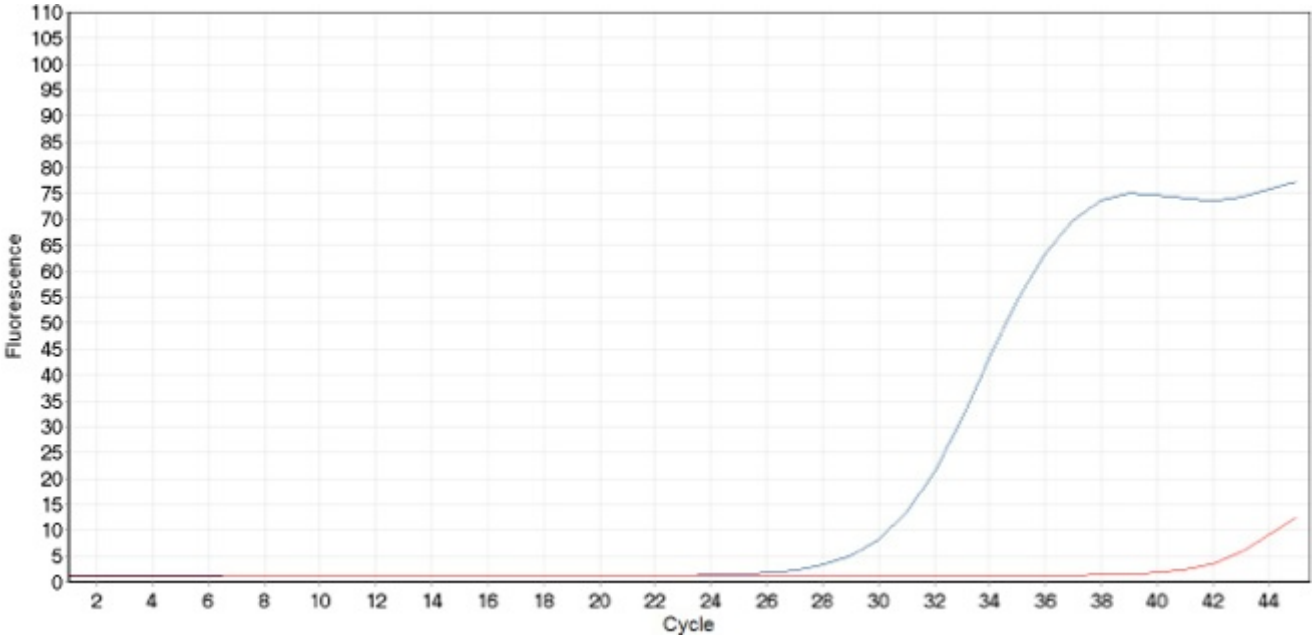

Figure 4. Cycling green fluorescence data from *Cryptosporidium parvum* stool sample DNA extracted using the QIAamp Fast DNA Stool Mini Kit

