Genetic diversity and development of a rapid molecular detection method for protozoan parasites in raw and treated wastewater

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Nonsikelelo Precios Hlongwa (nee Mthethwa)

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Supervisor: Professor Sheena Kumari Kuttan Pillai

Co-supervisor: Dr Isaac Dennis Amoah

Co-supervisor: Professor Poovendhree Reddy

Co-supervisor: Professor Faizal Bux

APPROVAL

I hereby approve the submission of the following thesis.

Supervisor: Professor Sheena Kumari Kuttan Pillai

Date: 31 January 2024

Co-supervisor: Dr Isaac Dennis Amoah

Date: 31 January 2024

....

Co-supervisor: Professor Poovendhree Reddy

Date: 31 January 2024

.....

Co-supervisor: Professor Faizal Bux

Date: 31 January 2024

ABSTRACT

Protozoan parasites can cause a wide range of diseases in both humans and animals. Despite this, little is known about their genetic diversity in the environment, particularly in wastewater. Current methods of detecting protozoan parasites are time-consuming and expensive, limiting our ability to monitor potential risks associated with their discharge into the environment. Consequently, this study aimed to determine the genetic diversity of protozoan parasites in wastewater treatment plants and to develop a rapid and affordable technique for their detection and quantification from environmental samples. Shotgun metagenomics and 18S rRNA gene sequencing were employed to assess the diversity of protozoan parasites in influent (untreated) and effluent (treated) wastewater samples collected from different geographical locations within South Africa. Furthermore, rapid fluorescent and colorimetric loop-mediated isothermal amplification (LAMP) methods were developed for their detection from different environmental matrices. The LAMP methods were then compared with the established methods, including quantitative PCR and digital PCR for their sensitivity and feasibility. Additionally, the study has also evaluated oocyst concentration and DNA extraction methods to maximize oocyst recovery from wastewater samples resulting in a recovery rate of 64.1%.

Using 18SrRNA analysis, it was found that protozoan diversity (Shannon index, *P*-value=0.003) and taxonomic composition (PERMANOVA, *P*-value=0.02) were significantly associated with WWTP location and treatment stage (*P*-value=0.003). An abundant number of free-living, parasitic, and potentially pathogenic protists was observed in the untreated wastewater samples, including Alveolates (Apicomplexa and Ciliophora), Excavata (Discoba and Parasalia), and Amoebozoa (*Entamoeba* and *Acanthamoeba*). In contrast, treated wastewater samples were found to be dominated by fungi and algae. In a subset of samples (n=3), shotgun metagenomics analyses revealed the presence of protozoa of public health importance, including *Cryptosporidium* spp. All untreated wastewater samples studied were found to contain *Entamoeba hystolitica*, *Blastocystis hominis*, *Naegleria gruberi*, *Toxoplasma gondii*, *Cyclospora cayetanensis*, and *Giardia intestinalis*. The functional pathways associated with pathogenic protozoa were classified into thiamine diphosphate biosynthesis III, Heme biosynthesis, methyl erythritol phosphate (MEP), Methylerythritol 4-phosphate pathway, and pentose phosphate pathway.

The optimized LAMP methods (colorimetric and fluorescent) successfully detected *Cryptosporidium parvum* (*GP60* gene) and the *Cryptosporidium* genus (*SAM* gene) from

environmental samples with 100% specificity. Both methods demonstrated a high sensitivity, with the same limit of detection (LOD) of 1.1 copies of *C. parvum* per 25 µl reaction (0.02 ng/µl). A comparison of LAMP, ddPCR, and qPCR revealed that ddPCR had the highest sensitivity, with a limit of detection (LOD) of 1 copy/reaction and 100% true positives followed by fluorescent LAMP with a LOD of 1.1 copies and 75% true positives, while qPCR was the least sensitive with a LOD of 14 copies and 100% true positives. All three methods showed good linearity (> R^2 =0.9) over a wide dynamic range of *C. parvum* concentrations. The study further revealed that fLAMP is the most affordable ($12.46/sample), followed by qPCR ($28.19/sample), and ddPCR ($67.29/sample). Using the optimized protocol, *C. parvum* and *Cryptosporidium* spp. were detected in 50–85% (n = 60) of environmental samples (treated and untreated wastewater, sludge, and surface water) in comparison to 58–98% (n = 60) detected by ddPCR. Additionally, these findings suggest that LAMP can be an effective and affordable method for monitoring protozoan parasites in the environment. The findings of this study provided valuable insights into the genetic diversity of protozoan parasites in wastewater, which is crucial for advancing our understanding of disease epidemiology, evolution, and ecology. Furthermore, the findings of this study have important implications for monitoring pathogens in wastewater, especially in countries with limited resources for monitoring and managing waterborne diseases.

DECLARATION

I certify that this thesis is my original work and contains no previously submitted material for academic qualification to the Durban University of Technology or any other institution. My dissertation is comprised of the work I've carried out since beginning my PhD studies. I further declare that a full reference list for all sources cited or quoted has been supplied.

Nonsikelelo Precios Hlongwa (nee Mthethwa) (Student number: 21649243)
Masters in Applied sciences, Biotechnology
Date: 2024

DEDICATION

This work is dedicated to my beloved grandmother, Margaret Thabile Mhlongo.

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Published papers:

1.Profiling pathogenic protozoan and their functional pathways in wastewater using 18S rRNA and shotgun metagenomics. Nonsikelelo P Mthethwa-Hlongwa, Isaac D Amoah, Andres Gomez, Sam Davison, Poovendhree Reddy, Faizal Bux, Sheena Kumari. Science of The Total Environment (2023). https://doi.org/10.1016/j.scitotenv.2023.169602.

2.**Mthethwa, N. P**., Amoah, I. D., Reddy, P., Bux, F., & Kumari, S. (2021). A review on application of next-generation sequencing methods for profiling of protozoan parasites in water: Current methodologies, challenges, and perspectives. *Journal of Microbiological Methods*, *187*, 106269.
https://doi.org/10.1016/j.mimet.2021.106269

3.Amoah, I. D., **Mthethwa, N. P**., Pillay, L., Deepnarain, N., Pillay, K., Awolusi, O. O., ... & Bux, F. (2021). RT-LAMP: a cheaper, simpler and faster alternative for the detection of SARS-CoV-2 in wastewater. *Food and environmental virology*, *13*, 447-456.
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4. **Mthethwa, N. P**., Amoah, I. D., Reddy, P., Bux, F., & Kumari, S. (2022). Development and evaluation of a molecular based protocol for detection and quantification of Cryptosporidium spp. in wastewater. *Experimental Parasitology*, *234*, 108216.
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5. **Mthethwa, N. P**., Amoah, I. D., Reddy, P., Bux, F., & Kumari, S. (2022). Fluorescence and colorimetric LAMP-based real-time detection of human pathogenic Cryptosporidium spp. from environmental samples. *Acta Tropica*, *235*, 106606.
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Conference attendance:

1. **International Water Association (IWA) conference on Water and Wastewater management with a special focus on Developing countries**. December 03-09 **2023**. Perth, Australia. Profiling pathogenic protozoan and their functional pathways in wastewater using 18S rRNA and shotgun metagenomics. **Nonsikelelo P Mthethwa-Hlongwa**, Isaac D Amoah, Andres Gomez, Sam Davison, Poovendhree Reddy, Faizal Bux, Sheena Kumari

2. **The South African Society of Microbiology Conference**. 4-6 May 2021. Development and evaluation of a molecular based protocol for detection and quantification of Cryptosporidium spp. in wastewater. **Nonsikelelo Precios Mthethwa**, Isaac Dennis Amoah, Poovendhree Reddy, Faizal Bux and Sheena Kumari

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1.0 INTRODUCTION

1.1 Background

South Africa is a water-scarce country with an arid to semi-arid climate in most parts (Oki and Quiocho, 2020, Nhemachena et al., 2020). The country faces many challenges with respect to its water resources, including high water demand, variable rainfall patterns, deteriorating water quality, and inadequate infrastructure (du Plessis and du Plessis, 2019). For example, the quality of water supplied to households is often poor due to high demand, limited treatment facilities and aging infrastructure (Nkosi et al., 2021). Many rural areas still lack access to clean water and proper sanitation facilities, which increases risk of waterborne diseases (Nkosi et al., 2021, Tseole et al., 2022, Murei et al., 2022). Moreover, many of the country's wastewater treatment plants are overburdened which results in a high number of untreated wastewater discharges into rivers and the ocean (Moloi et al., 2020, Rimayi et al., 2018). As a result of the preceding and other factors such as international travel, immunocompromised individuals (e.g., HIV/AIDS infected), poverty, domesticated animals, and more; waterborne protozoan parasite infections continue to be a substantial public health burden in South Africa.

Protozoan parasites are unicellular, eukaryotic, and heterotrophic organisms that belong to Kingdom Protista (CDC, 2022, Schnittger and Florin-Christensen, 2018). They are found in various environments, including soil, water, animal, and human hosts (Yaeger, 2019). About 15000 species of protozoan parasites have been reported around the globe (Omarova et al., 2018). Examples of waterborne protozoan parasites and those responsible for human infections include *Toxoplasma gondii*, *Cryptosporidium* spp., *Entamoeba hystolitica, Cyclospora cayetanensis, Isospora belli, Blastocystis homonis, Balantidium coli, Acantamoeba* spp., *Sarcostysis* spp., *Naegleria* spp., and *Giardia* spp. (Bridle, 2014, Garcia et al., 2017, Griffiths, 2017, Xiao et al., 2018, Ajonina et al., 2018). These parasites are major waterborne pathogens because they require low infectious doses, are chlorine resistant, and are very small (1–17 μm) (Widmer and Sullivan, 2012). Additionally, the (oo)cysts of these parasites are resistant to extreme environmental conditions and can persist in the environment for several months (Omarova et al., 2018).

The life cycle of protozoan parasites can vary greatly depending on the species. Some species have a complex life cycle that involves multiple hosts, while others have a relatively simple

life cycle that involves only one host (Yaeger, 2019, Sibley, 2011). In general, protozoan parasites can be transmitted to humans and animals through various routes such as ingestion, inhalation, or through contact with contaminated surfaces or vectors such as mosquitoes and ticks (CDC, 2022, WHO, 2019). They can cause a variety of diseases, from mild to life-threatening diseases, including giardiasis, cryptosporidiosis, amoebic dysentery, and toxoplasmosis (Hemphill et al., 2019). These diseases can be hazardous for vulnerable populations such as young children, the elderly, and immunocompromised individuals (Osman et al., 2016). For example, protozoan diarrheal diseases are the second leading cause of death in children under age five years worldwide (CDC, 2015, WHO, 2017, CIDRAP, 2018).

Protozoan parasites are commonly found in raw and treated wastewater, which poses a risk of environmental and public health contamination if not properly treated and removed (Omarova et al., 2018, Siwila et al., 2020, Blake and Betson, 2017). For example, according to WHO standards for the safe use of wastewater, greywater, and excreta, the presence of pathogens in untreated wastewater is an indication of fecal contamination and can constitute a significant health risk to individuals and communities (WHO,2018, Halalsheh et al., 2018). In untreated domestic wastewater, protozoan parasites can be found in large numbers originating from human and animal waste (Chahal et al., 2016, Berglund et al., 2017). The presence and diversity of these pathogens in untreated wastewater can indicate the health status of the community as well as the risk of waterborne illness (Hamilton et al., 2018, Zahedi et al., 2021). However, the presence of protozoan parasites in treated wastewater can pose a risk to public health if the treated wastewater is reused for purposes such as irrigation or drinking water (Prado et al.,2021). For instance, Omarova et al., (2018) highlighted that even after treatment protozoan parasites especially *Cryptosporidium* and *Giardia* can persist in the water supply.

Accurate and rapid detection of protozoan parasites in environmental samples is crucial (Momčilović et al., 2019). Environmental DNA (eDNA) approaches have been used to monitor the presence and diversity of protozoan parasites in wastewater or environmental samples (Farrell et al., 2021, Sengupta et al., 2022). Protozoan parasites exhibit a high level of genetic diversity (Bass et al., 2015, Strassert et al., 2019). Therefore, information on the variation in genetic makeup within and between populations of protozoans is essential for understanding their evolution, ecology, and adaptation to different host environments (Guo et al., 2017). This can assist to identify the source of an outbreak, trace the movement of the parasite from one

region to another, and determine the extent of genetic exchange between different strains or populations (Manoharan et al., 2021). Moreover, understanding genetic diversity can help identify the specific strains that are most prevalent and potentially harmful to human health (Manoharan et al., 2021, Lokmer et al., 2019).

Traditional methods to identify protozoan in environmental samples, such as microscopy and culture-based techniques, have several limitations. These includes low sensitivity and specificity, time-consumption, and labour-intensive (Vasavada et al., 2020, Marembo and Xu, 2020, Pepper and Gerba, 2015). Moreover, these methods often require specialized skills and equipment and may not be applicable to all protozoan species (Fletcher et al., 2012). PCR-based methods on the other hand (qPCR and droplet digital PCR) amplify specific DNA sequences of protozoan species, allowing their detection and identification (Adeyemo et al., 2018). However, these methods have limitations, such as the risk of false positives due to DNA contamination, the limited detection range, the high cost and some are only laboratory based and not portable for point of care analysis (Rajapaksha et al., 2019, Ramírez-Castillo et al., 2015). Loop-mediated isothermal amplification (LAMP), is an alternative molecular method that amplifies DNA under isothermal conditions (typically 60-65°C) (MacAulay et al., 2022) and has recently been shown promising as a method for pathogen detection from environmental and clinical samples. The method offers a rapid, cost-effective, and highly sensitive alternative to traditional detection methods (Li et al., 2017). Recently, several studies have focused on the development of LAMP assays for the detection of pathogenic protozoan parasites, viruses, and bacteria (John et al., 2021, Salamin et al., 2017, Nzelu et al., 2019, Seki et al., 2018).

Molecular methods based on the 18S rRNA gene and metagenomic profiling have been used for protozoans (Lefèvre et al., 2008, Freudenthal et al., 2022). The 18S rRNA gene is a widely used genetic marker for identifying and characterising protozoan parasites in environmental samples (Ríos-Castro et al., 2022). This gene is highly conserved among eukaryotes but also contains regions of variability that can be used to distinguish different species or genera (Lee and Muthu, 2021). Shotgun metagenomic profiling, on the other hand involves sequencing DNA from environmental samples, such as wastewater, to identify the microbial community present (Thoendel et al., 2016, Lokmer et al., 2019). This approach does not rely on the amplification of specific genes but rather on the sequencing of all DNA present in the sample

(Lokmer et al., 2019). The resulting data can be used to identify protozoan parasites in the wastewater and to assess their diversity and abundance.

1.2 Problem statement

Despite the significant health risks posed by these protozoan parasites, their genetic diversity and prevalence in wastewater samples in South Africa remain largely unknown (Sengupta et al., 2022). In order to fully understand the diversity of protozoan parasites in wastewater and the role that wastewater treatment plants play in their transmission, further studies are required. Moreover, current methods for detecting human pathogenic protozoan parasites from wastewater samples are often time-consuming and expensive. Therefore, developing a rapid and cheaper detection and semi-quantitative technique can help prevent the spread of these diseases and minimize the risk of outbreaks.

1.3 Aim

To assess the genetic diversity of protozoan parasites in wastewater from selected provinces in South Africa and to develop a rapid detection technique for human pathogenic protozoan parasites from wastewater samples.

1.4 Objectives

1. To develop and validate a molecular protocol for DNA extraction, efficient protozoan (oo)cysts recovery and quantification of protozoan parasites from wastewater.
2. To generate 18S rRNA and metagenomic profiles of protozoan parasites in raw and treated wastewater from selected South African provinces.
3. To develop a rapid, cost-effective Loop-Mediated Isothermal Amplification technique for the detection and semi-quantification of human pathogenic protozoan parasites species.
4. To apply the newly developed LAMP technique for the detection of pathogenic protozoan parasites in environmental samples (wastewater, surface water and sludge).
5. To compare the developed rapid molecular detection method (objective 2) with the established methods, such as quantitative PCR and droplet digital PCR.

1.5 Thesis layout

CHAPTER 1:
Introduction

CHAPTER 2:
Literature review of the study

CHAPTER 3:
Covers objective one of this study: The development of molecular protocol for DNA extraction, efficient oocyst recovery and quantification of protozoans from wastewater

CHAPTER 4:
Covers objective two of this study: genetic diversity of protozoan parasites in wastewater from selected provinces in South Africa

CHAPTER 5:
Covers objective 3 and part of objective 4: development of a rapid, low-cost Loop-Mediated Isothermal Amplification technique for the detection and semi-quantification of human pathogenic protozoan parasites in wastewater samples

CHAPTER 6:
Covers objective 4: Application of the newly developed LAMP technique for the detection of pathogenic protozoan parasites in environmental samples (wastewater, surface water and drinking water)
& Objective 5 : The sensitivity, specificity, applicability, and techno-economic viability of fLAMP, compared to ddPCR, and quantitative PCR (qPCR) for the detection of protozoan

CHAPTER 7:
Major conclusions and recommendations

2.0 LITERATURE REVIEW

2.1 Protozoan parasites

Protozoans are unicellular, eukaryotic, and microscopic organisms found in almost every habitat, including soil and water (Yaeger, 2011, CDC, 2022). While most protozoans are free-living, they can invade human and animal tissues and cause life-threatening diseases (Reyes-López et al., 2022). More than 50,000 free-living protozoan species, 10,000 parasitic species, and 80 human protozoan pathogens have been reported (Yaeger, 2011, Aronson and Magill, 2020). They are divided into groups based on their morphology, behavior, and molecular characteristics (Schwartz and Bonura, 2022). Sarcomastigophora, Labyrinthomorpha, Apicomplexa, Microspora, Ascetospora, Myxospora, and Ciliophora are among the seven phyla groups included in the classification (Omarova et al., 2018, 2015, CDC, 2022)(figure 1). The most important species causing human disease are members of the phyla Apicomplexa, Sacromastigophora and Cilliophora (Schwartz and Bonura, 2022, Hechenbleikner and McQuade, 2015)(figure 1).

Apicomplexa includes more than 500 diverse species, many of which are important pathogens of humans and other animals (David Sibley, 2011). They are distinguished by the presence of an apical complex, a specialized organelle involved in host cell invasion, as well as the lack of cilia or flagella (Morrissette and Gubbels, 2020). This phylum contains important pathogens, such as the genus *Cryptosporidium*, which infect the gastrointestinal tract and lungs of a wide variety of animals, including humans (Gerace et al., 2019). *Cryptosporidium* consists of 44 species and more than 120 genotypes (Ryan et al., 2021). *C. parvum*, *C. hominis*, *C. meleagridis*, *C. Canis*, and *C. felis* are all pathogenic to humans, and all are zoonotic (Weber, 2010). Other well-known members of Apicomplexa include *Babesia, Plasmodium,* and *Toxoplasma gondii* (Varberg, 2017). They cause severe illness in immunocompromised people, children, and animals, particularly in developing countries (Magana-Arachchi and Wanigatunge, 2020).

The phylum Sacromastigophora are characterized by the presence of flagella or pseudopods, and they include free-living *Euglenozoa*, and parasitic Kinetoplastids (*Trypanosoma brucei* and *Leishmania*), *Entamoeba hystolitica, Giardia* spp. and *Trichomonadidae vagilis* (Dixon, 2015). *Giardia* and *Entamoeba hystolitica* are one of the major causes of waterborne illness

and can be difficult to treat in some cases (Lalle and Hanevik, 2018, Griffiths, 2017). *Trypanosoma* and *Leishmania* spp. are also responsible for some of the most serious parasitic diseases in humans and other animals, such as sleeping sickness and leishmaniasis disease (Altamura et al., 2022).

Cilliophora is a complex phylum characterized by the presence of hair-like structures called cilia, which is used for locomotion and feeding (Warren et al., 2016). They include free-living and parasitic organisms such as *Balantidium coli*, *Ichthophthirius multifilis*, and *Tetrahymena pyriformis* (Berman, 2012). Only *Balantidium* and *Tetrahymenas* are significant to humans causing dysentery and respiratory infections, respectively (Thompson, 2015, Gerba, 2015). These organisms are mostly transmitted and spread through waterborne, vector-borne, foodborne, direct contact, environmental, and congenital transmission (Gerba, 2015, Griffiths, 2017). Most waterborne disease outbreaks in both developing and developed countries have been attributed to them. Therefore, understanding the biology of these pathogenic organisms leads to a better understanding of host-parasite interactions, advanced basic research, and protection of public health. For instance, it can help to inform measures to prevent the spread of infections, such as through water and sanitation management or insect vector control.

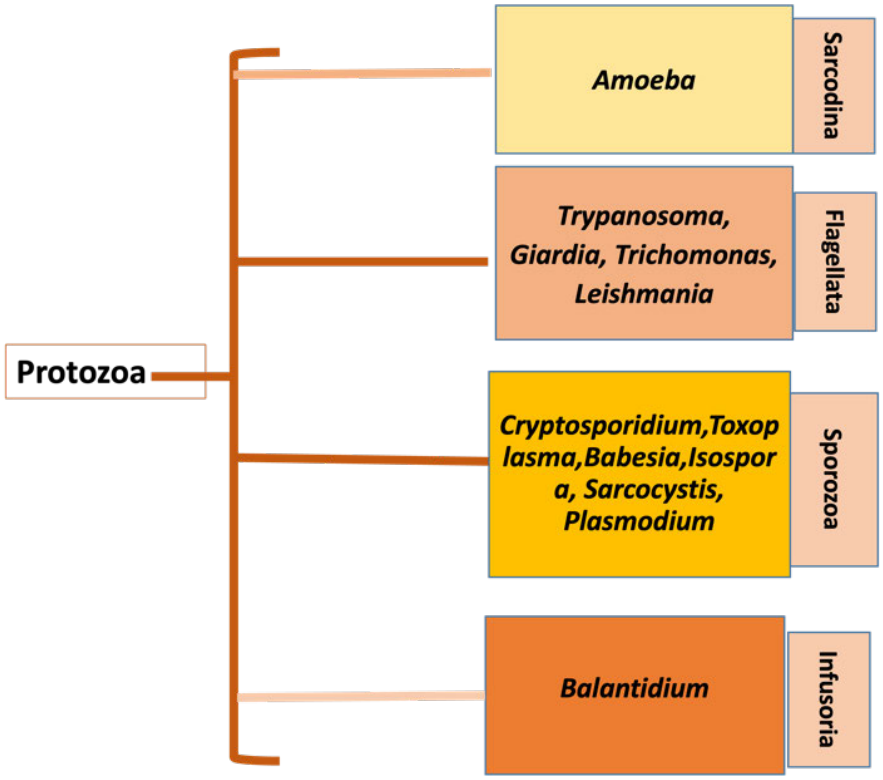

Figure 1. Classification of parasitic protozoa.

2.2 Life cycle of waterborne pathogenic protozoans

The life cycles of pathogenic protozoan parasites can vary depending on the specific species but typically involve multiple stages of growth and reproduction, with a definitive host (where sexual reproduction occurs), an intermediate host (where asexual reproduction occurs), and also involve direct life cycles (Auld and Tinsley, 2015). The general life cycle involves infection, attachment, reproduction, encystment, transmission, host colonization, disease progression, and treatment (Bhunia, 2018). These stages are critical to protozoans' ability to function as pathogens and infect various hosts. Therefore, understanding their life cycle is essential for prevention, accurate diagnosis, and effective treatment (Bogitsh et al., 2018).

A detailed life cycle of a protozoan parasite, *Cryptosporidium parvum* and *Cryptosporidium hominis* is depicted in figure 2. They infect mammal's small intestine epithelial cells, including humans and undergo the most complex life cycle, alternating between sexual and asexual reproductive stages (Gerba, 2015, Vaisusuk and Saijuntha, 2021). The oocyst is the infectious form of the parasite, and it is resistant to environmental conditions such as heat, chlorine, and UV light (Hassan et al., 2020). It is spherical in shape, measuring 4-6 μm in diameter, and is surrounded by a thick wall that protects the parasite from environmental stresses (Vaisusuk and Saijuntha, 2021). The sporozoite is the motile, infective form of the parasite that is released from the oocyst upon ingestion by a host (Pinto and Vinayak, 2021). The life cycle of *C. parvum* begins when the host ingests the oocysts through contaminated water or food (Gururajan et al., 2021). The oocysts pass through the stomach and enter the small intestine, where they release sporozoites (Pumipuntu and Piratae, 2018). The sporozoites attach to the cells lining the small intestine and begin to replicate asexually, producing more sporozoites (Pumipuntu and Piratae, 2018). These sporozoites then attach to other cells in the small intestine and continue to replicate (Gerace et al., 2019). As the sporozoites continue to replicate, they eventually form sexual forms of the parasite called gametocytes (Santin, 2020). The gametocytes then fuse together to form zygotes, which develop into oocysts (Santin, 2020). These oocysts are then excreted from the host's body through feces and can contaminate the environment, starting the life cycle anew (Siddique et al., 2021). The entire life cycle of *C. parvum* occurs within the host's small intestine and typically takes only a few days to complete (Siddique et al., 2021). The parasite can cause gastrointestinal illness, including diarrhea,

abdominal pain, and fever, in both humans and animals (Gerba and Pepper, 2019, McDougald et al., 2020).

Waterborne pathogenic protozoans can share similarities and differences in their life cycle; some have unique life cycles. For example, the life cycle of *Giardia intestinalis* shares some similarities with other enteric protozoa such as *Entamoeba histolytica* and *C. parvum* (Hemphill et al., 2019). All three protozoa have a faecal-oral transmission route and have two life cycle stages: a cyst stage and a trophozoite stage (Zhang et al., 2022, Gunn and Pitt, 2022). They can survive in the harsh conditions of the gastrointestinal tract and cause diarrhea by attaching to and damaging the lining of the small intestine (Martínez-Ocaña et al., 2020). However, there are differences in their life cycles, such as the distinct shape of the cyst stage of *G. lamblia* and the invasive stage of *E. histolytica* that can cause extra-intestinal disease manifestations (Hemphill et al., 2019).

Naegleria fowleri's and *Giardia*'s life cycles differ from that of other pathogenic protozoa because they lack an obligate intracellular phase (Reyes-López et al., 2022). *Naegleria fowleri* exists as a free-living amoeba in freshwater and soil habitats (Maciver et al., 2020). The infective stage of *N. fowleri* is the trophozoite stage, which enters the human host through the nasal canal when swimming or diving in warm freshwater (Hara et al., 2019). This amoeba then migrates to the brain via the olfactory nerve, creating a rare and severe central nervous system illness (Hara et al., 2019) (Kurup et al., 2021). The ability of *N. fowleri* to convert from trophozoite to cyst form helps it to persist under difficult environmental conditions until favourable conditions return (Krishnamoorthi et al., 2022).

Lastly, the life cycle of *Cyclospora cayetanensis* is also unique compared to other enteric protozoa in several ways. It involves asexual and sexual reproduction, with the infective stage being the sporulated oocyst (Almeria et al., 2019). These oocysts are excreted in the feces of the host and require days to weeks to sporulate in the environment before becoming infective (Almeria et al., 2019). After ingestion by a new host, the oocysts excyst in the small intestine, releasing sporozoites that invade the epithelial cells of the small intestine (Dubey et al., 2022, Lindsay, 2019). Unlike other enteric protozoa, *C. cayetanensis* does not have a cyst stage, it has an oocyst stage, just like *Cryptosporidium*, and its life cycle requires an extracellular phase in the environment for the oocysts to sporulate and become infective (Fletcher et al., 2012).

Additionally, the life cycle of *C. cayetanensis* is unique in that it has a narrow host range, infecting only humans and a limited number of primates (Solarczyk, 2021). Therefore, understanding the life cycle of protozoan diseases is crucial to establishing effective preventative and control techniques.

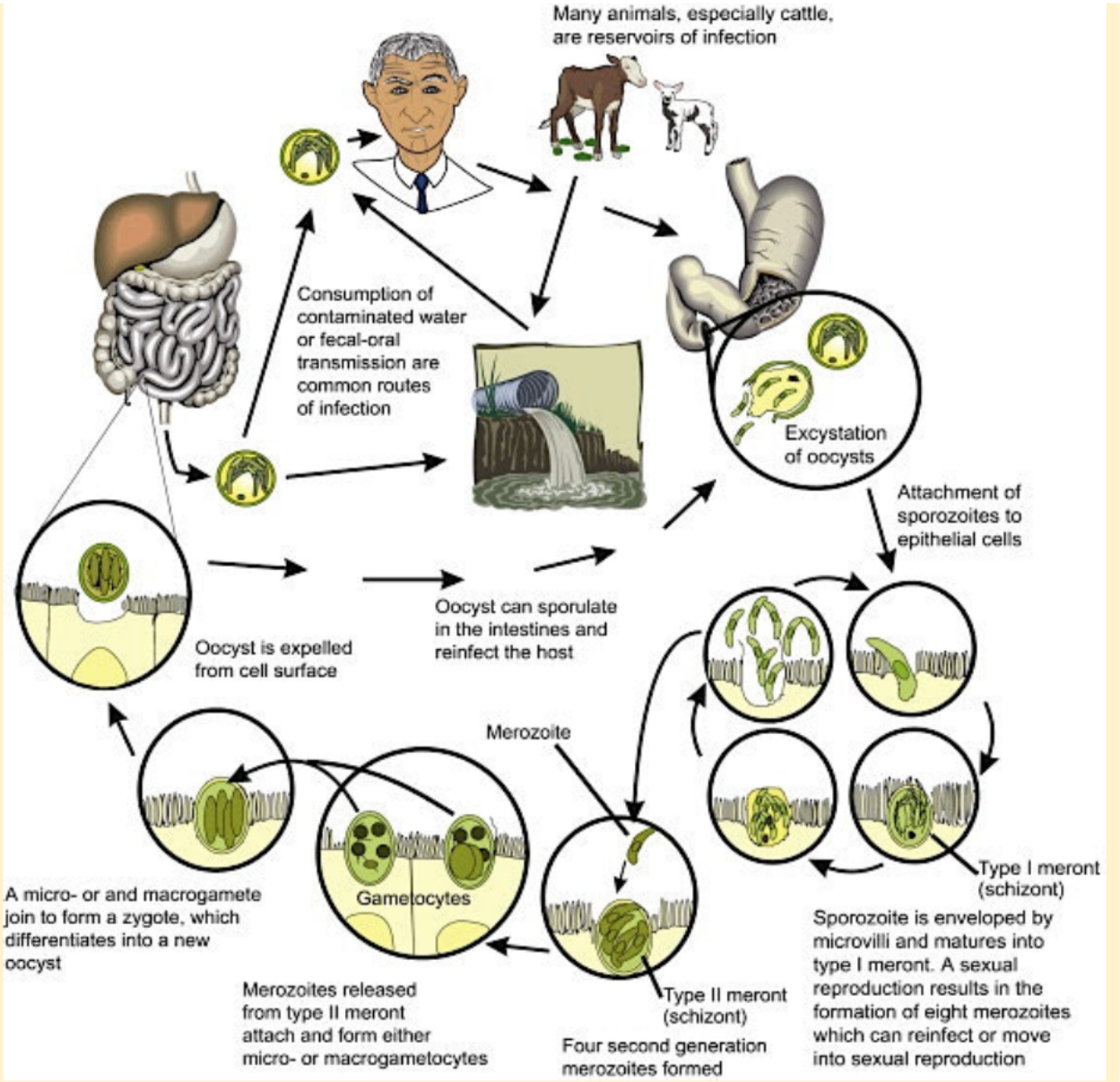

Figure 2 . Life cycle of *Cryptosporidium hominis* (Gerba, 2015).

2.3 Global distribution of waterborne protozoan infections

Waterborne protozoan infections are a global health concern, with prevalence varying widely across different regions, including both developing and developed countries (Omarova et al.,

2018). The distribution of these infections varies depending on a range of factors, including the availability and quality of drinking water, sanitation practices, and the prevalence of the parasites in the environment (Ma et al., 2022).

Developed countries have reported a high prevalence of waterborne protozoan infections, including the United States, Canada, Australia, and several European countries (Ma et al., 2022). In the United States, *Giardia* and *Cryptosporidium* are among the most common cause of infections and are responsible for most waterborne disease outbreaks (Zahedi and Ryan, 2020). For example, 141/251 waterborne protozoa outbreaks were observed in the United States (2017-2020), with 117 outbreaks caused by *Cryptosporidium* spp., 23 by *Giardia* spp. and 1 by *N. fowleri* (Ma et al., 2022). Another study conducted in the United States found that *Cryptosporidium* was responsible for 57.2% of all waterborne disease outbreaks between 2009 and 2017 (Gharpure et al., 2019), while *Giardia* was responsible for 111 outbreaks in 26 states (CDC, 2021). The United Kingdom reported 51 outbreaks between 2017 and 2020, of which 49 were caused by *Cryptosporidium* and two by *Giardia* (Ma et al., 2022). Naegleriasis is a waterborne protozoan infection that is most reported in warm regions of the world (Hoseinzadeh et al., 2021). A review of cases of naegleriasis reported between 1965 and 2018 found that most cases occurred in the southern United States, with smaller numbers of cases reported in Australia, Europe, and Asia (Güémez and García, 2021). Cyclosporiasis is waterborne protozoan infection that is most reported in tropical and subtropical regions of the world (Giangaspero and Gasser, 2019). A review of outbreaks of cyclosporiasis conducted between 2000 and 2019 found that most outbreaks occurred in North America, with smaller outbreaks reported in Europe, Asia, and South America (Almeria et al., 2019). *Toxoplasma gondii* is a protozoan parasite that can cause severe health problems in immunocompromised individuals (CDC, 2018). A study by Shapiro et al., (2019) reported the presence of *T. gondii* in surface water sources in several countries, including the United States, France, and China (Shapiro et al., 2019).

In developing countries such as sub-Saharan Africa and Southeast Asia (India, Nepal and Pakistan), waterborne protozoan infections are particularly prevalent due to poor sanitation and limited access to clean drinking water (Efstratiou et al., 2017, Siwila et al., 2020, Daud et al., 2017, Unicef, 2023). Cryptosporidiosis outbreaks have been reported in both urban and rural areas, particularly in communities with contaminated water supplies (Janssen and Snowden,

2021). A meta-analysis of studies conducted between 1990 and 2018 found that the prevalence of cryptosporidiosis was high in low-income countries, individuals with gastrointestinal symptoms, those younger than 5 years old, and residents not living in urban areas (Dong et al., 2020). For example, in Asia in 2020, the occurrence of *Cryptosporidium* and *Giardia* was found in biogas wastewater and their presence in vegetables (Tram et al., 2022). *Giardia* and *Cryptosporidium* infections in India have been reported in many areas, particularly in rural communities where access to clean water and sanitation facilities is limited (Daniels et al., 2016). Murugesan et al., (2017) found that over 70% of diarrhea cases in children under five years old were caused by waterborne protozoan parasites (Murugesan et al., 2017). The protozoan parasite *Entamoeba histolytica* was discovered in water sources in Bangladesh, indicating a high risk of transmission by polluted water (Singh et al., 2021). It causes amoebic dysentery and liver abscesses (Kantor et al., 2018). In China, waterborne protozoan infections have also been reported, with outbreaks of *Cryptosporidium* infections occurring in several provinces (Liu et al., 2020). A study conducted in Uganda found that over 50% of water sources tested positive for *Giardia* and *Cryptosporidium*, highlighting the extent of the problem in this region (Mugoya et al., 2019). In other African countries, including Ethiopia and Kenya, waterborne protozoan infections are a major public health concern (Squire and Ryan, 2017). *Cyclospora cayetanensis* and *Blastocystis hominis* are two other waterborne protozoan parasites that have been identified in South Africa (Siwila et al., 2020).

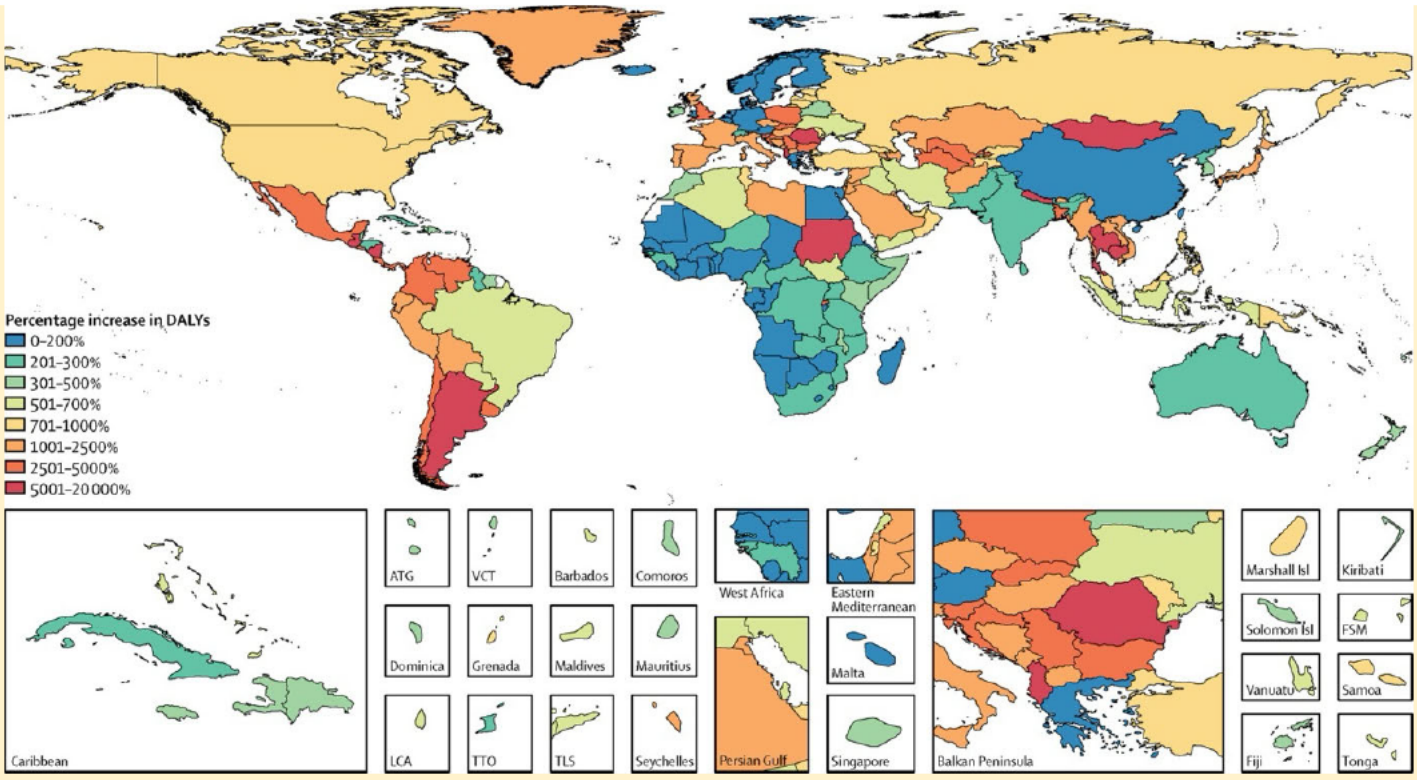

Figure 3. A chart shows the average percentage rise in disability-adjusted life-years (DALYs) caused by *Cryptosporidium* infection in 2016 (Khalil et al., 2018).

This chart illustrates the difference before and after considering the DALYs related to undernutrition in children under five years old. The study conducted by Khalil et al. in 2018 provides this information. The diagram includes data for Antigua and Barbuda (ATG), Saint Vincent and the Grenadines (VCT), Trinidad and Tobago (TTO), Timor-Leste (TLS), and the Federated States of Micronesia (FSM) (Khalil et al., 2018). Speculation of why DALYs are quite high in developed part of the world (from yellow to red).

2.4 Distribution of waterborne protozoan parasites in South Africa

Waterborne protozoan parasites are responsible for many instances of waterborne infections globally, especially in developing nations where access to safe drinking water is limited (Pal et al., 2018). South Africa is no different, with the distribution and prevalence of these parasites in the country being attributed to poor sanitation, inadequate water treatment, and agricultural activities (Edokpayi et al., 2017, Magwaza et al., 2020). The epidemiology of waterborne protozoan parasite infections in South Africa is complicated further by the high prevalence of HIV/AIDS infections, which increases susceptibility, and international travel, which enables migratory patterns of waterborne diseases from other African and developed countries (Squire and Ryan, 2017, Samie et al., 2014, Modjadji, 2021). Several research studies have been

conducted to determine the incidence of waterborne infections caused by protozoan parasites in South Africa. One such study conducted in rural and peri-urban areas of South Africa found that among patients diagnosed with gastroenteritis, 8% and 6% tested positive for infections caused by *Giardia* and *Cryptosporidium* species, respectively (Potgieter et al., 2023). Another study reported a prevalence of 19% for *Giardia* infections and 25% for *Cryptosporidium* infections in rural communities in Gauteng, South Africa (Ngobeni et al., 2022). In 22 studies, it was also found that the prevalence of *Cryptosporidium* spp. infection in Southern African countries was 16.8%, with a higher prevalence among HIV/AIDS patients, children, and diarrhoeic individuals. South Africa had the highest prevalence, while Zimbabwe had the lowest (Omolabi et al., 2022).

Overall, the global distribution of waterborne protozoan parasites is influenced by various factors, including water treatment and sanitation systems, environmental conditions, and socioeconomic factors. Effective prevention and control strategies are needed to reduce the burden of waterborne protozoan parasites on public health.

2.5 Public health relevance of waterborne parasitic protozoa

2.5.1 Impact on human health

Ingestion of protozoan pathogens contaminated water or food can lead to various illnesses, ranging from mild diarrhea to life-threatening diseases such as amoebic dysentery, cryptosporidiosis, and giardiasis (Dao et al., 2020, Vaisusuk and Saijuntha, 2021, Wiser, 2021). These infections can have significant health consequences, particularly in vulnerable populations such as young children, pregnant women, and immunocompromised individuals (Gerba and Pepper, 2019). For example, *Cryptosporidium* and *Giardia* infections can lead to chronic diarrhea, malnutrition, growth retardation in children and cognitive impairments (Nataro and Guerrant, 2017, Khalil et al., 2018). Cryptosporidiosis can also lead to severe illness in immunocompromised individuals, such as those with HIV/AIDS, resulting in chronic diarrhea, wasting syndrome, and death (Certad et al., 2017). Chronic *Giardia* infections have also been associated with an increased risk of irritable bowel syndrome and chronic fatigue syndrome (Litleskare et al., 2018). *Entamoeba histolytica* infection can lead to severe amoebic

dysentery, liver abscesses, abdominal pain, malnutrition, bloody diarrhea and death in some cases (WHO, 2017). Lastly, *Toxoplasma gondii* is a protozoan parasite that can cause severe health problems in pregnant women and immunocompromised individuals (Al-Malki, 2021). Infection during pregnancy can result in stillbirth, neonatal death, and congenital disabilities (Nasiru Wana et al., 2020). In immunocompromised individuals, *Toxoplasma* infections can cause encephalitis, pneumonia, and other severe complications (Zhou et al., 2021). Therefore, understanding risk factors associated with transmission of waterborne infections is important.

2.6 Risk factors associated with transmission

A variety of risk factors have been identified that increase the transmission of waterborne protozoan parasites to humans and animals, including exposure to contaminated water sources, poor sanitation and hygiene practices, exposure to animal feces, and travel to endemic areas (Omarova et al., 2018, Ahmed et al., 2018, Manetu and Karanja, 2021).

Contaminated water sources are a major risk factor for the transmission of waterborne protozoan parasites (Omarova et al., 2018). The World Health Organization (WHO, 2019) states that the primary transmission route for waterborne protozoan parasites is ingesting contaminated water. Protozoan parasites such as *Cryptosporidium* and *Giardia* are two of the most commonly found parasites in surface water sources (Pal et al., 2018). These parasites are resistant to chlorine disinfection and can survive for extended periods in water sources (Casini et al., 2018). Studies have shown drinking untreated surface water is a significant risk factor for contracting waterborne protozoan parasite infections (Reses et al., 2018). For example, a study conducted in Ethiopia found that rivers that were used for bathing and washing clothes were more likely to contain *Giardia* and *Cryptosporidium* parasites than rivers used for other purposes (Sitotaw et al., 2019). Another study conducted in Nepal found that water sources contaminated with fecal matter were more likely to contain these parasites than clean water sources (Shrestha et al., 2020).

Swimming in contaminated recreational water facilities is a risk factor for waterborne protozoan parasite infections (Pineda et al., 2020, Hassanein et al., 2023). *Cryptosporidium* can be found in contaminated recreational water facilities such as swimming pools and water parks (Xiao et al., 2018). Studies have shown that recreational water facilities such as lakes and water

parks can also be sources of transmission for waterborne protozoan parasite infections (Hoseinzadeh et al., 2021, Xiao et al., 2018). *Cryptosporidium* was detected in recreational lake water in Malaysia, highlighting the potential for recreational water sources to be contaminated with protozoan parasites (Ho et al., 2022).

Poor sanitation and hygiene practices are significant risk factors for waterborne protozoan parasite infections (Gupta et al., 2020). Poor sanitation practices, such as open defecation and inadequate handwashing, can contribute to the transmission of *Giardia* infections (CDC, 2019). Inadequate access to safe water sources can also increase the risk of waterborne protozoan parasite infections (Pal et al., 2018). Poor sanitation and hygiene practices can also lead to the transmission of other waterborne diseases, such as cholera, typhoid fever, and hepatitis A (WHO, 2019). Untreated and treated wastewater can be a significant risk factor for waterborne protozoan parasite infections (Al-Nihmi et al., 2020). Wastewater treatment processes can reduce the number of protozoan parasites, but some parasites may still survive and persist in the environment (Zhang et al., 2022, Benito et al., 2020). The discharge of untreated wastewater into water bodies can increase the risk of waterborne protozoan parasite infections in humans and animals (Bridle, 2021).

Animals can serve as reservoir hosts for protozoan parasites, and the discharge of their feces into water sources can contaminate the water with parasites that can cause infections in humans (Dufour and Bartram, 2012). For example, *Cryptosporidium* and *Giardia* can survive in animal feces and can contaminate water sources through runoff or leaching into groundwater (Alegbeleye and Sant'Ana, 2020). Climate change can also increase the risk of waterborne protozoan parasite infections by altering precipitation patterns, temperature, and other environmental factors that affect the survival and transmission of these parasites (Rupasinghe et al., 2022, Ikiroma and Pollock, 2021). For example, heavy rainfall can lead to the runoff of animal feces into water sources, which can increase the risk of waterborne protozoan parasite infections (Angelici and Karanis, 2019). The transmission of waterborne protozoan parasites to humans and animals is a complex issue influenced by a range of risk factors. Therefore, understanding and addressing these risk factors is essential for preventing the transmission of these parasites and developing methods for protecting human health.

