Occurrence and Variations of *Cryptosporidium* and *Giardia* in Wastewater Treatment and Receiving River Basins

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ABSTRACT

Wastewater disposal may be a source of environmental contamination of *Cryptosporidium* and *Giardia*. Releasing untreated wastewater into the environment may result in waterborne or foodborne outbreaks. The presence of *Cryptosporidium* and *Giardia* in wastewater is of major concern in human health when wastewater is used for irrigation. The infections influence morbidity with several cases of illnesses including diarrhoea and other gastrointestinal diseases. Mortality is of high incidence, especially among the immunocompromised. Worldwide, *Cryptosporidium* and *Giardia* are well-documented in outbreaks from drinking water, recreational water and food.

The aim of the study was to demonstrate the level of occurrence of *Cryptosporidium* and *Giardia* in selected wastewater treatment plants (WWTP) as well as receiving water bodies and to account for the use of wastewater in irrigation in Ethekwini, South Africa. The occurrence and reduction of *Cryptosporidium* and *Giardia* in the WWTPs were investigated, the species determined and their viability assessed. The correlations between *E. coli*, Enterococci and the concentration of the protozoan parasites were included. Additional specific objectives relate to the effect of effluent chlorination, the seasonal variation and the impact on crops based on the occurrence and quantities of *Cryptosporidium* and *Giardia* in the irrigated water.

Influent water concentrations reflect the endemicity level in the connected population. This together with water from the intermediate treatment steps and effluent samples in four wastewater treatment plants were investigated monthly (sampled September 2015-April 2016). Conventional filtration and centrifugation methods were used for the detection and enumeration of *Cryptosporidium* and *Giardia*. Immuno-magnetic separation (IMS) on concentrated water samples was performed using Dynal anti-*Cryptosporidium* and anti-*Giardia* beads and immune-magnetic antibodies and examined with epifluorescence microscopy. In addition, molecular methods were used to differentiate the species of *Cryptosporidium* and assemblages of *Giardia* based on polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR).

The influent counts for *Cryptosporidium* oocyst varied between 1.83 to 3.02 $\log_{10}$ oocysts/L, with a corresponding concentration of *Giardia* cysts ranged from 2.39 to 3.15 $\log_{10}$ cysts/L. The overall mean reduction over the wastewater treatment plants was in the range of 1.35 $\log_{10}$/L to 1.61 $\log_{10}$/L and 1.35 $\log_{10}$/L and 1.62 $\log_{10}$/L for *Cryptosporidium* and *Giardia* respectively. *Cryptosporidium* and *Giardia* were present in the effluents from all wastewater treatment plants. In general, weak negative to poor positive correlation was found between *E. coli*, faecal enterococci and the presence of *Cryptosporidium* and *Giardia* in all WWTPs. Both *Cryptosporidium* and *Giardia* were further detected at the downstream sampling points.

The PCR and qPCR speciation did not compare with the traditional specific antibody and microscopic counts. The 18s rRNA gene-based nested PCR resulted in 13 (27%) out of 48 wastewater sample positive for *Cryptosporidium*. *C. parvum* was the dominated species followed by *C. hominis.* In ß-giardin based qPCR, all wastewater samples were positive for *Giardia* Assemblage A and *Giardia* Assemblage B.

The impact of wastewater on the rivers signifies the possible health risks associated with the use of the effluents for agricultural purposes and the receiving water bodies' use for domestic and recreational activities. Crops irrigated with the river water from the study area gave concentrations of *Cryptosporidium* and *Giardia* in spinach samples of 67 to 480 and 3 to 27 per 100 g vegetable respectively. The contamination of the fresh vegetables analysed mainly arose from the use of the effluent from the WWTP B and the receiving river water used for irrigation.

In a pilot study researching the effect of chlorine and UV irradiation, distilled water and wastewater samples spiked with environmentally derived *Cryptosporidium* oocysts and *Giardia* cysts which were exposed to different doses of chlorine and UV with different exposure times. Viability quantification and detection was done with microscopy and flow cytometry using two vital dyes, namely Syto9+PI and DAPI+PI. *Giardia* was affected to a much higher degree than *Cryptosporidium,* both in relation to chlorination and UV, where in the latter case close to zero percent remained viable even after a low dose. *Cryptosporidium* was found to be resistant to chlorination but responded well to high UV doses. This is in line with similar earlier investigations done in drinking water. In the comparative assessment between the vital dyes, DAPI+PI dyes gave

a lower mean percentage viability value than Syto9+PI; and flow cytometry gave a higher mean percentage value than microscopy.

The presence of *Cryptosporidium* and *Giardia* in all treated effluents from all investigated wastewater treatment plants exceeded World Health Organisation's (WHO) risk-based values and South African National Standard's (SANS:241) limit for raw water and irrigation respectively. Enforced regulations and alternative wastewater treatment methods may ensure a higher level of safety for the environment. Additional information on the occurrence of *Cryptosporidium* and *Giardia* in wastewater samples, which is currently lacking or limited in South Africa, will further increase our understanding in formulating risk assessment strategies to map out the level of infection to exposed population.

DECLARATION

"I declare that the thesis herewith submitted for the degree Doctor of Philosophy: Health Sciences at Durban University of Technology is my original work and has not been previously submitted for a degree at any other institution of higher education, and that its only prior publication was in the form of conference papers, book chapter and/or journal articles. I further declare that all the sources cited or quoted are acknowledged and indicated by means of a comprehensive list of references".

	29/09/2019
Folasade E. Adeyemo	Date

We hereby approve the final submission of the thesis:

	29/09/2019
Supervisor: Prof. Thor Axel Stenström	Date

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This 29 day September 2019 at Durban University of Technology.

DEDICATION

To **Deborah Temitayo Adeyemo** and **Dorothy Temilade Adeyemo**, my two children. You have been a source of inspiration to me throughout this study period.

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Folasade Esther Adeyemo
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ABBREVIATIONS

ADWF	Average Dry Weather Flow
AE	Any Elution
ANOVA	Analysis of Variance
APHA	American Public Health Association
ATL	Aerosol Technology Laboratory
BNR	Biological Nutrient Removal
CC-IFA	Cell Culture- Immunofluorescence Assay
CFU	Colony Forming Unit
COWP	*Cryptosporidium* Oocyst Wall Protein
DALYS	Disability Adjusted Life Years
DAPI	4′,6-diamidino-2-phenylindole
ddPCR	Droplet Digital Polymerase Chain Reaction
DFA	Direct Fluorescent Antibody Staining
DIC	Differential Interference Contrast
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
dPCR	Digital Polymerase Chain Reaction
DWAF	Department of Water Affairs and Forestry
E. coli	*Escherichia coli*
EF1α	Elongation Factor 1 Alpha
ELISA	The Enzyme-Linked Immunosorbent Assay
EMA	Ethidium Monoazide
FALS	Forward Angle Light Scatter
FC	Flow Cytometry
FISH	Fluorescence *In Situ* Hybridization
FITC	Fluorescein Isothiocyanate
FRET	Fluorescence Resonance Energy transfer
GDH	Glutamate Dehydrogenase
GHS	General Household Survey

HAART	Highly Active Anti-Retroviral Therapy
HCT	Human Colon Carcinoma
HRM	High-Resolution Melting
HSP	Heat Shock Protein
HTH	High test hypochlorite
IFA	Immunofluorescence Assay
IMS	Immunomagnetic Separation
ISO	International Organization for Standardization
ITS	Internal Transcribed Spacer
LAMP	Loop Mediated Isothermal Amplification
LOD	Limits of Detection
MF	Membrane Filtration
ML1	Microsatellite Locus 1
ML2	Microsatellite Locus 2
MPN	Most Probable Numbers
NPCR	Nested Polymerase Chain Reaction
NWWTP	Next to Waste Water Treatment Plant
NTZ	Anti-Infective Nitazoxanide
PCR	Polymerase Chain Reaction
PCR-RFLP	PCR-Based Restriction Fragment Length Polymorphism
PI	Propidium Iodide
PMA	Propidium Monoazide
QC	Quality Control
qPCR	Quantitative Polymerase Chain Reaction
qRT-PCR	quantitative Reverse Transcriptase - Polymerase Chain Reaction
RFLP	Restricted Fragment Length Polymorphism
RNA	Ribonucleic Acid
rRNA	ribosomal RNA
RSD	Relative Standard Deviations
RT-qPCR	Reverse Transcription -qPCR
SALS	Side-Angle Light Scatter

SANS	South African National Standards
SARChI	The South African Research Chairs Initiative
SSU	Small Subunit
TDS	Total Dissolved Solids
TPI	Triosephosphate Isomerase
TRAP	Thrombospondin-Related Attachment Protein
UNICEF	United Nations Children's Fund
USEPA	United States Environmental Protection Agency
UCT	University of Cape Town
UV	Ultraviolet
UVT	Ultraviolet Transmittance
WHO	World Health Organisation
WWTP	Waste Water Treatment Plant

PUBLICATIONS

This work has resulted in the following manuscripts and publication:

Journal Article

Folasade Adeyemo , Gulshan Singh, Poovendhree Reddy & Thor Axel Stenström 2018. Methods for the detection of *Cryptosporidium* and *Giardia*: From microscopy to nucleic acid-based tools in clinical and environmental regimes. *Acta Tropica*. Vol 184, pp 15 – 28.

Conference Presentations and Abstracts

Folasade Adeyemo, Gulshan Singh and Thor Axel Stenström 2015. Prevalence and Incidence of *Cryptosporidium* and *Giardia spp*. in South Africa and Africa context. Abstract proceedings. YWP-IWA Conference 2015 (The 4th YWP-ZA Biennial, & 1st African IWA YWP Conference). CSIP International Convention Centre in Pretoria, South Africa.

Folasade Adeyemo, Gulshan Singh, Poovendhree Reddy and Thor Axel Stenström. Quantitative detection of *Cryptosporidium* and *Giardia* in four wastewater treatment plants and the effect of treated effluents discharge into receiving water bodies in KwaZulu-Natal. Human Capacity Building through Postgraduate Research for Sustainable Development in Africa Conference 2016. Durban University of Technology Durban South Africa from 03-05 October 2016. (1st Interdisciplinary Research and Postgraduate Conference 2016).

Folasade Adeyemo, Gulshan Singh and Thor Axel Stenström. Occurrence and Variations of *Cryptosporidium* and *Giardia* in Wastewater Treatment and Receiving River Basins Accounting for their Downstream Impact. STINT/NRF workshop from 17-21 October 2016. Durban University of Technology.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

Waterborne diseases are caused by pathogenic microorganisms which can be transmitted when contaminated water is consumed (Plutzer and Karanis, 2016, Xiao, 2010). Water meant for human consumption must be free from pathogenic microorganisms and concentrations of chemicals that may be hazardous to human health (WHO, 2015). The major pathogenic microbial contaminants found in water include viruses, bacteria, fungi, protozoa and helminths (USEPA, 2017). When present in drinking water, wastewater, environmental and water bodies for recreational and agricultural purposes, different pathogenic species may contribute to waterborne outbreaks of diseases (WHO, 2006b). Among the waterborne pathogens; *Giardia duodenalis* (syn. *G. lamblia, G. intestinalis*) and *Cryptosporidium* spp., are the most common parasitic protozoa found in wastewater (Efstratiou et al., 2017).

Different species of *Cryptosporidium* can infect the gastrointestinal tract of a wide range of vertebrates (Ryan and Xiao, 2014), causing the diarrheal disease called cryptosporidiosis. *Cryptosporidium* species are partly host specific in their infections, but some are zoonotic. The infectious dose is low and the ingestion of as little as 10 oocysts can cause infection in healthy persons (Yoder et al., 2012b). Infected persons shed 10^7-10^8 oocysts in 128g which is a single bowel movement (Saneian et al., 2010). While humans can excrete oocysts between 1-20 days after cessation of diarrhoea (Fayer, 2004), calves can excrete 10^{10} oocysts per day (WHO, 2011b). The excreted oocysts are resistant to adverse environmental conditions and are responsible for the infectious stage. The global release of *Cryptosporidium* oocysts to surface waters has been estimated at 3×10^{17} oocysts per year, with comparable contributions from human wastewater and manure from livestock. However, an excretion rate of 10^9 oocysts per person per year for ill persons and total excretion rates of 5×10^7 and 1×10^8 oocysts/person/year for developed and developing countries respectively has been estimated (Hofstra et al., 2013), which has adverse implication on public health. The acceptable limit (*Cryptosporidium*/ *Giardia* count/10 mL) for domestic, irrigation and recreational water, according to South Africa and WHO guidelines should have values less than 1 (DWAF, 1996b, SANS, 2015). The taxonomy of *Cryptosporidium* is constantly evolving. Currently, 31 *Cryptosporidium* species are considered valid (Kvac et al., 2016, Li et al.,

2015, Zahedi et al., 2016, Holubová et al., 2016). Among these, more than 20 *Cryptosporidium* spp. and genotypes have been reported in humans, although *C. parvum* and *C. hominis* remain the most common ones, affecting humans and a wide range of domestic and wild animal hosts (Zahedi et al., 2016, Xiao, 2010, Ryan and Xiao, 2014).

Giardia spp. is a flagellated protozoa that infects the gastrointestinal tract of humans and certain animals. The genus *Giardia* consists of six morphological distinct species, but human infection (giardiasis) is usually assigned to *G. intestinalis*, also known as *G. lamblia* or *G. duodenalis*, (WHO, 2011b), but also found in many other mammals including pets and livestock (Feng and Xiao, 2011). The infectious dose of human-derived *G. intestinalis* ranges from 25 to 100 cysts, but 10 cysts might be enough to trigger an infection (Cook et al., 2007, Mattila, 2013). The excreted cysts are the infectious stage of *Giardia* and are considered of importance in environmental transmission. *Giardia* infection is not invasive and often asymptomatic, where 50 - 75% may not develop any disease symptoms (Carmena et al., 2012). Symptoms generally include diarrhoea and abdominal cramps. However, malabsorption deficiencies in the small intestine may be present in severe cases, mostly in young children (WHO, 2011b).

These two-parasitic protozoans with *Cryptosporidium* oocysts and *Giardia* cysts share the same transmission routes and some other characteristics. The role of passive transmission by flies may also play an important role in the mechanical transmission of *Cryptosporidium* and *Giardia,* including human infectious species (Adenusi and Adewoga, 2013, El-Sherbini and Gneidy, 2012, Zhao et al., 2014, Fetene et al., 2011).

Worldwide, *Giardia* and *Cryptosporidium* are well-documented in outbreaks from drinking water, recreational water and food. *Cryptosporidium* and *Giardia* infections transmitted through water in the developed and developing countries represent a public health risk and they are the main cause of several outbreaks in the world (Efstratiou et al., 2017). In the United States alone, cryptosporidiosis and giardiasis are two of the most common waterborne infectious diseases resulting in diarrhoea. They are the most frequent protozoan agents with a total of 30,000 cases reported every year (Yoder et al., 2012a, Yoder et al., 2012b). *Cryptosporidium* is the most common diarrhoea-causing protozoan parasite worldwide (WHO/UNICEF, 2009). The mortality

rate in developing countries due to infectious diarrhoea in relation to *Cryptosporidium* and *Giardia* could be as high as 56% (WHO, 2010). A report by Mamo and Hailu (2014) shows that in Africa, diarrhoea has been estimated to be responsible for 25–75% of all childhood illnesses. Episodes of diarrhoea led to about 14% of outpatient visits and 16% of hospital admissions and accounted for an average of 35 days of illness per year in children aged less than five years (Samie and Ntekele, 2014). In the Global Enteric Multicenter Study (GEMS), the aetiology and population-based burden of paediatric diarrhoeal disease in sub-Saharan Africa were quantified, where *Cryptosporidium* was second only to rotavirus, as a causative agent of diarrheal disease among children under five years (Kotloff et al., 2013). In addition, Sow et al. (2016) estimated that 2.9 million cases of *Cryptosporidium* infections occur in children (< 24 months old) in moderate/high mortality regions in Sub-Saharan Africa. These *Cryptosporidium* attributable infections were found to be associated with mortality (<2-fold increase) in children in the age group between 12 to 23 months (Kotloff et al., 2013). Furthermore, the occurrence of cryptosporidiosis and giardiasis is probably higher than recorded as only one in 14 people with diarrhoea in South Africa seek formal treatment from a health practitioner, clinic or hospital every year, while approximately 43 000 South Africans have been estimated to die from diarrhoea every year (Dungeni and Momba, 2010, Jarmey-Swan et al., 2001). Official data from Statistics South Africa estimate that diarrhoea accounts for approximately 20% of children under-five mortality (STATS SA, 2012). Furthermore, the 2010 General Household Survey (GHS), a nationally supported representative inquiry into the livelihood of South Africans, showed that there were over 60,000 cases of childhood diarrhoea per month and approximately 9,000 child diarrhoeal deaths in the same year (STATS SA, 2012).

Oocysts and cysts may remain viable for several months under a range of environmental conditions (CDC, 2012a). *Cryptosporidium* oocysts and *Giardia* cysts can survive for weeks to months in warm water, and therefore may be present in wells and water systems where stagnant water sources, such as naturally occurring ponds, storm water storage systems, and rivers and even clean looking mountain streams, may be affected (Michael, 2010). Both *Cryptosporidium* and *Giardia* are resistant to disinfectants at the concentrations and exposure times commonly used (Carmena, 2010). They can remain viable under conventional drinking water treatment conditions (Xiao et al., 2004).

Giardia and *Cryptosporidium* are found in all types of water reflecting sources of contamination in the watershed, including surface water, sewage or treated effluents. Surface waters such as rivers, natural and artificial lakes serve as drinking water supplies in many areas. In developing countries, 90% of all wastewater are still untreated and discharged into local water bodies which limit a safe water supply (Breisha and Winter, 2010).

Urban wastewater or heavily polluted surface water, containing a wide range of enteric pathogens, is used to irrigate agricultural land in many developing countries including South Africa (Gumbo et al., 2010). In South Africa, most wastewater treatment plant effluents are directly discharged to the rivers or are used for different purposes such as irrigation of fruits and vegetables (Gumbo et al., 2010). This constitutes a significant risk of infection for the public through water, food or direct contact with the treated wastewater that might not be properly disinfected (Samie and Ntekele, 2014). Similarly, improper disposal of sewage and runoff of animal and/or human feces are sources of contamination of these protozoan parasites. Several studies have reported on the occurrence and the removal efficiency of *Cryptosporidium* and *Giardia* from wastewater treatment plants in different countries (Taran-Benshoshan et al., 2015, Fu et al., 2010, Ramo et al., 2017, Rodriguez-Manzano et al., 2012, Reinoso et al., 2011). **Chapter 4** presents the removal efficiency of *Cryptosporidium* and *Giardia* from selected WWTPs within KwaZulu-Natal. Hatam-Nahavandi et al. (2015) also reported on *Giardia* cysts overall removal efficiency. The occurrence of *Cryptosporidium* and *Giardia* has been documented in wastewater, but the documentation is sparse in South Africa in relation to their occurrence in wastewater and receiving water bodies. Therefore, production of high-quality effluent remains a major concern in developing countries such as South Africa. This study presents results essential for the understanding of human health risks as related to environmental transmission of *Cryptosporidium* and *Giardia* in wastewater.

Monitoring of *Cryptosporidium* and *Giardia* is carried out throughout the world. It has been used for risk assessment purposes, for evaluation of the reliability of water treatment system and also to assist with waterborne outbreak investigations (Betancourt and Rose, 2004). However, routine monitoring for *Giardia* and *Cryptosporidium* is not always practical because the available methods are time consuming and have various limitations (Sigudu, 2010). Existing techniques commonly used for the isolation of *Cryptosporidium* and *Giardia* from water is the USEPA

method 1623. This method is used for determination of the identity and concentration of *Cryptosporidium* and *Giardia* in water by filtration, immunomagnetic separation (IMS) and detection using an immunofluorescence assay (USEPA, 2005). There are other methods for the detection of *Cryptosporidium* and *Giardia* in water, but they have demonstrated lower recoveries and increased variance compared to the USEPA's Methods 1623 (Quintero-Betancourt et al., 2003). In this study, the revised method described by USEPA 1623.1 was used for the detection and enumeration of *Cryptosporidium* and *Giardia* from all water samples. **Chapter 3** provides information on available methods, advantages and limitations of advanced detection techniques like nucleic acid-based approaches for the detection of viable oocysts and cysts. Conventional and widely accepted detection techniques such as microscopy, antibody and enzyme-based procedures are also discussed.

To assess the risk to human health associated with the presence of *Cryptosporidium* and *Giardia* in water, species and genotypes of these protozoan present in water need to be identified. The use of molecular tools to genotype and subtype *Cryptosporidium* and *Giardia* parasites has contributed to improved understanding of the transmission of cryptosporidiosis and giardiasis in humans and animals. More recently, PCR based assays have extensively been adopted for the detection in diverse types of environmental samples (Guy et al., 2003, Castro-Hermida et al., 2015, Nguyen et al., 2016). PCR that has been applied for analysing environmental samples for the detection of both *Giardia* and *Cryptosporidium* needs attention in relation to the increased possible false positives (Elsafi et al., 2013). In addition to the wide application of PCR, nested PCR have been used to increase sensitivity (Castro-Hermida et al., 2015, Nikaeen et al., 2005). To accomplish **Objective 2** for molecular characterization of the *Cryptosporidium* spp. and *Giardia* spp., a nested PCR targeting the 18S rRNA gene of *Cryptosporidium* spp. was carried out, as previously described (Hadfield et al., 2011). ß-giardin P434 (P1) and ß-giardin P434 (H3) were used as adopted from Guy et al., 2003. In **Chapter 3,** a comprehensive review on different detection methods (both conventional and molecular) in clinical and environmental samples are presented.

The isolation techniques used for the recovery of *Cryptosporidium* and *Giardia* in this study, as well as the molecular methods (nested PCR) considered for the quantification and speciation of these two protozoan parasites are also presented in **Chapter 3**.

1.2 Research problem

Sewage discharges and poorly managed wastewater treatment plants are presently recognised as one of the major sources of water pollution in South Africa (Dungeni and Momba, 2010). Treated sewage is often discharged straight into water courses that also serve as a source of drinking water or which have recreational sites downstream. In the Republic of South Africa, *Giardia* cysts and *Cryptosporidium* oocysts were found in all types of water tested including surface water, sewage or treated effluents (Kfir et al., 2000). Surface waters such as rivers, natural and artificial lakes serve as drinking water supplies in some areas where access to potable water is scarce or where groundwater cannot be used appropriately for the production of drinking water. Urban wastewater or heavily polluted surface water is used to irrigate agricultural land in many developing countries including South Africa (Gumbo et al., 2010). Sewage water also serves as an alternative water resource in arid and semi-arid areas where there is scarcity of water.

A portion of the South African population lack access to safe water and a hygienic environment and therefore, rely on surface water without treatment. Farmers use the water for irrigation of their vegetable crops which poses risk to the consumers. Therefore, consumption of uncooked raw vegetables poses a significant health risk to the community. This increases their exposure to pathogens such as *Cryptosporidium* and *Giardia* and thus increases their risk of infection. Insight into the prevalence of *Cryptosporidium* and *Giardia* on the leafy vegetables irrigated with the effluent water discharged from one of the study areas is presented in **Chapter 4.**

The occurrence of *Giardia* and *Cryptosporidium* in receiving wastewater is a reflection of the level of infection in the connected population (morbidity and infection). It has been suggested as part of an early warning system in other countries. The effluent concentration defines the subsequent human health risks for downstream populations and activities. Chlorine is the most commonly used disinfectant worldwide and it is extremely effective against most bacteria and also against some viruses (WHO, 2006b). It is extensively used as a final treatment step in South African wastewater treatment plants. Due to production of hazardous oxidation by-products during chlorination and ozonation, the use of UV irradiation has gained more consideration (Hijnen et al., 2007) and has recently been evaluated in South Africa. Data regarding the effect of chemical disinfectant, such as chlorine and ozone, on *Giardia* cysts and *Cryptosporidium* oocysts is

currently lacking in South Africa. Conventional treatment processes are not designed to completely remove both protozoa from wastewater. Efficiencies of (oo)cyst removal varying from 75 to 100% for *Giardia* and 40 to 100% for *Cryptosporidium* have been reported (Nasser, 2016, Nasser et al., 2012). Furthermore, a number of studies have shown that reliance on bacterial indicators for measurement of good quality water do not correlate with the concentration of these protozoa. Pathogenic organisms such as *Cryptosporidium* and *Giardia*, which are resistant to chemical disinfection, can still be present in the absence of bacterial indicators (Ehsan et al., 2015, Keeley and Faulkner, 2008, Xiao et al., 2013). Additionally, WHO report that bacterial counts are not always a reliable tool to predict the presence/absence of these parasitic protozoa (WHO, 2011a).

1.3 Study aim and objectives

This research aimed to demonstrate the level of occurrence *Cryptosporidium and Giardia* have selected wastewater treatment plants, that are receiving water and account for its use. The work accounts for influent and effluent sampling and in addition from two to three intermediate treatment steps in the wastewater treatment plants.

The following objectives were pursued:

1. To investigate the occurrence and reduction of *Cryptosporidium* and *Giardia* in selected South African wastewater treatment plants in KwaZulu-Natal.

2. To assess the speciation and the viability of *Cryptosporidium* and *Giardia* in environmental samples with the specific aim of evaluating if effluent chlorination was efficient.

3. To assess the downstream effects in receiving water bodies and the relative impact of treated effluents.

4. To evaluate the impact of seasonal variation on the occurrence of *Cryptosporidium* oocyts and *Giardia* cysts in all sampling sources.

5. To investigate the impact on crops; based on the occurrence and quantities of *Cryptosporidium* and *Giardia* in the irrigated water.

1.4 Study outline

Chapter 1 gives an introduction of this thesis and highlights why it is essential to improve wastewater quality and the effluents from wastewater before being discharged into receiving water bodies.

Chapter 2 presents a literature survey of the work that has been done on the prevalence of *Cryptosporidium* and *Giardia,* more importantly on the African continent. It also focuses on the wastewater quality and infectious diseases, together with the history overview, biology and life cycle of *Cryptosporidium* and *Giardia* spp.

Chapter 3 presents methods for the detection of *Cryptosporidium* and *Giardia.* This includes discussing microscopy to nucleic acid-based tools in clinical and environmental regimes; and methodology and approaches used in this study as a comparison for the detection and quantification of *Cryptosporidium* and *Giardia.*

Chapter 4 reports on the results from a quantitative detection and enumeration of *Cryptosporidium* and *Giardia* in four wastewater treatment plants. It highlights immuno-magnetic separation and microscopy as well as with the qPCR speciation together with species sequencing analysis.

Chapter 5 reports on the effect of chlorine and UV on *Cryptosporidium* and *Giardia* based on the use of vital dyes.

Chapter 6 includes a short general discussion, conclusions and recommendations of the study.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Protozoa can be categorised as a varying group of unicellular eukaryotic organisms. The organisms include sporozoa (intracellular parasites; for example, *Cryptosporidium*, *Cyclospora*, *Isospora* and *Toxoplasma*), flagellates (these have a tail-like structure that is used for mobility; for example, *Giardia*), amoebae (they move by using temporary cell body, which is projected and called pseudopods; for example, *Entamoeba*, *Acanthamoeba*, and *Naegleria*) and ciliates (move by cilia which are multiple hair-like structures; for example, *Balantidium*) (Plutzer and Karanis, 2016). Waterborne parasitic protozoan diseases are distributed worldwide and are a public health risk in all countries and are responsible for different types of disease outbreaks (Baldursson and Karanis, 2011, Efstratiou et al., 2017). Some of these foodborne and waterborne protozoan parasites are well known, such as *Cryptosporidium* and *Giardia.* They are the main cause of human diarrhoeal morbidity as well as losses in livestock. Symptoms are gastroenteritis, diarrhoea, vomiting and anorexia (DWAF, 1996c). They affect humans as well as a wide range of domestic and wild animals (Fayer, 2004). They can shorten the life span of immunocompromised individuals (Reynolds et al., 2008, Smith and Paget, 2007) and cause chronic and debilitating illness in these individuals. However, immunocompetent individuals usually recover within a few weeks (Austin et al., 1990).

Various therapeutic drugs, such as anti-infective nitazoxanide (NTZ), paromomycin, and highly active antiretroviral therapy (HAART) have been used as an attempt to treat *Cryptosporidium* infection in humans but are usually unsuccessful. Currently, there is limited effective therapy for a *Cryptosporidium* parasite infection (Omoruyi, 2010). Conversely, metronidazole, tinidazole, and nitazoxanide have been used for treating *Giardia* infections (CDC, 2015b). Alternative medications proposed for *Giardia* infections include paromomycin, quinacrine, and furazolidone (The Medical Letter, 2013).

Surface water may contain oocysts of *Cryptosporidium* and cysts of *Giardia* related to the level of faecal pollution (Robertson and Lim Ai Lian, 2011). These environmentally robust (oo)cysts have an enhanced resistance to disinfectants that are used to treat water (Hansen and Ongerth, 1991).

Poor water quality has a major effect on human health, both through outbreaks of waterborne diseases and by contributing to the background rate of diseases (further dealt with in 2.2) (Redwan et al., 2008). When the oocysts are excreted in faeces, they sporulate and may be immediately infectious (Fayer et al., 2000, Tzipori and Ward, 2002, Yoder et al., 2010). These infections are usually self-limiting diarrhoea with severe abdominal pain in both immuno-suppressed and immuno-competent persons, including those who are HIV-infected persons and children (Areeshi et al., 2008, Fayer et al., 2000, Stark et al., 2009). The immunological competence of individuals will determine the duration of clinical symptoms (Leav et al., 2003). The diarrhoea infection can be life-threatening in HIV-infected patients who have limited access to good sanitation and quality of water and with poor compliance to highly active antiretroviral therapy (HAART) (Kucerova et al., 2011, Pupulin et al., 2009).

Poor water quality, lack of access to appropriate sanitation and poor hygiene account for diarrhoeal diseases which is considered to be one of the major contributors in terms of morbidity and mortality. Approximately 3.1% of all deaths worldwide and 3.7% of DALYs (disability adjusted life years) are caused by unsafe or inadequate water, sanitation and hygiene (WHO, 2015).

One of the 2015 Millennium Development Goal's was to provide access to quality sanitation facilities for about half of the global population without it. This target was missed by about 700 million people. It is estimated that 842 000 people within the low- and middle-income countries die due to inadequate sanitation and hygiene every year (Factsheet, 2014). This figure represents 58% of diarrhoeal deaths. Poor sanitation remains the cause of about 280,000 deaths making diarrhoea a major killer. This is preventable by improved sanitation. According to the Factsheet (2014), improved sanitation and health can prevent death of up to 361,000 children below the age of 5 every year. Waterborne diseases occur after the ingestion of water which is contaminated by pathogens from human excreta, animal faeces or urine (Leclerc et al., 2002). About 2.5 billion people do not have access to quality sanitation all over the world and also about 780 million people lack access to treated water (CDC, 2015a). This will contribute to incidences of illness and poor health. The presence and impact of *Cryptosporidium* and *Giardia* are in the forefront among the protozoan parasite contaminants but is less well documented in South African waters than the bacterial contaminants. Outbreaks of waterborne diseases caused by *Cryptosporidium* oocysts and

Giardia cysts, enteric viruses and other pathogenic microorganisms in aquatic environments due to poor sanitation are well documented (Craun et al., 2010).

2.2 Waterborne infectious diseases

Bacteria, viruses, helminths and protozoa are microbial groups that include some of the infectious agents that cause waterborne infections through ingestion, contact or are airborne. A large number of water supply systems in developing countries are unprotected and susceptible to contamination from surface runoff, debris, unsanitary collection methods, as well as human and animal faecal pollution (Naidoo, 2013, Moyo et al., 2004, WHO, 2015). It is difficult to control the risks posed by *Cryptosporidium* and *Giardia* infections in humans because of human exposure to contaminated water (Baldursson and Karanis, 2011). Their transmission may also be through the faecal-oral route and ingestion from contaminated water, food or through direct contact in situations where hygienic conditions are lacking (Barry et al., 2013). These multiple transmission routes, low infection doses and the documented high resistance to chlorine disinfection make *Cryptosporidium* and *Giardia* ideally suitable for transmission through water (Sigudu et al., 2014). As previously mentioned, infections are usually characterized by self-limiting diarrhoea associated with severe abdominal pain in both immuno-suppressed and immuno-competent people, such as HIV-infected adults and children (Areeshi et al., 2008, Fayer et al., 2000, Stark et al., 2009) with the clinical symptoms depending on the person's immunological competence (Leav et al., 2003).

In South Africa, it is estimated that death due to intestinal infectious diseases has increased to 37,398 in 2007 from 14,276 in 2000 (STATS SA, 2010). This represents a 61.8% increase in the death rate due to intestinal infections alone. This shows that South Africans are at high risk for intestinal infections. It is believed that many of these infections are waterborne and sanitation related, where the situation can be improved by providing safe drinking water and treated wastewater for other uses (STATS SA, 2010). Thus, factors responsible for these infections include sanitation and irregular access to safe water. However, factors like population growth and an increase in immunocompromised patients cannot explain the increase in the infection rate (Luyt et al., 2011). Some of these infections are also linked to infrastructural problems, unsatisfactory communal pipes, interruption of water supply, unskilled handling of water treatment works and

power supply interruptions resulting in poor water supply (STATS SA, 2011, Lewin et al., 2007, Haarhoff et al., 2008, Momba M N B et al., 2006).

A lack of appropriate infrastructure for providing safe drinking water in many poor developing countries has led to an increased prevalence of waterborne diseases. While the sanitary water supply systems and infrastructure may be generally effective in developed countries, waterborne infections are caused by poor catchment management and system failures (Smith and Nichols, 2010, Cummins et al., 2010). Water related infectious diseases remain a major concern worldwide, mainly in low income countries, where there is limited or no access to safe drinking water (Nath et al., 2006). Table 2.1 from WHO illustrates pathogens found in contaminated water and their related diseases and bring up their persistence and resistance to chlorine as well as their health significance.

Table 2.1: Example of waterborne pathogens, their resistance to chlorine and health significance

Pathogen	Type species/ genus/group	[1]Persistence in water supplies	[2]Resistance to chlorine	[3]Health significance*
Viruses	Enteroviruses, Parechoviruses, Hepatitis A virus	Long	Moderate	High
	Hepatitis E virus	Long	Moderate	High
	Noroviruses, Sapoviruses	Long	Moderate	High
	Rotaviruses	Long	Moderate	High
	Astroviruses	Long	Moderate	Moderate
	Adenoviruses	Long	Moderate	Moderate
Bacteria	*Salmonella typhi*	Moderate	Low	High
	Shigella (*S. dysenteriae*)	Short	Low	High
	Campylobacter (*C. coli C. jejuni*)	Moderate	Low	High
	Burkholderia pseudomallei	May multiply	Low	High
	Mycobacterium avium complex	May multiply	High	Low
	Escherichia coli, particularly enterohemorragic *E. coli* (*E. coli* O157)	Moderate	Low	High
Protozoa	*Entamoeba* (*E. histolytica*)	Moderate	High	High
	Giardia (*G. intestinalis*)	Moderate	High	High
	Cryptosporidium (*C. hominis/parvum*)	Long	High	High
	Cyclospora (*C. cayetanensis*)	Long	High	High
Helminths	*Ascaris lumbricoides*	Moderate	Moderate	High
	Schistosoma mansoni	Moderate	Moderate	High

Adapted from (WHO, 2017a).

[1]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.

[2]Resistance is based on 99% inactivation at 20 °C where, generally, low represents a Ct99 of < 1 min.mg/L, moderate 1–30 min.mg/L and high > 30 min.mg/L (where C = the concentration of free chlorine in mg/L and t = contact time in minutes) under the following conditions: the infective stage is freely suspended in water treated at conventional doses and contact times, and the pH is between 7 and 8.

[3]Health significance relates to the severity of impact, including association with outbreaks.

2.3 Short summation of epidemiology of pathogenic protozoa associated with human illness

Table 2.2 gives an overview of pathogenic protozoa associated with human illness. Many parasites are listed with their disease indications, host(s) and routes of transmission. Transmission through contaminated water is less likely for parasites such as *Dientamoeba fragilis* and *Microsporidia* where direct transmission from infected patients, ingestion of spores and zoonotic transmission are more common. Other parasites like *Cystoisospora belli, Giardia intestinalis* and *Balantidium coli* are transmitted through direct contact with infected person as well as through water and food. *Blastocystis* sp*.,* and *Cystoisospora belli* are also transmitted by faecal-oral route. *Cryptosporidium* spp., *Cyclospora cayetanensis*, *Giardia intestinalis, Entamoeba histolytica, Blastocystis* sp., *Cystoisospora belli* and *Balantidium coli* are transmitted by food ingestion and other means. *Cryptosporidium* spp. and *Giardia intestinalis* have routes of transmission through food ingestion, water, person-to-person and zoonotic and are the two most important pathogenic protozoa.

Progress in the detection of protozoan parasites from water has placed water quality analysis in a new perspective (Grabow, 2001). Protozoan parasitic cysts and oocysts have been found to be more resistant to certain water purification processes than bacterial indicators. The presence of *Cryptosporidium* and *Giardia* species in water, even in very low numbers, poses a high risk to the consumer (Rose et al., 1990). These protozoa have been reported to infect distal and proximal regions of the small intestine, occupying epicellular and extracellular niches respectively, which affect host-parasite interactions, pathophysiology and disease mechanisms (Reynolds et al., 2008, Ortega-Pierres et al., 2009).

Table 2.2: Illustration of the epidemiology of pathogenic protozoa associated with human illness

Parasite	Disease indication	Host(s)	Routes of transmission	References
***Cryptosporidium* spp.**	Diarrhoea	Humans, other mammals and birds	In water or from undercooked food; person to person; zoonotic	(Fletcher et al., 2012)
Cyclospora cayetanensis	Watery diarrhoea	Humans, primates and other mammals	Through contaminated water and food, also person to person	(CDC, 2012b)
Giardia intestinalis	Diarrhoea, abdominal cramps and malabsorption	Humans, other mammals and birds	Ingestion of contaminated water or food, person to person; zoonotic	(Helmy et al., 2014)
Entamoeba histolytica	Dysentery, diarrhoea, invasive colitis, liver abscess	Humans and other primates	Transmitted through contaminated food and water	(Ralston and Petri Jr, 2011)
***Blastocystis* sp.**	Abdominal pain and diarrhoea. Asymptomatic infections are common	Humans and other mammals. Pathogenicity sometimes questioned	Partly unclear. Transmitted via the faecal-oral route. Transmission through consumption of contaminated food or water is possible	(Coyle et al., 2012)
Dientamoeba fragilis	Abdominal discomfort, nausea and diarrhoea	Humans	Transmission partly unclear. Faecal-oral route by direct transmission from infected patients is the most likely	(Nagata et al., 2012, Stark et al., 2012)

			route of transmission. Environmentally sensitive	
Cystoisospora belli	symptoms include fever, watery diarrhoea, nausea, vomiting, abdominal pain	Humans, mainly immune-compromised. Mild symptoms in immune-competent	Faecally contaminated water or food	(Marathe and Parikh, 2013)
Balantidium coli	Diarrhoea, dysentery Often asymptomatic infections	Humans, pigs, nonhuman primates, cats, rodents	Ingestion of food or water contaminated by mammal faeces. Untreated or minimally treated water and on uncooked or undercooked food; zoonotic	(Bellanger et al., 2013, Fletcher et al., 2012)
Microsporidia **(group of species originally classified as protozoa, but nowadays among fungi)**	Persistent diarrhoea (varying other symptoms)	Humans and other mammals. More common among immunocompromised	Ingestion of spores; person to person; zoonotic	(Fletcher et al., 2012)

Source: Partly adapted from Fletcher et al. (2012)

In South Africa, it is estimated that 11 out of 110 children who are infected with cryptosporidiosis die (Momba and Dungeni, 2010). Many South Africans do not seek formal treatment and therefore the cases recorded may be lower than the actual figure. Approximately 43 000 people in South Africa are estimated to die from diarrhoea every year (Dungeni and Momba, 2010, Jarmey-Swan et al., 2001). Intestinal parasites occur at unacceptably high levels throughout South Africa (Samie et al., 2009). A report by Jarmey-Swan et al. (2001) on the prevalence of diarrhoeal disease caused by waterborne pathogens shows that *Cryptosporidium* and *Giardia* are endemic in the KwaZulu-Natal population with a prevalence of 39.3% in <1-year old individual and 38.5% in 3 to 4 year old group. The occurrence of cryptosporidiosis and giardiasis is probably higher than is officially recorded in the province (Jarmey-Swan et al., 2001).

Intestinal parasites were further detected in 560 samples (68% of the total samples) of patients in hospitals and schools in the Vhembe District of the Limpopo Province, South Africa (Samie et al., 2009). Samie et al. (2009) also found that among hospital attendees, which represent 34.2%, *E. histolytica/dispar* were the most prevailing organisms in their diarrhoeal stool samples, followed by *Cryptosporidium* (25.2%) and *Blastocystis hominis* (17.8%). The corresponding figures in non-diarrhoeal samples were 9.3%, 17.7%, and 1.9% respectively. *G. intestinalis* and *C. cayetanensis* were also associated with diarrhoea among hospital attendees. Adams cited by Samie et al. (2009) likewise obtained higher rates of infection of *Giardia* and other intestinal infections among children attending schools in a low-income but well-serviced community in Cape Town, South Africa. Similar results have been obtained from other part of Africa such as Nigeria and Tanzania (Alakpa and Fagbenro-Beyioku, 2002).

2.4 Biology of *Cryptosporidium* spp.

Cryptosporidium was first discovered by Edward Tyzzer in 1907 (Chalmers, 2014). *Cryptosporidium* is classified taxonomically as Apicomplexan Protozoans. *Cryptosporidium* species have a monoxenous life cycle (the cycle occurs in one host) which is primarily completed within the gastrointestinal tract of the host (Bouzid et al., 2013, Carreno et al., 2001a, Carreno et al., 2001b). *Cryptosporidium* presents unique characteristics which differentiate it from other coccidian. *Cryptosporidium* has a multi-stage life cycle which is characterised by six major stages in a single host. These stages are: excystation (a process where an excysting oocyst releases

sporozoites); schizogony (asexual reproduction); gametogony (formation of gametes); fertilisation of the macrogametocyte by a microgamete to form a zygote; oocyst wall formation; and sporogony (sporozoites form within the oocyst) (Rosales et al., 2005).

Round, thick-walled and environmentally stable oocysts constitute the most important stage in the *Cryptosporidium*'s life cycle. They have a size ranging between 4 and 6 μm in diameter (Ridley, 2012). This is known as the infective stage. One of the important features of the life cycle of *Cryptosporidium* is that it begins by ingesting the resistant oocysts. The parasite completes its cycle in the digestive tract when ingested by humans (Figure 2.1). Mature oocysts contain four sporozoites. The oocysts 'excyst' to release the sporozoites into the gastrointestinal tract. The excystation of oocysts is induced by the acid in the stomach and the presence of proteolytic enzymes. The excystation occurs further down in the gastrointestinal tract in the small intestine favoured by the presence of fatty acids, bile salts and neutral pH (CDC, 2015a).

The 'thin-walled' oocysts are formed from about 20% of zygotes that fail to develop a cell wall from ruptures. These ruptures were formed before the faecal passage and maintain the infection in the host. Most of the zygotes have thick and resistant walls. They are passed within the faeces after becoming mature oocysts (Health Canada, 2010). These thin-walled oocysts try to re-infect the epithelial cells of the gastrointestinal tract immediately after they have been released (CDC, 2015a).