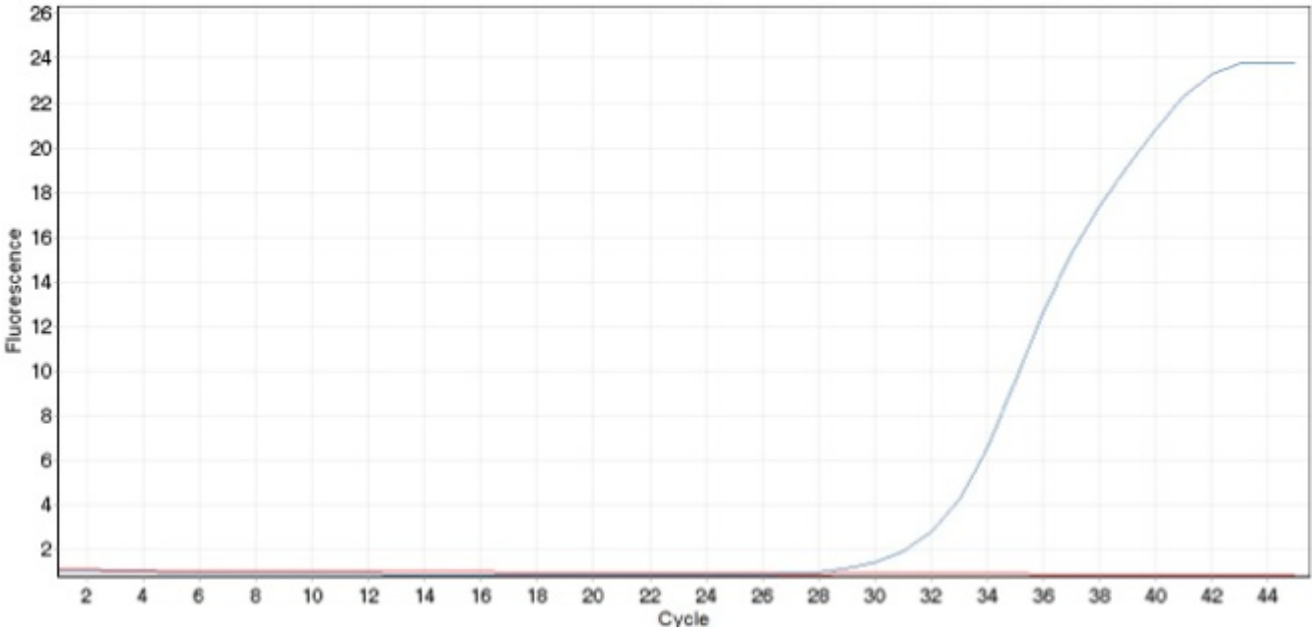

Figure 5. Cycling green fluorescence data from *Cryptosporidium parvum* stool sample DNA extracted using the QIAmp Fast DNA Stool Mini Kit in conjunction with bead beating and freeze-boil cycles.

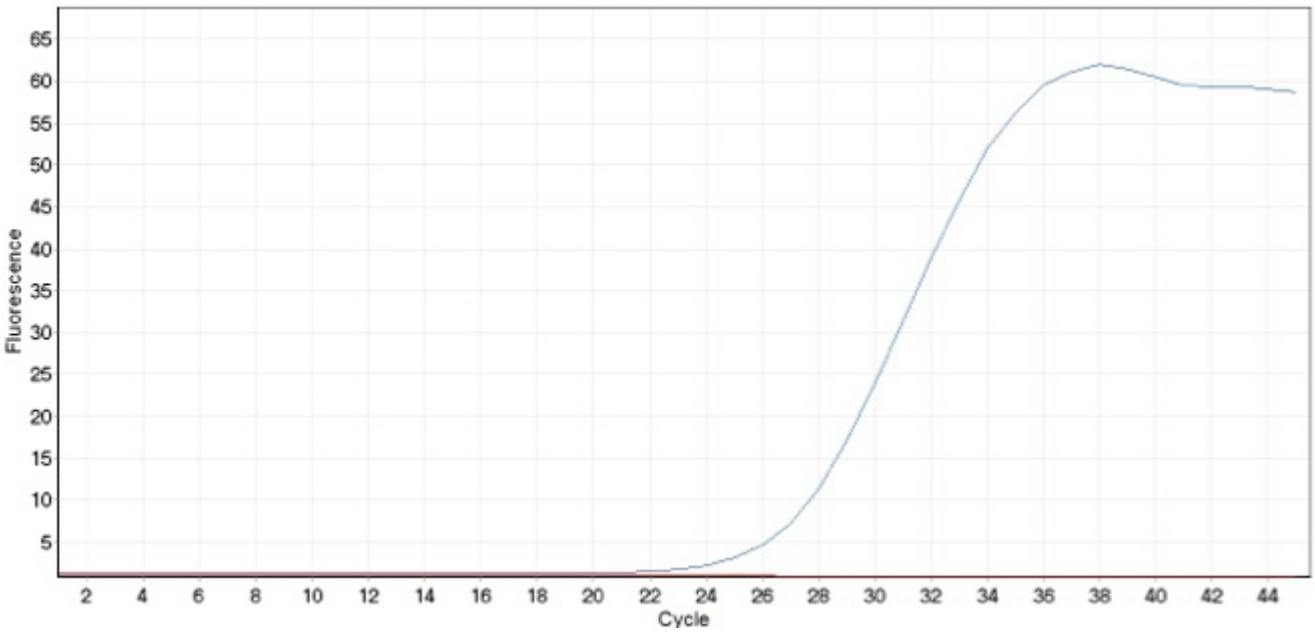

Figure 6. Cycling green fluorescence data from *Cryptosporidium parvum* stool sample DNA extracted using the QIAmp Fast DNA Stool Mini Kit and optimised qPCR conditions.