2.7 Prevention and control strategies

A range of measures is needed to prevent and control waterborne protozoan parasite infections, including improving sanitation, using a proper water treatment, implementing effective monitoring and surveillance methods, and educating the public (Cissé, 2019, Ma et al., 2022, Cairncross and Feachem, 2018). Investment in water and sanitation infrastructure, public health education, and advanced diagnostic methods such as molecular diagnostics is essential to reduce the burden of these infections (Mokomane et al., 2018, Bryan, 2020). Additionally, advanced wastewater surveillance techniques such as next-generation sequencing are necessary to decrease the prevalence of waterborne protozoan parasite infections (Zahedi et al., 2019, Zahedi et al., 2021).

2.7.1 Water and Wastewater treatment for control and prevention of protozoan parasites in the environment

Water treatment plants (WTPs) and wastewater treatment plants (WWTPs) play a significant role in controlling the spread of waterborne protozoan parasites (Oosthuizen, 2022, Couto et al., 2019, Crini and Lichtfouse, 2019). Several methods are available for water treatment, including physical, chemical, and biological processes (Crini and Lichtfouse, 2019). Physical treatment methods can remove protozoan parasites from wastewater by physical means such as sedimentation, filtration, and coagulation /disinfection (Maurya et al., 2020). Filtration is the most common physical treatment method used in wastewater treatment plants (WWTPs) and has been used to remove protozoan parasites from water sources (Shingare et al., 2019). The process removes microorganisms by forcing water through a filter bed, which traps the parasites (Shingare et al., 2019). WWTPs also use disinfection to eliminate protozoan parasites, although the infective form (oo)cysts are resistant to environmental conditions and disinfection (Abeledo-Lameiro et al., 2018). Chemical processes such as chlorination and ozonation have been applied to inactivate protozoan parasites (Kong et al., 2021, Kumar et al., 2020).

Biological processes, on the other hand such as activated sludge treatment and biofiltration, can also reduce the concentration of protozoan parasites in water sources (Gerba and Pepper, 2019). These methods involve using microorganisms such as bacteria and algae to remove parasites by breaking them down into harmless substances (Gerba and Pepper, 2019). While sewage and wastewater treatment systems play an important role in preventing the transmission

of waterborne protozoan parasites, there are several shortcomings associated with these strategies (Treacy, 2019).

One of the main shortcomings of sewage and wastewater treatment systems is their inability to completely remove protozoan parasites from wastewater (Giglio and Sabogal-Paz, 2018, Xiao et al., 2018). The use of conventional treatment methods such as sedimentation, filtration, and chlorination are not always sufficient to eliminate all protozoan parasites, particularly *G. duodenalis*, *Cryptosporidium* spp., *C. cayetanensis*, *T. gondii* and *E. histolytica* (oo)cysts (Putignani and Menichella, 2010, Almeria et al., 2019). They are highly resistant to conventional disinfectants and can survive in the environment for extended periods (Sánchez et al., 2018). The presence of biofilms in wastewater treatment systems can also provide a protective environment for protozoan parasites, allowing them to survive and even reproduce (Koul et al., 2022). Surveillance approaches are therefore essential to prevent and control waterborne protozoan parasite infections (Rosado-García et al., 2017, Omarova et al., 2018). Regular monitoring of water sources for the presence of protozoan parasites can identify potential sources of contamination and enable prompt intervention to prevent outbreaks (Teklehaimanot et al., 2014). Similarly, surveillance of reported cases of protozoan infections can identify trends in the incidence of infections and inform targeted prevention and control strategies (Sani Kalil et al., 2020).

2.8 Detection and quantitative surveillance methods

One key control and prevention strategy for protozoan parasite infections is the detection and quantitative analysis of water and wastewater for the presence of protozoan parasites (Sánchez et al., 2018, Pickering et al., 2019). This can be accomplished using a variety of techniques, including traditional methods (microscopy and immunofluorescence), polymerase chain reaction (PCR) technology (PCR, qPCR, and ddPCR), and molecular techniques like Loop mediated Isothermal technology (Zahedi et al., 2021, Cuetero-Martínez et al., 2023, Martins et al., 2019, Fusaro et al., 2022).

2.8.1 Conventional detection and quantitative microbiology methods

Microscopy is the most used method for the detection of waterborne protozoan parasites in water/environmental samples (Kahler et al., 2021). It involves the use of a microscope to

visualize the parasites in a water sample and can be done using various staining techniques, such as modified acid-fast staining, chromotrope 2R staining, and trichrome staining (WHO, 2019, Chaskes and Austin, 2021, Prakoeswa et al., 2022). Prior to utilizing microscopy for protozoan parasite detection in water or environmental samples, a concentration step is essential, with various methods, such as sedimentation, centrifugation, filtration, membrane filtration, flotation techniques, precipitation, and formalin-ethyl acetate sedimentation to enhance microscopic examination by concentrating parasites (Naidoo and Archer, 2022, Garcia, 2021, Momčilović et al., 2019). Several studies have successfully applied microscopy for pathogen detection due to its visual confirmation, low cost, and compatibility with different sample types and microscopes (Martinelli et al., 2015). However, microscopy has several limitations, including its low sensitivity and specificity. It requires skilled personnel and can be time-consuming (Shapiro et al., 2019, Adeyemo et al., 2018). A study by Kitajima et al. (2014) evaluated the effectiveness of microbiological surveillance in detecting *Cryptosporidium* spp. in effluent and influent wastewater in Arizona. The study found that microscopy had a low detection rate, while qPCR had a higher detection rate (Kitajima et al., 2014).

Immunofluorescence is another common method for detecting waterborne protozoan parasites in water samples (Galván et al., 2014). In this procedure, antibodies labelled with a fluorescent dye specific to the parasites of interest are utilized, allowing the parasites to be seen under a microscope (Rosado-García et al., 2017). Immunofluorescence has several advantages over microscopy, the most notable of which are rapid and ease to use (Tan et al., 2020, Maia et al., 2022). However, it has several drawbacks, including a lack of sensitivity compared to nucleic acid methods (Olech, 2022).

2.8.2 Advanced detection and quantitative methods

Advanced methods for microbiological surveillance of water samples for the presence of waterborne protozoan parasites have been developed to overcome the limitations of conventional methods (Nemati et al., 2023). These methods include molecular methods, flow cytometry, and biosensors (Olech, 2022). Molecular methods have become increasingly popular for the analysis of waterborne protozoan parasites due to their high sensitivity, specificity, and ability to detect low levels of parasites (Nemati et al., 2023).

2.8.3 PCR based detection methods

The traditional polymerase chain reaction (PCR) is a sensitive and specific method for detecting the DNA of waterborne protozoan parasites in water samples (Gallas-Lindemann et al., 2016). Several studies have shown that PCR can be used to detect waterborne protozoan parasites in water samples (Rosado-García et al., 2017). Al-Jawabreh et al., (2019) used PCR to detect *Cryptosporidium* spp. and *Giardia* spp. in water samples from the West Bank of Palestine. According to the findings, PCR was more sensitive than microscopy in detecting both parasites in water samples (Al-Jawabreh et al., 2019). Feng et al., (2019) used PCR to detect *Cryptosporidium* spp. and *Giardia* spp. in Shanghai, China from tap water samples. The study discovered that PCR could detect low levels of parasites in water samples (Feng et al., 2011). The application of PCR however has limitations, including insensitivity when the target DNA concentration is low, the inability to quantify, and the need for gel electrophoresis for results analysis (O'Leary et al., 2021, Agriculture et al., 2019, Hajia, 2018).

Real-time PCR on the other hand is a quantitative method that detects and quantifies waterborne protozoan parasites in real time (Sharma et al., 2023). The advantages of quantitative PCR over conventional PCR is that it is more sensitive, and specific, and results can be visualized in real time without the need for gel electrophoresis (Moreira et al., 2018). A study by Villamizar et al., (2019) used real-time PCR to detect *Blastocystis, G. duodenalis, C. parvum* and *Entamoeba*. The study found that real-time PCR had high sensitivity and specificity for detection compared to microscopy and conventional PCR (Villamizar et al., 2019). Another study used real-time PCR (qPCR) to efficiently monitor the removal of *Giardia* and *Cryptosporidium* from water samples collected from drinking water treatment plants (Moussa et al., 2023).

Droplet digital PCR (ddPCR) is a more advanced and a recently developed method for detection of microbes from environmental and clinical samples (Baltrušis et al., 2019). Several studies have been conducted to investigate the utility of ddPCR for detecting waterborne protozoan parasites which was shown to overcome most limitations associated with molecular methods (Rajapaksha et al., 2019, Mthethwa et al., 2022). For example, several previous studies found that ddPCR can detect a very low concentration of *Cryptosporidium* spp. and

Giardia in water samples better than conventional methods (Yang et al., 2014, Adeyemo et al., 2018). Another study also found that ddPCR was more sensitive and specific in detecting *C. felis* compared to nested PCR assays (Kao et al., 2021).

2.8.4 Loop mediated isothermal amplification (LAMP)

Unlike polymerase chain reaction (PCR), which requires thermal cycling to denature DNA and anneal primers, LAMP can amplify DNA at a single constant temperature, usually between 60-65°C making it a simpler and faster technique (Notomi et al., 2000).

2.8.4.1 The principle of LAMP

The principle of LAMP (figure 4) involves the use of a DNA polymerase with strand displacement activity, four to six primers that recognize six distinct regions on the target DNA, and a loop structure that allows for the amplification of multiple copies of the target sequence (Mishra et al., 2023). The LAMP reaction consists of an initial step of strand displacement and amplification, followed by a second step of elongation and amplification (Sivaprasad et al., 2021). In the first stage, the primers hybridize with the target DNA and initiate the amplification process. The primers consist of two different types of sequences, viz., inner and outer primers (Becherer et al., 2020). The inner primers (also known as the FIP and BIP) contain two regions that anneal to the target DNA in opposite orientations, forming a loop structure that facilitates amplification (Becherer et al., 2020). The outer primers (also known as the F3 and B3) anneal to the regions flanking the loop structure, ensuring that the amplification occurs in a specific and controlled manner (Huang et al., 2020). During the strand displacement and amplification stage, the DNA polymerase uses the primers to synthesize a complementary strand, which displaces the original strand, forming a loop structure that contains the target DNA sequence (James and Alawneh, 2020). This loop structure acts as a template for further amplification in the second stage of the reaction (Soroka et al., 2021). In the second stage, the DNA polymerase continues to elongate the loop structure, generating large amounts of amplified product (Soroka et al., 2021). LAMP can be accelerated using additional loop primers (Park, 2022) (loop forward & loop reverse). The loop structure allows for the amplification of multiple copies of the target sequence, resulting in a highly specific and sensitive amplification of the DNA/RNA of interest (Zhang et al., 2021). The amplified product can therefore be detected by various methods (detailed below).

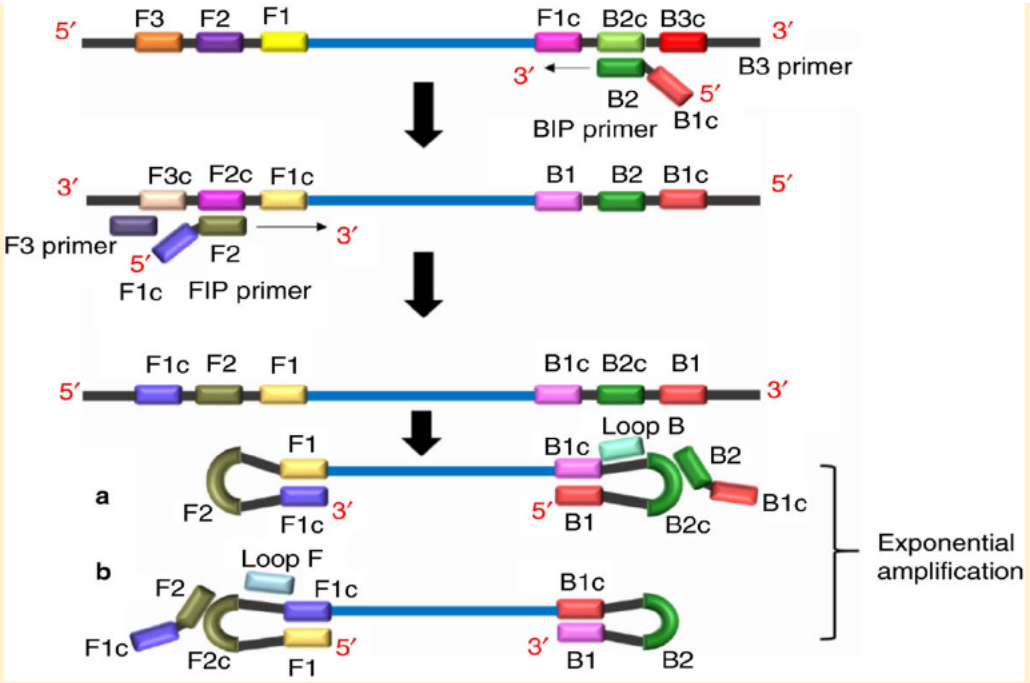

Figure 4. Showing principle of LAMP (Wong et al., 2018).

2.8.4.2 Different LAMP methods

Several LAMP techniques have been developed, each with unique features that make them suitable for specific applications. Reverse transcription LAMP (RT-LAMP) (Yang et al., 2020), loop-mediated amplification coupled with a lateral flow assay (LAMP-LFA) (Jawla et al., 2021), multiplex LAMP (mLAMP) (Fan et al., 2022), strand displacement amplification LAMP (SDA-LAMP) (Zhang et al., 2022), and LAMP using CRISPR-Cas12 (Selvam et al., 2021) are examples of these techniques.

2.8.4.3 Advantages and disadvantages of LAMP for protozoan

The LAMP technique is a powerful tool that addresses most challenges faced by the molecular methods discussed in the above previous sections. First, LAMP is recognized for its sensitivity and specificity, contributing to a lower likelihood of false positives. This is mainly because LAMP utilizes a set of four to six primers that can identify six different regions on the target DNA (Thompson and Lei, 2020). Several studies have compared LAMP to other nucleic acid amplification techniques, such as PCR, and found that LAMP is highly specific and sensitive, with a lower risk of false-positive results than PCR (Badparva et al., 2022, Dai et al., 2019).

For example, a study used LAMP in comparison to qPCR for the detection of *Toxoplasma gondii,* and the results showed high sensitivity to detect as few as 5 oocysts/5ml (Durand et al., 2020). The LAMP method can detect as few as 10 copies of the target DNA, making it a useful tool for the detection of low-abundance DNA (Craw and Balachandran, 2012). Furthermore, LAMP can amplify DNA rapidly, with results often available within 15-60 minutes, compared to several hours for conventional PCR (Wang et al., 2019, Fitri et al., 2022). It is cost effective due to that LAMP does not require thermal cycling, making it more convenient, cheaper, and simpler to perform than PCR (Amoah et al., 2021). LAMP products can be visualized simply by various methods, including the use of turbidity measurements, visual inspection, or fluorescent dyes (Wong et al., 2018, Park, 2022). However, the design of primers for LAMP can be challenging due to the need for specificity and avoidance of non-specific binding to other regions of the DNA template (Kang et al., 2022). Additionally, there is a significant risk of carryover contamination with LAMP, which can result in false-positive results in subsequent reactions (Hardinge and Murray, 2019).

2.8.4.4 LAMP analysis methods

There are various methods for analysing Loop mediated isothermal amplification (LAMP) products, depending on the specific application and the level of detection required. These include gel electrophoresis, turbidity measurements, fluorescent probes, and colorimetry. Gel electrophoresis is a commonly used method to analyse LAMP products by visualization using ethidium bromide staining (Green and Sambrook, 2019, Mousavi et al., 2020). However, this method is not preferable due to the use of carcinogenic ethidium bromide stain and that it is time-consuming, requires specialized equipment, and is not suitable for real-time analysis (Wong et al., 2018).

The turbidity measurement, on the other hand, is a simple and rapid method to monitor the LAMP reaction in real-time (Garg et al., 2022, Fischbach et al., 2015). The turbidity of the reaction mixture increases with the formation of insoluble magnesium pyrophosphate, which is produced during the amplification process (Park, 2022). The turbidity can be measured using a simple spectrophotometer or a turbidimeter (Fischbach et al., 2015). Another method involves the use of fluorescent probes/dyes to detect LAMP products in real time (Gadkar et al., 2018). These probes bind to the amplified products and emit a fluorescent signal that can be detected

using a fluorometer in real-time (Hardinge and Murray, 2019). Fluorescent probes are highly sensitive and specific and can detect low concentrations of target nucleic acid in a short time (Talap et al., 2022). Lastly, the colorimetric detection is a simple and rapid method for the visual detection of LAMP products (Zhang et al., 2022). It involves the use of pH-sensitive dyes or metal ion indicators that change colour in response to the accumulation of magnesium pyrophosphate during amplification (Zhang et al., 2022). Colorimetric detection is suitable for point-of-care testing and field-based applications since the colour change can be seen without specific equipment (Diaz et al., 2021).

Sections 2.7.5 to 2.7.10 have been published with the following details: "*Mthethwa, N. P., Amoah, I. D., Reddy, P., Bux, F., & Kumari, S. (2021). A review on application of next-generation sequencing methods for profiling of protozoan parasites in water: Current methodologies, challenges, and perspectives. Journal of Microbiological Methods, 187, 106269. https://doi.org/10.1016/j.mimet.2021.106269*" Full paper is attached as supplementary information (Appendix 2).

2.8.5 Next-generation sequencing methods

Next-generation sequencing (NGS) technologies have emerged as promising approaches for preventing and controlling of protozoan parasites infections. This section provides a review of current methods and recommends standardization of techniques to optimize metagenomic analysis for protozoan parasites, including sample pre-processing, DNA extraction, sequencing approaches, and analysis methods.

2.8.6 Optimization of sample preparation

2.8.6.1 Sample collection

Sample preparation before sequencing can significantly affect the quality, quantity, and accuracy of the metagenomic results (Thomas et al., 2012, Felczykowska et al., 2015). Different pre-processing techniques are optimized and validated, including sample collection, preservation, and DNA extraction (Felczykowska et al., 2015). The key reasons are to obtain a

representative environmental sample, reduce the impact on the chemical and physical integrity of the sample, and minimize downstream inhibitors (Skotarczak, 2009). Most of the reviewed articles on metagenomics profiling did not report the sample collection procedure used to ensure a representative sample (Table 1). However, the two commonly used methods for environmental sample collection are grab (snap sample) and composite sampling methods (Ma et al., 2009, Dong, 2015). The grab sampling approach is a single discrete sample collected over a time not exceeding 15 minutes (EPA, 2017). It gives snap microbial information specific to the sampling area, location, and time and has been applied for the investigation of microorganisms and conditions in wastewater, surface water, and soil sample (EPA, 2017). The composite sampling approach, either by time composite sampling or by flow proportional sampling, consists of equal volume, discrete sample aliquots collected at constant time intervals, or constant sample volume at varying time intervals into one container (Ort et al., 2010). A composite sample reflects the average characteristics of the sample matrix during the compositing process (Dong, 2015). The suitability of a sampling method depends on the area and type of samples being investigated. However, Quince et al., (2017) recommended that shotgun metagenomic studies use composite sample collection to achieve an average representation of microbiome from the same habitat overtime (Quince et al., 2017). For wastewater samples, a 24h composite sample could provide a better representation of parasites entering and exiting a treatment plant as opposed to single snapshot samples. Despite this recommendation, there is no available data to determine the best sample collection method for metagenomic profiling of protozoan parasites in environmental samples. This area, therefore, needs more attention, when considering metagenomic studies for profiling of protozoan parasites from the environmental samples.

2.8.6.2 Sample preservation

Sample preservation is key to maintain the integrity or characteristics of the collected sample. There are currently several promising preservation methods available for short-term storage at 4°C, ambient temperature, or in a cooler box (Crawley et al., 2016, Escotte-Binet et al., 2019, Zacharia et al., 2019) (See Table S1 Appendix I). This method is commonly used during transportation, on-field, and resource-limited laboratories (Lalonde and Gajadhar, 2016, Lear et al., 2018). The long-term preservation method includes storage at -20°C, -80°C, or -150 °C, and freeze-drying (Marquis et al., 2019, Ögren et al., 2020, Brumfield et al., 2020). This is one

of the commonly used preservation methods and is also applicable for short-term preservation. Other preservation methods include the use of buffers or chemicals such as potassium dichromate,10% formaldehyde, 70-95% ethanol, solitary use of silica gel beads, buffer DMSO-EDTA, DNA/RNA shield, etc (Wilke and Robertson, 2009, Kuk et al., 2012, Papaiakovou et al., 2018, Lear et al., 2018). The DNA/RNA shield (Zymo Research, Freiburg, Germany) and the RNA*later* (ThermoFisher Scientific, USA) are frequently used to preserve the genetic integrity and expression profiles of samples at ambient temperatures (no refrigeration for short term storage), additionally, the buffers, DNA/RNA shield, and RNA *later* completely inactivate infectivity of the agents present in the sample (Menke et al., 2017, Plauzolles et al., 2020). Menke., (2017) investigated self-made nucleic acid preservation buffer (NAP) against commercial buffers DNA/RNA Shield (Zymo Research, Freiburg, Germany) and RNA *later* (ThermoFisher Scientific, USA), together with freezing or storing at room temperature before sequencing. Results revealed that immediate freezing (-20°C) of samples yielded the best results. Moreover, the self-made nucleic acid preservation buffer (NAP) had better preservation qualities, was cheaper, and indicated applicability in microbiome studies compared to commercial RNA *later* and DNA/RNA Shield. Other studies recommended a simpler and common method to preserve microbial community and DNA, especially for resource-limited areas, to keep the sample at a cool temperature and perform DNA extraction within 48 hours or as soon as possible after collection (Borneff-Lipp and Duerr, 2013, Felczykowska et al., 2015, Lear et al., 2018, Wylezich et al., 2020).

2.8.6.3 Sample concentration

Concentration of protozoan (oo)cysts from environmental samples are needed to improve the chances of accurate representation of a target population. Moreover, protozoans are considered taxa that have high spatial heterogeneity, they are at low population density relative to the sample area and compared to bacterial (prokaryotes) population (Lear et al., 2018). The standard biomass concentration methods for protozoan parasites include flotation, which is based on the density of the species being targeted such as sucrose gradient flotation/ficoll gradient separation (Babaei et al., 2011, Andersson et al., 2015, Gallas-Lindemann et al., 2016, Escotte-Binet et al., 2019). These methods are commonly applied for the concentration of parasites *Cryptosporidium* or *Giardia* (oo)cysts (Lora et al., 2016, Sammarro Silva and Sabogal-Paz, 2020). However, because of the presence of foreign material in environmental

samples of similar density and size to (oo)cysts of interest, it can be attached to (oo)cysts or concentrated along with (oo)cysts and may lead to loss of parasites or carry-over contamination (Al-Sabi et al., 2015, Sammarro Silva and Sabogal-Paz, 2020). Other concentration methods commonly used are centrifugation under different centrifugal forces and time period (Felczykowska et al., 2015, Hendriksen et al., 2019). Filtration is also successfully used for the concentration of protozoan parasites. This involves concentrating protozoans by passing through different filter pore sizes ranging from 0.22 µm and 0.1 µm filters (Almeida et al., 2015, Brumfield et al., 2020), and application of both centrifugation (Bridle et al., 2010) and filtration (Mahmoudi et al., 2015) on the same sample. Based on the reviewed literature, commonly used methods included filtration and centrifugation and are thus recommended for the concentration of protozoans in environmental samples.

2.8.6.4 Nucleic acid extraction

Different DNA extraction methods have been reported in the literature, as presented in Table 1 and Figure S1(Appendix I), both commercial DNA extraction kits and custom DNA extraction protocols were used. These include the phenol-chloroform extraction method (Babaei et al., 2011, Mahmoudi et al., 2015), salting-out method (Sun, 2010), and modified UNEX protocol (Shields et al., 2013, Moreno et al., 2018), which are the most used to isolate and extract protozoan DNA from wastewater, surface water/irrigation water, and fecal samples. In addition, various commercial DNA extraction kits were also employed with varying efficiency (Almeida et al., 2015, Gallas-Lindemann et al., 2016, Shin et al., 2018, Javanmard et al., 2018, Maritz et al., 2019, Zahedi et al., 2019, Marquis et al., 2019, Brumfield et al., 2020, Rusiñol et al., 2020). Among the different kits used, QIAamp Fast DNA Stool Mini kit, Powersoil, and power water DNeasy extraction kits were mostly used for metagenomic projects from environmental samples. Their application revealed simplicity of the protocol, fast speed on overall metagenomic workflow, and high-quality DNA with no inhibitor and carryover interferents, moreover, the protocol allows for a larger number of samples to be analysed at a minimum time (Hamner et al., 2019, Hendriksen et al., 2019, Zahedi et al., 2019, Maritz et al., 2019).

It is often difficult to break the robust protozoan parasite's oocysts and to release the DNA (Sánchez et al., 2018, Wylezich et al., 2018). Therefore, to improve DNA extraction efficiency,

a variety of pre-treatment methods have been employed by different researchers, such as freeze-thaw, bead beating (Felczykowska et al., 2015, Wylezich et al., 2018), and addition of chemicals (such as ethylene diamine tetra acetic acid (EDTA), protease K, NaCl, NaH_2PO_4, and Na_2HPO_4) (Fang et al., 2014). In some instances, liquid nitrogen has been used to achieve rapid freezing of the samples to enhance the extraction of DNA (Mahmoudi et al., 2015). It is recommended that for each sample type, pretreatment and DNA extraction protocols are optimized, because the optimized method for one sample may not work for the other. Several studies have focused on the optimization of DNA extraction methods for protozoan parasites from samples such as stool, water, soil, green leaves, fruits, and urine (Lalonde and Gajadhar, 2016, Barbosa et al., 2017, Ackerman et al., 2019, Temesgen et al., 2020). For instance, Psifidi., (2015) compared 11 DNA extraction methods for optimum whole-genome analysis, these included commercial kits, modified commercial extraction protocols, and a custom magnetic beads protocol. Three of the modified commercial kits (Nucleospin Blood, nucleospin tissue, and Dx method) and the custom magnetic beads protocol indicated suitability for longer storage and high-throughput analysis. The modifications made for commercial kits included increased volume of lysis buffer, protease K, and as well as an increased incubation time with protease K. Another significant processing step required before sequencing is cellular-host DNA removal (Miller et al., 2013, Pereira-Marques et al., 2019). This is especially important for environmental samples that are often associated with human DNA (Lear et al., 2018).

Fractionation and selective lysis are common methods that have been used for the reduction of host DNA (Thomas et al., 2012, Oyola et al., 2013). However, based on current literature, there is no data to support a particular method for human host DNA degradation. Purification of extracted DNA is an additional step required before NGS analysis, as it removes inhibitors and chemicals left after DNA extraction to increase the efficiency and sensitivity of sequencing and analysis. DNA binding silica column purification is one such method used, it is quick, simple, and efficient (Ackerman et al., 2019). However, it increases overall expense, significantly reduces the DNA concentration, as some of the DNA gets trapped in the silica column thus a higher starting DNA concentration is always recommended (Andersson et al., 2015). Limited studies are reporting on available purification methods, which makes it difficult to ascertain the best method for metagenomics profiling of protozoan parasites.

Table 1: Commonly used methods for environmental sample preparation reported in the literature.

Sample type	Sample processing	DNA extraction method	Comment	Reference
1. Surface, irrigation, and wastewater effluent	Centrifugation, IMS-IFA	UNEX protocol	Both Illumina and qPCR analysis from the sample showed no PCR inhibition	(Moreno et al., 2018a)
2.Wastewater sludge, soil, stormwater, and sediments	N/A	Powersoil DNA extraction kit	The study concluded that the DNA extraction method used (bead-beating) may have been insufficient to break open *Cryptosporidium* oocysts	(Maritz et al., 2019)
3. Wastewater samples	Filtration (0.2 µm)	PowerWater Sterivex DNA	The DNA extraction efficiency in the present study was unknown.	(Zahedi et al., 2019)
4.Oocysts	N/A	QIAamp DNA Mini Kit	3-5 oocysts per gram of produce were reliably detected using the optimized isolation methods and qPCR MCA.	(Lalonde and Gajadhar, 2016)
5. Oocysts spiked in berries	N/A	PowerSoil kit	PowerSoil kit was the method of choice for extraction of DNA of coccidian oocyst and detection by using TaqMan probe qPCR protocols.	(Temesgen et al., 2020)
6. Soil sample	0.1% Tween80/PBS for dispersion, sucrose flotation, mechanical grinding,	Fast DNA spin kit	The best protocol used 0.1% Tween 80, sucrose gradient, and FastPrep DNA extraction. It accurately detects T. gondii	(Escotte-Binet et al., 2019)

			DNA in soil samples detection limit below 1 oocyst/g of fresh soil.	
7. River oyster sample	N/A	Omega Biotek E.Z.N.A. Tissue Kit	N/A	(Marquis et al., 2019)
8. Surface Water samples	Filtration using Envirocheck HV (Pall Gelman Laboratory) capsule	DNeasy PowerSoil	Recovery efficiency: Of the 39 samples analyzed 7.7% (3/39) were positive for *T. gondii*, and the occurrence of the oocysts was detected in 30% (3/10).	(Galvani et al., 2019)
9. Oocysts of *Cryptosporidium* and *Giardia*	N/A	DNeasy Blood and tissue kit	*C. parvum*, *G. lamblia*, and *C. cayetanensis* sp each parasite signal without interference or no	(Shin et al., 2018)
10. Wastewater and vegetables	Filtration (pore size 0.4 µm) and centrifugation	YTA DNA extraction kit for stool	N/A	(Javanmard et al., 2018)
11.Wastewater samples	Centrifugation and filtration (0.22 µm)	DNA isolation kit	N/A	(Ajonina et al., 2018)
12. Wastewater, surface waters, groundwater, drinking water	Filtration, flocculation, and centrifugation, and sucrose density gradient	QIAamp Mini Kit	*Cryptosporidium* spp. And *Giardia* was detected in samples (by IFA, nested PCR, and by LAMP without any inhibitors	(Gallas-Lindemann et al., 2016)
13. Treated tap water sample	Ultrafiltration-centrifugation for secondary concentration	Biofire Diagnostics 1-2-3 SWIPE Sample Purification	Alternative DNA extraction buffer, second spin column cycle, Tris–EDTA buffer enhanced detection sensitivity for *Cryptosporidium*	(Kimble et al., 2015)

14. Raw and treated water	Membrane filtration technique. cellulose acetate membranes that had a 47 mm diameter and 1.2 µm porosity	NucleoSpin Tissue	The sequencing showed *Cryptosporidium parvum* and *Giardia duodenalis* DNA	(Almeida et al., 2015)
15. River water	Filtration (0.45 µm) Then centrifugation	Phenol-chloroform method and QIAamp DNA Mini Kit	N/A	(Mahmoudi et al., 2015)
16. Water samples spiked with c.parvum oocysts	Liquid nitrogen	QIAamp DNA Tissue Mini Kit +liquid nitrogen	Extraction of DNA from *C. parvum* oocysts was most effective when it was preceded with cycles of liquid nitrogen/water baths incubation and with the use of lysate buffer and overnight proteinase K digestion. This resulted in a higher copy number of the 18S rRNA gene	(Adamska et al., 2012)
17. Water	Centrifugation and filtration (25 mm GF/C Whatman glass microfibre filter)	MasterPure Complete DNA and RNA extraction kit (Epicentre)	N/A	(Bridle et al., 2010)
18. Drinking water	Filtration 0.6 µm ,0.2 µm and 0.1 µm pore size pore size)	ZymoBIOMICSTM DNA Miniprep Kit	N/A	(Brumfield et al., 2020)
19.Drinking water, reservoir water, groundwater, and river water	Standardized SMF protocol	Fast DNA SPIN Kit for soil	N/A	(Rusiñol et al., 2020)

20. Urban sewage water	Centrifugation	QIAamp Fast DNA Stool Mini kit and Nucleospin RNA XS kit	N/A	(Hendriksen et al., 2019)
22. Water samples	Filtration (47 mm, 0.45)	PowerWater DNA isolation kit	DNA sequences indicated the presence of both Eukarya and Bacteria in the river water community, genera that are of potential concern to human health, including *Acanthamoeba, Leishmania, Candida, and Rhizomucor*, were identified in the analyses	(Hamner et al., 2019)

2.8.7 DNA sequencing approach

Both targeted and nontargeted (shotgun) sequencing approaches have been used to study protozoan diversity. In the nontargeted sequencing approach, all microbial genomes present in a sample are first sheared into smaller fragments before sequencing on NGS platforms. This approach then results in output sequence data of all genes from all domains of life (Miller et al., 2013, Lear et al., 2018). The targeted sequencing, on the other hand, is referred to as the second metagenomic approach; however, it is not entirely meta since it does not sequence all the microbial genomes present in a sample at the same time; instead, it is targeted to a single marker gene (Quince et al., 2017, Kibegwa et al., 2020). Thus, this approach employs taxon-specific primers for sequencing predefined domains of organisms in complex environmental samples, for example in the case of protozoans the 18S rRNA gene is the preferred area of interest (Ryan et al., 2017).

2.8.7.1 Shotgun metagenomics

Several published articles were retrieved on the shotgun metagenomic approach, focusing on the environmental microbiome (Table 2). This approach has enabled successful investigations of protozoan's diversity associated with sewage water in both urban (Maritz et al., 2019) and rural settlements (Hendriksen et al., 2019). It has also been used in a comparative analysis of the drinking water microbiome (Brumfield et al., 2020), identification of pathogens in river water (Hamner et al., 2019) as well as an assessment of microbial diversity in marine ecosystems (Kisand et al., 2012). These studies reported a diverse protist community dominated by free-living clades, seasonal and host differences in protist composition in urban New York sewage and revealed a significantly increased abundance and variations in *Cryptosporidium* spp. and *Giardia* in informal settlement sewage (Kenya) over time (Ryan et al., 2017). A shotgun sequencing approach was also used to analyse environmental microbiome (bacteria, eukaryote, virus, and archaea) in water samples of a coastal region, the results revealed a very low recovery rate of only 2.9 % of eukaryotes in the study data compared to bacterial diversity (Kisand et al., 2012).

Few other studies used a combination of the different sequencing platforms to get a better representation of the diversity and functional properties of the protozoan community in an ecosystem. For example, the pathogenic microbiome of the world's largest water reuse facility

Orange County Water District (OCWD) Advanced Water Purification Facility (AWPF) was investigated by a combination of shotgun metagenomics, transcriptomic and amplicon-based approach to provide a novel in-depth characterization of wastewater from influent to the final product (Stamps et al., 2018). This study showed that protists were in low abundance, representing less than 1 percent recovered metagenomic or meta-transcriptomic sequence data. A combination of different methods in a single run could offer both in-depth higher resolution of targeted genes and comprehensive primer-bias-free analysis of the dominant genes present in a sample (Lear et al., 2018). This approach, therefore, is becoming popular to researchers, as it is considered simpler due to the use of the same workflow techniques, same sample, and protocol simultaneously (Lear et al., 2018). Moreover, these methods can be complementary to each other, the limitation of one method can be overcome using the other. As per the reviewed literature (table 2), The shotgun approach shows a strong benefit in its applicability for protozoan parasite detection due to the following reasons: first A single sequencing-based test is used to capture all taxa present within a sample, providing a single output dataset on the microbial diversity and functional novelties associated with that environment (Quince et al., 2017) and second the approach works without an amplification stage, enabling all eukaryotes identification and by-passes the biases usually found in amplicon-based sequencing (Wylezich et al., 2019, Wylezich et al., 2020). Another advantage of the untargeted metagenomics approach is that no prior decision is needed for which protozoan specific gene marker or region to screen however, further research studies and tools are needed to improve protozoan gene resolution for better taxonomic assignments of sequences from metagenomics data.

2.8.7.2 Amplicon-based metagenomics

Amplicon-based metagenomics includes/involves several steps to analysis. The first step begins with designing PCR primers that target specific conserved gene regions or gene fragments. For example, protozoan studies often target the conserved 18S rRNA gene (Miller et al., 2013, Tanaka et al., 2014, Dulanto Chiang and Dekker, 2019), the whole intergenic transcribed spacers (ITS), or the large ribosomal subunit (LSU)/28S rRNA) gene (Uyaguari-Diaz et al., 2016). These targets are then used in PCR amplification to generate DNA sequences (amplicons). The generated amplicons are sequenced using NGS technologies. And lastly, resulting sequences are compared to a reference database using bioinformatic platforms for species and genus identification (Miller et al., 2013).

In addition, this approach allows for targeting multiple genes of different target regions to be studied simultaneously in a single reaction to achieve high coverage of protozoan parasites (Uyaguari-Diaz et al., 2016). In complex samples, such as environmental samples, this approach can provide even greater details of protozoan parasites without being dominated by other highly abundant microbes (Miller et al., 2013). This highly targeted approach enables researchers to efficiently discover, validate, and screen genetic variants within a specific genome (Dulanto Chiang and Dekker, 2019). It reduces sequencing costs and the intensive bioinformatics analysis task compared to broader approaches such as the shotgun metagenomic approach (Dulanto Chiang and Dekker, 2019).

The majority of reported studies captured in Table 2 used the deep amplicon-based metagenomic approach for analyses of protozoan parasite diversity from different environmental matrices. The commonly targeted regions of the genome are V4 (Moreno et al., 2018a, Stamps et al., 2018), V9 (Greay et al., 2018, Maritz et al., 2019), and V5 (Popovic et al., 2018) of the 18S rRNA gene. The 18S rRNA gene marker has nine variable regions (V1 to V9), which are commonly used for diversity studies (Hadziavdic et al., 2014). Analysis of water and wastewater samples has been conducted using 18S rRNA looking at different regions from V1-V9 within the taxonomic marker (Tanaka et al., 2014, Cooper et al., 2016, Popovic et al., 2018, Moreno et al., 2018). However, the V4 region is the longest and the most conserved variable region within 18S rRNA thus has been considered to have the highest resolution for protozoans (Hadziavdic et al., 2014, Pawlowski et al., 2016, Lear et al., 2018).

Analysis of most important protozoan parasites in surface water and wastewater was conducted using 18S rRNA gene amplicon sequencing the V4 hypervariable region and sequences of *Toxoplasma gondii, Entamoeba histolytica, Cryptosporidium* spp*. Acanthamoeba castellanii, Giardia intestinalis, Blastocystis* spp. were recovered (Moreno et al., 2018). Several other studies looked at the same 18S rRNA region V4 to analyze parasites in water samples and detected *Entamoeba, Blastocystis, Acanthamoeba* spp*., Cryptosporidium parvum, Giardia intestinalis, Toxoplasma gondii, and Trichomonad* spp*.* (Bradley et al., 2016, Maritz et al., 2017, Stamps et al., 2018, Maritz et al., 2019).

Other studies have combined various regions within the 18S rRNA gene to achieve high species resolution. For example, water and wastewater-associated microbiome were investigated using the V9 region together with V3 and V4 within the *Cryptosporidium* gene (Greay et al., 2018). Results revealed that the eukaryotic V9 region had inadequate sensitivity for intestinal parasite detection in wastewater. Other studies analysed water and wastewater protozoan parasite diversity by examining both V9 and V4 regions of the 18s rRNA to understand the protist pattern in sewage water (Maritz et al., 2019) and for characterization of the zoonotic and Trichomonad taxa (Maritz et al., 2017). The results revealed the mean relative abundance detected by both taxa to be 12.5% V4 and 15.4% V9. The V4 probe detected mostly common soil protist and V9 detecting kinetoplastids, additionally, intestinal parasites of vertebrates were present in low abundance. However, they further reported that the 18S rRNA regions selected did not provide a fine-scale zoonotic taxonomic resolution and could not define closely related species of trichomonas. Gene region combination of V9 and V1-V3 hypervariable regions of the 18S rRNA gene are commonly used for the investigation of protozoans in soil and fecal samples, although inconsistent results are sometimes reported (Geisen et al., 2015, Cooper et al., 2016, Vermeulen et al., 2016).

Bradley et al., (2016) investigated the effect of amplification biases on microbiome structure and diversity in freshwater, coastal, and wastewater samples using the V4 and V8-V9 regions. Results revealed V4 and V8-V9 regions had similar microbial community representations. Although the V9 section of the 18S rRNA gene is commonly used for the analysis of protists diversity (Ramirez et al., 2014), Lear et al., (2018) mentioned that this region tends to include organisms from other taxa. The community of soil protists associated with metazoan control DNA was assessed by Amplicon sequencing of SSU rDNA,18S rDNA (V1–V3 region) and the sequencing markers revealed different protist communities from those that were expected (Geisen et al., 2015). More limitations of this approach are included in section 3.6.

Table 2: Metagenomic studies focused on environmental samples, reported in the literature.

Sample type	Metagenomic approach	Sequencing-platform	Protozoan parasite	Comments	Reference
1.Surface irrigation water and wastewater	18S rRNA gene amplicon sequencing: V4 hypervariable region	Illumina MiSeq	*Toxoplasma gondii, Entamoeba histolytica, Cryptosporidium spp. Acanthamoeba castellanii, Giardia intestinalis, Blastocystis sp*	NGS methodology developed for identification of most important WPPs	(Moreno et al., 2018a)
2.Raw sewage, soil, stormwater, and sediments.	18S rRNA gene amplicon sequencing: V4 and V9 hypervariable region	Illumina HiSeq Rapid Run	Protist community (oligohymenophorea and ciliates) *Entamoeba* *Blastocystis*	Sewage contained a diverse protist community dominated by free-living clades, Seasonal differences in protist composition were observed, human and animal associated protist was also detected	(Maritz et al., 2019)
3.Raw sewage, soil, stormwater, and sediments.	Shotgun metagenomic				
4.Wastewater samples (influent to effluent)	Small subunit ribosomal RNA (SSU rRNA) gene	Illumina MiSeq and -Illumina HiSeq	*Acanthamoeba mauritaniensis, Acanthamoeba palestinensis* and more)	Provided the first in-depth characterization of water at a	(Stamps et al., 2018)

	sequencing (V4 hypervariable region), metagenomics, and metatranscriptomics			multibarrier potable reuse facility, from influent to final product water.	
5.Wastewater treatment plant samples (influent, intermediate and effluent)	Amplicon sequencing: 18S ribosomal DNA (V9) hypervariable of eukaryotic *Cryptosporidium*-specific 18S (V3 & V4) primers sequencing	Illumina MiSeq	*Cryptosporidium* species (zoonotic) *Blastocystis sp*. STs, *Endolimax* spp., *Entamoeba spp*.and *Iodamoeba* spp.	Eukaryotic V9 18S NGS had inadequate sensitivity for intestinal parasite detection in wastewater samples and six were identified by *Cryptosporidium*-specific NGS	(Greay et al., 2018)
6.Raw wastewater treatment sample	Amplicon based sequencing: 18S locus	Illumina MiSeq	*Cryptosporidium* species	*Cryptosporidium* was prevalent in the raw influent and a large diversity of *Cryptosporidium* species and genotypes was revealed.	(Zahedi et al., 2018)
7.Petroleum contaminate	18SrRNA SEQUENCING	Illumina MiSeq	*Naegleria*, *Vorticella Arabidopsis* , *Asarum Populus*, *Naegleria* , *Colpoda.*	Results demonstrated the ability of protozoa (61.90 - 62.04%) to adapt and survive	(Kachienga et al., 2018)

d water samples				in petroleum oil-polluted water sites.	
8.Water samples (of coastal regions of the The Mediterranean Sea)	Whole-genome sequencing	Roche 454 Genome Sequencer FLX	Archea-0.3 Bacteria-96.2 Eukaryotes-2.9 Viruses -0.5 Other=0.2	Analysis of the metagenomes revealed significant differences in both microbial diversities. and abundance between the two areas, reflecting their distinct ecological habitats and anthropogenic stress conditions	(Kisand et al., 2012)
9.Raw sewage samples from a private apartment building	Amplicon-based sequencing:18S. rRNA marker gene, V4 and V9,	Sanger Sequencing And Illumina MiSeq	*Cryptosporidium parvum*, *Giardia intestinalis*, *Toxoplasma gondii*, and *Trichomonad spp*.	A workflow for the detection and analysis of protists in sewage samples, with a focus on zoonotic and trichomonad taxa, based on high throughput. amplicon sequencing of existing 18S rRNA markers was experimentally validated	(Maritz et al., 2017)

10.Freshwater samples	Deep amplicon sequencing-viruses (g23 and RdRp), bacteria (16S rRNA and cpn60), and eukaryotes (V1–V3 regions of the 18S rRNA gene and internal transcribed spacer (ITS1/ ITS2)	Illumina MiSeq	Eukaryotes (Chlorophyta, Arthropoda, Streptophyta, Chytridiomycota, Apicomplexa, Nematoda, and Chordata), bacteria, and viruses)	The study developed a systematic approach to separate and characterize eukaryotic-, bacterial and viruses	(Uyaguari-Diaz et al., 2016)
11.Samples-freshwater, coastal, and wastewater	Amplicon sequencing (Full-length 18S rRNA gene sequences) (v8-v9,v4)	Illumina MiSeq	N/A	1.V8-V9 region provided the highest accuracy of the selected mock community as measured through a mean relative. abundance and beta-diversity measurements 2. V4 and V8-V9 regions showed similarities. overall representations of environmental samples and	(Bradley et al., 2016)

				trade-offs between hypervariable regions	
12.Drinking water	Shotgun metagenomics	Ion S5 XL Semiconductor Sequencer	Protozoa (*Acanthamoeba mauritaniensis* and *Acanthamoeba palestinensis*	Reported the complete microbiome (bacteria, viruses, fungi, and protists)	(Brumfield et al., 2020)
13.Sewage, drinking water, reservoir. water, groundwater, and river water	Amplicon sequencing targeting the hypervariable V4 region of the 18S rRNA gene	Illumina MiSeq platform.	*Acanthamoeba* spp, *Entamoeba coli*, *E. dispar*, *E. moshkovskii*, *Naegleria fowleri*, *N. australiensis*, *N. clarki* and members of the *Hartmannellidae* family	Underestimated protozoan by metagenomic approach compared to bacteria and virus	(Rusiñol et al., 2020)
14.Urban sewage	Shotgun metagenomic sequencing	Illumina HiSeq (bacterial and parasitic DNA) and	*Giardia* spp., *Plasmodium* spp., *Ascaris* spp., and *Blastocystis* spp. were the most abundant parasites throughout the study period	Data obtained from urban sewage illustrated the potential for this method to be used for future public health disease surveillance	(Hendriksen et al., 2019)

		MiSeq (DNA and RNA viruses)			
15. Water samples	Shotgun metagenomic sequencing	MinION sequencing platform (Oxford Nanopore)	N/A	Bacteria were more abundant compared to waterborne human pathogenic Eukaryotes	(Hamner et al., 2019)
16.Ten single-cell genomes	Full-genome sequencing.	Illumina MiSeq	*Cryptosporidium*	demonstrated the power of applying single-cell genomics to dissect infectious disease caused by closely related parasite species or subtypes	(Troell et al., 2016)
17.Soil metazoa: microarthropods, enchytraeids, earthworms and nematodes	Amplicon sequencing -SSU rDNA -18S rDNA: V1–V3 region,	Roche GS FLX 454	Amoebae, flagellates, ciliates	Ciliates Were generally over-represented in sequence numbers, while many amoeba and flagellate taxa were under-represented	(Geisen et al., 2015)

18. *Eimeria* oocysts	18S rRNA locus targeting Eimeria (hypervariable V4 region of the 18S rRNA locus)	Illumina MiSeq platform.	*Eimeria*	The study developed a data analysis pipeline for community analysis of eukaryotic organisms using *Eimeria* communities as a model. OTU assignment at at a lower threshold (95%) there was greater resolution between OTU consensus sequences	(Vermeulen et al., 2016)
19.Pre-diagnosed protozoan oocysts	Untargeted RNA metagenomics sequencing	Sequenced on the Ion Torrent S5XL platform	Protists and helminths	Demonstrated the applicability of untargeted RNA metagenomics for the parallel detection of parasites	(Wylezich et al., 2020)

2.8.8 DNA sequencing platforms used for metagenomic profiling of protozoan parasites

Next-Generation Sequencing systems facilitate deep amplicon sequencing and shotgun metagenomic studies by enabling massive parallel sequencing reactions and analysis of all DNA molecules in a sample. Different systems are employed by NGS including Roche 454, Pacific Biosciences, Ion Torrent, Illumina/Solexa, and Oxford Nanopore (Thomas et al., 2012). They all follow the same workflow of library preparation, sequencing, and raw data output (Escobar-Zepeda et al., 2015). The difference between the mentioned platforms is the sequencing methods listed in Table 3 which are: pyrosequencing, sequencing by synthesis, sequencing by ligation, and ion semiconductor sequencing (Kunin et al., 2008, Thomas et al., 2012, Ambardar et al., 2016, Goodwin et al., 2016). The selection of the appropriate NGS platform for sequencing depends on the metagenomic approach, questions being asked, costs, and genome length. Illumina platforms, for example, produce short read lengths, while PacBio and nanopore platforms produce longer read lengths, resulting in larger fragments, making bioinformatics easier (Shokralla et al., 2012, Ambardar et al., 2016). Therefore, PacBio and nanopore sequencing platforms can be advantageous when searching for larger genomes such as eukaryotes, however, Illumina could function best with species with smaller genomes, such as prokaryotes and short genome regions.