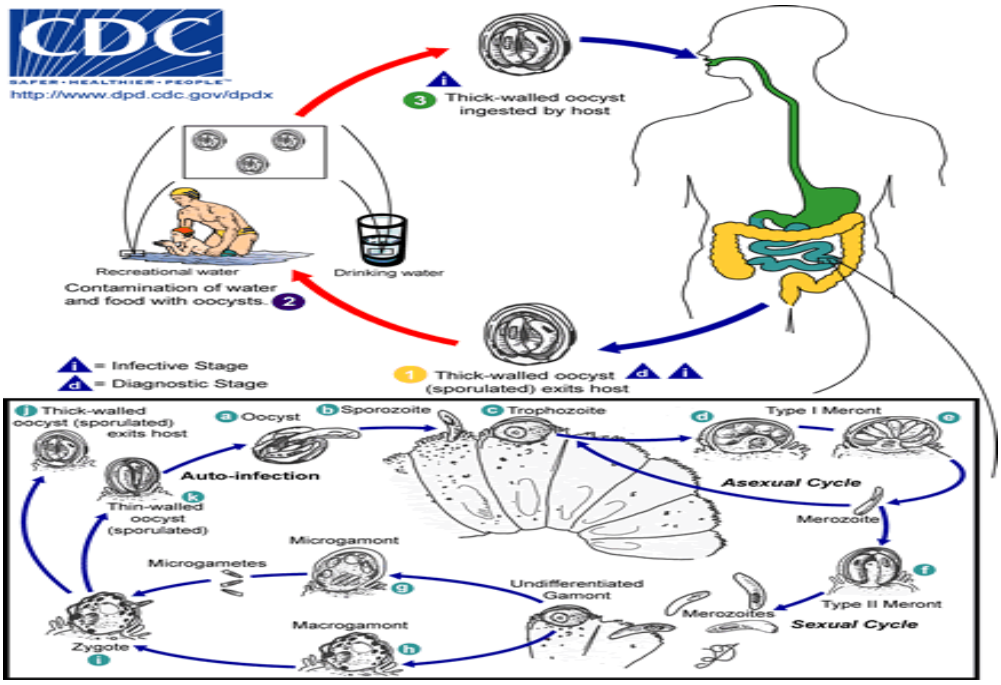

Figure 2.1: Life cycle of *Cryptosporidium*

Source: (CDC, 2012b)

2.5 Biology of *Giardia* spp.

Giardia was first discovered by Antonie van Leeuwenhoek in 1681 (Lappin, 2014). *Giardia* belongs to the subphylum of Sarcomastigophora, order of Diplomonadida. The 'diplo' is referring to two karyomastigonts which each have four flagella and two nuclei but no mitochondria and no Golgi complex. These binucleate organisms are asexual. There are two major development stages in the *Giardia* lifecycle, which are the cysts and the trophozoites. The cysts are oval in shape and the sizes range from 8 to 12µm long and 7-10µm wide. The cysts form part of the infectious stage and upon ingestion excystation occurs, releasing trophozoites. Excystation is induced by the acidic nature of the stomach lumen and the presence of enzymes and is affected similarly to the oocysts of *Cryptosporidium* (Morrissette and Sibley, 2002). Trophozoites have a nucleus and a well-developed cytoskeleton but lack mitochondria, peroxisomes and the components of oxidative phosphorylation. They have an endomembrane system with at least some characteristics of the

Golgi complex and an encoplasmic reticulum, which becomes more extensive in encysting organisms (Adam, 2001). The trophozoites release occurs in the small intestine, where the trophozoites multiply. The dividing trophozoites are carried to the colon and encyst along the way due to bile salts and other stimuli. Depending on the species and the host, cysts can appear in the faeces again from 3 days to 3 weeks after infection (CDC, 2017). Usually, excretion begins around the same time the first symptom of the infected host appears. In humans and animals, infections may last from a few days to several months, with intermittent shedding of the cyst. Most sources consider *Giardia* cysts to be immediately infectious when they are excreted in the faeces, but some cysts might become infectious after a maturation period of up to 7 days (CDC, 2012a). Figure 2.2 illustrates the life cycle of *Giardia.*

On the basis of DNA sequence analysis, *Giardia intestinalis* assemblages have been defined in categories (A-H) (Plutzer et al., 2010). Assemblages A and B are considered to be virulent for humans and acts as 'zoonotic' assemblages (Plutzer et al., 2010).

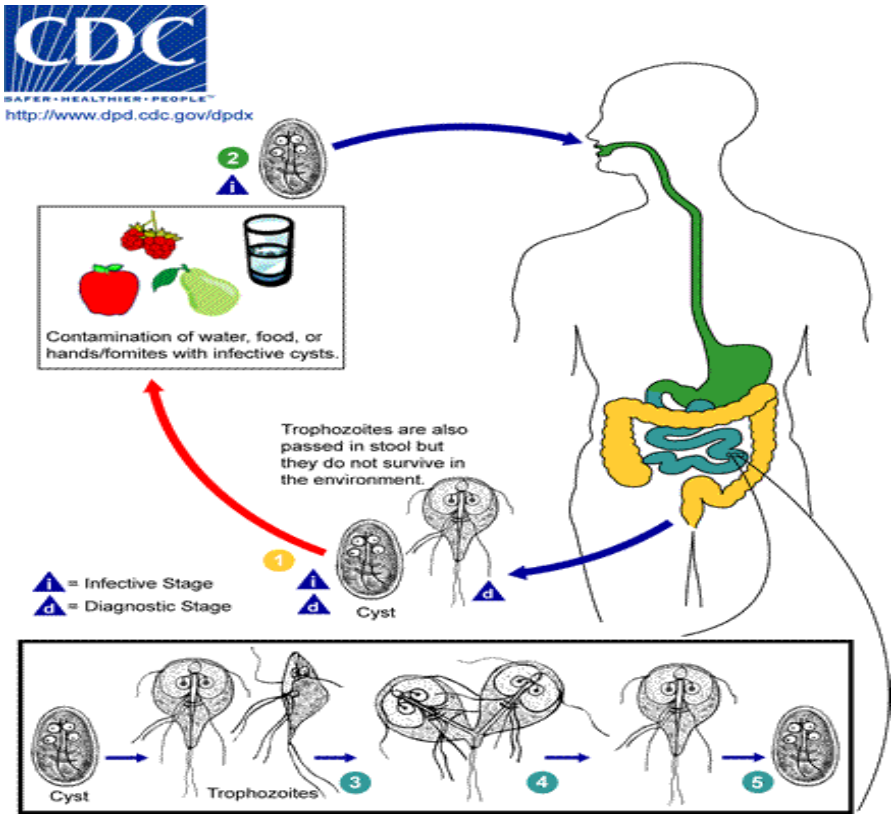

Figure 2.2: Life cycle of *Giardia*

Source: (CDC, 2017)

2.6 Species and major host for *Cryptosporidium* spp and *Giardia* spp

At least 29 *Cryptosporidium* species have been identified as valid. More than 17 *Cryptosporidium* species and genotypes have been reported to infect humans, where *C. hominis* and *C. parvum* are globally the most common. Other species such as *C. meleagridis, C. cuniculis, C. suis, C. muris, C. canis, C. felis, C. ubiquitum and C. andersoni* have been occasionally found in humans (Li et al., 2015, Squire and Ryan, 2017). Zoonotic infections in humans are responsible for the diarrhoea which has been a threat to water supply in South Africa. Related studies on *Cryptosporidium* spp. also suggest that *Cryptosporidium* of cattle are the most common and important zoonotic transmission. The genotypes of other companion animals are less important (Omoruyi, 2010).

In Table 2.4, 14 *Giardia* species are identified to be a cause of infection in humans. About five *G. intestinalis* assemblages have been recognised in humans with additional genotypes in animals (Squire and Ryan, 2017). The major hosts include human, livestock, mammals, pets, amphibians, fish, reptiles and birds. They are mostly transmitted through contaminated water and food. Pets, cattle and other hoofed livestock can be re-infected from their fur when grooming. Some can be found in humans when in contact with diarrhea infected persons. Tables 2.3 and 2.4 illustrate the hosts and major *Cryptosporidium* spp. and *Giardia* spp.

Table 2.3: Some species and major hosts for *Cryptosporidium* spp.

Cryptosporidium species	*Major hosts*	Comments
C. hominis *C. parvum*	Humans, monkeys Cattle, other ruminants, humans	Can be transmitted through contaminated water and contaminated food.
C. meleagridis	Turkeys, humans	*C. meleagridis* occur sporadically, especially in groups of travellers.
C. andersoni and C.bovis *C. muris* *C. suis*	Cattle and sheep Rodents Pigs	Rare occurrence in humans
C. felis *C. canis*	Cats Dogs	Found in humans but less common. (all species which can infect humans can be transmitted person-to-person)
C. wrairi	Guinea pigs	Not associated with human cryptosporidiosis, but the route of infection could likely be faecal-oral with ingestion of oocysts in feces
C. bailey	Poultry	Not associated with human cryptosporidiosis
C. galli	Finches, chicken	Not associated with human cryptosporidiosis
C. serpentis and C. saurophilum	Reptiles and Lizard	Not associated with human cryptosporidiosis
C. molnari and C. scophthalmi	Fish	

Adapted from (Squire and Ryan, 2017, Zahedi et al., 2016, Ryan and Xiao, 2014, Thompson et al., 2016)

Table 2.4: Some species and major host for *Giardia* spp.

Giardia Species	*Major host*	Comments
G. intestinalis *G. intestinalis* *Assemblage B (G. enterica)* *G. intestinalis*	Humans, livestock Humans Humans and other mammals	Transmitted through contaminated water and contaminated food faecal oral-route (poor hygiene practices).
G. intestinalis (G. canis)	Dogs	Animals like dogs can also be re-infected from their fur when they groom
G. intestinalis (G. bovis)	Cattle, other hoofed livestock	Can also be re-infected from their fur when they groom
G. intestinalis (G. cati) *G. intestinalis (G. simondi)* *G. agilis*	Cats Rats Amphibians	They can also be found in humans but less common. Contact with another person with diarrhoea. (all species which can infect humans can be transmitted person to person*)*
G. muris	Rodents	Not associated with human giardiasis, but the route of infection could likely be faecal-oral with ingestion of cysts in faeces
G. microti	Muskrats, voles also found in Fish	Not associated with human giardiasis
G. psittaci	Birds	Not associated with human giardiasis
G. ardeae	Birds	Not associated with human giardiasis
G. varani	Reptiles	Unique specie recently found in lizards

Adapted from (Squire and Ryan, 2017, Zahedi et al., 2016, Ryan and Xiao, 2014, Thompson et al., 2016, Feng and Xiao, 2011)

2.7 Transmission and environmental persistence

Water contamination with *Giardia* cysts and *Cryptosporidium* oocysts is documented from wells, different types of water systems and stagnant water sources like naturally occurring ponds as well as storm water storage systems and clean-looking mountain streams (CDC, 2012b). The cysts and oocysts are resilient and will survive in a wide temperature range and including, to some extent, desiccation. Cysts survive in lake or tap water for approximately two months at 0-8°C (tap water - 2 weeks at 20-28°C; lake water - 1 month at 17-20°C (CDC, 2012a). Cysts also remain viable in river water for up to three months at 0-4°C, one month at 20-28°C and survive in seawater for up to 2 months at 4°C (CDC, 2017). In addition, *Giardia* cysts and *Cryptosporidium* oocysts can survive for some weeks and up to months in warm water (about 25°C). The time frames are not fixed and vary between authors. Auerbach (2012), for example, states that cysts can stay infectious for up to three months in cold water. Oocysts of some species, such as *Cryptosporidium parvum,* can remain infectious after excretion with faeces in wet conditions for more than six months (Fayer, 2010).

Furthermore, oocysts have been found to be extraordinarily resistant to common water treatments including chlorination (Hijnen et al., 2007). They can survive under the standard range of concentrations of chlorine used in water treatment plants. Protozoan parasitic cysts and oocysts are more resistant to different water purification processes than bacterial indicators. There is a high risk to water consumers even with a low number present of *Cryptosporidium* and *Giardia* in water (Rose et al., 1990). Risks are shown during swimming, swallowing of water and contact with recreational fresh water.

Cryptosporidium and *Giardia* are contagious after being excreted in feaces. The (oo)cysts can be infectious even at low dose. Even ingestion as small as 10–30 (oo)cysts can cause infection in healthy persons (Baldursson and Karanis, 2011). Re-infection is also possible in animals with *Giardia* through their fur when they groom (CDC, 2012a). People that are infected have been shown to shed 10^7–10^8 oocysts per gram and they have been revealed to excrete oocysts for up to 50 days after cessation of diarrhoea (Saneian et al., 2010).

2.8 *Cryptosporidium* and *Giardia* in wastewater treatment plants

An efficient and proper treatment of wastewater is now becoming an acceptable option to mitigate the waterborne or foodborne outbreaks of *Cryptosporidium and Giardia.* As mentioned earlier, both *Cryptosporidium* and *Giardia* have been reported to be resistant to the chlorine-based disinfectants at the concentrations and exposure times used in the water industry (Pereira et al., 2008). To reduce the levels of pathogens and risks associated with the wastewater discharge, appropriate and efficient treatment of wastewater is needed. *Cryptosporidium* spp. and *Giardia intestinalis* are commonly detected in wastewater (McCuin and Clancy, 2006) as exemplified in Table 2.5.

In order to achieve removal of contaminant, a combination of steps occurs in wastewater treatment, namely: primary, secondary, and tertiary treatment (UN, 2003). These include a series of processes, such as coagulation, flocculation, sedimentation and filtration. The most common form of wastewater treatment is activated sludge which utilises a consortium of mixed microbial communities through an aerobic biological process to degrade wastewater components (LaPara et al., 2000, Leu et al., 2012).

Primary treatment involves the removal of solid materials while secondary treatment is used to digest organic materials such as nitrogen and phosphorus. Tertiary treatment is aimed at disinfection and pathogenic microorganisms' removal. This may be chemical, physical or irradiation. Tertiary treatment presents some differences which can involve sand filtration combined with UV treatment/chlorine. The effectiveness of the wastewater treatment plants depends on the type of treatments carried out (Ramo et al., 2017, Nasser, 2016, Taran-Benshoshan et al., 2015).

A study by Taran-Benshoshan et al. (2015) showed that secondary activated sludge treatment removed *Giardia* cysts by 3.28 $\log_{10}$/L as compared to the removal of *Cryptosporidium* oocysts with 0.94 $\log_{10}$/L. The sedimentation velocity of Giardia is higher than for faecal coliform and *Cryptosporidium* oocysts. The removal efficiency of *Giardia* during the activated sludge process may be attributed to the attachment of its cysts to the particles of the sewage. *Cryptosporidium* oocysts low removal efficiency is caused by slower settling velocity of its oocysts in the wastewater effluent (Taran-Benshoshan et al., 2015). In the tertiary sand filtration treatment, it was found that this treatment was inefficient for the removal of

Cryptosporidium and *Giardia*. This may reflect, to some extent, the conditions under which the study was carried out due to poor optimization of the filtration process (Taran-Benshoshan et al., 2015). On the other hand, membrane ultrafiltration has been reported to show more efficiency in removing *Cryptosporidium* and *Giardia* than conventional treatment processes like flocculation, sedimentation and sand filtration processes (Fu et al., 2010).

Table 2.5: Examples of concentration of *Cryptosporidium* and *Giardia* in untreated and treated wastewater in different countries

Country	oocysts/L	cysts/L	Water type	Reference
Arizona, US	Influent: 40–160 Effluent: <4–36	Influent: 2700–9900 Effluent: <4.0–150	Wastewater	(Kitajima et al., 2014)
China	0.365	1.258	Recreational lake water	(Xiao et al., 2018)
China	33–600	130–3,600	Untreated wastewater	(Fu et al., 2010)
	67–333	533–2,033	Primary treatment effluents	
	0- 9	0- 32	Secondary treatment effluent	
	0-0.4	0-2.1	Tertiary treatment effluents	
Poland	28.5	113.6	Effluent sewage treatment	(Sroka et al., 2013)
Israel	4–125	10–12,225	Raw wastewater	(Taran-Benshoshan et al., 2015)
	9.94	0.93	Tertiary effluent	
Sweden	8–158	250–12,500	Untreated wastewater	(Ottoson et al., 2006)
Florida, US	0.1–1,000	1–10,000	Untreated wastewater	(Harwood et al., 2005)
Norway	100–1,100	100–13,600	Untreated wastewater	(Robertson et al., 2006)
Spain	1.38-2.6	0.6-1.7	Effluent wastewater	(Domenech et al., 2018)
Asia	60,000	100,000	Untreated wastewater	(Hamilton et al., 2018)
Philippines	0.06 ± 0.19	0.02±0.06	Treated wastewater	(Kumar et al., 2016)
Thailand, Malaysia and Philippines	0.13±0.18 to 0.57±1.41	0.12±0.3 to 8.90±19.65	Untreated water	(Kumar et al., 2016)

2.8.1 Chlorination and UV for the removal of Cryptosporidium and Giardia in wastewater

Chlorine remains as the most widely used product to disinfect conventional wastewater during treatment processes especially at the tertiary treatment stage. Disinfection is highly important at the tertiary treatment stage. Both *Cryptosporidium* and *Giardia* are judged as highly chlorine resistant (Wu et al., 2016) and treatment under South African conditions would potentially be inefficient In wastewater treatment, *Cryptosporidium* was earlier judged as more resistant to removal and inactivation by chlorine and other conventional wastewater treatment than *Giardia* (Cacciò et al., 2003). Due to the *Cryptosporidium* resistance to chlorine, other alternative disinfectants have been suggested, like chlorine dioxide, ozone and ultraviolet (UV) disinfection. Pereira et al. (2008) experimented with chlorine, chlorine dioxide and ozone. They used 2 x 10^4 ooysts/mL purified from calves' faeces for their experiment. With hypochlorous acid, inactivation of 49.04% was found after 2 hours. At 5ppm, 90.56% inactivation rate was found for chlorine dioxide while ozone was the most efficient with 100% inactivation at a concentration of 24 mg/L.

UV disinfection has become a preferred alternative in wastewater treatment. UV has been growing extremely fast in the water industry since it has been demonstrated to be highly effective in removing *Cryptosporidium* oocysts and *Giardia* cysts. It is therefore appropriate to investigate the performance of UV in comparison with chlorine in wastewater treatment as well. UV disinfection is much more efficient than chlorination for *Cryptosporidium* (Ashbolt, 2004). The efficacy of UV irradiation in inactivation of *Cryptosporidium* was assessed by Morita et al. (2002). They presented the effect of UV irradiation in the inactivation of *Cryptosporidium*. The viability was measured based on two methods; the ability to infect animals and through the excystation of oocysts, which were exposed to different UV doses. Based on the investigations it was established that when the UV dose increased, the infectivity decreased exponentially. Two (2)-log reduction, which is 99% inactivation, required about 1.0 mWs/cm^2 at 20°C. *Cryptosporidium* showed high resistance to UV irradiation which required 230 mWs/cm^2 for 2-log excystation reduction. This dosage is considered high. There was very low excystation at low UV doses below 100mWs/cm^2. UV is preferred because they have no hazardous by-products and maintenance cost is low. UV is effective for bacterial and viral disinfection in wastewater treatment (Morita et al., 2002).

Many studies have presented the viability of chlorine and UV in water treatment. The use of chlorination with sunlight may be advantageous in improving *Cryptosporidium* inactivation. It may also be low-cost, simple and an effective method of *Cryptosporidium* inactivation, especially in wastewater treatment. Zhou et al. (2014) presented the inactivation of *Cryptosporidium* using free available chlorine and solar irradiation. It was found that exposure to 60 minutes of simulated sunlight caused <0.5 log inactivation. This methodology may be applied to wastewater treatment in order to reduce the concentration of *Cryptosporidium* and *Giardia*. However, exposure to simulated sunlight for the same time (60 mins) in combination with free available chlorine (FAC) of 8 mgL^{-1} as Cl_2 resulted in >2 log inactivation. This clearly shows that s combination of treatment plans can result in higher inactivation of protozoan parasites. They further concluded that employing FAC photolysis may be advantageous in treating wastewater. It can also be extended to engineered wetlands, provided NH_3 and DOC levels are low enough to maintain FAC residues. This research may have proffered solutions to disinfection of *Cryptosporidium* in outdoor swimming pools treated with FAC or other products containing chlorine to prevent health risks for the community.

2.8.2 Water quality standards and public health safety

Microbial contaminants, emanating from fecal contamination including wastewater, are used as indicators of water quality; hence their detection, isolation and identification in water have always been difficult and expensive. To avoid such problems, indicator organisms are used in determining the relative risk of the presence of pathogens in contaminated water (WHO, 2017b). Bacteria, such as coliforms, *Escherichia coli*, and faecal streptococci are used as indicators of quantitative faecal contamination in water sources. The presence and risk of *Giardia* and *Cryptosporidium* does not correlate with faecal bacteria indicators and is analysed directly if required. Their quantitative presence can also serve as an indicator of contamination with other protozoan parasites. Accordingly, WHO have developed water quality guidelines and countries have individual created water quality standards to protect public health which reflect both fecal contamination based on the presence of indicator bacteria like *E coli* and faecal streptococci (enterococci), but not specifically the presence of parasitic protozoa. However, SANS and DWAF have included standard values for these in the South African National legislation (see Table 2.6) which relates to domestic water, irrigation and recreational water.

The community health risk is measured using the concentration of indicator organisms like faecal coliforms (DWAF, 1996c) and *Escherichia coli* (SABS, 2006). In general, there is a high microbial risk in consuming contaminated water containing faeces from humans or animals. The major source of faecal microorganisms and pathogens is the wastewater discharges in coastal seawaters and fresh waters (Grabow, 2001, Fenwick, 2006, WHO, 2008). However, river water quality in South Africa has been negatively affected by discharges from wastewater treatment plants which usually contain high microbial loads (Luyt et al., 2012, Teklehaimanot, 2013). Faecal pollution of recreational water leads to health problems because of the presence of infectious microorganisms. These are usually found in human sewage or animal sources (WHO, 2006b). Therefore, monitoring microbial surface water quality in South Africa becomes a vital tool in public health protection (Luyt et al., 2012).

Table 2.6: Microbiological requirements for domestic, irrigation and recreational water (SANS and WHO guidelines)

Indicator Organisms	**Water Use**	**Limit**	**References**
Heterotrophic plate count/1 mL	Drinking water	0-100	(SANS, 2015)
Total coliform count/ 100 mL	Drinking water	≤ 10	(SANS, 2015)
Faecal coliform count/ 100 mL	Drinking water	≤ 1	(SANS, 2015)
Faecal coliform count/ 100 mL	Drinking water	0	(WHO, 2011a)
Faecal coliform count/ 100 mL	Irrigation water	≤ 1,000	(WHO, 2006a)
Escherichia coli count/ 100 mL	Drinking water	0	(SANS, 2015, WHO, 2011a)
Escherichia coli count/ 100 mL	Irrigation purposes	≤ 1	(DWAF, 1996a)
Cryptosporidium count/ 10 mL	Drinking water	0	(SANS, 2015)
Giardia count/10 mL	Drinking water	0	(SANS, 2015)
Cryptosporidium/ *Giardia* count/10 mL	Recreational water	≤ 1	(DWAF, 1996b)

2.9 The prevalence of *Cryptosporidium* and *Giardia* in wastewater and irrigation water from different water sources

The presence of *Cryptosporidium* and *Giardia* in wastewater is a main concern for all types of downstream activities from the effluent point and for the indirect and direct water reuse for irrigation (Domenech et al., 2018). Table 2.6 shows a few selected reports on the concentration of *Cryptosporidium* and *Giardia* in different wastewater treatment plants and effluents of the world to illustrate the concentrations.

Reuse of reclaimed wastewater has been adopted as an important option for an alternative water source for irrigation (Mekala and Davidson, 2016). Recently, non-potable water reuse for irrigation and crop processing has become a subject of research in order to mitigate the consequences of increasing scarcity of water (Hachich et al., 2013). However, wastewater reuse can result in serious threat to animal and human health if the pathogen load and reduction is not clearly accounted for due to treatment or in a multi-barrier approach (Ma et al., 2016, Plutzer and Karanis, 2016).

Pathogens present in the environment can be mobilised by heavy rainfalls which can cause the increased run-off into the wells, coaster waters and rivers (Cann et al., 2013). Both temperature and rainfall have considerable impact on viability, survival and dissemination of parasitic protozoa (Schijven et al., 2013). For example, after a heavy rainfall a high density of these protozoans occurred in water supplies in Brazil (Neto et al., 2010). The relationship between the climate change and parasitic protozoa goes beyond the development level of countries. People, regardless of where they live, are exposed to a wide range of pathogens including parasites, viruses, bacteria and algae through recreational water usage, food and drinking water consumption (Schijven et al., 2013). Additionally, lack of access to appropriate sanitation and potable water locally and regionally affects the environment through flooding. Flooding leads to disastrous conditions, causes damage and additional risk to crops, livestock and water.

Irrigation of agricultural land is done in many developing countries, including South Africa, using urban wastewater (Gumbo et al., 2010). Scott et al. (2000), among many others, proposed that wastewater that is rich in nitrates and phosphates can result in improved and increased crop yields without using inorganic fertilisers. On the negative side, heavy metals, organic compounds and a wide range of enteric pathogens are present in wastewater which is

detrimental to both human health and the environment (Scott et al., 2000). In this context, the prevalence of protozoan in wastewater and assessment of wastewater treatment and removal efficiencies is important to safeguard human health risk.

Since *Giardia intestinalis* and *Cryptosporidium* spp. are commonly found in wastewater, they are also frequently associated with waterborne outbreaks worldwide (Efstratiou et al., 2017, Gertler et al., 2015). Their occurrence in wastewater and effluents are exemplified in Table 2.5. The exemplified concentrations, even with extensive dilution afterwards in the recipient, can cause serious illness for both animals and humans (Kitajima et al., 2014, Gallas-Lindemann et al., 2013, Taran-Benshoshan et al., 2015).

Additionally, several studies have reported the presence of *Cryptosporidium* oocysts and *Giardia* cysts in a variety of African water sources. For example, irrigation water in Burkina Faso (Kpoda et al., 2015), wells, springs, stream and lakes in Cameroon (Ajeagah et al., 2007, Ajeagah, 2013), as well as wastewater in Côte d'Ivoire (Yapo et al., 2014). They have also been found in wastewater treatment plants, canals, tanks and swimming pools in Egypt (El-Kowrany et al., 2016, Youssef et al., 1998). Also, occurrence has been documented in water sources such as surface and well, treated water storage tanks in Ethiopia (Atnafu et al., 2012, Fikrie et al., 2008), treated and untreated effluents, as well as sewage and roof-harvested rainwater in South Africa (Dungeni and Momba, 2010, Samie and Ntekele, 2014). Samie and Ntekele (2014) also reported the occurrence of *Giardia* in municipal wastewater samples collected in the Vhembe District in South Africa. Another report by Dobrowsky et al. (2014) on rainwater from 29 rainwater tanks in Kleinmond, South Africa shows that *Giardia* spp. were detected in about 25% of the sampled eight tanks of water (Dobrowsky et al., 2014).

Surface water that is used for irrigation in South Africa has been reported to contain pathogens and be of variable quality (Duhain, 2011). The occurrence of *Cryptosporidium* and *Giardia* are highly likely although not frequently quantified. Surface water of poor microbiological quality could therefore frequently contaminate irrigated vegetables where the contaminated water is deposited on the surface of the crops during irrigation (Robertson and Gjerde, 2001). The pathogens present in this water will be transmitted onto fresh produce and may cause human infections (Duhain, 2011).

In fresh wastewater, *Cryptosporidium* oocysts and *Giardia* cysts pervasiveness and concentration can be influenced by some features like sanitary conditions, socioeconomic status, the amounts of rainfall every year and also how sensitive the detection methods are (Nasser et al., 2012). The issue of water scarcity in South Africa, together with the complexity of hydrological situations, make it extremely difficult to get adequate water for the general development of living standards (Schreiner and Hassan, 2011). The water scarcity in the country has also contributed to an increase in use of wastewater and nearby available surface water for several recreational, agricultural and aquaculture purposes (Duhain, 2011, DWA, 2011).

The department of environmental affairs (DEA) highlights that South Africa receives only 450 mm rainfall per year as compared to the world average of 870 mm per year which makes South Africa the 30th driest country in the world (DEA, 2011). Increase in the preservation of water whilst maintaining water quality and efficiency in water use is important in the country considering the low rainfall per year (DEA, 2011).

The declining water quality is of major concern in South Africa. Water quality in many rivers in South Africa keeps deteriorating and this affects its availability and use. This is also the situation for the rivers in the eThekwini Metropolitan Area, in Kwazulu-Natal where this study has been conducted (Naidoo, 2013). Discharges of inadequately treated industrial, agricultural and domestic wastewater into rivers largely contributes to the poor quality of rivers (Naidoo, 2013).

An increase in foodborne infection outbreaks is associated with raw vegetable consumption world-wide (Alhabbal, 2015, Olyaei and Hajivandi, 2013). Changes in consumers dietary habits is one of the most important causes of this increase. The consumption of fresh or minimally processed vegetables and fruits has drastically increased among people recently (Polak, 2010). Awareness on the need to eat fresh products without any chemical preservatives has increased among people. Microbial contamination may occur and are commonly from faeces of humans and animals; irrigation and cleaning water; and from soil (Alade et al., 2013, Nazemi et al., 2012, Tomass and Kidane, 2012). Contamination could also occur with the rinsing and sprinkling of fresh vegetables with contaminated water (Alade et al., 2013, Olyaei and Hajivandi, 2013). *Cryptosporidium* and *Giardia* can survive on both washed and unwashed

vegetables. An increase in the case of foodborne illnesses has been recently reported which is mainly linked to eating fresh vegetables (Alhabbal, 2015, Olyaei and Hajivandi, 2013, Sunil et al., 2014). In both developed and developing countries, there is a high report of illness caused by intestinal parasitic infections from eating raw vegetables (Haq et al., 2014, Nazemi et al., 2012). This can be linked to inadequate personal hygiene and poor sanitation (Tefera et al., 2014).

2.10 The prevalence of *Cryptosporidium* spp. and *Giardia* spp. in animals in an African context

Contamination of water sources and rivers can occur due to the closeness and access of different animals, both farmed and domestic including wildlife and birds, to the water sources during their activities. Different types of animals, including mammals, birds, reptiles, amphibians and fish have been found to be hosts to *Cryptosporidium* and *Giardia* globally (CDC, 2012b, CDC, 2016, Ryan and Xiao, 2014, Squire and Ryan, 2017, Yang et al., 2010). Presently, about 150 mammalian species have been identified as hosts for different *Cryptosporidium* species (Ryan and Xiao, 2014). Usually, younger animals are more susceptible to these parasites than adults and thus may be the main carriers. Animals kept as pets play an important role in the zoonotic transmission of the parasites because of their proximity to humans and the large quantity of parasites (cysts/oocysts) excreted. Animals are known to harbour both zoonotic and host-specific *G. intestinalis* assemblages that are morphologically identical and require sensitive typing tools to trace transmission (Feng and Xiao, 2011).

Samra et al. (2013) detected *Cryptosporidium* spp. among the wildlife animals and the native domestic calves from the interface of wildlife/livestock of the Kruger National Park, South Africa. *Cryptosporidium* spp. (identified as *C. andersoni* (2/4) and *C. bovis* (2/4)) occurred in 8% (4/51) of the calves. Four out of the 214 wildlife samples tested positive for *Cryptosporidium* with a prevalence of 2.8% each in impala and buffalo. *Cryptosporidium ubiquitum* was detected in two impala and one buffalo and *C. bovis* in one buffalo (Samra et al., 2013). Another study of villages in Mafikeng, North West province of South Africa revealed the overall prevalence of *Cryptosporidium* to be 80% in pigs in the whole of Mafikeng (Samra et al., 2012). Previous research in Nigeria supported the presence of *Cryptosporidium*

bovis, *C. ryanae* and *C. andersoni* as commonly encountered species which are affecting cattle (UN, 2003, Maikai et al., 2011).

Cryptosporidium parvum is recognised as a significant cause of life-threatening neonatal diarrhoea in calves (Maikai et al., 2011). *C. bovis* (7.2%), *C. ryanae* (4.1%), and *C. andersoni* (2.5%) were reported in native cattle in Northern Nigeria. Another study from Nigeria by Maikai et al. (2011) found 78.1% of suckling calves up to 3 months of age infected with *Cryptosporidium*. According to a study conducted by Sak et al. (2013) in the Central African Republic, phylogenetic analyses based on small subunit ribosomal ribonucleic acid (SSU rRNA) sequences showed that *Cryptosporidium* originating from a habituated gorilla was 100% similar to the *C. bovis* reference sequence listed in GenBank (AY741305). *Cryptosporidium* spp. was only detected in a single case (1 out of 201 samples examined) from both gorillas and 1 out of 191 that were examined from other wild and domestic animals (Sak et al., 2013).

Prevalence of *Giardia* spp. has also been reported in some other parts of Africa. In Central African Republic, a single-species infection was detected in all western lowland gorillas with the exception of one co-infection with *E. bieneusi* genotype D and *G. intestinalis* apparent in a gorilla from the Makumba group (Sak et al., 2013). Chimpanzees in Guinea-Bissau were found to harbour *Giardia intestinalis* (Sá et al., 2013). Ethiopia had prevalence of 2.3% of *Giardia intestinalis* infection and 7.8% of *Cryptosporidium* infection (Wegayehu et al., 2013). An occurrence of *Giardia intestinalis* was also reported in Nigeria in non-biting synanthropic flies (*M. domestica*) (Adenusi and Adewoga, 2013). *Giardia* spp. was also retrieved in lions from a study conducted in Zimbabwe. It was reported that sources of some oocysts were probably from prey species for the lions (Mukarati et al., 2013). Occurrence of *Giardia* spp. was also reported in Bamboo lemurs, Eastern rufous mouse lemurs in Ranomafana National Park, Madagascar (Rasambainarivo et al., 2013), and in Volcanoes National Park Rwanda (Hogan et al., 2014). Ruminant's livestock were also found to harbour *G. intestinalis* in Egypt from a study conducted on livestock where 53% (424/804) prevalence was revealed in all the livestock (Helmy et al., 2014). *Giardia* spp. was also revealed in stray cats with 91% (102/113) prevalence in Egypt, especially in the Northern Region of Nile Delta (Khalafalla, 2011).

2.11 Conclusion

Access to different sites where pathogens are present can be limited to prevent exposure to waterborne pathogens. This can be achieved by preventing access and exposure to wastewater treatment plants. Combined sewer overflows can have limited access as well. In the case of surface water for recreation, sources of pathogens can be controlled by reducing pathogen concentrations. Throughout the world, giardiasis and cryptosporidiosis are common. Mostly, inadequate supply of treated wastewater and poor sanitation are the factors leading to the spread of these infections, and people that are more vulnerable are the immunocompromised ones like children, HIV/AIDS sufferers, pregnant women and geriatrics. Outbreaks and prevalence of these two protozoan parasites (*Cryptosporidium* and *Giardia*) have been investigated in different parts of the world and these can be extended to developing countries like South Africa. Due to the size and frequency of these outbreaks, cryptosporidiosis and giardiasis became a serious public health issue worldwide and prompted re-evaluation in developing countries, especially in South Africa.

CHAPTER 3: METHODOLOGY AND APPROACHES FOR THE DETECTION OF *CRYPTOSPORIDIUM* AND *GIARDIA* IN ENVIRONMENTAL SAMPLES AND LABORATORY ASSESSMENTS

3.1 Introduction

This chapter focuses on detection of *Cryptosporidium* and *Giardia* methods. It is divided into two main sections. Section A summarises the methods specifically used in this study for the occurrence and reduction of *Cryptosporidium* and *Giardia* in selected wastewater treatment plants, receiving waters and on crops in Durban, South Africa. In addition, the chapter presents methods used for the viability assessment of *Cryptosporidium* and *Giardia* in chlorine and UV treatment experiments. Section B is a published review (Adeyemo, F. E., et al. 'Methods for the detection of *Cryptosporidium* and *Giardia*: From microscopy to nucleic acid-based tools in clinical and environmental regimes' Acta Tropica **184**: 15-28) (Adeyemo et al., 2018). It presents an overview of methods used globally for the detection of *Cryptosporidium* and *Giardia* in clinical and environmental water samples. The advantages and limitations of the methods and advanced detection techniques are provided. Some techniques, like acid-based approaches, used for the detection of viable oocysts and cysts are discussed. Enzyme, microscopy and antibody based techniques, which are the widely accepted conventional techniques, are also presented.

Chapters 4 and 5 are presented as individual entities. To ensure the proper flow of presentation and minimize some repetitions, there is cross-referencing of methodology in these chapters and throughout the thesis.

SECTION A: Sampling methodology to quantify *Cryptosporidium* and *Giardia* in wastewater treatment plants, recipients and on crops in this thesis from Durban, South Africa.

Details of sampling sources and collection and characteristics of wastewater treatment plants used in the study are fully presented in section 4.2 of Chapter 4. Figure 3.1 presents an overview of treatment stages of the wastewater treatment plant with trickling filter treatment and different sampling sources used for this study. Figure 3.2 similarly presents an overview of treatment stages of wastewater treatment plants with activated sludge treatment (aeration tank) and different sampling points.

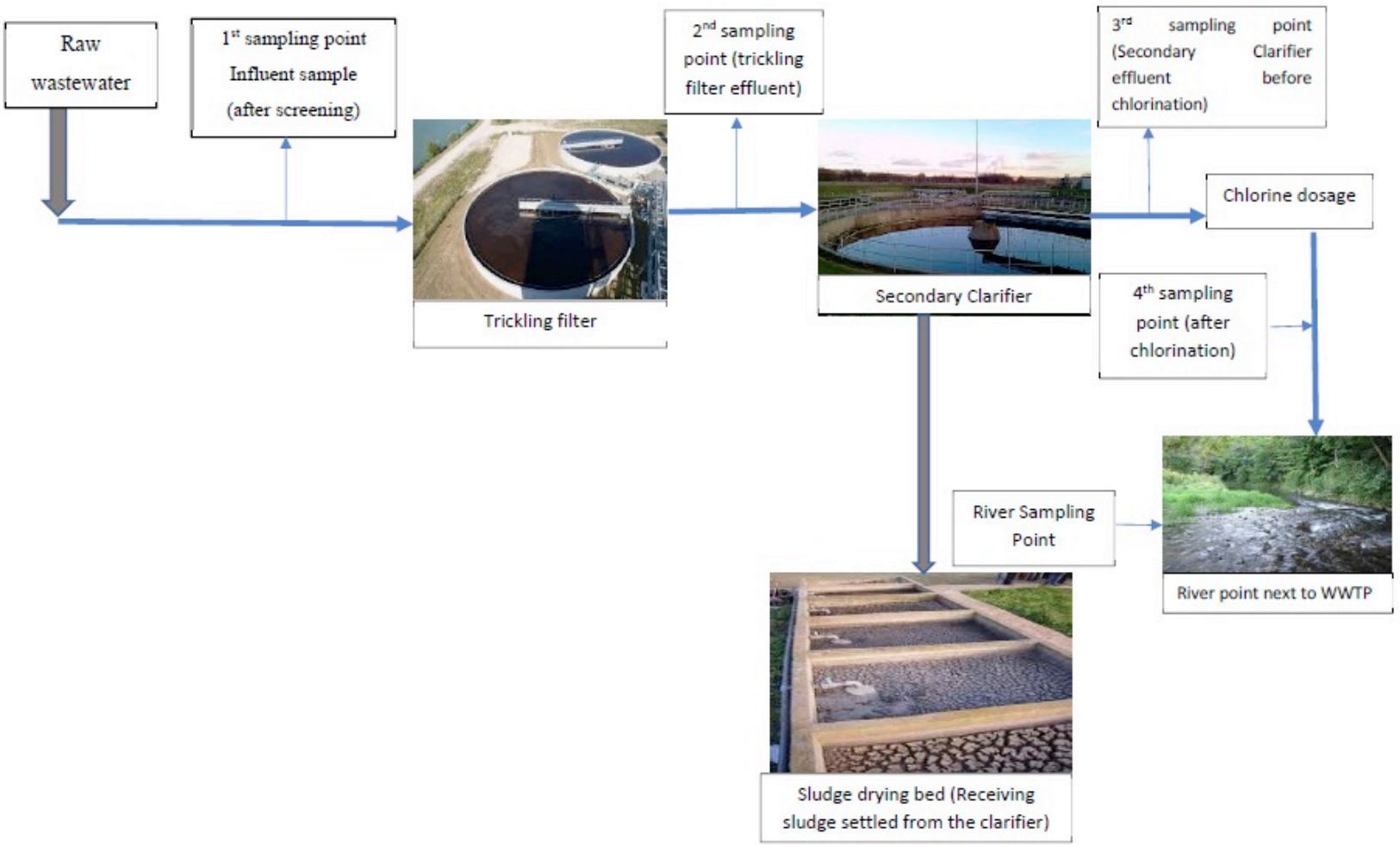

Figure 3.1: Overview of treatment stages of WWTP showing trickling filter treatment and different sampling sources

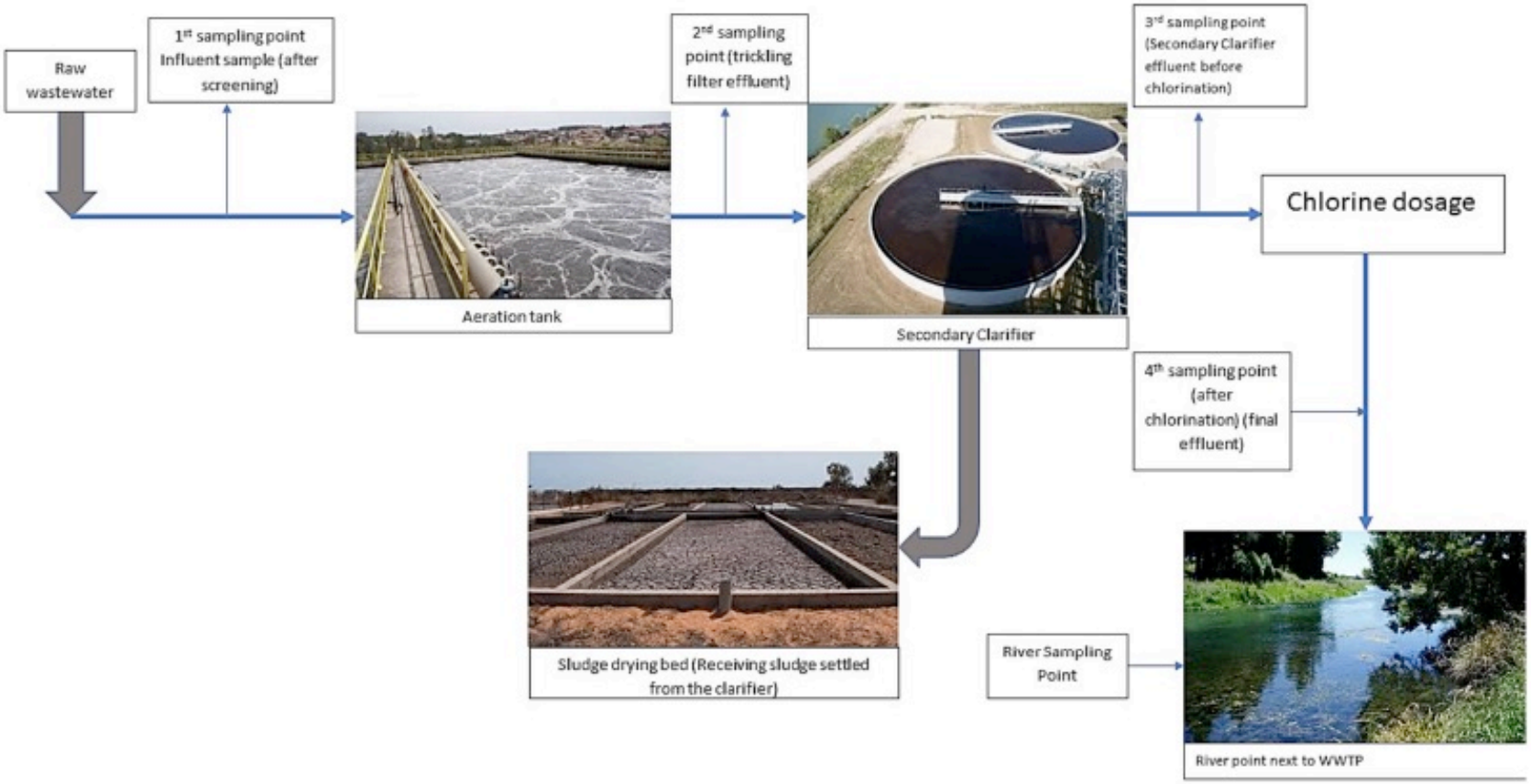

Figure 3.2: Treatment stages of WWTP showing aeration tank and different sampling sources

3.2 Enumeration of faecal enterococci and *Escherichia coli*

The detection and enumeration of faecal enterococci and *Escherichia coli* in environmental water samples was performed using the conventional spread plate method on selective agar. This was analysed according to the standard method (APHA, 2005, APHA, 1998). Agar was prepared according to the manufacturer's instruction. Appropriate serial dilutions were performed on all waste water samples. Dilutions were prepared with sterile distilled water, and 0.1 mL of the respective dilution was inoculated and spread on the appropriate selective media in triplicate. Brilliance *E. coli*/coliform selective agar (Oxoid Product – CM1046) was used to detect the presence of *E coli*/coliform in the samples. The culture petri dishes were incubated for 24 hours at 37°C and all purple colonies were counted as *Escherichia coli*. Briefly, faecal enterococci analysis was performed on Slanetz & Bartley medium (Oxoid Product– CM0377) for the enumeration of enterococci in water samples in triplicates and incubated at 35°C for 4 hours and then 44-45ºC for 48 hours. All the red or maroon colonies were counted as enterococci and expressed as colony forming units per 100 mL (CFU 100mL^{1}). This is calculated using equation (1).

$$CFU\ /100mL = \frac{number\ of\ colonies \times dilution\ factor}{sample\ volume} x100 \qquad (1)$$

Water samples were also assessed for various physicochemical parameters like pH, Temperature (^{O}C), Total dissolved solids (TDS mg/L) and dissolved oxygen per milligram (DO, mg/L). These parameters were determined on site at the time of sampling with a YSI Model 556 MPS Handheld Multi-parameter Water Quality Meter.