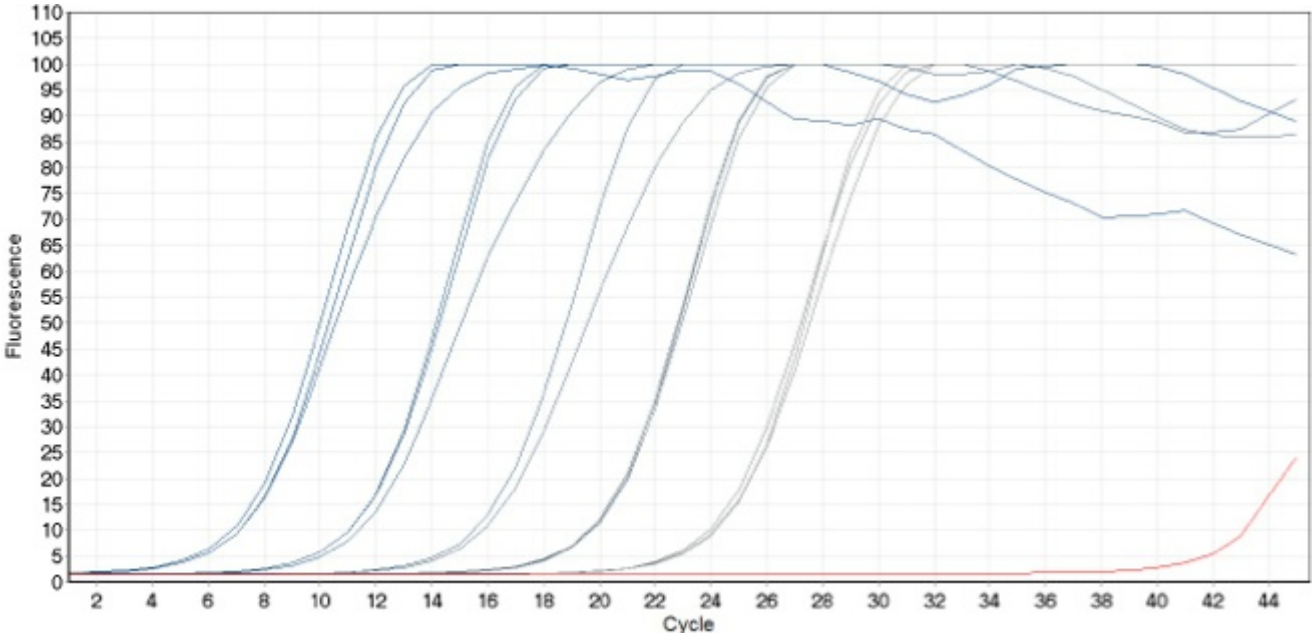

Figure 7. Cycling green fluorescence data from the tenfold serial dilution of the *Cryptosporidium parvum* COWP gBlocks Gene Fragment ranging from $1x10^{-1}$ ng/µl to $1x10^{-5}$ ng/µl.

Appendix 3B

Table 1. Evaluation of qPCR-HRM assay of river water samples collected upstream and downstream of the ERWAT Hartebeesfontein WWTP at three different time intervals

Sample source	**Melting temperature (Tm) of peaks**	**Description**	**Identity**	**Accession number**
Cryptosporidium parvum Positive Control	79.59°C	*Cryptosporidium parvum* oocysts wall protein gene, partial cds	99%	AF481960.1
First Sampling (03/09/2015) Upstream	79.48°C	*Cryptosporidium parvum* oocysts wall protein gene, partial cds	99%	AF481960.1
First Sampling (03/09/2015) Downstream	79.64°C	*Cryptosporidium parvum* oocysts wall protein gene, partial cds	100%	AF481960.1
Second sampling (07/09/2015) Upstream	79.01°C	*Cryptosporidium parvum* oocysts wall protein gene, partial cds	98%	AF481960.1
Second sampling (07/09/2015) Downstream	78.93°C	*Cryptosporidium parvum* oocysts wall protein gene, partial cds	99%	AF481960.1
Third sampling (16/10/2015) Upstream	79.24°C	*Cryptosporidium parvum* oocysts wall protein gene, partial cds	99%	AF481960.1
Third sampling (16/10/2015) Downstream	78.96°C	*Cryptosporidium parvum* oocysts wall protein gene, partial cds	99%	AF481960.1