Illumina MiSeq was previously used to investigate the most important waterborne protozoans in water and wastewater targeting the V4, V9 (150bp) regions of the 18S rRNA (Moreno et al., 2018) and the primers were very compatible with Illumina resulting in a total of 9034 raw sequences however only a total sequence percentage from 0.026 to 1.396% represented pathogenic protozoa. The MiSeq platform is also commonly used to sequence combinations of variable regions in the 18S rRNA gene targeting protozoans. For example, it was used to sequence regions V4-V5 (Popovic et al., 2018) and the V1-V3 hypervariable regions (Maritz et al., 2017, Moreno et al., 2018, Stamps et al., 2018, Greay et al., 2018, Zahedi et al., 2019). Illumina MiSeq was also used as a sequencing platform of choice for the development of a novel detection method for protozoans targeting the V9 region (200bp) and analysis of the protozoan community in other environmental sample targeting the V4 and V5 regions (Audebert et al., 2016, Vermeulen et al., 2016, Hino et al., 2016, Kounosu et al., 2019). Target regions obtained were not more than the maximum read length for MiSeq of (2 x 300bp), and

total throughput of 1.5–2 Gb per run (Shokralla et al., 2012). Illumina NextSeq has a larger output and a read length (2x150bp) shorter than MiSeq (2x300bp) (Ambardar et al., 2016). Illumina HiSeq platform is also used for the shotgun metagenomics approach, for example, it was used to determine and for whole community sequencing of Kenya urban sewage to monitor the presence of protozoan pathogens (Hendriksen et al., 2019). The results indicated that pathogenic parasites had significantly higher and increasing read abundances over time. HiSeq X has the highest throughput of 800-900GB, can sequence the whole genome within a day (Shokralla et al., 2012, Goodwin et al., 2016, Lokmer et al., 2019).

Ion Torrent platform employs the sequencing by detection of hydrogen ion method and offers single read length-end of up to an average of 400bp with 1-1.78 % error rate (Zhang et al., 2015, Goodwin et al., 2016). Moreover, the system has a fast run time of 2-7hours (Glenn, 2011, Goodwin et al., 2016). This high throughput sequencing platform was used for comparative analysis of the complete water microbiome (Brumfield et al., 2020) and DNA of opportunistic plant and animal pathogens was identified including protozoa *Acanthamoeba mauritaniensis* and *Acanthamoeba palestinensis*.

MinIon platform (produced by Oxford Nanopore technologies) is one of the real-time, third-generation sequencing technologies together with Pacific Bioscience and is commonly used for shotgun metagenomics. Moreover, they are non-PCR-based methods and generate long reads (Miller et al., 2013, Brown et al., 2017). MinIon sequencer is one of the most advanced novel platforms and has been used for identification of pathogenic microbial contamination of waterways (Hamner et al., 2019), for sequencing of the protozoan parasite, *Trypanosoma cruzi* (Díaz-Viraqué et al., 2019), and demonstration of a novel diagnostic assay for malaria (Imai et al., 2017). MinIon nanopore is the first portable sequencing device, thus it is applicable on the field, can generate up to 98 kb long reads, and is cheaper than conventional sequencers (Laver et al., 2015, Jain et al., 2016). Although it has a very fast run time with less complicated sample preparation (Miller et al., 2013), the error rate is extremely high, up to 38% (Ambardar et al., 2016).

Other sequencers that have not been commonly used for protozoan research include the first NGS real-time sequencing-by-synthesis pyrosequencing technology, Roche 454 Genome Sequencer FLX which was used for whole-genome sequencing in water (Kisand et al., 2012).

The NGS: SOLiD 5500xl Lifescope Genomic (Hanevik et al., 2015) platform and Sanger sequencing were used for investigating the genetic diversity of *Cryptosporidium hominis* by whole genome sequencing (Gilchrist et al., 2018).

Table 3: High throughput sequencing platforms.

NGS methods	Systems	Advantages	Disadvantages	Read length (bp)	Reference
Pyrosequencing	Roche Gs Titanium x	-Long read length compared to Sanger. -Short run time	-Inaccurate homopolymer sequencing -High error rate - High cost	500-1000	(Shokralla et al., 2012, Miller et al., 2013, Ambardar et al., 2016)
Sequencing by synthesis	Illumina (HiSeq and MISeq)	-Overcome homopolymer due to terminator nucleotides/accurate sequencing of homopolymer regions. -High output /run compared to pyrosequencing. -relative short-read length because of optical signal decay and dephasing. -Low cost	-Increased error rate with increased length -Long run time -short read	36-250(HISeq), 150 (MiSeq)	(Shokralla et al., 2012, Miller et al., 2013, Ambardar et al., 2016, Raza and Ahmad, 2019)
	Pacific Biosciences SMRT DNA sequencer	-Non-PCR based-3rd generation. -Fast run time -Long read	-High DNA input -High error rate -expensive	2000-15000	

	Oxford Nanopore-MinION	-Non-PCR based-3rd generation. -Fast run time -No sample preparation	N/A	48000	
Sequencing by ligation	SOLiD sequencer Applied biosystem 5500 xl genetic analyzer.	-Overcome homopolymer due to terminator nucleotides/accurate sequencing of homopolymer regions -High output /run compared to pyrosequencing. -Oligonucleotides used instead of DNA polymerase	-Short reads -Increased error rate with increased length	N/A	(Huang et al., 2012, Liu et al., 2012)
Ion semiconductor	Life Technologies Ion Torrent	-Fast run time -Cost-effective and time-efficient.	-High error -Short read length	200-400	(Bragg and Tyson, 2014, Fujimoto et al., 2014)

2.8.9 Bioinformatic analysis tools

Different bioinformatics tools are being used to analyze the sequencing data obtained using shotgun and amplicon sequencing. This includes QIIME (Kounosu et al., 2019), MetaPhlAn (Kibegwa et al., 2020), MetaPhyler (Liu et al., 2011, Beisser et al., 2017), and Mothur (Almeida and De Martinis, 2019) which are used for targeted metagenomics and map the fewest reads (Lindgreen et al., 2016). The tolls such as CLAssifier, Genomic Origins Through Taxonomic, RIEMS tools (Scheuch et al., 2015), CHAllenge, Kraken, Genometa, MG-RAST, and MEGAN are used for analysing all sequences present in each data set and commonly for untargeted metagenomics (Neves et al., 2017, Kibegwa et al., 2020). Some of the reference databases for protozoans include SILVA (most used database for classification of 18S Illumina reads in QIIME) (Moreno et al., 2018), Eukaryotic Pathogen Genomics

Database Resource (EuPathDB)(Aurrecoechea et al., 2017), PR2 protist ribosomal database (Guillou et al., 2013, Moreno et al., 2018), VEuPathDB.org, GeneDB.org and ProtVirDB (Ramana and Gupta, 2009).

Recently, Moreno et., al (2018) reported the use of Software QIIME 1.9.1 and PR2 protist ribosomal database to analyse Illumina data and the results revealed the accuracy of the tool for taxonomic classification and identification of most waterborne protozoans down to species level. RIEMS is another software pipeline used for sensitive and detailed taxonomic classification of untargeted metagenomics dataset readings and was previously applied for parasites related sequencing. Their results provided clear parasite species allocation and subtyping with reads related to *Blastocystis* spp. and *Entamoeba* spp. (Scheuch et al., 2015, Wylezich et al., 2020). A new bioinformatics pipeline ContamFinder for protozoan parasites was developed by (Borner and Burmester, 2017) and was able to identify apicomplexan contaminations in NGS sequencing data. Limitations are discussed in the section below.

Presented in Figure 5 is a flow of the common and recommended steps used in the metagenomics profiling of protozoan parasites in environmental samples.

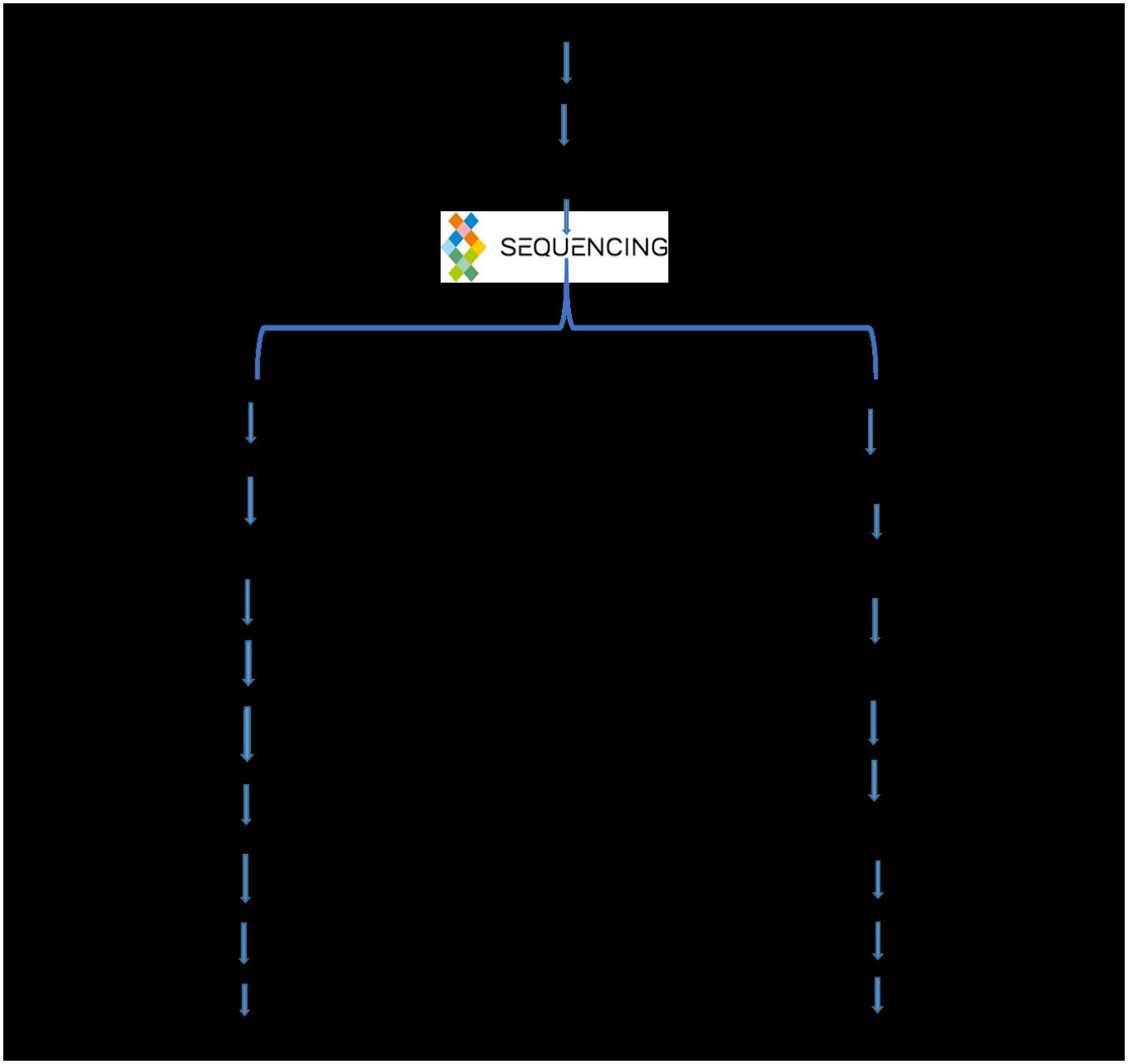

Figure 5. Workflow from sampling to bioinformatics.

2.8.10 General limitations

Despite the number of advantages mentioned for shotgun metagenomics, we noted common limiting factors associated with the shotgun metagenomics approach for protozoan biodiversity studies. This approach has been reported to result in limited depth coverage of protozoan parasites compared to other taxa present in environmental samples. For example, Lear et al.,

(2018) mentioned that this approach favors full genome coverage of small and less complex prokaryotes or viral genomes than large and complex eukaryotes. Protozoan parasites are reported to be poorly represented in reference databases due to fewer studies compared to prokaryotes, large eukaryote genome sizes, and non-coding regions found in eukaryotes genes (Bik et al., 2012, Marcelino et al., 2020). This is one of the frequently reported disadvantages which limits the application of the whole genome shotgun metagenomics.

Additionally, whole-genome shotgun sequencing results in a large amount of sequence data that is often time-consuming and challenging due to the complex bioinformatics analyses (Miller et al., 2013, Quince et al., 2017). Although the bioinformatics platforms have been developed for the analysis of generated data, they still require larger reference databases with complete sequences and larger genome sizes (Quince et al., 2017). Another challenge associated with amplification-free DNA library preparation is the requirement for an increased concentration of DNA (not less than 250-500ng) (Thomas et al., 2012, Dulanto Chiang and Dekker, 2019). Certain type of samples yields a lower quantity of DNA which requires PCR amplification to increase the DNA concentration, and this results in the introduction of biases such as over-amplification of some fragment, over others, interfering with the accuracy of measured abundance and microbial diversity (Miller et al., 2013, Dulanto Chiang and Dekker, 2019).

Based on reviewed studies (Table 2) on the deep amplicon-based metagenomics approach, we noted that even though the approach offers higher species resolution, more target-specific analysis, and is less costly, several limitations were encountered. Firstly, this approach offers limited analysis and estimation of the protozoan parasite's biodiversity, it does not offer universal detection of protozoan parasites, mainly due to sequence length restrictions on available sequencing technologies (Hadziavdic et al., 2014). The use of different target regions instead of the whole taxonomic gene tends to exclude some protozoan parasites which may be of public health importance (Hadziavdic et al., 2014, Maritz et al., 2017, Maritz et al., 2019). Standard universal primers targeting all protozoans or eukaryotes are not yet available. The non-universal primers are subject to taxonomic biases during amplification, and inclusion of non-targeted domains such as bacteria or archaea, which further interfere with quantification accuracy or population structure (Hadziavdic et al., 2014). Pseudogenes and the presence of multiple copies of eukaryotic genes may also interfere with quantification. For instance,

Cryptosporidium parvum has 5 copies in the 18S rRNA gene and *Entamoeba histolytica* has 200 copies (Zahedi et al., 2018). Therefore, this may lead to overestimation or underestimation of microbes that are present as well as their proportions. At present, no standard primers are targeting the entire protozoan taxonomy or the entire eukaryotes domain. To avoid exclusion of some of the important pathogenic protozoans, we recommend the use of general eukaryote primers designed to exclude other taxa, such as those reported to include 0 (archaea),0 (bacteria), and 96/100% (eukaryote) (Table 5 appendix). We also recommend the use of combined variable regions of the 18S rRNA to increase the chances of detecting more protozoans, for instance, V4 and V9 have been reported for most previous studies (Table 2).

The main challenge with bioinformatic analysis for protozoan studies is the insufficient reference sequences in available databases for identification/profiling (Lopes et al., 2017). Contaminations in the draft incomplete genomes (by fragments of sequences from other species) is another challenge in the bioinformatic analysis that often leads to errors in results (Borner and Burmester, 2017, Orosz, 2017, Fleetwood, 2018). Some studies reported that the SILVA database was limited and not broad enough to retrieve and identify waterborne protozoans in their sequence dataset (Tanaka et al., 2014, Moreno et al., 2018). A previous study utilized a PR2 database to classify taxonomically the sequences obtained and they were able to achieve an accurate identification down to species level. However, no sequence of *Cryptosporidium* was recovered from Illumina data, though they were detected during their initial screening in PCR amplification with specific primers. Therefore, this raised concerns of their designed 18S rRNA gene specificity into discriminating very closely related species such as *C. parvum* and *C. hominis* which have 97% sequence similarity (Moreno et al., 2018).

3.0 DEVELOPMENT AND EVALUATION OF A MOLECULAR BASED PROTOCOL FOR DETECTION AND QUANTIFICATION OF *CRYPTOSPORIDIUM* SPP. IN WASTEWATER

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3.1 Introduction

Protozoan parasites are among the key waterborne pathogens globally and remain a major public health problem in developing countries (Fletcher et al., 2013; Kotloff et al., 2013; GBD, 2017). *Giardia intestinalis*, *Cryptosporidium* spp., *Cyclospora cayetanensis*, *Toxoplasma gondii,* and *Entamoeba histolytica* are the common human pathogenic protozoan parasites (Yaeger, 1996; Lane and Lloyd, 2002; Insulander et al., 2013; Omarova et al., 2018). These protozoan parasites have been detected in most surface waters in concentrations related to the level of fecal pollution (Hansen and Ongerth, 1991; Vermeulen et al., 2019). Their cystic forms are persistent in the environment and are resistant to the commonly used disinfection methods in wastewater and water treatment (Tomass and Kidane, 2012; Okojokwu et al., 2014). Furthermore, protozoan parasites have a low infectious dose, from 1 to 100 (oo)cysts or less (Steiner et al., 1997; Okhuysen et al., 1999; Fayer, 2004; Okojokwu et al., 2014).

The protozoan parasite *Cryptosporidium* is well documented as a major waterborne pathogen and the fourth leading cause of death from gastrointestinal diseases worldwide (Donnelly and Stentiford, 1997; Carmena, 2010; Hassan et al., 2020). In 2015, it accounted for roughly 12.1 percent of all deaths among children under the age of five worldwide (Khan et al., 2018; Hassan et al., 2020). *Cryptosporidium* spp. are distinguished from other protozoans by their ability to self-infect, their inherent resistance to disinfectants, and complex life cycle (Leitch and He, 2011; Ghazy et al., 2015). Human infections are caused by *C. andersoni, C. muris*, *C. suis*, *C. wrair*i, and *C. felis*, with *C. hominis* and *C. parvum* accounting for roughly 90% of the

infections (Helmy et al., 2013; Khan et al., 2018). Monitoring and detection of *Cryptosporidium* spp. in environmental samples such as water can be difficult due to their low numbers and the complexity of the environmental sample matrix (Bilung et al., 2017). Therefore, sensitive, and reliable detection methods are critical.

Various techniques are available for the detection and quantification of protozoan parasites, these include microscopic examination (Abazaj et al., 2016; Ahmed and Karanis, 2018), antibody (IMS-IFA based), and enzyme-based analysis (Den Hartog et al., 2013), as well as molecular methods (Adeyemo et al., 2018). Molecular based methods such as polymerase chain reaction (PCR) (Ögren et al., 2020), restriction fragment length polymorphism (PCR-RFLP) (Azami et al., 2007; El-Alfy et al., 2019), real-time PCR (Bonilla et al., 2015) have become the most used methods in recent years due to their specificity and sensitivity. Another PCR-based technology that has recently attracted a lot of attention is the droplet digital PCR (ddPCR) (Xue et al., 2018; Mauvisseau et al., 2019). ddPCR is a relatively new method that uses water-oil emulsion droplet technology (Yang et al., 2014; Deprez et al., 2016). This method has shown higher sensitivity, precision, less sensitivity to inhibitors (Mauvisseau et al., 2019), and offers absolute quantification in molecular diagnosis (Basu., 2017). It has been used in diagnostic laboratories for viral, bacterial, and parasite analysis due to its advantages (Taylor et al., 2015; Maheshwari et al., 2017; Olmedillas-López et al., 2017; Cheng et al., 2019). While molecular-based methods can detect and quantify protozoans specifically, and sensibly, there is a lack of reliable and standardized preparation workflows.

The development of useful molecular diagnostic assays depends on the high precision of sample processing and extraction of high quality and quantity of DNA from various sample matrices. For this reason, the development of a molecular assay for the detection of protozoan parasites in environmental samples is very challenging, and results can be inconsistent due to several factors. These include (1) samples require a concentration step to get sufficient biomass containing (oo)cysts for DNA extraction, and (2) DNA extraction from protozoan parasites is often difficult due to the robust (oo)cysts cell wall that is not easy to lyse (Hawash, 2014; Shapiro et al., 2019), (3) lastly, environmental samples contain a higher concentration of inhibitors that can interfere with polymerase activity during PCR (Schrader et al., 2012; Hedman and Rådström, 2013). Therefore, optimization of sample processing procedures has been necessary before DNA extraction and amplification in PCR.

Recovery and concentration of (oo)cysts in water and wastewater is usually achieved through centrifugation and filtration (Moreno et al., 2018; Galvani et al., 2019). However, other less commonly used methods include immunomagnetic separation (De Jong, 2017), salt flotation (Kotian et al., 2014; Wells et al., 2016), glucose flotation (Norris et al., 2018), and formol-ether concentration (Parameshwarappa et al., 2012). Most of these methods are not specific for concentration of (oo)cysts, except the immunomagnetic separation method, therefore, it is possible to concentrate a large amount of foreign material of the same size, as the (oo)cysts, which could subsequently interfere with downstream molecular analysis. Additionally, the rigid cell wall of the (oo)cysts is a challenge for DNA extraction. Therefore, mechanical, and chemical pretreatments such as freeze-thaw (Wells et al., 2016), sonication, and bead-beating have been applied as additional steps in commercial DNA extraction protocols to assist in (oo)cysts wall disruption (Psifidi et al., 2015; Felczykowska et al., 2015). Some of the commercial DNA extraction kits used for molecular analysis of protozoan parasites in environmental samples include DNeasy PowerSoil (Galvani et al., 2019), QIAamp Stool Mini Kit (Menu et al., 2018), MoBio PowerWater DNA Isolation kit (Djurhuus et al., 2017), DNeasy blood and tissue kit (Daniels et al., 2015) and YTA Stool DNA Isolation mini kit (Asgarian et al., 2018). Additionally, other studies have used modified or custom extraction procedures, such as phenol-chloroform (Djurhuus et al., 2017; Rosenbaum et al., 2019), salting-out method (Sun, 2010), UNEX protocol (Moreno et al., 2018), and MagnaPure 96 with mechanical grinding (Galvani et al., 2019). All these modifications are aimed at improving the efficiency of molecular methods in detecting protozoan parasites in environmental samples, such as wastewater or water. However, there are inconsistencies in their efficiency in extracting the DNA from oocysts.

This chapter presents a workflow optimization to achieve maximum (oo)cysts recovery from wastewater samples, improve DNA extraction and successful quantification using the advanced ddPCR platform. This presents a protocol that can be adopted to improve the sensitivity and accuracy of molecular techniques for protozoan parasite detection in the water environment.

3.2 Methodology

Figure 6 gives an overview of the experimental design of the study involving different phases. Phase 1 focused on optimization of droplet digital PCR (ddPCR) assay for the detection of *Cryptosporidium parvum* based on reference DNA (*C. parvum*). The second phase was to determine the best method for extraction of DNA from *C. parvum* using standard oocysts of known concentrations. Phase 3 focused on determination of the lowest number of oocysts that can be extracted using the best method determined in Phase 2. Phase 4 assessed the impact of sample centrifugation and filtration for enhancing the recovery of the oocyst from wastewater. Finally, the last phase of the study applied the optimized methods to determine the presence of *Cryptosporidium* spp. in different wastewater.

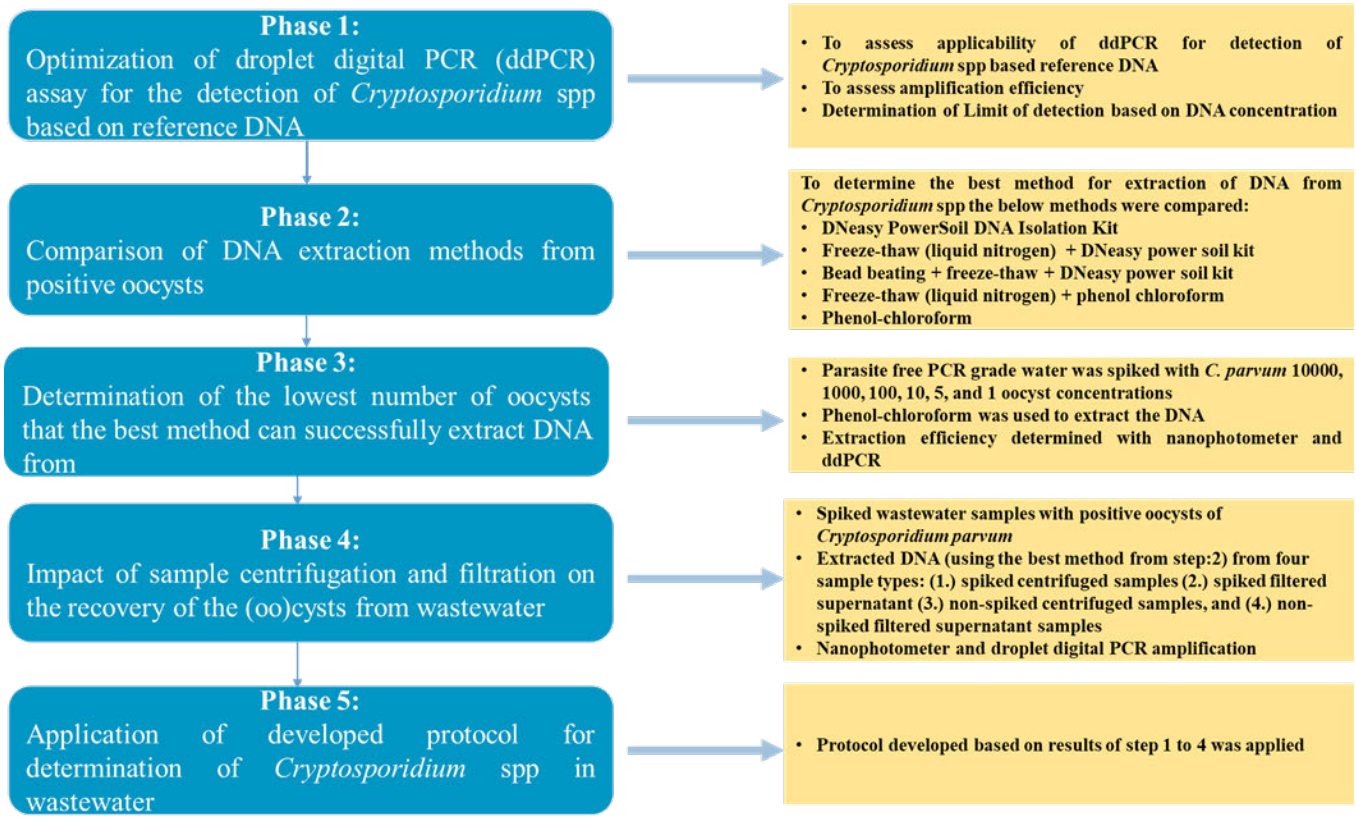


Figure 6: Experimental design of the study

3.2.1 Optimization of droplet digital PCR (ddPCR) assay for the detection of *Cryptosporidium* spp.

The ddPCR amplification protocol and the detection limit were first optimized using synthetic DNA of *C. parvum* purchased from Microbiologics Inc. ($1.1x10^7$ copies/vial). The synthetic DNA was amplified using QX200 Droplet Digital PCR system (Bio-Rad). Each ddPCR assay mix was prepared in a final volume of 20 µL, containing 10 µL of 2× evagreen ddPCR supermix, 0.5µm forward and reverse primers (Table 1), and 1µl of template DNA (4000 copies of synthetic DNA in a reaction volume). A no template control (NTC) was also included in the reaction. The generation of droplets was performed using QX200 Droplet Generator (Bio-Rad). PCR amplification was carried out on a T100 thermal cycler (Bio-Rad), using the conditions: 10 min at 95°C, denaturation at 95°C during 15 s and annealing at 56°C-60°C for 1 min, 72°C for 1 min, final extension at 72°C for 10min, 50 cycles. The plates were analysed using the QX200 Droplet Reader and QuantaSoft software (Bio-Rad). The threshold line was based on the negative and positive droplets separation. The synthetic DNA was further serially diluted using a 1:10 dilution factor to determine the detection limit of the ddPCR protocol.

3.2.2 Specificity of primers used

This study used droplet digital PCR for the detection and amplification of the targets using the generic 18S SSU rRNA and species-specific gp60 primer pair (Table 4). Primers were optimized and confirmed with gel electrophoresis to ensure specificity, and the results revealed single bands indicating specific amplification. Furthermore, the specificity of the SYBR green assay was also assessed with real time PCR, single specific melt curves were observed. The specificity of both primers using the ddPCR SYBR green assay was also confirmed by looking at good droplet separation of the negative and positive droplets (figure S2 appendix I), which is usually seen when there is nonspecific binding noise in between separation. Additionally, when testing environmental samples, a positive synthetic DNA was included to confirm the amplified product of interest and primer specificity. Following primer specificity analysis, only the gp60 primer assay was used to optimize the methodology in this study (phase1 to phase 4). In phase 5, the generic 18s rRNA assay was performed and applied to see if the assay can target other *Cryptosporidium* species when needed.

3.2.3 Oocysts estimation based on the copy numbers per reaction volume

C. parvum oocyst concentrations were calculated directly from the ddPCR data, which has the advantage of providing more accurate and absolute quantification (Deprez et al.). Briefly, the *C. parvum* gene copy numbers contained in individual samples were converted to oocyst number estimates, based on published evidence that there are five copies of the rDNA unit per haploid genome (Le Blancq et al., 1997), four haploid sporozoites per oocyst (Zahedi et al., 2018) and thus 20 copies of the *C. parvum* per oocyst.

The oocysts concentration was calculated using the formula (Equation 1):

Equation 1 (oocysts concentration)

$$oocysts = \frac{x}{y}$$

Where "x" is the copy number measured per 20µl reaction volume and "y" is the number of copies of the gene per one oocyst (in this case).

3.2.4 Comparison of DNA extraction methods from standard *C. parvum* oocysts (Experiment 1)

Five different DNA extraction protocols were compared for maximum DNA recovery from oocysts. This includes a standard DNeasy Powersoil extraction kit (Qiagen, Norway) and a conventional DNA extraction (phenol-chloroform) and modified protocols as described below. The PowerSoil extraction protocol was chosen based on previous literature that this extraction method is most suitable and gave the best results in terms of purity and speed when extracting DNA from parasites (Temesgen et al., 2020, Barbosa et al., 2017). The phenol-chloroform was chosen because it provides a cheaper alternative to the commercial kits that are expensive. Moreover, it has been reported efficient in DNA extraction from protozoan (oo)cysts especially from water samples where protozoan parasites are mostly found in lower concentration (Rosenbaum et al., 2019).

The efficiency of these methods in extracting DNA from *C. parvum* oocysts was determined by spiking the oocysts into 500µl nuclease-free water. The concentration of the oocysts was $2.5x10^2$ and $2.5x10^3$. The parent stock of $1x10^6$ *C. parvum* oocysts purchased from Waterborne Inc. was prepared and guaranteed 99% pure by the supplier. According to Waterborne Inc, they

were purified by sucrose percoll gradient and water washes. Each suspension was then viewed microscopically. The stock volume was then calculated to give $2.5x10^2$ and $2.5x10^3$ of the oocysts and was spiked in the nuclease free water and was used as a starting material for different DNA extraction methods. The purity of the extracted DNA was determined based on the OD260/OD280 and OD260/OD230 ratios measured using the Nanophotometer (Implen GmBH, NP80, Germany). The details of the extraction protocols are described in detail below.

3.2.4 Extraction method 1 (M1)- DNeasy power soil kit

The first genomic DNA extraction method used was the DNeasy PowerSoil DNA Isolation Kit (Qiagen, Norway) as per the manufacturer's recommendation without any modifications. In this method, the oocysts were mechanically lysed using provided bead tubes in the kit with a lysis buffer solution followed by vortexing. The final DNA was suspended in a 50µl elution buffer.

3.2.5 Extraction method 2 (M2)-Freeze-thaw (liquid nitrogen) + DNeasy power soil kit

The samples of different concentrations of *C. parvum* oocysts were transferred into the bead tubes provided by DNeasy PowerSoil Kit. Lysis buffer was added to the tubes and briefly vortexed to mix. The samples were then set to freeze in liquid nitrogen for 5 min and thawed at 65°C for 5 minutes. After 10 cycles of freeze-thaw (Wells et al., 2016), the remainder of the DNA isolation protocol was followed as per the DNeasy PowerSoil manufacturer instruction.

3.2.6 Extraction method 3 (M3)- Bead beating+ freeze-thaw + DNeasy power soil kit

The samples were subjected to the DNA extraction protocol as described in Method 2 (M2) with an additional mechanical lysis step. Briefly, the power bead tubes containing the cysts and the lysis buffer provided by the manufacturer were subjected to bead beating at high speed for e 60s (two cycles). To achieve higher lysis efficiency, the bead beating step in the extraction kit was replaced with a stronger homogenization (higher velocity) with Bead Rupture 12 Homogenizer (Omni International, USA). After bead beating, the samples were subjected to freeze-thaw as described in (M2), followed by the DNeasy Power Soil extraction kit using as per the manufacturer's instructions.

3.2.7 Extraction method 4 (M4)-freeze-thaw (liquid nitrogen) + phenol-chloroform

DNA was extracted using the phenol-chloroform method and freeze-thaw (liquid nitrogen) pretreatment. Briefly, the oocyst of *C. parvum* (2.50E+02 and 2.50E+03) were suspended in 500 µl of lysis buffer (10 mmol/L Tris/HCl pH 8.0, 10 mmol/L EDTA, 0.1 mol/L NaCl, 2% SDS pH 8.0) and 100 µl protease K) followed by incubation for 2 hours at 55 °C - 60°C in a water bath (Djurhuus et al., 2017)(Awolusi, 2016). The samples were subjected to pretreatment using 10 cycles of freeze-thaw steps (Wells et al., 2015, Wells et al., 2016, Babaei et al., 2011) as described in M2. After the pretreatment the proteins and other impurities were removed by adding phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v), followed by phenol removal by adding an equal volume of chloroform. The gDNA was then precipitated by adding 0.6 X volume of isopropyl alcohol and incubated overnight at -20°C (Javadi et al., 2014, Butler, 2012, Djurhuus et al., 2017). The DNA was then washed with 70% ethanol and suspended in 1X Tris–EDTA buffer.

3.2.8 Extraction method 5 (M5)- phenol-chloroform

The total gDNA was extracted from samples as described in method 4 (M4) with modifications. In this the additional pretreatment step was avoided and followed the standard phenol-chloroform methods described by Djurhuus et al., 2017 and Awolusi, 2016.

3.2.9 Determination of the lowest number of oocysts required for DNA extraction, detection and quantification (Experiment 2)

The limit of detection was determined to ascertain the lowest concentration of *C. parvum* oocysts that the optimized phenol-chloroform protocol can successfully extract DNA from. The protocol was applied to triplicate nuclease free water samples spiked with *C. parvum* oocysts (Figure 8). Parasite-free PCR-grade water samples were spiked with a variable number of oocysts. The concentration of *C. parvum* oocysts used were 10000, 1000, 100, 10, 5, and 1 oocyst, spiked into 500µl nuclease-free water. All spiked samples and negative control (without spiked oocysts) were subjected to the optimized phenol-chloroform DNA extraction protocol and the extracted DNA was analysed using both nanophotometer and droplet digital PCR amplification.

3.2.10 Impact of sample centrifugation and filtration on the recovery of the (oo)cyst from wastewater (Experiment 3)

To ascertain the impact of filtration and centrifugation steps on the recovery of (oo)cysts from wastewater, the optimized DNA extraction protocol was applied on influent wastewater samples. Wastewater samples were obtained from the treatment plants in different cities within South Africa. Approximately, 2L of the samples (raw wastewater) were taken from the head of works in sterilized sample bottles and kept at room temperature and transported to the laboratory for analysis. In the laboratory (Figure 7), the samples were split into half (1L each), the first potion was spiked with 12500 oocysts of *C. parvum*. The spiked samples were concentrated by centrifugation (Hermle Labortechnik GmbH) at 3500 rpm (max 4 x 340 g) for 10 minutes and the total DNA was extracted from the pellet using the phenol-chloroform method as described above (M5). In addition, the supernatant from after the initial centrifugation step was also filtered using 0.1µm pore size cellulose acetate filter paper. The membrane filter paper was placed into a centrifuge tube, eluted using PBS as in the USEPA 1623 method. The DNA was extracted from residual material on the filter paper using the same method as above. This was done to see if there are any residual (oo)cysts in the supernatant. Several past studies have either centrifuged (Moreno et al., 2018, Hendriksen et al., 2019) or filtered (Galvani et al., 2019, Zahedi et al., 2019) their samples.

The procedure described above was then repeated with non-spiked wastewater samples. In this study, we then extracted DNA from four types of samples (in triplicate): (1.) spiked centrifuged samples (2.) spiked filtered supernatant (3.) non-spiked centrifuged samples, and (4.) non-spiked filtered supernatant samples. The extracted DNA from the set of spiked and non-spiked samples were analysed for DNA quality and quantity and subsequently amplified using ddPCR targeting *C. parvum* gp60 gene. Therefore, the percentage recovery of spiked oocysts (12500 oocysts) was determined using the formula (equation 2):

Equation 2 (oocysts % recovery)

$$\boldsymbol{P\%} = \frac{\boldsymbol{y}}{\boldsymbol{x}} \times \mathbf{100}$$

Where "x" is the total number of spiked oocysts and "y" is the total oocyst recovered in the spiked sample minus the total oocysts recovered in the non-spiked sample.

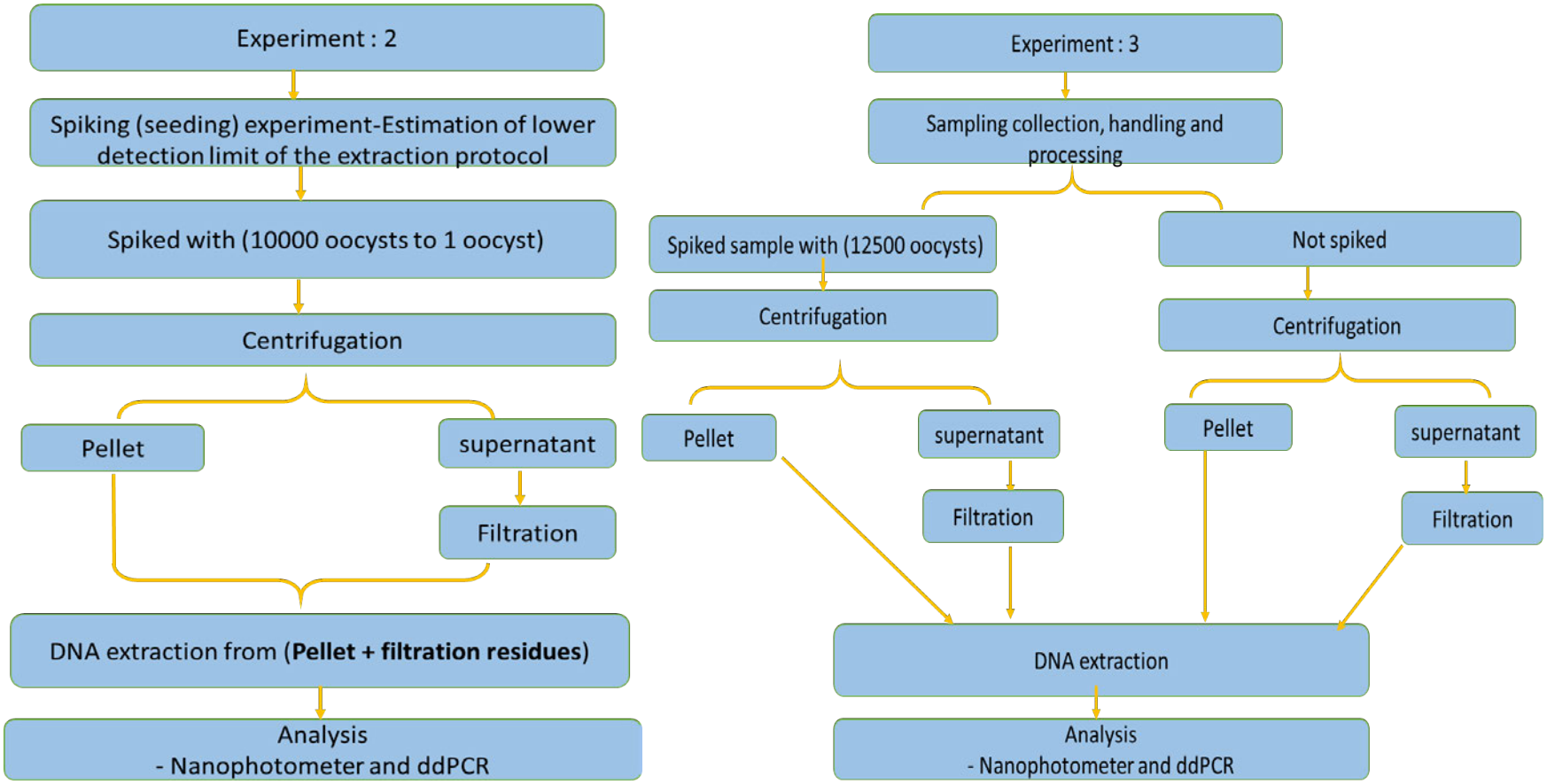


Figure 7. Methodological flow for determination of limit of detection and sample handling experiment.

3.2.11 Application of developed protocol for determination of *Cryptosporidium* spp. in wastewater

The outcomes of the three experiments above (1,2 and 3) were evaluated for the detection of *Cryptosporidium* spp. using the 18S SSU rRNA assay in wastewater from three geographical regions in South Africa using the optimized protocols. The extracted DNA was quantified using ddPCR following the protocol as described in section 1 using the primers listed in Table 4.

Table 4: Primers used in the amplification of extracted DNA for the detection of *Cryptosporidium* spp.

Primers	Forward	Reverse	Size (bp)	Target	References
Cryptosporidium 18S	GTTTTCATTAATCAAGA ACGAAAGTTAGG	GAGTAAGGAACAACC TCCAATCTCTAG	100	All known *Cryptosporidium* species (21)	(Burnet et al., 2013)
Cryptosporidium (gp60)	GCCGTTCCACTCAGAGGAAC	CCACATTACAAATGAAGTG CCGC	150	*C. parvum*	(Sánchez et al., 2018, Hunter et al., 2007)

3.3 Results

3.3.1 Optimization of droplet digital PCR for the detection of *Cryptosporidium parvum* based on reference DNA

The ddPCR assay was successfully optimized for *C. parvum* using primer in Table 4. The copy number of *C. parvum* measured by ddPCR was 3342.39 copies/20µl reaction volume with clear separation of positive and negative droplets, and without any sign of inhibitors or nonspecific binding. When diluted through the 1:10 dilution factor, the decreasing droplet concentration was observed (figure S2 appendix). The limit of detection was evaluated by determining the lowest copy number concentration of *C. parvum* that can be detected from the volume of sample added to the reaction. Serial dilutions of 1:10 were performed starting with 3342.39 copies/20µl reaction volume. Based on the obtained result in table 5, the limit of detection of the ddPCR assay for *C. parvum* is 0.07 copies /µl in a reaction (1.32 copies in 20µl reaction). Further dilutions indicated a “no-call” or undetectable copies.

Table 5: Limit of detection for the droplet digital PCR assay using reference DNA.

Dilution	Copies detected in ddPCR (20 µl reaction volume)	Detection rate (X detected/Y (2) replicates)
10^{-1}	3342.39	2/2
10^{-2}	270.5	2/2
10^{-3}	29.39	2/2
10^{-4}	6.03	2/2
10^{-5}	3.09	2/2
10^{-6}	2.77	2/2
10^{-7}	1.32	2/2
10^{-8}	0	0/2

3.3.2 Comparison of DNA extraction methods from positive oocysts

Genomic DNA extracted from positive *C. parvum* oocysts using all methods as shown in tables 6 had a detectable nucleic acid concentration using the nanophotometer measurement, except for the DNeasy PowerSoil kit (M1) and the Bead beating+ freeze-thaw + PowerSoil kit (M3). The phenol-chloroform extraction protocol showed the best performances with an average DNA concentration of 223 (± 0.7) ng/µl (Table 5). The liquid nitrogen + DNeasy power soil kit extraction method extracted the lowest DNA concentration, with an average DNA yield of 0.275 (±0.01) ng/µl. The other methods tested (Table 6) in this study displayed varying detectable DNA concentrations, ranging from 1.25 – 100 (±0.01- ±0.07) ng/µl. Furthermore, the purity of the detectable DNA concentrations ranged between 1.5-2 (OD260/OD280) and 1.5-2.2 (OD260/OD230). However, the best purity of 1.8 (260/280 ratio) and 1.9-2 (260/230 ratio was achieved with both the DNeasy PowerSoil extraction kit and phenol-chloroform extraction kit.

The *C. parvum* (GP60) gene was detected from all the samples irrespective of the extraction protocol used, including from those samples where the DNAs were not detectable using a nanophotometer (Table 6). For instance, the PowerSoil isolation kit resulted in an undetected DNA concentration based on the nanophotometer reading (Table 6). However, 72.38 (± 0.18) copies/reaction volume of the *C. parvum* gene were detected using the ddPCR (Table 6).

Phenol-chloroform method without any pretreatment resulted in the highest copies of 1807.15 (± 0.30) copies/reaction based on the ddPCR analysis. Therefore, the phenol-chloroform method was chosen as the best performing method for further experiments.