3.3 Sample concentration and microscopic examination

3.3.1 Conventional methods

There are different conventional methods which have been developed for the detection of *Cryptosporidium* and *Giardia* in raw source waters, wastewater and drinking water. These conventional methods, compared to the USEPA's Methods 1623, have lower recoveries but variance increase (Quintero-Betancourt et al., 2002). An Information Collection Rule was developed by USEPA where large municipal water supplies are required to use a specified

method in recovering and detecting *Cryptosporidium* oocysts in water sources (USEPA, 1996). This method was improved in order to enhance the detection and recovery of *Cryptosporidium* oocysts in water samples; both raw and finished. This is necessary because of small sample volumes involved and variable recovery rates (Simmons et al., 2001). The USEPA's Methods 1623 (Quintero-Betancourt et al., 2002) with additional updates (EPA Method 1623) was applied in this thesis (USEPA, 2012). This includes four main steps: initial filtration to capture oocysts from a 10-liter sample of water; immunomagnetic separation (IMS) to concentrate and purify the oocysts washed from the filter; fluorescent-antibody staining and 4′,6-diamidino-2-phenylindole (DAPI) counterstaining of the IMS product; and microscopic examination and enumeration of the sample by epifluorescent and differential interference contrast (DIC) microscopy (USEPA, 1997). The method of detection can be summarised as immune-magnetic separation (IMS), and immunofluorescence assay (IFA) microscopy. The two methods, IMS and FA, can be used in identifying the genera, *Cryptosporidium* or *Giardia*, but cannot be used for species specifications (USEPA, 2012).

Other methods used in concentrating (oo)cysts in water samples are also reported which are based on filtration using different filters (Simmons et al., 2001, Fayer et al., 1998, Morales-Morales et al., 2003). Also, in environmental samples, direct PCR methods have been developed for the detection of *Cryptosporidium* and *Giardia* (Hörman et al., 2004, Mayer and Palmer, 1996, Xiao et al., 2000), although not quantitative.

3.3.1.1 *Immunomagnetic separation*

Samples were analysed according to the USEPA 1623.1 revised method (USEPA, 2012). Water samples were filtered by using a flat-bed membrane filtration technique (Millipore Corp., Bedford, MA 01730, with 142 mm filter holder) with added color seeds as an internal standard which is an internal positive control for *Cryptosporidium* and *Giardia*, (ColourSeed C&G, BTF Pty Ltd, Sydney, Australia). ColourSeed is a suspension containing exactly 100 *Cryptosporidium* oocysts and 100 *Giardia* cysts. They are modified using an attachment of a Texas Red analogue to the cell wall. This allows them to be differentiated from unmodified oocysts and cysts by the use of fluorescence microscopy (Warnecke et al., 2003)**.** Relative standard deviation (RSD) and Mean percent recoveries were calculated for statistical analysis,

which are used to correct the counts. ColorSeed C&G containing *Giardia* cysts and *Cryptosporidium* oocysts was vortexed for one minute and was added to the samples according to the manufacturer's instructions. Oocysts and cysts in the water samples were concentrated on the surface of the cellulose acetate membrane filter with 0.45 µm pore size and 142 mm diameter (Merck Millipore, USA). The concentrated samples were scraped off the filter and washed using 50 mL of PBS-Tween-80 (0.1%), followed by centrifugation at 1,500 x-g for 15 minutes. The supernatants were aspirated to 5-mL above the pellet, and Immunomagnetic separation (IMS) was performed. The cysts and oocysts were captured from the remaining 5 mL of the supernatant using Dynalbead anti-*Giardia* and anti-*Cryptosporidium* immunomagnetic antibodies (GC-Combo Dynabeads kit, Life Technologies AS, Norway).

3.3.1.2 *Direct Fluorescent Antibody Staining (DFA)*

DFA was performed using commercial test Aqua-Glo™ G/C Direct Comprehensive Kit (Waterborne Inc., USA). 50 µL aliquot of the purified suspension containing the captured oocysts was air-dried on a well-slide at room temperature. Samples were then fixed with 50 µL of methanol and allowed to dry. After methanol fixation, 50 µL of 4', 6"-diamidino-2-phenylindole solution (DAPI) was added for 5 minutes at room temperature. The slides were rinsed off DAPI by adding 100 µL wash buffer and left for 1 minute, then stained with fluorescein isothiocyanate (FITC)–conjugated *Giardia/Cryptosporidium* monoclonal antibody reagent (Aqua-Glo G/C Kit Waterborne Inc, USA) for 30 minutes at 37 °C. The slides were examined by using epifluorescence microscopy at 40X magnification using an Axio Carl Zeiss epifluorescence microscope (Carl Ziess, RSA). *Giardia* cysts (~6-µm) were identified based on their size, shape and the pattern and intensity of immunofluorescent assay staining (bright green fluorescence of the cyst wall). *Cryptosporidium* oocysts (~4-µm) were identified based on their size, shape and the presence of a suture on the oocyst wall. Figure 3.3 presents the schematic diagram of the methodology used for the quantification of *Cryptosporidium* oocysts and *Giardia cysts* from all water and crop samples.

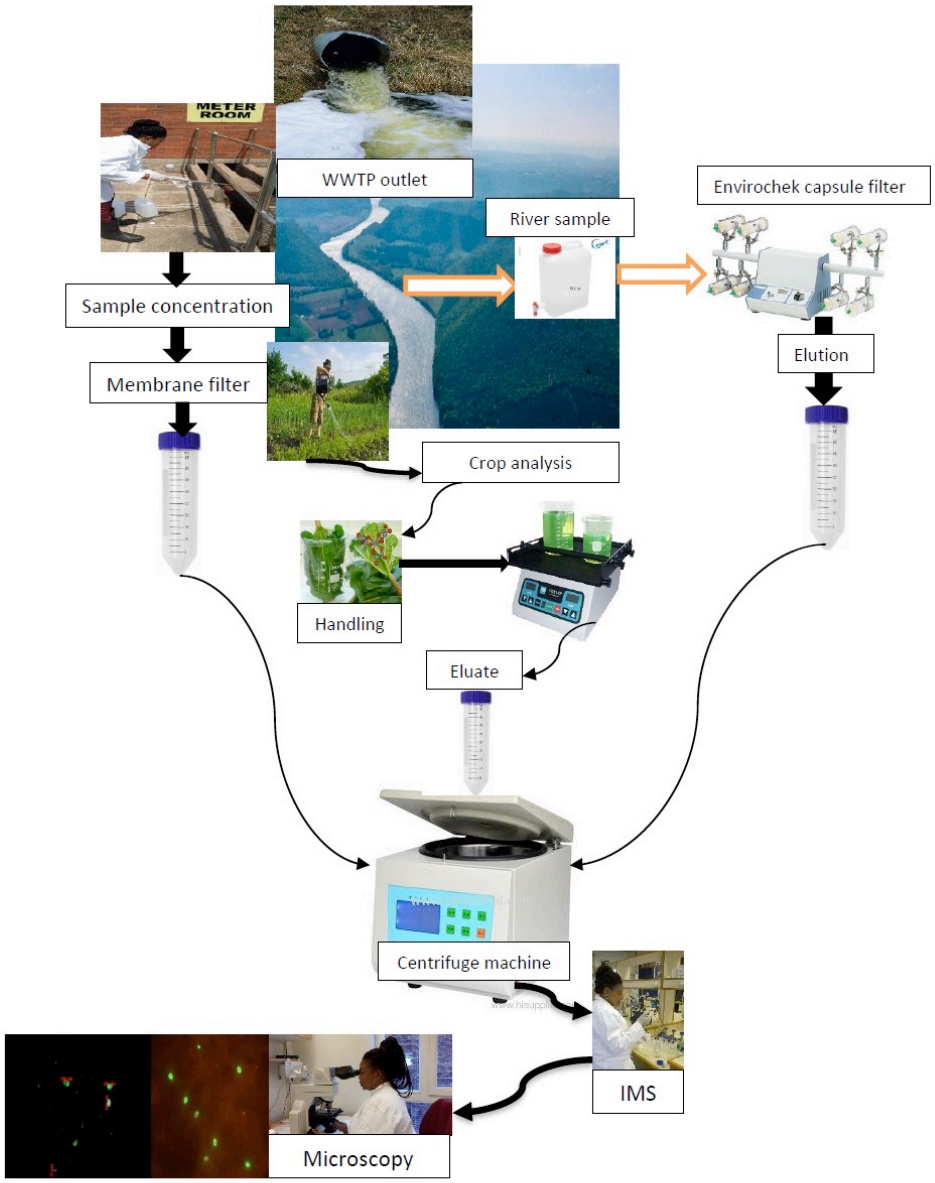

Figure 3.3: Sampling collection processes and handling from different water sources

The wastewater effluent to the river is at the point where farmers take water for irrigation purposes. Samples of spinach irrigated with the river water were taken from the farm next to the river. Wastewater samples collected from the treatment plant and its effluent were filtered using membrane filtration. The river samples were filtered through the envirochek capsule filter and the filters were filled with elution buffer. The capsule filters were installed on the pump and shaken for about 15 minutes. The capsule filters were scraped and washed in elution buffer, followed by centrifugation of the eluate at 1,500 x-g for 15 minutes. The oocysts, cysts and organic and inorganic debris materials were retained on the filter. Materials on the filter were eluted and the eluate was concentrated by centrifugation at 1,500 x-g for 15 minutes. The oocysts and cysts were stained on the slides with fluorescence labelled monoclonal antibodies and DAPI. The stained samples were examined using a fluorescence microscope. Quantification was done by scanning the full slide surface for the characteristics or morphology of oocyst and cysts through their sizes and shapes.

All water samples were subjected to DNA extraction for molecular characterization. DNA was extracted from wastewater samples using the QIAamp DNA Mini Kit according to the manufacturer's instructions, incorporating a modification added to the protocol (Qiagen GmbH, Hilden, Germany). The Polymerase chain reaction (PCR) and quantitative PCR (qPCR) were used to confirm the identities of the presumptive oocysts and cysts as explained in sections 3.3.2 and 3.3.3.

3.3.2 qPCR-based detection of Cryptosporidium and Giardia specific genes in environmental samples

3.3.2.1 Molecular techniques for the analysis of Cryptosporidium and Giardia

The microscopic-based methods produce total counts of live and dead *Cryptosporidium* oocysts in water samples, without distinguishing species or genotypes that can infect humans (Brescia et al., 2009). Several methods rely on the *in situ* hybridization of probes to particular genetic loci within *Cryptosporidium* oocysts or *Giardia* cysts, while most rely on the specific amplification of one or more loci from small amounts of DNA by the PCR (Girones et al., 2010).

3.3.2.2 DNA extraction and genotyping of Cryptosporidium and Giardia in wastewater

DNA was extracted from wastewater samples collected from four wastewater treatment plants in Durban. All wastewater samples collected were subjected to DNA extraction for molecular characterization. DNA was extracted from wastewater samples using the QIAamp DNA Mini Kit according to the manufacturer's instructions, incorporating a modification added to the protocol (Qiagen GmbH, Hilden, Germany). The samples were subjected to ten cycles of freeze–thaw followed by nucleic acid extraction and purification using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The freeze-thaw, protocol was performed according to Nichols et al. (2003) with few modifications. Briefly, the samples (Influent: 50/100 mL; Effluent: 300/600mL) were suspended in 180 µL of ATL lysis buffer (Qiagen GmbH, Hilden, Germany) and an initial step of 10 freeze-thaw cycles (freezing in liquid nitrogen for 10 min and heating at 56°C for 10 min) was incorporated followed by the addition of 20 µL of proteinase K per tube and the tubes were incubated overnight at 56°C. The DNA was extracted from (oo)cysts and the purification of DNA through the column was done according to the manufacturer's protocol. Thereafter, the DNA was eluted from the columns in AE buffer (any elution buffer). The quality and quantity of extracted DNA was analysed by Nano drop Spectrophotometer (Nano drop Technologies).

3.3.3 PCR and quantitative PCR assay for the detection of Cryptosporidium and Giardia from wastewater samples

The nested PCR (primers) used for the presence of *Cryptosporidium* amplifying a region within the 18S rRNA gene for the detection of *Cryptosporidium* were adopted from a previously reported nested PCR (Xiao et al., 2001). The first amplification reaction contained 1x AmpliTaq Gold Buffer (Thermo Fisher Scientific, Waltham, United States), 3 mM $MgCl_2$, 0.2 mM dNTP mix, 0.16 mg/mL bovine serum albumin (BSA), 200 µM of forward and reverse primer (5'-TTCTAGAGCTAATACATCCG-3′ and 5'-CCCATTTCCTTCGAAACA GGA-3'), 1 unit of AmpliTaq Gold Polymerase (Thermo Fisher Scientific, Waltham, United States) and 2 µL of diluted MDA product in a total volume of 25 µL. Subsequent to an initial denaturation for 5 minutes at 95 °C, there are 35 cycles, comprising 94°C for 45 sec, 56°C for 45 sec and 72°C for 1 minute, followed by a final extension at 72°C for 7 minutes. For the second amplification reaction, 2 µL from the first reaction was used as a DNA template and

added to a reaction mix identical to the first with the exception that BSA had been omitted and secondary primers were used. The reaction conditions were also the same as in the first reaction; with the exception that it consisted of 40 cycles with an annealing temperature of 58°C. Amplified products were detected by UV trans-illumination after electrophoretic size separation on 1% agarose gels. Samples considered *Cryptosporidium* positives were those that resulted in bands around 840 bp. According to Xiao et al. (2001) it varies between 826-864 bp. The marker used was GeneRuler 1kb Plus DNA Ladder and the positive control was obtained from a previous MoBio DNA extraction of a *C. parvum* faecal sample.

ß-giardin P434 (P1) and ß-giardin P434 (H3) primers specific for *Giardia* spp.*,* assemblage A and *Giardia* spp.*,* assemblage B respectively were used and adopted from Guy et al. (2003). The qPCR assay was performed using CFX96 Touch^{TM} Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA). Briefly, the reaction mixture contained Maxima SYBR Green/ROX qPCR Master Mix (12.5 µL), primers (0.4 µM each, 1 µL each) and 5 µL DNA template (10 to 10^7 GC per PCR) in a final volume of 25 µL. A mixture of all PCR reagents containing 5 µL sterile Milli-Q® water instead of the DNA template in each qPCR assay served as a negative control (Millipore, Billerica, MA, USA). qPCR amplification protocol for the targeted genes consists of initial denaturation for 5 min at 95 °C, followed by 40 cycles of two steps consisting of 15 secs at 95 °C and 1 min at 60 °C. The fluorescence signals were measured at the end of each annealing/extension combined step. At the completion of PCR amplification, melting temperature (Tm) analysis of the products was performed. This was done by reducing the temperature to 65ºC and then heating to 95ºC at a rate of 0.2 1C/S. Fluorescence was monitored continuously to confirm amplification specificity. The Tm peaks were automatically generated by the CFX Manager™ Software v3.1 and calculated based on the initial fluorescence curve (F/T). This was done by plotting the negative derivative of fluorescence over temperature versus temperature (dF/dT versus T). The successful qPCR amplification or the contamination of qPCR was monitored by using two positive DNA controls and two negative DNA controls (PCR water), respectively. The samples were considered positive or negative for *Giardia* Assemblage A or *Giardia* Assemblage B on the basis of amplification and melt curve. The sample was considered negative if the fluorescent signal did not increase within 40 cycles.

3.3.3.1 Sanger sequencing of wastewater samples positive for Cryptosporidium through nested PCR

Samples considered either positive or borderline positive by showing bands of appropriate size (around 840 bp) after the nested PCR, amplified product had been loaded onto the 1% agarose gel and subjected to gel electrophoresis. Nine of the samples showing clear bands on the agarose gel were selected for Sanger sequencing.

3.3.3.2 Purification of product through ExoSap and Sanger Sequencing

From each of the nine sample, 5 µL of product was mixed with 0.5 µL Exonuclease I (Exo I, 20 u/µL) and 1 µL SAP (Fermentas FastAP Thermosensitive Alkaline Phosphatese 1u/µL) and incubated at 37°C for 15 minutes, followed by 85°C for an additional 15 min. The purified PCR-product (2 µL) was added to a sequencing mix consisting of 1.0 µL BD stock, 0.5 µL 5X buffer and 1.5 µL of forward internal primer (5pmol/ µL), with a total reaction volume of 5 µL. The product was amplified in 9800 Fast Thermal Cycler for 25 cycles of 96°C for 10 seconds, 50°C for 10 seconds and 60°C for 30 seconds. It was subsequently cleaned with Montage Seq96 Sequence Reaction Cleanup kit (Merck Millipore, Billerica, United States) according to the user guide (Millipore, 2016) before sequencing in forward direction with 3130xl genetic analyzer (Applied Biosystems, Foster City, United States). Base calling was successful for all samples apart from one influent sample (Sample MR3). Data was generated in AB1 file format. Due to the complexity of the sample material, a large amount of background base calls was seen, and the sequences were interpreted with caution (See also section 4.4 for further background).

3.4 Collection and extraction of (oo)cysts from vegetable samples

Extraction of *Cryptosporidium* and *Giardia* from vegetable samples was performed according to the method described by (Nguyen et al., 2016) and (Cook et al., 2006) with some modifications. About 250 g of randomly selected spinach consisting of stems and leaves were collected with a sterile plastic bag from a farm. This farm is situated near WWTP B and the farmers use the effluent from the plant for irrigation. The 250 g sample was stored in a cooling box and transported to the laboratory for the analysis of protozoan parasites. In the laboratory,

a 30 g portion of vegetables (stems and leaves) was weighed in a plastic weighing boat. The sample was soaked in 200 mL of 0.01% TWEEN80 in distilled water. The samples were subsequently shaken on the orbital shaker for 1 hour and the liquid phase separated by centrifugation at 2500 ×-g for 10 min and *Cryptosporidium* and *Giardia* concentrated by immunomagnetic separation (IMS) (See further in section 3.4.1).

3.4.1 Immunomagnetic separation

The supernatants from each sample were decanted into clean glass beakers, leaving approximately 1 mL fluid above the pellets. These were transferred into the Leighton tube using Pasteur pipette. The centrifuge tubes were rinsed with approximately 3 mL of the decanted supernatant and then added to the Leighton tube containing the sample. This washing procedure was repeated until the volume in the Leighton tube was approximately 10 mL. The pellet was resuspended by vortexing, prior to IMS. Immunomagnetic separation was done to separate *Cryptosporidium* and *Giardia* from the concentrated debris using Dynabead GC Combo Kit (GC-Combo Dynabeads kit, Life Technologies AS, Norway), according to the manufacturer's instructions. The final suspension comprising of 50 µL was pipetted onto a microscope well slide. The sample slides were left to dry at room temperature.

3.4.2 Immunofluorescence staining

Air-dried concentrates were fixed in absolute methanol for 10 min. This enhances (oo)cyst attachment onto microscope slides, before being stained with a fluorescein isothiocyanate (FITC)-conjugated anti-*G. lamblia a*nd anti-*C. parvum* monoclonal antibodies to stain the cell wall of *Cryptosporidium* oocyst and *Giardia* cysts using Aqua-Glo G/C Kit for 30 minutes at 37^{o}C and 4′6-diamidino-2-phenyl indole (DAPI).

3.4.3 Microscopy

The slides were examined, and (oo)cysts were visualised by the use of an epifluorescence microscope, using an Axio Carl Zeiss epifluorescence microscope (Carl Ziess, RSA). All assessments of the presence of fluorescent nuclei and internal morphology were taken at either ×40 or ×100 magnification. All (oo)cysts in each well were counted.

3.5 Effect of chlorine and UV on *Cryptosporidium* and *Giardia* viability assessed with vital dyes

This pilot experiment was aimed at assessing the efficiency of chlorine and ultraviolet (UV) irradiation in the inactivation of viable *Cryptosporidium* and *Giardia* in contaminated water. The contaminated water contained a specific spiked amount of *Cryptosporidium* and *Giardia*.

3.5.1 Purification of oocysts

Isolation and purification of oocysts were done from fresh raw wastewater effluent using the glucose gradient centrifugation technique and flotation in glucose-NaCl solution (Maddox-Hyttel et al., 2006). Samples were filtered through metal mesh-sieves 100 µm to remove debris and large particles, and later centrifuged at 3000 x g for 10 min. The sediments were suspended in PBS-Tween 80. The specific gravity of the flotation fluid was 1.07 g/mL. The flotation fluid was prepared by adding 50 g of glucose per 100 mL saturated NaCl solution and mixing thoroughly. Purified water (150 mL) (such as Milli-Q or Super-Q) was added and mixed thoroughly until the solution was homogenous and transparent. Flotation fluid was carefully added by inserting a borosilicate glass Pasteur pipette into the same tube containing the sample so that the tip rests against the bottom of the tube. This would allow it to rise from below with a transparent layer with the sample material (mixed with PBS-Tween 80) above it. The tubes were centrifuged at 750 rpm for 10 min. Thereafter, supernatant above the layer of flotation fluid and the pellet was transferred into a new tube. After that, the sample was washed by filling the tube up with purified water and then centrifuged at 3000 rpm for 10 minutes. The supernatant was aspirated down to 5 mL. The samples were then washed two more times (3 x 10 min centrifugation in total) or more depending on how dirty the sample was. The final volume was 5 mL of purified (oo)cysts suspension that was then left in the tube which was vortexed and stored in the fridge at 4°C for future use.

3.5.2 Chlorine treatment of (oo)cysts

Spiked solutions were prepared with wastewater and distilled water. The distilled water represents the control for the experiment. The water samples (both distilled water and wastewater) were chlorinated using HTH (High test hypochlorite 65% chlorine) at concentrations of 0.5ppm, 2ppm and 5ppm. The chlorine and UV experiments were performed

according to the methods described by Wu et al. (2016) with modifications. In a 50-mL polyethylene bottle containing the chlorinated distilled water (control) or wastewater, a portion (1mL) of purified (oo)cysts suspension, approximately 74,600 oocysts per mL were added to each bottle and kept in the dark for adsorption at varying time intervals (15mins, 30mins, 60mins and 120mins). The samples were collected at different time intervals, and the chlorine reaction was quenched with sodium thiosulphate ($Na_2S_2O_3$) at a concentration ratio of $Na_2S_2O_3$ to chlorine of 4:1 (Elmolla and Chaudhuri, 2010, Qin et al., 2014). The same amount of (oo)cysts spiked in chlorinated distilled water and wastewater was added to the controls test (untreated), which was incubated and processed the same way as the chlorine treated bottles were. This aimed at determining the concentration of (oo)cysts that may be present in the sample (distilled water and wastewater) before spiking, that might contribute to an increase in concentration of the spiked samples, particularly in the wastewater. The untreated control was also run to determine the extent of permeability shown by the (oo)cysts to the stains. The pH and total dissolved solid of the spiked samples (distilled water and wastewater) were measured after filtration and before spiking with the respective (oo)cysts. The pH was 7.6 and 6.8 respectively. The total dissolved solid (TDS) was 35.5 mg/L and 250 mg/L. The wastewater used was clear to the naked eye though its turbidity was not measured.

Distilled water and wastewater samples, at different chlorine concentrations (0.5ppm, 1ppm, 2ppm, 5ppm) were spiked with (oo)cysts suspension and tested at various time intervals (15, 30, 60, and 120min) for chlorine residual. An iodometric method was used for the residual chlorine test. The starch-iodide titration was used to monitor the concentration level of the chlorine during the reaction time (Wu et al., 2016). Sodium thiosulphate ($Na_2S_2O_3$) titration was used against the residual chlorine concentration in the sample. The volume of $Na_2S_2O_3$ consumed was used to calculate the concentration of residual chlorine in the sample with equation (2).

$$\text{Residual chlorine} = \frac{\text{volume of } Na_2S_2O_3 \times \text{normality of } Na_2S_2O_3 \times 34.45 \times 1000}{\text{volume of sample taken}} \qquad (2)$$

For the UV experiments, both samples (distilled water and wastewater) were exposed to UV irradiation. A portion (1mL) of purified (oo)cysts suspension, approximately 74,600 oocysts

per mL was added to each bottle containing distilled water or wastewater. The experimental bottles used were 50-mL polyethylene bottles, round and transparent (Nasser, 2016) with the height and diameter of 91 mm and 32 mm respectively. The surface area exposed to UV was 804 mm^2. The experimental bottles were placed under a medium pressure mercury lamp (Heraeus GPH 212T5L/4, 10 W) and the temperature was maintained in a controlled water bath (model: D-91126, Schwabach FRG, Germany) at 25(±0.2) ^oC. The samples were collected at different time intervals; 10 sec, 20 sec, 40 sec, 80 sec and 160 sec corresponding to the UV doses of 5.2mJ/cm^2, 10.4 mJ/cm^2, 20.8 mJ/cm^2, 41.6 mJ/cm^2 and 83.2 mJ/cm^2 respectively. Controls without UV application were incubated and processed the same way as the UV treated bottles. The reduction in viability was evaluated according to Pereira et al. (2008) by comparison between the viability of the oocysts exposed to disinfectants and oocysts in the control experiment. The oocysts in the control experiment are considered 100% viable. UV doses were measured in millijoules per cm^2 (mJ/cm^2) and were calculated as follows in equation (3):

$$UV\ dose = \left(\frac{I}{UVT}\right) x\ t \qquad (3)$$

Where, I = Intensity measured in milliwatts per cm^2 (mW/cm^2), UVT = UV-Transmittance, T = Exposure time (t) (seconds)

UV dose is the product of UV light intensity and time. Dose is sometimes referred to as fluence. DOSE is equal to Intensity x Time= millijoules/(sec)(cm^2) x time= mJ/cm^2. Therefore, the UV fluence was calculated as in equation (4) (Rosenfeldt et al., 2006, Tian et al., 2014, Yuan et al., 2009).

$$UV\ dose = UV\ intensity\left(\frac{mj}{(\text{sec})(cm^2)}\right) x\ time(\text{sec}) = mJ/cm^2 \qquad (4)$$

3.5.3 Viability Test

The viability of the (oo)cyst after treatment with chlorine and UV was assessed and determined using vital dye Syto-9+Propidium iodide, and with the inclusion of DAPI+ Propidium iodide staining. Stained (oo)cysts were counted with a flow cytometer and epifluorescence microscope. The nucleic acid dyes were supplied by Molecular Probes (Eugene, OR, U.S.A.). Live/Dead viability kit (L-34856) for flow cytometry and Live/Dead viability kit (L-7012) for microscopy and quantification assays were used twice (double-staining). The methodology

used is explained in the next sections similar to what is obtainable in the literature with minor modifications (Ramo et al., 2017, Nasser et al., 2012, Sroka et al., 2013, Vesey et al., 1994, Montemayor et al., 2005, USEPA, 2012).

3.5.4 Staining procedure with Syto-9 and PI

The Live/Dead BacLight Viability assay utilizes mixtures of SYTO® 9 green fluorescent nucleic acid stain and the red fluorescent nucleic acid stain, propidium iodide. According to an explanation given by Netuschil et al. (2014), the two stains vary both in their spectral characteristics and their ability to enter healthy bacterial cells. The Syto-9 stain labels bacteria with both intact and damaged membranes when used alone. Whereas, propidium iodide permeates only bacteria with damaged membranes, competing with the Syto-9 stain for nucleic acid binding sites when both dyes are present. When mixed in recommended proportions, Syto-9 stain and propidium iodide produce green fluorescent staining of bacteria with intact cell membranes and red fluorescent staining of bacteria with damaged membranes (Netuschil et al., 2014).

Viability of the (oo)cysts were determined by double-staining of (oo)cysts by using Live/Dead BacLight kit (Invitrogen) according to Sroka et al. (2013) adapted from Taghi-Kilani et al. (1996) with minor modifications. The Live/Dead BacLight kit was removed from -20^{o}C dark storage and allowed to thaw at room temperature. Equal volumes of the two reagents (SYTO9 and propidium iodide) were combined in a 1.5 mL Eppendorf tube and mixed. The tube was wrapped with aluminium foil in order to avoid light penetration into the tube containing the staining solution. Three µL of this mixture were then added to 97µL of DW, containing 2×10^4 oocysts. After incubation at 37 °C for 60 min, 10 µL of aliquots were placed on a slide and microscopical observations were made. Samples were observed under an Axiolab Zeiss fluorescence microscope, using either 400X or 1000X magnification. Colour and intensity of live (dark green) and dead (bright red or orange/yellow) (oo)cysts staining were visually assessed. Another aliquot from staining solution 100 µL was in the fluidic sample tube and analysed with flow cytometer. A green filter block was used to examine the Syto-9 and PI (propidium iodide) stained (oo)cysts. Non-viable (oo)cysts were fluorescent red, while viable (oo)cysts were fluorescent green.

3.5.5 Reagent quality and performance check of the fluorogenic dyes

Method performance of the dyes was checked to confirm if the specific dye was staining what they were meant to stain and penetrate into. This was to assess the usefulness of these dyes as indicators of *Cryptosporidium* oocysts and *Giardia* cysts. These stains vary both in their spectral characteristics and in their ability to penetrate into the cells. Therefore, samples were also analysed separately for each stain (DAPI only, PI only and Syto-9 ONLY) along with all samples analysed with DAPI+PI and Syto-9 + PI. This was performed to compare and to establish performance criteria of each stain in order to determine if the results of analyses from each stain was correct.

3.5.6 Staining procedure DAPI and PI

Incubation of oocysts with DAPI and PI was performed according to the protocols described by Campbell et al. (1992). Working solutions of DAPI (2 mg/mL in absolute methanol) and PI (1 mg/mL in 0.1 M PBS, pH 7.2) were prepared and stored at 4°C in the dark. 100 µl of test suspension containing approximately 60.1 x 10^3 to 74.6 x 10^3 were incubated simultaneously with 10 µl of DAPI working solution and 10 µl of PI working solution at 37°C for 2h. (Oo)cysts were viewed by fluorescence microscopy and also analysed by flow cytometry.

3.5.7 Microscopy

Ten-microliter aliquots of oocyst suspension was viewed under an epifluorescence microscope equipped with a UV filter block (365-nm excitation, 445-nm emission) for DAPI and a green filter block (500-nm excitation, 630-nm emission) for PI. Images were captured using a Zeiss AxioCam MRc (Carl Zeiss, Germany) camera and image quantification was carried out using the Zeiss AxioVision Release 4.6 (12-2006) imaging software. Proportions of ruptured (ghost), PI-positive (PI+), DAPI positive/PI positive (DAPI+PI+), DAPI-positive/PI-negative (DAPI+ PI-), DAPI negative PI-negative (DAPI- PI-) oocysts were quantified in each sample.

An epifluorescence microscope was used at either 400× or 1000× magnification to examine all samples. *Cryptosporidium* oocysts were defined as being spherical in shape, 4-7 µm in diameter, with a surface fold sometimes visible, highly retractile with phase contrast, and with sporozoites or cytoplasm visible with DIC. *Giardia* cysts were defined as oval or spherical in

shape, 6-16 µm in diameter, highly retractile with phase contrast, with diagnostic internal structures (trophozoite nucleii) (Vesey et al., 1994).

3.5.8 Flow Cytometry

The flow cytometry analysis was performed on FACSCalibur BD Biosciences, Sydney standard model, with three PMTs equipped with standard filters (FL1: LP 695/40 nm; FL2: LP 585/42 nm; FL3: LP 488/10 nm; FL4: LP 780/60 nm; FL5: LP 616/23 nm FL6: LP 530/30 nm). The machine was fitted with an argon ion laser operating at 488 nm Argon Laser and with cell Quest Pro software (version 4.0.2, BD Biosciences, Sydney). Acquisition settings were defined using a no stained sample (autofluorescence), adjusting the PMTs voltage to the first logarithmic (log) decade. Instrument controls followed standard procedures adapted from Barbosa et al. (2008). Sheath fluid consisted of 2.0 mM potassium phosphate buffer (pH 6.8). The detectors used were forward angle light scatter (FALS) and side-angle light scatter (SALS).

The instruments were calibrated daily with Coulter ImmunoCheck fluorescent beads, according to the manufacturer's instructions. The laser output, high voltage, and gains of the detectors were adjusted to obtain the tightest populations of purified oocysts and cysts with maximum separation from background debris in samples. To determine what was represented by each population that appeared on the Log FSC vs Log SSC scatter-plot, cell sorting was performed, and samples of each population sorted on to microscope slides were examined microscopically (Vesey et al., 1994).

SECTION B: Methods for the detection of *Cryptosporidium* and *Giardia*: From microscopy to nucleic acid-based tools in clinical and environmental regimes (Review paper publication I).

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Review

Methods for the detection of *Cryptosporidium* and *Giardia*: From microscopy to nucleic acid based tools in clinical and environmental regimes

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ABSTRACT

The detection and characterization of genotypes and sub genotypes of *Cryptosporidium* and *Giardia* is essential for their enumeration, surveillance, prevention, and control. Different diagnostic methods are available for the analysis of *Cryptosporidium* and *Giardia* including conventional phenotypic tools that face major limitations in the specific diagnosis of these protozoan parasites. The substantial advancement in the development of genetic signature based molecular tools for the quantification, diagnosis and genetic variation analysis has increased the understanding of the epidemiology and preventive measures of related infections. The conventional methods such as microscopy, antibody and enzyme based approaches, offer better detection results when combined with advanced molecular methods. Gene based approaches increase the precision of identification, for example, many signatures detected in environmental matrices represent species/genotype that are not infectious to humans.

This review summarizes the available methods and the advantages and limitations of advance detection techniques like nucleic acid-based approaches for the detection of viable oocysts and cysts of *Cryptosporidium* and *Giardia* along with the conventional and widely accepted detection techniques like microscopy, antibody and enzyme based ones. This technical article also encourages the wide application of molecular methods in genetic characterization of distinct species of *Cryptosporidium* and *Giardia*, to adopt necessary preventive measures with reliable identification and mapping the source of contamination.

1. Introduction

Cryptosporidium and *Giardia* are the most commonly occurring enteric protozoan parasites, responsible for gastrointestinal disorders that may lead to nutritional imbalances and severe health problems, particularly among children in developing countries (Thompson and Ash, 2016). They could cause chronic and debilitating illness in immune-compromised individuals (Carmena et al., 2012). Their environmental transmission poses significant risks to human health and these protozoan parasites have repeatedly been recovered in surface waters (Ehsan et al., 2015; Chuah et al., 2016; Kumar et al., 2016) and wastewater (Sroka et al., 2013; Ma et al., 2016). The resistance against chlorine disinfection of *Cryptosporidium* and *Giardia* is a major public health challenge for the water industry (Carmena et al., 2012). *Cryptosporidium* transmitted through water has been the cause of multiple diarrhoeal outbreaks in the United States, Sweden, and United Kingdom as well as in both developed and developing countries (Insulander et al., 2005; Ignatius et al., 2012; Samie and Ntekele, 2014). *Giardia* additionally infects humans and animals and has been reported to be responsible for 2.8×10^8 cases of intestinal infections per annum worldwide (Thompson 2004; Squire and Ryan, 2017).

The specific diagnosis and genetic characterization of *Cryptosporidium* and *Giardia* is a pre-requisite to understand the associated epidemiological risks and is necessary to trace the variants present in a particular population (Thompson and Ash, 2016). Traditional methods based on microscopy, antibodies and enzymes have limitations in the specific diagnosis of protozoan parasites (see detailed reviews by Jex et al., 2008; Koehler et al., 2014). Therefore, further development occurs targeting both speed, sensitivity and specificity, since the conventional methods in addition may be tedious, costly and partly non-species specific. Traditionally, the diagnosis of *Cryptosporidium* and *Giardia* is mainly based on the detection of the typical morphological characteristics of oocysts/cysts in stool specimens, either by using acid-fast staining (Garcia et al., 1983) or by an indirect immunofluorescence assay procedure with monoclonal or polyclonal antibodies (Shams et al., 2016). These traditional methods are used frequently in developing countries, but lack of discrimination between distinct species based on morphology and or host occurrence. This affects the

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comparative potential between regions with a need to apply novel discriminatory and rapid analytical methods for fast and accurate identification of these protozoan parasites adaptable both for developed and developing regions and representative for both clinical and environmental samples.

A wider application and advancement of molecular methods would be especially valuable in developing countries, where the health impact due to local poor sanitation and hygiene is of concern. Based on previously published literature, most studies done in developing regions (for example, African countries), were based on conventional techniques for the detection of parasitic protozoans, except a few where advanced molecular methods have been adapted often through cooperation with partners from industrialized countries.

In this review, different methods in general with their pros and cons, their application in developing regions is exemplified but not limited to the African continent.

In African countries, at least 13 species of *Cryptosporidium* and five *G. duodenalis* assemblages in humans have been identified with additional genotypes in animals (Squire and Ryan 2017). Detailed description of different genotypes of *Cryptosporidium* and *Giardia* and their epidemiology and prevalence in the human population, among animals, and in water reservoirs have been described in a recent review by Squire and Ryan, 2017. The most prominent genotypes of these parasitic protozoans are summarized in Table 1 and the most commonly used technologies adopted to detect *Cryptosporidium* and *Giardia,* on the African continent are summarized in Table 2.

According to reports provided by the Global Enteric Multicenter Study (GEMS), *Cryptosporidium* is second only to rotavirus as a causative agent of diarrheal disease during the first 5 years of life in Sub-Saharan Africa (Kotloff et al., 2013). Its impact is seconded by Sow et al. (2016), who estimated that 2.9 million cases related to *Cryptosporidium* infections occurs in children (< 24 months age) in Sub-Saharan Africa. These *Cryptosporidium* attributable infections were found to be associated with mortality (< 2 fold increase) in children in the age group between 12 to 23 months (Kotloff et al., 2013).

In this article, the most commonly used techniques (selected based on published reports) involving microscopy, immunology and advance nucleic acid based approaches are addressed, highlighting their advantages and disadvantages. The review briefly describes the strategies adopted for the detection of viable *Cryptosporidium* ooysts and *Giardia* cysts and emphasizes the wide application of molecular methods for genetic characterization of *Cryptosporidium* and *Giardia* to provide suitable preventative measures.

It was evident from these studies that the conventional methods were mainly used, while the use of advanced molecular tools is applied partly in cooperation with partners outside the African continent. Still the implementation to increase sensitivity, specificity and genetic characterization of the targeted parasitic protozoa is not widespread. Investigations of their occurrence in environmental samples are limited.

2. Conventional techniques

2.1. Microscopic methods

The microscopy based methods have been extensively used for the detection of *Cryptosporidium* oocysts and *Giardia* cysts in environmental water, food, faecal and/or tissue samples (Quintero-Betancourt et al., 2003; Soares and Tasca, 2016). The morphological features of both, but especially in the case of *Cryptosporidium* cannot form the base for identification and differentiation using light microscopy (Fall et al., 2003). Therefore, different staining procedures have been used to aid in the clear distinction of especially *Cryptosporidium* oocysts from co-existing protists and for excluding similarities from the environmental or faecal debris.

2.1.1. Conventional staining methods for the detection of Cryptosporidium and Giardia

Different staining methods such as the traditional Ziehl-Neelsen, “negative staining technique” of Heine, Kinyoun's acid-fast technique, and less traditional applications such as trichome stain of different fluorescent-based stains such as auramine phenol and Safranin methylene blue staining (SMB) have been used for the detection of *Cryptosporidium* (Vohra et al., 2012). Acid-fast staining is usually the method of choice for the clinical microbiology laboratories. Some studies have incorporated dimethyl sulfoxide as a modification of producing oocysts with a brilliant pink to fuchsia colour against a pale green background (Vohra et al., 2012). Variability in stain uptake may occur both due to the stain itself and the age of the oocysts after prolonged

Table 1
Cryptosporidium and *Giardia* species prominent in African Continent (adapted and modified from Zahedi et al., 2015, Thompson and Ash 2016, and Squire and Ryan 2017).

Cryptosporidium Species	Host	Reported in Africa
C. parvum	*Bos taurus* (Cattle)	commonly reported in humans, but also reported in animals
C. hominis	*Homo sapiens* (Human)	most common species in humans but also reported in animals
C. meleagridis	*Meleagris gallopavo* (Turkey)	commonly reported in humans but also reported in animals
C. bovis	*Bos taurus*(Cattle)	reported in animals with rare reports of occurrence in humans
C. viatorum	*Homo sapiens* (Human)	reported in humans
C. felis	*Felis catis* (Cat)	reported in animals with rare reports of occurrence in humans
C. canis	*Canis familiaris* (Dog)	commonly reported in animals but also reported in humans
C. xiaoi	*Ovis aries* (Sheep)	reported in animals with rare reports of occurrence in humans
C. muris	*Mus musculus* (House mouse)	reported mainly in animals but also reported in humans
C. suis	*Sus scrofa* (Pig)	reported mainly in animals but also reported in humans
C. andersoni	*Bos taurus*(Cattle)	reported both in humans and animals
C. cuniculus	European rabbit	reported mainly in animals but also reported in humans
C. ubiquitum	*Bos taurus*(Cattle)	commonly reported in humans and animals
C. ryanae	*Bos taurus*(Cattle)	reported in animals
C. erinacei	*Erinaceus europaeus*(European hedgehog)	reported in animals with rare reports of occurrence in humans
C. baileyi	*Gallus gallus domesticus*(Chicken)	reported in animals

Giardia species	Host	Reported in Africa
G. duodenalis Assemblage A	Human other animals	Animals/humans
G. duodenalis Assemblage B		Animals/humans
G. duodenalis Assemblage C	Dogs and other canids	Animals/humans
G. duodenalis Assemblage D	Dogs and other canids	Animals
G. duodenalis Assemblage E	Cattle and other hoofed animals	Animals/humans
G. duodenalis Assemblage F	Cats	reported in animals with rare reports of occurrence in humans

Table 2
Techniques adopted in the African continent to determine the prevalence of protozoan parasites.