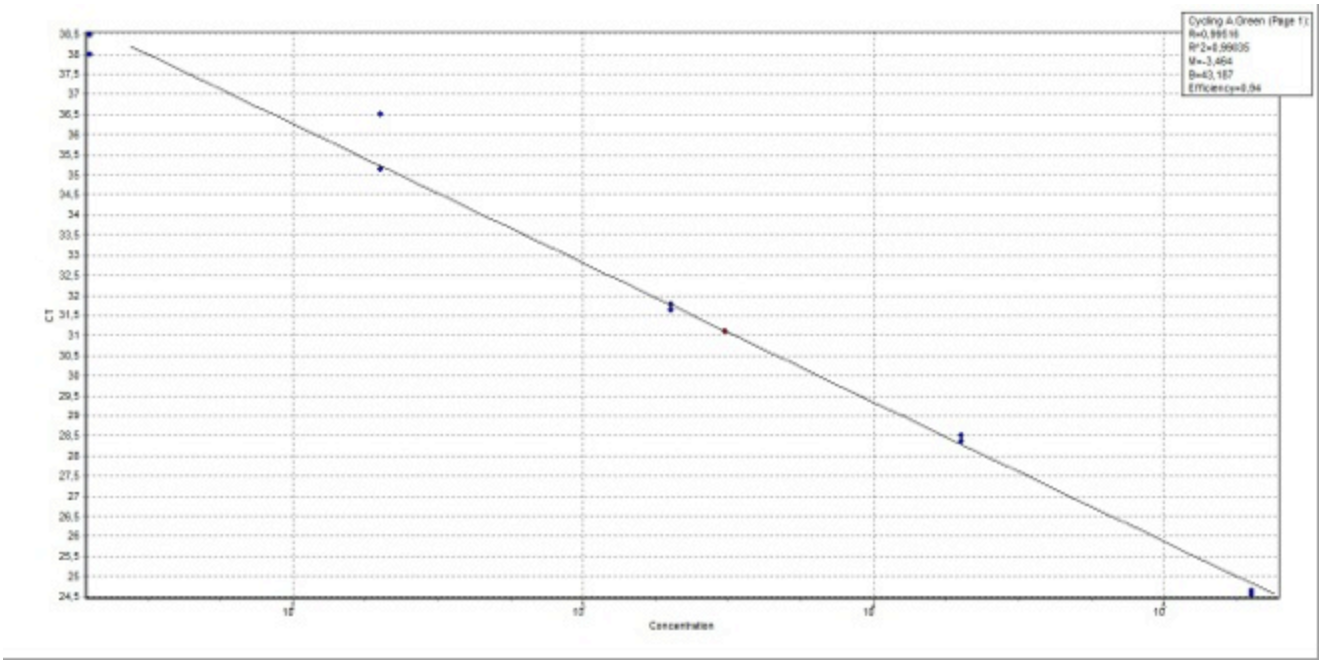

Figure 1. Real-time PCR detection of the tenfold serial dilution of the *Cryptosporidium* genome Genesig Kit DNA ranging from 20 copies/µl to $2x10^5$ copiesµl.

Appendix 3C

Table 1. Environmental sample concentration costs

Reagent or Consumable	**Cost per unit (excl. VAT)***	**Required**	**Total Cost (excl. VAT)***
Falcon tube	R0.80	1	R0.80
0.45 µm nitrocellulose membrane	R10.62	2	R21.24
2 ml microcentrifuge tube	R0.25	1	R0.25
1000 µl filter tip	R0.67	1	R0.67
Total cost for reagents or consumables			R22.96
Total cost of labour			R55.00
Time required			30 minutes
Total cost for sample preparation			R77.96

*VAT = Value added tax at 14 %

Table 2. DNA Extraction costs

Reagent or Consumable	**Cost per unit (excl. VAT)***	**Required**	**Total Cost (excl. VAT)***
2 ml microcentrifuge tube	R0.25	1	R0.25
1,5 ml microcentrifuge tube	R0.25	2	R0.50
1000 µl filter tip	R0.67	10	R6.70
200 µl filter tip	R0.67	1	R0.67
10 µl filter tip	R0.67	2	R1.34
QIAamp Fast DNA Stool Minikit	R95.99	1 reaction	R95.99
Total cost for reagents or consumables			R105.45
Total cost of labour			R275.00
Time required			150 minutes
Total cost for DNA extraction			R380.45