Table 6: DNA concentration and purity based on the nanophotometer and ddPCR measurements.

DNA extraction methods	ng/µl (±SD)	*Cryptosporidium parvum* copies/20 µl reaction (ddPCR) (±SD)	260/280 ratio	260/230 ratio
(M1) 2.50 × 10^3 PowerSoil kit	–	71.57 (±0.01)	–	–
(M2) 2.50× 10^2 (cyst) liquid nitrogen + PowerSoil kit	1.25 (±0,01)	34.52 (±0.38)		
(M2.1) 2.50 × 10^3 (cysts) liquid nitrogen + PowerSoil kit	0.275 (±0.01)	103.08 (±0.42)	–	–
(M3) 2.50× 10^2 (cysts)- Bead beating + freeze-thaw + PowerSoil kit	–	72.38 (±0.18)	–	–
(M3.1) 2.50 × 10^3 Bead beating + freeze-thaw + PowerSoil kit	0.45 (±0.02)	127.41 (±0.33)	–	–
(M4) 2.50× 10^2 (cyst) liquid nitrogen + phenol chloroform	3.05 (±0.01)	184.84 (±0.79)	1.8	1.9
(M4.1) 2.50× 10^3 (cysts) liquid nitrogen + phenol chloroform	4.5 (±0.04)	1310.73 (±0.26)	1.8	1.9
(M5) 2.50× 10^2 (cyst) phenol chloroform	100 (±0)	465.80 (±0.18)	1.8	2
(M5.1) 2.50× 10^3 (cyst) phenol chloroform	223 (±0.71)	1807.15 (±0.30)	1.8	2

--- Undetected.

3.3.3 Determination of a lower detection limit of the phenol-chloroform extraction protocol for *C. parvum* oocysts in nuclease-free water

The results of the LOD assessment indicated high sensitivity of the phenol-chloroform extraction protocol with successful DNA extraction from all spiked oocysts concentration (10 000 -1 oocyst). DNA measured using a nanophotometer indicated a concentration range from 31 (±0.7) to 230 (± 0.35) ng/µl. Furthermore, DNA extracted from the lowest spiked oocysts (1 oocyst of *C. parvum*) was subsequently detected using ddPCR assay obtaining 5.93 copies/reaction volume (Table 7).

Table 7: LOD phenol-chloroform DNA extraction.

Spiked oocysts	Copies of *Cryptosporidium parvum*/20 µl reaction volume
10 000	1169.81
1000	529.31
100	320.00
10	35.80
5	17.16
1	5.93

3.3.4 Impact of sample centrifugation and filtration on the recovery of the (oo)cysts from wastewater

Total extracted DNA and *C. parvum* copies were found in significant concentrations from both samples that were taken through centrifugation alone and filtered supernatant after centrifugation. For instance, in the non-spiked wastewater samples, that were centrifuged, 281.32 (±0.31) ng/µl of DNA was extracted, when spiked, the concentration of DNA extracted was 669.47 (±0.81) ng/µl. However, when the supernatant was further filtered, 52.33 (±0.71) and 281,32 (±0,31) of DNA were extracted in the non-spiked and spiked samples, respectively. *C. parvum* gene detected via the ddPCR analysis indicated a similar trend of 167592.4 (±1.2) copies/L of wastewater in the spiked sample and 33528.56 (± 0.8) copies/L of wastewater in the non-spiked sample. Corresponding to a recovery of approximately 8379 of oocysts per liter of wastewater for the spiked samples and approximately 1676 oocysts per liter of wastewater for the non-spiked samples (Table 8). Further filtration of the supernatant after centrifugation resulted in the ddPCR *C. parvum* copies of 31392.20 (±0.63) copies/ L of wastewater for the spiked samples and 5136.45 (±0.22) copies/L of wastewater for the non-spiked wastewater samples (Table 8). Based on these measurements, we were able to determine that further filtration of the supernatant after centrifugation improves recovery of the oocysts by 10.5% (Table 8).

Table 8: Measured *C. parvum* copies (ddPCR) in both centrifuged only samples and filtered supernatant after centrifugation.

Samples	Mean copies gene/L	SD	Total recovered oocysts/L	Difference	%Oocysts recovery
Spiked centrifuged sample	167592.40	±1.2	8379.62		
Non-spiked, centrifuged sample	33528.56	±0.8	1676.43	6703.19	53.63
Spiked filtered supernatant sample	31392.20	±0.63	1569.61		
Non-spiked filtered supernatant sample	5136.45	±0.22	256.82	1312.79	10.5

3.3.5 Evaluation of a molecular protocol for detection of *Cryptosporidium* spp. in wastewater samples

Among the (9) samples tested from different wastewater treatment plants from different locations in South Africa, all showed positive amplification when 1ng of DNA was used as template DNA concentration for ddPCR (Table 8). The mean (±SD) concentrations measured were 5375.78 (±6.38) copies/reaction (Cape town), 196.054 (±0.44) copies/reaction (Johannesburg) and 54,58 (±2.59) copies/reaction (Durban). This translates to an estimated *C. parvum* oocysts concentration of approximately 268.79, 9.80 and 2.73 oocysts in 1ng added, respectively.

Briefly, the workflow established (figure 8) begins with composite sampling for a complete representation of the microbial population from source water, followed by application of both centrifugation and filtration of supernatant, after centrifugation, to increase oocysts recovery. DNA extraction using the phenol-chloroform protocol and lastly amplification in ddPCR (figure 9).

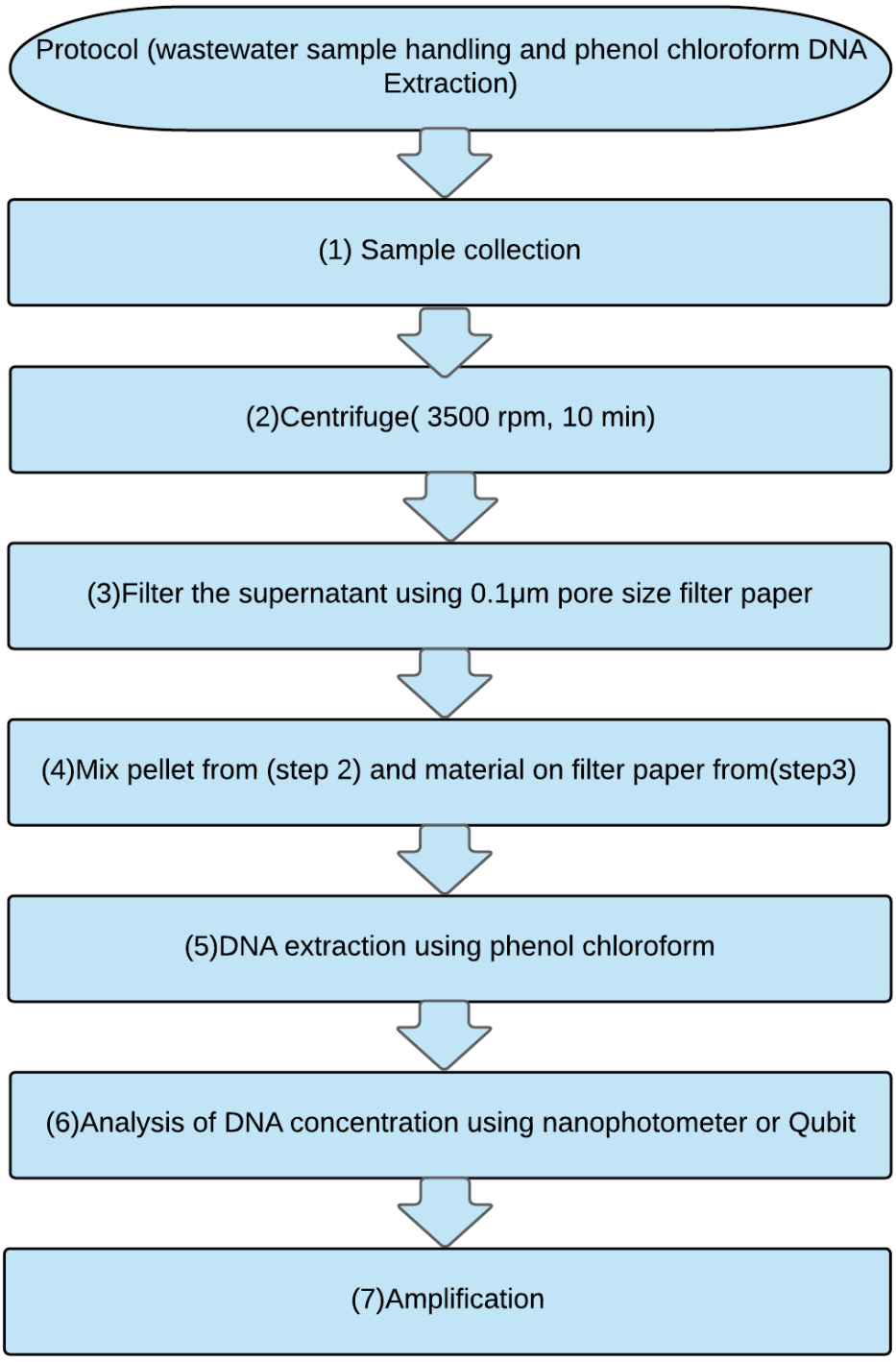


Figure 8. Wastewater processing, DNA extraction, and amplification using ddPCR

3.4 Discussion

Molecular detection of microorganisms in environmental samples is largely dependent on the extraction of the nucleic acid material and other co-factors (sample collection and preparation). In our study, the phenol-chloroform DNA extraction method comprised of the lysis step (500 µl cell lysis buffer (10 mmol/L Tris/HCl pH 8.0, 10 mmol/L EDTA, 0.1 mol/L NaCl, 2% SDS pH 8.0) (Djurhuus et al., 2017)(Awolusi, 2016) was determined to be the best method for the extraction of the protozoan parasite's DNA. The findings indicated consistency in DNA yield from *Cryptosporidium parvum* oocysts, with the highest concentration of 223 ng/µl and high purity of 1.8 (260/280) and 2 (260/230) (Table 6). The DNA extracted with phenol-chloroform proved to be compatible with ddPCR, showing good amplification with high copy numbers and no sign of inhibition. The high DNA yield achieved by the phenol-chloroform extraction method could be linked to the lysis step being effective in breaking the oocyst shell and releasing enough genetic material to be isolated. This could be due to the SDS and proteinase K added to the lysis buffer, SDS assists in cell membrane digestion, facilitate the solubilization of nucleic acid complexes, and lead to improved precipitation of the nucleic acids (Djurhuus et al., 2017, Barbier et al., 2019). Additionally, the protease K breaks down the proteins that protect the DNA molecules while they are in chromosomes (McKiernan and Danielson, 2017). The phenol-chloroform method has been previously recommended for recalcitrant species such as the robust protozoan oocysts (Butler, 2012, Barbier et al., 2019).

It was also observed that removing the freeze-thaw (liquid nitrogen) modification step from the phenol chloroform method (Table 6 method: liquid nitrogen + phenol chloroform) improved the results as the DNA concentration increased (Table 6 method: Phenol chloroform), This implies that the method was too harsh for the cells and contributed to gDNA degradation. A similar study found that freeze-thaw during extraction caused damage to Giardia cysts, resulting in a low gDNA yield (Babaei et al., 2011).

The phenol-chloroform extraction protocol used in this study further showed high sensitivity with successful DNA extraction from as low as 1 oocyst of *C. parvum*. Additionally, the DNA extracted directly from 1 cyst spiked into 500µl of nuclease-free water was subsequently detected by ddPCR, demonstrating the ddPCR's sensitivity in detecting such a small starting quantity, which is essential in water quality monitoring of protozoan parasites since the

infectious dose of protozoans needed varies from as low as 10 to 100 (oo)cysts depending on the infecting species (Zacharia et al., 2019). The lowest detection limit of each ddPCR with the extraction protocol was 5,93 copies equivalent to ≤ 1 oocyst per reaction. The number of oocysts that can be detected (LOD) in wastewater, surface water, or drinking water is imperative for public health protection. The operating water quality standards for *Cryptosporidium* spp. and *Giardia* spp. in drinking water, for example, are less than 1 (oo)cyst/10 L and 1 cyst/10 L, respectively, according to the South African National Standard (SANS) 241-1 (Sigudu et al., 2014). According to WHO Guidelines, the operational water quality value for (oo)cysts in raw water are 10 oocysts/ L and in wastewater, the 8.89 log reduction value is required (WHO, 2017). Hawash., (2014) reported the LOD with the Qiagen mini stool extraction protocol to be 2 oocysts per reaction. Their LOD is similar to our study, however, the difference is that their extraction was directly from feces. Another study reported *C. parvum* DNA extraction from stool samples with less sensitivity, with LODs of 10 and 50 oocysts for a real-time PCR assay, and Quick DNA Fecal/Soil Microbe-Miniprep kit, respectively (Valeix et al., 2020).

The phenol-chloroform method has been utilized for many years and is reported to remain one the most effective, reliable, and efficient DNA extraction methods especially for high molecular weight DNA or eukaryotes such as protozoans (Butler, 2012, Sessions, 2013, McKiernan and Danielson, 2017). For instance, Rosenbaum et al., (2019) reported that phenol-chloroform extraction method showed a better performance compared to the other eight DNA extraction techniques tested. However, the phenol-chloroform method is time-consuming (McKiernan and Danielson, 2017), and the phenol used is hazardous (Hawash, 2014). Therefore, further studies are required to optimize this approach for a shorter time and proper phenol handling to reduce the risks of exposure.

Before DNA extraction, one of the most critical steps in the detection of protozoan parasites in wastewater is the concentration of the (oo)cysts. (oo)cysts losses are known to occur at the sample handling and processing stages of filtration, regular centrifugation, and density gradient centrifugation (Al-Sabi et al., 2015, Razakandrainibe et al., 2020). Alternative methods such as alkaline and acid flocculation (Sammarro Silva and Sabogal-Paz, 2020), new filtration units (Al-Sabi et al., 2015), centrifugation at 1550 rcf for 10 min (Razakandrainibe et al., 2020) have been suggested and assessed. However, they are time-consuming and reduce recovery rate,

thus there is still no standard oocysts recovery protocol applicable before DNA extraction. We observed that further filtering of the supernatant after centrifugation resulted in additional oocysts recovery (Table 8). For instance, 8379.62 oocysts were recovered from spiked samples that were only centrifuged and a total of 1569.61 oocysts were recovered from the filtered supernatant (Table 8). This could mean that when the samples were concentrated with centrifugation alone at 3500 rpm (max 4 x 340 g) for 10 minutes, some oocysts with lower density remained in the supernatant. Centrifugation alone may lead to an underestimation of oocysts concentration; thus, our approach is necessary for improving oocysts recovery in water samples. The recovery of oocysts in the supernatant through filtration could be due to the filter paper pore size of 0.1µm used for filtration able to capture all oocysts left in the supernatant after centrifugation.

Finally, the established workflow (figure 8) was applied to wastewater samples from different areas. *Cryptosporidium* spp. were detected in all wastewater samples from the different parts of South Africa with varying concentrations. The copy number concentrations were in ranges but slightly higher than previously detected *Cryptosporidium parvum* copies (3084 copies/µl) from environmental water samples analysed using the qPCR kit (Lombard, 2016). The findings show that the new protocol (figure 8) when applied to wastewater samples is applicable for monitoring protozoan parasite *Cryptosporidium* spp. in the environment, especially in wastewater. This could be attributed to the improved oocysts recovery via the use of both centrifugation and filtration of the supernatant, DNA extraction with the phenol-chloroform method which has been shown to improve DNA extraction and finally amplification with the ddPCR.

3.5 Conclusions

This study successfully developed and evaluated a protocol for protozoan parasite detection in wastewater using ddPCR. The use of ddPCR to detect *Cryptosporidium parvum* demonstrated high sensitivity, with a limit of detection of 1.32 copies/20 µl reaction volume.

The workflow approach proved that filtration of the supernatant after centrifugation leads to enhanced (oo)cysts recovery by 10.5%, bringing the total recovery to approximately 64.1%. This means that relying on only centrifugation could potentially lead to underestimation of protozoan concentration in wastewater and other water matrices. The use of phenol-chloroform DNA extraction protocol without any prior pretreatments achieved efficient high DNA yield with a sensitive LOD of as low as 1 cyst per DNA extraction. Therefore, the optimized method in this study could be very important in water safety monitoring, which is critical for the design and implementation of effective measures aimed at reducing waterborne protozoan infections.

4.0 METAGENOMIC PROFILING OF PROTOZOAN PARASITES IN RAW AND TREATED WASTEWATER IN SOUTH AFRICA

This chapter has been published with the following details: Profiling pathogenic protozoan and their functional pathways in wastewater using 18S rRNA and shotgun metagenomics. Nonsikelelo P Mthethwa-Hlongwa, Isaac D Amoah, Andres Gomez, Sam Davison, Poovendhree Reddy, Faizal Bux, Sheena Kumari. Science of The Total Environment (2023). https://doi.org/10.1016/j.scitotenv.2023.169602.(Appendix 4)

4.1 Introduction

Infectious disease accounts for a large proportion of death and disability worldwide, and in certain regions, it remains the most serious cause of ill health (WHO, 2020). Traditional public health and sanitation measures have long been effective in limiting many pathogens that spread through contaminated water or vectors (Maritz et al., 2019; Zacharia et al., 2018). However, there is a need for more effective surveillance to reduce the disease burden, and spread of infectious disease and mitigate health risks based on analyses of microbial diversity (Garcia et al., 2017). Determining the genetic diversity and geographical distribution of microbes is important for tracking pathogen adaptation and evolution. In addition, it is critical to understand the relationship between environmental factors and microbial community structure (Wu et al., 2019; Feng et al., 2018).

Recent advances in metagenomic sequencing have revolutionized the understanding of complex microbial communities, including analyses of the human gut, and environmental (soil-water) systems. Nevertheless, there are few available data on the diversity and biogeography of eukaryotic microbial communities in wastewater treatment plants. Several studies have indicated that wastewater treatment plants are a significant reservoir of human and animal

pathogens, posing a severe threat to public health and the environment (Yashas and Udayashankara, 2017). The relative abundance of these pathogens in untreated wastewater may also vary depending on the location and source (Ahmed et al., 2018; Omarova et al., 2018). However, compared to bacteria, the genetic diversity and distribution of protozoa in wastewater have received little attention, especially in African countries with high disease burdens. Enteric pathogenic protozoa found in water are a major cause of severe human disease, causing epidemics and endemics in developed and developing countries (Roth et al., 2018). Cryptosporidiosis, Giardiasis, Toxoplasmosis, Cystoisosporiasis, intestinal amoebiasis, cyanosporiasis, Blastocystosis, Balantidiasis, and Granulomatous Encephalitis (GAE) are among the diseases caused by waterborne protozoans that are of public health concern (Bridle, 2014; Garcia et al., 2017; Griffiths, 2017; Xiao et al., 2018; Ajonina et al., 2018). These pathogenic infections are spread directly through the fecal-oral route or indirectly through contaminated water or food (Omarova et al., 2018). These pathogens are also resistant to disinfectants like chlorine, and are environmentally robust, with some being small enough (1–17 μm) to avoid water treatment (Widmer and Sullivan, 2012). Therefore, a full understanding of the diversity of these parasites in the environment will provide insights into their infection patterns and potential measures to reduce transmission.

A reliable detection and identification method is essential for determining the prevalence and distribution of protists in sewage samples. The current advanced methods for profiling eukaryotic diversity in complex environmental samples rely on sequencing the 18S rRNA eukaryotic gene and shotgun metagenomic analysis. The 18S rRNA short amplicon-based sequencing method amplifies and sequences a specific variable region of the 18S rRNA gene in wastewater microbial DNA. The 18S rRNA gene is conserved in all eukaryotic organisms, including protozoans. Pathogenic protozoans can therefore be identified by comparing

wastewater sequences to a database (Miller et al., 2013; Maritz et al., 2019). On the other hand, shotgun metagenomic sequencing sequences all the DNA fragments in a wastewater sample without prior knowledge of the microorganism present (Quince et al., 2017). Both techniques have potential advantages, however, their applications are limited, especially when it comes to identifying protozoan parasites, which are found in low numbers in wastewater samples (Wang et al., 2014; Maritz et al., 2017; Marzano et al., 2017; Maritz et al., 2019). The lack of metagenomic studies on protozoan parasites has been attributed to the complexity of eukaryotic genomes, their large genome size, higher variability of the genome, limited reference databases, computational challenges, and low abundance compared to prokaryotes (Castellanos-Gonzalez et al., 2018, Zahedi et al., 2019, Zahedi et al., 2018, Huang et al., 2016). Therefore, fewer studies and limited complete genome sequences are available in reference databases for environmental samples. It is necessary to optimize methods and advance sequencing technology, reference databases, and computational tools in order to meet these challenges.

This study focused on profiling protozoan parasites from treated and untreated sewage collected at ten area points across three major South African cities: Cape Town, Durban, and Johannesburg. These regions are high-risk areas due to their popularity as tourist destinations, large populations living in informal settlements, and inadequate access to safe water, sanitation, and healthcare (Corburn and Sverdlik, 2019). Additionally, protozoan infections are common as a large part of the population is immunocompromised e.g., HIV positive (de Jong, 2017). To monitor the prevalence of infection risks associated with protozoan pathogens in these different areas, the genetic profiles of important human and non-human pathogenic protozoans in wastewater treatment plant samples were evaluated using 18S rRNA short amplicon sequencing and shotgun metagenomic sequencing approaches.

4.2 Materials and methods

4.2.1 Description of Wastewater Treatment Plants (WWTPs) and sample collection

Wastewater samples (n=10) were collected from three of South Africa's largest and most populated cities, namely, Cape Town (CPT, n=4), Durban (DBN, n=4), and Johannesburg (JHB, n=2) (Figure 9(a-b). The study limited the sample size to only domestic wastewater treatment plants (WWTPs) in order to minimize the variability in untreated wastewater composition. Two WWTPs, close to each other, were selected from each city. Both treated (n=4) and untreated (n=6) wastewater samples were collected within each WWTP. A pooled composite sample of approximately 2L was taken from each sampling point within the wastewater treatment plant at different time intervals of 15/120 minutes. The samples from different locations were then transported overnight (in a cooler box) to the laboratory for analysis. Sample details are provided in Table S5 Appendix I).

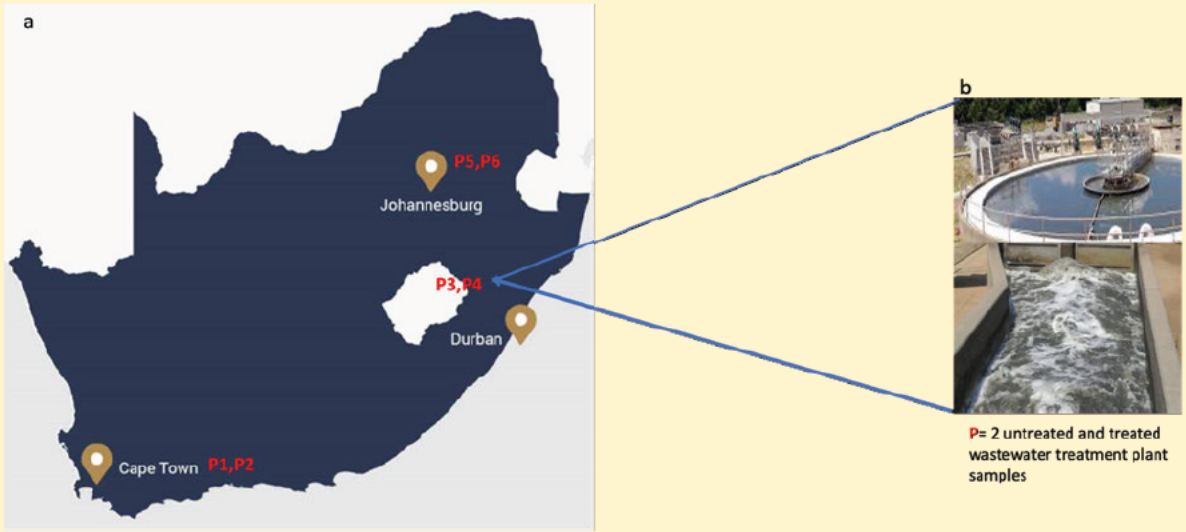


Figure 9. Geographical location of the samples collected. Samples were collected from two wastewater treatment plants in each location, both untreated and treated. A total of 12 samples were collected (2L each).

4.2.2 Sample preparation and DNA extraction

The DNA was extracted in accordance with our previous study (Mthethwa et al., 2022). Briefly, the wastewater samples were subjected to centrifugation at 3500 rpm (max 4 × 340 g) for 10 min, followed by filtration of the supernatant using a 0.1µm pore size cellulose acetate filter paper. Total DNA was extracted from both the pellets obtained after centrifugation and the residual material on filter paper following the phenol-chloroform method (Mthethwa et al., 2022; Awolusi, 2016). A suspension of the pellet as well as residual material on the filter paper was prepared in lysis buffer (10 mmol/L Tris/HCl pH 8.0, 10 mmol/L EDTA, 0.1 mol/L NaCl, 2% SDS, pH 8.0) and then treated with protease K. The mixture was incubated at 55 to 60 °C in a water bath for two hours (McKiernan and Danielson, 2017; Awolusi, 2016), followed by five consecutive freeze-thaw cycles. After that, Tris-saturated Phenol-Chloroform-Isoamyl alcohol (25:24:1) was added, mixed well, and centrifuged for two minutes at 12000 rpm (Awolusi 2016; Wells et al.,2016; Babaei et al., 2011). An equal volume of Chloroform was then added to the supernatant, followed by centrifugation at 12000 rpm for two minutes. The supernatant was transferred to a new tube and precipitated with isopropyl alcohol following centrifugation at 3000rpm for 2 minutes. This was followed by an ethanol (70%) wash. The final product, DNA, was air-dried and stored in TE buffer at -20°C for further analysis.

4.2.3 18S rRNA gene amplification and Illumina sequencing

The extracted DNA was quantified using a Nanophotometer (Implem, United Scientific, South Africa). Thereafter the DNA was sent to a commercial laboratory (Inqaba Biotech, South Africa) for high-throughput sequencing and analysis. Briefly, for amplicon sequencing, two published primers (Hadziavdic et al., 2014) were used to target the largest hypervariable (V4-V5) region of the Eukaryotes 18S rRNA gene. The forward (5'-CAGCAGCCGCGGTAATTCC-3') and reverse (5'-CCCGTGTTGAGTCAAATTAAGC-3')

primers with adapter amplicon lengths compatible with Illumina MiSeq specifications (2 × 300nt pair-end reads) were synthesized and used for amplification. The PCR reactions were carried out under the following conditions: initial denaturation at 95°C for 15 minutes, followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 1 minute. The amplification was completed with a final extension step at 72°C for 10 minutes. The resulting PCR products were purified using a column-based PCR purification kit (Thermo Fisher Scientific, South Africa), separated on gel electrophoresis to verify the size and quality, and the PCR products were quantified using the Qubit (Thermo Fisher Scientific, South Africa) instrument. Indexing PCR, Ampure bead purification, Equimolar pooling, and Sequencing on Illumina Miseq was performed at the commercial lab (Inqaba Biotech, South Africa).

4.2.4 Shotgun metagenomic sequencing

Shotgun metagenomic sequencing for all untreated samples was performed in parallel with 18S rRNA short amplicon sequencing. This was done to increase the taxonomic resolution of each organism detected down to species and strain level. Additionally, the shotgun procedure was used to obtain functional profiles of the Eukaryotic microbial community present in the samples. The metagenomic libraries were constructed at the Inqaba Biotech company in South Africa using a Library Prep kit (gDNA, amplicon, plasmids). High-throughput DNA sequencing was performed using the Illumina NexSeq platform.

4.2.5 Data analysis

4.2.5.1 Bioinformatics processing

The 18S rRNA raw paired-end reads were subjected to quality control, which included trimming of primers, sequencing adapter, and elimination of low quality (Q=30) and short length reads using cutadapt (Zamora-Terol et al., 2020). Paired-end reads were merged using BBMap (Brandt et al., 2021). High-quality reads were considered for downstream analysis using the DADA2 plunging within QIIME2 (qiime2.org) to produce unique amplicon sequence variants (ASV's). Taxonomic assignment of the ASVs was carried out by first training a SILVA reference database classifier (clustered at 99% sequence identity) according to the primer used and was then applied to generate a taxonomy frequency and abundance table. Relative taxonomic abundances were calculated for all wastewater samples and displayed in the stacked bar plot for phylum, class, and genera distributions. Additionally, the percentage distribution of phylum, class, and genera across all samples are also represented using pie charts in the supplementary section. The relative abundance of the Eukaryote taxa in different samples at the phylum, class, and genus level (ASVs) was calculated using indicator species analyses (Dufrêne and Legendre, 1997) and visualized using boxplots.

Shotgun metagenomic bioinformatics processing was done by removing reads mapping to the human genome using kneaddata (Langmead and Salzberg, 2012). Basic quality control was performed by trimming reads with quality scores below 20, and shorter reads (90bp) using Trimmomatics (Bolger et al., 2014). Taxonomic classification and relative abundance estimation were done using Kraken 2 (Wood et al., 2019). Lastly, HUMAnN 3.0 was used to profile microbial pathways and virulent gene families. The databases used included Metaphlan4, Uniref 90 full, and Chocophlan (Maritz et al., 2019). Each pathway derived from the shotgun approach was screened and only those pertaining exclusively to eukaryotes and

protozoans were discussed. All bioinformatics analyses were conducted using resources from the University of Minnesota's Supercomputer Institute (MSI).

4.2.5.2 Statistical analyses

All microbial community analyses were carried out using the R statistical interface (Maritz et al., 2019). The R vegan package was used to calculate alpha diversity (Shannon, and richness) and Beta diversity (Bray Curtis distances) (Jin et al., 2017, Liu et al., 2021). Weighted and unweighted Bray Curtis Principal Coordinate Analysis (PCoA) distances were computed. The permutational multivariate analyses of variance (PERMANOVA) test was used to determine statistically significant differences in microbial community composition. False discovery rate (FDR)-adjusted Kruskal-Wallis tests for multiple comparisons (q 0.05) and species indicator analysis (indicator values) implemented in the labdsv R package (Maritz et al., 2019) were used in tandem to detect taxa that were differentially abundant at different geographical locations and between treated and untreated samples. The stats, vegan, heatmap, and ggplots2 R packages were used to create the graphs (Jin et al., 2017, Liu et al., 2021). Venn diagrams were generated to identify sample similarities(core) and unique species (Chen et al., 2021)

4.3 Result and Discussion

A total of 669 617 (1 339 234 PE) 18S rRNA paired-end raw amplicon reads were obtained from 10 wastewater samples, resulting in 337 249 high-quality reads mapped in the SILVA reference database (Table S5 Appendix I). The mean sequencing depth was 33 724.9 reads/sample, identifying a total of 438 species. Shotgun metagenomic sequencing of untreated wastewater samples from selected locations produced 23 826 052 paired-end raw reads. Using a Kraken2 standard plus Eukaryotes reference database, 19 493 187 (78.8% - 84.45%) high-quality reads were mapped. Of the 82.11% averaged mapped reads, only 1.02 % average reads

were of Eukaryotes with a mean sequencing depth of 66 816 reads/sample (Table S6 Appendix I).

4.3.1 Alpha diversity of protist communities in different wastewater samples

Alpha diversity analyses (Shannon index) of the 18S rRNA gene sequencing results revealed significantly higher Eukaryote diversity in both untreated and treated wastewater samples from DBN compared to CPT and JHB samples (Kruskal-Wallis test, *P*-value=0.03) (Figure 10a). Microbial richness (Figure 10b), which only considers the presence or absence of taxonomic groups, revealed a pattern similar to that in Figure 10a, but no statistical significance was observed (Kruskal-Wallis test, *P*-value=0.17). Due to the limited number of samples collected for each type of wastewater (treated or untreated), statistical differences were not determined between the types of wastewater Despite this, some potential differences were visually apparent. Figure 2b, for example, illustrates that untreated samples may exhibit greater microbial diversity. Rarefaction curves (Figure S4a Appendix 1) supported the diversity patterns observed in Figure 10a-b, accounting for sequencing depth. Notably, DBN samples exhibited higher microbial richness, whereas CPT samples showed lower richness, but relatively better coverage and sequencing depth. Analysis of shotgun metagenomic data for alpha diversity revealed no significant differences in community richness and diversity between samples (Figure S5 (a-b) Appendix 1). Rarefaction curves (Figure S5c) suggested that metagenomic sequencing depth adequately captured diversity, reaching asymptotic values as the number of sequences increased. Results from our study indicate that different factors could influence protozoan diversity within WWTPs, including wastewater source, sewage infrastructure, geographical location, environmental factors, and microbial adaptations as

demonstrated by various other authors (Xu et al., 2018, Goux et al., 2015, McLellan and Roguet, 2019, Manaia et al., 2018).

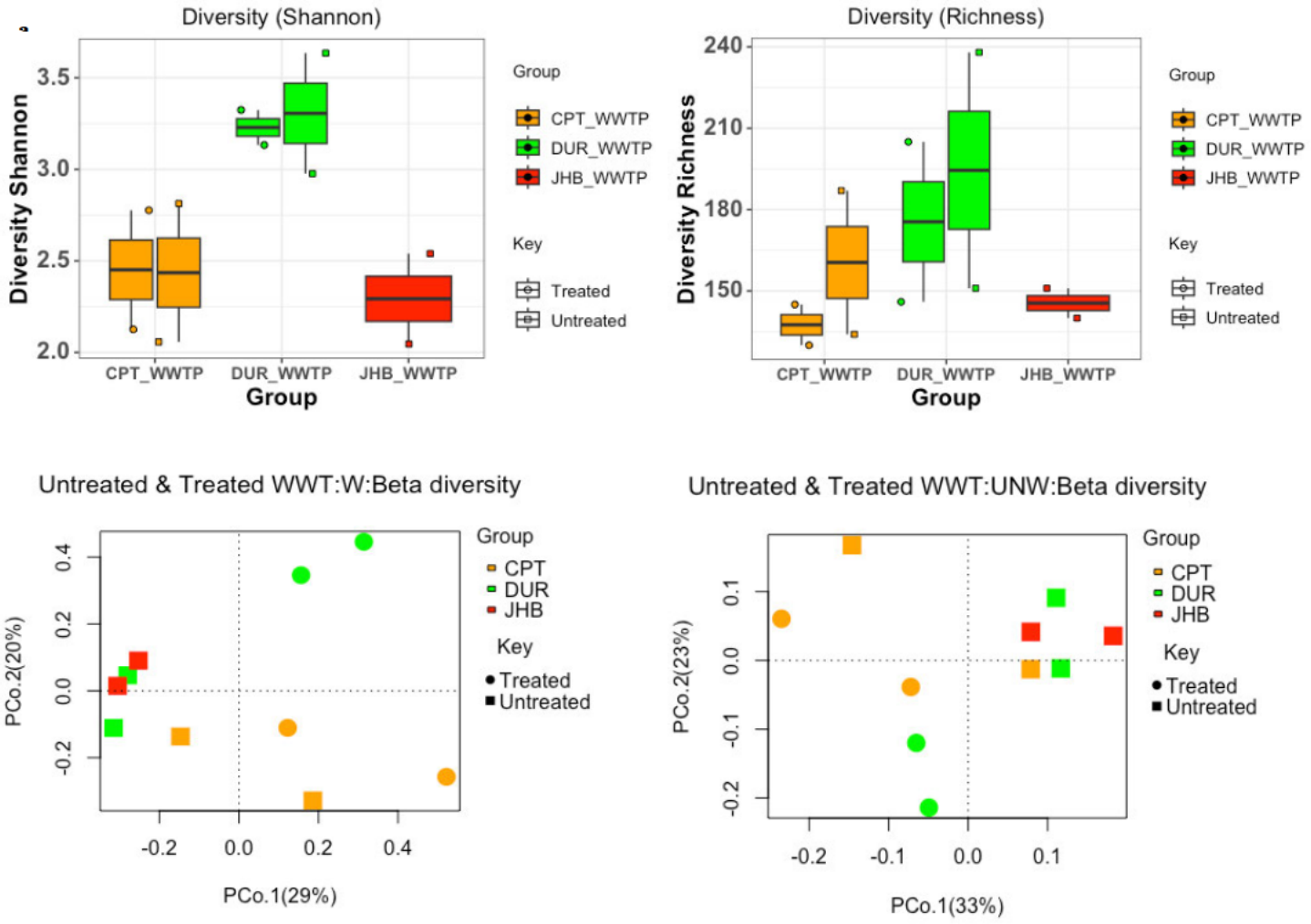


Figure 10(a-d). Eukaryotes diversity showing treated wastewater and untreated wastewater sampled from **CTP_WWTP**= Cape town wastewater treatment plants, **DUR_WWTP**=Durban wastewater treatment plants and **JHB_WWTP**=Johannesburg wastewater treatment plants.

A Kruskal-Wallis rank sum test was used for testing the box plot distributions with *P*-value adjustment method: Benjamini-Hochberg (BH). (**a**)Boxplot of alpha diversity as measured by Shannon diversity index, showing higher diversity in samples from Durban compared to CPT and JHB geographical locations (*P*-value 0.03). (**b**) Alpha diversity showing higher eukaryotes species richness in DBN location with no significant difference between all three sampled locations (*P*-value=0.17). The pairwise-Wilcox test showed no significant differences between both location groups nor type of wastewater (treated or untreated). (**c-d**). Principal coordinates analysis (PCoA) plot of Beta diversity as measured by Bray Curtis dissimilarity matrices is reported and the proportion of variation explained by each axis is listed in parentheses. (**c**) Weighted PCoA showing significant difference in microbiome composition across water types and the geographical locations (**R^2= 0.28, pseudo-F=1.82, *P*<0.022**) and across the type (Key) of a wastewater (**R^2= 0.21, pseudo-F=2.27, *P*<0.003**) according to PERMANOVA tests and ANOSIM (**R=0.17, *P*<0.041**). (**d**) Unweighted Beta diversity showing significant

difference in microbiome composition across water types and geographical locations (**R^2= 0.32 pseudo-F=1.82, *P*<0.040**) and type of wastewater (**R^2= 0.181, pseudo-F=2.10, P<0.035)** according to PERMANOVA and ANOSIM **(R=0.26, P<0.011)**.

4.3.2 Beta diversity analyses across different types of wastewater

Principal coordinates analysis (PCoA) of Beta diversity as measured by weighted and unweighted Bray Curtis dissimilarity matrices for the 18S rRNA gene sequencing approach is shown in Figure 10(c-d). These analyses revealed noticeable similarities and distinctions in the microbial community patterns influenced mainly by the type of wastewater (Figure 10c-d). Treated, and untreated wastewater samples were clearly separated, with dissimilarities being more prominent after treatment. For instance, the treated samples from CPT and DBN formed distinct clusters, separated from the untreated cluster, while also being positioned apart from each other along axis 2 (Figure 10c-d). The untreated wastewater samples also showed a significantly different composition from the treated samples along axis 1 (Figure 10c-d). Axis one of this PCoA model explained 29% and 38% of the variation in the data set for both weighted and unweighted PCoA, respectively (Figure10c and 10d). Permutational multivariance analysis of variance (PERMANOVA) using the adonis2 function in R (vegan package) corroborated statistical differences in microbial composition in different sample types, regardless of the location (R^2= 0.21, pseudo-F=2.27, *P*<0.003 and: R^2= 0.181, pseudo-F=2.10, P<0.035) for weighted and unweighted Bray-Curtis distances, respectively. The analysis of similarities (ANOSIM) also confirmed significant differences in microbial communities between sample types (R: 0.52, *P*< 0.02) and (R: 0.44, *P*< 0.02) for unweighted and weighted distances, respectively. Various factors such as the treatment process used, the source of influence, and the geographic location may contribute to the specific microbiome profiles of treated wastewater samples (Oluseyi Osunmakinde et al., 2019, Thobejane et al., 2023, Giwa et al., 2020, Verburg et al., 2021). In a previous study, sewage microbiome patterns

were examined at various stages (influent, activated sludge, and effluent) in Hong Kong's largest wastewater treatment plant, and their PCoA profiles also revealed a dispersed pattern among effluent samples (treated) and overlapping influent samples (untreated), as well as a distinct separation between treated and untreated samples. (Cai et al., 2014). The study also observed that all untreated samples from the wastewater treatment plants were clustered together, with the exception of one sample. The increased similarity in the microbiome patterns observed among the analyzed untreated samples may be attributed to consistent influent (untreated) wastewater composition, shared microbial sources from human and environmental origins, limited treatment efficiency, and resilient microbial populations (Cai et al., 2014, Fletcher et al., 2012).

4.3.3 Differentially abundant taxa in untreated and treated wastewater sample type

Indicator species analysis (Indval) identified particular taxa distinguishing untreated and treated samples (Figure 11a-f)(Table S7 Appendix1). Untreated wastewater samples specifically exhibited a significant presence of phyla Alveolata, Stramenopiles, Apusomonadidae, and Nucletmycea (Figure 11d-e). On the other hand, the phyla Tubulinea, Rhizaria, and Discoba were largely detected in the treated water samples (Figure 11f-h), whereas Chloroplastida and Holozoa appeared in both treated and untreated samples (Table S7). The abundance of Holozoa and Tubulinea in the treated samples may be attributed to their feeding habits, as they consume bacteria or act as hosts for prokaryotic symbionts (Betat et al., 2015). Other studies have also reported increased viruses and certain eukaryotes in treated wastewater samples compared to untreated samples (Azli et al., 2022, Yasir, 2020). According to those studies, these microorganisms displayed high resistance to sedimentation and inactivation throughout the entire treatment process. As a result, even after being discharged,

the remaining microorganisms, such as viruses and larger eukaryotes, can continue to reproduce, grow, and thrive in the treated water (Azli et al., 2022).

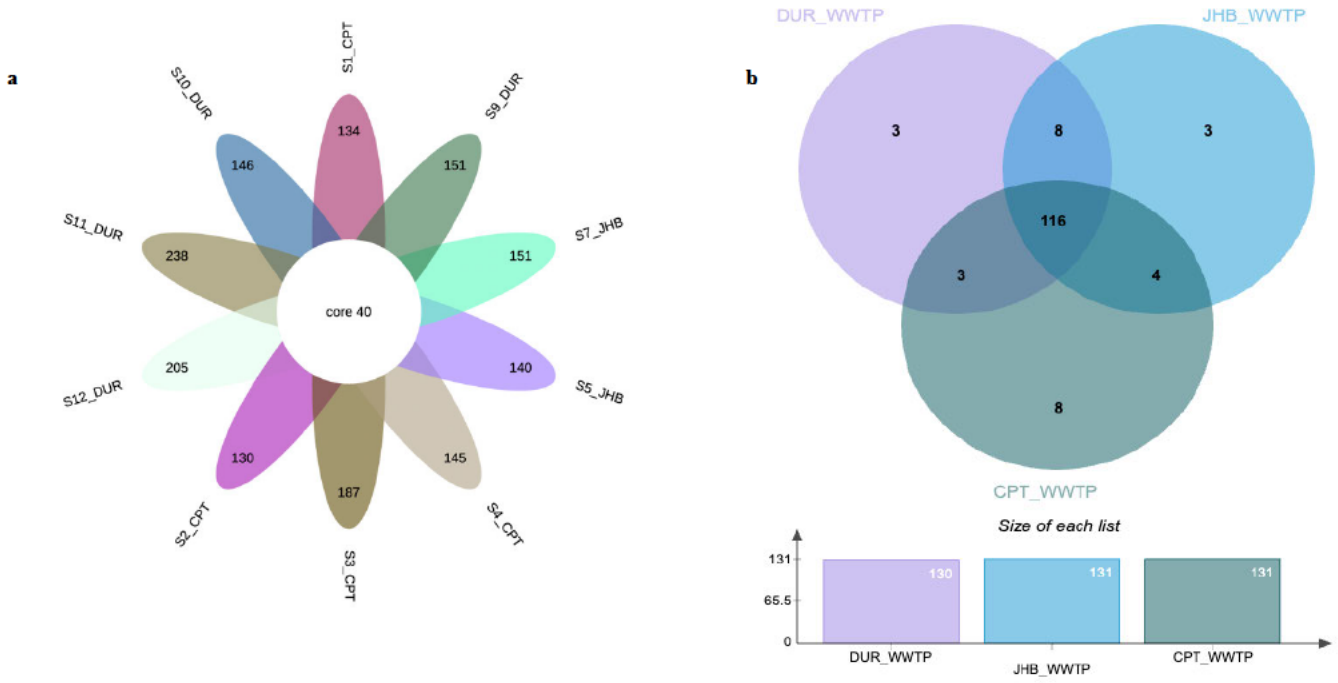


Figure 11(**a**) 18S rRNA short amplicon sequencing:

Venn flower diagram showing core and unique species across all wastewater treatment plant samples. The central circle shows the number of species common to all samples while the petals show the number of species in addition to the core set unique. Abbreviated sample names (S1-S2) are given outside each petal, and details are given in Table S5 Appendix 1). **(b)** Shotgun metagenomic: Venn diagram depicting the number of shared and unique number of species from the geographical locations.

4.3.4 Similarities and variations observed in different geographical locations

While the type of wastewater was primarily responsible for patterns of differentiation in microbial communities, similarities and variations across geographical regions were also evident (Figure 10c-d & Figure S5(d-e)). Microbial analysis of samples from Johannesburg (JHB), Durban (DBN), and Cape Town (CPT) revealed distinct patterns, with JHB and DBN showing less variation and clustering, while one CPT sample differed significantly (Figure 10c-d). Additional shotgun sequencing data further confirmed unique microbial compositions in

each city(Figure S10(d-e)). Despite the limitation of one representative sample per site, principal coordinate analysis (PCoA) suggested greater variation in the CPT sample along PCo axis 1 (95% and 75% variation) (Figure S10(d-e). These findings could suggest that the geographical proximity between human communities plays a role in shaping the microbial composition of wastewater (Kim et al., 2021). These findings agree with a previous study where activated sludge community similarity decreased as a result of the distance decay relationship (Kim et al., 2021). PERMANOVA and ANOSIM analyses based on location show differences in environmental microbial communities across the cities, with CPT samples showing distant microbial profiles from those in JHB and DBN samples (test R^2= 0.28, pseudo-F=1.82, P<0.022 and ANOSIM's R=0.17, P<0.05 for weighted analyses and R^2= 0.32 pseudo-F=1.82, P<0.040 and ANOSIM's R=0.18, P<0.02 for unweighted distances). The interaction between the location group samples and the type of wastewater showed no statistical significance according to the weighted (R^2= 0.11 pseudo-F=1.42, P<0.16) and unweighted PCoA PERMANOVA test (R^2= 0.08 pseudo-F=0.88, P<0.58). However, in this study, further research is recommended with larger sample sizes to confirm these observations.