Country	Techniques	References
Niger	Direct stool examination, Ritchie & Ziehl-Neelsen technique	Gay-Andrieu et al. (2007)
Chad Central Africa	Light microscopy using direct smear in lugol's solution	Korzeniewski and Bochniak (2011)
Sierra Leone	DMSO modified Acid-Fast and Trichrome-PLUS stain	Gbakima et al. (2007)
Maputo, Mozambique	Direct microscopic observation using Ritchie's concentration technique	Fonseca et al. (2014)
Guinea-Bissau	Microscopic examination	Centeno-Lima et al. (2013)
Malawi	Microscopic methods and PCR-RFLP	Morse et al. (2007)
Southern Ethiopia	Microscopic analysis	Abossie and Seid (2014)
Ethiopia	Culture based method and microscopic examination	Gebreyesus et al. (2014)
Ethiopia	Direct microscopy of the smear in saline and Lugol's iodine for parasite detection	Ramos et al. (2014)
Gambia	Immunofluorescence Microscopic Analysis, Fecal Neopterin, Lactulose Mannitol Permeability	Campbell et al. (2004)
Sudan	Microscopy	Saeed et al. (2015)
Nigeria	PCR, Restriction fragment length polymorphism analysis and DNA sequencing	Ayinmode et al. (2012)
Uganda	Direct light microscopy, PCR and sequencing	Ankarklev et al. (2012)
Senegal	Ziehl-Neelsen modified staining, ELISA using the "*Cryptosporidium* Antigen Detection Microwell ELISA kit	Faye et al. (2013)
Tanzania	Direct smear and formol-ether concentration method	Barda et al. (2014)
Zimbabwe	Laboratory based methods	Simango and Mutikani (2004)
Madagascar	Modified Ziehl–Neelsen method and examined, by bright-field microscopy and PCR	Areeshi et al. (2008)
Madagascar	Basic laboratory method and PCR	Randremanana et al. (2012)
Rwanda	Immunochromatographic assay in comparison with light microscopy and PCR	Ignatius et al. (2014)
Rwanda	qPCR	Kabayiza et al. (2014)
Swaziland	Anti-*Cryptosporidium* immunofluorescence and nuclear staining technique (after formal-ether concentration	Dlamini et al. (2005)
Kenya	Direct saline and formal-ether-sedimentation techniques	Obala et al. (2013)
Democratic Republic of São Tomé and Príncipe	Parasitological and molecular methods	Lobo et al. (2014)
Zambia	Duplicate Kato-Katz thick smears immunofluorescence kit	Siwila et al. (2010)
Ghana	Conventional culture, polymerase chain reaction	Opintan et al. (2010)
Tetouan (Morocco)	PCR-RFLP	El Fatni et al. (2014)
Four province of South Africa	PCR-RFLP	Samra et al. (2013)
The Gambia, Mali, Mozambique, Kenya,	Individual commercial immunoassays	Kotloff et al. (2013)
Eastern Cape Province, South Africa	Formalin ethyl acetate concentration technique	Nxasana et al. (2013)
KwaZulu-Natal, SouthAfrica	Immunomagnetic separation and the ammonium bicarbonate protocol	Trönnberg et al. (2010)
Egypt	Copro-antigen RIDA®QUICK test and by real-time PCR	Helmy et al. (2014)
Egypt	Microscopic examination after modified Ziehl-Neelsen staining and detection of *Cryptosporidium* coproantigen by enzyme-linked immunosorbent assay	Mousa et al. (2014)
Egypt	Microscopically by direct smear method and after concentration using formol-ethyl acetate, multiplex qPCR	Nazeer et al. (2013)
Botswana	Culture based methods and microscopy	Rowe et al. (2010)

storage (Vohra et al., 2012). Although the modified Ziehl-Neelsen staining is the main technique for *Cryptosporidium* spp. by clinical laboratory technologists, it may lack in sensitivity and specificity (Chartier et al., 2002). Experienced analysts are required for the interpretation of the results and the procedure is time consuming (taking about 30–45 min per sample) (Vohra et al., 2012). *Giardia* cysts are not stained with traditional acid- fast staining procedure, however, with the modified Ziehl-Neelsen or Kinyoun white-light stains, cysts are stained with the methylene blue counterstain and can be identified in stained smears. While analysing *Cryptosporidium* in carbol fuschin/methylene blue stained smears of feces, the possible presence of *Giardia* cysts should be kept in mind (Harrington, 2008).

The negative staining technique of Heine is a simple, inexpensive, and efficient way of screening for *Cryptosporidium* spp. (Potters and Van Esbroeck, 2010; Khanna et al., 2014). This is a proficient way of screening for coccidian parasites of the intestinal tract in general and especially used for *Cryptosporidium*. Modifications in the original Heine staining technique have been attempted to increase the sensitivity by staining stool smears separately using malachite green, methylene blue and crystal violet, in place of carbol fuchsin (Khanna et al., 2014). The most successful improvements were with malachite green and crystal violet, which enhanced the refractivity of the structures the most, while methylene blue was less suitable and reduced the refractivity (Khanna et al., 2014).

The modified Heine staining technique, using malachite green, is a practical, safe, and sensitive method to detect (oo) cysts in stool specimens. It further enhances the ease with which they can be differentiated from yeasts and is more suitable for diagnosis by less experienced microbiological analysts. The sensitivity of this technique can be further increased by phase-contrast microscopy and/or examination at 400× magnification (Khanna et al., 2014). Advantages and disadvantages with the conventional staining methods for the detection of *Cryptosporidium* are shortly summarized in Table 3.

For the detection of *Giardia*, simple stains that have been used includes iodine (Smith and Paget, 2007), iron-haematoxylin (Garcia, 2007), Giemsa (Wolfe, 1990) and trichrome (Thornton et al., 1983). *Giardia* cysts can be concentrated by various methods such as formalin-ether or formalin-ethyl acetate (Smith and Paget, 2007).

2.1.2. Other available staining methods for the detection of Cryptosporidium and Giardia

Malachite green is considered as a safe and sensitive stain for detecting *Cryptosporidium* (oo) cysts in stool specimens. Yeasts will not take up the stain to the same extent, which reduce false positive diagnosis (Shams et al., 2016). Similarly, negative staining with nigrosin does not stain yeasts either (Shams et al., 2016). Negative staining can be used for initial screening of slides, which later can be confirmed if necessary by the modified Ziehl-Neelsen staining or other classical techniques (Potters and Van Esbroeck, 2010). Giemsa staining has been used, but proven problematic due to decolourization and poor colour disparity, which necessitates oil-immersion microscopy (Shams et al., 2016). Brar et al. (2016) suggested the easy use of Leishman's stain in the diagnosis of *Cryptosporidium*, but this stain is less readily available. *Giardia* trophozoites and cysts can also be examined by direct wet

Table 3
Comparison of commonly used conventional staining methods.

Types of technique	Advantages	Disadvantages	Comments	Reference
Modified Ziehl-Neelsen	Simple, low cost, suitable for screening substantial number of samples Due to the permanent stain, uncertain or confusing positive sample can possibly be confirmed.	Variability in stain uptake may occur, related to the stain itself and the age of the oocysts after storage	Traditional well proven method generally available in most clinical laboratories both in developed and developing countries The modified Ziehl-Neelsen stained smears shows 70–78% sensitivity and specificity respectively	Vohra et al. (2012) Shams et al. (2016)
Negative staining technique of Heine	It is simpler, cheaper and needs less time than the modified Ziehl-Neelsen staining	Thickness of the smear badly affects the visibility of the protozoan parasites. [a]Inferior results are caused by Köhler illumination.	The sensitivity of this technique can be increased by using phase-contrast microscopy or examination at 400X magnification	Vohra et al. (2012) Khanna et al. (2014)
Dimethyl Modified Acid-fast staining sulfoxide	It is a versatile and widely applicable, reliable, fast and simple method	Lack of specificity	Formalin-fixation may be used with acid-fast stains.	Vohra et al. (2012)

[a] In this process, only the straight rays of light are allowed to pass through the optical system. This can block rays of light and can cause the negatively stained oocysts to lose their refractivity.

mount microscopy.

2.2. Immunological assays

Immunological methods offer several advantages over light microscopy in terms of sensitivity and specificity for the detection of *Cryptosporidium* oocysts and *Giardia* cysts in diverse types of samples (Jex et al., 2008; Koehler et al., 2014). Direct fluorescent-antibody (DFA/DIF), Immunofluorescence assays (IFA), combined with immunomagnetic separations (IMS) and fluorescent-monoclonal-antibody detection have all been used frequently for the detection of these parasitic protozoans (Jex et al., 2008; Koehler et al., 2014).

2.2.1. Immunofluorescence assay utilizing immunomagnetic separation purification

Immunomagnetic separation is a purification procedure that in the current application with *Cryptosporidium* and/or *Giardia* uses microscopic, magnetically responsive particles coated with an antibody targeted to react with these specific pathogens.

The beads are mixed in test samples to bind *Cryptosporidium* and/or *Giardia*, and after a magnetic field is applied, beads and the associated sorbed cysts or oocyst can be isolated (Lai, 2009). These pathogens are selectively removed from other debris using the magnetic field, and further analysed by different immunofluorescence assays, based on preferences.

This method is frequently used for the isolation and detection in environmental samples like surface water and wastewater. In the USEPA method (USEPA, 2012), a cartridge is used for filtration of initial large volume water samples. The eluted concentrate is exposed, and the cysts/oocysts fished out by immunomagnetic separation. The final counts are done with immunofluorescence microscopy, where *Cryptosporidium* and *Giardia* can further be stained for viability assessment using 4′,6-diamidino-2-phenylindole (DAPI) and viewed using differential interference contrast (DIC) microscopy (USEPA, 2012). Even though there are high recovery efficiencies [62–100% reported by Rochelle et al. (1999)] and precision with this technique, the antibody-based method as well as others do not provide species or genotype identification. The inability to assess the infectivity of the quantified organism, is another limitation of the assay (Allen et al., 2000; Simmons et al., 2001; Brescia et al., 2009).

Immunomagnetic separation and immunofluorescence assays have been criticized for being time-consuming, labour-intensive, expensive and for requiring a high level of analytical expertise (LeChevallier et al., 2003). However, the application is much better than traditional methods and their application is far more precise.

2.2.2. Direct and indirect immunofluorescence assay

DFA tests based on fluorescein-labelled antibodies directed against cell wall antigens of *Giardia* cysts and *Cryptosporidium* oocysts allow visualization of the intact parasites, providing a definitive diagnosis in faecal samples (Johnston et al., 2003). Commercial DFA tests are available, such as the widely used MERIFLUOR® *Cryptosporidium/Giardia* test from Meridian Biosciences, which is more sensitive than conventional staining techniques and is easy to perform (Johnston et al., 2003). This test produces excellent specificity, improved sensitivity, takes less time and requires less skill. Additionally, the high quality of the reagents results in minimal background fluorescence or nonspecific staining and enhances identification (Vohra et al., 2012). The sensitivity and specificity of the most commonly used commercial DFA test, the MERIFLUOR DFA has been reported to be in range of 96–100% and 99.8–100%, for *Giardia* and *Cryptosporidium* respectively. This test has a better sensitivity than traditional examination of permanent smears for *Giardia* and *Cryptosporidium* (Traub et al., 2009). DIF has a high positive predictive value (PP) (90.7%) when used to test human samples, which indicates that this test may be used to diagnose or exclude *Giardia* infections without the need for three consecutive

stool samples to improve the diagnostic sensitivity of microscopic examination (El-Nahas et al., 2013). DIF showed 100% sensitivity and specificity in human validation studies and has been used as a reference standard in several other studies (El-Nahas et al., 2013). The high sensitivity and specificity makes DIF ideal for diagnostic verification when an infection is suspected, but the causative agent cannot be demonstrated, even if more expensive fluorescent microscopes are required (El-Nahas et al., 2013).

Indirect immunofluorescence makes use of two antibodies and the primary antibody in this case is unconjugated, whereas, the second fluorophore-conjugated antibody is used for acting against the primary antibody for the detection of the targeted pathogen. The differences between direct and indirect immunofluorescence include the shorter time utilized in direct immunofluorescence since it only requires one tagging step and further non-specific binding is also reduced due to the use of conjugated primary antibodies. At the same time, samples comprising of endogenous immunoglobulins, are expected to showcase high background with indirect methods.

2.2.3. Fluorescent-monoclonal-antibody detection

Antibodies are widely used to target and label specifically extra- or intracellular antigens within cells and tissues (Bauer, 2014). Monoclonal antibody (mAbs) based methods can be used with high specificity in (oo)cyst detection (Lai, 2009). When different classes of mAbs (IgG1, IgG3, and IgM) were used for environmental monitoring of *Cryptosporidium,* their results revealed that staining with IgG1 antibodies generally produced fewer unwanted fluorescent particles than staining with IgG3 and IgM antibodies (Lai, 2009). It was also reported that IgG1 antibodies had high affinity and specificity towards (oo)cysts in water concentrates (Lai, 2009). However, mAbs with higher avidity and specificity to oocysts in water concentrates have significantly improved their detection and enumeration (Lai, 2009). Monoclonal and polyclonal antibodies were compared for their abilities to react with *Giardia* cysts and *Cryptosporidium* oocysts after storage in environmental samples and some other tested media (Rose et al., 1989). Three monoclonal antibodies against *Cryptosporidium* and two monoclonal antibodies and a polyclonal one directed against *Giardia* cysts were evaluated and no significant difference in antibodies was found in preliminary studies in environmental waters (Rose et al., 1989). mAbs-based immunofluorescence assays have also proven to be valuable in detecting (oo) cysts in fecal samples in addition to the water concentrates. When compared to enzyme linked immuno sorbent Assay (ELISA) it was confirmed that fluorescent monoclonal methods increased the sensitivity and specificity of *Giardia/Cryptosporidium* (oo)cyst detection and hence provide excellent screening techniques and offer useful data for epidemiological studies, and consequently control of the parasites (Omoruyi et al., 2014). The examples of commonly used antibody-based/microscopic staining methods have been presented in Table 4.

2.2.4. Vital dyes used in microscopy to address the viability of Cryptosporidium and Giardia

Vital dyes are fluorogenic, which makes them amenable to the IFA technique and microscopy. They can be useful for evaluating the viability of small numbers of oocysts found in environmental samples (Campbell et al., 1992). The use of propidium iodide (PI) (Campbell et al., 1992; Bukhari et al., 2000) and other nucleic acid dyes, such as SYTO-9 (Lai, 2009) may indicate viability through cell-wall integrity. The vital dye detection methods are applicable to individual cysts/oocysts and provides the ability for the identification of these in water and environmental samples (Campbell et al., 1992). Moreover, incorporation of PI staining (as a counterstain) with IFA detection helps in improving detection and viability determination of the cells. However, the vital dye method is not directly correlated with the infective potential of cysts/oocysts since these can be damaged to an extent that they cannot initiate or maintain infection, but is apparently intact and with dye permeability patterns identical to that of infective ones (Roberston and Gjerde, 2007). An overrating will thus result.

2.3. Enzyme-linked immunosorbent assays

Enzyme immunoassays (EIAs) use antibodies for the qualitative detection of *Giardia* and *Cryptosporidium* specific antigens in preserved stool specimens (Johnston et al., 2003; Elgun and Koltas, 2011). However, the use of an enzyme based immunoassay does not exclude the need to analyse multiple stool specimens for sensitive detection of *Giardia* (Elsafi et al., 2013). These tests are more sensitive than conventional staining techniques and are easier to perform as well as more economical with shorter detection times. ELISA method was first developed in the 1970s using chromogenic substrates and has been used extensively thereafter among immunoassays methods. The ELISA assay can be particularly useful in laboratories not accustomed to diagnosing *Giardia* or *Cryptosporidium* regularly in epidemiologic studies as well as in need of diagnostic standardization. The drawbacks include the cost involved and the occurrence of false- positive results (Vohra et al., 2012).

In general, most of the immunoassays are not sensitive for environmental samples due to the poor recoveries and insufficient recognition of targeted antigen by various antibodies. This is due to complex matrices present in environmental water. Thus, interpretation of environmental data based upon immunoassays must be made with the limitations of these methods in mind.

2.3.1. Antigen detection tests

Rapid antigen detection assays that are more precise, simple and cost-effective offer a relevant alternative for routine examination (Shams et al., 2016). These assays are superior to microscopic examination (Elgun and Koltas, 2011) but require multiple reagent additions, washing steps and incubations. Moreover, the newer immune-chromatographic technology provides additional diagnostic options (El-Moamly and El-Sweify, 2012). However, despite their advantages, there are reports claiming that these rapid immune-chromatographic assays are not as sensitive as the classic immunoassays and can have higher rates of false-positives (El-Moamly and El-Sweify, 2012).

2.3.2. ImmunoCard STAT

This commercial immunoassay developed by Meridian Biosciences, Inc have been reported to be successful in detecting *Cryptosporidium* and *Giardia.* Most of the commercially available immunoassays are based on the enzyme immunoassay format, requiring addition of many reagents as well as incubation- and washing steps (see also Section 2.3.1). Alternatively, ImmunoCard STAT is a non-enzymatic rapid immunoassay capable of distinguishing between these two organisms in one quick test (Sadaka et al., 2015). ImmunoCard STAT has proven to be simple and time saving and can be used as a rapid investigation tests for Giardiasis and Cryptosporidiosis or even integrated with other diagnostic profiles to confirm the diagnosis (Elsafi et al., 2013). The assay is easy to use, without the high expertise needed to perform or interpret its results (El-Moamly and El-Sweify, 2012). It was also reported that no confirmation may be needed for this assay because of the high positive predictive value (Elsafi et al., 2013).

In addition, non-enzymatic rapid immunoassays as ImmunoCard STAT for the detection of *Giardia* and *Cryptosporidium* antigens in aqueous extracts of fecal specimens was developed. The assay simultaneously distinguishes between the two organisms with one rapid test (Garcia et al., 2003).

2.4. Cell culture immunofluorescence assay

The cell culture immunofluorescence assay (CC-IFA) detection can directly visualize and quantify infections by enumerating the number of infectious foci that develop on each monolayer of a receptive cell-culture seeded with the parasitic protozoa (Johnson et al., 2011).

Table 4
Examples of commonly used Antibody-based/Microscopic staining methods.

[a]Types of technique	Advantages	Disadvantages	Comments	Reference
Immunomagnetic separation (IMS) coupled to IFA	Less debris and better clarification of samples when compared to methods using flotation	Cannot determine the viability or infectivity of detected oocysts and cysts	IMS, coupled with an IFA increases recovery rates for oocysts	USEPA (2012)
		Method is very sensitive, but IMS is too cumbersome and costly to use on large numbers of samples in labs with limited resources		Manser et al. (2013)
Fluorescent-monoclonal-antibody	Less background from staining of sections and cells.	Requires skilled operators and expensive equipment.	Short fluorescent decay times have an impact on the sensitivity of detection	Lai (2009)
	Specifically detecting one target epitope, which means that they are less likely to cross-react with non-target proteins.			
Direct immunofluorescence assay	Provide a definitive analysis. This test has a higher sensitivity than traditional examination of permanent smears.	Cross reactivity might be a problem- often difficult to develop the monoclonal antibody that will work the best	99.8 to 100% sensitivity and specificity for *Cryptosporidium* reported by using DFA test and MERIFLUOR DFA test	Shams et al. (2016)
Enzyme immunoassays (EIA)	Does not depend on microscopy skills	Cross-reactivity might occur with the secondary antibody, resulting in nonspecific signal.	Although potentially useful as a screening test, further development and refinement of the method is required before it could replace microscopy in the diagnosis of *Cryptosporidium* and *Giardia.*	Vohra et al. (2012)
	Highly sensitive and specific and is useful for testing large numbers of specimens	Cost and multiple step procedure may be limiting factors for the EIA use		El-Moamly and El-Sweify (2012)
Immuno-chromatographic lateral-flow immunoassays	Eliminate the need for trained microscopists.	Not reliable as routine methods.	The rapid assays for both *Giardia* and *Cryptospori- dium* have become popular diagnostic tools. More precise, rapid, simple and cost-effective modality	Johnston et al. (2003)
	No need of costly equipment examination, can be completed in 10 min rather than the 1–2 h required to perform DFA tests or EIAs.			Vohra et al. (2012)
				Elsafi et al. (2013)
Rapid immunoassays (ImmunoCard STAT)	Simple and time saving.	Interpretation of the ImmunoCard STAT results from the Specimens could sometimes be problematic because of the low intensity of the bands produced in the test	Beneficial in everyday diagnosis and assessment for *Giardia* and *Cryptosporidium*	El-Moamly and El-Sweify (2012)
	Can be used for rapid screening tests for Giardiasis and cryptosporidiosis.	Not as sensitive as the classic immunoassays and can have higher rates of false-positives.		Johnston et al. (2003)
	Does not need special training or expensive equipment			

[a] IMS: Immunomagnetic separation, EIA: Enzyme immunoassays and DFA/DIF: Direct immunofluorescence assay.

Importantly, both freshly confluent and aged HCT-8 cell (human ileocecal adenocarcinoma) monolayers (see also Section 2.4.1) can be used for the CC-IFA, making it logistically feasible for water quality and utility laboratories to measure the viable fraction (Sifuentes and Di Giovanni, 2007). CC-IFA has not been widely used by the water industry, although it generates the lowest number of false positives when tested with inactivated oocysts and have a detection limit of 1–3 oocysts per monolayer (Johnson et al., 2011). It also performed better than the other assays with oocysts recovered from spiked filters using Method 1623 (USEPA, 2005). CC-IFA accurately predicted the number of infectious oocysts in blind coded samples of purified oocyst suspensions (Bukhari et al., 2007).

2.4.1. Tissue culture infectivity

Most detection methods do not measure if cysts/oocysts are viable or infectious (Lai, 2009). The tissue culture method is used to study the organism in an environment simulating the *in vivo* situation, but does not require the use of animal models (Woods et al., 1995; Yang et al., 1996). This method is based on the use of cell lines, such as HCT-8 or Caco-2, which are very susceptible to infection to low levels of *Cryptosporidium* oocysts (LeChevallier et al., 2003). The HCT-8 cell line has the advantage of being a robust cell culture that is highly permissive to *Cryptosporidium* infections (LeChevallier et al., 2003). Moreover, it has been successfully used for determining the infectivity of *Cryptosporidium* oocysts in water (Slifko et al., 1997). However, it has been reported that the method was not capable of determining the infectivity when *Cryptosporidium* oocysts were present in low counts in the samples (Lai, 2009). In addition, oocysts must be isolated and cleaned of any fecal residue before infectivity test. The factors such as oocyst age, animal source, oocyst collection and purification techniques may affect oocyst viability (Coulliette et al., 2006). Although different studies suggested that the tissue culture method can determine oocyst viability, it is not a practical method for oocyst detection in water samples due to the limitation factors as stated above.

2.5. Flow cytometry, basic principle and application

Flow cytometry has the advantages of automation, objectivity, and can analyze many thousands of cells per second. The flow cytometry quantitatively measures the optical characteristics of cells (or other particles) as they are presented in a single file in front of a focused light beam. In measurements, the cells are introduced into the flow cell into a fast-flowing fluid stream termed the sheath flow. The starting point of the flow is a jet of isotonic sheath fluid (which is about 100 µm in diameter) that travels at roughly 20 km h^{-1} into which samples infuse at a controlled rate, typically between 10 and 60 µL min^{-1}. The slower moving sample is hydro-dynamically focused at the center of this rapid flow stream where it creates the flow of cells which moves in a single file into the cytometer. It is claimed that this method would be beneficial in the *in-vivo* evaluation of possible anti-cryptosporidial agents, where sensitive detection of treatment-persuaded parasite load variation is required (Shams et al., 2016). *Cryptosporidium* oocysts and *Giardia* cysts as isolated particles, can be detected by flow cytometry if labelled with a suitable fluorescent label. Analyses of the stool samples from persistently infected mice confirmed that the flow cytometry method was almost 10 times more sensitive than conventional immunofluorescent assay (Shams et al., 2016).

An optimized FC protocol for *Giardia* provides an accurate, fast, simple and automated detection method for clinical diagnosis and water analysis. Furthermore, identification of *Giardia* cysts was facilitated in a flow cytometer due to an improved staining of cysts caused by exposure of epitopes to the monoclonal antibody in suspension (El-Nahas et al., 2013). Another advantage is that larger volumes of samples can be analyzed, providing a more accurate description of the samples compared to fluorescence microscopic examination (El-Nahas et al., 2013).

A flow cytometric detection approach along with IMS was used for the detection of *Giardia* cysts in spiked water samples and a 90% recovery, was reported (Keserue et al., 2011).

Flow cytometry has been coupled with qPCR to quantitate the concentrations of different pathogens along with *Cryptosporidium* and *Giardia* in wastewater and its receiving river water (Wang et al., 2016). The authors suggested that the used strategy can be employed to access the wastewater treatment efficiency and its ecological impact on receiving surface water.

3. Nucleic acid based detection methods

Nucleic acid based detection of pathogens offers potential advantages over conventional methodologies. They may provide an accurate, sensitive and robust identification of parasitic protozoans in environmental samples. Their advantages are:

i) The identification of the host-specificity of the recovered cysts and oocysts strains/species. The further potential risks largely depend on the mode of transmission, whether it is zoonotic or anthroponotic.
ii) The identification of the specific source and severity of infections in epidemiological surveillance studies, and most importantly, the species genotyping for employing preventive measures, predominantly in the case of any outbreak.

A wide range of molecular methods have been used extensively for the detection and genotyping of *Cryptosporidium* and *Giardia* species in environmental samples, like:

- Fluorescence *in situ* hybridization (FISH) (relying on the specific *in situ* hybridization of nucleic acid probes to particular genetic loci within *Cryptosporidium/Giardia* cysts and oocysts),
- Polymerase Chain reaction (PCR) and
- Quantitative polymerase chain reaction (qPCR) (amplifying gene fragments from small amounts of genomic DNA).

These methods performed well in the detection of genetic variation within protozoan parasite populations from diverse types of samples, such as environmental water and faecal samples. The potential benefit of nucleic acid-based methods are high throughput rates, increased precision and reliability. The most commonly used molecular methods are included in the following sections.

3.1. Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) is based on the hybridization of fluorescently labelled oligonucleotide or probes to the specific region in the DNA or RNA of targeted organism. The FISH technique has been considered as a powerful tool for phylogenetic, ecological, diagnostic and environmental studies in microbiology. This method can help in revealing the mechanisms of survival and infection at the cellular level. The method, based on species specific probes, has been applied for the detection of *Cryptosporidium* and *Giardia* (oo)cysts in environmental samples (Lemos et al., 2005; Graczyk et al., 2008). rRNA provides a potential target for nucleic acid probes, as elevated levels of rNRA is found in viable cells of the targeted organism (Smith et al., 2004). In case of *Giardia* spp., most FISH assays targeted the variable region of the small subunit (SSU) of the nuclear ribosomal RNA (rRNA) gene (Lemos et al., 2005; Bednarska et al., 2007). The use of FISH and MAbs for species-specific identification and viability of *Giardia* spp., and *Cryptosporidium* spp., (*C. parvum*, and *C. hominis*) in human fecal and water supply samples was also reported (Lemos et al., 2005). Enrichment steps are frequently required, (either cultural pre-enrichment or a magnetic bead based enrichment or both) to enhance sensitivity (Girones et al., 2010). Moreover, FISH has the advantages of detecting and identifying pathogenic *Cryptosporidium* species in clinical,

water and environmental samples within a 3-h time frame. Therefore, it is a dependable alternative to PCR and RFLP analyses. FISH probes have an additional advantage over mAbs because they provide an indication of viability and are species specific. FISH can well distinguish between the two-major *Cryptosporidium* species specifically involved in human infections (Vohra et al., 2012). According to Smith et al. (2004), the use of FISH to determine the viability of *C. parvum* oocysts is complicated because rRNA from viable cells may be degraded during sample processing, thus producing false-negative FISH results. Moreover, extended preservation of rRNA in nonviable oocysts, either through insufficient RNase activity or non-permeabilizing inactivation conditions may produce false-positive FISH results.

3.2. Polymerase chain reaction, nested PCR, PCR restricted fragment length polymorphism

The polymerase chain reaction and advanced quantitative polymerase chain reactions (qPCRs) are powerful molecular tools that can amplify the gene sequence corresponding to the desired target to multiple detectable copies. PCR offers several advantages over conventional methods and has been used extensively for the genotyping of *Cryptosporidium* and *Giardia*. PCR based assays has been adopted frequently for the detection of *Cryptosporidium* and *Giardia* in diverse types of environmental samples (Guy et al., 2003; Castro-Hermida et al., 2015; Nguyen et al., 2016). PCR is automated and includes large quantity processing. The cost of PCR could easily be reduced with a few modifications to reduce hands-on time, such as the use of 96-well plates for PCR setup and amplification. A non-homologous internal modification could easily be constructed with commercially available kits such as the PCR mimic construction kit (Clontech, Palo Alto, Calif.) which would result in the amplification of a different-sized band with the *Cryptosporidium* diagnostic primers. This internal control could then be used as a tool for monitoring the success of each PCR and would also reduce the assay to a one-tube test, thereby rendering it more cost-effective (Vohra et al., 2012). The advantage of this method is the sensitivity of the technique over conventional methods and that genotyping can be performed. The limitations include, lack of standardised methods, cost involved and expensive instrumentation. Even though, PCR is a preferred method in analysing environmental samples for the detection of both *Giardia* and *Cryptosporidium*, its application needs attention in relation to the increased possible false positives (Elsafi et al., 2013). In addition to the wide application of PCR, nested PCR have been used to increase sensitivity (Nikaeen et al., 2005; Castro-Hermida et al., 2015). Nested PCR uses two sets of primers like in standard PCR reaction, where the first primer set binds to sequences outside the target DNA and region amplified serves as a template for the second pair of primers. The specificity of the reaction is increased, because if there is nonspecific binding of the first pair of primers, it is very unlikely that the second set of primers will also bind to the non-specifically amplified DNA template.

Nested PCR has been used to determine *Giardia* and *Cryptosporidium* spp., in surface water, water catchments and wastewater samples (Monis and Saint 2001, Osaki et al., 2013; Prystajecky et al., 2014; Ulloa-Stanojlovic et al., 2016). PCR-based restriction fragment length polymorphism (PCR-RFLP) techniques utilizing specific primer pairs for the selective amplification of different genetic loci, followed by enzymatic cleavage or sequencing, have been used to characterize and classify *Cryptosporidium* and *Giardia* species or "genotypes (Xiao et al., 2001; Coupe et al., 2006; Almeida et al., 2010).

3.3. Quantitative PCR

Quantitative PCR (qPCR) has been used extensively in detection and quantification of pathogens in environmental as well as clinical samples (Singh et al., 2010; Hanabara and Ueda, 2016; Singh et al., 2016). qPCR allows the real-time monitoring of DNA amplification and offers practical advantages over conventional PCR. Compared to the end point analysis of conventional PCR, quantification of genetic targets over a wide dynamic range is one of the advantages of qPCR.

There exists a proportional relationship between threshold cycle termed as C_T and the initial copies of the target gene. A standard cure generated with a known amount of gene copies is utilized to compare the C_T value of unknown sample for estimating the accurate gene copy numbers of the targeted gene (Jex et al., 2008). The increased sensitivity and specificity, shorter time and less labour make qPCR a preferred choice for the detection of pathogens. Different qPCR chemistries (Molecular Beacon probes, Taqman probes, Scorpian probes, FRET probes and intercalating dyes like SYBR Green) alone or in combination with other high throughput techniques have been used widely for the detection of pathogens like bacteria and viruses (Singh et al., 2010; Aw and Gin 2010; Singh et al., 2012, 2016, 2017a). The qPCR has been used for the determination and quantification of *Giardia* cysts and *Cryptosporidium* oocysts in environmental samples (Guy et al., 2003; Staggs et al., 2013; Nguyen et al., 2016).

qPCR assays have previously been described to target the SSU rRNA gene to detect all members of the *Cryptosporidium* genus (Hadfield et al., 2011). The amplified SSU rRNA region covers the major region of interspecies/genotype variability in the gene, allowing identification of nearly all *Cryptosporidium* spp. and genotypes through sequence analysis of the PCR product (Hadfield et al., 2011).

A multiplex TaqMan-probe based qPCR assay was developed for the quantification of *Cryptosporidium* (COWP gene) and *Giardia* (β-giardin gene) in environmental waters (Guy et al., 2003). The developed assays have been adopted by many researchers for the quantification of these parasitic protozoans.

A comparative study involving ten qPCR assays was reported, for detection of *Cryptosporidium* species in which 6 qPCRs were already published and 4 were designed by Staggs et. al., 2013. It was found that *Cryptosporidium* assay targeting 18S rRNA gene was able to detect all *Cryptosporidium* species with some cross amplification of *T.gondii*, algae and dinoflagellate (Staggs et al., 2013).

Fluorescence resonance energy transfer (FRET) probes and melt curve analysis have been used for the identification of *Cryptosporidium* oocysts (Li et al., 2015). Three qPCR assays based on the small-subunit (SSU) rRNA gene (18S-LC1 and 18S-LC2 assays) and *hsp90* gene were used. It was found that the one of 18S-LC1 assay targeting (SSU) rRNA gene and *hsp90* assays could differentiate between common human pathogenic species such as *C. hominis, C. meleagridis* and *C. parvum* (Li et al., 2015). The 18S-LC2 assay could differentiate non-pathogenic species (for example, *C. andersoni*) from human-pathogenic species commonly found in source water. Different genetic targets used for the detection of *Cryptosporidium* and *Giardia* (irrespective of the types of samples reported) are summarized in Tables 5 and 6.

3.4. Viability strategies based up on quantitative reverse transcriptase PCR and qPCR coupled with propidium monoazide dye

The viability of pathogens is a crucial factor to assess potential health risk. Described viability tests for *Cryptosporidium* and *Giardia* can be differentiated as follows; i) animal infectivity, ii) cell culture, iii) *in vivo* excystation, iv) vital dye staining, v) qRT-PCR and vi) fluorescence *in situ* hybridization (FISH).

Quantitative PCR and qRT-PCR have an elevated level of sensitivity and specificity. These methods are rapid, cost-effective and produce substantial information on the presence, quantity and distribution of viable pathogens in water and food. For successful PCR reactions, good quality DNA is a prerequisite and DNA extraction from environmental matrices is a challenge due to presence of PCR inhibitors. Different DNA extraction methods followed by viable PCR/qPCR/qRT-PCR have been described in a few studies.

One example is a qRT-PCR study that was developed to detect viable *Giardia intestinalis* cysts, *Cryptosporidium* spp. and *Toxoplasma gondii*

Table 5
Genetic targets used to detect *Cryptosporidium* oocysts through molecular methods in environmental and other types of samples.

Sample Type	Methods Used	Genetic targets	References
Environmental Water Samples and Sewage	qPCR primers and TaqMan probe	*Cryptosporidium* oocyst wall protein (COWP) gene	Guy et al. (2003)
Environmental Soil and Vegetables	qPCR and DNA sequencing.	*C.parvum SWI2/SNF2 ATPase*, Rad16 ortholog gene	Hong et al. (2014)
Drinking Water treatment plants	Nested PCR,	Small subunit (SSU) of nuclear ribosomal RNA (18s rRNA)	Castro-Hermida et al. (2015)
Human Samples	Nested PCR	60 kDa glycoprotein (gp60)	Rahmouni et al. (2014)
Human Samples	PCR-RFLP	Microsatellite locus 1 (ML1) and Microsatellite locus 2 (ML2)	Hunter et al. (2007)
Human Samples	PCR	Thrombospondin-related attachment protein (TRAP) genes	Mojarad et al. (2011)
Human samples	PCR RFLP	70 kDa heat shock protein (hsp70) gene	Gobet and Toze (2001)
Human environmental and animal samples	Nested PCR RFLP	90-kDa heat shock protein (Hsp90) genes	Feng and Xiao (2011)
Calf fecal samples	Real time PCR	Actin gene	Homem et al. (2012)
Fecal samples from humans	PCR-coupled high-resolution melting-curve (HRM) analysis method	Second internal transcribed spacer of nuclear ribosomal DNA (ITS-2)	Pangasa et al. (2009)

oocysts (Travaillé et al., 2016). A mechanical rupture of (oo)cysts followed by mRNA extraction and subsequent amplification targeting *β-giardin, hsp70* and *SporoSAG* for the detection of *Giardia, Cryptosporidium* and *T. gondii* respectively, showed that the qRT-PCR, could detect viable, but not infectious parasites compared to *in vivo* methods as references (Travaillé et al., 2016).

A TaqMan reverse transcription-PCR was used to detect viable *Cryptosporidium* oocysts in soil samples (Liang and Keeley, 2011). In this study, different types of mRNA extraction methods were compared for the detection of *Cryptosporidium* oocysts from different types of soil. Among all methods examined, the PowerSoil kit based method had the following limits of detection (LOD) for *Cryptosporidium* oocysts per gram of 1.5×10^2 (sandy soil), 1.5×10^4 (clay) and 1.5×10^3 (loamy soil) (Liang and Keeley, 2011). The obtained sensitivity was however not judged as sufficient for risk based assessments. Nam and Lee (2010) developed a duplex qRT-PCR based on the *hsp* 70 mRNA gene for the detection of *Cryptosporidium* and *Giardia* oocysts and cysts respectively. In this method, viable oocysts (10/100 µL) and *Giardia* cysts in the range of 10^3/100 µL were detected. The detection sensitivity was further improved by heat shock stimulation and resulted in 10^0 and 10^1 (oo) cysts/100 µL respectively.

Nucleic acid binding dyes based methods, for example, use of propidium monoazide (PMA) and ethidium monoazide (EMA) dyes to address viability has gained a lot of attention (Nocker et al., 2006, 2007; Singh et al., 2013; Alonso et al., 2014; Ma et al., 2016). Propidium monoazide is a membrane-impairment dye that penetrates only membrane-damaged cells. Once inside the cell, PMA covalently cross-links to DNA upon photoactivation, and PCR amplification is strongly inhibited. PMA dye was used in combination with qPCR for detecting viable *Cryptosporidium* oocysts (Brescia et al., 2009). This CryptoPMA-PCR assay could detect viable oocysts in surface water spiked with *Cryptosporidium* oocysts. This proof of concept study suggested that the CryptoPMA-PCR assay could be a potential method for the detection of *Cryptosporidium* oocysts in water.

To differentiate between viable populations of *Cryptosporidium* and *Giardia* from non-viable ones (in this case, heat killed) in wastewater, PMA qPCR was employed (Alonso et al., 2014).

In PMA-qPCR for *Giardia*, three gene targets were considered, β-giardin (amplicon length: 75 bp), triosephosphate isomerase (*tpi*) (amplicon length: 75 bp), and glutamate dehydrogenase (*GDH*) (amplicon length: 75 bp) and it was found that DNA from dead cysts were not amplified with large product size of primers targeting *Giardia* genes. In case of *Cryptosporidium*, a 150-bp COWP assay could quantify viable oocysts, and failed to amplify non-viable ones. It was further reported that both qPCR assay coupled with PMA dye could detect *Cryptosporidium* and *Giardia* oocysts and cysts in the range of $< 10^3$ oo (cysts)/L in wastewater samples. PMA based qPCR was also used for viability assessment of *Cryptosporidium* and *Giardia* in wastewater samples in China (Ma et al., 2016).

Liang and Keeley (2012) compared qPCR coupled with PMA (*hsp 70* gene) to reverse transcription (RT)-qPCR (*hsp70* mRNA) in oocysts spiked water samples. Two disinfection methods involving ammonia or hydrogen peroxide were used to evaluate the efficacy of both approaches to address the viability of *Cryptosporidium*. In the case of ammonia disinfection, qPCR-PMA (DNA based) was found to be inconsistent, possibly due to the presence of solid particles in the samples that will further reduce the activation of dye. However, qPCR- PMA (DNA based) and (RT)-qPCR (*hsp70* mRNA based) were found to be consistent in the detection of viable oocysts when hydrogen peroxide was used as disinfectant, which alludes to better reproducibility of mRNA based methods.

3.5. Digital polymerase chain reaction

The absolute quantification of pathogens by qPCR in environmental samples is challenging. Firstly, the generation of standard curves are often a troublesome process and lack of reference standard curves confounds inter laboratory comparison of qPCR results. Secondly, the use of qPCR in quantifying genetic signatures representing different pathogens in complex environmental matrices is quite challenging

Table 6
Genetic targets used to detect *Giardia* cysts through molecular methods in environmental and other types of samples.

Sample Type	Methods Used	Genetic targets	References
Human samples	PCR-RFLP	small subunit rRNA	Hawash et al. (2016)
Natural Water	PCR	*β-giardin* gene	Adamska (2015)
Human, animals, samples	PCR	5.8 S gene and Internal transcribed spacers ribosomal geneITS1 and ITS2	Caccio et al. (2010)
Wastewater	Nested PCR	Triosephosphate isomerase (TPI)	Sike et al. (2011)
Surface Water	Nested PCR	glutamate dehydrogenase GDH	Mahmoudi et al. (2013)
Surface water and drinking water	Loop-mediated isothermal amplification	elongation factor 1 alpha (EF1α) gene	Koloren and Ayaz (2016)

(Sidstedt et al., 2015). Smaller quantities of PCR inhibitors may delay the threshold cycle (Cq) value which leads to the underestimation of the template copy number (Sidstedt et al., 2015). The development of the relatively new, digital polymerase chain reaction has the potential to overcome problems related to qPCR absolute quantification (Baker, 2012; Pavšič et al., 2016; Cao et al., 2017). In digital PCR, a sample is diluted and partitioned into a mass of compartments so that each contains one or zero copies of the sequence of interest (Baker, 2012; Cao et al., 2017) prior to PCR amplification. The exact number of copies of target DNA in samples can be determined by counting positive versus negative partitions. However, even under the best conditions (sufficient partitioning of samples), the probability of having more than one molecule per reaction, always exist (Majumdar et al., 2015; Debski and Garstecki, 2016). Two types of digital PCR platforms have been used i) Digital PCR on chips, ii) Digital PCR on droplets (Baker, 2012). Droplet digital PCR (ddPCR) based on water emulsions offers added advantages over qPCR and eliminates the need for external standard curves (Hindson et al., 2011; Pinheiro et al., 2012). Several studies have reported the utility of ddPCR in quantifying bacteria and virus together within different clinical and environmental samples (Kim et al., 2014; Lui and Tan, 2014; Rački et al., 2014; Devonshire et al., 2015; Zhao et al., 2016; Singh et al., 2017b) but its environmental applications have been very limited. Cao et al. (2016) have defined the use of digital PCR in water monitoring applications and sees it as a molecular method for field applications. The use of ddPCR has not been explored extensively to detect *Cryptosporidium* and *Giardia* and there is limited information available. Yang et al. (2014), compared ddPCR and qPCR for the quantification of *Cryptosporidium* targeting the 18S rRNA and actin gene. Different DNA template of *Cryptosporidium* was used to compare linearity, sensitivity, precision and cost of both methods. qPCR and ddPCR showed significant linearity and ddPCR precision was better than qPCR as measured by the relative standard deviation. ddPCR precision decreases with decrease in DNA concentrations while qPCR was unaffected by template concentration. It was also observed that ddPCR was less affected by the presence of PCR inhibitors but the overall cost of ddPCR was found to be twice that of qPCR. The authors advocated the use ddPCR for exact quantification of serial dilutions needed to prepare standard curve for qPCR. Zahedi et al. (2016) also used ddPCR to quantify the exact copy number of *Cryptosporidium* in standard dilutions of oocysts to be used in qPCR.