*VAT = Value added tax at 14 %

Table 3. Real-Time PCR assay coupled with HRM assay costs

Reagent or Consumable	**Cost per unit (excl. VAT)***	**Required**	**Total Cost (excl. VAT)***
1.5ml microfuge tube	R0.25	1	R0.25
100µl filter tip	R0.67	2	R1.34
200µl filter tip	R0.67	4	R2.68
10µl filter tip	R0.67	4	R2.68
Nuclease free water	R0.004 per µl	100µl	R0.40
SensiFast HRM kit	R13.50 per reaction	1 reaction	R13.50
Oligo nucleotides	R2.50 per µM	0.4µM	R1.00
Quality control:	**Negative control (NTC)**		
Reagent or consumable:	**Cost per unit (excl. VAT)*:**	**Required:**	**Total cost (excl. VAT)*:**
1.5ml microfuge tube	R0.40	1	R0.25
100µl filter tip	R0.67	2	R1.34
200µl filter tip	R0.67	4	R2.68
10µl filter tip	R0.67	4	R2.68
Nuclease free water	R0.004 per µl	100µl	R0.40
SensiFast HRM kit	R13.50 per reaction	1 reaction	R13.50
Oligo nucleotides	R2.50 per µM	0.4µM	R1.00
Quality control:	**Positive control (PC)**		
Reagent or consumable:	**Cost per unit (excl. VAT)*:**	**Required:**	**Total cost (excl. VAT)*:**
1.5ml microfuge tube	R0.40	1	R0.25
100µl filter tip	R0.67	2	R1.34
200µl filter tip	R0.67	4	R2.68
10µl filter tip	R0.67	4	R2.68
Nuclease free water	R0.004 per µl	100µl	R0.40
SensiFast HRM kit	R13.50 per reaction	1 reaction	R13.50
Oligo nucleotides	R2.50 per µM	0.4µM	R1.00
Total cost for reagents or consumables			R65.55
Total cost of labour			R55.00
Time required for set up			30 minutes

Time required for qPCR run	152 minutes
Total cost of qPCR-HRM assay	R120.55

*VAT = Value added tax at 14 %

Table 4. Genesig Kit costs

Reagent or Consumable	**Cost per unit (excl. VAT)***	**Required**	**Total Cost (excl. VAT)***
1.5ml microfuge tube	R0.25	1	R0.25
100µl filter tip	R0.67	2	R1.34
200µl filter tip	R0.67	4	R2.68
10µl filter tip	R0.67	4	R2.68
Nuclease free water	R0.004 per µl	100µl	R0.40
Genesig Kit	R67.76 per reaction	1 reaction	R67.76
Oasig 2 x qPCR Mastermix	R18.61 per reaction	1 reaction	R18.61
Quality control:	**Negative control (NTC)**		
Reagent or consumable:	**Cost per unit (excl. VAT)*:**	**Required:**	**Total cost (excl. VAT)*:**
1.5ml microfuge tube	R0.40	1	R0.25
100µl filter tip	R0.67	2	R1.34
200µl filter tip	R0.67	4	R2.68
10µl filter tip	R0.67	4	R2.68
Nuclease free water	R0.004 per µl	100µl	R0.40
Genesig Kit	R67.76 per reaction	1 reaction	R67.76
Oasig 2 x qPCR Mastermix	R18.61 per reaction	1 reaction	R18.61
Quality control:	**Positive control (PC)**		
Reagent or consumable:	**Cost per unit (excl. VAT)*:**	**Required:**	**Total cost (excl. VAT)*:**
1.5ml microfuge tube	R0.40	1	R0.25
100µl filter tip	R0.67	2	R1.34
200µl filter tip	R0.67	4	R2.68
10µl filter tip	R0.67	4	R2.68
Nuclease free water	R0.004 per µl	100µl	R0.40
Genesig Kit	R67.76 per reaction	1 reaction	R67.76

Oasig 2 x qPCR Mastermix	R18.61 per reaction	1 reaction	R18.61
Total cost for reagents or consumables			R281.16
Total cost of labour			R55.00
Time required for set up			30 minutes
Time required for qPCR run			152 minutes
Total cost of qPCR-HRM assay			R336.16

*VAT = Value added tax at 14%