4.3.5 Core eukaryote community

A core eukaryote community was evident across all wastewater treatment plants sites in South Africa, comprising of 40 different species of the 18S rRNA short amplicon data (Figure 11a) and 116 species of the shotgun metagenomics data (Figure 11b) (Table S8). This included both disease-causing and non-disease-causing organisms. Some of the 116 core (common) pathogenic species as presented in Table S8 included *Cryptosporidium hominis* TU502, *Trypanosoma brucei gambiense* dal972, *Leshmania donovani*, *Cryptosporisdium parvium lowa* II and *Girdia intestinalis*. Several factors may contribute to the core pathogenic community, including travelers, shared facilities, as well as similar environmental and socio-

economic conditions between different regions within the country (Fletcher et al., 2012). For instance, DBN and CPT are two of the most populated, busiest, and premier South African tourist destinations. Infection transmission/carryover between the two cities could be caused by a large number of travellers and shared facilities like public swimming pools, beaches, public toilets, and restaurants. Additionally, a previous report by Garcia et al., (2017) suggests that the global distribution of protozoa, such as *Cryptosporidium* and *Giardia*, could potentially be attributed to factors like domesticated animals and traveling (Garcia et al., 2017), both of which South Africa is known for, thus potentially influencing the prevalence of parasites in the region. However, additional long-term studies involving more WWTPs from various regions are needed to validate these findings.

4.3.6 Taxonomic profiles: 18S rRNA short amplicon sequencing and shogun metagenomics sequencing approach

The 18S rRNA short amplicon sequencing results revealed a diverse Eukaryote protist community in all samples. The dominant phylum, labeled as "Other" in the plot, constituted 48% of the community and encompassed low-prevalence protists (below 1% abundance) and non-protozoan Eukaryotes (Figure S6a & Figure 12a). The Alveolate phylum, known for pathogenicity (Liu et al., 2017, Plattner et al., 2012), was the most common, constituting 27% of the total Eukaryote relative abundance (Figure S6a) (supplementary). Ciliophora and Apicomplexa, major classes within Alveolates, exhibited relative abundances of 25.86% and 1.44%, respectively (Figure 12b). The members of the Alveolate group were predominant in all samples, with the highest prevalence of Ciliates in CPT, particularly in untreated as compared with treated samples (Figure 12a&b). Furthermore, the Ciliates *Teletrochidium*

(15.16%), *Conthreep* (2.35%), *Heliophrya* (1.84%), and *Petrichia* (1.11%) dominated at the genus level in all untreated and treated WWTP samples from CPT and DBN, except for *Heliophrya* which was not detected in JHB samples. On the other hand, the Apicomplexan *Cryptosporidium* genus was more prevalent in DBN treated and untreated WWTP samples compared to other locations (CPT & JHB).

The second most abundant protozoan phylum was the diplomonads Excavata, which are important parasites of humans in both in their free-living and symbiotic forms. (Figure 12a) (Yubuki et al., 2016). Notable members included the class Discoba (3.65%) and the genus *Petalomonas* (1.10%) (Figure S6b, Figure S3c, and Figure 12b). Amoebozoa and Rhizaria were the least abundant among the top six eukaryote phyla (2.84% and 1.31%, respectively) (Figure S6a). Amoebozoa, with Tubulinea (0.74%)(Figure S6c), was detected only in 50% of the samples with high prevalence in the DBN untreated wastewater samples (Figure 11c). A common soil protist, Cercozoa, and an intestinal protist, *Blastocystis*, both occurred in low abundance in all samples with higher abundance in DBN (Figure 12a-c).

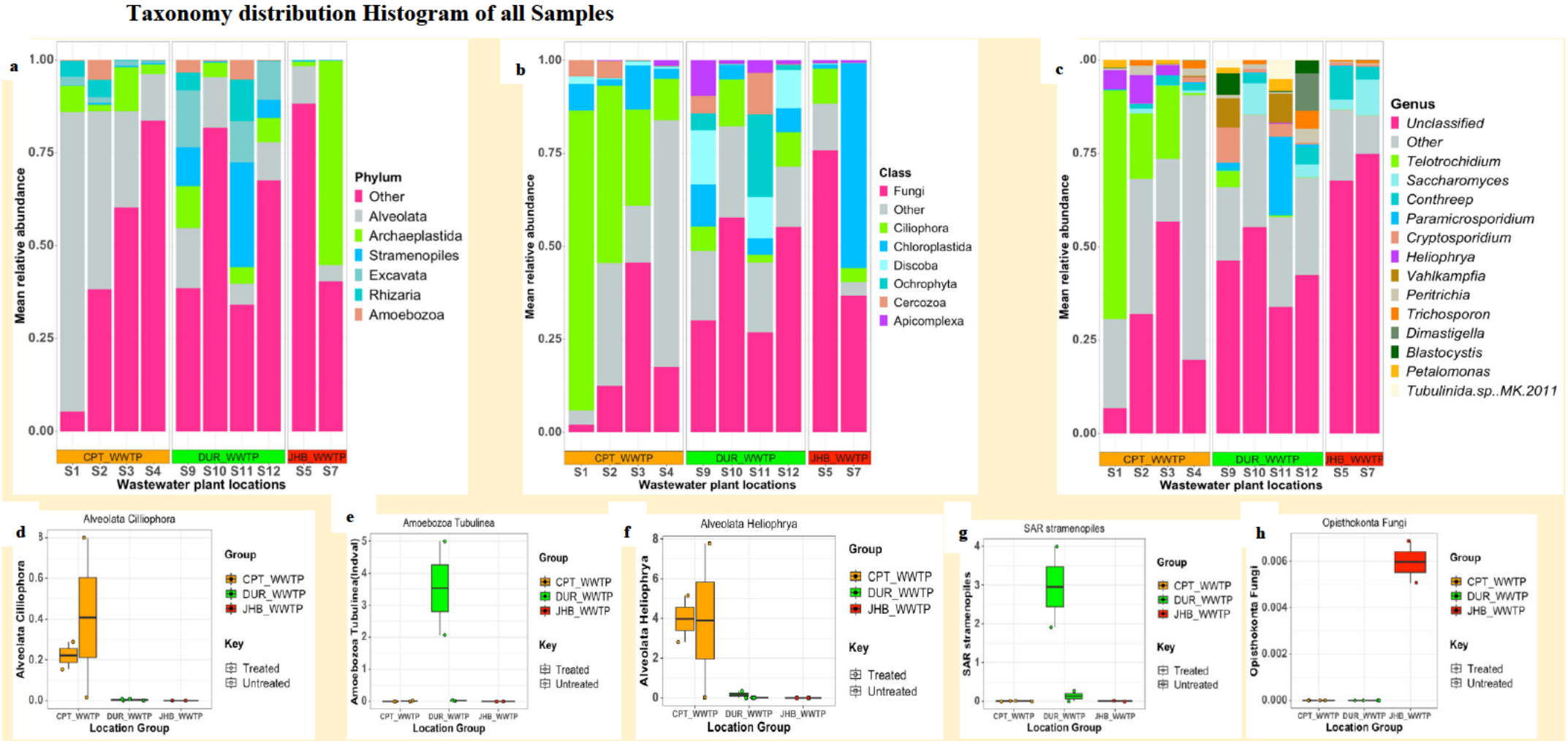


Figure 12.(a-h) Eukaryote relative abundance of individual wastewater samples (S1 to S12) from different locations.

(a) Phylum-level Relative abundance of individual samples. The main groups in wastewaters consist of Alveolata, Excavata, Rhizaria and Amoebozoa known to contain pathogenic waterborne species. (b) Relative abundance of the Eukaryote at the Class level per location per sample. (c) Relative abundance of the Eukaryote at Genus level per location per sample. (d-h)Differential relative

abundance analysis of the eukaryote taxa associated with wastewater samples collected from different geographical in treated and untreated .Indicator value 'IndVal' analysis-based box plots showing the most abundant taxa associated with each group. **(d)** Phylum Alveolata significantly abundant in Cape town Untreated wastewater, **(e)** Amoebozoa significantly abundant in Durban treated wastewater samples. **(f)** Alveolata Heliophrya significantly abundant in both treated and untreated Cape Town wastewater samples **(g)** SAR significantly abundant in Durban samples. **(g)** Fungi significantly abundant in JHB untreated wastewater samples.

Shotgun metagenomic sequencing further revealed the microbial composition at phylum, genus, and species levels in Figure 13a-c. Bacteria dominated in all locations, accounting for 78.8% to 84.45% (Table S6). Assignments to eukaryotes were the next most frequent, with 0.99%,1.03%, and 1.05% for S1 (CPT), S2 (JHB), and S3 (DBN) sewage samples, respectively. Several protozoan taxa within the Eukaryote domain with potential concern to human health were detected, including Apicomplexa, Euglenozoa, Evosea, Discosea, Cercozoa, Parabasalia, and Fornicata were identified in the phylum level (Figure 13a). Apicomplexa was the highest abundant phylum in all samples studied, with CPT showing the highest percentage, followed by DBN and then JHB. Sequences assigned to Apicomplexa were identified as *Eimeria* spp. (0.34%), *Cryptosporidium* spp. (8.57%), *Toxoplasma* (55.58%), *Babesia* (3.34%), *Cyclospora* (0.06%), and *Plasmodium* (15.52%), at the genus level (Figure 13b).

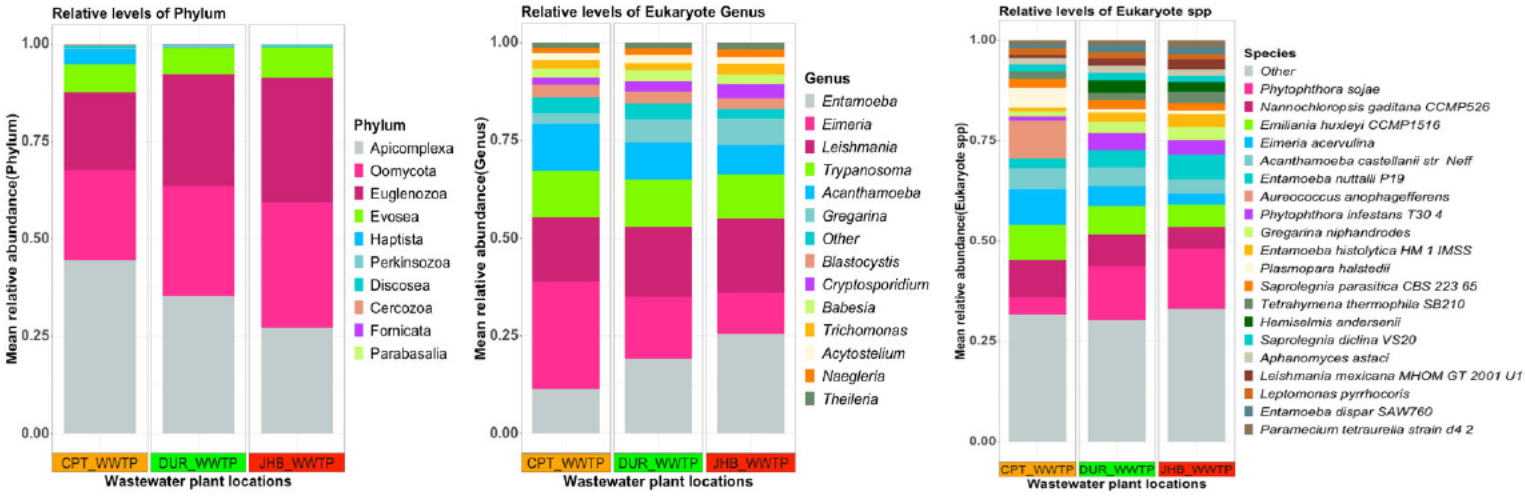


Figure 13(a-c).Shotgun generated Eukaryote relative abundance of individual wastewater samples,

CPT_WWTP = Cape town, **JHB_WWTP**=Johannesburg, and **DUR_WWTP**=Durban. **(a)** Phylum-level Relative abundance of individu samples. **(b)** Relative abundance of the Eukaryote at the Genus-level per location per sample. **(c)** Species-level Relative abundance of the mo prevalent Eukaryotes.

The higher taxonomic resolution provided by the shotgun metagenomic sequencing approach revealed that all areas had a significantly high prevalence of human and zoonotic pathogenic protozoan species (Figure 13c). For instance, *Eimeria acervuline* (16.64%), *Acanthamoeba castellani* (13.36%), *Entemoeba nuttalli* (12.93%), and *Aureococcus anophagefferens* (9.47%) were highly prevalent. Given the dominance of potentially pathogenic protists, the abundance of high-priority pathogenic waterborne protozoan was profiled, as declared by public health officials around the world, such as the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) (Figure 14a-b). The potentially pathogenic protozoa detected included *Cryptosporidium parvum Iowa II* (0.31%), *C. hominis.TU502* (0.16%), *C. muris* RN66 (2.56%), *Giardia intestinalis* (0.31%), *Entamoeba histolytica* (6.58%), *Naegleria gruberi* (2.37%), and *Toxoplasma Gondii* (1.98%). In comparison to all other identified human waterborne protozoans, *Acanthamoeba castelli, Entamoeba nutalli, and Entamoeba hystolitica* were the most common pathogenic species, accounting for 75% of the high-priority human waterborne species abundance across all geographical areas (Figure 14a).

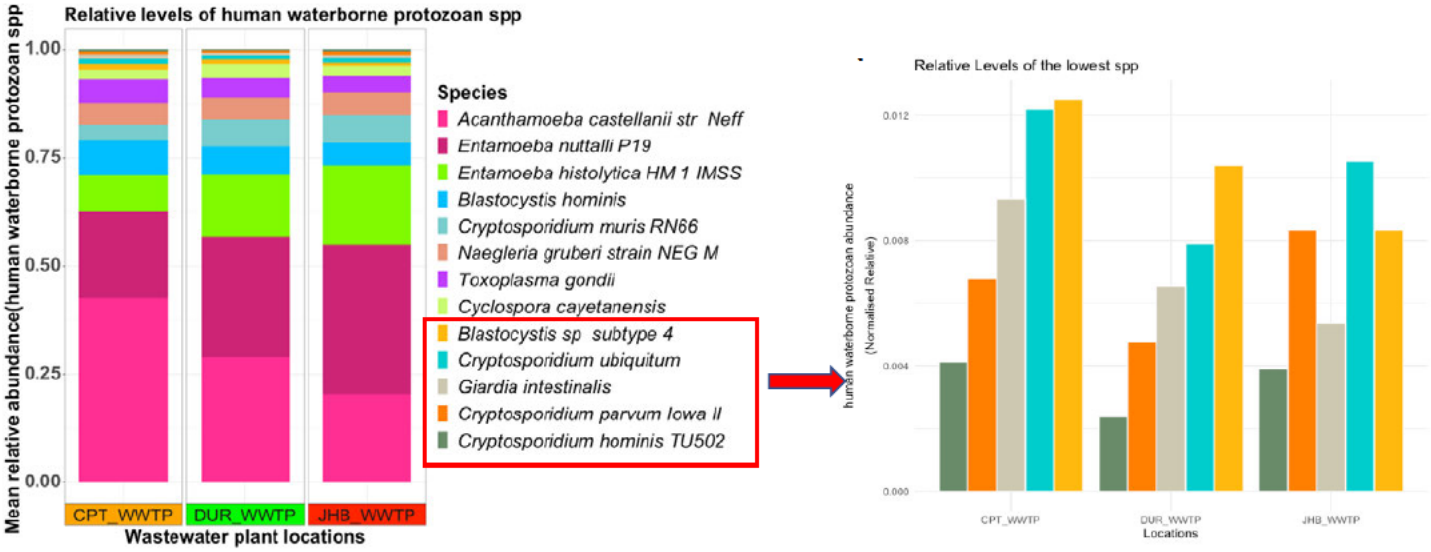


Figure 14(a-b).The relative abundance of the most important human waterborne protozoa in each city is shown, as per WHO and CDC guidelines/priority.

The above-identified protists are typically found in humans and animals, either in a free-living state or as parasites, leading to various diseases. A similar study involving the metagenomic profiling of raw sewage samples from New York (Maritz et al., 2019) found the presence of protist communities such as Ciliophora, *Petrichia*, Fungi, Cercozoa, and Excavata, which are also demonstrated in this study. Moreover, the identification of priority waterborne protozoa emphasizes the potential for severe and life-threatening protozoan-related illnesses within the community, such as primary amoebic meningoencephalitis (PAM), and respiratory and gastrointestinal infections (Lee et al., 2018, Garcia-R et al., 2020, Güémez and García, 2021). Such infections are generally spread by infected water sources, food, animals, or person-to-person contact (Ma et al., 2022, Gerba, 2015). Thus, underlines the necessity of effective wastewater treatment and suitable disposal methods as well as the need for monitoring their prevalence in treated wastewater. The prevalence of pathogenic protozoa, including *Acanthamoeba* spp., which are also associated with *Acanthamoeba keratitis* and severe central nervous system infections, raises concerns for public health (Yoder et al., 2012, Chalmers, 2014, CDC., 2012). These pathogens are difficult to treat and can survive in various environmental conditions, their presence in wastewater is a potential health concern (Omarova et al., 2018). They have been found in river waters, soils, and untreated surface irrigation water mixed with wastewater (Mahmoudi et al., 2015, Moreno et al., 2018). They are the third leading cause of waterborne protozoal contamination across ten African countries (Uganda, Tunisia, Sudan, South Africa, Namibia, Guinea Bissau, Ethiopia, Egypt, Central African Republic, and Benin), accounting for more than a third of all documented cases (Ben Ayed et al., 2019). Additionally, the identified Amoebozoa can act as a host and carrier for several other bacterial pathogens such as *Salmonella enterica*, *Pseudomonas* spp., *Mycobacterium*, *Vibrio*, and

Legionella pneumophila (Juárez et al., 2018, He et al., 2021). Additionally, these pathogens exhibit resistance to common disinfection methods such as chlorine treatment (He et al., 2021)

Regional variations were observed, with certain pathogenic species more abundant in specific areas. For example, *C. parvum* was more prevalent in JHB, while *Blastocystis* spp., *C. ubiquitum*, *G. intestinalis*, and *C. hominis* were more abundant in CPT. This observation could reflect the high human and zoonotic-related disease burden in this region. These pathogens have been shown to cause a significant proportion of reported cases of waterborne disease, with cryptosporidiosis being the leading cause of worldwide waterborne protozoan outbreaks (WHO, 2020, Garcia et al., 2017, Ma et al., 2022). These findings are consistent with previous findings in South Africa (Omoruyi et al., 2011) and other developing countries such as Kenya (Delahoy et al., 2018), Ghana (Alum et al., 2014, Sampson et al., 2017), and Nigeria (Jombo et al., 2010), where diarrheal infections are primarily linked to *Cryptosporidium* spp. De Jong et al. (2017) investigated three WWTP in Durban, KwaZulu Natal, and found that *Cryptosporidium* spp. dominated all of them. Furthermore, Abu Samra et al (2013) discovered the presence of *Cryptosporidium* in four other South African regions (Gauteng, KwaZulu Natal, Mpumalanga, and Northwest). This study analyzed clinical stool samples from hospitals, indicating their prevalence among the populations in the area (Abu Samra et al., 2013).

Additionally, all WWTP locations showed the least abundance of other protozoans of public importance, including *Blastocystis hominis*, *Naegleria gruberi*, *Toxoplasma gondii*, *Cyclospora cayetanensis*, and *Giardia intestinalis* (Figure 14a-b). The highest counts were found in CPT and JHB untreated WWTPs, likely reflecting public health patterns in the surrounding community at the time of sampling.

4.3.7 Functional pathways and virulent gene families distinguishing WWTPs microbiome

Virulence gene families and enriched functional pathways linked to protozoa have been identified in wastewater samples collected from diverse locations (Figure 15). These findings indicate the widespread presence of protozoan parasite and corroborate the above taxonomic profile results, as illustrated in Figures 15a and b. Specific observations include an increased abundance of certain genes in JHB, such as serine/threonine protein phosphatase and mucin-desulfating sulphatase, and elevated levels of TRAP Transporter in DBN (Figure 15a). The exclusive presence of specific gene families, such as Trypsin-Like Serine and Cysteine Protease in JHB highlights potential regional distinctions in protozoan parasite-associated virulence factors. Previous studies have identified potential virulence factors linked to *Cryptosporidium* spp*.,* and other protozoa and elucidated the genes and proteins crucial for parasite survival, replication, and potential host infection. These studies have highlighted serine protease, aminopeptidase, CSL, gp900, gp60, and a sporozoite and merozoite cell surface protein complex (gp15/40/60), which may be involved in excystation, adhesion, and invasion (Bouzid et al., 2013, Audebert et al., 2020, Wang et al., 2022, Xu et al., 2020, David Sibley, 2011).

The results of shotgun metagenomic analysis further confirmed the presence of functional pathways associated with pathogenic waterborne protozoa in all wastewater treatment plants (WWTPs) (Figure 15b). These pathways include amino acid biosynthesis, peptidoglycan maturation, adenosine and branched-chain amino acid biosynthesis, and the Calvin-Benson-Bassham cycle. Notably, protozoa such as *Cryptosporidium parvum*, *Toxoplasma gondii*, and *Giardia intestinalis* rely on various amino acids for energy and protein synthesis (Bouzid et al., 2013, Krishnan and Soldati-Favre, 2021). Polyamine pathways (spermidine biosynthesis) are important compounds for the growth and replication of pathogenic protozoa such as

Leishmania donovani and *Trypanosoma cruzi* (Verdaguer et al., 2019). Additionally, essential pathways for eukaryotes such as heme B biosynthesis, glycolysis and chitin were also found. While heme B biosynthesis pathways are actively involved in the degradation of organic matter, some waterborne protozoan parasites rely on heme for their survival and growth (Verdaguer et al., 2019). Pathogenic protozoa such as *Entamoeba histolytica* and *Trichomonas vaginalis* use glycolysis for their energy metabolism (Verdaguer et al., 2019) and were observed in all WWTP in this study. Chitin is also a major component of the cyst walls of the protozoan parasites *A. Castellani* and *E. histolitica*, which utilize chitin for their survival and virulence (Michael, 2016, Verdaguer et al., 2019). Lastly, pentose phosphate pathway (non-oxidative branch) II, specifically linked to *Entamoeba hystolitica*, was found in all WWTP. This suggests that the protozoan pathogens may show metabolizing and growing functional potential within the WWTPs. This could lead to an increased risk of virulence and transmission to humans and animals if the treated wastewater is not properly disinfected before being released into the environment. Accordingly, this study emphasizes the need to regularly monitor WWTPs for the presence of pathogenic protozoa and to take measures to ensure that treated wastewater can be discharged without risk.

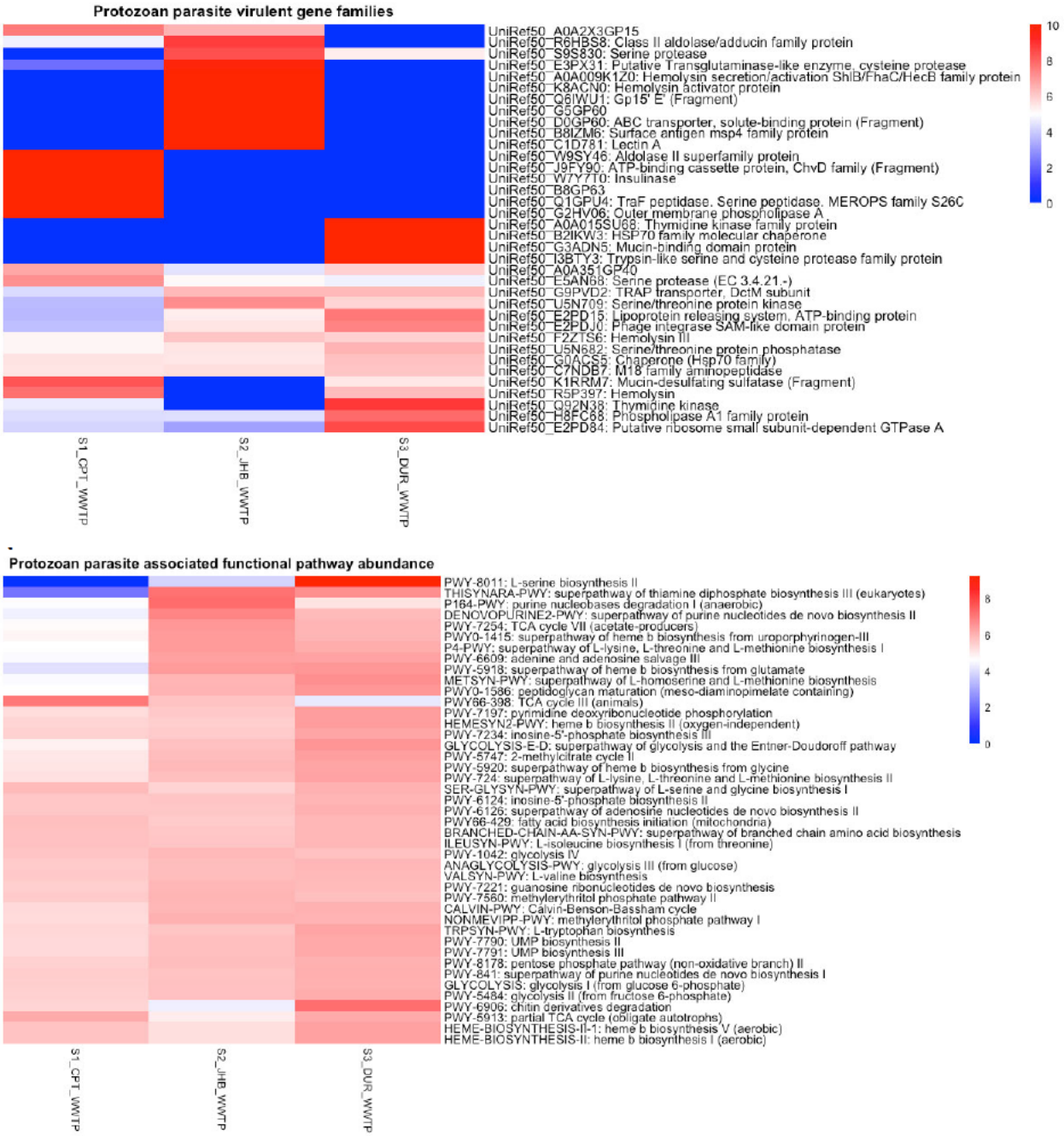


Figure 15 Heatmaps showing (a) Protozoan parasite virulent gene families and (b) Functional pathways abundance of the influent (untreated) metagenomes in each city.

4.4 Conclusions

Using 18S rRNA gene amplicon sequencing and shotgun metagenomic profiling, this study explored the genetic diversity of human and non-human protozoan parasites in South African wastewater treatment plants (WWTPs). There was a significant difference in the microbial diversity structure depending on the type of wastewater (treated or untreated) as well as the treatment process used at each geographical location. In addition, all WWTPs shared a core community. Several protozoa of public health importance were found in the untreated wastewater samples, including *Cryptosporidium* spp., *Entamoeba histolitica*, *Blastocystis hominis*, *Naegleria gruberi, Toxoplasma gondii*, *Cyclospora cayetanensis*, and *Giardia intestinalis*. Thus, these findings contribute to the understanding of the incidence of recognized and emerging protozoan pathogenic species with specific relevance to South African environments. Although this study provides an overview of possible protozoan pathogens in the region, further research is still necessary to better understand their prevalence. For a more comprehensive understanding of the protozoan species in wastewater samples and the impact of different factors on their prevalence, additional longitudinal research based on next generation approaches should be conducted, including a larger sample size within an extended geographical scope. This approach could aid in the development of intervention approaches to address specific protozoans of public health importance and reduce the spread of waterborne diseases in countries with similar public health challenges.

5.0 DEVELOPMENT OF A RAPID, QUANTITATIVE LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) TECHNIQUE FOR THE DETECTION AND SEMI-QUANTIFICATION OF HUMAN PATHOGENIC PROTOZOAN PARASITES-*CRYPTOSPORIDIUM*

This chapter, objective 2 and part of objective 4 of this thesis **has also been published with the following details: "Mthethwa, N. P**., Amoah, I. D., Reddy, P., Bux, F., & Kumari, S. (2022). Fluorescence and colorimetric LAMP-based real-time detection of human pathogenic Cryptosporidium spp. from environmental samples. *Acta Tropica*, *235*, 106606. https://doi.org/10.1016/j.actatropica.2022.106606." Full paper is attached as supplementary information (Appendix 5).

5.1 Introduction

Infections caused by *Cryptosporidium* spp. are a serious public health issue that can be transmitted by contaminated food, water, and wastewater (Hassan et al., 2020). The parasites have been linked to several large outbreaks of water-borne disease, including the largest known outbreak in 1993, which affected more than 400 000 people (WHO, 2019). High rates of *Cryptosporidium* spp. infections occur in developing countries, where sanitation is inadequate and safe drinking water is scarce (Kurenzvi et al., 2020). Reports indicate that 75% and 88% of global *Cryptosporidium* spp. infections and deaths occur in Africa, with Nigeria and the Democratic Republic of Congo (DRC) contributing the greatest number of cases (Wanyiri et al., 2014, Kombo Mpindou et al., 2021). The resistance of *Cryptosporidium* spp. to commonly used water disinfection methods such as chlorination, exacerbates the public health concern (Omarova et al., 2018). Their infective oocysts can survive in harsh environmental conditions for months, and the dose required to infect humans (1-10 oocysts) is very low (WHO, 2017). Thus, water treatments are governed by a set of published guidelines issued by environmental protection agencies, such as the United States Environmental Protection Agency (EPA), the World Health Organization (WHO), and provincial regulators, in order to prevent disease-causing organisms from entering water supplies at levels that are safe for the environment and public health (Feng et al., 2021, WHO, 2019).

In order to monitor the reduction of potential infections or oocyst concentrations to health standards, an efficient and rapid method for the detection and quantification of *Cryptosporidium* spp. oocysts is essential. Nevertheless, due to their low numbers and the complexity of the sample matrix, their detection in environmental samples such as wastewater can be challenging (Bilung et al., 2017). The current diagnosis of *Cryptosporidium* spp. relies on microscopic, immunological, and advanced molecular methods for quantitative and qualitative testing (Adeyemo et al., 2018). However, these methods have certain limitations. For example, the microscope technique is time-consuming, requiring extensive experience and highly skilled personnel, and does not provide specific identification information (Hassan et al., 2020, Ryan et al., 2017). While immunological techniques such as immunochromatography and enzyme-linked immunosorbent assay (ELISA) are rapid, they possess limited specificity and sensitivity (Ryan et al., 2017). Although molecular techniques such as polymerase chain reaction (PCR), real-time PCR (RT-PCR), and droplet digital PCR (ddPCR) overcome most of the constraints mentioned above, they are expensive, and they are not readily available, especially in low-income countries where disease burden is high (Keikha, 2018, Silva et al., 2020, Ryan et al., 2017). Therefore, methods with increased speed, sensitivity, specificity, reproducibility, automation, and low cost are required to facilitate the detection and monitoring of infections. A promising candidate is the isothermal amplification technique since it allows detection of a nucleic acid target sequence without the use of thermal cyclers and high temperatures required to denature double-stranded DNA (Liu et al., 2019, Rahimi Esboei et al., 2022).

These methods use a strand displacement polymerase to enable primer binding and initiation of amplification reaction at a single constant temperature (25-65°C) (Wang et al., 2015). Examples of isothermal amplification methods are: loop mediated isothermal amplification (LAMP), whole genome amplification (WGA), strand displacement (SDA), helicase dependant amplification (HAD), recombinase polymerase amplification (RPA) and nucleic acid sequences based amplification (NASBA) (Ahmad and Hashsham, 2012). All these methods have gained significant attention in molecular diagnosis, and some have been used to provide point-of-care diagnosis in resource-limited settings (Wilisiani et al., 2019). The LAMP is a promising candidate for the detection of protozoan parasites, owing to several good features discussed below. The LAMP method was developed by Notomi et al., (2000) and is both simple and fast: under ideal conditions, the reaction can be completed in less than an hour (Notomi et

al., 2000, Lu et al., 2020, Keikha, 2018, Fallahi et al., 2020). This method is considered sensitive, because it can detect DNA at very low concentrations and it is highly specific: six distinct regions/sequences on the target gene are recognized by four specific primers (Liu et al., 2019, Tavares et al., 2011). Moreover, one of the key advantages of LAMP is its ability to process complex samples such as water or wastewater with minimal effort as the *Bst* polymerase is reported to be highly resistant to inhibitor molecules (Keikha, 2018). Additionally, it allows the use of regular water baths to regulate the temperature of amplification reactions without the need for costly thermal cyclers (Ahmad and Hashsham, 2012, Silva et al., 2020). LAMP efficient amplification has been widely applied for rapid diagnosis of different plant pathogens (Bühlmann et al., 2013, Lu et al., 2015), pathogenic parasites (Fallahi et al., 2015, Fallahi et al., 2018, Gallas-Lindemann et al., 2013), malaria causing pathogen (Picot et al., 2020), *E.coli* (Liu et al., 2019), *Mycobacterium* spp. (Yashiki et al., 2019) and recently SARS-CoV-2 (Lu et al., 2020, Zhang et al., 2020, Park et al., 2020, Amoah et al., 2021). A prior study demonstrated the utilization of the traditional LAMP approach, using magnesium pyrophosphate and UV light for results analysis, in detecting waterborne protozoan parasites (*Cryptosporidium* spp*., Giardia* spp*., and Toxoplasma* spp.) in environmental samples (Sotiriadou, 2012).

Using a combination of calorimetric and real-time fluorescence methods, this study sought to develop and evaluate rapid, sensitive, and specific LAMP methods for the detection of *Cryptosporidium* spp. from environmental matrices. The first assay was based on the colorimetric LAMP method, which relies on instant visual detection of amplification results, eliminating the need for further gel electrophoresis and the use of hazardous ethidium bromide. It has the potential to reduce workflow time and costs, enabling it to be used at the point-of-care or on the field. The second method used a real-time LAMP assay that enabled the monitoring of results in real time. With this method, a fluorescent dye (SYBR green) was added to the reaction, which allowed results to be monitored in a qPCR thermocycler machine or on a connected fluorometer screen. All tests were conducted alongside established Droplet digital PCR as a reference method, for method evaluation and validation.

5.2 Methodology

5.2.1 Reference Oocysts and DNA

The LAMP assays were optimized by using non-viable *Cryptosporidium* spp. (*Cryptosporidium parvum* and *Cryptosporidium muris)* oocysts obtained from Waterborne Inc. (New Orleans, USA). A stock of $1x10^6$ oocysts of *Cryptosporidium* spp. were prepared and certified to be 99% pure by the supplier. DNA was extracted from the reference oocysts according to a modified Phenol-chloroform extraction method chapter 3(III). LAMP type-specific primer sets that target the *SAM* gene (GenBank accession number: AY161084) and *GP60* gene (GenBank accession number: AB237136) found in *C. parvum, Cryptosporidium hominis* and *Cryptosporidium meleagridis* were used for optimization of both LAMP (table 9) and ddPCR method (Table 10). Melting temperature value, primer length, primer dimers, and specificity of the primers were first assessed using the Basic Local Alignment Search Tool (BLAST) for sequence comparison from the National Centre for Biotechnology Information (NCBI). A synthetic DNA of *C. parvum* purchased from Microbiologics Inc. ($1.1x10^7$ copies/1.5ml) was also used as a positive control for all reactions.

5.2.2 Optimisation of LAMP methods for detection of *Cryptosporidium* spp.

LAMP methods were first developed using the colorimetric LAMP technique (cLAMP), which allows for visual interpretation of the results through the changes in colour. The second was the fluorescent LAMP (fLAMP), which utilizes a DNA double strand intercalating fluorescent dye for the detection and semi-quantification of positive amplifications. The following sections provide more details about these two methods.

5.2.3 Colorimetric/visual LAMP method (phenol red indicator)

The methods were carried out at 60 °C, and 65 °C to determine the optimal reaction temperature of the primers. These temperatures were chosen based on the melting temperature of the primer and previous literature recommendations. On the basis of specific binding, brightness, and sharpness of the ladder-like pattern of the LAMP products in gel electrophoresis, the concentrations of different LAMP reagents such as mixture of dNTPs, MgSO4, and betaine were optimized. The assays were also run for various time intervals ranging from 15 to 60 minutes, with results taken every 5 minutes, to determine the best time for amplification.

Following optimization, the reaction was carried out at 65 °C using the heating block and the mixture consisted of the following as per individual method:

The cLAMP was performed in a 25µl total reaction volume which consisted of 12.5µl WarmStart cLAMP 2X Master Mix (WarmStart isothermal amplification buffer, Bst 2.0 Warm- Start DNA Polymerase) (New England Biolab, USA), Phenol red indicator (for visual colour), LAMP Primer Mix (10X) (2µM F3 primer, 2Mm B3 primer,16µM FIP, 16µM BIP, 8 µM LoopF and 8µM LoopB), 2µl template DNA, and 8µl of nuclease-free water was added. The phenol red indicator used for analysis allows for a clear visual detection of amplification. It relies on the production of protons and a decrease in pH that occurs due to extensive DNA polymerase activity in a LAMP reaction, resulting in a colour change from red/pink to yellow (Tanner et al., 2015). A visual inspection of the results was immediately conducted following removal of the tubes from the heating block. The preliminary results were confirmed by gel electrophoresis. In order to determine the minimum time required for a positive reaction to occur, results were taken every five minutes during the optimization phase.

5.2.4 Fluorescent Real-time LAMP (fluorescent dye)

The fluorescent Real-Time LAMP (fLAMP) reactions were performed in a QuantStudio™ 3 Real-Time PCR System (Thermofisher, USA). The 25µl fLAMP reaction mixture contained 12.5µl of WarmStart amplification kit which contained the same reaction mix components and conditions as described in the colorimetric/visual LAMP method above. The only difference was the replacement of the phenol red indicator with 50X SYBR green (fluorescent dye), which binds to dsDNA for real-time detection of LAMP. Fluorescence intensity was measured every minute, and the time it took for a sample (reaction) to cross the 400 000 threshold was recorded to estimate the time for positive amplification. The melting analysis was then performed, enabling the generation of derivative melting curves. The LAMP tests were performed in triplicate and negative (no template) controls were included.

5.2.5 Specificity and sensitivity of both cLAMP and real time fLAMP

The specificity of optimised cLAMP and fLAMP *C. parvum* methods was evaluated using closely related species of the same genus (*C. muris*), other waterborne protozoan parasites (*Giardia intestinalis and Giardia muris*), and a group of bacteria commonly found in the

environment, such as soil and water (*Escherichia coli,* and *Aeromonas* spp.). The limit of detection was determined by serial dilutions of *C. parvum* DNA template up to 0.004ng/µl, starting at 20ng=1132copies/µl for both cLAMP and fLAMP. For fLAMP, after setting the threshold line to 400 000, the assay was then tested for the lowest concentration of template DNA that could be detected without false positive amplification.

5.2.6 Confirmation of cLAMP and fLAMP methods

After amplification and electrophoresis on agarose gel, LAMP products were confirmed by cutting the lowest band from the 2 % agarose gel of LAMP reaction and purified using QIAquick Gel Extraction Kit (Quiagen, Hilden, Germany). The pure amplicons were then cloned and sequenced by a commercial lab (Inqaba Biotec, SA) (Bakheit et al., 2008, Bilgiç et al., 2017, Choi et al., 2018). The Sequencing Analysis 5.2.0 program (Applied Biosystems, CA, USA) was used to convert the raw sequencing data to chromatograms. The Bioedit (version 7.2.5.0) and NCBI database were used for further analysis and identification.

5.2.7 Droplet digital PCR (ddPCR) method

The ddPCR reactions were performed on all samples using the QX200 Droplet Digital PCR system (Bio-Rad). Each assay mix was prepared in a final volume of 20µl, containing 2× evagreen ddPCR Supermix (10µl), 0.5µm forward (1µl) and reverse primers (1µl) (Table 10) and template DNA (2µl). Generation of droplets was performed by the QX200 Droplet Generator (Bio-Rad). PCR amplification was carried out on a T100 thermal cycler (Bio-Rad), using the conditions: 10 min at 95°C, followed by 50 cycles of denaturation at 95°C for 15 s, annealing at 56°C-60°C for 1 minute, and extension at 72°C for 1 minute, and a final step of extension at 72°C for 10 minutes. After amplification in thermal cycler the samples were analysed using the QX200 Droplet Reader and QuantaSoft software (Bio-Rad). Based on the No Template Control (NTC) and positive control, a threshold line was drawn.

5.2.8 Detection of *Cryptosporidium parvum* and *Cryptosporidium* spp. from environmental samples

We further evaluated the efficacy of the optimized fLAMP method using environmental samples for the identification of *C. parvum* and *Cryptosporidium* spp. using the *GP60* and *SAM* genes. The fLAMP method was chosen because of its sensitivity, high speed, and real-time

analysis of results. A total of 60 samples were collected from the province of KwaZulu-Natal in South Africa for this study, comprising untreated wastewater (15), treated wastewater (15), surface water (15), and sludge (15) (Figure S4 Appendix I). Sample collection, processing, and DNA extraction were done in accordance with our previously optimized protocol (Mthethwa et al., 2022). In brief, 2L of samples from different matrices (untreated and treated wastewaters, sludges, and surface waters) were collected at different time interval of 15/ 120 minutes using the composite sampling technique. This was done in order to obtain a complete representation of the microbial community in the studied source water. The samples were kept on ice box in sterile sampling bottles before being transported to the laboratory for analysis. In the laboratory, the samples were concentrated by centrifugation (Hermle Labortechnik GmbH) at 3500 rpm (max 4 x 340 g) for 10 minutes plus filtration of supernatant for the maximum recovery as optimised in chapter 3 (III). The supernatant was filtered using 0.1µm pore size cellulose acetate filter paper. The total DNA was then extracted from both the pellets and the residual material on the filter paper mixed using the phenol-chloroform method (Djurhuus et al., 2017, Awolusi, 2016). The extracted DNA was subsequently amplified using fLAMP as described above. The ddPCR method described above was applied to the same samples as a reference method. The primers listed in Tables 9 and 10 were used.

Table 9: Primer sequences used in LAMP amplification experiments.

Cryptosporidium parvum GP60* gene	
Primer type	**Sequence 5'-3'**
F3,	TCG CAC CAG CAA ATA AGG C
B3	GCC GCA TTC TTC TIT TGG AG
FIP	ACC CTG GCT ACC AGA AGC TTC AGA ACT GGA GAC GCA GAA
BIP	GGC CAA ACT AGT GCT GCT TCC CGT TTC GGT AGT TGC GCC TT
LF (loop primer) LF1	GTACCACTAGAATCTTGACTGCC
LB (loop primer) LB1	AACCCACTACTCCAGCTCAAAGT
Cryptosporidium* spp. *SAM gene*	
F3,	ATTTGATRGACAAAGAAACTAG
B3	CGATTGACTTTGCAACAAG

FIP	TTGCGCCCTGTTAATCCAGCATTAATTAATCCATCTGGCAGRTTT
BIP	TTGTAGATACATACGGAGGATGGGTCTACTTTAGTTGCATCTTTCC
LF (loop primer) LF1	CTGCTGGCCCMCCAATTG
LB (loop primer) LB1	CATGGRGGTGGTGCATTTAG

* **(Sotiriadou, 2012)**

Table 10: Primer sequences used in ddPCR amplification experiments.

Primers	Forward 5'-3'	Reverse 5'-3'	Size (bp)	Reference
Cryptosporidium spp. (*SAM* gene)	GTTTTCATTAATCAAGA ACGAAAGTTAGG	GAGTAAGGAACAACC TCCAATCTCTAG	100	(Burnet et al., 2013)
C. parvum (*GP60*)	GCCGTTCCACTCAGAGGAAC	CCACATTACAAATGAAGT GCCGC	150	(Wu et al., 2000)

5.3 Results

5.3.1 Optimization of cLAMP method for the detection of *Cryptosporidium* spp.

The cLAMP method was successfully carried out at 65°C with both *Cryptosporidium* spp. and *C. parvum* using species-specific primers. Positive reaction results were characterized by a change from red/pink to yellow, whereas negative controls remained pink (Table 11). Moreover, agarose gel electrophoresis (Table 11) indicated a ladderlike pattern that indicated stem loops with inverted repeats, confirming the positive LAMP product. As indicated by a change in colour, the amplification of DNA template samples began within 15 minutes of incubation, and the efficiency declined after 45 minutes with one false positive NTC. Furthermore, a non-specific amplification or false positive was detected in one of the three NTC reaction tubes after an increase in reaction time beyond 45 minutes (table 11). Consequently, the incubation time and input DNA concentration were optimized in order to eliminate false positives results. Table 4 shows that all DNA template positive reaction samples displayed 100% amplification and no amplification in NTC after 25 to 30 minutes of incubation when the template concentration was 20 ng/µl (1132 copies of *C. parvum*). Thus, in this study, 30 minutes was chosen as the maximum reaction time without any false positives/non-specific binding, that is, the cut-off time.

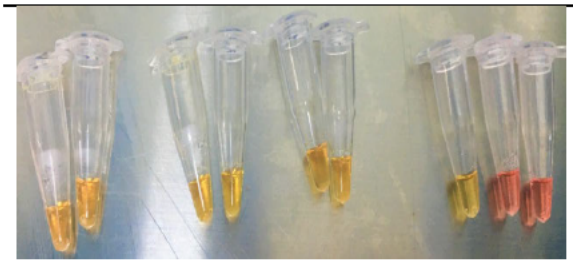
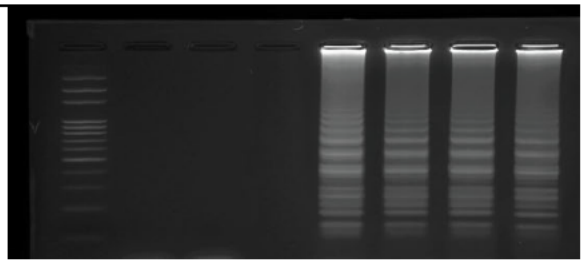
5.3.2 Detection limits and specificity of the cLAMP method

The cLAMP method was further evaluated for its limit of detection on the basis of *C. parvum* copy number and input concentration of DNA template for 30 minutes incubation at 65°C. A positive result was considered to be obtained from at least two of the three replicates examined (75%) for each sample. In Table 12, it is shown that the *C. parvum* LOD is 1.1 copies per 25µl reaction (0.02ng/µl), which is the lowest input amount that was detected with 75% efficiency. The results also revealed that 9 copies/25µl reaction (0.16ng/µl) was the lowest input DNA template concentration at which 100% efficiency was obtained. Based on the results presented in Table 12, template DNA at 0.6 copies/25µl reaction and lower failed to amplify.

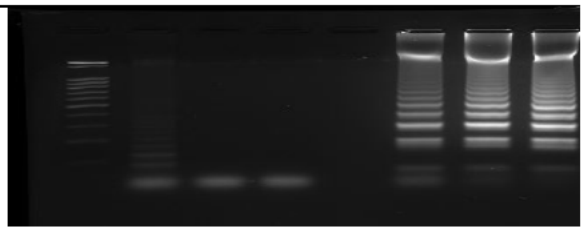
Furthermore, the colorimetric LAMP method for *C. parvum* was noted to be highly specific, with no cross reactivity observed for other species lacking the GP60 gene. DNA from closely related species and other pathogens such as *C. muris*, *G. lamblia*, *G. muris*, *E. coli*, and Aeromonas spp. were not detected by the assay (Table S4 Appendix I).