3.6. Loop mediated isothermal amplification

To improve PCR amplification and to reduce the challenges associated with residual co-amplification of non-specific sequences, a novel and simple method termed as loop mediated isothermal amplification (LAMP), was developed by Notomi et al. (2000). The method is driven by *Bst* DNA polymerase and a set of four carefully designed primers (inner primers and outer primers) that recognize a total of six distinct sequences on the target DNA was used. The LAMP method can amplify copious quantities of DNA with high specificity and sensitivity under isothermal conditions. It has been developed for different pathogens, including bacteria, viruses, fungi and protozoan parasites (Yoshida et al., 2005; Bao et al., 2014; Duan et al., 2014; Mahittikorn et al., 2015). The method was first used for *Cryptosporidium* by Karanis et al. (2007) and later for *Giardia* (Plutzer and Karanis, 2009; Plutzer et al., 2010).

Bakheit et al. (2008), compared LAMP methods targeting SAM-1, *hsp*, *gp60* genes with nested PCR targeting 18 small subunit rRNA gene for the detection of *Cryptosporidium* in different fecal samples originating from cattle, sheep and horses in South Africa. The fecal samples (one third) were found to be positive in the LAMP method, while all samples were found negative by nested PCR. The LAMP assay based on three genes could differentiate between *Cryptosporidium* species and results were further confirmed by sequencing. In this study, the LAMP method performed well in comparison to nested PCR for the detection of *Cryptosporidium* in fecal samples and proved its usefulness in detecting low levels of *Cryptosporidium* (Bakheit et al., 2008).

Cryptosporidium oocysts were detected in diverse types of water samples, for example, river water, well and fountain water through immunofluorescence based microscopy and further by LAMP and nested PCR (Koloren et al., 2011). As compared to immunofluorescence and nested PCR, the LAMP method proved to be more effective in the detection of *Cryptosporidium* oocysts (25.7% positive). In another study Koloren et al. (2013), used an adenosylmethionine synthetase (SAM) gene based LAMP method to assess *Cryptosporidium* spp. in sea and tap water samples. In comparison to the nested PCR (positive for 31% of samples), the LAMP method could detect *C. parvum*, *C. hominis* and *C. meleagridis* in 65.5% of the samples.

Cryptosporidium and *Giardia* were detected in surface water samples by utilizing immunofluorescence assay, PCR and LAMP (Mahmoudi et al., 2013). Approximately, 37.5% of surface water samples were positive for targeted protozoan parasites by one or the other techniques used. An interesting result was observed where the IFA negative samples for *Cryptosporidium* were found to be positive by the LAMP method. However, in the case of *Giardia*, both methods performed similarly. The authors suggested that the use of a combination of methods could be a better option for mapping water contaminated with these protozoan parasites (Mahmoudi et al., 2013).

4. Conclusion and future perspectives

The review provides a brief understanding of commonly used methods for the detection of *Cryptosporidium* and *Giardia* in clinical and environmental regimes. Advantages and disadvantages of different techniques, including, microscopy, and immunology based methods, flow cytometry and molecular tools have been discussed for the identification of *Cryptosporidium* and *Giardia* species. However, it was envisaged that selection of appropriate technique would be very important as per research objective and type of sample analysed. The detailed understanding of advanced molecular tools used for the detection and quantitation of *Cryptosporidium* and *Giardia* genotypes will provide opportunities to design and develop novel, rapid, diagnostic, and cost-effective intervention methods to reduce the risk to human health.

Besides, commonly used detection methods discussed in this review, the use of other molecular approaches such as genome wide sequencing and nanotechnology based biosensors will further open a new avenue to provide wider prospects for the identification and designing of appropriate genetic markers of these protists.

In the future, research should be expanded to less privileged countries, where cryptosporidiosis and giardiasis are prevalent in human populations and chance of zoonotic transmission of these parasitic protozoans is high. In developing countries, a variety of traditional methods are used frequently for the detection of these water borne protozoan parasites. However, applicability of advanced molecular methods is still in infancy and used partially in cooperation with several research partners outside the continent, due to insufficient research funding and lack of expertise. Therefore, global research collaboration programs integrating a consortium with expertise in this field should be established/promoted to disseminate the skills and bridging the knowledge gaps between developed and developing countries. Furthermore, an intensive program of scientific visits, exchange of students, and technical workshops will be highly appreciated and will be extremely helpful for researchers from developing countries to explore advanced methodologies and unravel the research questions in the field of detection of *Cryptosporidium* and *Giardia*.

Conflict of interest

No conflict of interest declared.

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CHAPTER 4: OCCURRENCE AND REDUCTION OF *CRYPTOSPORIDIUM* AND *GIARDIA* IN SELECTED WASTEWATER TREATMENT PLANTS IN DURBAN, SOUTH AFRICA

4.1 Introduction

Despite the advancement in water technology in many developed and developing countries, waterborne outbreaks are still a global threat. A lack of knowledge about the epidemiology and transmission dynamics of waterborne pathogens, especially from animals ranging within the catchments, leads to poor management practices for drinking water catchments (Smith and Nichols, 2010, Cummins et al., 2010). Oocysts of *Cryptosporidium* and cysts of *Giardia* reflect and relate to the level of faecal pollution from humans and animals or may be due to other anthropogenic activities in a catchment (Hansen and Ongerth, 1991, Robertson and Lim Ai Lian, 2011). This relates to their occurrence in different areas such as aquatic habitats, sludge, effluents from wastewater treatment plants (WWTPs) or direct faecal pollution from low cost or informal settlements in the developing areas of third world countries. Their occurrence in influents to wastewater treatment plants are a reflection of infected individuals or transmission of these pathogens among the connected population. Since the oocysts and cysts are environmentally robust and persistent in aquatic habitats, the efficiency of wastewater treatment will further reflect their occurrence in the effluents and be a determinant of further downstream exposure in recreational areas and drinking water intakes. The risk may be further exacerbated in food consumption if treated wastewater, highly polluted recipient water and/or biosolids are applied to agricultural lands as irrigation water or as part of fertilization (Sroka et al., 2013, Chalmers and Davies, 2010, Graczyk et al., 2008, Hachich et al., 2013).

Cryptosporidium and *Giardia* are further found in animals including mammals, reptiles, birds, amphibians and fishes (Rinaldi et al., 2012, Yang et al., 2015). These may serve as additional sources in the catchment or as part of the stormwater fraction entering wastewater treatment plants. Conventional treatment processes are not designed to completely remove protozoa from wastewater. In developed countries, the occurrence of *Cryptosporidium and Giardia* in raw wastewater may be expected to be in concentrations of over 13,000 oocysts/L and 1,000 cysts/L respectively (Taran-Benshoshan et al., 2015, Kitajima et al., 2014, Cacciò et al., 2003, McCuin and Clancy, 2006), but depends on country and socio-economic conditions, as is reflected in

Table 2.5. Efficiencies of (oo)cyst removal varying from 75.3 to 100% for *Giardia* and 40 to 100% for *Cryptosporidium* have been reported (Nasser, 2016, Nasser et al., 2012). Commonly used bacterial indicators of the hygienic quality of wastewater do not correlate with the concentration of these protozoa (Bonadonna et al., 2002, Keeley and Faulkner, 2008) even though *Escherichia coli* and enterococci has been used to increase the assurance of wastewater quality when monitoring for faecal pollution.

The aim of this chapter of the thesis was to investigate the prevalence of *Cryptosporidium* and *Giardia* at four municipal wastewater treatment plants (WWTPs) and receiving wastewaters in Durban, South Africa. This included investigating the correlations between *E coli*, Enterococci and protozoan parasites concentrations. The removal efficiency at each wastewater processing stage was evaluated, including the effect of their biological treatment and other treatment steps. Seasonal variations were addressed.

4.2 Materials Methods

4.2.1 Sampling sources and collection

Samples of untreated and treated wastewater were collected from four wastewater treatment plants (WWTPs) situated in Durban, a city situated in KwaZulu-Natal, South Africa. The characteristics of the selected WWTPs are summarized in Table 4.1. The treated effluent from WWTP A discharges to a maturation pond and further to the recipient river. The wastewater treatments (WWTPs C and D) share the same post chlorination and final effluent tanks after the treatment from separate clarifier tanks. Water samples were collected from different treatment steps (sampling points indicated with * in Table 4.1) at the plants. The samples are made up of a pooled number of subsamples to a final volume of 250 mL from influent and 2000 mL from the biological treatment step, secondary clarifier tank, post chlorination and effluents from all treatment plants. A total volume of 2000 mL was also collected from all river sampling points near WWTP A and WWTP B (Next to wastewater treatment plant (NWWTP), Upstream, Downstream and River Mouth). These are the only WWTPs that discharge directly into rivers in close proximity. This gives the opportunity to directly determine the impact of the wastewater effluents on the river quality.

The samples were collected monthly between 16th September 2015 to 29th April 2016 from WWTP A, 28th September 2015 to 8th March 2016 from WWTP B, 12th October 2015 to 25th April, 2016 from WWTP C and WWTP D. Samples, including river water samples that are connected to WWTP A and WWTP B, were processed and analysed according to the USEPA 1623.1 revised method (USEPA, 2012) and immunomagnetic separation applied (USEPA, 2012). Further, PCR and qPCR analysis were made (Methods described in sections 3.3.2 and 3.3.3) to confirm the presence of *Cryptosporidium* and to determine their genetic diversity in wastewater.

4.2.2 Characteristics of wastewater treatment plants used in the study

The treatment steps in the WWTPs include: an initial screening step (aimed at removal of large particles), a grit removal chamber, a trickling filter or aeration tank, and finally, clarifiers and post chlorination. In some instances, a maturation pond is used before the water is released to the recipient. The characteristics for the selected WWTPs are specified in Table 4.1.

WWTP A is designed to treat 3.1 Ml/d Average Dry Weather Flow (ADWF) but the plant is operating at 10% above capacity at 3.4 Ml/d Average Dry Weather Flow (ADWF). WWTP A comprises of two process steps. The first one is the biological nutrient removal (BNR) and the plant is of a UCT (University of Cape Town) process configuration (Wang et al., 2013) and is receiving its influent mainly from domestic wastes. This is followed by an extended aeration which is designed to treat 2.75 Ml/d ADWF of screened and degritted raw sewage (Ramdhani, 2012, Naidoo et al., 2002). The wastewater is then clarified, and the water is chlorinated in the chlorination tank after which it is released to the connected river as final effluent.

WWTP B has a design capacity of 18.80 ML/d, and operates at a level of 10.98 ML/d. The plant is a biological treatment plant, which is based on trickling filters with four primary settling tanks, six trickling filters, six secondary clarifiers and six 2000 m^3 anaerobic digesters (unheated and unmixed) for the primary and secondary sludge. Digested sludge is dried on 54 drying beds and stored onsite or disposed of by application on agricultural land. Samples were taken from the influent (the raw wastewater), effluent of the biological filters, effluent of the secondary clarifier and the final effluent after the chlorination tank.

The **WWTP C** and **D** are located in the same vicinity but treat wastewater from different sources. **WWTP C** receives an average of 2,000 m^3/d which is entirely domestic wastewater while **WWTP D** receives an average of 8,000 m^3/d wastewater of which about 30% is industrial and 70% domestic. Influents are pumped into equalisation tanks and screened before going through the grit chambers. From the grit chambers the water enters the aeration tanks followed by the clarification process. The effluent from the **WWTP C** is combined with the effluent from **WWTP D** and dosed together with chlorine before it is released into the recipient river (Mhlanga et al., 2009). Sampling was done at the influents after screening (the raw wastewater), effluent of aeration tank, the effluent of clarifier tanks and the effluent of the chlorination tank (final effluent).

Table 4.1: The characteristics of selected wastewater treatment plants

Plant	Type of plant	Population served	Primary treatment	Secondary treatment	Tertiary treatment (Disinfection	Design capacity (ML/d)	Operating Capacity (ML/d)	Composition
WWTP A	Domestic/Industrial	26,000	Screening, grift removal, SED	*AT, AS, *SC SED (settling)	*Chlorination	11.07	4.67	(80%) domestic and (20%) industrial
WWTP B	Domestic	73,500	Screening, grift removal, SED	*trickling filter (biological filter), AS, *SC and SED	*Chlorination	18.8	11	Receives 100% domestic wastewater
WWTP C	Domestic	29,000	Screening, grift removal, SED	*AT, AS, *SC, SED	*Chlorination	10.8	2	Receives an average of 2 ML/d which is entirely domestic wastewater
WWTP D	Domestic and Industrial	56,000	Screening, grift removal, SED	*AT, AS, *SC, SED	*Chlorination	14.8	8	8 ML/d wastewater of which about 30% is industrial and 70% domestic.

[a]ADWF: Average Dry Weather Flow; SED: Sedimentation; AS: Activated sludge; AT: Aeration tank; SC: Secondary clarifier, *: Sampling points
[b]Domestic wastewater: made up of toilet wastewater (black water) and greywater from kitchens, water from laundry (washing) and bathrooms. The quantity and concentration of the flow will depend on the socioeconomic behaviour of the population.
[c]Industrial wastewater: Wastewater discharges from industrial and commercial sources. It contains by-product of industrial or commercial activities.

4.3 Sample concentration and Immuno-magnetic separation

Samples were analysed according to the USEPA 1623.1 revised method (USEPA, 2012). The step by step details are given in section 3.3 in Chapter 3.

A 50 µL aliquot of the purified suspension containing the captured (oo)cysts was air-dried on a well-slide (Invitrogen Dynal AS, Oslo, Norway) and stained with fluorescein isothiocyanate (FITC)–conjugated *Giardia*/*Cryptosporidium* monoclonal antibody reagent using (Aqua-Glo G/C Kit, Invitrogen, USA) and 4', 6"-diamidino-2-phenylindole solution for 30 minutes at 37°C. The slides were examined by using an Axio Carl Zeiss epifluorescence microscope at 40X magnification (Carl Ziess, RSA). Colour seed correction factor was used to adjust the counts, Texas Red filter set (Zeiss filter set 00) was used to identify ColourSeeds (used as internal standards). The percent recovery varied between 33-77% for *Cryptosporidium* and 50-96% for *Giardia*. Further details of sampling collection processes and handling from different water sources are given in Section 3.3 of Chapter 3 of this thesis.

4.4 DNA extraction and genotyping of *Cryptosporidium* and *Giardia*

All the influent and final effluent wastewater samples collected were subjected to DNA extraction for molecular characterization. DNA was extracted from wastewater samples using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, incorporating a modification added to the protocol. The samples were subjected to ten cycles of freeze–thaw (− 80 °C for 10 minute and 56 °C for 5 minute), followed by nucleic acid extraction and purification using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). The quality and quantity of extracted DNA was analysed by Nanodrop Spectrophotomer (Nanodrop Technologies) for the PCR and quantitative PCR assay for the detection of *Giardia* and *Cryptosporidium*. Detailed molecular analysis and qPCR detection procedure of *Cryptosporidium* and *Giardia* specific genes in environmental samples is presented in the methodology in Chapter 3 (Section 3.3.2).

4.5 Statistical analysis

The data was entered in Microsoft Excel 2016 (Microsoft Corporation, California, USA) and summary statistics performed. The data was transformed to Log_{10} and presented in the tables and graphs were plotted to give better representation and ease of data presentation. The data was then checked for normality using the Shapiro-Wilk's Normality test, with a 95% confidence interval. The data was determined to be non-parametric based on the normality tests, therefore to compare three or more categories, such as the difference in oocysts/cysts concentration at the different treatment steps within each WWTP and between WWTPs, the Kruskal-Wallis test was chosen and to compare two categories, such as the difference in the oocysts/cysts concentration in the influent and effluent, the Mann-Whitney U-test was used to compare differences in concentrations of *Cryptosporidium* and *Giardia.* Spearman correlation was used to ascertain the relationship between the bacterial organisms (*E coli* and Enterococci) and oocysts and cysts of respectively.

4.6 Results and discussion

4.6.1 Bacteriological assessment of wastewater

It is important to have increased awareness and knowledge about the treatment processes efficiency in relation to microbiological contamination. As water is an essential resource; especially in Africa; for drinking, recreation and bathing, it is extremely important to maintain water quality that is acceptable in all water sources like ponds, rivers and lakes. Better understanding of bacteriological quality of treated and untreated wastewater is necessary. The *E coli* values and faecal enterococci quantities found in the influent samples were in the range of 10^5 to 10^6 MPN/100mL. These values are within a similar range as earlier reported in the literature. *E coli* influent concentration was in the range of 9.1 x 10^6 MPN/100mL to 14 x 10^6 MPN/100mL (Lucas et al., 2014) and between 10^5/100mL and 10^7/100mL for *E coli* in raw wastewater in other studies (Garcia-Armisen and Servais, 2007, Madoux-Humery et al., 2013). The bacteriological assessment (*E. coli* and Enterococci) was carried out and are compiled in Tables S1-S5 in Appendix 2. There was no recovery of faecal enterococci in final effluent from **WWTP A** water samples throughout the six-consecutive monthly sampling. *E coli* was only recovered in the final effluent of 2nd, 4th, 5th and the 6th month sampling. **WWTP B** effluent water samples from all six trials had no *E coli* and no faecal enterococci occurrence except in

the first and second month of sampling, but the reduction could not be calculated for the first trial as the influent concentration was too numerous to count even at a 5-fold dilution. Water samples from **WWTP C** and **WWTP D** also showed no occurrence of faecal enterococci and *E coli* in final effluent samples, however, *E coli* was only observed at the 1st, 3rd, 4th and 5th months samples from both **WWTP C** and **WWTP D** wastewater treatment plants.

The faecal enterococci and *E. coli* quantities of the intake raw water samples reduced over each treatment step in all the treatment plants (Appendix 2). Faecal enterococci were not detected in the final effluent from **WWTP A** water samples throughout the six-consecutive monthly sampling. *E coli* was recovered in the final effluent of second, fourth, fifth and the sixth sampling month. **WWTP B** effluent water samples from all six trials had no presence of *E coli* and faecal enterococci except in the first and second month of sampling, but the reduction could not be calculated for the first trial as the influent concentration was too numerous to count even at a 5-fold dilution. Faecal enterococci and *E coli* were also not detected in the final effluent water samples from **WWTP C** and **WWTP D**, however, *E coli* was observed at the first, third, fourth- and fifth-month samples from both **WWTP C** and **WWTP D**. There are differences between the sewer network characteristics. Some of the sewers receive influent from domestic wastes and others from industrial wastes. For example, **WWTP A** receives 80% and 20% of its influent from domestic and industrial sources respectively. **WWTPs B** and **C** receive their influent from domestic sources while **WWTP D** receives 30% and 70% of its influent from industrial and domestic sources respectively. This agrees with the literature that the concentrations of faecal indicators were significantly higher in WWTPs that receive industrial effluents when compared with WWTPs that receive water from domestic effluents (Manaia et al., 2010, Madoux-Humery et al., 2013). Another contributing factor could be the differences in WWTP configurations, dimensions and the demographic characteristics of the regions (Lucas et al., 2014). There was a reduction of faecal enterococci concentration from influent water samples to clarifier tank in all the WWTPs. The same was observed for *E coli*. There was gradual reduction of *E coli* and faecal enterococci from influent to aeration tank/trickling filters, clarifier and post chlorination (final effluent) in all the WWTPs. This is similar to the study of Servais et al. (2007) that concludes that primary and secondary treatment processes remove up to 99% of faecal indicator bacteria. However, in some points along the trials, both *E coli* and faecal enterococci were not detectable. The quality of the raw wastewater in terms

of E *coli* and faecal enterococci presence may have little or no influence on the level of these indicators in the effluent as concluded by Manaia et al. (2010). Lucas et al. (2014) supports this by concluding that it is difficult to determine if the domestic and industrial water sources could have impact on the faecal indicator bacterial levels present in raw wastewater because there are obvious differences in the WWTPs due to their populations.

The detection of *E coli* and faecal enterococci followed the same trend for river samples where upstream values were higher than the downstream values across all the trials. This shows that despite the presence of *E. coli* and faecal enterococci in the final effluents from the WWTPs, this did not result in an increase in the concentration of these bacteria in the rivers. Chlorination is the main impacting factor in the reduction of *E coli* and faecal enterococci. This will also affect the effluent values and is a probable main determinant of the reduced values when comparing the higher values of the river samples upstream with the lower ones downstream. WWTPs can produce effluents that are the main source of pathogens for natural water bodies (Wéry et al., 2008). The treatment plants examined are efficient in removing both *E coli* and faecal enterococci from wastewater in agreement with the study by Nasser (2016) where 9.6 log reduction of faecal coliform was found from the tertiary treatment using chlorine.

4.6.2 Occurrence of Cryptosporidium and Giardia spp. in wastewater

Cryptosporidium oocysts and *Giardia* cysts were found in all the influent and effluent samples from all four treatment plants as well as in receiving waters (Tables 4.2 and 4.4). The average log concentrations of *Cryptosporidium* and *Giardia* in the raw influents throughout the six consecutive trials ranged from 2.10 log oocysts/L to 2.45 log oocysts/L and from 2.59 log oocysts/L to 2.72 log oocysts/L respectively. The *Giardia* concentrations were thus higher than the *Cryptosporidium* concentrations. Earlier reported values depend on the local conditions and the prevalence in the connected population, ranging between 0.78-2.54 log oocysts/L and 1.95-3.92 log cysts/L. This is ranging from 103 to 139 oocysts per litre, with a minimum of 40 and maximum of 340 oocysts per litre found in all the treatment plants considered (Montemayor et al., 2005, Castro-Hermida et al., 2015, Galván et al., 2014). The values obtained in this study are similar to what are reported in the literature. Ramo et al. (2017) found an average of 96

oocysts/L (1.98 log) in the raw influent and average of 31 cysts/L (1.49log) in effluent in a study from Spain.

The individual influent log concentrations of *Cryptosporidium* oocysts obtained from all four WWTPs during the period September 2015-March 2016 gives the temporal consistency and individual variability as presented in Figure 4.1. Lim et al. (2007) concludes that the number of parasites that are found in the influent sewage can be associated with the population size served by the WWTP. The same trend was found in this study as all the four WWTPs assessed serve different population size (Table 4.1). A comparison of the number of oocysts was made between the different sampling occasions in all influents. The corresponding individual influent log concentrations of *Giardia* cysts obtained from the different sampling sites from all four WWTPs are presented in Figure 4.2. The log concentrations of *Cryptosporidium* found in WWTP influents were consistent, where the individual values only varied less than one log/L, in the range 1.83 $\log_{10}$/L to 3.02 $\log_{10}$/L except for the influent water sample collected from **WWTP B** during the last sampling round in March 2016 (Figure 4.1). *Giardia* cysts log concentrations from all influents were consistent except for **WWTP A** and **WWTP B** January values. The values are in the range 2.39 $\log_{10}$/L to 3.15 $\log_{10}$/L (Figure 4.2). This same trend is found in previous studies (Castro-Hermida et al., 2015, Hachich et al., 2013, Ramo et al., 2017). Highest concentration of *Giardia* was observed in **WWTP A** during the month of January (summer period) (Figure 4.2). This agrees with the results of Ramo et al. (2017) where (oo)cysts concentration was at the peak during summer months. High prevalence of these protozoans in the influent may also be due to the prevalence of livestock in the area (Quílez et al., 2008, Quilez et al., 2008).

Table 4.2: Mean log values ± SD of *Cryptosporidium* and *Giardia* between six parallel sampling trials for influent and effluent in all sampling sites (n=6)

Sampling sites	INFLUENT				FINAL EFFLUENT				LOG REDUCTION		P value*	
	Cryptosporidium		*Giardia*		*Cryptosporidium*		*Giardia*		*Cryptosporidium*	*Giardia*	*Cryptosporidium*	*Giardia*
	Oocysts/L		Cysts/L		Oocysts/L		Cysts/L					
WWTP A	189	2.19±0.28	575	2.70±0.23	6	0.70±0.22	17	1.15±0.30	1.50±0.32	1.54±0.32	**0.0022**	**0.0022**
WWTP B	359	2.45±0.31	597	2.72±0.24	9	0.83±0.36	13	1.09±0.12	1.61±0.31	1.62±0.16	**0.0022**	**0.0012**
WWTP C	163	2.16±0.24	553	2.71±0.20	6	0.75±0.17	17	1.22±0.11	1.41±0.19	1.49±0.20	**0.0022**	**0.0022**
WWTP D	133	2.10±0.16	411	2.59±0.14	6	0.75±0.17	17	1.22±0.11	1.35±0.18	1.38±0.15	**0.0020**	**0.0018**

*P-values < 0.05 using Kruskal-Wallis

Table 4.3: Comparison of removal of oocysts/cysts between influent and effluent final stages of the wastewater treatment plants (n=6).

	WWTP A vs WWTP B	WWTPA vs WWTP C	WWTPA vs WWTP D	WWTP B vs WWTP C	WWTP B vs WWTP D	WWTP C vs WWTP D
Cryptosporidium	0.1797	0.9372	0.6304	0.1320	**0.0152**	0.5887
Giardia	0.6991	0.4848	0.6991	0.8182	0.3095	0.3939

Comparison of the concentrations between the influent and effluents showed a significant difference in their concentrations (p-value < 0.05) (Tables 4.2 and 4.3), which implies a significant reduction in concentrations of both *Cryptosporidium* and *Giardia* during treatment in all the wastewater treatment plants. When comparing the removal of (oo)cysts achieved by each plant, no significant difference between the plants were found, except for the removal of *Cryptosporidium* between WWTP B and WWTP D. This could be attributed to the difference in plant configuration. WWTP B has a trickling filter treatment step as opposed to an aeration tank in WWTP D.

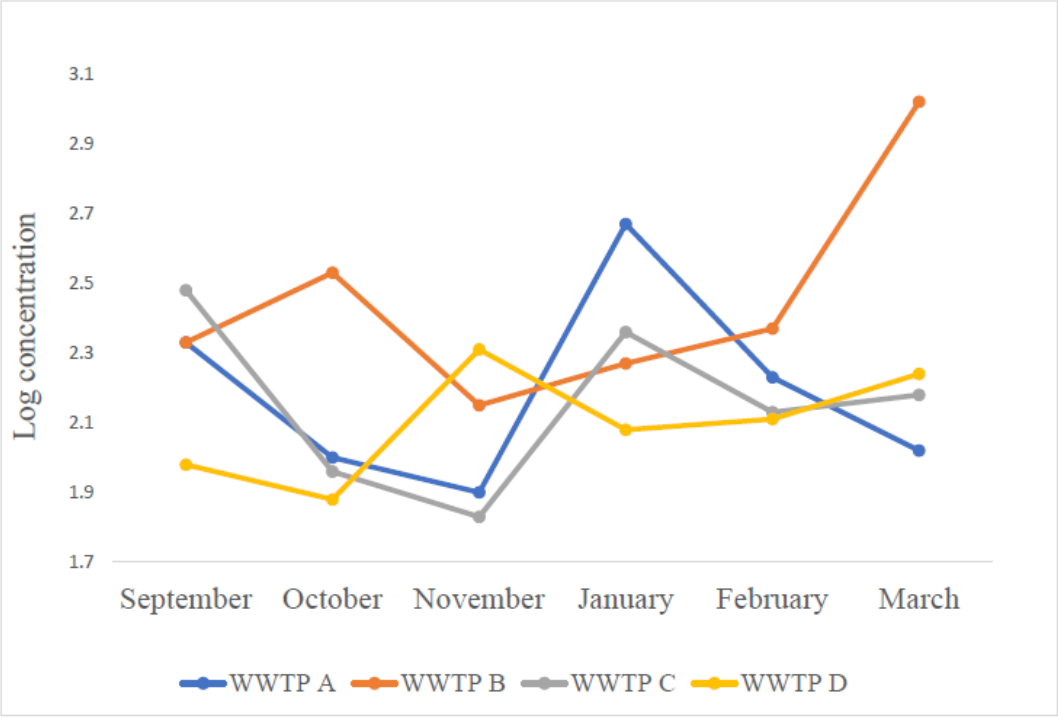

Figure 4.1: Temporal *Cryptosporidium* log concentrations (oocysts/L) in influent samples A, B, C and D

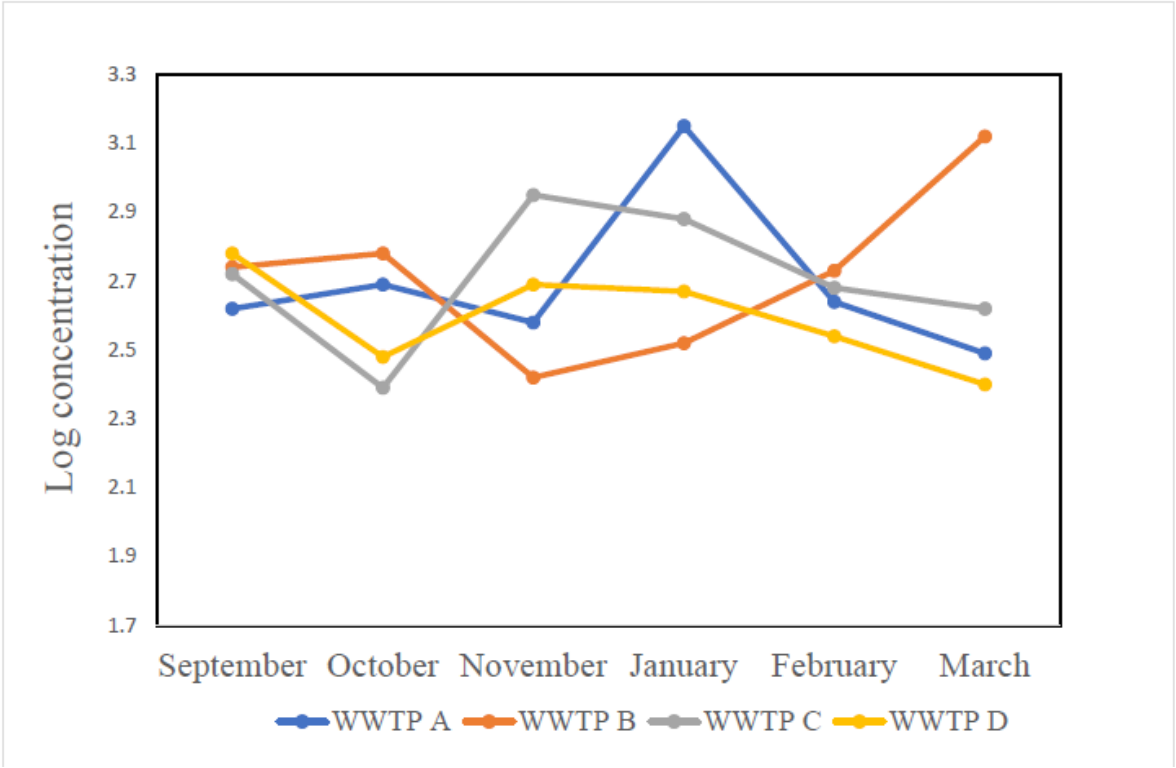

Figure 4.2: Temporal *Giardia* log concentrations (oocysts/L) in influent samples A, B, C and D.

The concentration of *Cryptosporidium* and *Giardia* differed significantly when comparing all the different treatment steps (p-value < 0.05). However, this difference occurred after specific treatment steps/stages. For instance, the trickling filter and aeration tank treatment steps resulted in significant reductions in both *Cryptosporidium* and *Giardia* concentrations (p-value < 0.05), based on comparison of concentrations in the influent samples and concentrations after the trickling filter and aeration tank treatment steps. In addition, at WWTP C, concentrations of both oocysts/cysts differed significantly after the clarifier treatment step, and in WWTP D only *Giardia* concentrations had significant reductions after this treatment step. Comparison of the reduction over the rest of the individual treatment steps showed no significant difference (p-value > 0.05) (refer to Table 4.5). Thus, for both parasites, irrespective of the treatment plant, the biological treatment (aeration tank or trickling filter followed by the clarifier) was the significant step/s for the reduction.

4.6.3 Removal of Cryptosporidium and Giardia in different treatment steps

Tables 4.2 and 4.4 summarises the mean log concentrations, standard deviation and log reduction of *Cryptosporidium* and *Giardia* from the different treatment steps. No complete removal in any of the WWTPs occurred as also was the case in a previous studies (Fu et al., 2010), however the concentrations in the influent and effluent differed significantly (p-value < 0.05) (Table 4.4). Table 4.5 presents statistical difference in concentration of *Cryptosporidium* and *Giardia* from different treatment steps for all WWTPs (P values)**.** Both *Cryptosporidium* and *Giardia* were reduced to an almost similar degrees in the full treatment (*Cryptosporidium* between 1.34 - 1.63 $\log_{10}$/L and *Giardia* between 1.38 - 1.67 $\log_{10}$/L) (Table 4.2). Ramo et al. (2017) found the removal efficiency of *Cryptosporidium* to be in the range of 0.35 log/L to 1.8 log/L and for *Giardia* between 1.06 log/L to 2.34 log/L in all the WWTPs assessed. These broader ranges of removal are in line with what was found in this study also with similarities in the description of the treatment plants and population served. This shows that the four WWTPs examined were all efficient in reducing the concentration of *Cryptosporidium* and *Giardia* in wastewater. Similar studies produced same trend of removal (Taran-Benshoshan et al., 2015). The mean log values of river samples compared with the effluents from WWTPs are presented in Tables 4.8 and 4.10.

In Table 4.4, the log reduction per litre achieved over the aeration tank/trickling filter step for all the four plants was in the range of 1.08 - 1.19 and 1.38 to 1.62 for *Cryptosporidium* and *Giardia* respectively. The removal of *Giardia* was slightly higher than for *Cryptosporidium* over the activated sludge treatment and significantly better over the trickling filter treatment step. Over this treatment step, log reductions per litre of 1.08 to 1.19 and 1.14 to 1.37 for *Cryptosporidium* and *Giardia* respectively was achieved. In this study, the influent samples were taken before the grit removal. Therefore, the measured reduction in oocysts/cysts concentrations is a combination of both the removal achieved during the grit removal step and the aeration tank/trickling filter treatment step. The overall reduction over these steps is in line with other reported findings (Fu et al., 2010, Ramo et al., 2017, Taran-Benshoshan et al., 2015) accounting for primary treatment with the grit removal step. Over the clarifier treatment step, the log reduction per litre was marginal and in the range of 0.12 to 0.21 and 0.11 to 0.21 for *Cryptosporidium* and *Giardia* respectively. The reduction here is most probably a reflection of the particle settling efficiency, but is still in line with the ranges of those reported in other studies, showing a superior removal during the clarifier treatment step (Fu et al. (2010). The clarifier step is reported to remove about one third of (oo)cysts (Fu et al., 2010). In the study of Fu et al. (2010), the individual step log reductions per litre for *Cryptosporidium* found for the primary, secondary and tertiary treatment steps were between 1.53-2.43, 1.83-2.51 and 0-0.93 respectively. *Giardia* had log reductions per litre of 2.60-3.20, 2.73-3.30 and 0-1.04 for primary, secondary and tertiary treatment respectively. It was also found that WWTP B with a total log reduction per litre of 1.61 for Cryptosporidium *and* 1.62 for *Giardia* in all the treatment steps is the best performing plant wastewater. This may be attributed to the difference in plant configuration. WWTP B has a trickling filter treatment step as compared to the aeration tank used by WWTP D. Trickling filters have been reported to result in higher removal of (oo)cysts (Ramo et al., 2017, Kitajima et al., 2014, Sroka et al., 2013), which may have accounted for the higher efficiency in WWTP B.

After the clarifier treatment step, about one third of oocysts can still be alive (Fu et al., 2010), therefore, advanced treatment may be required. Such treatments may include physical treatments like membrane ultrafiltration, high-sand filtration and chlorination for water reuse (Fu et al., 2010, Taran-Benshoshan et al., 2015). The effect of chlorination is shown in table

4.4, expressed as the log reduction per litre values from after clarifier to effluent and discussed in Section 4.6.4 below.

Table 4.4: Mean log values ±SD concentrations after the different treatment steps and the log reduction/L over the individual treatment steps (n=6).

Cryptosporidium												
Sampling sites	**Influent**		**Aeration tank/ Trickling filter***		**Clarifier**		**Chlorination**		**Log reduction/L**			
									Aeration tank/ Trickling filter	**Clarifier**	**Chlorination**	**Total Log Reduction**
	Oocysts/L		Oocysts/L		Oocysts/L		Oocysts/L					
WWTP A	189	2.19±0.28	13	1.02±0.31	8	0.88±0.18	6	0.70±0.22	1.17±0.14	0.14±0.14	0.18±0.15	1.50±0.32
WWTP B	359	2.45±0.31	20	1.25±0.23	14	1.11±0.18	9	0.83±0.36	1.19±0.14	0.15±0.11	0.27±0.33	1.61±0.31
WWTP C	163	2.16±0.24	13	1.08±0.15	8	0.87±0.13	6	0.75±0.17	1.08±0.10	0.21±0.14	0.12±0.07	1.41±0.19
WWTP D	133	2.10±0.16	10	1.00±0.13	8	0.87±0.13	6	0.75±0.17	1.10±0.12	0.12±0.11	0.12±0.07	1.35±0.18
Giardia												
	Cysts/L		Cysts/L		Cysts/L		Cysts/L					
WWTP A	575	2.70±0.23	39	1.51±0.30	23	1.32±0.20	17	1.15±0.30	1.19±0.17	0.19±0.17	0.16±0.16	1.54±0.32
WWTP B	597	2.72±0.24	24	1.34±0.19	19	1.24±0.21	13	1.09±0.12	1.37±0.10	0.11±0.04	0.15±0.10	1.62±0.16
WWTP C	553	2.71±0.20	32	1.48±0.14	19	1.27±0.10	17	1.22±0.11	1.22±0.17	0.21±0.11	0.05±0.02	1.49±0.20
WWTP D	411	2.59±0.14	30	1.46±0.13	19	1.27±0.10	17	1.22±0.11	1.14±0.16	0.19±0.09	0.05±0.02	1.38±0.15

*WWTPs A, C and D have an aeration tank and WWTPs B has a trickling filter.

Note: The log reduction per litre after the aeration tank/trickling filter will include reduction achieved during the primary treatment (grit removal) which was not explicitly measured and accounted for in this study.

Table 4.5: Statistical difference in concentration of *Cryptosporidium* and *Giardia* from different treatment steps for all WWTPs (P values)

Cryptosporidium			
Sampling sites	**Influent vs Aeration tank/Trickling filter***	**Aeration tank/Trickling filter* vs Clarifier**	**Clarifier vs Effluent (after chlorination)**
WWTP A	0.0022	0.3751	0.1962
WWTP B	0.0022	0.2281	0.2615
WWTP C	0.0022	0.0260	0.1994
WWTP D	0.0022	0.0898	0.1994
Giardia			
Sampling sites	**Influent vs Aeration tank/Trickling filter***	**Aeration tank/Trickling filter* vs Clarifier**	**Clarifier vs Effluent (after chlorination)**
WWTP A	0.0022	0.3095	0.4848
WWTP B	0.0022	0.3939	0.1488
WWTP C	0.0022	0.0260	0.3768
WWTP D	0.0022	0.0370	0.3768

*WWTPs A, C and D have an aeration and WWTPs B has a trickling filter.

4.6.4 Removal of Cryptosporidium and Giardia using chlorination in all the WWTPs

Tertiary treatment or disinfection of the wastewater after the clarifier treatment step is required in South Africa. The impact of chlorination as a tertiary treatment step gave varying results. Chlorination was successful in removing only 3.6% to 10.38% of total *Giardia* and 8% - 17% *Cryptosporidium* of concentration in all the four treatment plants. This is partly expected, due to the level of resistance against chlorination, where, however, the impact of additional physico-chemical factors and variability between samples also may impact on the results.

Disinfection of wastewater using chlorine is done with the use of gaseous chlorine (Cl_2) or hypochlorite salts. Chlorine in all its forms reacts with water and hypochlorous acid (HOCl). Hypochlorous acid then dissociates to form a hypochlorite ion in relation to this reaction: $HOCl \leftrightarrow OCl^- + H^+$. Chlorine is also found in the form of monochloramine and dichloramine. This is in addition to HOCl and (OCl^-). The combination of many parameters like pH, ammonia concentration and temperature will determine the form of chlorine that will be dominant. The proportion of hypochlorite ions relative to hypochlorous acid will increase with pH increase and monochloramine will also increase with higher ammonia concentrations. It is very important to know the dominant form of chlorine in any disinfection process. Of all the four oxidising forms of chlorine, hypochlorous acid (HOCl) is the most potent (Lim et al., 2010).

After chlorination (between 3rd to 4th sampling point), WWTP A showed a slight reduction of *Giardia* (0.16 log reduction) while WWTP C and D virtually had no effect for *Giardia* (0.05 log reduction). Since, all the treatment steps in the WWTPs were unable to remove *Giardia* and *Cryptosporidium* completely from wastewater, the effluent water discharged to the river still contains these parasites in variable concentrations. Advanced treatment step like chlorination, UV and membrane ultrafiltration are necessary for *Cryptosporidium* further reduction for water reuse to be possible (Ramo et al., 2017). It can be concluded that the four WWTPs only marginally reduced the concentration of *Cryptosporidium* over the chlorination step (range of 8% - 17%), which is a valuable documentation from the South African perspective, where there is heavy reliance on chlorination. This effect may be due to chlorination but other factors like further sedimentation or oxidation may also have affected the total concentration of *Cryptosporidium* removed.

4.6.5 Occurrence of Cryptosporidium and Giardia in downstream river

In Table 4.6, the mean log concentration of oocysts upstream the effluent discharge point of WWTP A was 1.00 (cysts 1.34) while the corresponding values for the effluent was 0.70 (1.15) and downstream 0.81 (1.06). This shows that the effluent discharge to the river had little or no appreciable effect on the concentration of *Cryptosporidium* or *Giardia* in the river samples downstream. This river was chosen because it receives effluent discharge from WWTP A, providing a great opportunity to study the impact of these effluents on the water quality in relation to these parasites. The trend of higher upstream values was consistent and represented all the six investigation runs except one. Although the difference in (oo)cysts concentrations was not significant when comparing the upstream values with the downstream one and the effluents (p-value of 0.123 for *Cryptosporidium* and 0.1126 for *Giardia*) this trend is evident. Based on the p-values alone the concentration of *Cryptosporidium* upstream and in the effluent was significantly different. This was not the case when upstream and downstream were compared. For *Giardia*, concentrations in the upstream and downstream were significantly different. (Table 4.7). This may be due to the higher concentration of the oocysts in the effluent. Comparison of the upstream concentrations with the downstream concentrations did not show any significant difference. Although the effluents had high oocysts concentrations, it did not significantly influence the baseline concentrations in the river.

While at upstream, additional pronounced unidentified pollution sources may occur. The effluent discharge will also represent a lower contribution in volume as compared to the river water volume and flow. Dilution effects of protozoans from wastewater effluent entering the river was seen by Lucas et al. (2014), even though WWTP effluents represent a point-source of pathogen for downstream river impacts (Wéry et al., 2008). The concentrations of both *Cryptosporidium* and *Giardia* continue to decrease as river flows downstream. Reduction of parasite concentrations along the rivers may further be due to sedimentation of particles with attached parasites.

Such sedimentation plays an important role in the reduction of (oo)cysts as observed by others (Kitajima et al., 2014, Ramo et al., 2017, Sroka et al., 2013). Additional pollution sources downstream will also affect the concentrations. This is evident from the increase in the

concentrations of oocysts and cysts registered in all sampling points at the fourth sampling trial (Summer Season) in the river receiving effluent from WWTP B (Table 4.8). The criteria used on selecting River A (receiving effluents from WWTP A) was used here as well. In addition, there are a number of informal settlements along this river (River B), this gives an opportunity to possibly compare the impact of these informal settlements on the river quality as well. There was significant difference in the oocysts/cysts concentrations at the various sampling points in this river (p-value < 0.05). High concentrations of both the oocysts and cysts occurred in the effluent but were not significantly different compared to the upstream concentrations. Comparison between the upstream and downstream concentrations of both oocysts and cysts also showed no significant difference (See Table 4.9). The effluent, thus, did not have a significant impact on the load of parasites in the river.

The concentrations of *Cryptosporidium* and *Giardia* were increasing consistently along the river flow path from effluent to the downstream river mouth. Diffuse pollutions sources from the dense informal settlements or runoff into the river will be the potential added sources of pollution. Naturally different locations of sampling and timing of sampling have an effect on the concentration of (oo)cysts (Oragui, 2003). It can also generally be attributed to the land use in the watershed that may contribute to river pollution (in this case close to WWTP B) as the concentration of (oo)cysts is linked to the disease prevalence and to the population size that WWTP serves (Lim et al., 2007).

Table 4.6: Log concentrations of *Cryptosporidium* and *Giardia* at the river sampling points from WWTP A

Sampling	UPSTREAM		EFFLUENT		DOWNSTREAM	
	oocysts/L	cysts/L	oocysts/L	cysts/L	oocysts/L	cysts/L
1st	0.78	1.11	0.95	1.32	0.48	0.95
2nd	1.33	1.79	0.93	1.48	1.08	1.61
3rd	1.04	1.24	0.54	1.11	0.85	0.93
4th	0.93	1.24	0.65	1.16	0.88	0.93
5th	1.06	1.42	0.70	1.23	0.95	1.08
6th	0.88	1.22	0.40	0.60	0.65	0.88
Mean log ±SD	1.00±0.19	1.34±0.24	0.70±0.22	1.15±0.30	0.81±0.22	1.06±0.28

Table 4.7: Statistical difference in concentrations of *Cryptosporidium* and *Giardia* at the river sampling points from WWTP A (p values)

	UPSTREAM vs EFFLUENT	UPSTREAM vs DOWNSTREAM	EFFLUENT vs DOWNSTREAM
Cryptosporidium	0.0542	0.2615	0.4696
Giardia	0.4217	0.0450	0.2971

Table 4.8: Log concentration of *Cryptosporidium* and *Giardia* at four river sampling points next to WWTP B

Sampling Schedule	UPSTREAM		EFFLUENT		NTWWT[a]		DOWNSTREAM		RIVER MOUTH	
	oocysts/L	cysts/L	oocysts/L	cysts/L	oocysts/L	cysts/L	oocysts/L	cysts/L	oocysts/L	cysts/L
1st	1.39	1.11	1.00	1.15	1.11	1.11	1.33	1.38	1.24	1.30
2nd	1.02	1.26	1.13	1.08	0.95	1.27	1.31	1.53	1.28	1.52
3rd	0.90	1.23	0.78	0.90	0.95	1.15	1.08	1.28	1.00	1.30
4th	1.90	2.05	0.18	1.11	1.84	1.81	1.50	2.11	1.36	1.77
5th	0.81	1.15	0.78	1.06	0.78	1.23	0.93	1.20	1.27	1.46
6th	0.93	1.23	1.15	1.26	0.95	1.19	0.85	1.13	0.95	1.34
Mean log +SD	1.16±0.41	1.34±0.35	0.83±0.36	1.09±0.12	1.10±0.38	1.29±0.26	1.17±0.26	1.44±0.36	1.18±0.17	1.45±0.18

[a]NTWWT: Next to wastewater treatment plants (downstream)

Table 4.9: Statistical difference in concentration of *Cryptosporidium* and *Giardia* at four river sampling points next to WWTP B (p values)

	UPSTREAM vs EFFLUENT	UPSTREAM vs NTWWT	UPSTREAM vs DOWNSTREAM	UPSTREAM vs RIVER MOUTH	NTWWT vs DOWNSTREAM	NTWWT vs RIVER MOUTH	DOWNSTREAM vs RIVER MOUTH
Cryptosporidium	0.3095	0.9357	0.7483	0.5887	0.6868	0.1922	1.0000
Giardia	0.0761	0.9355	0.3776	0.0651	0.3095	0.0649	0.5887

4.6.6 Effect of seasonal variation on the concentrations of Cryptosporidium and Giardia

The highest *Cryptosporidium* concentration was observed from WWTP B during autumn (March) and the highest *Giardia* concentration from WWTP A in the month of January (Summer in South Africa). The increase in *Cryptosporidium* concentration in the influent from WWTP B during the autumn season could be due to the influence of stormwater runoff. Oragui (2003) stated that the measured density range is a representation of snapshots of raw sewage full variability. This may be attributed to location and timing differences of sample collection, which may greatly influence the faecal indicators density from one measurement to the other. Moreover, wastes are being generated and released into WWTP B from upstream areas which contributed to its increase. Around WWTP B, informal settlements occurred as well as the presence of illegal dumping around the plant which affects the river quality but not directly the impact of the wastewater quality or effluent. However, when connected either directly or through stormwater impact, different watersheds with highly variable land uses can present very different concentrations of organisms in the raw sewage (Madoux-Humery et al., 2013).

The increase in concentration of oocysts and cysts from WWTP B at the last sampling round (during autumn) could have been due to rainfall prior to and during the time of sampling. The presence of contaminants in wastewater can be increased to up to 10 to 1000 times during storm events as compared to dry periods (Characklis et al., 2005). This increase may be more noticeable depending on the catchment characteristics and storm intensity. It was observed that the WWTP water was very turbid and runoff could have contained different contaminants. This observation can be supported by the findings of Koompapong et al. (2014) where higher contamination of oocysts was reported during rainy season. An increase in number of oocysts and cysts reported in river samples in this study during the wet season could also be due to the runoff that carries parasites into the river. Similar results have been reported by Koompapong and Sukthana (2012) and (Atherholt et al., 1998). Higher concentration of oocysts observed from WWTP B could also be due to high concentration of local settlements, since the plant serves about 73,487 people from different social strata, which populations wise is higher than the population that other plants serve (WWTP A: 25,822, WWTP C and WWTP D: 55,744).

In the influent water samples collected from **WWTP A**, the number of oocysts and cysts (468 oocyst/L and 1,416 cysts/L respectively) were higher in the summer period during the fourth sampling time than at the other sampling events. This finding could be due to higher turbidity, as was noticed with the samples and which was in line with the findings of Sayed et al. (2016). These authors found higher water contamination from a hospital during summer than recorded in winter, leading to an attachment of particles which may affect the reduction (Sayed et al., 2016). This is also supported by the findings reported by Khalifa et al. (2014) who reported a 22.6% prevalence in winter and 66.7% in summer due to differences in the turbidity. The seasonal patterns vary greatly due to location. According to a previous research in an Arabian country, seasonality was found in the rate of human infection which could reflect the occurrence of the parasite in the environment according to season and community practice (Areeshi et al., 2007). In the present study lower concentration of oocysts was recorded during the third trial and first trial analysis from WWTP B and its recipient. There was a heavy rainfall the day before sampling in WWTP B during the first and third trials. This rainfall might have resulted in a significant lower concentration of oocysts due to dilution. This low concentration of oocysts is supported by similar findings of Martinez and Merino (2011) where less quantities of parasites was observed after heavy rain that washed away contaminants. In a combined sewer, runoff water input can be an important dilution of contaminant densities (Passerat et al., 2011). During rainfall, wastewater entering the WWTPs may be diluted by street washing waters. The reduction in the contamination in raw sewage may be attributed to this dilution (Lucas et al., 2014). It can be concluded that WWTPs should have different treatment procedures which will be dependent on seasonality to increase their treatment efficiency. The capacity of the treatment plants and number of process units during rainfall should be addressed. Apart from the quantity of influent, quality of influents also can vary, and this will affect the WWTP efficiency. WWTP efficiency and management is dependent on the variability, quality and characteristics of influents.

4.6.7 Relationship between Cryptosporidium and Giardia counts versus E. coli/Enterococcus concentrations

Association between the bacterial counts and concentration of (oo)cysts differed depending on the sampling point. Correlation was done with only counts from samples taken at the influent, after the clarifier treatment step and after chlorination. The decision to use counts from these points was

due to the significant reduction in the concentration of these microorganisms seen from the log reductions (Section 4.6.3 to 4.6.4). Despite high counts of all these microorganisms in the influent, there was weak positive correlation between *E. coli* and both parasites (*Cryptosporidium* and *Giardia*) (r = 0.029 and 0.081; p values = 0.902 and 0.733 respectively) and negative correlation with Enterococci (r = -0.122; p value = 0.609). These correlations were however not significant. The presence of large particulate matter in the samples at this sampling point could have resulted in the uneven distribution of these microorganisms. These large particles may lead to attachment of some of these microorganisms and therefore their concentration in the samples taken could be affected. Microbial partitioning in water has been reported extensively, for example, Jeng et al. (2005) reported that *E. coli* and Enterococci attachment to particles in water ranged from 22-30% and 8-12% respectively. Therefore, their concentration in samples that contain higher particulate matter will be different. This may have accounted for the weak or no correlation reported in this study. These levels of relationships could be influenced by the turbidity of the samples, the influent and biofilter samples are more turbid than the final effluents, therefore with high turbidity, the concentration of the oocysts and cysts may be higher.

Samples taking after the clarifier, showed a much clearer association between the microorganisms. *E. coli* correlated positively with all the other three microorganisms (r = 0.447, 0.508 and 0.350 for Enterococci, *Cryptosporidium* and *Giardia* respectively). These correlations were significant for all except for *Giardia* (p values = 0.029, 0.011 and 0.094 for Enterococci, *Cryptosporidium* and *Giardia* respectively) (Table 4.10). A similar correlation trend was seen between the Enterococci and *Cryptosporidium* at this sampling point except for correlation with *Giardia* which was negative although not significant (Table 4.10). Correlation between the two protozoan parasites was however significantly positive at this sampling point (r = 0.448; p value =0.028). At this point the number of large particles and organic content is considerably low and therefore the concentration of these microorganisms could show the natural association between these. The same trend was observed for the post chlorinated wastewater samples. Based on these findings only *Giardia* did not have any significant positive correlation with the two bacterial indicators and the correlation was especially weak for the post chlorinated samples. Chlorination has been reported in this study (Section 4.6.4) as well as other studies to have minimal impact on *Giardia*. Therefore, the weak correlation between the bacterial indicators and the *Giardia* could be

attributed to this difference in impact. *Cryptosporidium* is impacted by chlorination to a lesser extent as compared to the bacteria but similar to *Giardia*, which could account for the significantly positive correlation between the *Cryptosporidium* and the other microorganisms in the post chlorinated samples.

The results from this study are in contrast to other findings where poor correlation between the faecal indicators and the presence of pathogens was seen (Harwood et al., 2005). Duhain (2011) and Ehsan et al. (2015) neither found any significant correlation between the level of faecal coliforms and *E coli* and the presence of *Cryptosporidium* and *Giardia*. This agrees with the study of Xiao et al. (2017) where it was concluded that there was no correlation between the two parasites and faecal bacterial indicators. The difference in these correlation studies could be due to the difference in the type of samples taken. Hogan et al. (2012) found that *E. coli* counts greater than 400 MPN/100 mL resulted in higher protozoa counts while total coliforms exceeding 10,000 MPN resulted in lower (oo)cysts concentrations. The Hogan et al. (2012) study therefore supports the findings from this study due to the high *E. coli* counts reported.

The inconsistency in reports on correlation between bacterial indicators and other pathogens reiterate that the use of indicators in prediction of pathogens should take into consideration the type of sample. For instance, in samples with large particulate matter or chlorinated samples, bacterial indicators should not be used to predict the concentration of other pathogens especially protozoan parasites like *Giardia*.

Table 4.10: Correlation between the concentration of bacterial indicators (*E coli* and Enterococci) and *Cryptosporidium* and *Giardia*

	E. coli			Enterococci			*Cryptosporidium*			*Giardia*		
	Influent	Clarifier	Post chlorination	Influent	Clarifier	Post chlorination	Influent	Clarifier	Post chlorination	Influent	Clarifier	Post chlorination
E. coli				-0.122	**0.447**	**0.585**	0.029	**0.508**	**0.412**	0.081	0.350	0.094
Enterococci	-0.122	**0.447**	**0.585**				-0.035	**0.422**	**0.432**	-0.276	-0.134	0.078
Cryptosporidium	0.029	**0.508**	**0.412**	-0.035	**0.422**	**0.432**				0.248	**0.448**	**0.674**
Giardia	0.081	0.350	0.094	-0.276	-0.134	0.078	0.248	**0.448**	**0.674**			

Note: Bolded correlation values indicate significant correlation based on the P values (using Kruskal-Wallis).

4.6.8 Occurrence of Cryptosporidium and Giardia in spinach irrigated with the effluent water discharged from WWTP B

Leafy vegetable (spinach) samples were collected from farmers next to the WWTP B as presented in Section 3.4, Chapter 3 and analysed for the presence of *Cryptosporidium* and *Giardia*. The infectious protozoan is not the focus for investigations from fresh vegetables but may play a major role in the transmission. Parasitic food-borne illnesses linked to the consumption of the fresh vegetables are generally documented (Hassan et al., 2012, Al-Megrm, 2010). *Cryptosporidium* and *Giardia* were detected on the surfaces of the irrigated crops. In general, high numbers of the (oo)cysts on the surfaces of the crops were recorded (Table 4.11).

Table 4.11: The number of oocysts/100g and cysts/100g found in spinach irrigated with treated effluent discharged from WWTP B

Trials	Oocysts/100g	Cysts/100g
1	480	27
2	67	3
3	263	10

* The values represent three independent trials with single values.

Cryptosporidium and *Giardia* were generally present in water used for irrigation of crops around the study area (WWTP B and connected River, Table 4.6 and Table 4.10). The degree of contamination of the fresh vegetables analysed in this study was due to the use of the out flowing water from the Wastewater Treatment Plant for irrigation. The concentration of *Cryptosporidium* oocysts and *Giardia* cysts in the spinach samples collected were high, and the number of oocysts higher than the number of cysts in all the sampling trials (Table 4.11). The concentrations of *Cryptosporidium* and *Giardia* in spinach samples ranged from 67 to 480 per 100 g vegetable and 3 to 27 per 100 g vegetable respectively. From Table 4.11, there are three independent trials presented with the individual number of oocysts/100g and cysts/100g present in the spinach sampled.

The occurrence of intestinal parasitic infections from developed and developing countries linked to raw vegetables has been reported (Nazemi et al., 2012, Haq et al., 2014) as well as through

contaminated water used for irrigation or washing process and during the post-harvest handling and processing (Ishaku et al., 2013). The degree of contamination of fresh vegetables mostly depends on treated or untreated wastewater use for irrigation. Another way of contamination may be through the water supply contaminated with sewage for irrigation. Additionally, the unhygienic conditions for the preparation of food especially in home settings can also cause contamination (Amoah et al., 2008).

The consumption pattern has changed and the level of consumption of raw vegetable has also increased worldwide. The consumption of salad vegetables may lead to the ingestion of intestinal parasites that in turn result in disturbances in human health such as diarrhoea, dysentery, bloating and flatulence, obstruction, fatigue and anaemia. Further information of the detection and enumeration of these parasites is highly important (Rahman et al., 2014).

4.6.9 PCR and qPCR for detection of Cryptosporidium and Giardia

4.6.9.1 Nested PCR for Cryptosporidium and qPCR for *Giardia*

Presence of *Cryptosporidium* and *Giardia* DNA in the 42 wastewater samples (listed in Table 4.12) was assessed through amplification of the 18S rRNA gene region through a nested PCR and *Giardia* Assemblage A and B specific qPCR assay. In the *Cryptosporidium* nested PCR, a representative agarose gel for the amplified PCR products is exemplified in Figure 4.3 (additional gels Figures 4.4 – 4.9 are added for clarity in Appendix 1). Samples considered *Cryptosporidium* positives (Table 4.12) were those that resulted in clear bands of around 840 bp (Xiao et al., 2001) (826-864 bp were considered). Out of 42 wastewater samples, only 19 samples (45.23%) were either positive or borderline positive and only 9 samples were positive (21.43%) for 18s rRNA gene based nested PCR for *Cryptosporidium*.

In *ß-giardin* based qPCR (presence absence test) for *Giardia,* all the 42 samples tested were positive for *Giardia* Assemblage A and *Giardia* Assemblage B. The samples were considered positive for Assemblage A and B because their threshold cycle (Cq) lies within 40 cycles, similar to the positive control *Giardia* DNA (kindly provided by Karin Troell, of National Veterinary Institute, Sweden, Uppsala**)** and their melt curve also overlaps with positive control *Giardia* DNA.

Table 4.12: Extracted DNA samples from wastewater collected between September 2015 and March 2017 at four different Wastewater Treatment Plants (WWTPs).

DESIGNATED NAME	WWTP ORIGIN	SAMPLE TYPE
K1(1)	WWTP A	Effluent 1 (300 mL)
K1(2)	WWTP A	Post Cl_2 (300 mL)
K1(3)	WWTP A	Clarifier tank (300 mL)
K1(4)	WWTP A	Raw influent 1
K2(1)	WWTP A	Effluent (600 mL)
K2(2)	WWTP A	Post Cl_2
K2(3)	WWTP A	Clarifier tank
K2(4)	WWTP A	Raw influent (100 mL)
K3(1)	WWTP A	Effluent (600 mL)
K3(2)	WWTP A	Post Cl_2
K3(3)	WWTP A	Clarifier tank
K3(4)	WWTP A	Raw influent (100 mL)
K4(1)	WWTP A	Effluent (600 mL)
K4(2)	WWTP A	Post Cl_2
K4(3)	WWTP A	Clarifier tank
K4(4)	WWTP A	Raw influent (100 mL)
K5(1)	WWTP A	Effluent (600 mL)
K5(2)	WWTP A	Post Cl_2
K5(3)	WWTP A	Clarifier tank
K5(4)	WWTP A	Raw influent (100 mL)
K6(1)	WWTP A	Effluent (100 mL)
K6(2)	WWTP A	Post Cl_2
K6(3)	WWTP A	Clarifier tank
K6(4)	WWTP A	Raw influent (100 mL)
IS1	WWTP B	Raw influent 2 (50 mL)
IS2	WWTP B	Raw influent (100 mL)
IS3	WWTP B	Raw influent (100 mL)
IS4	WWTP B	Raw influent (100 mL)
IS5	WWTP B	Raw influent (100 mL
IS6	WWTP B	Raw influent (100 mL)
SH1	WWTP C	Influent
SH2	WWTP C	Influent 2
SH3	WWTP C	Influent (100 mL)
SH4	WWTP C	Influent (100 mL)
SH5	WWTP C	Influent (100 mL)
SH6	WWTP C	Influent (100 mL)
MR1	WWTP D	Influent
MR2	WWTP D	Influent 2
MR3	WWTP D	Influent (100 mL)
MR4	WWTP D	Influent (100 mL)
MR5	WWTP D	Influent (100 mL)
MR6	WWTP D	Influent (100 mL)

*Samples that were either positive or borderline positive for 18s rRNA gene based nested PCR for *Cryptosporidium* are highlighted in blue colour and those that were positive are highlighted in red colour.

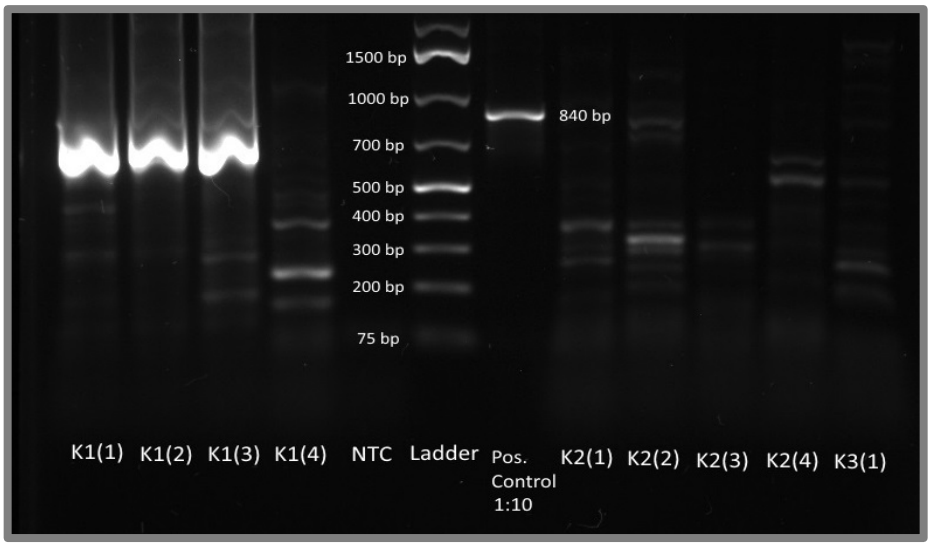

Figure 4.3. PCR products from the samples denoted below the bands. Samples were considered positives based on presence of a clearly visible band around the size of 840 bp: K1(1), K1(2), K1(3). K2 (2) was also considered as a potential positive, although being weak.

4.6.9.2 Genotyping of Cryptosporidium DNA positive wastewater samples

Nine of the samples showed clear bands on the agarose gel and some samples were considered borderline positives, for instance, K6 (4), K5 (4) and a few others (The designated name and origins are presented in Table 4.12). The reason might be that the presence of inhibitors in these samples decreases the quality of DNA during the extraction process. The quantity of DNA loaded on the agarose gels could be another reason for this. Nine *Cryptosporidium* positive wastewater samples were subjected to Sanger sequencing. The taxonomy of wastewater from IS4 (WWTP B: Influent sample) obtained after NCBI Blast search of nucleotide sequences, reveals that, out of 680 hits of *Cryptosporidium*, 397 hits were from *C. parvum* and 161 from *C. hominis*. The high proportion of *C. parvum* does not necessarily mean that there is an animal (such as dairy cattle) influx on the wastewater. Furthermore, two hits of *C. donkey* genotype with a 96% similarity of Accession number KU200955.1, KU200954.1 representing *C. donkey* genotype and one hit with 96% similarity with Accession number: HQ397716.1 representing *C. rabbit genotype* indicate the presence of the same type of *Cryptosporidium* genotypes in South African wastewater. Both human and zoonotic genotypes of (oo)cysts were recorded. Similar finding has been reported by Ehsan et al. (2015), where human and zoonotic genotypes of *Cryptosporidium* and *Giardia* were reported in surface water at all investigated catchment sites. Earlier presence of *C. donkey* genotype have been reported in donkeys in Algeria (Laatamna et al., 2015) and *C. rabbit genotype* has been reported in human samples in Nigeria (Molloy et al., 2010, Molloy et al., 2011). From a global scenario, a waterborne outbreak in the UK caused by *C. cuniculus* from rabbits has highlighted the importance of wildlife in the dissemination of *Cryptosporidium* to drinking water sources and the associated human health risk (Puleston et al., 2014). In WWTP A samples, (K4: Influent samples), out of 679 NCBI blast hits, 391 belongs to *C. parvum* and 161 belongs to *C. hominis* and 33 hits belongs to *C. cuniculus*. The sequence was showing 90% similarity with the Accession number KP098560.1, MG5166739.1 representing *C. cuniculus* isolate. In WWTP D samples (MR3: Influent samples), out of 637 NCBI blast hits, 374 belongs to *C. parvum* and 184 belongs to *C. hominis* with 10 hits of *C. cuniculus* with 2 hits of *C. donkey* and 2 hits of *C. chipmunk* genotype *I*. In WWTP A samples, (K4: Influent samples), out of 679 NCBI blast hits, 391 belongs to *C. parvum* and 161 belongs to *C. hominis* and 33 hits belongs to *C. cuniculus*. The sequence was showing 90% similarity with the Accession numbers KP098560.1, MG516739.1 representing *C.*

cuniculus isolate. In WWTP D samples (MR3:Influent samples), out of 637 NCBI blast hits, 374 belongs to *C. parvum* and 184 belongs to *C. hominis* with 10 hits of *C. cuniculus* with 2 hits of *C. donkey* and 2 hits of *C. chipmunk genotype I* (89 % similarity, Accession number: MF411074.1, MF411073.1) In WWTP C sample (SH4 Influent samples) out of 653 NCBI Blast hits, 358 belongs to *C. parvum,* 168 belongs to *C. hominis*, 33 hits belong to *C. cuniculus*, 12 hits of *C. meleagridis* were found with 2 hits of *C. donkey genotype* (91% similarity to Acession number : KU200955.1, KU200954.1 and one hit of C. rabbit *genotype* (90 % similarity to HQ397716.1). Based up on our NCBI blast results, *C. parvum* like isolates dominates in targeted WWTPs followed by *C. hominis.* Possibility of the presence of rare *Cryptosporidium* genotypes in wastewater is high, for example, *C. donkey* and *C. rabbit* genotype*, C. chipmunk* genotype, (which were initially found in animal and human samples). This needs further detailed investigation because at times it is very difficult to differentiate between various genotypes, as the sequences often resemble each other in similarity and query coverage, for example in sample SH4, blast results showed similar hits for *C. hominis*, *C. parvum* and other genotypes. A phylogenetic tree neighbour joining tree (Mega 6 software) generated by aligning the sequences obtained in sequencing is presented in Appendix 3.

4.7 Conclusion and recommendations

The following was found:

- The results showed the presence of *Cryptosporidium* and *Giardia*, including human and zoonotic genotypes.
- *Giardia* cysts were found to be more dominant than *Cryptosporidium* in all the water samples collected in all four WWTPs
- There was a significant difference between the presence of *Cryptosporidium* and *Giardia* in all treatment steps from all WWTPs ($P < 0.05$).
- No direct seasonal variation occurred.
- Novel strains that have never been found in Africa before were detected, which are *Cryptosporidium* spp. (Donkey genotype) and *Cryptosporidium* spp (Cuniculus).

- Weak negative correlation was found between the level of *E. coli* and the presence of *Cryptosporidium* and *Giardia* in influent water samples from WWTP B, but weak to moderate positive relationship was found between *E. coli* and the presence of *Cryptosporidium* and *Giardia* in the rest of the treatment steps in the plant. There was no relationship between *E. coli* and the presence of *Giardia* in the final effluent from this water source. A weak correlation shows that the increase in the level of *E. coli* had a very low likelihood of the presence of *Cryptosporidium* and *Giardia.* Weak negative correlation shows that the presence of *E. coli* does not necessarily mean *Cryptosporidium* and *Giardia* are present in any water sample and verse versa. In summary, faecal indicators cannot be used as a tool to predict the presence or absence of *Cryptosporidium* and *Giardia* in any water sample.
- In general, weak negative to poor positive correlation was found between *E. coli*, faecal *enterococci* and the presence of *Cryptosporidium* and *Giardia* in all WWTPs. Lack of correlation or little correlation between the indicator organisms (Faecal enterococci and *E. coli*) and the protozoan parasites (*Cryptosporidium* and *Giardia*) indicates that wastewater monitoring needs not to be done only on indicator organisms but needs to be investigated further for the presence of protozoan parasites because of their high resistance to chemical disinfectant. This is because indicator organisms may not be adequate to accurately envisage the safety of water in terms of *Cryptosporidium* and *Giardia* as they may occur in the absence of the indicator organisms.
- *Cryptosporidium* and *Giardia* are known as important enteric protozoan pathogens and have challenged water and health authorities by their ability to resist chlorine disinfection and filtration. This study showed reduction of the protozoan's contaminants from all treatment points which might have been the results of proper chlorination treatment at the selected plants in this study. Nevertheless, the selected treatment plants and rivers did not achieve a complete removal of the target protozoan parasites. It is well known that the infective dose for protozoan parasites is low and the ingestion of one (oo)cyst is sufficient to cause infection and disease that takes the form of gastroenteritis and diarrhea. Thus, the presence of these parasites in wastewater effluents represents a threat to public health upon downstream exposure. Moreover, the occurrence of *Cryptosporidium and Giardia* in incoming wastewater is a reflection of the level of infection or transmission in the

connected population and the outgoing concentrations defines the subsequent human health risks for downstream populations and activities.

- A reduction of the parasites from the influent to the final effluent occurred before discharge to the recipients. Despite the wastewater treatment plants removal efficiencies *Cryptosporidium* and *Giardia* also occur from upstream source of pollution to rivers. The use of the effluents or the receiving water bodies are a potential health risk for recreational and agricultural purposes.
- Open defecation downstream from the effluent point was observed at one of the sampling sites. It is recommended that wastewater or environmental and recreational water should be jointly treated by government and non-governmental organizations. Secondly, rivers and wastewater should be monitored regularly, and test should be conducted for the presence of protozoan parasites (*Cryptosporidium* and *Giardia*) consistently. Lastly, epidemiological studies and quantitative risk analysis of the presence of these target protozoans (*Cryptosporidium* and *Giardia*) should be carried out in order to provide suitable control strategies against these waterborne protozoan parasites.

CHAPTER 5: EFFECT OF CHLORINE AND UV IN THE INACTIVATION OF *CRYPTOSPORIDIUM* AND *GIARDIA* IN WASTEWATER

5.1 Introduction

Wastewater from wastewater treatment plants may contain many different pathogenic microorganisms including *Cryptosporidium* and *Giardia* (Abeywardena et al., 2015). *Cryptosporidium* and *Giardia* are resistant to many chemical treatments and methods and are thus removed to a lower extent than many other pathogens (CDC, 2016).

As pointed out in Chapter 2, the protozoan parasites, *Cryptosporidium* and *Giardia*, are known causative agents for gastrointestinal diseases both in the normal population and especially amongst the immunocompromised (Cama and Mathison, 2015). The mode of transmission is through direct contact, contact with animals or through the ingestion of contaminated water or food (Juranek, 1997).

Both *Cryptosporidium* oocysts and *Giardia* cysts are very resilient and may survive in water for months. In addition, they are well known to be resistant to chemical disinfection (Campbell et al., 1995), which has always posed challenges for water treatment authorities, wastewater treatment plant operators and catchment authorities. Chlorination has been widely used as a disinfectant in water and wastewater treatment against many pathogens. However, for *Cryptosporidium* spp especially, the resistance against chlorine is a serious challenge in standard water treatment processes (Craun et al., 2010). Furthermore, *Giardia* are judged as highly chlorine resistant whereby both protozoan parasites render treatment under the South African conditions inefficient (Zhou et al., 2014). When used, low doses of chlorine are globally encouraged, since high chlorine doses may cause ecological side effects from chlorinated hydrocarbons (Pereira et al., 2008). Due to the resistance of *Cryptosporidium* to chlorine, alternative disinfectants such as chlorine dioxide, ozone and ultraviolet (UV) disinfection have been suggested and are in use in many countries (USEPA, 2012).

UV offers an alternative for the removal of *Cryptosporidium* and *Giardia* from both water and wastewater treatment plants. The use of UV irradiation has been growing extensively in water

treatment due to its demonstrated high efficiency in the inactivation of *Cryptosporidium* and *Giardia* (Hijnen et al., 2006). UV, as well as chlorination treatment is normally applied as the last step in wastewater treatment, but the security and reliability normally depends on the combination of treatment steps, where the multi-barrier approach enhances the treatment robustness. The advantages of UV over chlorine as a preferred technique in addition to the inactivation efficiency is the lack of disposal problems, as well as it being compact with a robust design, enhancing the durability in handling and transition. Some of the limitations of UV in developing countries especially include dependency on constant and secure power supply, which is not always available in many parts of developing countries (Vilhunen et al., 2009).

As few as 10 cysts or oocysts can cause infection. Therefore, the detection methods need to be sensitive and reliable. Effective methods of detection need to be developed for the detection of (oo)cysts in wastewater (Vesey et al., 1994). Therefore, the sensitivity and reliability of methods of detection of *Giardia* and *Cryptosporidium* are important. The requirements of detection of 1 cyst per 10 to 100 litres of water are expressed for *Giardia* (Keserue et al., 2011). Flow cytometry (FCM) methods for detection have been proposed with the use of many staining methods. Some of these staining methods produce false-positive results (Hsu et al., 2005). While the microscopy method is laborious with many hours of laboratory experiments, flow cytometry offers a better alternative (Vesey et al., 1994, Nie et al., 2016). Microscopy may also be affected by mineral particles, algae and plant that may interfere with the results in environmental samples. Additionally, skilled operators are required for microscopy procedures, with the need to process many samples at once (Nie et al., 2016, Vesey et al., 1994). Similarly, flow cytometry may also be disadvantageous in its ability to distinguish between oocysts and some auto-flourescent plants, algae and mineral particles (Nie et al., 2016).

In the current study, different doses of chlorine and varying exposure times were used with distilled water and wastewater spiked with (oo)cysts from wastewater samples. In parallel, same samples were assessed for UV irradiation treatment effect at different UV doses. Microscopy and flow cytometer were used for the quantification and detection of *Cryptosporidium* and *Giardia* in wastewater. Syto9+PI and DAPI+PI dyes were used for staining the collected wastewater samples and results were compared. This pilot experiment in this study aimed at assessing the effect of

chlorine and ultraviolet (UV) irradiation in the inactivation of *Cryptosporidium* and *Giardia* in contaminated water. “Inactivation” has here been defined, for the purpose of this study, as the specific change when stained with Syto9+PI and DAPI+PI dyes and “viability” as the remaining unchanged (oo)cysts.

5.2 Methodology

In this study, different doses of chlorine and varying exposure times were used with distilled water and wastewater spiked with (oo)cysts from wastewater samples. In parallel, the same samples were treated with UV irradiation at different UV doses. Microscopy and flow cytometry were used for the detection of *Cryptosporidium* and *Giardia,* where Syto9+PI and DAPI+PI dyes were used for staining the wastewater samples for comparative assessments. Purification of oocysts was done using the methods described in Chapter 3 in section 3.5.1. Chlorine treatment of (oo)cysts was done as explained in section 3.5.2 in Chapter 3. The section also describes the method of calculating the residual chlorine doses used and a description of the UV doses and their method of calculation. The viability test used for (oo)cyst after treatment with chlorine and UV using vital dye Syto-9+Propidium iodide, and with the inclusion of DAPI+ Propidium iodide staining, is explained in section 3.5.3 of Chapter 3. The staining procedure with Syto-9 and PI is given in section 3.5.4 of Chapter 3. The descriptions of reagent quality and performance check of the fluorogenic dyes, staining procedure of DAPI and PI, microscopy and flow cytometry are given in sections 3.5.4, 3.5.5, 3.5.6 and 3.5.7 respectively.

5.2.1 Data Analysis

Summary statistics were performed on the data after entering them into Microsoft Excel 2016 (Microsoft Corporation, California, USA). The percentage viability after exposure of the *Cryptosporidium* and *Giardia* to varying doses of UV and chlorine was calculated. The concentration of viable (oo)cysts was captured and its normality checked using the Shapiro-Wilk’s Normality test, with a 95% confidence interval. A comparison of viability after exposure to all the different doses of UV and chlorine, as well as the impact of varying durations of exposure, was done with the Kruskal-Wallis test. Further comparisons between specific doses and duration (two doses) were done with the Mann-Whitney U-tests. The effect of microscopy and flow cytometry

in the quantification of different concentrations of (oo)cysts was also compared using the Mann-Whitney U-tests. The check for normality and comparison tests were done in GraphPad Prism (Version 5, GraphPad Software, California, USA).

5.3 Results and discussion

The effects of UV as an indicator of viability at different doses are presented and discussed in section 5.3.1, while section 5.3.2 presents the effect of different doses of chlorine and duration of exposure on viability. In both sections, comparisons between UV and chlorination are presented and discussed. In section 5.3.3, the effect of turbidity on viability and comparisons between the results obtained using the two different dyes, Syto9+PI and DAPI+PI, are presented and discussed. Finally, Section 5.3.4 presents the comparison between the microscopy and flow cytometry results during the experiment.

5.3.1 Effect of UV on viability

Table 5.1 presents the effect of the UV dose on the *Giardia* cysts and *Cryptosporidium* oocysts as measured with microscopic counts using DAPI+PI and Syto9+PI. Table 5.5 presents the same effect as measured with flow cytometry counts using the same dyes (DAPI+PI and Syto9+PI). The comparative impacts on the in distilled water versus in wastewater are further presented in Figure 5.1 and the effect due to different stains in Figure 5.2. Overall, the UV effect is higher on *Giardia* than *Cryptosporidium,* but with a common cut-off value of 20mJ/cm^2 based on selected doses (Table 5.1). A comparison of all the doses of UV showed significant impact on test results, with decreasing values as the dose increases (p values of 0,0234 for *Cryptosporidium* and 0,0148 for *Giardia*). However, comparisons between two specific doses as presented in Table 5.4 showed that from 10.4 mJ/cm^2, the difference differed significantly from the least dose of 5.2 mJ/cm^2. Qian et al. (2005) reported that UV irradiation usually inactivates *Cryptosporidium* by attacking the nucleic acid, thereby preventing multiplication of the parasite. *Giardia* is thus less resistant to UV irradiations than *Cryptosporidium,* in line with the conclusion by Rahdar and Daylami (2016) that *Giardia* responded very well to UV irradiation. It can be observed that UV irradiation has more of an effect on *Giardia* than chlorine (Table 5.1). Die-off of the (oo)cysts is directly proportional to the increase in exposure time and the UV doses (Sutthikornchai et al., 2016). The sensitivity of

Giardia to UV irradiation was higher than *Cryptosporidium,* as found in the obtained results (Table 5.1) and was also observed by Rahdar and Daylami (2016). Campbell and Wallis (2002) showed that UV irradiation at 20 to 40 mJ/cm^2 is capable of killing 99.9% of *Giardia* cysts, which supports the findings in this thesis. However, the current findings as presented below deviate partly from the results obtained by Rahdar and Daylami (2016) and Campbell and Wallis (2002), probably due to differences in test regimes and methods used. Rahdar and Daylami (2016) counted dead cysts using conventional microscope. The (oo)cysts used in this study were extracted from environmental water samples, while Rahdar and Daylami (2016) used (oo)cysts extracted from feces of chronic infected patients. This may account for the different results generated in this present experiment.

Morita et al. (2002) found slightly contradicting concentrations. In their experiments, a two-log reduction in *Cryptosporidium* excystation required a high UV dose of 230mWs/cm^2 for the fresh faecal samples they tested in purified water. The UV doses required for 1-, 2-, and 4-log reduction in infectivity in the purified water with fresh faecal samples were 0.48, 0.97 and 1.92mWs/cm^2 respectively, which are higher than the doses used in the present study. Moreover, a 200 times higher dose will be required for a 2-log reduction in viability than reduction in infectivity as assessed by *in vitro* excystation. This demonstrates that if *Cryptosporidium* oocysts are exposed to low doses of UV irradiation, they may still be able to excyst in the purified water with faecal samples (Morita et al., 2002).

In Table 5.1, the mean percentages and standard deviation of *Cryptosporidium* and *Giardia* at different UV doses gave a remaining 1±1% of viable *Cryptosporidium* at 83.2mJ/m^2. At 20.8 mJ/m^2, all *Giardia* were already dead. Prolonged UV exposure could also be more potent than short exposure, as reported by Sutthikornchai et al. (2016). Moreover, at low UV doses, a fraction of viable *Cryptosporidium* and *Giardia* were found dead (Table 5.1), where a remaining 47% and 25% of *Cryptosporidium* and *Giardia* were counted as viable respectively at a 5.2mJ/cm^2 dose. It can further be established that UV irradiations have more effect on *Giardia* than *Cryptosporidium* in all the trials as reported (Table 5.1; Figures 5.1 and 5.2). *Cryptosporidium* reduction in viability due to UV exposure in wastewater is encouraging and also in agreement with King et al. (2008), who found that solar UV can rapidly inactivate *Cryptosporidium* in environmental waters.

Figure 5.1 illustrates the reduction in mean percentage viability of *Cryptosporidium* and *Giardia* as related to treatment with different UV doses. The results presented in Figure 5.2 represent the mean percent viability using both dyes for the 5 trials (that is Syto9+PI in trials 1 to 3 and DAPI+PI in trials 4 to 5). All *Giardia* were eliminated at the 20.8mJ/cm^2 of UV dose, which confirms its efficiency in removing *Giardia* from wastewater. Less than 2% of *Cryptosporidium* was counted as viable after 83mJ/cm^2 UV exposure. An increase in exposure dose can significantly reduce the viability of both *Cryptosporidium* and *Giardia* in water. The obtained results of this study reiterated earlier findings that UV disinfection is much more effective than chlorination (see for example Ashbolt (2004)). This agrees with previous studies where the efficacy of UV over chlorine has been demonstrated in the removal of *Cryptosporidium* and *Giardia* in wastewater (Hijnen et al., 2006, King et al., 2008, Morita et al., 2002, Würtele et al., 2011).

5.3.2 Effect of chlorination

Table 5.1 presents the mean percentages of *Cryptosporidium* and *Giardia* after exposure to 0.5ppm, 2ppm and 5ppm of chlorine during different exposure times. For 0.5ppm, *Giardia* became non-viable as assessed by the dyes after 60 minutes of exposure, except in trial four where they were extinct after 30 minutes. Half of the *Cryptosporidium* were still counted as viable (42 to 47% in distilled water and 42 to 51% in wastewater) after 120 minutes of exposure time. The difference after exposure to the different doses and varying durations was significant. Comparisons of specific durations of exposure at the various doses showed that irrespective of the dose, exposure of *Giardia* for 15 and 30 minutes did not result in any significant difference in their viability, except for exposure to 2 ppm of chlorine (Table 5.2). The findings of Rahdar and Daylami (2016) concur that lower concentrations of chlorine result in limited elimination of oocysts. Since the reduction in distilled water and in wastewater were similar, the data has been treated jointly. Thus, the counted mean viable fraction of *Cryptosporidium* decreased from 86±2% after 15 minutes' exposure to 0.5 ppm of chlorine to 46±4% viability after 120 minutes. However, 0.5 ppm of chlorine was not judged as effective for *Cryptosporidium* removal, even after a long exposure time (120 minutes) when compared to the effect on *Giardia* in wastewater (Figure 5.3). A low dose of chlorine (0.5ppm) with about 60 minutes of exposure time may be sufficient for *Giardia* removal as all *Giardia* present were counted as non-viable after 60 minutes. These results were corroborated in the World Health Organisation study (WHO, 2002) which reported that *Giardia*

is less resistant to disinfection including chlorination. In Table 5.1, at 2 ppm, it was found that all *Giardia* became non-viable after 15 minutes in all the four trials (2 to 5), except in trial one where an extended time of up to 30 minutes was needed. A total reduction of *Giardia* was obtained with a chlorine dose of 0.5 ppm after 60 minutes (as for 2 ppm after 15 minutes of exposure time). The effectiveness can thus be either enhanced by increased exposure time or through an increase in dose (Omarova et al., 2018). The statistical significance in the difference in viability after these different durations of exposure and doses supports this (Tables 5.2 and 5.3).