Table 11: Results of colorimetric methods after amplification and electrophoresis on 2% agarose gel.

Detection of *Cryptosporidium* spp. using the *SAM* gene

Colorimetric LAMP products	Gel electrophoresis of colorimetric LAMP products
P P P P P P N N	L N N P P P P
	

Detection of *C. parvum* using the *GP60* gene

N N N N P P P	N N N N P P P
	

*** N= No Template Control (NTC) and P= DNA template samples (positive amplification), L=DNA ladder**

Table 12: Limit of detection based on input *Cryptosporidium parvum* DNA copy number for 30 minutes incubation at 65°C.

DNA copies /25µl reaction	1132	226.4	45.28	**9.05**	4.5	2.3	**1.132**	0.6	0.2
Positive reaction	100%	100%	100%	**100%**	75%	75%	**75%**	0%	0%

5.3.3 Optimization of the fLAMP method and the limit of detection and specificity of the fLAMP method

Using the real-time fLAMP results shown in figure S3 Appendix I, it was demonstrated that all samples tested had been successfully amplified in 28 minutes at 65°C for a particular sample. The amplification curves appeared and crossed the threshold line of 400 000 within 26 minutes and reached the plateau phase within 28 minutes of the limits of detection for fLAMP were further tested. Table 13 shows that the lowest concentration of *C. parvum*/25µl reaction that crossed the threshold within 28 minutes was 1.10 target copies (0.0175ng/µl). At a concentration of 0.035ng/l, the detection efficiency dropped below 100%, with some replicate samples crossing the threshold after 28 minutes. In *C. parvum*, the time required for positive amplification was dependent upon the number of target copies (table 13); the higher the target copies, the shorter the time required for positive amplification specificity tests (figure 16) demonstrating that no amplification curves were observed for other *Cryptosporidium* species or pathogens lacking the *GP60* gene. Figure 16b illustrates the melting curve plot which indicates the presence of only one product and the high specificity of the method (figure 16b).

Table 13: Limit of detection for the real time fLAMP (based on *C. parvum* copy number/input concentration of DNA template) incubation at 65°C.

DNA copies /25µlreaction	1132	566	283	141	70.75	35	17	8	4	2	1.10	0.55
DNA concentration in ng/µl	20	10	5	2.5	1.25	0.625	0.31	0.156	0.07	0.035	0.0175	0.008
Positive reactions	100%	100%	100%	100%	100%	100%	100%	100%	100%	75%	75%	0%
Obtained LAMP CT values	14,97 (±0)	16,335(±0 .01)	16,804(±0 .09)	17,65(±0. 3)	18,04(±0.05)	18,7 (±0)	19,35(±0.3)	21,97(±1.07)	22,1(±2.11)	26,67(±0.31)	27,833 (±3.35)	32,9 (±3.31)

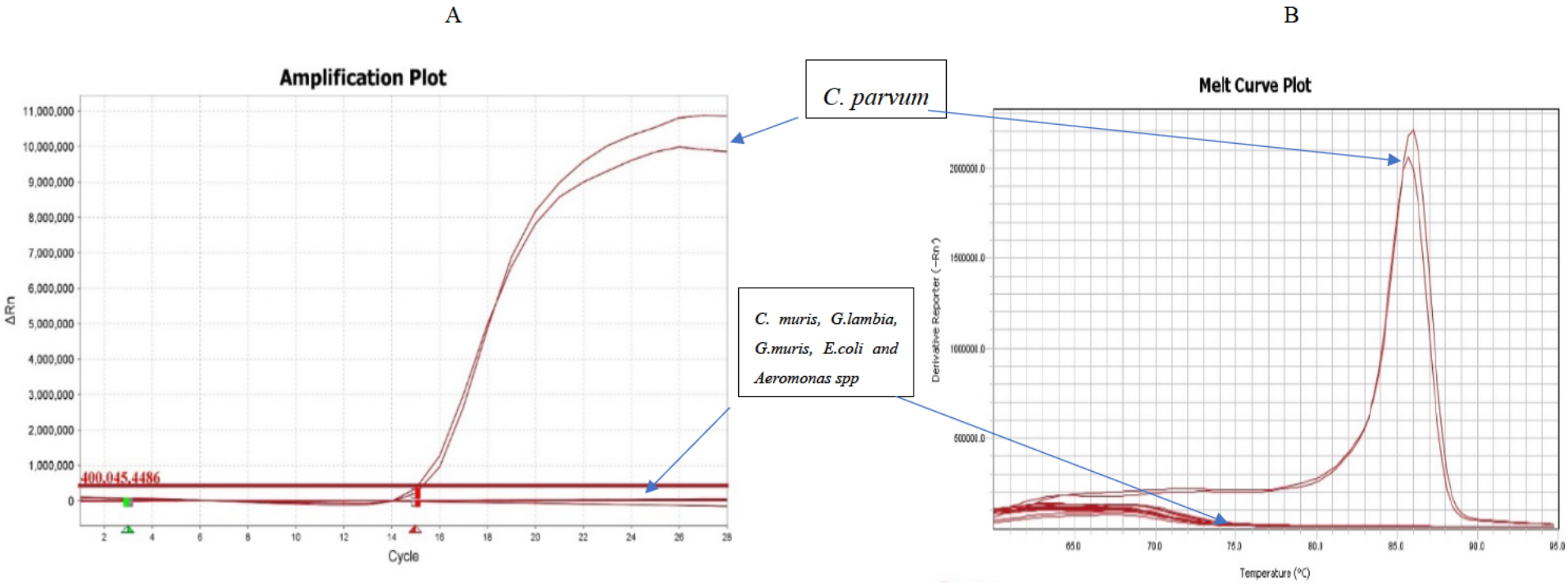


Figure 16. Results demonstrating the specificity of the GP60 gene against pathogens found in the soil/water environment using *C. parvum*. (a) showing amplification curves and (b) showing melting curves.

5.3.4 Evaluation and confirmation of the optimised LAMP method for the detection of *C. parvum* and *Cryptosporidium* spp. in wastewater samples

In further tests, the optimized method was applied to different environmental samples and was found to be successful in detecting *Cryptosporidium* spp. up to 50% to 60% of all samples tested with a concentration of 1ng/ul template DNA (Table 14). The high prevalence of *Cryptosporidium* spp. was observed in untreated wastewater samples with 67-100%. However, surface water had the lowest prevalence of 13% (2/15). Results also indicated that when fLAMP was applied in different water matrices, it achieved a total detection rate of 85% for primer 1 and 52 % for primer 2. While the reference method, ddPCR achieved a total detection rate of 98% for primer 1 and 58% for primer 2 (table 14). Correspondence in detection was not statistically significant, with a p value of 0.56 between ddPCR and fLAMP method. Droplet digital PCR successfully detected *Cryptosporidium* spp. in all wastewater sample types with slightly better sensitivity than fLAMP (Table 14) ranging from 03/15 to 15/15 (number of positive amplification/tested samples). Surface water samples also showed the lowest prevalence for ddPCR when primer 2 was used, which was consistent with the results obtained in the fLAMP analysis noted earlier.

Table 14: Detection of *Cryptosporidium* spp. in environmental samples using both fLAMP and ddPCR.

	Number of detected positive reactions /total number of tested samples			
	Primer 1(*Cryptosporidium 18s rRNA*)		Primer 2 (*C. parvum)*	
Sample type	fLAMP	ddPCR	fLAMP	ddPCR
Untreated wastewater	15/15	15/15	10/15	13/15
Treated wastewater	13/15	15/15	09/15	10/15
Sludge	13/15	14/15	10/15	09/15
Surface water	10/15	15/15	02/15	03/15
Total detection rate	85%	98%	52%	58%

5.4 Discussion

Cryptosporidiosis is one of the most important emerging and commonly recognised waterborne infections, which affect humans worldwide. Monitoring of wastewater for reuse or discharge into surface water bodies may contribute significantly to reducing *Cryptosporidium* spp. related diarrheal infections. As a result, the development of accurate, sensitive, and cost-effective methods could contribute to achieving this goal.

The cLAMP and fLAMP technologies have been utilized successfully for the detection and identification of viral (Quoc et al., 2018, Amoah et al., 2021) and microbial infections (Huang et al., 2018, Dea-Ayuela et al., 2018). In this study, both the cLAMP and real time fLAMP assays proved to be effective in detecting *Cryptosporidium* spp. and yielded results in a shorter period of time compared to PCR. The extra two forward and backward loop primers used in the assays are known as amplification reaction accelerators, which reduces the reaction time required for LAMP (Nzelu et al., 2019). However, when the LAMP reactions were incubated for more than 45 minutes during optimisation stage, nonspecific products or false positives were observed in one of the no templates controls (NTC) for both cLAMP and fLAMP products. In this context, it is possible that when the reaction is incubated for too long or the conditions are not fully optimized, an uneven reagent ratio, primer dimers, or nonspecific amplifications occur, resulting in false positives (Aoki et al., 2021). Similar results were obtained in previous studies when the Lateral flow Loop-Mediated Isothermal Amplification test reaction was left for 70 minutes resulting in the formation of primer dimers as well as the binding of the intercalating dye (SYBR green 1) to both products and primer dimers (Mamba et al., 2018).

The fLAMP method was found to be slightly faster than the colorimetric method in this study. This may be due to the intercalating fluorescent dye used in fLAMP, which only binds to double-strand DNA and allows the results to be seen in real time (Quyen et al., 2019). The colorimetric assay on the other hand relies on time for the phenol red indicator to change colour for positive results, which is dependent on the release of enough protons for the pH to change. Similar observations were previously reported, where real-time LAMP positive amplification of *Xanthomonas gardneri* DNA was first observed after 15 minutes, but amplification of the same DNA was visible in the colorimetric assay after 30 minutes (Stehlíková et al., 2020). A

recent study by Amoah et al., (2021) has reported a similar amplification time of 35 minutes for the Reverse Transcription cLAMP method for detection of SARS-CoV-2 in wastewater (Amoah et al., 2021). Another study used LAMP with a cut-off time of 40 minute to identify SARS-CoV-2 virus in clinical samples (Lu et al., 2020), further demonstrating the rapidity of the LAMP assay in detecting pathogens from environmental samples.

The cLAMP and fLAMP methods were highly sensitive and specific for detecting *Cryptosporidium* species with a limit of detection of 1.1 copies/25µl reaction (0.02ng/µl) with 75% efficiency. This indicates that the developed assays can be applied to environmental samples that contain pathogens, even at low concentrations such as diluted samples. However, for 100% detection efficiency, the concentration of template DNA must be at a high level, or a confirmation method, such as DNA sequencing or real-time PCR, must be used. We also observed a decrease in cLAMP efficiency as the number of copies was reduced, which is in agreement with previous findings that observed fading changes in colour when fewer DNA/RNA template copies were used (Lu et al., 2020). It should be noted that, as demonstrated in this study, all reactions should be performed in triplicate or more for accuracy. Previous research has shown that the LAMP is sensitive and capable of starting amplification with a small amount of DNA (10-100 copies) when used for library preparation prior to sequencing (Imai et al., 2017). The LOD observed in this study for Mycobacterium spp. is consistent with previous research that indicated LOD for *Mycobacterium avium* LAMP tests to be 0.4 pg. of genomic DNA per reaction, which is 100-1000 times lower than the LOD for PCR testing (Yashiki et al., 2019). Furthermore, Daskou et al. (2019) reported that cLAMP had a limit of detection of 10 copies of HPV16, whereas PCR had a limit of detection of 100 copies, indicating higher and sometimes similar sensitivity than some conventional molecular methods.

The optimized LAMP methods (fLAMP and cLAMP) in this study also demonstrated high specificity for *Cryptosporidium* specific primers, with no positive reactions for other pathogens (*E. coli and Aeromonas* spp.) commonly found in soil and water, indicating that the method is reliable and accurate for the detection of protozoa parasites from environmental samples. The specificity of the methods relies on the use of four primers, which recognizes six distinct sequences of target DNA. The fLAMP assay specificity was also assessed using melting curve analysis of amplified products (figure 20), and only peaks of the same melting temperature

observed for *C. parvum*. This provided an advantage over colorimetric assay as it ensures the assay's accuracy and reliability by allowing for easy discrimination of non-specificity through the observation of melting curves. Furthermore, the amplicons for both assays were subjected to DNA sequencing and analysis, which confirmed the specificity by indicating the presence of only the genes of interest.

The detection of *C. parvum* and *Cryptosporidium* spp. in different environmental matrices using fLAMP demonstrated great potential applicability in complex environmental samples, with only a slight difference of 13% (*SAM* gene) and 6% (*GP60* gene) detection rate when compared to ddPCR. The obtained higher detection percentage in ddPCR could be attributed to improved LOD and reduced inhibitor impact because of partitioning micro-reactions via droplet formation (Dingle et al., 2013). Previous study also demonstrated higher prevalence of SARS-CoV-2 in wastewater with ddPCR technique compared to RT-LAMP (Amoah et al., 2021). Influent wastewater samples tested for *C. parvum* and other *Cryptosporidium* spp. using both fLAMP and ddPCR showed the greatest prevalence of all screened samples. This may be due to their abundance in influent wastewater samples as a result of the high disease burden in the community around them. In contrast, both fLAMP and ddPCR revealed a very low prevalence of *C. parvum* in surface water compared to primer one (*SAM* gene) which targets a wide range of *Cryptosporidium* spp. This could also be attributed to lower copy numbers of *C. parvum* in extracted DNA resulted from less concentrated surface water volume and lower biomass. Detection of *Cryptosporidium* spp. in surface water has been reported to be challenged by very low oocysts concentrations which requires large volumes of water collection (> 10L) for concentration step (Hassan et al., 2020).

5.4.1 The significance of the findings for public health protection

The two LAMP methods optimised in this study demonstrates a great potential as an environmental and laboratory surveillance tool with the following reasons. First, both fluorescent and colorimetric LAMP protocol used proved to be very sensitive in detecting as few as 1.10 target copies of *C. parvum* equivalent to one oocyst, which is well within the water quality guidelines discussed above as well as published acceptable range of (10-100 oocyst) for infection monitoring and prevention (Dixon et al., 2011, Zacharia et al., 2019). The use of highly sensitive detection tools for pathogens in water quality monitoring is one of the key

fundamental principles for decision-making regarding water distribution system infrastructure, the best water treatment, and prevention of waterborne outbreaks (Ramírez-Castillo et al., 2015). Second, the developed LAMP assays can be performed at a single constant temperature of 65°C for less than 60 minutes. According to Caliendo et al., (2013) new tests should be simple to use and provide a quick result (ideally within an hour) to have a positive impact on infection care, pathogen detection, pathogen discovery, and disease surveillance (Caliendo et al., 2013).

The optimized protocol in this study provides an even faster time as well as other excellent features that are critical in public health protection. This includes the easier visual interpretation of results with fast turnaround time due to elimination of additional time needed for gel electrophoresis or the use of carcinogenic ethidium bromide or UV exposure. Furthermore, when using either colorimetric or fluorescent LAMP, the use of constant temperature (65°C) for amplification eliminates the need for expensive thermal cycling equipment; the instrument required is a heating block, water bath or fluorometer for fLAMP, both of which are portable and commonly available in most laboratories. As a result, the cost is reduced even further, allowing it to be used in resource-limited countries and as a portable field device. Therefore, the adoption of these assays could play significant roles in public health protection.

5.5 Conclusions and recommendations

Despite ongoing efforts to ensure water safety, outbreaks of waterborne diseases continue to occur. The use of test methods that adhere to the World Health Organization's (WHO) guidelines for developing countries, specifically ASSURED (Affordable, Specific, User-Friendly, Rapid, Robust, Equipment-Free, and Deliverable to End Users), are crucial for reducing disease burdens and protecting public health. This study was able to successfully develop and evaluate a highly specific, sensitive, and rapid LAMP assay for the detection of human pathogenic *Cryptosporidium* species. The method has been validated by its application to environmental samples and comparison with the more advanced ddPCR. With the following findings:

The methods optimised can be carried out in a heating block, water bath, or thermal cycler at constant temperature of 65°C for 30 minutes for colorimetric LAMP and 28 minutes for the

real time fluorescent LAMP. The results can easily be interpreted in real time by colour change from red/pink to yellow, or by use of fluorescent dye. A limit of detection (LOD) of 1.1 copies per 25µl reaction volume input template DNA was obtained.

Future research should focus on the optimization and development of direct LAMP, which eliminates the need for a separate DNA extraction step. This will further reduce the time required to achieve the desired results as well as the cost involved. As a precautionary measure, we recommend that further research be conducted to improve the LAMP quantification method. The overall outcome of this study contributes to and addresses Sustainable Development Goals (SDGs) 3 and 6 by providing *Cryptosporidium* LAMP methods as a tool for promoting better health through effective water management, water-related disease surveillance, and outbreak management related to drinking water supply.

6.0 COMPARATIVE EVALUATION AND TECHNO-ECONOMIC FEASIBILITY OF FLAMP ASSAY AND PCR-BASED ASSAYS (DDPCR AND QPCR) FOR DETECTION OF *CRYPTOSPORIDIUM* SPP. IN ENVIRONMENTAL SAMPLES

6.1 Introduction

The use of molecular methods has become increasingly popular as precise and accurate means for regular monitoring of microorganisms and assisting in the surveillance of public health (Paruch, 2022). Among these methods, Polymerase chain reaction (PCR) based strategies have been widely used, from conventional PCR to real-time Quantitative PCR, and recently droplet digital PCR has gained a lot of interest due to its advantages such as sensitivity (Sresung et al., 2023).

However, the PCR-based methods have limitations, such as the high cost of instruments, reagents, and analysis software (Parihar et al., 2020, Augustine et al., 2020). Another disadvantage is the requirement for highly trained and qualified personnel to perform assay design, sample handling, and data analysis (Luka et al., 2022). As a result, these methods are not widely adopted for routine environmental monitoring or during outbreaks where large numbers of samples need to be analyzed (Yao et al., 2021). They are also not affordable for more end-users, especially those in resource-limited countries (Silva Zatti et al., 2020, Li et al., 2018). To address these challenges, alternative cheaper, faster, and point-of-care testing methods are in demand (Nzelu et al., 2019, Kang et al., 2022).

Loop mediated isothermal amplification (LAMP) is believed to have the potential to overcome some of the challenges associated with PCR-based strategies (Nguyen et al., 2020, Park, 2022). LAMP is a non-cycling, one-step procedure that amplifies the target DNA/RNA sequence at a constant temperature using a strand-displacement polymerase, eliminating the need for a thermal cycler (Park, 2022). It also provides high specificity because it uses more than four primers per reaction, has a high reaction speed, is simple to operate, and lowers the overall testing costs (Ding et al., 2019, Thompson and Lei, 2020). Different types of LAMP assays, including colorimetric, turbidity, and fluorescent-based LAMP, have been developed (Almasi et al., 2013, Garg et al., 2022). Further studies are ongoing to develop LAMP toward point-of-care testing for infectious diseases, as well as to develop LAMP kits in a ready-to-use state,

portable devices, and as an absolute quantitative method (Yang et al., 2018, Park, 2022, Umesha and Manukumar, 2018, Nguyen et al., 2020).

Chapter 5 (V) focused on developing and testing colorimetric and fluorescent LAMP methods for the rapid detection of human pathogenic *Cryptosporidium* spp. in environmental samples. Both LAMP-based methods successfully detected *C. parvum* and the *Cryptosporidium* genus from environmental samples with 100% specificity and no cross-reactivity. The developed protocols were able to detect *C. parvum* and *Cryptosporidium* spp. in 50–85% of environmental samples and showed substantial agreement with the reference method (ddPCR) used. However, to date, there have been limited studies comparing the efficiency and techno-economic viability of fLAMP, ddPCR, and qPCR for the detection of *Cryptosporidium* spp. in environmental samples. Therefore, a comparative evaluation is necessary to determine the potential of fLAMP as a viable alternative to qPCR and ddPCR. Furthermore, conducting an additional techno-economic feasibility assessment in comparison to established molecular methodologies could be beneficial.

Therefore, this chapter presents the comparative evaluation of qPCR, ddPCR and fluorescent LAMP molecular based platforms. In addition, this study investigated the suitability, economic viability, and technical feasibility of each method to determine the technique that is most appropriate for routine microbial assessment and public health surveillance.

6.2 Methodology

6.2.1 Source of DNA: Sample collection, processing, and DNA extraction

Sample description is represented in table 15. The first batch of sample include included: untreated wastewater (n=15), treated wastewater (n=15) and sludge (n=15) from 4 WWTPs, surface water from 4 rivers (n=15), within KwaZulu Natal province South Africa (figure S7 Appendix I). A total 60 environmental samples, of various matrices were collected, which comprised of 15 samples each for the four different matrices. DNA was extracted and subsequently kept in -20ºC for further analysis. Sample collection, processing, and DNA extraction were done in accordance with the optimized protocol in chapter 3(III). Briefly, approximately 2L of influent and effluent wastewater and surface water samples were collected at different time intervals of 15/120 minutes using the composite sampling technique in 5 replicates each. In 5 replicates, wastewater sludge samples (50g) were also collected from sludge drying beds using the composite sampling approach (Amoah et al., 2018). The samples were kept in cooler boxes and transported to the laboratory for analysis. For the influent, effluent and surface water, the samples were thereafter concentrated by centrifugation (Hermle Labortechnik GmbH) at 3500 rpm (max 4 x 340 g) for 10 minutes plus filtration of supernatant for the maximum recovery as optimised in chapter 3.

For sludge sample (oo)cysts were isolated and purified from sludge using the glucose gradient centrifugation method and flotation in a glucose-NaCl solution (Adeyemo et al., 2019) . Briefly, the process involved suspending 40g of sludge in PBS-Tween 80 (0.1%), preparing a flotation fluid with a specific gravity of 1.07 g/mL, carefully adding it to the sample using a borosilicate glass Pasteur pipette, and centrifuging the tubes at 750 rpm for 10 minutes. The resulting supernatant was transferred to a new tube and washed several times with purified water through centrifugation at 3000 rpm for 10 minutes each time. The final volume of purified oocysts suspension was used for DNA extraction following method described in chapter 3.

The second set/batch of 6 untreated wastewater samples (table 15) were collected from four WWTPS in Cape town, Johannesburg and Durban South Africa as described in chapter 4 (IV). *Cryptosporidium parvum* synthetic DNA with known copy numbers, purchased from Microbiologics was utilized as a positive control and as a template DNA for sensitivity tests.

Table 15: Sample description.

Batch	Name of sample	Location / type of WWT sample	Sample size (n)
First	Untreated WWT	Shallcross(n=5), Isipingo(n=5), Amanzimtoti(n=5) & Kingsburgh(n=5) WWTPs in Durban KwaZulu Natal	15
	Treated WWT	Shallcross(n=5), Isipingo(n=5), Amanzimtoti(n=5) & Kingsburgh (n=5) WWTPs in Durban KwaZulu Natal	15
	Sludge	Shallcross(n=5), Isipingo(n=5), Amanzimtoti(n=5) & Kingsburgh(n=5) WWTPs in Durban KwaZulu Natal	15
	Surface water	Shallcross(n=5), Isipingo(n=5), Amanzimtoti(n=5) & Kingsburgh(n=5) in Durban KwaZulu Natal	15
Second	WWTP1	Cape town influent 1	1
	WWTP2	Cape town influent 2	1
	WWTP3	Johannesburg influent 1	1
	WWTP4	Johannesburg influent 2	1
	WWTP5	Durban influent 1	1
	WWTP6	Durban influent 2	1

6.2.2 Comparative evaluation of fLAMP, qPCR and ddPCR

The same primer sets described in Table 9 and 10 of chapter 5(V) were used for detecting *Cryptosporidium* from different samples using different methods (LAMP, qPCR and ddPCR). All tests were conducted in 20 µl reaction volume. The quality control for all methods used in this study involved the addition of positive and negative (no-template controls to each experimental) run during the amplification step. The positive control was a synthetic DNA targeting *C. parvum*. Sterile nuclease-free water was used as the no-template control. Primers targeting the same gene: *SAM* gene for the 18S rRNA *Cryptosporidium* gene and the *C. parvum* GP60 gene were used for all assays. Different experiments were performed as described below.

6.2.3 Fluorescent Real-time LAMP

The fLAMP reactions were performed as described and optimised in chapter 5 (V). In this chapter, the reactions were performed in a QuantStudio 3 Real-Time PCR System (Thermofisher, USA) to enable the measurement of the fluorescence during amplification. A final volume of 20µl WarmStart fLAMP reaction Mix (New England Biolabs) contained:

WarmStart isothermal amplification buffer, *Bst* 2.0 WarmStart DNA Polymerase, 50X Fluorescent dye, LAMP Primer Mix (10X) (2 µM F3 primer, 2 µM B3 primer,16 µM FIP, 16 µM BIP, 8 µM LoopF and 8 µM LoopB), 2 µl template DNA, and PCR-grade water. The fluorescent intensity was measured every minute, and the time it took for a reaction (sample) to cross the cycle threshold, set at 400 000, was recorded to establish the time required for positive amplification. Melting analysis was then performed, allowing the generation of derivative melting curves. All LAMP tests were performed in triplicates.

6.2.4 Droplet digital PCR (ddPCR) assay

Droplet digital PCR assay for detection and quantification of *C. parvum* from wastewater was performed as described in chapter 3 and 5. Briefly, the ddPCR reaction mix was prepared in a final volume of 20 µl, containing 2× evagreen ddPCR Supermix (10 µl), 0.5 µm forward (1µl) and reverse primers (1µl) and template DNA (2 µl). The droplets were generated using a QX200 Droplet Digital generator system (Bio-Rad). Amplification was carried out on a T100 thermal cycler (Bio-Rad), using the conditions: 10 min at 95°C, followed by 50 cycles of denaturation at 95°C for 15 s, annealing at 56°C-60°C for 1 min, and extension at 72°C for 1 min, and a final step of extension at 72°C for 10min. After amplification in thermal cycler the samples were analysed using the QX200 Droplet Reader and QuantaSoft software (Bio-Rad). Based on the No Template Control (NTC) and positive control, a threshold line was drawn.

6.2.5 Real time PCR

Real time PCR assays were conducted in a QuantStudio™ 3 Real-Time PCR System (Thermofisher, USA) using the amplification conditions (thermal cycling conditions) as in ddPCR method. Each 20µl of qPCR reaction consisted of 2 x SYBR green qPCR master mix (10µl), 0.5µm forward (1µl) and reverse primers (1µl) and template DNA (2µl). A standard series was produced using synthetic *C. parvum* DNA copies derived from 10^9 diluted in 10-fold dilution increments down to 10^0 copies/µl. Two template replicates were included in each concentration of the standard series. A negative control sample was also included in each assay. Estimate of target copy numbers (concentrations) were from Cq values. A Cq value of 40 cycle was selected as a cut-off point, and values lower than the cut-off were not accurately quantifiable data.

6.2.6 Specificity and sensitivity of LAMP, ddPCR and real time PCR

Specificity was measured to determine how accurately the methods (fLAMP, real time PCR, and ddPCR) detect the target *C. parvum* while avoiding the detection of non-targets (negative samples). The specificity was assessed using closely related species of the same genus, as described in our previous study (*C. parvum* and *C. muris*). Waterborne protozoan parasites (*Giardia intestinalis*) and a group of bacteria usually found in the environment, such as soil and water (E. coli, *Aeromonas* spp., and *Acinetobacter* spp.), were also included.

The sensitivity of fLAMP, qPCR and ddPCR was determined to measure its ability to correctly identify the presence of the target (*C. parvum*) in a sample (positive results). This was done by serial dilution of *C. parvum* reference DNA and reported the limit of detection (LOD), which is the lowest concentration of target nucleic acid (*C. parvum*) that each assay can reliably detect. The sensitivity of each method was also described by including the percentage of true positive results obtained from a set of known *C. parvum* positive samples.

6.2.7 Linearity of LAMP, ddPCR and real time PCR

Linearity was measured to see how well each method (fLAMP, qPCR, and ddPCR) detects changes in the target *C. parvum* over a range of concentrations. To determine the linearity of each approach, a standard curve was created using a known concentration of the target analyte *C. parvum*. The standard was then diluted in a series of predetermined concentrations, and the assay was performed on each dilution. The resulting data were plotted as a graph with the logarithm of the known concentration on the x-axis and the assay signal (fluorescence) on the y-axis. For qPCR and fLAMP the standard curve was generated by plotting the logarithm of the initial target concentration against the threshold cycle (Ct) values. While for ddPCR the partition coefficient was generated by plotting the logarithm of the initial target concentration against the fraction of positive droplets. The resulting curves generated the relationship equation and calculated the correlation coefficient (R^2) between the target concentration and the amplification signal (fluorescent).

6.2.8 Cost comparison of LAMP, ddPCR and real time PCR

To determine the cost per test, various factors such as equipment, reagents, consumables, and labour were considered. The cost of reagents and consumables depended on the specific protocol used in this study. A list of the required materials for each protocol, along with their cost per unit and amount required per sample were created in this study, as shown in the table 16 below.

For qPCR, the cost of equipment included a QuantStudio 3 Real-Time PCR System (qPCR) (Applied Biosystems), and a data analysis computer. A qPCR instrument (Applied Biosystems), which costs approximately $11,000 on average. Reagent costs included the cost of SYBR Green master mix ($2.62 per reaction) and primers ($0.10 per reaction), while consumables included the cost of PCR plates ($6.56/plate), microplate sealing film ($108/per pack of 100) and pipette tips ($0.15 per tip).

For ddPCR, the cost of equipment used included the auto droplet generator, droplet reader, and a PCR instrument. An automated droplet digital PCR system from Bio-Rad, which costs around $30,000 for the droplet generator, $20,000 for the droplet reader, and $10,000 for the PCR instrument. Reagent costs included the cost of Auto QX200 Droplet reader Oil, Droplet Generation Oil for EvaGreen, Supermix for evaGreen, and primers. Consumables included the cost of disposable droplet generation cartridges, PCR plates, ddPCR microplate sealing foil, ddPCR plate, and pipette tips.

For LAMP, the cost of equipment required included the LAMP incubator/heating block and a fluorometer/qPCR thermocycler for fLAMP analysis detection. The cost of a LAMP heating block is typically around $600 to $1000, while the PCR instrument costs $10,000. Reagent costs included the cost of Warmstart master mix (New England Biolab, USA) and primers, while consumables included the cost of reaction tubes and pipette tips.

The cost of labour was also estimated for qPCR, ddPCR, and LAMP based on the time and expertise required for sample preparation, running the assay, and data analysis. According to payscale.com, the average hourly wage for a molecular Biology Technician in South Africa is $7.49 per hour, which is higher than the national average hourly wage rate for non-agricultural

workers ($1.55/hour). However, this figure is based on estimates and can vary based on other factors such as location, experience, and job responsibilities.

Therefore, to estimate the total cost of each method, the following formula was used:

Equation 3 (Cost estimation)

$$\text{Total cost} = (\text{Equipment cost} + \text{Reagents cost} + \text{Consumable cost}) \text{ x Number of reactions} + \text{Labor cost}$$

Where:

-Cost of reagents per sample was calculated by summing the total cost per sample for each reagent

- Consumable cost is the sum of all consumables per sample.

- Cost of equipment= was determined by cost of purchase/5-year life span. If analysing 1000 samples/year, then the depreciation cost per sample would be depreciation cost/year divide by 1000.

- Cost of labor, is the sum of time required for sample preparation, instrument setup, and data analysis.

The exchange rate between South African Rands (ZAR) and United States Dollars (USD) was determined by the fluctuating foreign exchange market and calculated by using the following formula:

Equation 4 (Exchange rate)

$$\text{Exchange rate} = \text{Spot rate } \left(\frac{\text{ZAR}}{\text{USD}}\right) \times (1 + \text{ZAR interest rate}) \div (1 + \text{USD intterest rate})$$

Where:

-Spot rate is the current exchange rate between ZAR and USD (May 2023).

-ZAR interest rate is the interest rate in South Africa.

-USD interest rate is the interest rate in the United States.

Table 16: Showing list and cost of reagents, consumables, instrument ad cost of Labour for qPCR, fLAMP and ddPCR.

Platform	Reagents, Equipments and Consumables	Cost per unit	Amount per sample	Total cost per sample
qPCR	SYBR Green qPCR master mix	$2.62/reaction	12 µl	$1.58
	Primers	$0.10/µl	2 µl	$0.20
	qPCR plates	$6.56/plate	1plate	$0.10
	Pipette tips	$0.14/tip	4tips	$0.56
	microplate sealing film	$108/ pack of 100	1 seal	$1.08
	qPCR instrument plus analysis software computer	$11,000		$2.20
	Labor	$7.49/hour	3 hours	$22.47
ddPCR	ddPCR Droplet reader Oil	$0.87/ml	20 µl	$0.02
	Automated ddPCR Droplet Generation Oil for EvaGreen	$5.8/ml	20 µl	$0.12
	ddPCR Supermix for EvaGreen	$1.2/reaction	12 µl	$0.72
	Primers	$0.10/µl	2 µl	$0.20
	Automated Droplet Generator Cartridges	$69.8/catridge	1	$17.45
	ddPCR plates	$7.84/ plate	1well	$0.10
	PCR 96 well plates	$6.56/plate	1well	$0.10
	Pipette tips	$0.01/ tip	4tips	$0.56
	ddPCR microplate sealing foil	$249/ pack of 50	1 seal	$4.98
	microplate sealing film	$108/ pack of 100	1 seal	$1.08
	Droplet generator	$30,000		$6
	Droplet reader	$20,000		$4
	PCR instrument	$10,000		$2
	Labor	$7.49/hour	4 hours	$29.96
LAMP	Warmstart master mix	$2.29/reaction	12 µl	$1.37
	Primers	$0.20/reaction	2 µl	$0.40
	Reaction tubes 02.ml with caps	$135/120 tubes	1 tube	$1.13
	Pipette tips	$0.01/ tip	4tips	$0.04
	Heating block	$600		$0.12
	PCR instrument (fluorescent measure)	$10,000		$2
	Labor	$7.49/hour	1 hour	$7.50

6.2.9 Applicability/Performance comparison of fLAMP, ddPCR and qPCR in environmental samples

6.2.9.1 fLAMP and ddPCR for detection of *C. parvum* and *Cryptosporidium* spp. using the first batch collected samples

To assess quantitative performance and precision of fLAMP and ddPCR, untreated and treated wastewater, surface water and sludge samples were used. ddPCR was chosen instead of qPCR

as a comparative and reference method due to its high sensitivity and absolute quantification without the need for standard curve. A total of 60 samples were used to assess the performance of the fLAMP method. This was done by applying the fLAMP on 15 untreated and 15 treated wastewater samples, 15 sludge samples and 15 surface water samples. Thereafter the same sample were analysed in ddPCR to compare obtained results. Total DNA was extracted from 2 L samples as described in the sample processing section and 2 µl of extracted DNA was analysed.

6.2.9.2 fLAMP, qPCR, and ddPCR for detection of *C. parvum* using the second batch of collected samples

fLAMP, qPCR and ddPCR were evaluated for the detection of *C. parvum* in six influent WWTPs samples (listed in Table 15) obtained from three different geographical regions in South Africa. The assessment involved quantification of 1ng/2ul of the extracted DNA using optimized protocols as described above and utilizing the primers listed in Table 9 and 10 of chapter 5(V).

6.2.10 Statistical analysis

To ascertain whether there were notable differences in the positivity rates obtained for three methods (fLAMP, qPCR, and ddPCR), a statistical analysis was conducted. Pairwise comparisons between the methods were calculated using the Kruskal Wallis test and the pairwise.wilcox.test function in R studio, with corrections for multiple testing. A *P*-value of less than 0.05 was considered significant, and it was used to determine whether there were significant differences among the methods.

6.3 Results

6.3.1 Sensitivity, specificity and linearity and cost comparison of fLAMP, ddPCR and qPCR

6.3.1.1 Sensitivity

The sensitivity of each method is reported as the limit of detection (LOD) as well as included percentage of true positive results obtained from a set of known positive samples. The LOD of qPCR was 14 copies of *C. parvum* per reaction volume with the Ct values ranging from 4 to 29. The fLAMP assay's detection limit was the second most sensitive, closer to that of ddPCR by 1.1 copies, however, with 75% efficiency. At 100% true positive results, 8 copies of target *C. parvum* were observed per reaction. The LOD for fLAMP was as low as 1.1 copies of *C. parvum* per reaction, with 75% true positives. ddPCR assay showed the highest sensitivity compared to fLAMP and qPCR with LOD of 1 copy of *C. parvum* per reaction with 100% true positives. In comparison to qPCR's limit of detection, the LOD for fLAMP was closer, with 8 copies per reaction volume with 100% efficiency.

6.3.1.2 Specificity

The fLAMP results demonstrated a high specificity with no false positives. The method detected only the target *C. parvum* and produced no amplification products or cross-reactions with the non-target or closely related sequences (*C. parvum* and *C. muris*) found in wastewater. Additionally, only a single melt curve for target *C. parvum* was observed. The qPCR specificity results also showed high specificity, with no incidence of false positive results, and a single melt curve for target *C. parvum* was observed. Lastly, ddPCR had high specificity with only positive droplets for target *C. parvum* and negative droplets for non-target sequences.

6.3.1.3 Linearity

The Linearity for fLAMP, qPCR, and ddPCR showed good results in measuring and detecting changes in the target *C. parvum* concentration over a range of concentrations (table 17). fLAMP revealed good linearity over the tested wide dynamic range of *C. parvum* concentrations. A standard curve was generated by plotting the copy numbers of synthetic *C. parvum* DNA against the threshold cycle (Ct) values. The correlation coefficient between the

target copy numbers and the Ct values was R2 = 0.997. A direct correlation was observed; the higher the input *C. parvum* copy numbers, the faster and earlier the fluorescent curve crossed the threshold within 28 minutes cut off time, with (y = 39393) e -0.472x, P = 0.008. qPCR also showed excellent linearity over a wide dynamic range of *C. parvum* concentrations, with a slope = -3.623, y-intercept = 38, R2 = 0.998, efficiency = 89%, and error rate = 0.067. The standard curve was generated by plotting the logarithm of the initial target concentration against the threshold cycle (Ct) values. Droplet digital PCR resulted in a clear separation of positive and negative droplets with no amplification inhibition, showing excellent linearity over the range of target concentrations. A linear relationship between the fraction of positive droplets and the logarithm of the *C. parvum* target concentration over the range of dilution concentrations demonstrates the linearity of the assay with a correlation coefficient $R^2 = 0.99$.

Table 17: Sensitivity, specificity and linearity comparison of LAMP, ddPCR and qPCR comparison.

	Sensitivity (Limit of detection per reaction) & % of true positive results	Specificity %	Linearity (R^2) (Correlation coefficient)
fLAMP	1.1copies (75%)	100	0.97
cLAMP	8 copies (100%)	100	
ddPCR	<1 copy (100%)	100	0.99
qPCR	14 copies (100%)	100	0.99

6.3.2 Cost comparison of LAMP, ddPCR and qPCR

Table 18 : Comparison of LAMP methods (cLAMP and fLAMP), qPCR and ddPCR.

	Direct cost		Indirect cost		
Assay	**Total cost of reagents per sample**	**Total cost of consumables per sample**	**Cost of instrument (Depreciation cost per sample)**	**Cost of labor per sample**	**Total cost per sample**
fLAMP	$1.77	$1.17	$2.00	$7.50	$12.44
cLAMP	$1.77	$1.17	$0.12	$7.50	$10.56
ddPCR	$1.06	$24.27	$12.00	$29.96	$67.29
qPCR	$1.78	$1.74	$2.20	$22.47	$28.19

This study calculated the direct and indirect cost per sample for LAMP (both fluorescent and colorimetric), qPCR, and ddPCR, and presented the findings in table 18. The calculation was done using the equation in the methodology section of this chapter and the input values were based on the requirements of the protocols and reagents utilized in this objective.

The analysis showed that the total cost per sample for LAMP was the cheapest compared to qPCR and ddPCR. However, cLAMP had a lower cost per sample at $10.56 compared to fLAMP ($12.44) due to the need for a fluorometer or qPCR thermal cycler for fLAMP analysis. Nevertheless, the total cost of reagents, consumables, and labour was estimated to be the same for both fLAMP and cLAMP.

qPCR was found to be the second most affordable technique, with a total cost per sample estimated to be $28.19. The cost increase for qPCR was mainly due to the total cost of labour ($22.47) and equipment. Lastly, ddPCR was found to be the most expensive technique among the three, with a total cost per sample estimated at $67.29. The total cost per consumable ($24.27), instrument ($12), and labour ($29.96) contributed to the increased cost.

6.3.3 Evaluation of fLAMP's performance and applicability in comparison to ddPCR and qPCR in environmental samples

6.3.3.1 fLAMP, qPCR, and ddPCR for detection of *C. parvum*

Figure 17 displays successful detection of *C. parvum* in all untreated wastewater (first batch) samples examined using fLAMP, qPCR, and ddPCR. The positivity rate for *C. parvum* detection was 100% for all methods used in the influent WWTPs samples. However, each technique produced different concentrations of *C. parvum* copy numbers with a comparable detection pattern. Figure 17 shows that qPCR recorded the highest concentration of *C. parvum in* all samples tested compared to fLAMP and ddPCR. The copy numbers ranged from 8202 (±10.2) copies in WWTP 2 to 326 (±17) copies in WWTP 5. The results for fLAMP and ddPCR followed the same trend, with the highest and lowest *C. parvum* concentrations observed by each method in the same WWTP. For example, ddPCR detected the highest *C. parvum* concentration of 5665.83 (±4) copies/reaction in WWTP 2 and the lowest of 72.66

(±1.3) *C. parvum* copies/reaction in WWTP 5. Similarly, fLAMP results indicated a high concentration in WWTP 2, with 879 (±2.1) *C. parvum* copies/reaction, and a lower concentration in WWTP 5, with 367 (±2.8) *C. parvum* copies.

6.3.3.2 fLAMP and ddPCR for detection of *C. parvum* and *Cryptosporidium* spp.

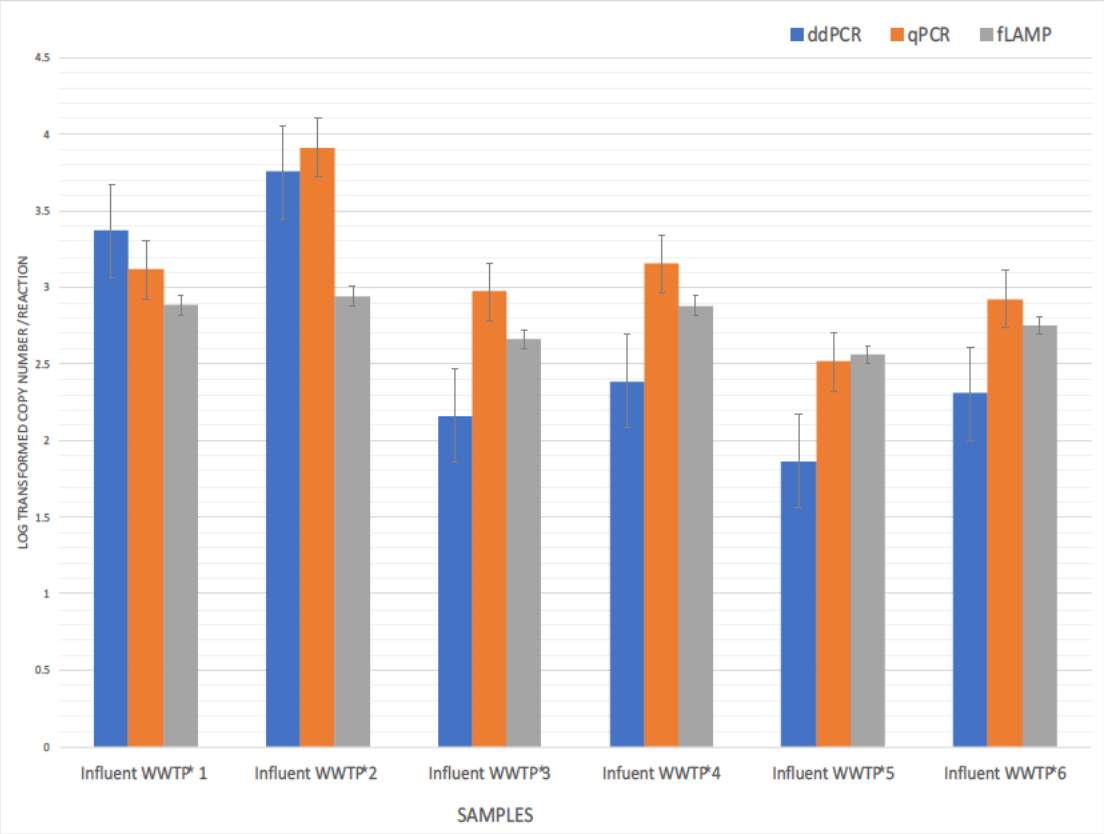
To further assess the applicability of semi-quantitative fLAMP, the technique was utilized to measure the levels of *Cryptosporidium* spp. in various matrices (wastewater, surface water and sludge) and compared to the results obtained by ddPCR. In the previous section, only the detection rate was evaluated without quantification. Additionally, ddPCR was chosen as the reference method instead of qPCR, due to its ability to provide absolute quantification.

The findings showed that some samples (>10) could not be quantified or measured accurately by fLAMP but were successfully measured by ddPCR at lower levels of parasite prevalence. Out of the 60 samples tested, the prevalence of *Cryptosporidium* spp. detected by LAMP and ddPCR were 76.7% (46 out of 60) and 88.3% (53 out of 60), respectively. Positive fLAMP samples contained between 43.17 to 656 copies/reaction of *Cryptosporidium* spp., while positive ddPCR samples contained between 8.53 to 145 copies/reaction (as shown in Figures 18 and 19). The prevalence of *C. parvum* detected by fLAMP and ddPCR were 53% (32 out of 60) and 70% (42 out of 60), respectively. The number of copies of *C. parvum* per reaction volume detected by fLAMP ranged from below the limit of detection (0.10) to 9 copies, while the positive ddPCR samples ranged from 0.02 copies per reaction volume (below the limit of detection) to a maximum of 11 copies per reaction volume.

In general, the results showed that both fLAMP and ddPCR had similar patterns of prevalence across different sample matrices, with untreated wastewater samples showing the highest concentration of target *Cryptosporidium* spp. and *C. parvum*, followed by sludge samples, surface water, and treated wastewater samples showing the lowest prevalence. Furthermore, qPCR and ddPCR assays yielded inconsistent results for samples with lower concentrations that were below the limit of detections.

The statistical analysis consisted of conducting a Kruskal test and a pairwise comparison test to determine whether there were any significant differences in the performance of three

methods (fLAMP, qPCR, and ddPCR) in detecting *Cryptosporidium* in different wastewater samples. The results of the Kruskal test showed that the differences in the application of the different assays in quantifying the *Cryptosporidium* genus in various wastewater samples were not significant (*P*-value = 0.429). Similarly, the differences were also not significant for the *C. parvum* species primer (*P*-value = 0.443). The pairwise comparison also supported the findings of the Kruskal test, revealing that there were no significant differences (with *P*-values of 0.88 and 0.34) among the three methods for detecting *C. parvum* and *Cryptosporidium* genus in different wastewater samples.