Cryptosporidium is smaller and more robust than *Giardia* and withstands the chlorine doses that are used in the water treatment processes. Their resistance has resulted in several waterborne outbreaks worldwide (Squire and Ryan, 2017, Efstratiou et al., 2017, Craun et al., 2010, Craun et al., 1998). Water authorities need to combine multiple treatment barriers for efficient *Cryptosporidium* removal in wastewater (Bukhari et al., 2000).

The results presented in Figure 5.3 compares the mean percentage viability using direct microscopy with Syto9+PI in trials 1 to 3 and DAPI+PI in trials 4 to 5. In Figure 5.4(c), there is a steady reduction in the viability of *Giardia* until after 30 minutes, when all *Giardia* were dead for 5ppm. A further increase in the duration of exposure will not really influence the viability of *Giardia,* as found when comparing after 50 minutes for both 0.5ppm and 2ppm (Figures 5.3(a) and (b)) and 30 minutes for 5ppm (Figure 5.3(c)). For *Cryptosporidium*, a reduction in viability did occur which was dependent both on concentration and exposure time, as seen by comparisons of Figure 5.3 (a), (b) and (c). In conclusion, an increase in exposure time may be significant up to a threshold, in this case at 100 minutes for 5ppm and 50 minutes for 0.5ppm and 2ppm, after which increases in exposure time may not have any appreciable reduction in viability. This points out that *Cryptosporidium* is more resistant to chlorine than *Giardia* when exposed under the same environmental conditions (Bukhari et al., 2000, Korich et al., 1990, Betancourt and Rose, 2004). In summary, an increased dose is more effective in the inactivation of both *Cryptosporidium* and *Giardia* than exposure duration. However, chlorine at higher doses may not be recommended due to toxic by-products such as trihalomethanes (THMs) (Rook and Evans, 1979). Higher doses above 2ppm concentrations are not used in conventional water treatment and are also not

economically viable in relation to water treatment stations (Pereira et al., 2008), and by extension wastewater treatment.

Table 5.1: Mean and SD of viability (%) after exposure to chlorine and UV irradiation using microscopy (n=5)

Duration of Exposure(min)	Chlorine dose			UV	
	0.5 ppm	2 ppm	5 ppm	UV dose (mJ/cm^2)	Mean±SD
Cryptosporidium					
15	86±2	80±6	42±8	5.2	47±5
30	70±6	67±4	32±2	10.4	22±7
60	55±6	59±5	23±2	20.8	5±1
120	46±4	50±6	15±3	41.6	3±2
				83.2	1±1
Giardia					
15	57±32	7±0	7±0	5.2	25±7
30	30±21	ND	ND	10.4	7±9
60	ND	ND	ND	20.8	ND
120	ND	ND	ND	41.6	ND
				83.2	ND

*ND means undetectable.

Table 5.2: Statistical difference in the reduction of viability from varying durations of exposure to doses of chlorine

Duration of exposure to 0.5 ppm Chlorine dose (minutes)						
	15 vs 30	15 vs 60	15 vs 120	30 vs 60	30 vs 120	60 vs 120
Cryptosporidium	0.1161	0.0079	0.0079	0.0079	0.0079	0.0157
Giardia	0.2420	0.0014	0.0012	0.0022	0.0024	-
Duration of exposure to 2 ppm Chlorine dose (minutes)						
	15 vs 30	15 vs 60	15 vs 120	30 vs 60	30 vs 120	60 vs 120
Cryptosporidium	0.1161	0.0079	0.0079	0.0079	0.0079	0.0157
Giardia	0.0128	0.0102	0.0140	-	-	-
Duration of exposure to 5 ppm Chlorine dose (minutes)						
	15 vs 30	15 vs 60	15 vs 120	30 vs 60	30 vs 120	60 vs 120
Cryptosporidium	0.0079	0.0079	0.0079	0.0079	0.0079	0.0119
Giardia	0.1068	0.0114	0.0088	-	-	-

Table 5.3: Statistical difference in the reduction of viability from varying doses of chlorine for the same duration of exposure

Chlorine dose for 15 minutes of exposure (ppm)			
	0.5 vs 2	0.5 vs 5	2 vs 5
Cryptosporidium	0.8642	0.0456	0.0484
Giardia	0.0156	0.0154	-
Chlorine dose for 30 minutes of exposure (ppm)			
	0.5 vs 2	0.5 vs 5	2 vs 5
Cryptosporidium	0.9682	0.0428	0.0498
Giardia	0.0012	0.0012	-
Chlorine dose for 60 minutes of exposure (ppm)			
	0.5 vs 2	0.5 vs 5	2 vs 5
Cryptosporidium	0.8280	0.0354	0.0412
Giardia	-	-	-
Chlorine dose for 120 minutes of exposure (ppm)			
	0.5 vs 2	0.5 vs 5	2 vs 5
Cryptosporidium	0.7140	0.0058	0.0062
Giardia	-	-	-

Table 5.4: Statistical difference in the viability of *Cryptosporidium* and *Giardia* for different UV doses (mJ/cm^2)

	5.2 vs 10.4	5.2 vs 20.8	5.2 vs 41.6	5.2 vs 83.2	10.4 vs 20.8	10.4 vs 41.6	10.4 vs 83.2	20.8 vs 41.6	20.8 vs 83.2	41.6 vs 83.2
Cryptosporidium	0.0079	0.0079	0.0079	0.0079	0.0079	0.0079	0.0079	0.0079	0.0079	0.0119
Giardia	0.0060	0.0059	0.0218	0.0060	0.0060	0.0060	0.0328	0.0079	0.0079	0.0218

Table 5.5: Mean and SD of viability (%) of (oo)cysts after exposure to chlorine and UV irradiation using flow cytometry

Number of Exposure time		Chlorine dose			Number of Exposure time	UV	
	Duration of Exposure (min)	0.5 ppm	2 ppm	5 ppm		UV dose (mJ/cm^2)	
1	15	82±3	1	41±1	1	5.2	43±1
2	30	79±1	2	33±1	2	10.4	12±1
3	60	71±1	3	21±1	3	20.8	10±0
4	120	66±3	4	12±0	4	41.6	8±0
					5	83.2	3±0

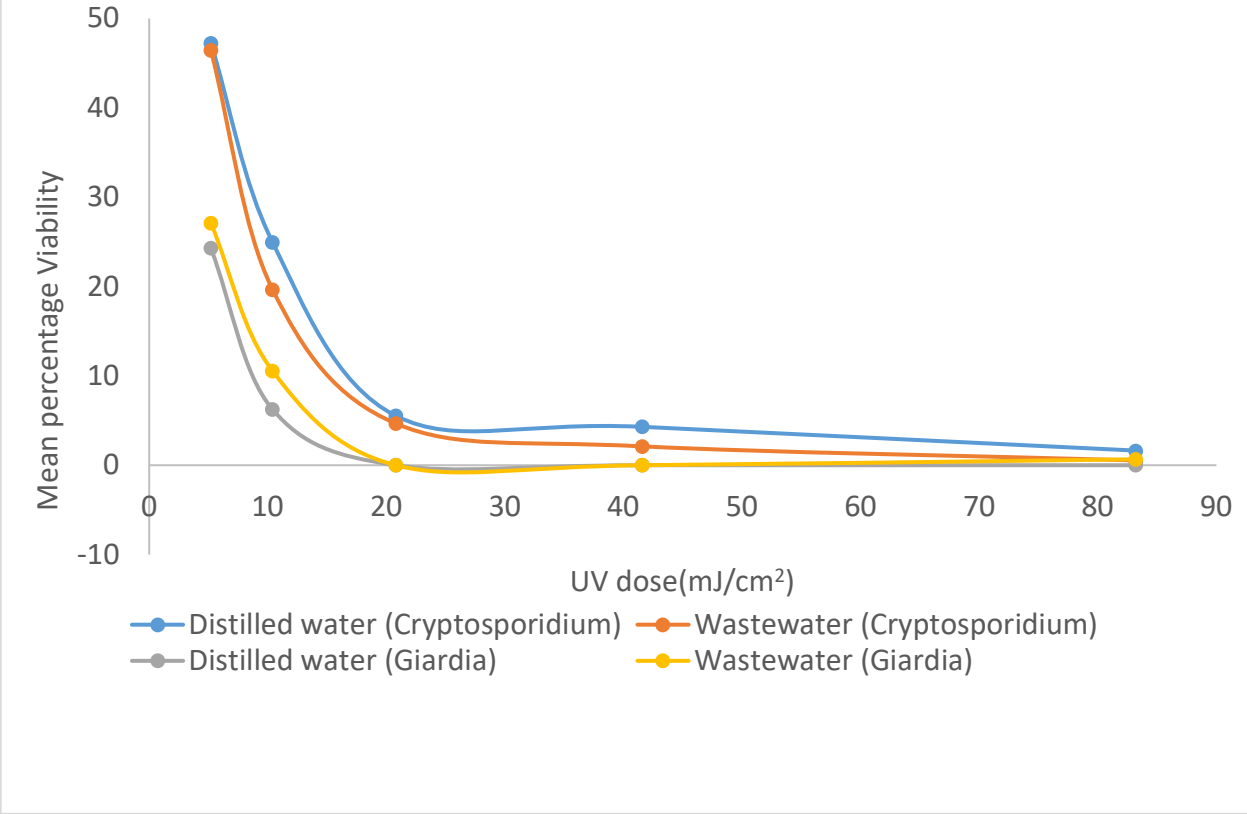

Figure 5.1: Mean percentage viability of *Cryptosporidium* and *Giardia* related to UV doses in treatment with spiked samples in distilled water and wastewater samples

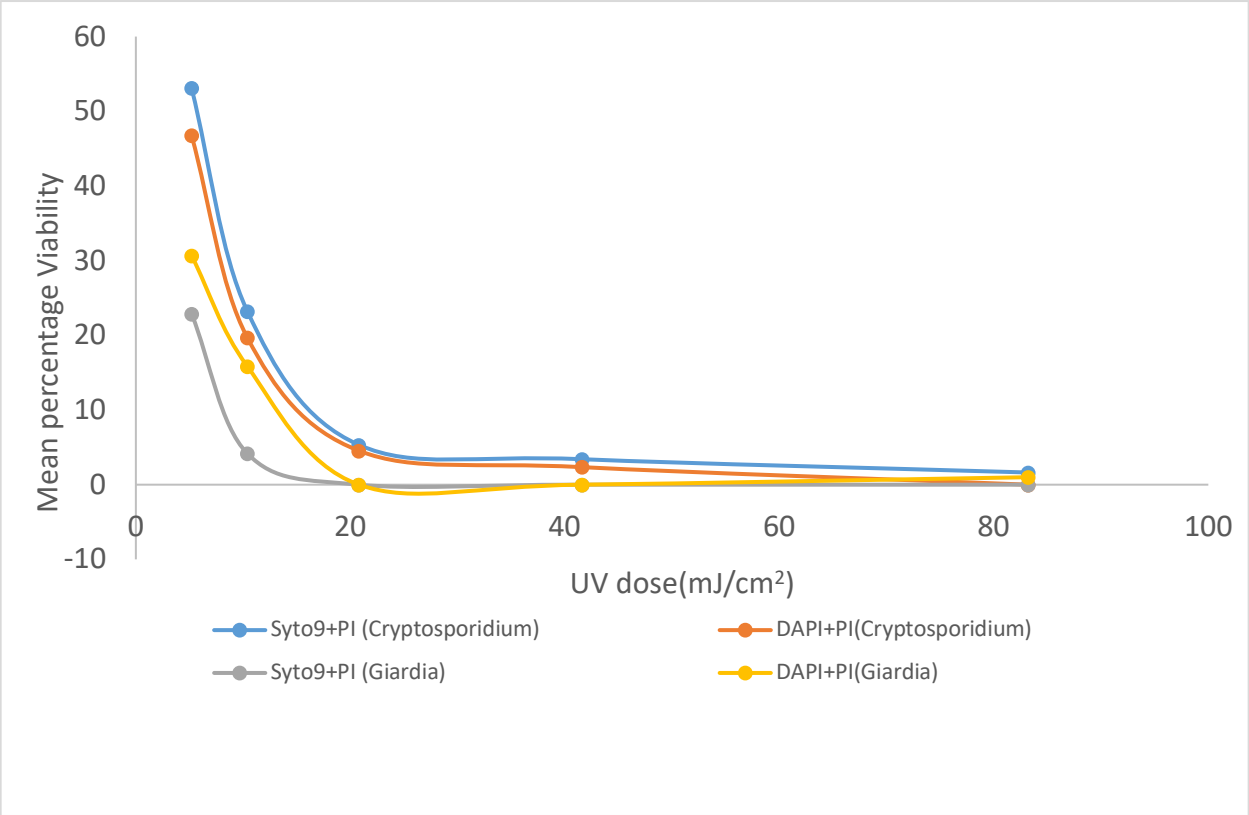

Figure 5.2: Mean percentage viability of *Cryptosporidium* and *Giardia* at different UV doses using Syto9+PI and DAPI+PI dyes

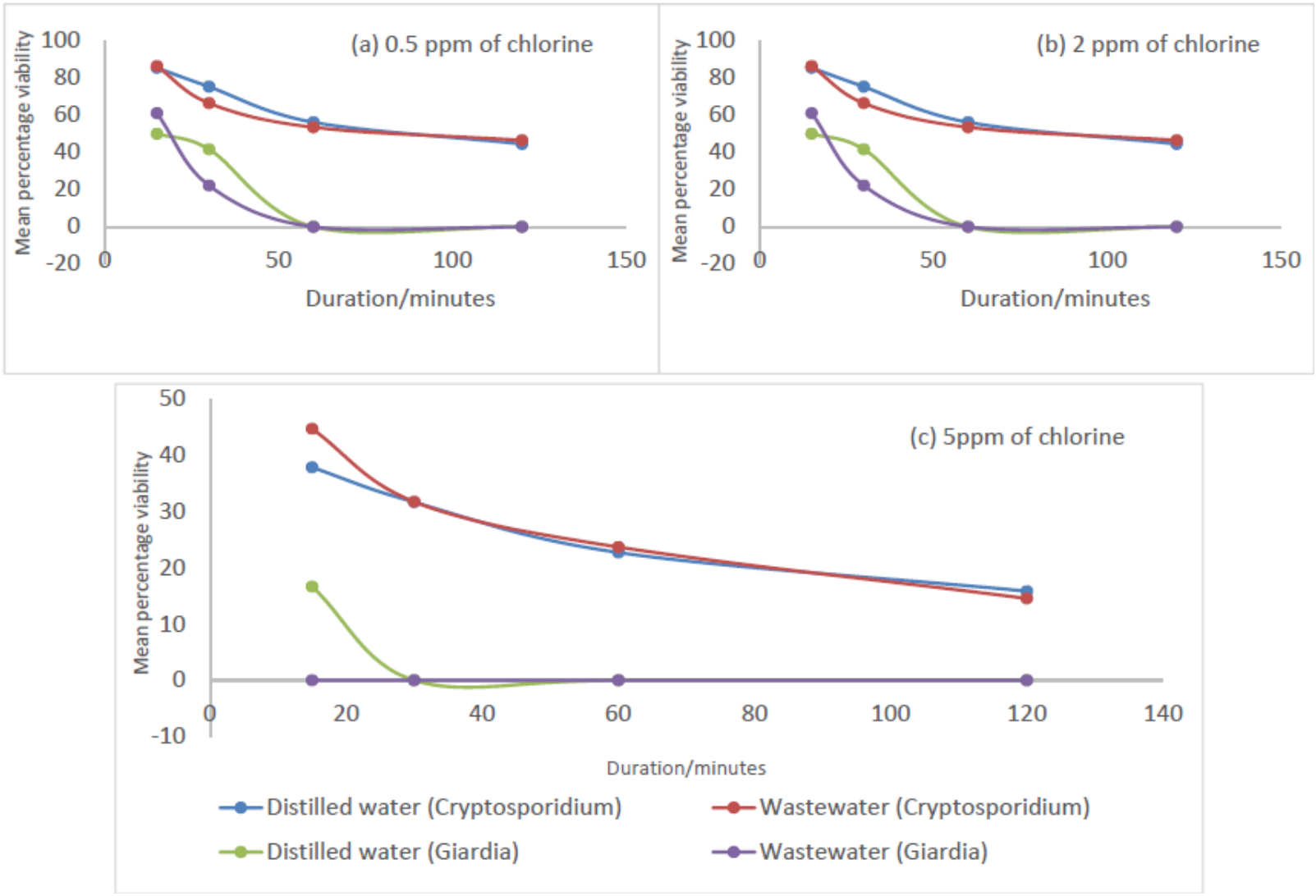

Figure 5.3: Mean percentage viability of *Cryptosporidium* and *Giardia* after prolonged exposure to 0.5ppm, 2ppm and 5ppm of chlorine treatments for distilled water and wastewater

5.3.3 Effect of Turbidity

When comparing the results between the distilled water and the wastewater experiment, a slightly higher fraction of counted viable (oo)cysts were obtained in the wastewater samples, especially in chlorine treatment (Table 5.6). The turbidity would affect the water treatment efficiency using UV irradiation, as depicted in Table 5.6 when both *Cryptosporidium* and *Giardia* are considered together. The mean percentage viabilities for distilled water and wastewater for all UV exposures were comparable for *Cryptosporidium*. This shows that viability was not really affected by opacity for *Cryptosporidium* removal. For *Giardia*, higher values were recorded for wastewater in many of the trials, showing that higher turbidity causes increase in viability due to UV opacity. The mean percentage viability for distilled water (Trials 1 and 2) and wastewater (Trials 3 to 5) are however comparable with values for wastewater slightly higher at lower exposure durations (15 to 30 minutes), especially for *Giardia*. Turbidity thus slightly affected the treatment efficiency, especially when low doses of chlorine were applied. *Cryptosporidium* percentage viability for distilled water and wastewater were comparable. Water with low turbidity responds better to chlorine treatment than turbid water (wastewater).

5.3.4 Comparison between Microscopy and Flow cytometry

When assessing the results obtained using microscopy as compared to flow cytometry for UV irradiations (in Table 5.6), the results for both methods showed decreasing values with increasing irradiation. The results are presented for both microscopy and flow cytometry in Table 5.5. It can be observed further that microscopy has a higher mean percentage values at 15 minutes, while flow cytometry has higher percentage values at 30 minutes, 60 minutes and 120 minutes for 0.5ppm. This shows that flow cytometry gives higher mean percentage values than microscopy, especially at 0.5ppm chlorination for both distilled water and wastewater. In Table 5.6, trials two and three show that *Cryptosporidium* and *Giardia* have higher mean percentage values at Trial two (distilled water) than Trial three (wastewater), using the same dye (Syto9+PI) for staining. This shows that under the same condition and staining, chlorination may have lower effect on wastewater than distilled water. Therefore, turbidity plays an important role of the effectiveness of chlorination.

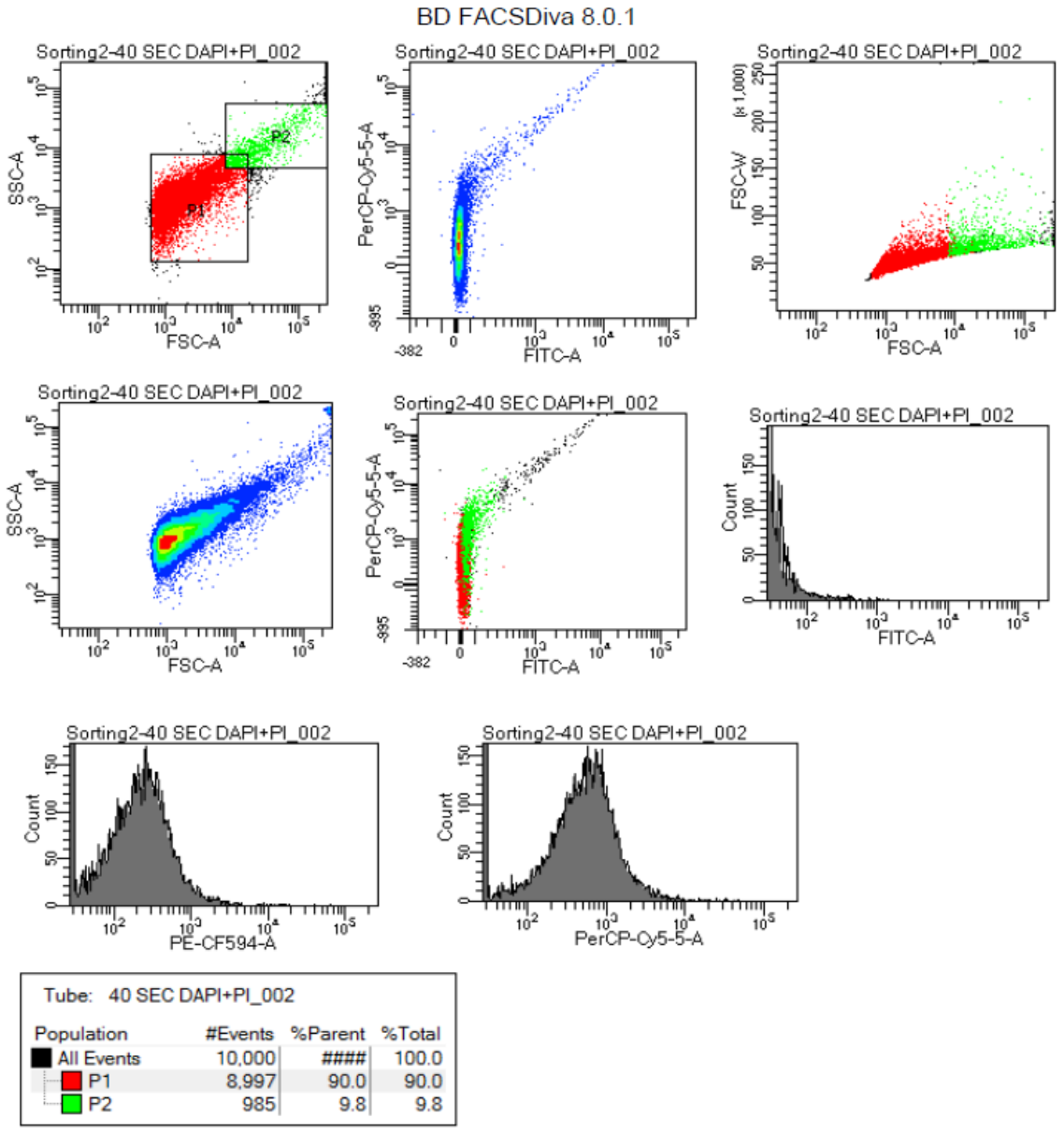

Figure 5.4: Illustration of flow cytometry histogram and dot plots showing viability of *Cryptosporidium* and *Giardia* at 40 seconds after exposure to UV irradiation

Note: Samples of Region P1 and P2 represent gates for non-viable and viable (oo)cysts with P1 as side-scatter (SSC) against forward side-scatter (FSC).

Samples of flow cytometry histograms and dot plots showing dye viability response of *Cryptosporidium* and *Giardia* are illustrated and exemplified in Figure 5.4. Samples of Region P1 and P2 represent gates for non-viable and viable (oo)cysts with P1 as side-scatter (SSC) against forward side-scatter (FSC). Histograms represent percentages of non-viable (oo)cysts plotted with count against PE-CF594-A and PerCP0-Cy5-5-A. The gates P1 and P2 were used to illustrate a subgroup of data. Statistics was generated using the population and also to limit the number of events collected. The gated region shows the sample of mixed population of cells. The quantitative results of all the cytometry histogram and dot plots are presented in Table 5.6 for chlorine and Table 5.7 for UV.

Comparison of the concentration of oocysts/cysts detected using both the microscopy and flow cytometry showed that detections at 30, 60 and 120 minutes of exposure showed a significant difference. At 5.2 mJ/cm^2 of UV, the concentrations detected by both microscopy and flow cytometry did not vary significantly, however with increasing doses there was significant difference in the concentrations. This may be due to the reduction in (oo)cysts concentration that may affect the limits of detection or the sensitivity of these techniques. This shows that the concentrations detected differed between the microscopy and flow cytometry may be due to difference in the detection techniques. The difference in concentrations detected by these two techniques could also be attributed to chance or the uneven distribution of these oocysts/cysts in the samples.

5.3.5 Comparison between Syto9+PI and DAPI+PI Vital Dyes

Tables 5.6 and 5.7 present the comparison between the results of microscopy and flow cytometer. While the first three trials are the results using Syto9+PI stains, the last two trials present results using DAPI+PI stains. The type of stain used for the experiment can have effect on the results as is evident in Figure 5.2. The figure presents the results of the two dyes independently. Syto9+PI was used for trials one to three and DAPI+PI was used for trials four to five. Syto9+PI dyes gives slightly higher percentages than DAPI+PI. Therefore, Syto9+PI is slightly more sensitive than DAPI+PI for *Cryptosporidium* and *Giardia.* This is notable because vital dyes may overestimate *Cryptosporidium* and *Giardia* in wastewater (Huffman et al., 2000).

Largely, the results of mean percentage values using Syto9+PI stains (trials 1 to 3) for (oo)cysts in Table 5.7 were slightly higher than those using DAPI+PI stains (trials 4 to 5) at the initial stages of the experiment (Trials 1 to 3) and became comparable at Trials 4 and 5 because of overestimation of *Cryptosporidium* and *Giardia* as mentioned earlier (Huffman et al., 2000). The percentages of flow DAPI and PI of (oo)cysts are presented for different doses and exposure time for chlorine (Table 5.6). At 0.5ppm, 85±3% reacted as viable at 15 minutes and 17% as dead. This decreased to 66±3% counted viable and 34% dead at the end of the trials at 120 minutes. The same results were recorded at 2 ppm. Nonetheless, at 5ppm, 41±7% was counted viable and 59% dead at 15 minutes and 12±0% and 88% dead at the end of the trial (120 minutes). In Table 5.7, the efficacy of UV was amply demonstrated in the trials that only 3% were counted viable at 83.2mJ/cm^2 (160 minutes) and 97% dead. At 10.4mJ/cm^2 (20 seconds), 11.0% were alive, which is lower than the highest dose of 5ppm of chlorine (41%) at 15 minutes. Meanwhile after 20 seconds, there was a slight reduction in percentage alive with UV exposure unlike a significant decrement in percentage from 0 to 10 seconds and 10 seconds to 20 seconds which was 56% and 88% respectively. Surprisingly, increase in the duration afterwards was not significant. Morita et al. (2002) suggested oocysts has high resistance to UV irradiation and high UV dose may be required. Then again, UV may not remove all incidences of (oo)cysts as 3% was still alive after 83.2 mJ/cm^2 (160 seconds).

Table 5.6: Comparison between the percentages of viability of (oo)cysts when using microscopy and flow cytometer for chlorine exposures

	0.5 ppm							
DURATION /min	15#		30*		60*		120*	
Trials	Microscopy	flow cytometer	Microscopy	flow cytometer	Microscopy	flow cytometer	Microscopy	flow cytometer
	83	85	75	76	51	71	40	67
	85	82	73	80	58	72	46	67
	82	77	68	81	45	70	41	63
	89	83	70	79	57	69	50	69
	86	81	58	78	54	72	45	66
MEAN± SD	**85±3**	**82±3**	**69±6**	**79±1**	**53±6**	**71±1**	**44±4**	**66±3**
2 ppm								
	79	85	63	76	57	71	46	67
	77	82	70	80	61	72	53	67
	70	77	61	81	52	70	44	63
	77	83	70	79	63	69	54	69
	84	81	62	78	54	72	45	66
MEAN± SD	**77±6**	**82±3**	**65±5**	**79±1**	**57±5**	**71±1**	**48±5**	**66±3**
5 ppm								
	42	40	33	31	24	21	14	12
	32	42	28	32	20	21	16	13
	42	41	33	33	24	21	11	12
	39	42	30	35	21	21	14	13
	50	42	30	33	24	23	17	13
MEAN± SD	**41±7**	**41±1**	**31±2**	**33±1**	**23±2**	**21±1**	**15±3**	**12±0**

Note: Trials 1 to 2: Distilled water. Trials 3 to 5: Wastewater. Trials 1 to 3 were performed using Syto-9+PI stains and Trials 4 to 5 were performed using DAPI+PI stains.
#No significant difference between concentrations detected by Microscopy and Flow cytometry
* Significant difference between concentrations detected by Microscopy and Flow cytometry

Table 5.7: Comparison between the percentages of viability of (oo)cysts when using microscopy and flow cytometer for UV irradiations

UV dose (mJ/cm^2)	5.2 mJ/cm$^{2\#}$		10.4 mJ/cm^{2}*		20.8 mJ/cm^{2}*		41.6 mJ/cm^{2}*		83.2 mJ/cm^2	
	Microscopy	Flow cytometer	Microscopy	Flow cytometer	Microscopy	Flow cytometer	Microscopy	Flow cytometer	Microscopy	Flow cytometer
	50	43	30	12	6	9	6	8	2	3
	40	43	17	12	4	10	2	8	1	3
	44	45	18	12	5	10	2	9	2	3
	46	43	20	11	5	10	2	9	0	3
	43	45	19	12	5	10	5	9	2	3
Mean± SD	**45±4**	**44±1**	**21±5**	**12±1**	**5±0**	**10±0**	**3±2**	**8±0**	**1±1**	**3±0**

Note: Trials 1 to 2: Distilled water. Trials 3 to 5: Wastewater. Trials 1 to 3 were performed using Syto-9+PI stains and Trials 4 to 5 were performed using DAPI+PI stains.
#No significant difference between concentrations detected by Microscopy and Flow cytometry
* Significant difference between concentrations detected by Microscopy and Flow cytometry
Note: Difference in concentrations detected after UV dose of 83.2 mJ/cm^2 was not determined due to very low concentrations and same concentrations detected by Flow cytometry for the for all trials.

5.4 Conclusion and recommendations

This study presented the reactions of *Cryptosporidium* oocysts and *Giardia* cysts to UV irradiations and chlorine at different doses and exposure time. It has been found that the effect of UV on *Giardia* cyst is more pronounced than the effect of chlorine at 0.5 ppm, 2 ppm and 5 ppm. More *Giardia* cysts became non-viable at lower chlorine concentration compared to *Cryptosporidium* oocysts. Longer exposure time for *Cryptosporidium* oocysts is needed for reduction in viability. Increasing the chlorine doses resulted in higher reduction of viability for *Cryptosporidium* and *Giardia*. This same trend was evident with UV irradiations. However, longer exposure time can also play significant role in the reduction in viability of *Cryptosporidium* and *Giardia* in wastewater treatment notably up to a threshold exposure time. After that further exposure has little or no effect on the viability. It was concluded that *Cryptosporidium* oocyst is more resistant to both chlorine and UV irradiations than *Giardia* in wastewater treatment which is in line with earlier investigations mainly done in drinking water. It was also suggested that since *Cryptosporidium* are resistant to chlorine treatment a combined treatment with UV and chlorine may be needed to eradicate *Cryptosporidium* in water treatment when shorter exposure times are applied. It should be pointed out that the viability methods based on dyes as indicators of the effects from UV and chlorine may not produce absolute values, since variations may occur. Cell tests would probably have given further and larger variations. Additionally, we have not measured infectivity because it was beyond the scope of this study.

This study, therefore, investigated the use of two different vital dyes for determining the viability of *Cryptosporidium* and *Giardia* which are Syto9+PI and DAPI+PI. It was found that Syto9+PI gives higher mean percentage viability values than DAPI+PI in wastewater. This shows that Syto9+PI is more sensitive and may be better than DAPI+PI for estimating the presence of *Cryptosporidium* and *Giardia* in wastewater. The viability of *Cryptosporidium* and *Giardia* were determined after treatment through using microscopy and flow cytometer. It was found that when flow cytometer determinations were made, higher percentage values were obtained than for microscopy at higher doses for both chlorine and UV irradiations. Based on the obtained results flow cytometer may be more sensitive than microscopy in detecting microorganisms especially *Cryptosporidium* and *Giardia*. It was further found that the treatment regime was more effective

in the control where distilled water was used than in the wastewater. It was found that turbidity may affect water treatment using both chlorine and UV irradiation at different doses.

Further studies are suggested in evaluating the effect of water temperature on the UV irradiation for wastewater treatment. Furthermore, there is a need to perform additional experiment on wastewater samples at different pH. Different strains of *Cryptosporidium* and *Giardia* should further be compared. This will provide clarity on reactions of different strains of *Cryptosporidium* and *Giardia* to different doses and exposure time of chlorine and UV. More vital dyes should be evaluated to establish the accuracy of the results obtained in wastewater treatment using chlorine and UV and compared with excystation and infectivity tests for consistency.

CHAPTER 6: GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

6.1 General Discussion

This study presents the demonstration of the level of occurrence of *Cryptosporidium* and *Giardia* in selected wastewater treatment plants and receiving water. The concentrations before treatment and reduction after treatment at each treatment step were investigated. It was found that the treatment steps used in South Africa reduced the concentrations of *Cryptosporidium* and *Giardia* to acceptable levels but no complete removal of the protozoan parasites, especially *Cryptosporidium,* was achieved. Chlorine was shown to not be efficient in removing these protozoan parasites from environmental water samples. The reduction of the concentration at each of the treatment steps were found which shows that many of the parasites were removed during mechanical and biological treatment processes and finally by chlorination to a lesser extent. In this study, the mechanical treatment process was not explicitly measured. However, measurements of reductions achieved after the aeration tanks or trickling filters may include reductions achieved during the mechanical treatment.

Different *Cryptosporidium* and *Giardia* strains were detected in this study, including human and zoonotic genotypes. Novel strains found in this study include the *Cryptosporidium* spp, (Donkey genotype) and *Cryptosporidium* spp (Cuniculus). These have never been reported in Africa. This shows a possible influence of animal waste on the wastewater treatment systems and a possible impact on surface water quality.

Tertiary treatment of wastewater effluents by chlorination is widely practiced in South Africa, however chlorination has been shown to have less impact on (oo)cysts. In this study, it was shown that increasing the dose of chlorine has an effect on the reduction in viability of *Cryptosporidium* and *Giardia.* Additionally, UV may also be used as a tertiary treatment option. Increasing the dose of UV also plays an important role in the reduction of viability of these protozoan parasites. Vital dyes were found to influence the detection of these organisms during the experiment. The distinction between flow cytometry and microscopy was investigated. Flow cytometry was found to be more sensitive than microscopy in detecting the organisms.

The main aim of this research was to demonstrate the level of occurrence of *Cryptosporidium and Giardia* in selected wastewater treatment plants and receiving water and account for its use, for example, in irrigation. The major outcomes and discussion points based on the objectives of the study are presented below;

Research objective 1: To investigate the occurrence and reduction of *Cryptosporidium* and *Giardia* in selected South African wastewater treatment plants in KwaZulu-Natal.

All the four WWTPs investigated in this study had varying concentrations of *Cryptosporidium* and *Giardia* in the final effluents. Reduction in concentration of these parasites was achieved in all the WWTPs, however there was no complete removal. Correlation between faecal *enterococci*, *E. coli* and the protozoan parasites (*Cryptosporidium* and *Giardia*) was measured before and after chlorination. In all treated effluents, a relationship between faecal *enterococci* and *E. coli* scarcely occurred. Low to no correlation was found between the presence of *Cryptosporidium* and *Giardia* and the presence of faecal *enterococci* and *E. coli*. The reason could have been due to the high impact that the chlorine has on the microbial cells but not on the parasitic cells. This study shows that faecal *enterococci* and *E. coli* cannot be used to envisage the presence of *Cryptosporidium* and *Giardia* in water. The results of this objective are presented in Chapter 4 of this thesis.

Research objective 2: To assess the speciation and the viability of *Cryptosporidium* and *Giardia* in environmental samples with the specific aim of evaluating if effluent chlorination affects the viability.

The study employed molecular techniques to identify the species of *Cryptosporidium* and *Giardia* in the water samples. The results showed both human and zoonotic genotypes of *Cryptosporidium* and *Giardia* in the wastewater samples. The genotyping results suggest that the presence of animals might have contributed. NCBI blast results showed that *C. parvum* isolates is the dominate species in the targeted WWTPs followed by *C. hominis.* Novel strains that have never been found in Africa before were detected. These strains are *Cryptosporidium* spp. (Donkey genotype) and

Cryptosporidium spp. (Cuniculus). For *Giardia*, in *ß-giardin* based qPCR (presence absence test), all the 42 samples tested were positive for *Giardia* Assemblage A and *Giardia* Assemblage B.

Furthermore, a pilot scale analysis was performed to assess the effect of chlorine and UV on *Cryptosporidium* and *Giardia* viability. Many *Giardia* cysts became non-viable at lower chlorine concentration than *Cryptosporidium* oocysts. Longer exposure time can play an important role in the reduction in viability of *Cryptosporidium* and *Giardia* in wastewater treatment as shown in Chapter 5. At that threshold exposure time, further exposure has little or no effect on the viability. It was observed and concluded that the *Cryptosporidium* oocyst is more resistant to both chlorine and UV irradiations than *Giardia* in wastewater treatment. The application of two different vital dyes for determining the viability of *Cryptosporidium* and *Giardia* showed that Syto9+PI (The Live/Dead BacLight) gives a higher mean percentage viability value and is more sensitive than DAPI+PI with wastewater. Comparison between flow cytometer and microscopy shows that flow cytometer gives higher mean percentage viability values than microscopy, especially at 0.5ppm chlorination for both distilled water and wastewater and also at higher doses for both chlorine and UV irradiations. The results of this objective are presented in Chapters 4 and 5 of this thesis.

Research objective 3: To assess the downstream effects in receiving water bodies and the relative impact of treated effluent

Objective 3 addressed the downstream effects in receiving water bodies and the relative impact of treated effluent. The concentrations of *Cryptosporidium* and *Giardia* found in the river samples indicate a risk to human health as the treated effluents that were being discharged to the connected river contained *Cryptosporidium* and *Giardia*. Moreover, the presence of *Cryptosporidium* and *Giardia* in the upstream of the river can also contribute to the concentration downstream of the river. Activities around the river could also be the source of contamination to the downstream areas as there are informal settlements living around the river's sampling points. Contaminants brought to the river through rainwater runoff from informal settlements, and also the livestock faeces, represent non-point sources of pollution. All these factors negatively impact the watercourses

when they are discharged to the water system. The results of this objective are presented in Chapter 4 of this thesis.

Research objective 4: To evaluate the impact of seasonal variation on the occurrence of *Cryptosporidium* oocyts and *Giardia* cysts in all sampling sources.

A comparison between different seasons was done for *Cryptosporidium* and *Giardia* in the influent to the wastewater treatment plants and in environmental samples. The occurrence of *Cryptosporidium* and *Giardia* were not significantly affected by the seasonality as there was no direct seasonal variation in the concentrations. Thus, the impact from the connected human population seems to be fairly constant. The results are discussed in Chapter 4 of this thesis.

Research objective 5: To investigate the impact on crops based on the occurrence and quantities of *Giardia* and *Cryptosporidium* in the irrigated water.

Cryptosporidium and *Giardia* were present in the water used for irrigation purposes. The results revealed that the fresh vegetables analysed were contaminated with *Cryptosporidium* and *Giardia* due to the use of the out flowing water from the wastewater treatment plant for irrigation. There was a high concentration of *Cryptosporidium* oocysts and *Giardia* cysts on the crop samples collected and this can be of major health concern. It has been documented in the literature that the infective dosage for protozoan parasites is low and sufficient to cause infection and disease resulting in gastroenteritis and diarrhoea. The presences of *Cryptosporidium* and *Giardia* in these water samples being used for irrigation purposes and on the crops show a potential health risk to the consumers of the crop. The results are discussed in Chapter 4 of this thesis.

6.2 General Conclusion

- As earlier stated in Chapter 4, the presence of *C. donkey* genotype has been reported in donkeys in Algeria and *C. rabbit genotype* was reported in human samples in Nigeria.

However, two hits of *C. donkey* genotype and one hit of *C.* rabbit genotype indicate the presence of similar *Cryptosporidium* genotypes in South African wastewater. Another *Cryptosporidium* species, *C. cuniculus,* which has not been detected in wastewater in Africa, was detected in the selected wastewater samples. This discovery highlights the importance for determining the potential role of animals in distributing zoonotic *Cryptosporidium* to water sources. Wildlife interference in water sources can be a danger in transmitting *Cryptosporidium* in drinking water. An example is an outbreak in the UK as mentioned in Chapter 4.

- There were no recent publications in South Africa that deal with the combination of flow cytometer and microscopy with two vital dyes to assess the viability of *Cryptosporidium* and *Giardia* in environmental samples, which this study has documented.
- This study has documented the occurrence of *Cryptosporidium* and *Giardia* and relatively high and constant values of effluent water in wastewater treatment in South Africa which will be used for wastewater regulation and control.
- It has been documented that crops irrigated with water from the recipient river can also be a source of contamination when consumed.

6.3 Recommendations

Based on the observation during the field investigation, the following are recommended for further studies:

- Presence of rare *Cryptosporidium* genotypes in wastewater is likely to occur, for example, *C. donkey* and *C. rabbit* genotype*, C. chipmunk* genotype, (which were primarily found in animal and human samples). This needs further detailed investigation since it is very difficult to differentiate between various genotypes, as the sequences often resemble each other.
- It was found that *Cryptosporidium* may be more resistant to chlorine treatment. Combined treatment of UV and chlorine may be needed to eradicate *Cryptosporidium* in water treatment in a shorter duration. This has to be investigated further.

- It is recommended that further studies should investigate the sensitivity of flow cytometry over microscopy in detecting *Cryptosporidium* and *Giardia* in water samples. Flow cytometry may be more sensitive than microscopy in detecting microorganisms, especially *Cryptosporidium* and *Giardia,* as reported in this thesis. Flow cytometry gave higher percentage values than microscopy in most of the results obtained in this experiment.
- Investigation on different species of *Cryptosporidium* and *Giardia* should be experimented on and compared. This will give clarity on reactions of different species of *Cryptosporidium* and *Giardia* to different doses and exposure time of chlorine and UV. More vital dyes should be experimented to establish the accuracy of the results obtained in wastewater treatment using chlorine and UV.

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APPENDICES

Appendix 1: Amplification of the 18S rRNA gene region through a nested PCR visualized through gel electrophoresis (Chapter 4).

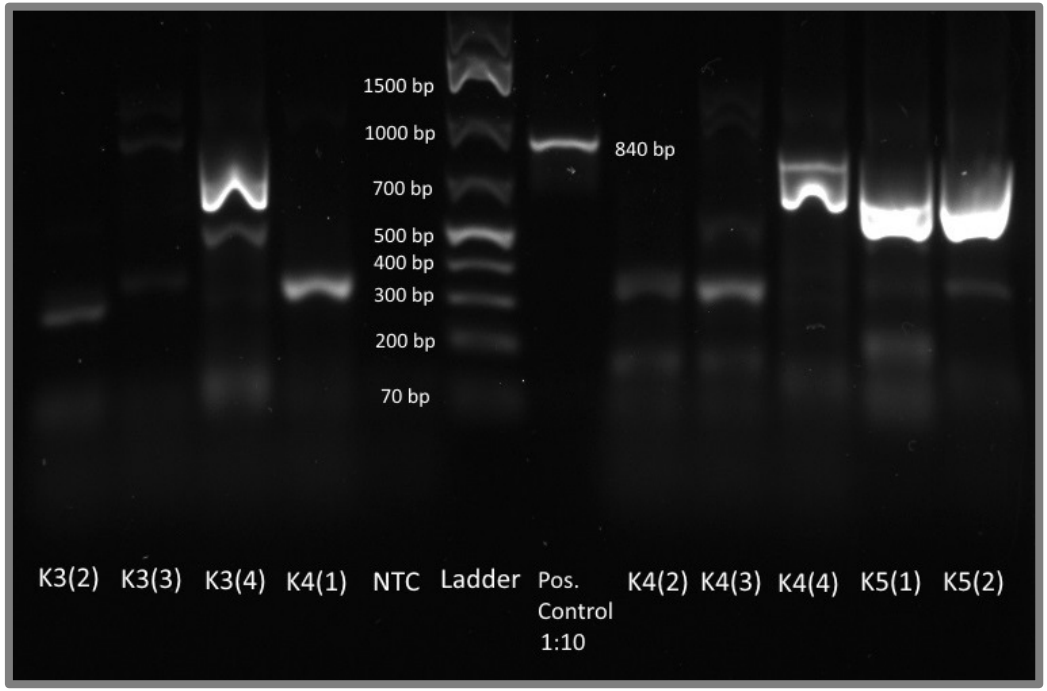


Figure 4.4. PCR products from the samples denoted below the bands. Samples were considered positives based on presence of a clearly visible band around the size of 840 bp: K3(4), K4(4), K5(1), K5(2).

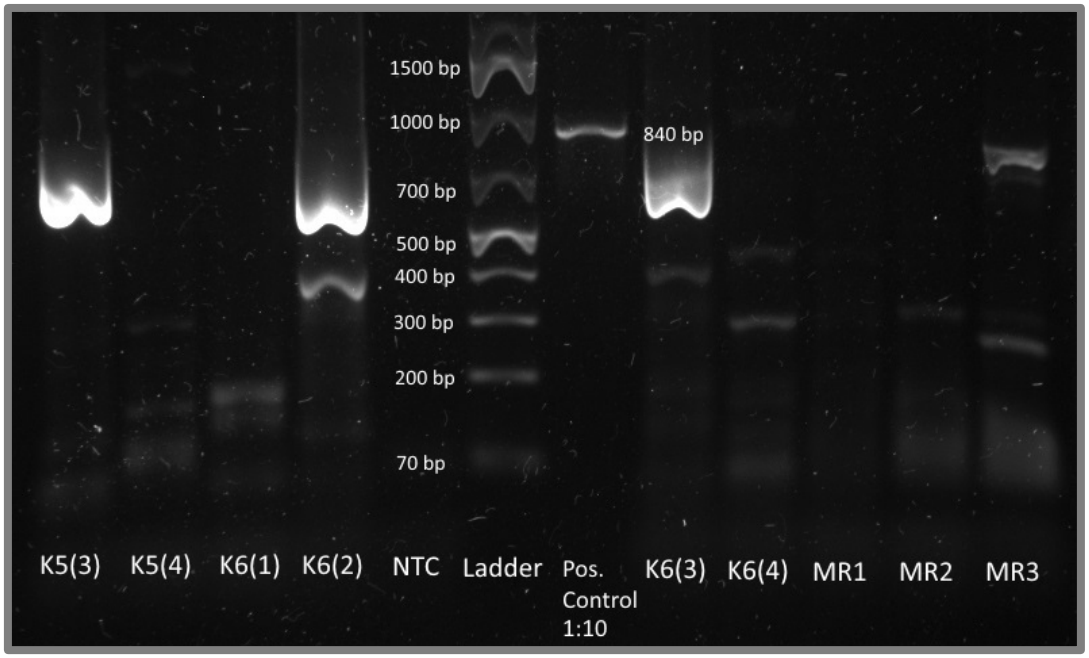


Figure 4.5. PCR products from the samples denoted below the bands. Samples were considered positives based on presence of a clearly visible band around the size of 840 bp: K5(3), K6(2), K6(3), MR3.

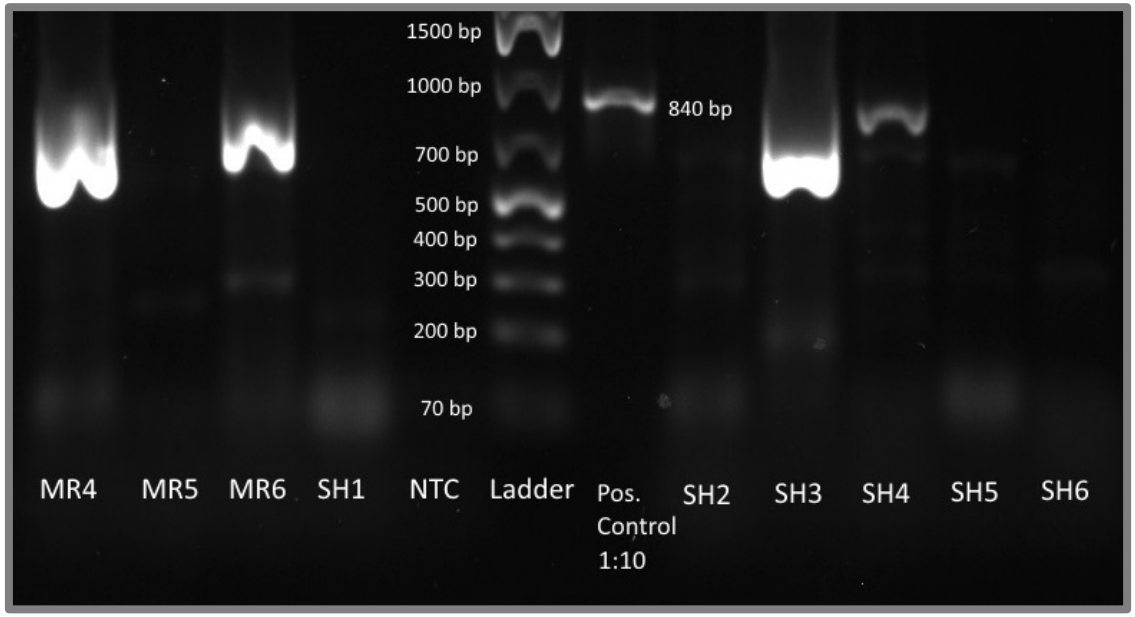


Figure 4.6. PCR products from the samples denoted below the bands. Samples were considered positives based on presence of a clearly visible band around the size of 840 bp: MR4, MR6, SH3, SH4

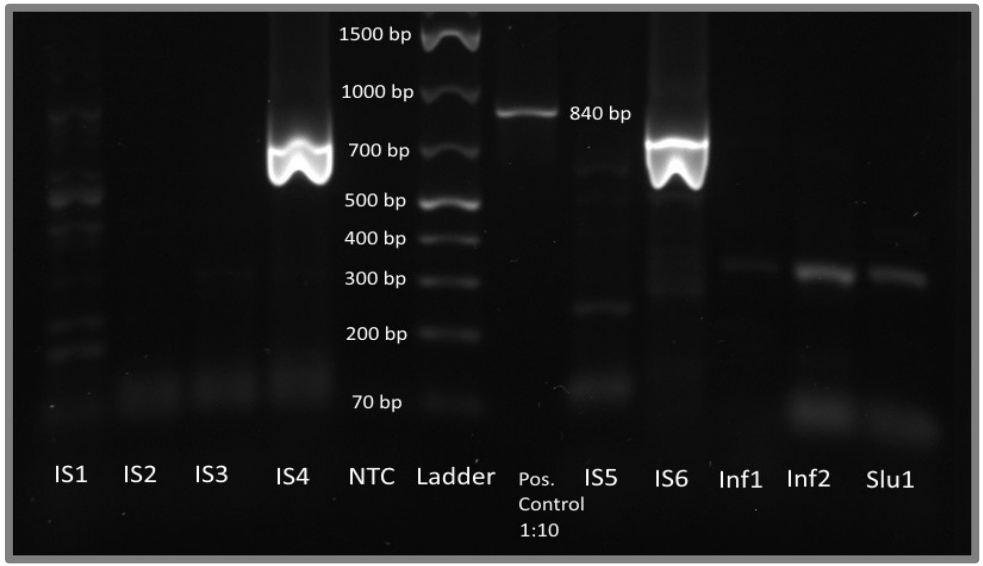


Figure 4.7. PCR products from the samples denoted below the bands. Samples were considered positives based on presence of a clearly visible band around the size of 840 bp: IS4, IS6.

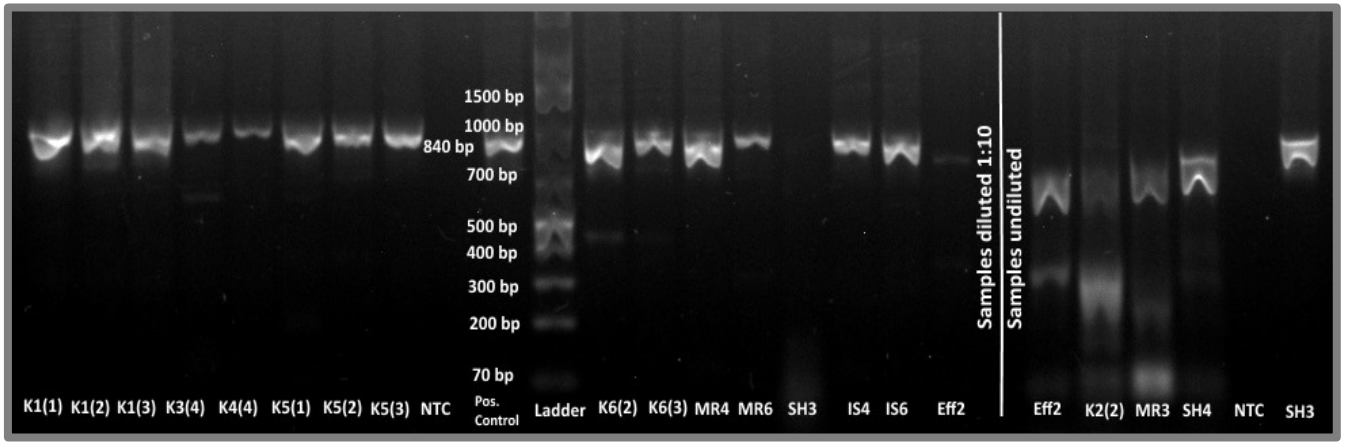


Figure 4.8. PCR products from the samples considered positives, visualized once again on agarose gel. A majority of samples were diluted 1:10 before loading, however the bands for EFF2, K2 (2), MR3 and SH4 were weaker and these samples were thus loaded undiluted. Sample SH3 was loaded both diluted 1:10 and undiluted.

Appendix 2: The bacteriological assessment of different sampling points

Table S1

WWTP A: Slanetz and Bartley for faecal enterococci count in waste water samples CFU/100mL												
Sampling point	1st Trial		2nd Trial		3rd Trial		4th Trial		5th Trial		6th Trial	
	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)
Influent	19500000	7.29	17500000	7.24	765000	5.88	221000	5.34	46500	4.67	100500	5.00
Clarifier Tank	5500	3.74	1500000	6.18	N/A	N/A	19000	4.28	4500	3.65	3500	3.54
Post Cl2	N/A	N/A	N/A	N/A	N/A	N/A	6000	3.78	N/A	N/A	N/A	N/A
Final Effluent	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
WWTP A: Brilliance *E coli* for *Escherichia coli* count in waste water samples CFU/100mL												
Influent	17700000	8.25	1835000000	9.26	152500	6.18	127500000	8.11	178500000	8.25	10050000	7.00
Clarifier Tank	162500	5.21	35000	4.54	13000	4.11	256000	5.41	640000	5.81	515000	5.71
Post Cl2	N/A	N/A	6500	3.81	28000	4.45	N/A	N/A	N/A	N/A	N/A	N/A
Final Effluent	N/A	N/A	3000	3.48	N/A	N/A	8500	3.93	35500	4.55	9000	3.95

Table S2

WWTP A: Slanetz and Bartley for faecal enterococci count from the river samples CFU/100mL												
Sampling point	1st Trial		2nd Trial		3rd Trial		4th Trial		5th Trial		6th Trial	
	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)
Upstream	N/A	N/A	2500	3.40	1000	3.00	1500	3.18	1500	3.18	3000	3.48
Downstream	1200	3.08	2900	3.46	800	2.90	1300	3.11	1600	3.20	2800	3.45
WWTP A: Brilliance *E coli* for *Escherichia coli* count in river samples CFU/100mL												
Upstream	21950	4.34	180500	5.26	209000	5.32	102500	5.01	10500	4.02	11000	4.04
Downstream	20100	4.30	156000	5.19	340000	5.53	120000	5.08	9300	3.97	13200	4.12

Table S3

WWTP B: Slanetz and Bartley for Faecal enterococci coliform count in waste water samples CFUs/100mL												
Sampling point	1st Trial		2nd Trial		3rd Trial		4th Trial		5th Trial		6th Trial	
	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)
Influent	TNTC	N/A	32000000	7.51	1510000000	9.18	237000	5.37	365000	5.56	2510000	6.40
Biofilter Tank	78500	4.89	800000	5.90	120000	5.08	52500	4.72	39000	4.59	38000	4.58
Humus Tank	56000	4.75	400000	5.60	60000	4.78	8500	3.93	1000	3.00	1500	3.18
Final Effluent	12500	4.10	65000	4.81	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
WWTP B: Brilliance *E coli* for *Escherichia coli* count in waste water samples CFU/mL												
Influent	TNTC	N/A	630000000	8.80	TNTC	N/A	2540000	6.40	2795000	6.45	155000000	8.19
Biofilter Tank	102000	5.01	20000000	7.30	42500000	7.63	199000	5.30	223000	5.35	2405000	6.38
Humus Tank	53500	4.73	14000000	7.15	8000000	6.90	138000	5.14	153000	5.18	193500	5.29
Final Effluent	10500	4.02	180000	5.26	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

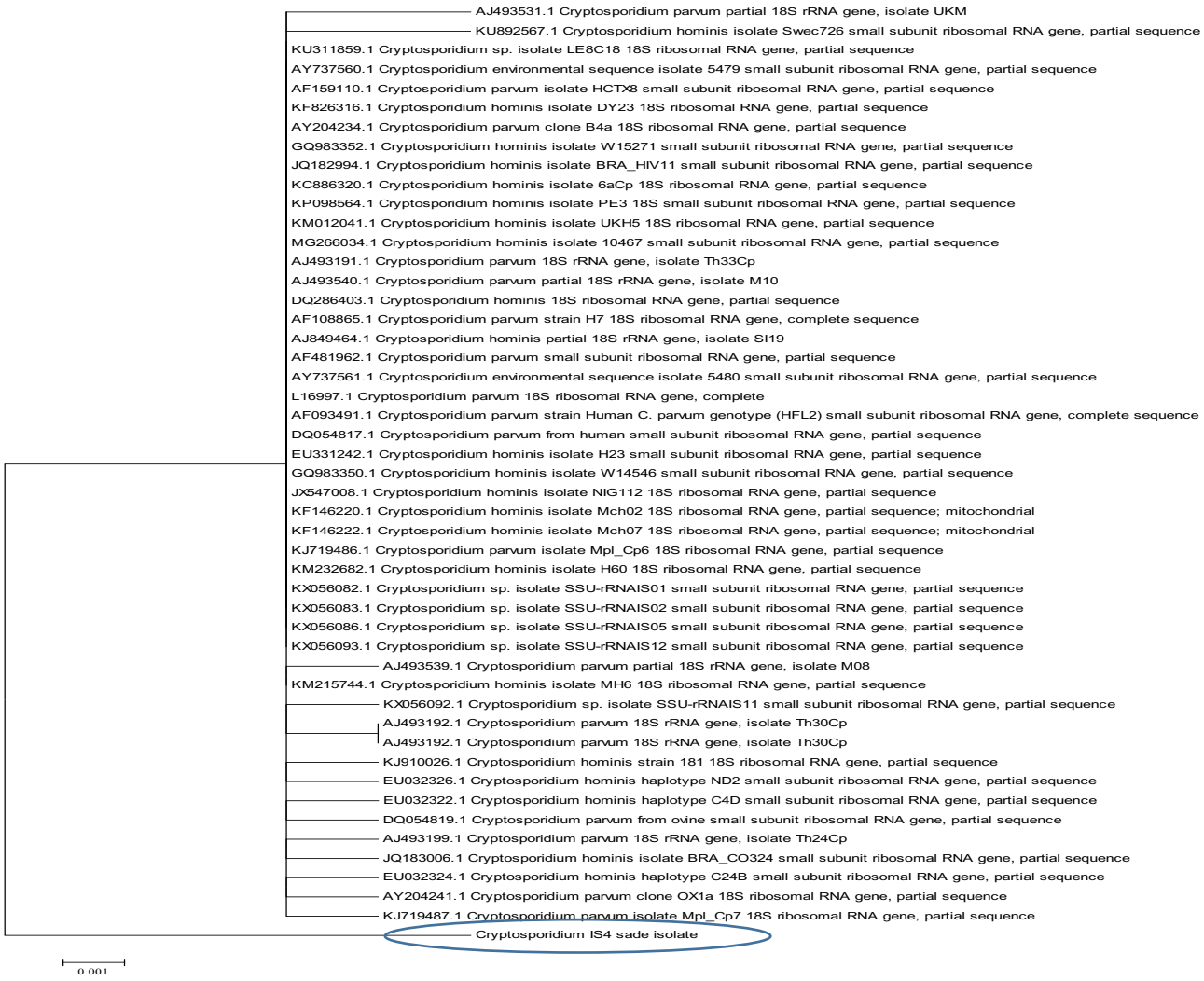
Table S4

WWTP B: Slanetz and Bartley for Faecal enterococci count in river water samples CFU/100mL												
Sampling point	1st Trial		2nd Trial		3rd Trial		4th Trial		5th Trial		6th Trial	
	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)
Upstream	N/A	N/A	5500	3.74	19000	4.28	8500	3.93	8500	3.93	19000	4.28
NTWWTP	N/A	N/A	N/A	N/A	21000	4.32	25000	4.40	6500	3.81	54500	4.74
Downstream	N/A	N/A	13500	4.13	5000	3.70	4500	3.65	2000	3.30	41000	4.61
River mouth	N/A	N/A	34000	4.53	31000	4.49	34000	4.53	4500	3.65	2000	3.30
WWTP B: Brilliance *E coli* for *Escherichia coli* count in river water samples CFU/100mL												
Upstream	9000000	6.95	155500	5.19	1260000	6.10	425000	5.63	615000	5.79	840000	5.92
NTWWTP	12000000	7.08	168000	5.23	1420000	6.15	1295000	6.11	885000	5.95	1510000	6.18
Downstream	11500000	7.06	127500	5.11	1200000	6.08	615000	5.79	850000	5.93	760000	5.88
River mouth	41000000	7.61	115000	5.06	225000	5.35	2470000	6.39	1615000	6.21	1735000	6.24

Table S5

WWTP C and D: Slanetz and Bartley for faecal enterococci count in waste water samples CFU/100 mL												
Sampling point	1st Trial		2nd Trial		3rd Trial		4th Trial		5th Trial		6th Trial	
	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)	Conc	Log (conc)
Influent WWTP C	12500000	7.10	57500000	7.76	1185000	6.07	115000	5.06	2740000	6.44	730000	5.86
Clarifier WWTP C	N/A	N/A	N/A	N/A	7000	3.85	N/A	N/A	274000	5.44	5000	3.70
Influent WWTP D	37500000	7.57	54000000	7.73	1580000	6.20	1840000	6.26	1870000	6.27	810000	5.91
Clarifier WWTP D	1000	3.00	2500	3.40	60500	4.78	3000	3.48	1000	3.00	5500	3.74
Post Cl2	N/A	N/A	N/A	N/A	N/A	N/A	6500	3.81	N/A	N/A	N/A	N/A
Final Effluent	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
WWTP C and D: Brilliance *E coli* for *Escherichia coli* count in waste water samples CFU/100mL												
Influent WWTP C	116500000	8.07	TNTC	N/A	1760000	6.25	299000000	8.48	126500000	8.10	183500000	8.26
Clarifier WWTP C	55000	4.74	4000000	6.60	77500	4.89	270000	5.43	199000	5.30	256500	5.41
Influent WWTP D	1340000	6.13	TNTC	N/A	21950000	7.34	298500000	8.47	104000000	8.02	253500000	8.40
Clarifier WWTP D	10500	4.02	67000000	7.83	210500	5.32	76500	4.88	150500	5.18	254000	5.40
Post Cl2	N/A	N/A	N/A	N/A	N/A	N/A	4450000	6.65	10000	4.00	47000	4.67
Final Effluent	7000	3.85	1000	3.00	1000	3.00	3450000	6.54	60000	4.78	N/A	N/A

Appendix 3: Phylogenetic tree neighbour joining tree generated by aligning the sequences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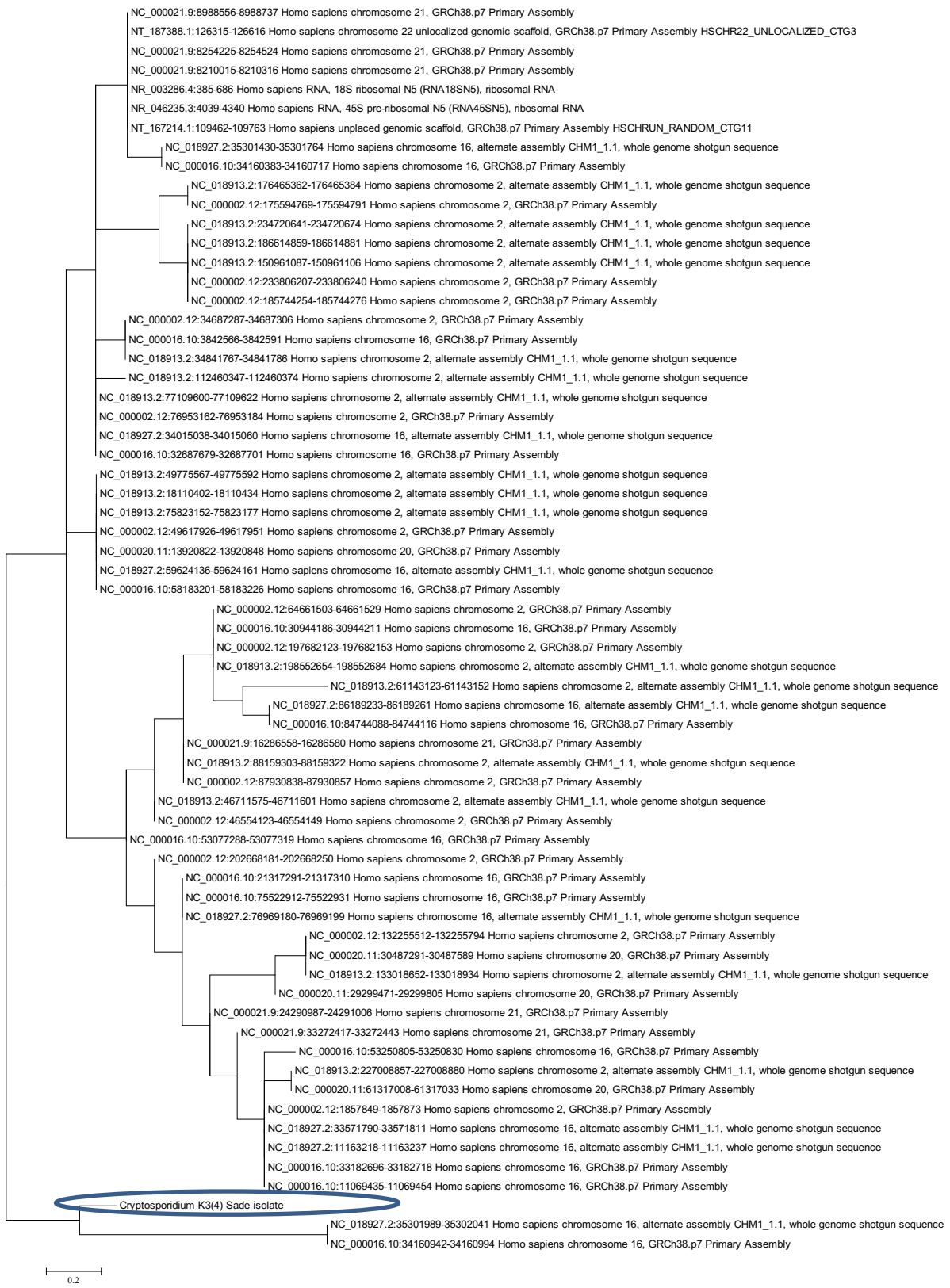
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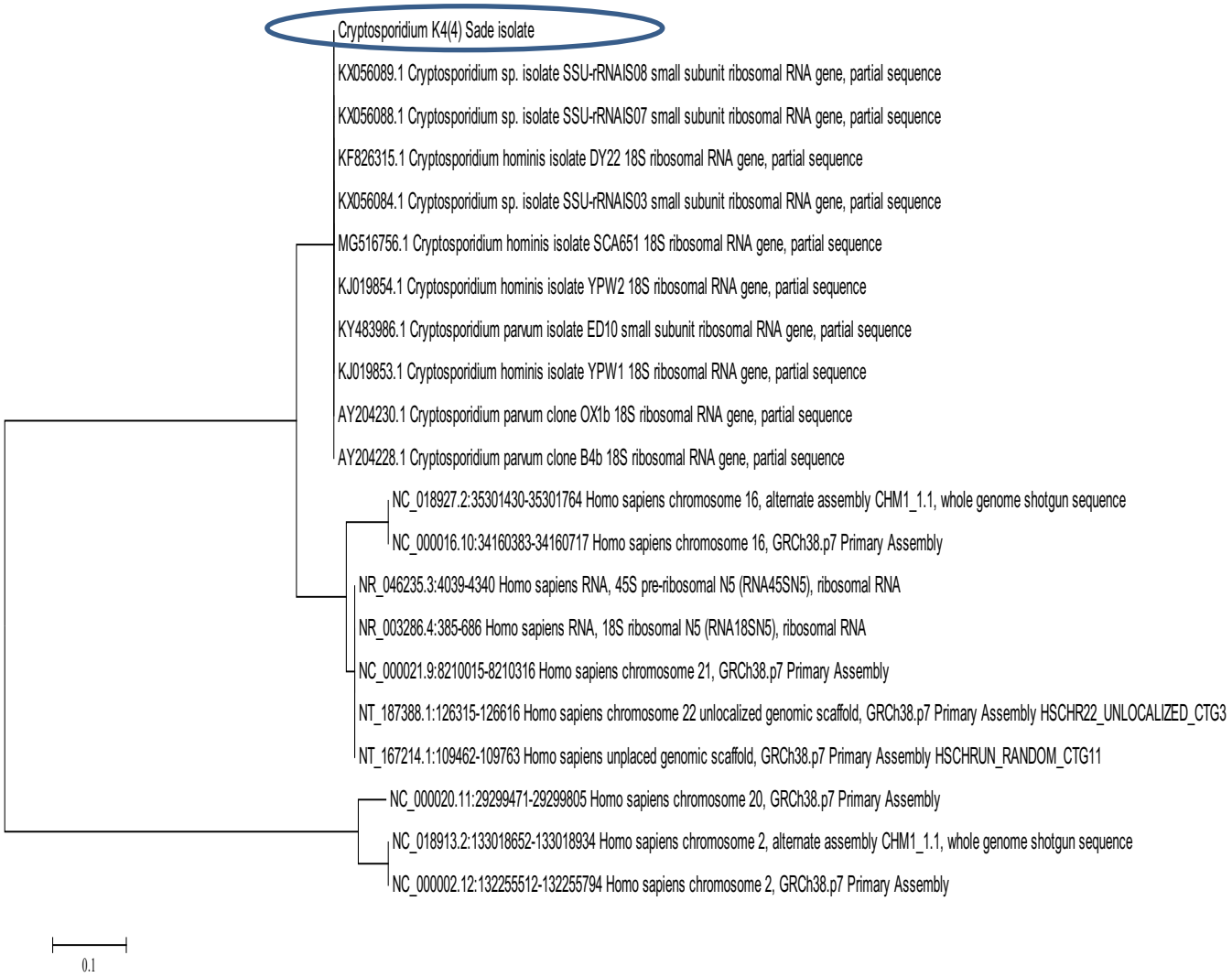
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NR_046235.3:4039-4340 Homo sapiens RNA, 45S pre-ribosomal N5 (RNA45SN5), ribosomal RNA
NR_003286.4:385-686 Homo sapiens RNA, 18S ribosomal N5 (RNA18SN5), ribosomal RNA
NC_000021.9:8210015-8210316 Homo sapiens chromosome 21, GRCh38.p7 Primary Assembly
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NC_000020.11:29299471-29299805 Homo sapiens chromosome 20, GRCh38.p7 Primary Assembly
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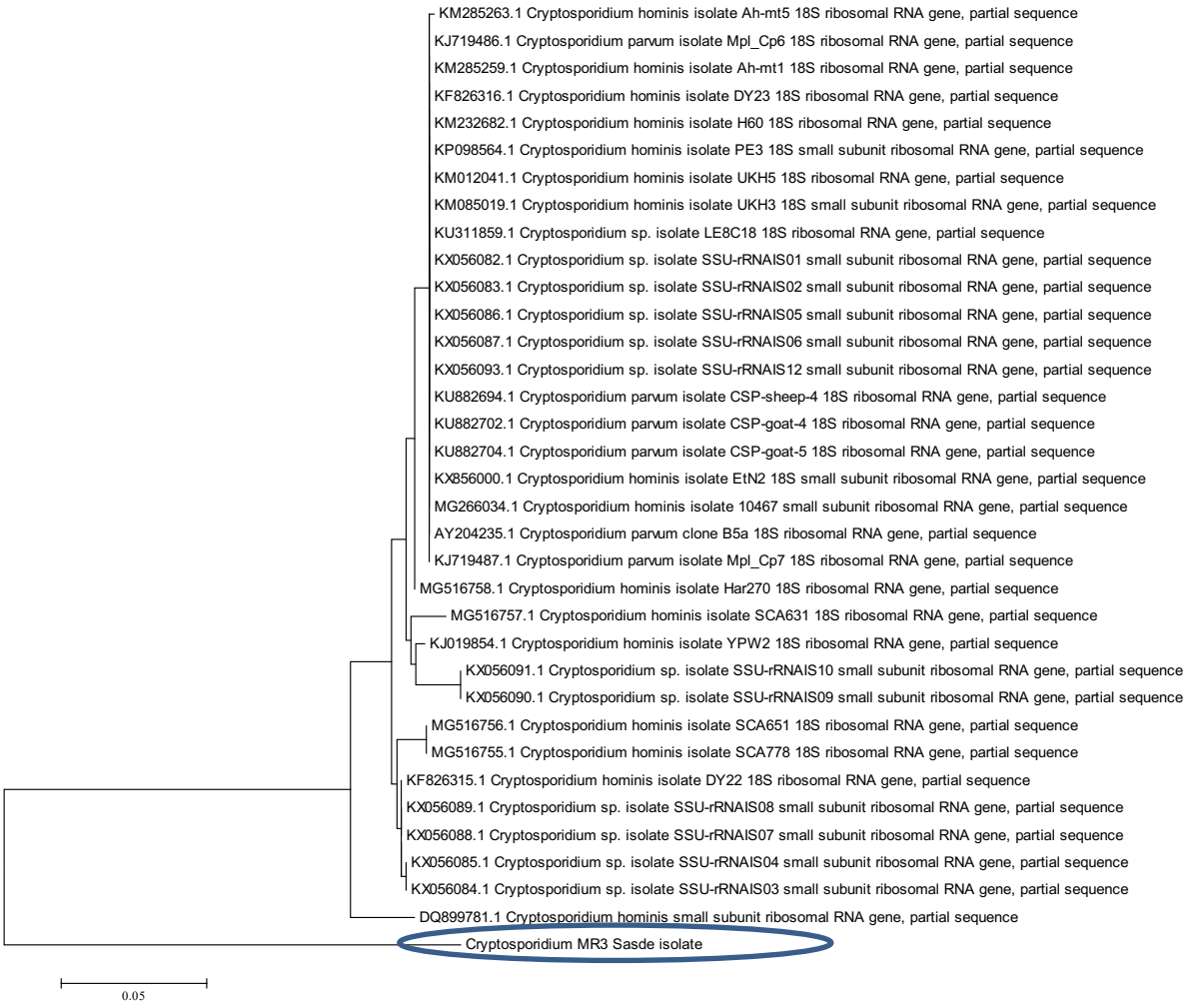
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Cryptosporidium MR3 Sasde isolate
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KC886320.1 Cryptosporidium hominis isolate 6aCp 18S ribosomal RNA gene, partial sequence
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KF146222.1 Cryptosporidium hominis isolate Mch07 18S ribosomal RNA gene, partial sequence; mitochondrial
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Cryptosporidium MR4 Sade isolate
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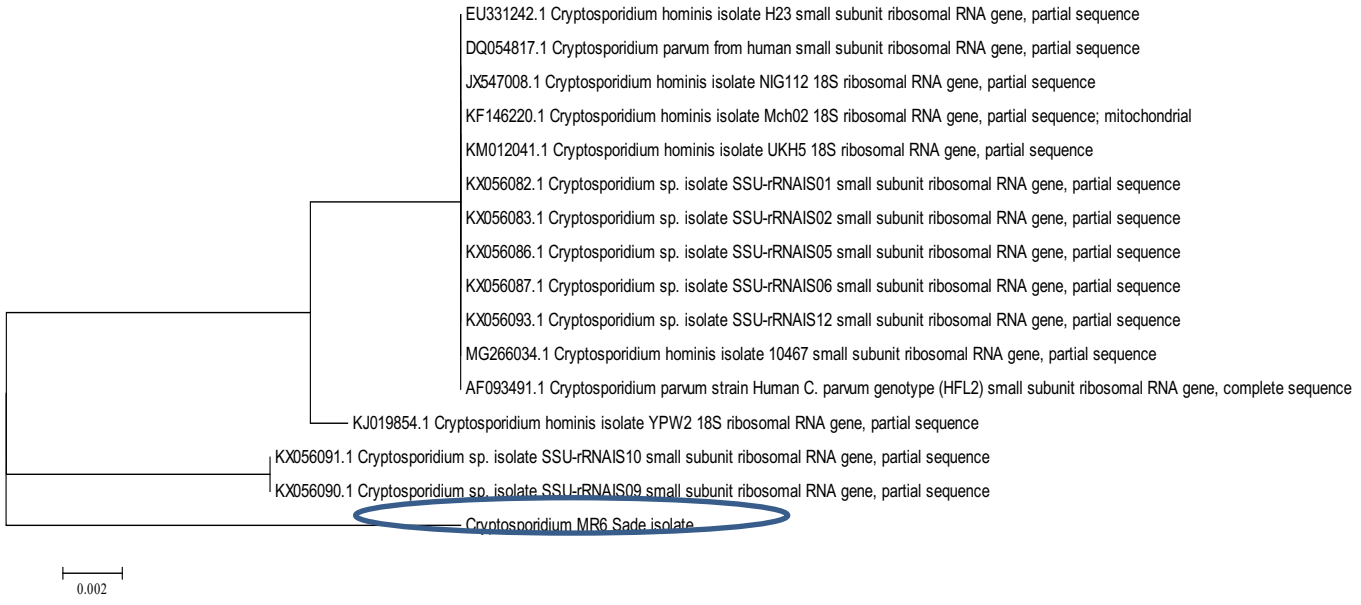
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MG266034.1 Cryptosporidium hominis isolate 10467 small subunit ribosomal RNA gene, partial sequence
AF093491.1 Cryptosporidium parvum strain Human C. parvum genotype (HFL2) small subunit ribosomal RNA gene, complete sequence
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Cryptosporidium MR6 Sade isolate
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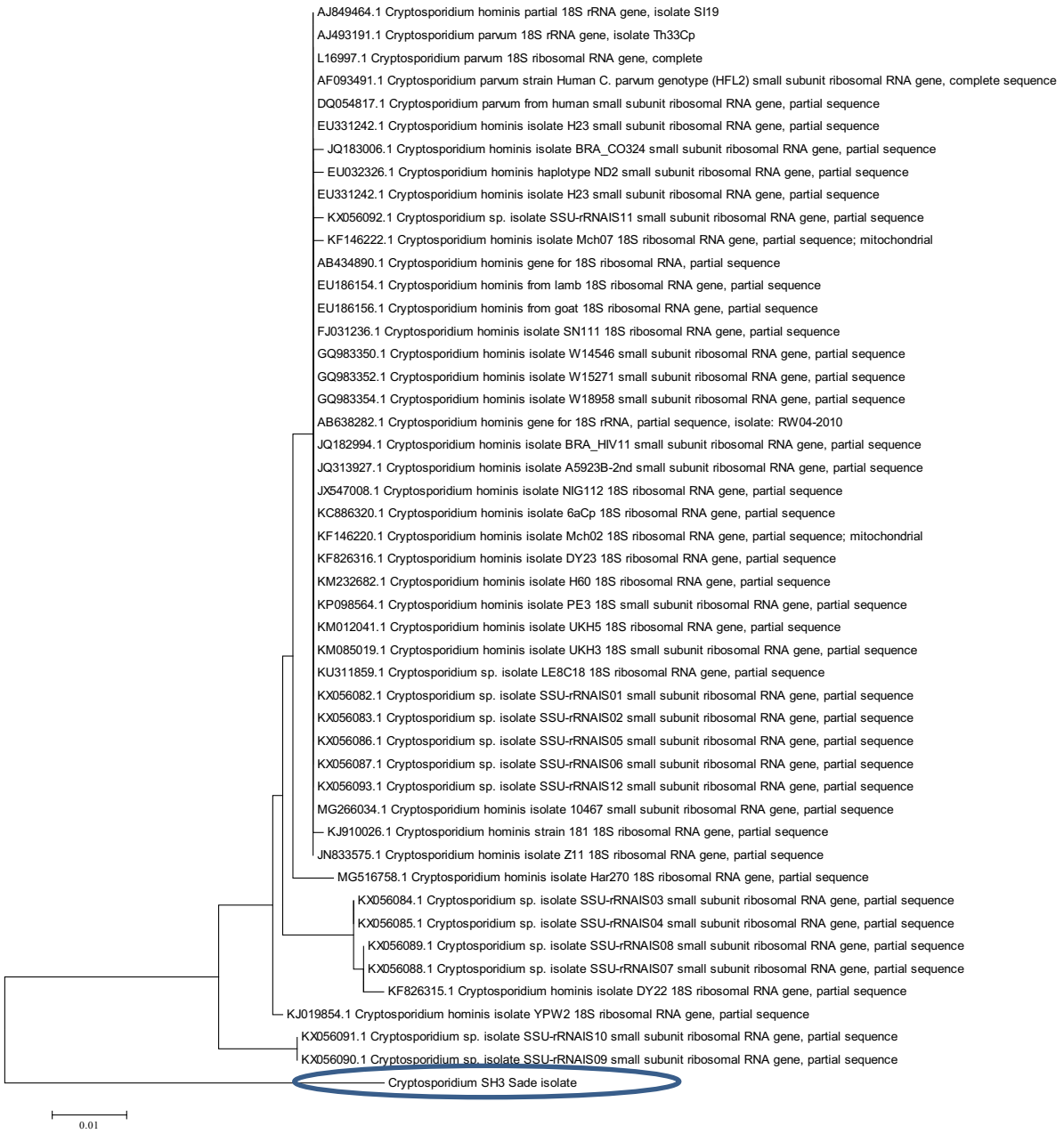
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KJ019854.1 Cryptosporidium hominis isolate YPW2 18S ribosomal RNA gene, partial sequence
KX056091.1 Cryptosporidium sp. isolate SSU-rRNAIS10 small subunit ribosomal RNA gene, partial sequence
KX056090.1 Cryptosporidium sp. isolate SSU-rRNAIS09 small subunit ribosomal RNA gene, partial sequence
Cryptosporidium SH3 Sade isolate
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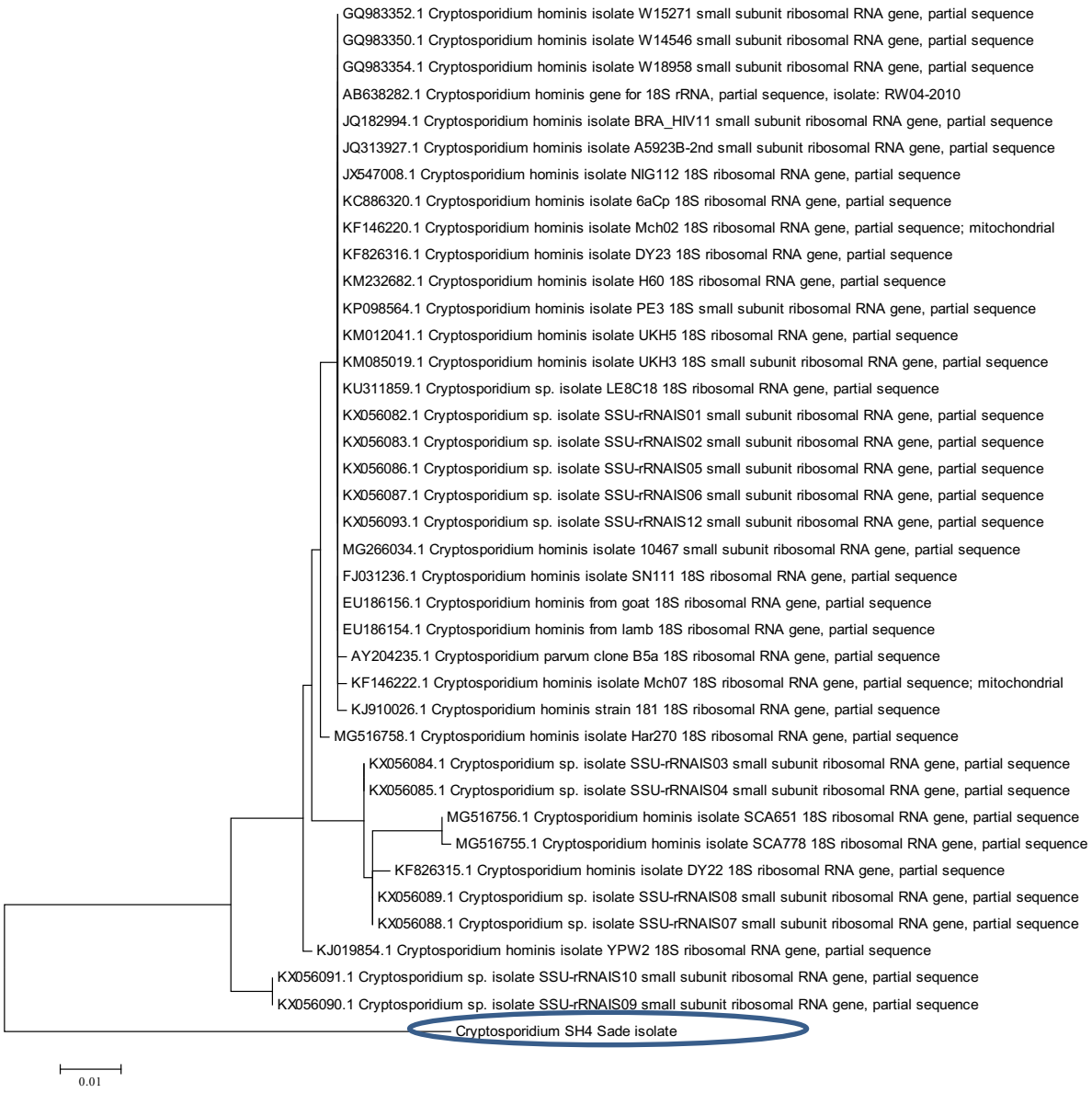
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AB638282.1 Cryptosporidium hominis gene for 18S rRNA, partial sequence, isolate: RW04-2010
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Cryptosporidium SH4 Sade isolate
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