***Wastewater treatment plant**

Figure 17: Depicts a comparison of fLAMP, qPCR, and ddPCR for the detection and quantification of *Cryptosporidium parvum* (data was normalised using the log transformation for clear presentation).

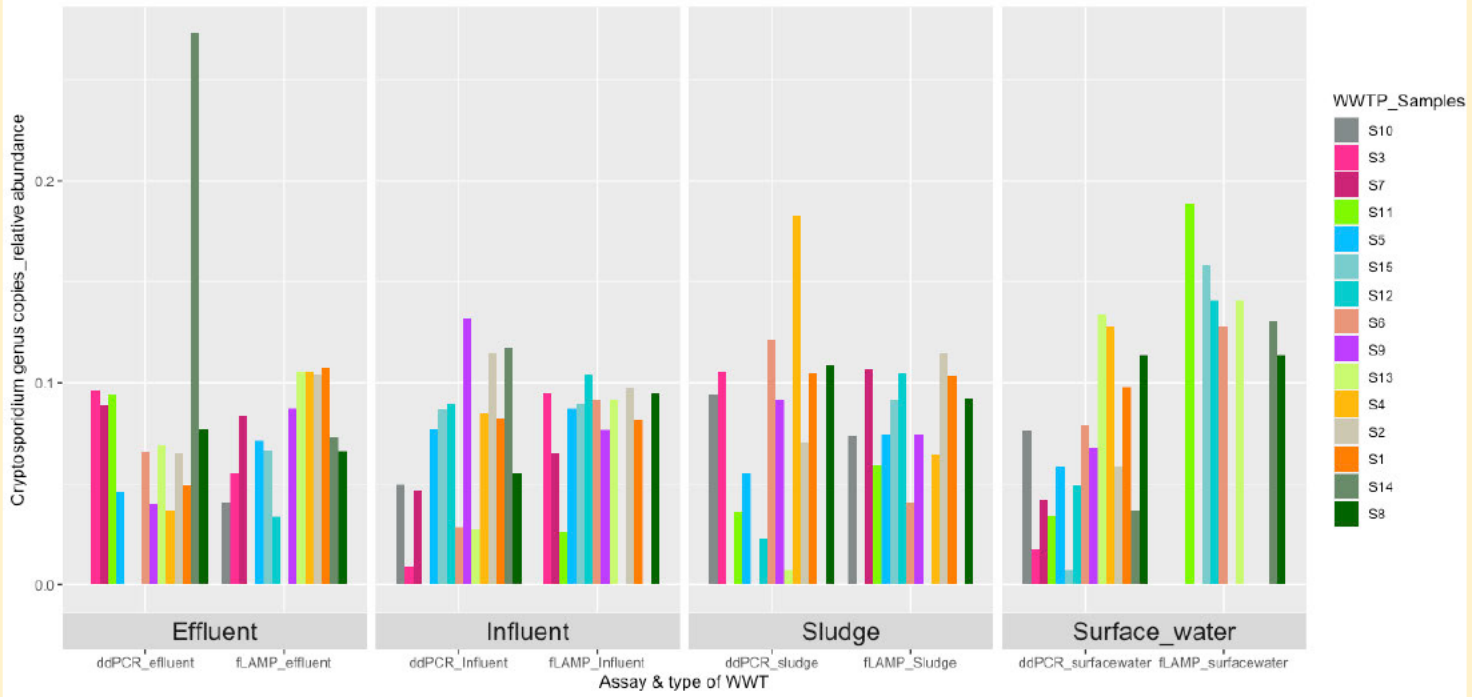


Figure 18. Table showing performance evaluation of *Cryptosporidium* spp. fLAMP in different sample matrices, confirmed with ddPCR .

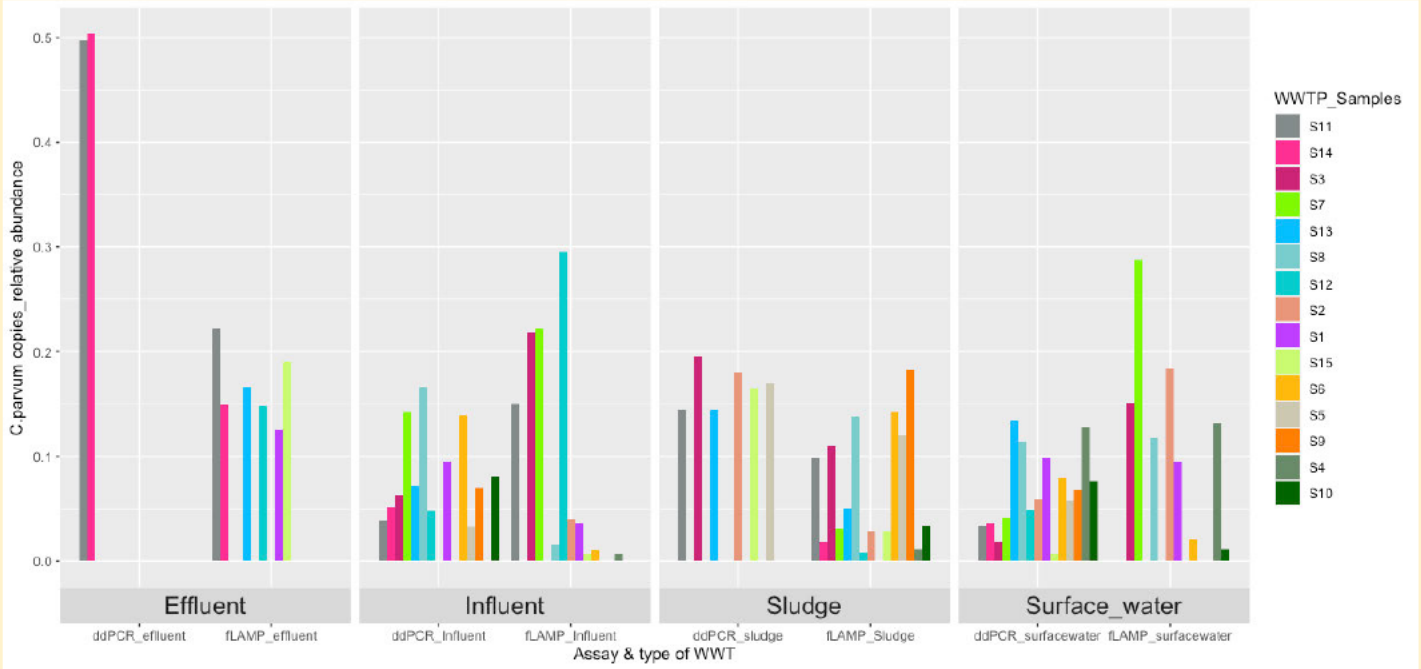


Figure 19. Performance evaluation of *C. parvum* fLAMP in different sample matrices, confirmed with ddPCR.

6.4 Discussion

The current study is the first, to our knowledge, to conduct a technical and economic assessment of fLAMP compared to ddPCR and qPCR for the detection of *Cryptosporidium* spp. in wastewater samples. Previous studies have focused on comparative evaluations of LAMP and qPCR for *Toxoplasma gondii* (Durand et al., 2020), ddPCR and qPCR for *Cryptosporidium* spp. (Yang et al., 2014), and *Plasmodiophora brassicae* (Gossen et al., 2019). Comparisons between real-time PCR, LAMP, and RT qPCR for *Salmonella* spp. (Zhang et al., 2011) and detection of *Alternaria solani* using LAMP, nested PCR, and qPCR (Khan et al., 2018).

Since complex environmental samples contain lower protozoan oocysts compared to bacterial species, evaluating sensitivity is a crucial step in developing detection techniques and assuring the public's safety of treated and surface water. The fLAMP demonstrated slightly higher sensitivity compared to qPCR and was less sensitive compared to ddPCR in this study. The higher sensitivity of LAMP compared to qPCR may be explained by two key factors. Firstly, LAMP uses a *Bst* polymerase that exhibits high strand displacement activity, which can generate exponential products equivalent to 30 cycles in a PCR assay, starting from as few as 10 template copies (Milligan et al., 2018). Secondly, the inclusion of loop primers (both forward and reverse loop primers) in the LAMP technique accelerates the reaction and enhances sensitivity (Seki et al., 2018).

Several studies have shown that LAMP has a high sensitivity compared to other methods (Nliwasa et al., 2016, Khan et al., 2018, Seki et al., 2018). According to a previous study, the faster an assay is, the more sensitive it becomes as errors are eliminated (Sadeghi et al., 2021). In line with this, Sadeghi et al. (2021) statistically demonstrated a sensitivity of 96.6% and 95.6% for LAMP and qPCR, respectively (P<0.001). Based on the analysis, LAMP can be used to reliably confirm the presence of *Cryptosporidium* spp. in environmental samples with its high sensitivity. However, it is still necessary to determine the LOD (limit of detection) for other pathogenic protozoans present in wastewater to establish a standard LOD for the method.

In chapter 3 and 5 of this study, the specificity of LAMP and ddPCR for *Cryptosporidium* was determined. The results of this study confirm that the specificity between LAMP, ddPCR, and qPCR is consistent with our previous findings in chapter 5. A high specificity with no cross-

reactivity in non-*Cryptosporidium* samples, and positive samples were correctly identified with single melt curves for both qPCR and LAMP. The high specificity of fLAMP assay could be due to the use of multiple primers that bind eight different regions within the target sequence in a single reaction. LAMP has been widely used for pathogen detection and has shown comparable specificity to PCR (Mahittikorn et al., 2017, Sadeghi et al., 2021). Some studies have even reported higher LAMP specificity than qPCR (Khan et al., 2018). Recent studies have also supported the high specificity of LAMP in detecting other pathogens such as *Giardia intestinalis* (Lalonde et al., 2021), *Blastocystis* spp. (Badparva et al., 2022), and SARS-CoV-2 (Bhadra et al., 2021).

The assays for fLAMP and qPCR were linear, with a strong correlation between serial dilutions of DNA template and measured *Cryptosporidium* copy numbers, as evidenced by R^2 values ranging from 0.9 to 0.99. The tests were also found to have an efficiency of over 89%, which falls within the acceptable range for good efficiency (Klymus et al., 2020). However, when these assays were applied to untreated wastewater samples, the detection efficiency varied, with differences in copy number despite a similar pattern. This discrepancy could be due to the differing platform principles of fLAMP, qPCR, and ddPCR, with fLAMP amplifying at a constant temperature and in a single run, while ddPCR and qPCR rely on cycling through different temperatures and incubation times. Additionally, the accuracy of the linear equation generated for qPCR and fLAMP may lead to the overestimation of target copy numbers, as reported by Yang et al., (2014). To address these issues, future studies should investigate various amplification factors and find ways to compare and equilibrate the platforms without introducing any bias.

Both fLAMP and ddPCR showed similar prevalence patterns and applicability in different sample matrices. Based on both fLAMP and ddPCR results, untreated wastewater samples had the highest prevalence of *Cryptosporidium* spp. and *C. parvum*, followed by sludge samples and surface water, which is consistent with potential sources of contamination (Hamilton et al., 2018). The lowest prevalence was observed in treated wastewater samples suggesting that the treatment processes effectively reduced the level of the protozoans (Nasser, 2015). The consistency between the results from both fLAMP and ddPCR supports the validity of the findings and either method can be used to reliably detect *Cryptosporidium* spp. and *C. parvum* in environmental samples. A study conducted in Malaysia found that untreated wastewater had

the highest prevalence of *Cryptosporidium* spp. and *C. parvum* (Kumar et al., 2016) however this study used qPCR method. While another found a higher prevalence of Cryptosporidium spp. in wastewater samples compared to surface water (Hamilton et al., 2018). ddPCR demonstrated a higher detection efficiency compared to fLAMP, with prevalence differences of 11.6% and 17% for *Cryptosporidium* spp. and *C. parvum*, respectively. This is likely due to the higher sensitivity, precision, and absolute quantification characteristics of ddPCR. As a result, when working with samples containing low *Cryptosporidium* oocysts, it is recommended to combine fLAMP with an established method, such as ddPCR assay, for confirmation and absolute quantification.

Compared to qPCR and ddPCR, fLAMP was the most affordable method when considering all costs, including direct and indirect costs per sample. This makes it a viable option for analyzing large numbers of samples at a low cost, particularly in low-income countries, commercial laboratories, and during outbreaks. Previous research has also emphasized the need for low-cost intervention and monitoring methods to prevent and treat diarrheal disease caused by *Cryptosporidium* spp. (Chola et al., 2015). Water contamination by pathogens results in an estimated annual global economic loss of nearly USD 12 billion (Alhamlan et al., 2015, Paruch, 2022b). Therefore, the use or development of rapid cost-effective surveillance methods, such as fLAMP, could save millions or billions of dollars associated with diarrheal infection in South Africa and worldwide especially during outbreaks.

6.5 Conclusions

In conclusion, this chapter aimed to compare the sensitivity, specificity, applicability, and techno-economic viability of fluorescent real-time loop-mediated isothermal amplification (fLAMP), droplet digital PCR (ddPCR), and quantitative PCR (qPCR) for the detection of *Cryptosporidium* in environmental samples. The major conclusions are as follows:

- fLAMP demonstrated slightly higher sensitivity than qPCR but was less sensitive than ddPCR.

- The specificity of LAMP, ddPCR, and qPCR was consistent with previous studies, and there was no cross-reactivity in non-*Cryptosporidium* samples.
- The tests were linear, and the efficiency was over 89%, which falls within the acceptable range for good efficiency.
- fLAMP and ddPCR showed similar prevalence patterns and applicability in different sample matrices.
- fLAMP is the most affordable ($12.46/sample), followed by qPCR ($28.19/sample), and ddPCR ($67.29/sample) is the most expensive.
- Compared to the PCR-based methods used, the fLAMP assay enabled faster, less expensive, and comparatively accurate detection of Cryptosporidium spp. in environmental samples. Therefore, fLAMP could be a valuable alternative for routine microbial assessment and public health surveillance.

7.0 CONCLUSIONS AND RECOMMENDATIONS

7.1 MAJOR CONCLUSIONS

The present study addressed crucial aspects of the detection and analysis of protozoan parasites in wastewater, offering significant findings pertaining to both the methodology and prevalence of these pathogens. In this study, a molecular-based protocol was developed and validated, allowing for efficient recovery, DNA extraction, and quantification of protozoan (oo) cysts in wastewater samples. The phenol-chloroform extraction method was found to be the most effective in terms of yield and sensitivity. This study also found that analyzing both the filtered supernatant and pellets after centrifugation improved the recovery efficiency of oocysts from wastewater by 10.5%, resulting in a total recovery of 64.1%.

Additionally, this study investigated the genetic diversity of human and non-human protozoan parasites in selected South African wastewater treatment plants (WWTPs) using 18S rRNA gene amplicon sequencing and shotgun metagenomic profiling for the first time. The eukaryote microbial diversity structure observed in WWTPs was significantly influenced by geographical separation or distance as well as the type of treatment stage (treated vs untreated). However, there was also a core community that was consistent across all plants.

This study also confirmed the presence of public health importance protozoa, including *Cryptosporidium* spp., *Entamoeba hystolitica*, *Blastocystis hominis*, *Naegleria gruberi*, *Toxoplasma gondii, Cyclospora cayetanensis,* and *Giardia intestinalis* in all untreated wastewater samples studied. Pathogenic protozoans were also found to inhabit the studied wastewater treatment plants, which were also confirmed by the presence of associated functional pathways.

Furthermore, this study has successfully developed and evaluated a highly specific, sensitive, and rapid LAMP assay for the detection of human pathogenic *Cryptosporidium* species from different environmental matrices. The optimized methods demonstrated high sensitivity and specificity (100%), with a limit of detection (LOD) of 1.1 copies of *C. parvum* per 25 µl

reaction (0.02 ng/µl). The LAMP reaction can be completed within 28 minutes for real-time fluorescence monitoring, and within 30 minutes for visual detection.

Comparative analysis with other established methods, such as ddPCR, and qPCR highlighted the potencies of the LAMP assay, showing slightly higher sensitivity than qPCR but being less sensitive than ddPCR. All three tests showed consistent specificity and no cross-reactivity in non-*Cryptosporidium* samples. fLAMP and ddPCR showed similar prevalence patterns and applicability in different sample matrices, with fLAMP being a promising and cost-effective method for detecting *Cryptosporidium* spp. in environmental samples.

In conclusion, this study makes a substantial contribution to the field by not only improving molecular detection techniques but also by highlighting the genetic diversity, prevalence, and potential health risks associated with protozoan parasites in wastewater. This study's results have significant implications for wastewater management and public health, emphasising the need for reliable diagnostic tools and ongoing monitoring to protect water resources.

7.2 Recommendation

- Additional research, with larger sample sizes and a broader geographic scope, is recommended to improve understanding of the protozoan species present in wastewater samples. Furthermore, the use of OMICS, which includes metagenomics, transcriptomics, proteomics, and metabolomics, could provide a more in-depth understanding of diversity and ecophysiology of pathogenic protozoa in the wastewater environment.

- It is recommended that more research be conducted in order to determine the suitability of LAMP technology for the detection and quantification of diverse human pathogenic protozoan parasites.

- Future studies should also focus on improving and developing direct LAMP, which does not require DNA extraction. Consequently, both time and cost can be reduced in order to achieve the desired results.

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9.0 APPENDICES

9.2 Appendix 1

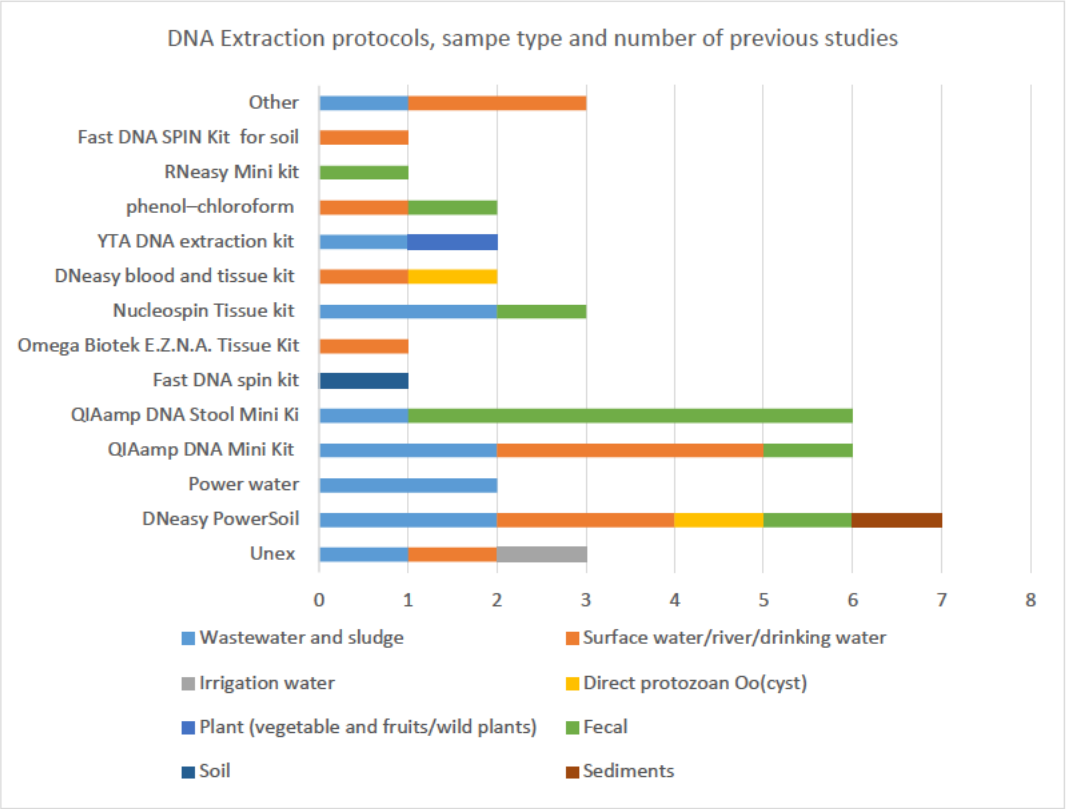
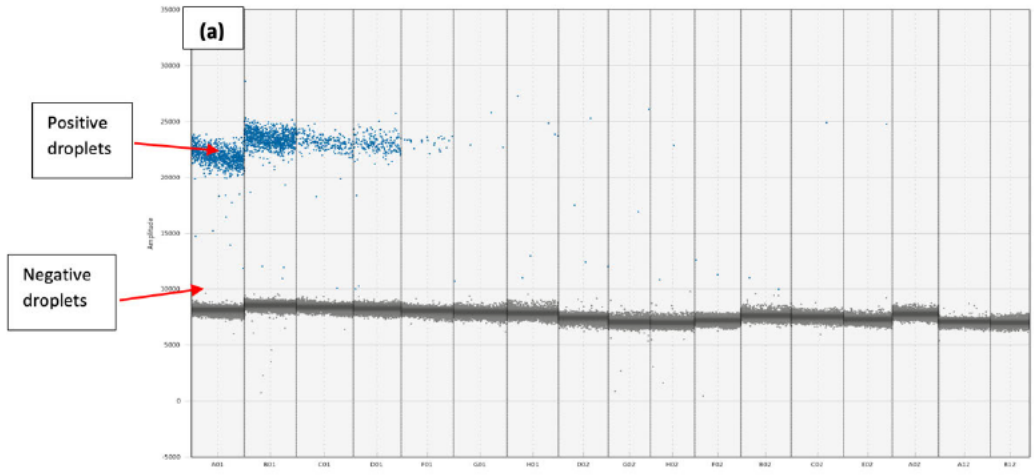


Figure S1: Different DNA extraction methods, sample type that have been reported in the literature

Table S1: Preservation methods

Method	Publications
Ambient cooling to 4°C, 23°C -(for short period	(Lalonde and Gajadhar, 2016, Crawley et al., 2016, Frey et al., 2019, Escotte-Binet et al., 2019, Marquis et al., 2019, Galvani et al., 2019, Javanmard et al., 2018, Ajonina et al., 2018, Sommer et al., 2015, Bridle et al., 2010, Zacharia et al., 2019)
freezing samples at -20, -80,−150°C /Liquid nitrogen	(Lalonde and Gajadhar, 2016, Brumfield et al., 2020, Ögren et al., 2020, Frey et al., 2019, Escotte-Binet et al., 2019, Marquis et al., 2019, Seixas et al., 2019, Galvani et al., 2019, Shin et al., 2018, Lee et al., 2018a, Javanmard et al., 2018, Gallas-Lindemann et al., 2016, Sommer et al., 2015, Babaei

	et al., 2011, Wylezich et al., 2020, Hendriksen et al., 2019, Hino et al., 2016)
Ethanol/alcohol with buffers	(Papaiakovou et al., 2018)
potassium dichromate	(Kuk and Cetinkaya, 2012)
10% formal aldehyde	(Zacharia et al., 2019)



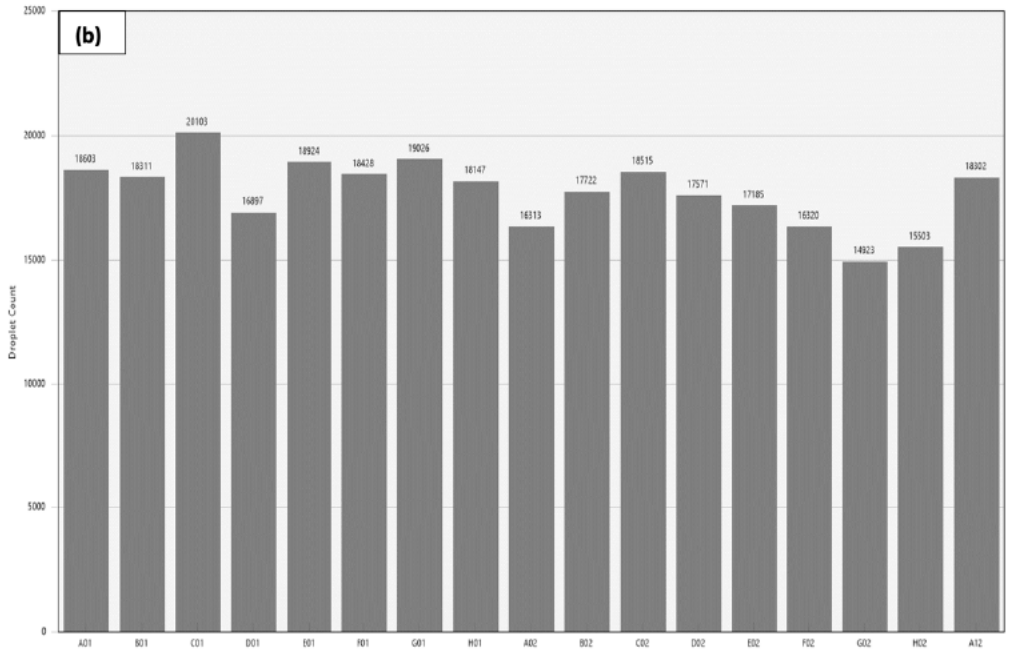
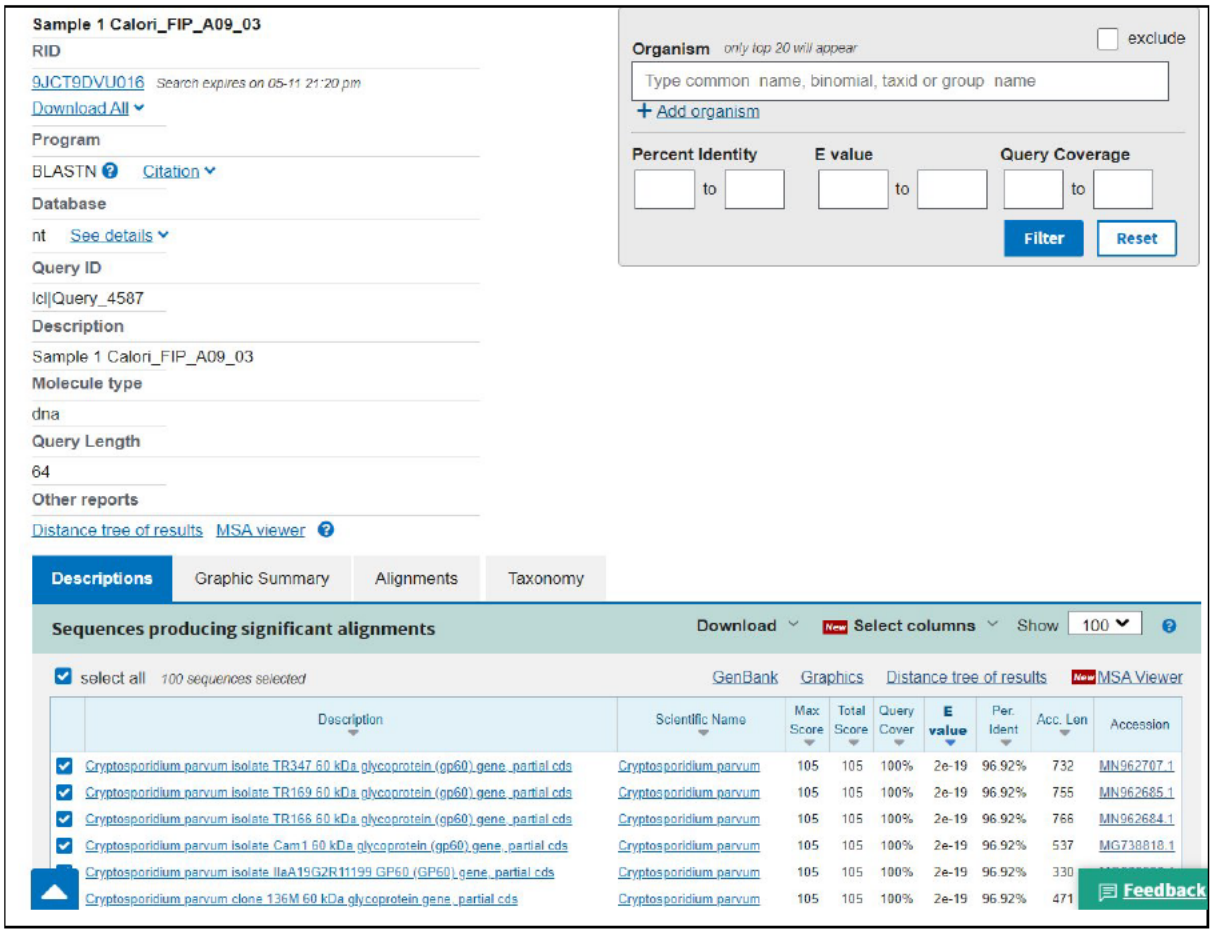
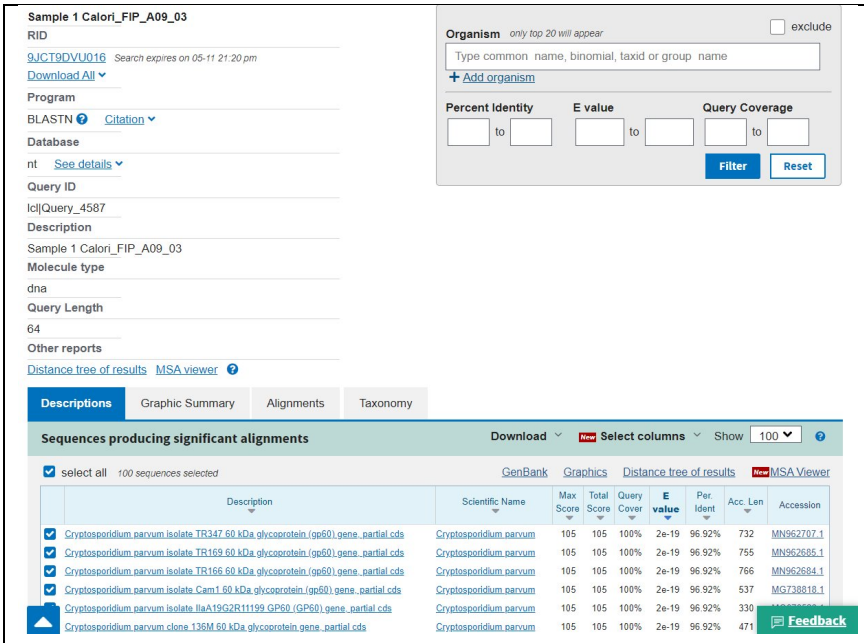
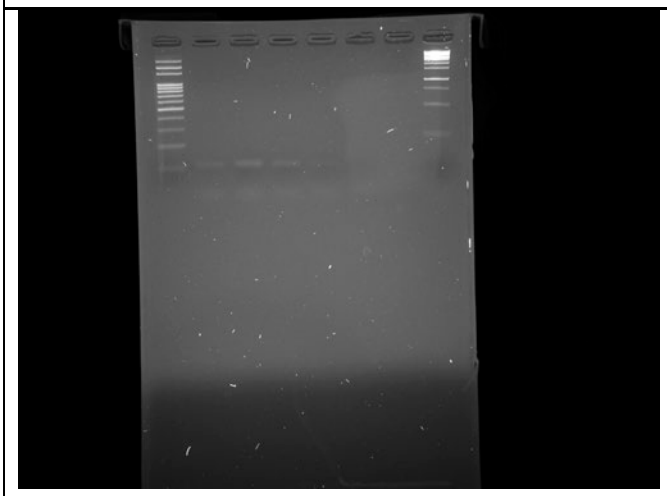


Figure S2: Droplet digital PCR, Phase 1 results showing limit of detection for the GP60 assay. (a) Decreasing copy number concentration. (b)The total number of accepted droplets. Y-axis: shows amplitude/droplet count. X-axis: shows sample wells.

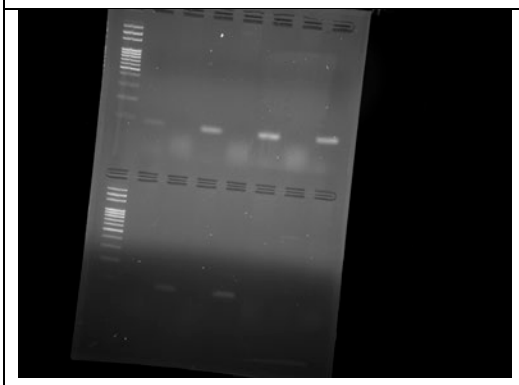
Appendix: Confirmation of primers





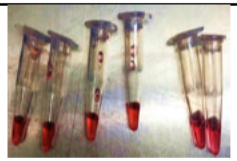
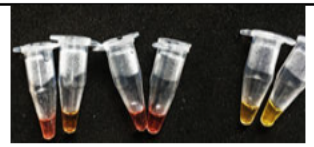
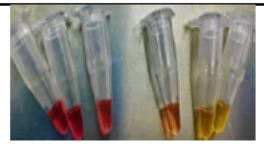


***Cryptosporidium* 18s rRNA (100bp)**



***Giardia gp60* (150bp)**

Table S2: Optimisation of incubation time at 65°C

Reaction incubation Time	Before incubation	10 minutes	15 minutes	25 minutes	30 minutes	35 minutes
P Detection rate for P=x(detected)/ y (replicates)	0/2	0/2	1/2	2/2	2/2	2/2
Color change						
Positive reaction	0%		25%	100%	**100%**	75%

* N= No Template Control (NTC) and P= unknown samples (positive amplification)

Table S3: Limit of detection (based on *Cryptosporidium parvum* copy number and input concentration of DNA template) for 30 minutes incubation at 65°C

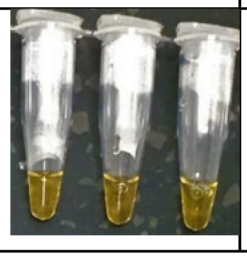
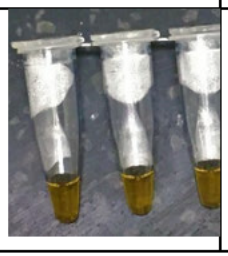
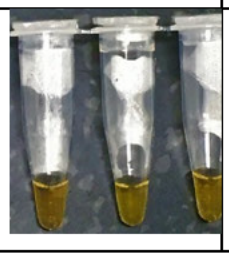
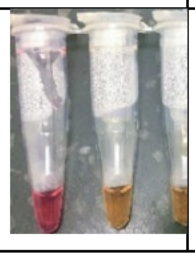
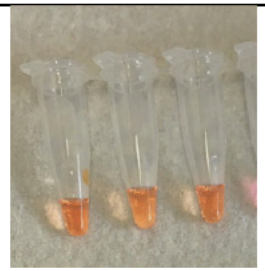
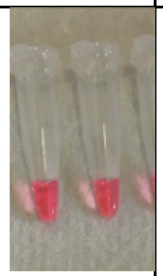
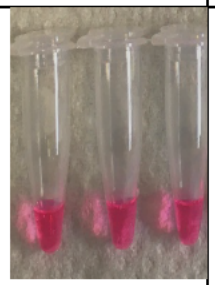
Detection rate= x(detected)/y (replicates)	3/3	3/3	3/3	3/3	3/3	3/3	2/3	1/3	0/3
Copies/25µl reaction	1132	226.4	45.28	9.05	4.5	2.3	1.132	0.6	0.2
Color change									

Table S4: Results demonstrating the specificity of the *C. parvum* cLAMP against pathogens found in the soil/water environment

	Cryptosporidium parvum	*Cryptosporidium muris*	*Giardia Lambia*	*Giardia muris*	*E. coli*	*Aeromonas*	NTC
Results							

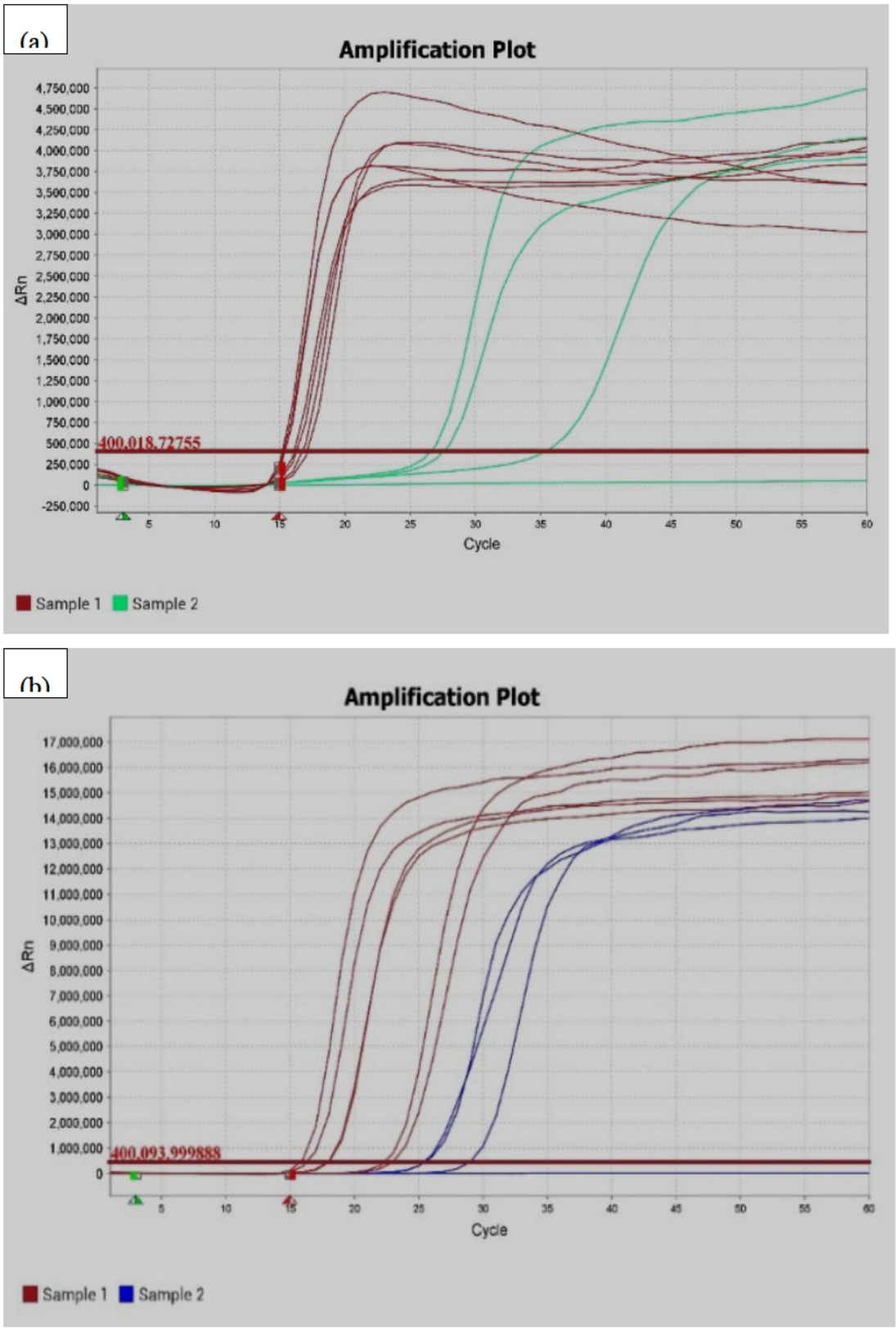


Figure S3: Showing real time fLAMP results after amplification, with x-axis label cycles as time in minutes and y-axis measures the fluorescent intensity. Label: Sample 1= DNA

template of *Cryptosporidium* spp., sample 2 =NTC. (a) showing SAM gene results, (b) showing GP60 gene.

Table: **S5.** 18S rRNA(V4V5) gene amplicon sequencing results. The number of samples(pooled) per wastewater treatment plant location, as well as the number of raw reads are shown with total reads mapped in SILVA database. S1 =sample

Sample id and name	Total raw reads (PE)	Number of Eukaryote taxonomic reads mapped in SILVA database & used in the analysis	%recovery	Unclassified reads%
S1=Cape Town Untreated wastewater	70824	62998	88.95	11.05
S2=Cape Town Treated wastewater	58595	23787	40.60	59.40
S3=Cape Town Untreated wastewater	66858	36332	54.34	45.66
S4=Cape town Treated wastewater	77508	36524	47.12	53.88
S9=Durban Untreated wastewater	57812	20007	34.61	65.39
S10=Durban Treated wastewater	55480	20346	36.67	63.33
S11=Durban Untreated wastewater	66672	41158	61.73	38.27
S12=Durban Treated wastewater	68878	27534	39.98	60.02
S5=Johannesburg Untreated wastewater	64509	29106	45.12	54.88
S7=Johannesburg Untreated wastewater	82481	39457	47.84	52.16

Table S6. Shotgun metagenomic sequencing results. The number of samples(pooled) per wastewater treatment plant location, as well as the number of raw reads are shown with total reads mapped in Protozoan & Eukaryote Kraken database.

Untreated wastewater Samples	Raw reads (paired-end)	Total Mapped reads (248,566 standard + Protozoa & Eukaryote database entries)	Eukaryotes spp used in R analysis	Filtered human waterborne pathogenic protozoa spp (WHO priority)	Diversity Shannon	Diversity Richness
S1(Cape town wastewater treatment plant)	9372262	7386326 (78.8%)	77248(1.05%)	9432(12.21%)	3.631260	96
S2(Johannesburg wastewater treatment plant)	7110301	6004945(84.45%)	62226(1.03%)	11020(17.71%)	3.761067	91

S3 (Durban wastewater treatment plant)	7343489	6101916(83.09%)	60976(0.99%)	9623(15.78%)	3.696070	92

Table S7 Differential relative abundance analysis of the eukaryote taxa associated with type of wastewater samples (untreated and treated) across different geographical locations

Taxonomy	Type of wastewater	Indicator value	Kruskal-wallis test (P-value)
Chloroplastida	Treated	0.989	0.03
Discoba	Treated	0.968	0.01
Holozoa	Treated	0.941	0.03
Rhizaria	Treated	0.742	0.04
Tubulinea	Treated	0.729	0.04
Nucletmycea	Untreated	1	0.01
Holozoa	Untreated	0.979	0.01
Alveolata	Untreated	0.97	0.01
Chloroplastida	Untreated	0.863	0.03
Stramenopiles	Untreated	0.859	0.03
Apusomonadidae	Untreated	0.833	0.02

Table S8: Showing common and unique shotgun generated species of the Venn diagram

DUR_WWTP	JHB_WWTP	CPT_WWTP	DUR_WWTP|JHB_WWTP	DUR_WWTP|CPT_WWTP	JHB_WWTP|CPT_WWTP	DUR_WWTP|JHB_WWTP|CPT_WWTP
Theileria equi	Neospora	Plasmodium inui San Antonio 1	Cryptomonas	Leishmania infantum JPCM5	Theileria annulata	Dictyostelia
Geminigeraceae	Leishmania infantum	Plasmodium fragile	Plasmodium knowlesi	Plasmodium vinckei vinckei	Plasmodium falciparum 3D7	Dictyosteliaceae
Acytosteliaceae	Plasmodium vinckei	Leishmania panamensis	Plasmodium inui	Toxoplasma gondii ME49	Evosea	Plasmodium Haemamoeba
		Cryptomonas parameciu m	Leishmania braziliensis species complex		Hymenostomatida	Peronosporaceae
		Guillardia theta	Phytophthora infestans			Theileria
		Phytophthora	Apusomonadidae			Trypanosoma with unspecified subgenus
		Thecamonas trahens ATCC 50062	Pyrenomonadales			Piroplasmida
		Aureococcus anophagef ferens	Eustigmatales			Dictyostelium discoideum
						Cryptosporidium hominis TU502
						Trypanosoma brucei gambiense DAL972
						Aphanomyces
						Babesia microti strain RI
						Cryptosporidium
						Blastocystis
						Theileria equi strain WA
						Leishmania donovani
						Cryptosporidium parvum Iowa II
						Trypanosoma brucei brucei TREU927
						Babesia
						Babesia sp Xinjiang
						Giardia intestinalis
						Babesia bovis T2Bo
						Sarcocystidae
						Plasmodium gaboni
						Plasmodium reichenowi
						Trypanosoma
						Cryptosporidium ubiquitum
						Plasmodium sp gorilla clade G2
						Aconoidasida
						Theileria parva strain Muguga

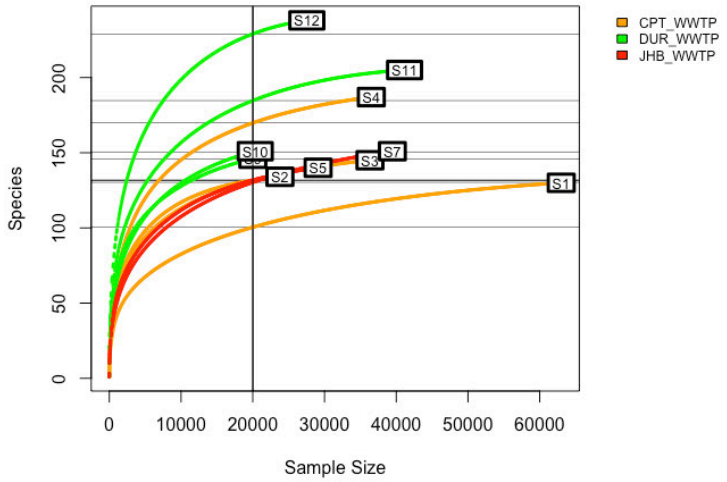


Figure S4(a) Alpha diversity of wastewater microbial communities. Rarefaction curve of the sequenced reads for all samples. Rarefied to 20007(lowest coverage). The plot shows different wastewater samples named S1 to S12 belonging to different wastewater treatment plant locations. The left side of the steep slope indicates that a large fraction of the species diversity remains to be discovered. All curves(samples) are slightly becoming flatter to the right but did not reach a plateau, reasonable number of individual samples have been taken, indicating that more intensive sampling is likely to yield a few more additional species.

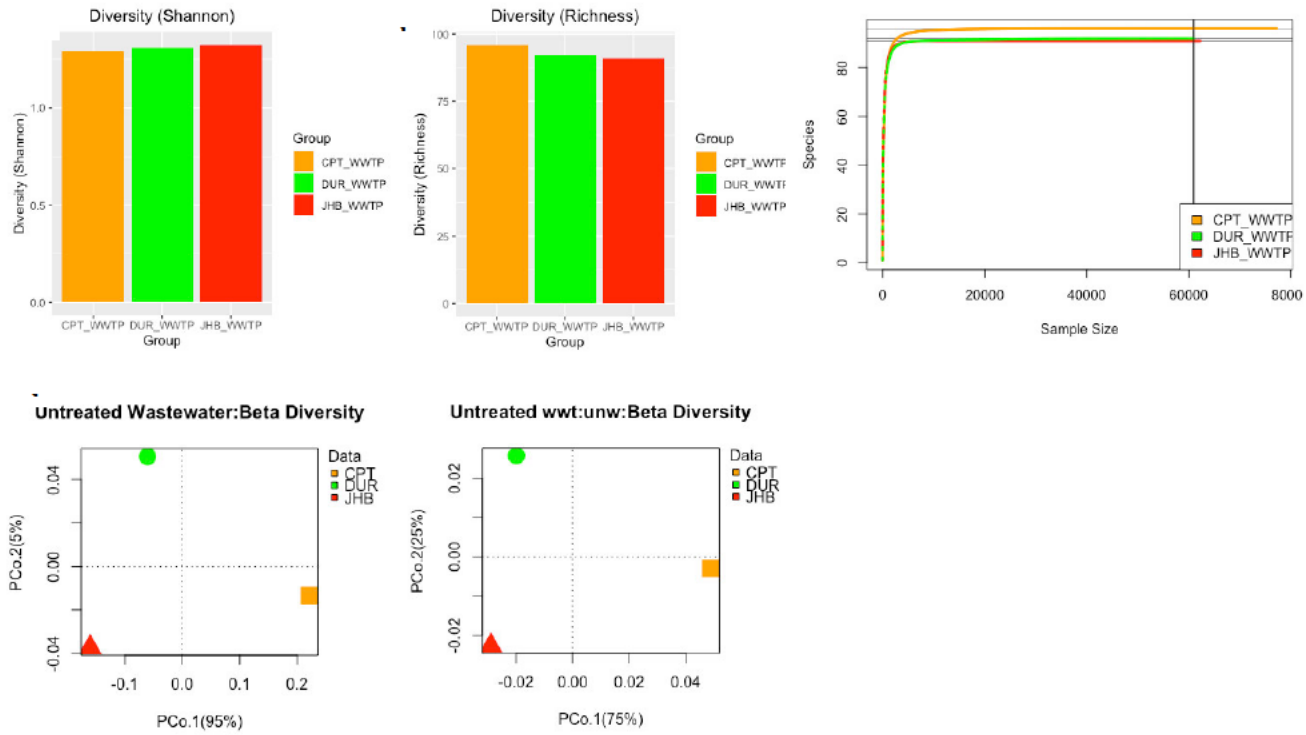


Figure S5(a-e). Alpha and beta **diversity of wastewater samples determined using shotgun sequencing**. The plots show wastewater samples from different locations (**CPT_WWTP** = Cape Town, **JHB_WWTP**=Johannesburg, and **DUR_WWTP**=Durban). (**S5(a-b)**) The sample from CPT showed higher species richness. The number of different species reached a plateau with an increasing number of sequences (sample size). **(S5c)** Rarefaction curve of the sequenced reads for all samples. (**S5d-e**) Principal coordinates analysis (PCoA) plot of beta diversity as measured by Bray Curtis dissimilarity matrix is reported and the proportion of variation explained by each axis is listed in parentheses The plot shows Eukaryote microbial differences in Untreated wastewater samples from different group locations. (**S5d**) Weighted PCoA showing 95% variation in environmental microbiome across the geographical. **(S5e)** Unweighted Beta diversity showing 75% variation in environmental microbiome across the geographical, however with no statistical power.

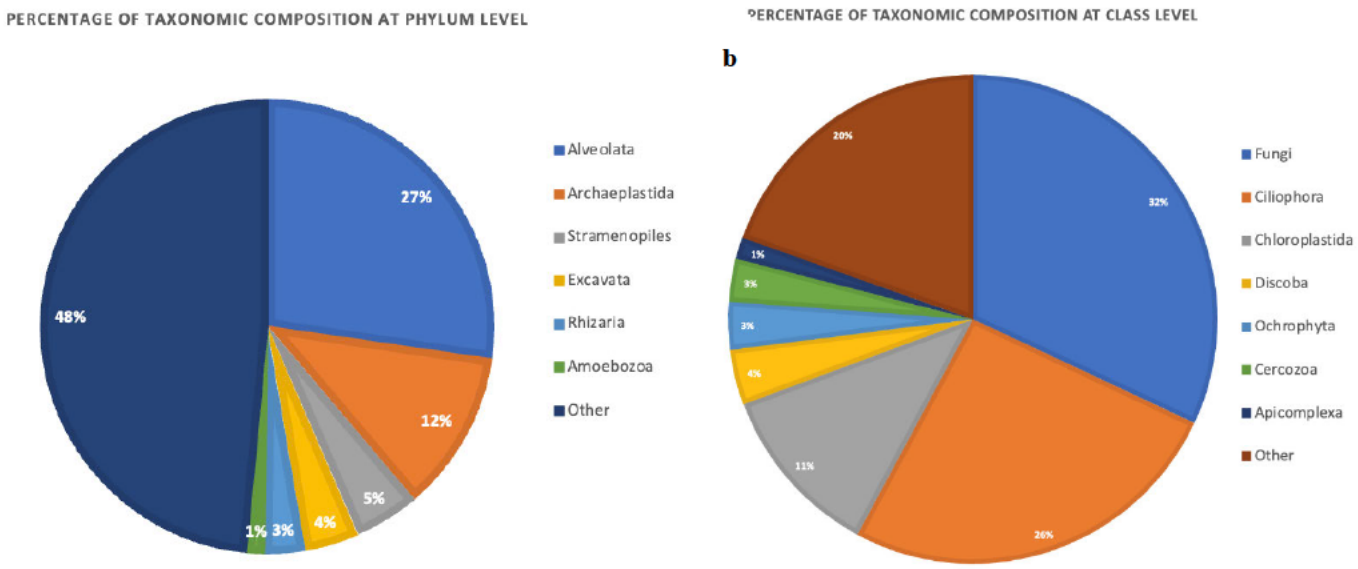


Figure S6(a-b). Percent abundance of taxa composition across all samples (**S6a**) at phylum and (**S6b**) class level.

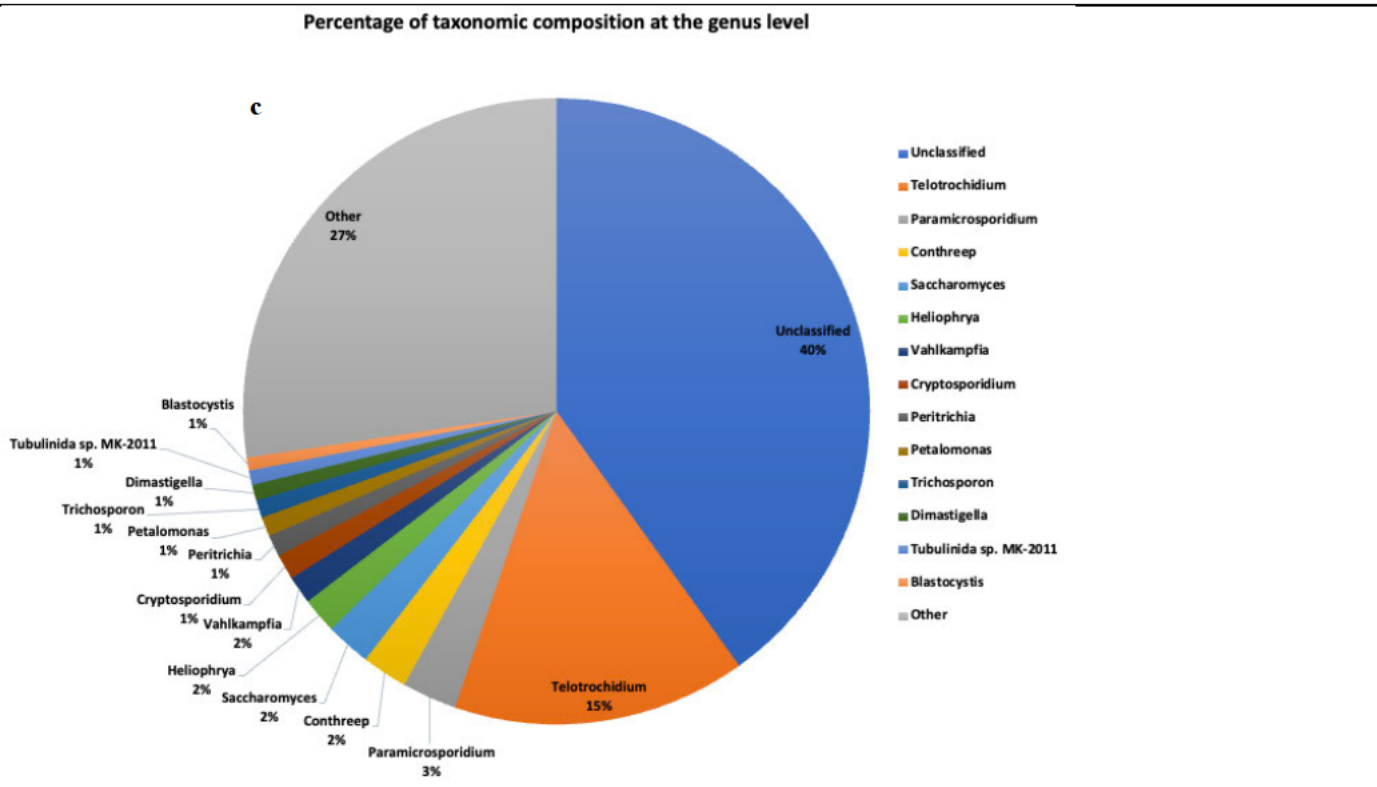


Figure S6c. Percent abundance of taxa composition at the genus level across all samples

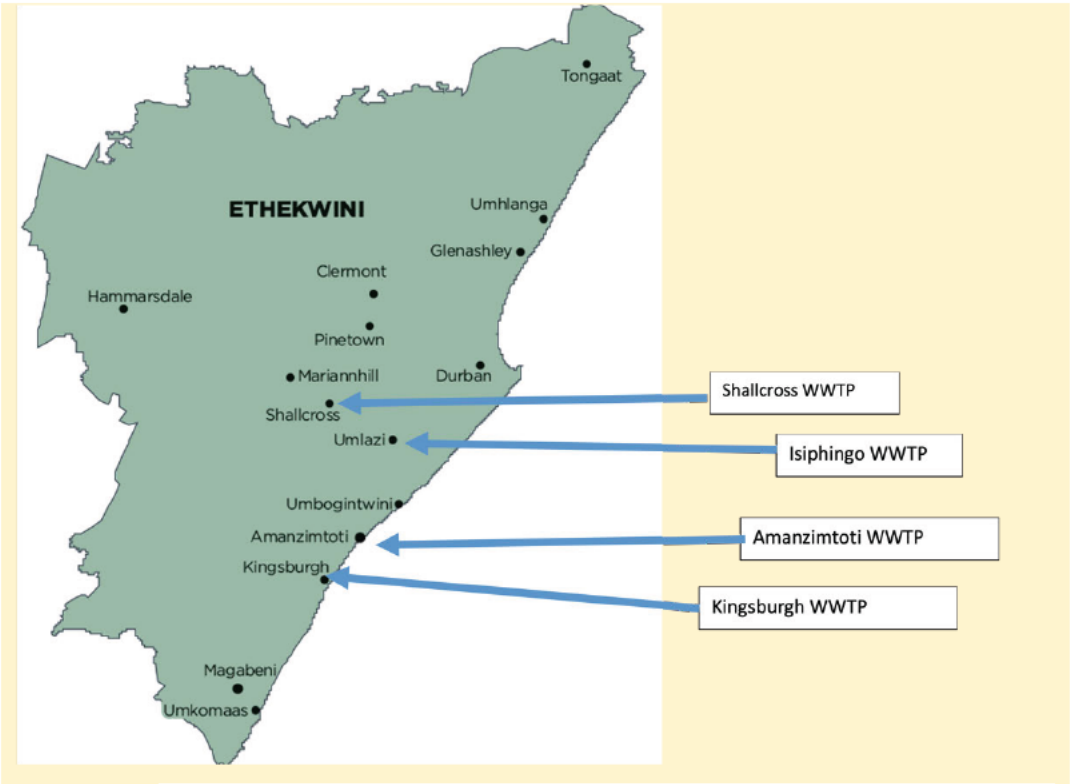


Figure S7. Map of South Africa indicating, eThekwini, KwaZulu-Natal province wastewater/surface water samples collection points.

9.2 Appendix 2

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Review

A review on application of next-generation sequencing methods for profiling of protozoan parasites in water: Current methodologies, challenges, and perspectives

N.P. Mthethwa [a,b], I.D. Amoah [a], P. Reddy [b], F. Bux [a], S. Kumari [a,*]

[a] Institute for Water and Wastewater Technology, Durban University of Technology, Durban 4000, South Africa
[b] Department of Community Health Studies, Faculty of Health Sciences, Durban University of Technology, Durban 4000, South Africa

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ABSTRACT

The advancement in metagenomic techniques has provided novel tools for profiling human parasites in environmental matrices, such as water and wastewater. However, application of metagenomic techniques for the profiling of protozoan parasites in environmental matrices is not commonly reported in the literature. The key factors leading to the less common use of metagenomics are the complexity and large eukaryotic genome, the prevalence of small parasite populations in environmental samples compared to bacteria, difficulties in extracting DNA from (oo)cysts, and limited reference databases for parasites. This calls for further research to develop optimized methods specifically looking at protozoan parasites in the environment. This study reviews the current workflow, methods and provide recommendations for the standardization of techniques. The article identifies and summarizes the key methods, advantages, and limitations associated with metagenomic analysis, like sample pre-processing, DNA extraction, sequencing approaches, and analysis methods. The study enhances the understanding and application of standardized protocols for profiling of protozoan parasite community from highly complexe samples and further creates a resourceful comparison among datasets without any biases.

1. Introduction

Intestinal protozoa are one of the major causes of parasite-induced diarrhea in healthy humans, animals, and immunocompromised individuals (Roth et al., 2018). Diarrheal diseases are the second leading cause of death in children under 5 years globally (CDC, 2015; WHO, 2017; CIDRAP, 2018). About 88% of diarrhea-associated deaths are attributable to treated or untreated water, inadequate sanitation, and insufficient hygiene (CDC, 2015; Kapwata et al., 2018). There are about 15,000 species of protozoan parasites reported around the globe (Omarova et al., 2018). Examples of waterborne protozoan parasites responsible for human infections include *Toxoplasma gondii, Cryptosporidium* spp., *Entamoeba hystolitica, Cyclospora cayetanensis, Isospora belli, Blastocystis homonis, Balantidium coli, Acantamoeba* spp., *Sarcostysis* spp., *Naegleria* spp., and *Giardia* spp. (Bridle, 2014; Garcia et al., 2017; Griffiths, 2017; Xiao et al., 2018; Ajonina et al., 2018). These parasites are major waterborne pathogens because they require low infectious doses, are chlorine resistant, and are very small (1–17 μm) (Widmer and Sullivan, 2012). Additionally, the (oo)cyst of these parasites are very resistant to environmental conditions and can persist in the environment for several months (Yaeger, 1996; Omarova et al., 2018). Therefore, a full understanding of the diversity of these parasites in the environment will give an insight into their infection pattern and potential measures that can be employed to reduce transmission.

Targeted molecular methods such as Sanger sequencing and traditional methods such as microscopic observation and enzyme/staining techniques have been useful for protozoan parasite detection (Miller et al., 2013). However, as stand-alone methods, they are very time-consuming, labor-intensive, and less specific (Ndao, 2009; Ahmed and Karanis, 2018; Adeyemo et al., 2018). Moreover, Sanger sequencing requires prior knowledge of the target sequence, can only analyze single fragments, and can only determine short sequence lengths (300–1000 bases) (Slatko et al., 2018; Dulanto Chiang and Dekker, 2019). Protozoan parasite species are closely related and host-specific, therefore, are not easily differentiated using conventional methods (Ricciardi and Ndao, 2015). For instance, DNA sequences of *Cryptosporidium parvum* and *Cryptosporidium hominis* are reported to be 95–97% identical (Widmer and Sullivan, 2012; Feng et al., 2018), which complicates

* Corresponding author.
E-mail address: sheenak1@dut.ac.za (S. Kumari).

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0167-7012/

differentiation. Additionally, some species possess very similar morphological characteristics. Therefore, when multiple species infect a host, they cannot be differentiated by traditional methods and may lead to misdiagnosis, e.g. *Entemoeba histolytica* and *Entemoeba dispar* are morphologically identical (Ricciardi and Ndao, 2015). Furthermore, (oo)cysts of the same species may show variation in morphology, such as oocysts of the *Eimeria* species (Vermeulen et al., 2016). Novel methods for the detection and profiling of protozoan parasites are thus in demand for understanding genetic diversity.

Metagenomics assisted by Next Generation Sequencing (NGS) technologies has been intensively applied in microbiome studies. It can be applied without prior knowledge of microbial communities present in a sample. It is not culture-dependent, enables detection of microbial communities at very low abundance in complex populations, and offers faster microbial assessment and recovery of novel species (Alves et al., 2018). Two main approaches have been employed for metagenomic profiling: (1) Deep amplicon metagenomics, also called Metaprofiling/amplicon-based sequencing, which involve Polymerase Chain Reaction (PCR) amplification of a target gene marker before NGS sequencing (Miller et al., 2013; Maritz et al., 2019), and (2) shotgun metagenomic approach which involves sequencing of total nucleic acid present in a sample by first shearing the DNA sequences into short fragments (Quince et al., 2017).

Despite, the widespread use of these different metagenomic techniques, there is limited application of metagenomics on eukaryotes, especially protozoan parasites, compared to prokaryotes (Wang et al., 2014; Maritz et al., 2017; Marzano et al., 2017; Maritz et al., 2019).

The lack of metagenomic studies on protozoan parasites has been attributed to the complexity of eukaryotic genomes, their large genome size, higher variability of some genes, and their presence in multiple copies. For instance, multiple gene copy numbers in the18S small subunit ribosomal DNA gene has been reported for *Toxoplasma gondii* (110 copies), *Cryptosporidium parvum* (5 copies) (Zahedi et al., 2018), and *Acanthamoeba castellanii* (600 copies)(Torres-Machorro et al., 2010). Moreover, eukaryotic genomes have a repetition of non-coding DNA sequences (Huang et al., 2016). All these factors contribute to the difficulty in developing accurate standard protocols for metagenomic profiling of protozoans, resulting in fewer studies, and limited complete genome sequences in reference databases.

Advances in NGS technologies such as sequencing longer reads (> 20 Kbp) (Vernet, 2017), simplified DNA sample preparation process, PCR free library preparation or less PCR induced errors, the requirement for less material and shorter workflows, has allowed for overcoming the shortcomings of conventional NGS platforms (Quail et al., 2012; Raza and Ahmad, 2019). For example, Pacific Biosciences's SMRT (3rd generation NGS) is single-molecule real-time sequencing technology, it provides significantly faster and longer read lengths (1000-15,000 bp) (Rhoads and Au, 2015), allows detection of nucleotide modification and highly accurate DNA sequence, which is typically not provided by other sequencing platforms (Lee et al., 2016; Pavlovic et al., 2020). The average reads of current Pacific Biosciences' instruments are close to 100 times longer than other NGS platforms such as Illumina, Ion Torrent, and Roch 454 (Raza and Ahmad, 2019).

Several factors could affect the NGS process for protozoan parasites. The first includes the lack of universal primers for the entire eukaryotes gene or protozoans for taxonomical classification and identification, making it difficult to monitor the entire population in a single assay using the 18S rRNA metagenomics approach (Demone et al., 2020). The smaller population of eukaryotes relative to the prokaryotes in mixed environmental samples could also affect the NGS process for protozoans (Lear et al., 2018). Furthermore, many parasites also lack completely sequenced and annotated reference genomes, restricting the use of DNA-based sequencing and reference mapping methods and leading to the lack of accurate and reliable reference databases (Marzano et al., 2017; Wylezich et al., 2020). Other factors include the presence of inhibitors in the environmental samples that could affect the isolation process or DNA extraction from protozoan oocysts (Felczykowska et al., 2015). The lack of a standardized way of sample processing, collection, and preservation make it difficult for comparison studies and to determine the appropriate metagenomic approach (Lear et al., 2018). This paper provides an overview of the current state of knowledge on the availability and application of NGS approaches in the profiling of protozoan parasites from a broad range of environmental matrices.

2. Methodology

The literature search was performed using the following keywords and search strings "Metagenomics OR next-generation sequencing OR Amplicon sequencing OR deep sequencing OR HiSeq/MiSeq Illumina OR Roche 454 OR Ion torrent OR Proton/PGM, SOLiD OR PacBio OR advanced Oxford nanopore AND "protozoans OR protozoan parasites OR *Giardia spp*, OR *Cryptosporidium* spp, OR *Blastocystis sp*, OR *Trichomonad* spp, OR *Toxoplasma gondii* OR *Entamoeba histolytica*" AND "environmental samples OR wastewater OR Water OR surface water OR soil. The databases searched were Web of Science, HINARI, Science direct, Google scholar, World Health Organisation, and United Nations Children Emergency Fund (UNICEF). The inclusion criteria considered articles published in English with no restriction on the country of origin. Articles focusing on clinical samples were excluded from the study.

3. Results

A total of 184 articles were retrieved (Fig. 1) using the search strategy outlined above. Nineteen articles contained information specific to the metagenomics profiling and 7 of these articles reported on shotgun metagenomics, and 15 on targeted /deep amplicon metagenomics. Out of the total articles retrieved, 48 were included for DNA extraction, sample processing, and preservation. Twenty-five (25) were included for bioinformatics analysis and NGS technologies.

3.1. Sample preparation: collection, preservation, pre-processing, nucleic acid extraction

3.1.1. Sample collection

Sample preparation before sequencing can significantly affect the quality, quantity, and accuracy of the metagenomic results (Thomas et al., 2012; Felczykowska et al., 2015). Different pre-processing techniques are optimized and validated, including sample collection, preservation, and DNA extraction (Felczykowska et al., 2015). The key reasons are to obtain a representative environmental sample, reduce the impact on the chemical and physical integrity of the sample, and minimize downstream inhibitors (Skotarczak, 2009). Most of the reviewed articles on metagenomics profiling did not mention the sample collection procedure they have followed to ensure a representative sample (Table 1). However, the two commonly used methods for environmental sample collection are grab (snap sample) and composite sampling methods (Ma et al., 2009; Dong, 2015). The grab sampling approach is a single discrete sample collected over a time not exceeding 15 min(EPA, 2017). It gives snap microbial information specific to the sampling area, location, and time and has been applied for the investigation of microorganisms and conditions in wastewater, surface water, and soil sample (EPA, 2017). The composite sampling approach, either by time composite sampling or by flow proportional sampling, consists of equal volume, discrete sample aliquots collected at constant time intervals, or constant sample volume at varying time intervals into one container (Ort et al., 2010). A composite sample reflects the average characteristics of the sample matrix during the compositing process (Dong, 2015). The suitability of a sampling method depends on the area and type of samples being investigated. However, Quince et al. (2017) recommended that shotgun metagenomic studies use composite sample collection to achieve an average representation of microbiome from the same habitat overtime (Quince et al., 2017). For wastewater samples, a

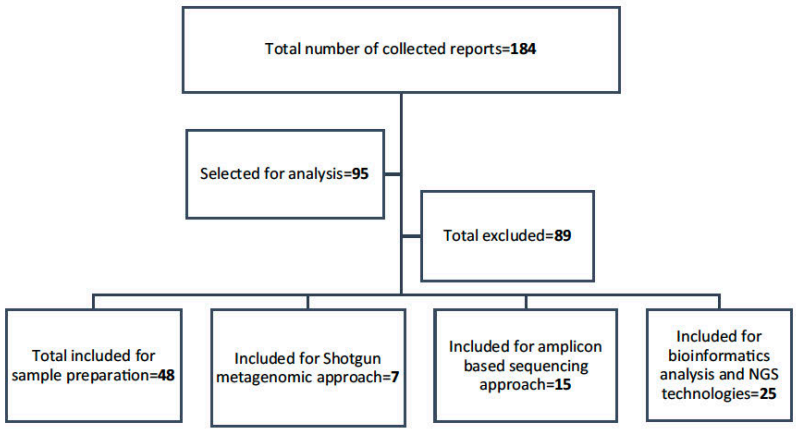

Fig. 1. Articles obtained through the search process.

24 h composite sample could provide a better representation of parasites entering and exiting a treatment plant as opposed to single snapshot samples. Despite this recommendation, there is no available data to determine the best sample collection method for metagenomic profiling of protozoan parasites in environmental samples. This area, therefore, needs more attention, when considering metagenomic studies for profiling of protozoan parasites from the environmental samples.

3.1.2. Sample preservation

Sample preservation is key to maintain the integrity or characteristics of the collected sample. There are currently several promising preservation methods available for short-term storage at 4 °C, ambient temperature, or in a cooler box (Crawley et al., 2016; Escotte-Binet et al., 2019; Zacharia et al., 2019) (See Table S4 Appendix I). This method is commonly used during transportation, on-field, and resource-limited laboratories (Lalonde and Gajadhar, 2016; Lear et al., 2018). The long-term preservation method includes storage at −20 °C, −80 °C, or −150 °C, and freeze-drying (Marquis et al., 2019; Ögren et al., 2020; Brumfield et al., 2020). Other preservation methods include the use of buffers or chemicals such as Potassium dichromate,10% formaldehyde, 70–95% ethanol, solitary use of silica gel beads, buffer DMSO-EDTA, DNA/RNA shield, etc. (Wilke and Robertson, 2009; Kuk et al., 2012; Papaiakovou et al., 2018; Lear et al., 2018). The DNA/RNA shield (Zymo Research, Freiburg, Germany) and the RNA*later* (ThermoFisher Scientific, USA) are frequently used to preserve the genetic integrity and expression profiles of samples at ambient temperatures (no refrigeration for short term storage), additionally, the buffers, DNA/RNA shield, and RNA*later* completely inactivate infectivity of the agents present in the sample (Menke et al., 2017, Plauzolles et al., 2020). Menke et al. (2017) investigated self-made nucleic acid preservation buffer (NAP) against commercial buffers DNA/RNAShield (Zymo Research, Freiburg, Germany) and RNA*later* (ThermoFisher Scientific, USA), together with freezing or storing at room temperature before sequencing. Results revealed that immediate freezing (−20 °C) of samples yielded the best results. Moreover, the self-made nucleic acid preservation buffer (NAP) had better preservation qualities, cheaper, and indicated applicability in microbiome studies than commercial RNA*later* and DNA/RNA Shield. Other studies recommended to keep the sample at a cool temperature and perform DNA extraction within 48 h or as soon as possible after collection (Borneff-Lipp and Duerr, 2013; Felczykowska et al., 2015; Lear et al., 2018; Wylezich et al., 2020).

3.1.3. Sample concentration

Concentration of protozoan (oo)cysts from environmental samples is needed to improve the chances of accurate representation of a target population. Moreover, protozoans have a high spatial heterogeneity, t low population density relative to the sample area and compared to bacterial (prokaryotes) population (Lear et al., 2018). The standard biomass concentration methods for protozoan parasites include flotation, such as sucrose gradient flotation/ficoll gradient separation, which is based on the density of the species being targeted (Babaei et al., 2011; Andersson et al., 2015; Gallas-Lindemann et al., 2016; Escotte-Binet et al., 2019). These methods are commonly applied for the concentration of *Cryptosporidium* or *Giardia* (oo)cysts (Lora et al., 2016; Sammarro Silva and Sabogal-Paz, 2020). However, foreign materials in the environmental samples, of similar density and size to (oo)cysts of interest, can attached to the (oo)cysts or concentrated along with these (oo)cysts and may lead to loss of parasites or carry-over contamination (Al-Sabi et al., 2015; Sammarro Silva and Sabogal-Paz, 2020). Other concentration methods commonly used are centrifugation, under different centrifugal forces and time (Felczykowska et al., 2015; Hendriksen et al., 2019). Filtration has also been successfully used for the concentration of protozoan parasites. This involves concentrating protozoans by passing through different filter pore size filters, ranging from 0.1µm to 0.22 µm (Almeida et al., 2015; Brumfield et al., 2020). Additionally, some studies apply both centrifugation and filtration on the same sample (Bridle et al., 2010; Mahmoudi et al., 2015). Based on the reviewed literature, commonly used methods included filtration and centrifugation and are thus recommended for the concentration of protozoans in environmental samples.

3.1.4. Nucleic acid extraction

Different DNA extraction methods have been reported in the literature, as presented in Table 1 and Fig. S3 (Appendix I), these include both commercial DNA extraction kits and custom DNA extraction protocols . These include the phenol-chloroform extraction method (Babaei et al., 2011; Mahmoudi et al., 2015), salting-out method (Sun, 2010), and modified UNEX protocol (Shields et al., 2013; Moreno et al., 2018), which are the most commonly used to isolate and extract protozoan DNA from wastewater, surface water/irrigation water, and fecal samples. In addition, various commercial DNA extraction kits were also employed with varying efficiency (Almeida et al., 2015; Gallas-Lindemann et al., 2016; Shin et al., 2018; Javanmard et al., 2018; Maritz et al., 2019; Zahedi et al., 2019; Marquis et al., 2019; Brumfield et al., 2020; Rusiñol et al., 2020). Among the different kits used, QIAamp Fast DNA Stool

Table 1
Commonly used methods for environmental sample preparation reported in the literature.

Sample type	Sample processing	DNA extraction method	Comment	Reference
1. Surface, irrigation, and wastewater effluent	Centrifugation, IMS-IFA	UNEX protocol	Both Illumina and qPCR analysis from the sample showed no PCR inhibition	(Moreno et al., 2018a)
2.Wastewater sludge, soil, stormwater, and sediments	N/A	Powersoil DNA extraction kit	The study concluded that the DNA extraction method used (bead-beating) may have been insufficient to break open *Cryptosporidium* oocysts	(Maritz et al., 2019)
3. Wastewater samples	Filtration (0.2 μm)	PowerWater Sterivex DNA	The DNA extraction efficiency in the present study was unknown.	(Zahedi et al., 2019)
4.Oocysts	N/A	QIAamp DNA Mini Kit	3–5 oocysts per gram of produce were reliably detected using the optimized isolation methods and qPCR MCA.	(Lalonde and Gajadhar, 2016)
5. Oocysts spiked in berries	N/A	PowerSoil kit	PowerSoil kit was the method of choice for extraction of DNA of coccidian oocyst and detection by using TaqMan probe qPCR protocols.	(Temesgen et al., 2020)
6. Soil sample	0.1% Tween80/PBS for dispersion, sucrose flotation, mechanical grinding,	Fast DNA spin kit	The best protocol used 0.1% Tween 80, sucrose gradient, and FastPrep DNA extraction. It accurately detects T. gondii DNA in soil samples detection limit below 1 oocyst/g of fresh soil.	(Escotte-Binet et al., 2019)
7. River oyster sample	N/A	Omega Biotek E.Z.N.A. Tissue Kit	N/A	(Marquis et al., 2019)
8. Surface Water samples	Filtration using Envirocheck HV (Pall Gelman Laboratory) capsule	DNeasy PowerSoil	Recovery efficiency: Of the 39 samples analyzed 7.7% (3/39) were positive for *T. gondii*, and the occurrence of the oocysts was detected in 30% (3/10).	(Galvani et al., 2019)
9. Oocysts of *Cryptosporidium* and *Giardia*	N/A	DNeasy Blood and tissue kit	*C. parvum*, *G. lamblia*, and *C. cayetanensis* specifically detected each parasite signal without interference or non-specific signal	(Shin et al., 2018)
10. Wastewater and vegetables	Filtration (pore size 0.4 μm) and centrifugation	YTA DNA extraction kit for stool	N/A	(Javanmard et al., 2018)
11.Wastewater samples	Centrifugation and filtration (0.22 μm)	DNA isolation kit	N/A	(Ajonina et al., 2018)
12. Wastewater, surface waters, groundwater, drinking water	Filtration, flocculation, and centrifugation, and sucrose density gradient	QIAamp Mini Kit	*Cryptosporidium* spp. And *Giardia* was detected in samples (by IFA, nested PCR, and by LAMP without any inhibitors	(Gallas-Lindemann et al., 2016)
13. Treated tap water sample	Ultrafiltration-centrifugation for secondary concentration	Biofire Diagnostics 1–2-3 SWIPE Sample Purification	Alternative DNA extraction buffer, second spin column cycle, Tris–EDTA buffer enhanced detection sensitivity for *Cryptosporidium*	(Kimble et al., 2015)
14. Raw and treated water	Membrane filtration technique. Cellulose acetate membranes that had a 47 mm diameter and 1.2 μm porosity	NucleoSpin Tissue	The sequencing showed *Cryptosporidium parvum* and *Giardia duodenalis* DNA	(Almeida et al., 2015)
15. River water	Filtration (0.45 μm) Then centrifugation	Phenol-chloroform method and QIAamp DNA Mini Kit	N/A	(Mahmoudi et al., 2015)
16. Water samples spiked with c.parvum oocysts	Liquid nitrogen	QIAamp DNA Tissue Mini Kit +liquid nitrogen	Extraction of DNA from *C. parvum* oocysts was most effective when it was preceded with cycles of liquid nitrogen/water baths incubation and with the use of lysate buffer and overnight proteinase K digestion. This resulted in a higher copy number of the 18S rRNA gene	(Adamska et al., 2012)
17. Water	Centrifugation and filtration (25 mm GF/C Whatman glass microfibre filter)	MasterPure Complete DNA and RNA extraction kit (Epicentre)	N/A	(Bridle et al., 2010)
18. Drinking water	Filtration 0.6 μm,0.2 μm and 0.1 μm pore size pore size)	ZymoBIOMICSTM DNA Miniprep Kit	N/A	(Brumfield et al., 2020)
19.Drinking water, reservoir water, groundwater, and river water	Standardized SMF protocol	Fast DNA SPIN Kit for soil	N/A	(Rusiñol et al., 2020)
20. Urban sewage water	Centrifugation	QIAamp Fast DNA Stool Mini kit and Nucleospin RNA XS kit	N/A	(Hendriksen et al., 2019)
22. Water samples	Filtration (47 mm, 0.45)	PowerWater DNA isolation kit	DNA sequences indicated the presence of both Eukarya and Bacteria in the river water community, genera that are of potential concern to human health, including *Acanthamoeba, Leishmania, Candida, and Rhizomucor*, were identified in the analyses	(Hamner et al., 2019)

Mini kit, Powersoil, and Powerwater DNeasy extraction kits were mostly used for metagenomic studies from environmental samples. Their application revealed simplicity of the protocols, fast speed on overall metagenomic workflow, and high-quality DNA with no inhibitor and carryover interferents, moreover, the protocols allowed for a larger number of samples to be analyzed at a minimum time (Hamner et al., 2019; Hendriksen et al., 2019; Zahedi et al., 2019; Maritz et al., 2019).

It is often difficult to break the robust protozoan parasite's oocysts and to release the DNA (Sánchez et al., 2018; Wylezich et al., 2018). Therefore, to improve the DNA extraction efficiency, a variety of pre-treatment methods have been employed by different researchers, such as freeze-thaw, bead beating (Felczykowska et al., 2015; Wylezich et al., 2018), and addition of chemicals (such as ethylene diamine tetraacetic acid (EDTA), protease K, NaCl, NaH_2PO_4, and Na_2HPO_4) (Fang et al., 2014). In some instances, liquid nitrogen has been used to achieve rapid freezing of the samples to enhance the extraction of DNA (Mahmoudi et al., 2015). It is recommended that for each sample type, pretreatment and DNA extraction protocols are optimized, because the optimized method for one sample may not work for the other. Several studies have focused on the optimization of DNA extraction methods for protozoan

parasites from samples such as stool, water, soil, green leaves, fruits, and urine (Lalonde and Gajadhar, 2016; Barbosa et al., 2017; Ackerman et al., 2019; Temesgen et al., 2020). For instance, Psifidi et al. (2015) compared 11 DNA extraction methods for optimum whole-genome analysis, these included commercial kits, modified commercial extraction protocols, and a custom magnetic beads protocol. Three of the modified commercial kits (Nucleospin Blood, nucleospin tissue, and Dx method) and the custom magnetic beads protocol indicated suitability for longer storage and high-throughput analysis. The modifications made for commercial kits included increased volume of lysis buffer, protease K, and an increased incubation time with protease K. Another significant processing step required before sequencing is cellular-host DNA removal (Miller et al., 2013; Pereira-Marques et al., 2019). This is especially important for environmental samples that are often associated with human DNA (Lear et al., 2018). Fractionation and selective lysis are common methods that have been used for the reduction of host DNA (Thomas et al., 2012; Oyola et al., 2013). However, based on current literature, there is no data to support a particular method for human host DNA degradation. Purification of extracted DNA is an additional step required before NGS analysis, as it removes inhibitors and chemicals left after DNA extraction to increase the efficiency and sensitivity of sequencing and analysis. DNA binding silica column purification is one such method used, it is quick, simple, and efficient (Ackerman et al., 2019). However, it increases overall expenses, significantly reduces the DNA concentration, as some of the DNA gets trapped in the silica column, therefore a higher starting DNA concentration is always recommended (Andersson et al., 2015). Literature on the application of purification methods before further analysis is limited, this makes it difficult to ascertain the best purification method for metagenomic profiling of protozoan parasites.

3.2. Metagenomics approach

Both targeted and nontargeted (shotgun) sequencing approaches have been used to study protozoan diversity. In the nontargeted sequencing approach, all microbial genomes present in a sample are first sheared into smaller fragments before sequencing on NGS platforms. This results in output sequence data of all genes from all domains of life (Miller et al., 2013; Lear et al., 2018). The targeted sequencing, on the other hand, is referred to as the second metagenomic approach; however, it is not entirely meta since it does not sequence all the microbial genomes present in a sample at the same time; instead, it is targeted to a single marker gene (Quince et al., 2017; Kibegwa et al., 2020). Thus, this approach employs taxon-specific primers for sequencing predefined domains of organisms in complex environmental samples, for example in the case of protozoans the 18S rRNA gene is the preferred area of interest (Ryan et al., 2017).

3.2.1. Shotgun metagenomics

Several published articles were retrieved on the shotgun metagenomic approach, focusing on the environmental microbiome (Table 2). This approach has enabled successful investigations of protozoan diversity associated with sewage water in both urban (Maritz et al., 2019) and rural settlements (Hendriksen et al., 2019). It has also been used in a comparative analysis of the drinking water microbiome (Brumfield et al., 2020), identification of pathogens in river water (Hamner et al., 2019) as well as an assessment of microbial diversity in marine ecosystems (Kisand et al., 2012). These studies reported a diverse protist community dominated by free-living clades, seasonal and host differences in protist composition in urban sewage (New York, USA), and revealed a significantly increased abundance and variations in *Cryptosporidium* spp. and *Giardia* spp., in informal settlement sewage (Kenya) over time (Ryan et al., 2017). A shotgun sequencing approach was also used to analyze environmental microbiome (bacteria, eukaryote, virus, and archea) in water samples of a coastal region, with the results revealing a very low recovery rate of only 2.9% of eukaryotes in the study data compared to bacterial diversity (Kisand et al., 2012).

Few other studies used a combination of the different sequencing platforms to get a better representation of the diversity and functional properties of the protozoan community in an ecosystem. For example, the pathogenic microbiome of the world's largest water reuse facility, Orange County Water District (OCWD) Advanced Water Purification Facility (AWPF), was investigated by a combination of shotgun metagenomics, transcriptomics and amplicon-based approaches to provide a novel in-depth characterization of wastewater from influent to the final product (Stamps et al., 2018). This study showed that protists were in low abundance, representing less than 1% recovered metagenomic or metatranscriptomic sequence data. A combination of different methods in a single run could offer both in-depth higher resolution of targeted genes and comprehensive primer-bias-free analysis of the dominant genes present in a sample (Lear et al., 2018). This approach, therefore, is becoming popular to researchers, as it is considered simpler due to the use of the same workflow techniques, same sample, and protocol simultaneously. (Lear et al., 2018). Moreover, these methods can be complementary to each other, the limitation of one method can be overcomed by the other method.

As per the reviewed literature (Table 2), The shotgun approach shows a strong benefit in its applicability for protozoan parasite detection due to the following reasons: (1) A single sequencing-based test is used to capture all taxa present within a sample, providing a single output dataset on the microbial diversity and functional novelties associated with that environment (Quince et al., 2017). (2) the approach works without an amplification stage, enabling identification of all eukaryotes and by-passes the biases usually found in amplicon-based sequencing (Wylezich et al., 2019; Wylezich et al., 2020). Another advantage of the untargeted metagenomics approach is that no prior decision is needed for which protozoan specific gene marker or region to screen. However, further research studies and tools are needed to improve protozoan gene resolution for better taxonomic assignments of sequences from metagenomic data.

3.2.2. Amplicon-based metagenomics/ deep amplicon sequencing/ targeted metagenomics/ metaprofiling

Amplicon-based metagenomics include/involve several steps to analysis. The first step begins with designing PCR primers that target specific conserved gene regions or gene fragments. For example, protozoan studies often target the conserved 18S rRNA gene (Miller et al., 2013, Tanaka et al., 2014, Dulanto Chiang and Dekker, 2019), the whole intergenic transcribed spacers (ITS), or the large ribosomal subunit (LSU)/28S rRNA) gene (Uyaguari-Diaz et al., 2016). These targets are then used in PCR amplification to generate DNA sequences (amplicons). The generated amplicons are sequenced using NGS technologies. And lastly, resulting sequences are compared to a reference database using bioinformatic platforms for species and genus identification (Miller et al., 2013).

In addition, this approach allows for targeting multiple genes of different target regions to be studied simultaneously in a single reaction to achieve high coverage of protozoan parasites (Uyaguari-Diaz et al., 2016). In complex samples, such as environmental samples, this approach can provide even greater details of protozoan parasites without being dominated by other highly abundant microbes (Miller et al., 2013). This highly targeted approach enables researchers to efficiently discover, validate, and screen genetic variants within a specific genome (Dulanto Chiang and Dekker, 2019). It reduces sequencing costs and the intensive bioinformatics analysis task compared to broader approaches such as the shotgun metagenomic approach (Dulanto Chiang and Dekker, 2019).

The majority of reported studies captured in Table 2 used the deep amplicon-based metagenomic approach for analyses of protozoan parasite diversity from different environmental matrices, usually targeting the 18S rRNA region. The 18S rRNA gene marker has nine variable regions (V1 to V9), which are commonly used for diversity studies

Table 2
Metagenomic studies focused on environmental samples, reported in the literature.

Sample type	Metagenomic approach	Sequencing-platform	Protozoan parasite	Comments	Reference
1.Surface irrigation water and wastewater	18S rRNA gene amplicon sequencing: V4 hypervariable region	Illumina MiSeq	*Toxoplasma gondii, Entamoeba histolytica, Cryptosporidium spp. Acanthamoeba castellanii, Giardia intestinalis, Blastocystis sp*	NGS methodology developed for identification of most important WPPs	(Moreno et al., 2018a)
2.Raw sewage, soil, stormwater, and sediments. 3.Raw sewage, soil, stormwater, and sediments	18S rRNA gene amplicon sequencing: V4 and V9 hypervariable region Shotgun metagenomic	Illumina HiSeq Rapid Run	Protist community (oligohymenophorea and ciliates) *Entamoeba* *Blastocystis*	Sewage contained a diverse protist community dominated by free-living clades, Seasonal differences in protist composition were observed, human and animal associated protist was also detected	(Maritz et al., 2019)
4.Wastewater samples (influent to effluent)	Small subunit ribosomal RNA (SSU rRNA) gene sequencing (V4 hypervariable region), metagenomics, and metatranscriptomics	Illumina MiSeq and -Illumina HiSeq	*Acanthamoeba mauritaniensis, Acanthamoeba palestinensis* and more)	Provided the first in-depth characterization of water at a multibarrier potable reuse facility, from influent to final product water.	(Stamps et al., 2018)
5.Wastewater treatment plant samples (influent, intermediate and effluent)	Amplicon sequencing: 18S ribosomal DNA (V9) hypervariable of eukaryotic *Cryptosporidium*-specific 18S (V3 & V4) primers sequencing	Illumina MiSeq	*Cryptosporidium* species (zoonotic) *Blastocystis sp.* STs, *Endolimax* spp., *Entamoeba spp.*and *Iodamoeba* spp.	Eukaryotic V9 18S NGS had inadequate sensitivity for intestinal parasite detection in wastewater samples and six were identified by *Cryptosporidium*-specific NGS	(Greay et al., 2018)
6.Raw wastewater treatment sample	Amplicon based sequencing: 18S locus	Illumina MiSeq	*Cryptosporidium* species	*Cryptosporidium* was prevalent in the raw influent and a large diversity of *Cryptosporidium* species and genotypes was revealed.	(Zahedi et al., 2018)
7.Petroleum contaminated water samples	18SrRNA SEQUENCING	Illumina MiSeq	*Naegleria, Vorticella Arabidopsis, Asarum Populus, Naegleria, Colpoda.*	Results demonstrated the ability of protozoa (61.90–62.04%) to adapt and survive in petroleum oil-polluted water sites.	(Kachienga et al., 2018)
8.Water samples (of coastal regions of the The Mediterranean Sea)	Whole-genome sequencing	Roche 454 Genome Sequencer FLX	Archea-0.3 Bacteria-96.2 Eukaryotes-2.9 Viruses −0.5 Other = 0.2	Analysis of the metagenomes revealed significant differences in both microbial diversities and abundance between the two areas, reflecting their distinct ecological habitats and anthropogenic stress conditions	(Kisand et al., 2012)
9.Raw sewage samples from a private apartment building	Amplicon-based sequencing:18S. rRNA marker gene, V4 and V9	Sanger Sequencing And Illumina MiSeq	*Cryptosporidium parvum, Giardia intestinalis, Toxoplasma gondii*, and *Trichomonad spp.*	A workflow for the detection and analysis of protists in sewage samples, with a focus on zoonotic and trichomonad taxa, based on high throughput. amplicon sequencing of existing 18S rRNA markers was experimentally validated	(Maritz et al., 2017)
10.Freshwater samples	Deep amplicon sequencing-viruses (g23 and RdRp), bacteria (16S rRNA and cpn60), and eukaryotes (V1–V3 regions of the 18S rRNA gene and internal transcribed spacer (ITS1/ITS2)	Illumina MiSeq	Eukaryotes (Chlorophyta, Arthropoda, Streptophyta, Chytridiomycota, Apicomplexa, Nematoda, and Chordata), bacteria, and viruses)	The study developed a systematic approach to separate and characterize eukaryotic-, bacterial and viruses	(Uyaguari-Díaz et al., 2016)
11.Samples-freshwater, coastal, and wastewater	Amplicon sequencing (Full-length 18S rRNA gene sequences) (v8-v9,v4)	Illumina MiSeq	N/A	1.V8-V9 region provided the highest accuracy of the selected mock community as measured through a mean relative abundance and beta-diversity measurements 2. V4 and V8-V9 regions showed similarities overall representations of environmental samples and trade-offs between hypervariable regions	(Bradley et al., 2016)
12.Drinking water	Shotgun metagenomics	Ion S5 XL Semiconductor Sequencer	Protozoa (*Acanthamoeba mauritaniensis* and *Acanthamoeba palestinensis*	Reported the complete microbiome (bacteria, viruses, fungi, and protists)	(Brumfield et al., 2020)
13.Sewage, drinking water, reservoir water, groundwater, and river water	Amplicon sequencing targeting the hypervariable V4 region of the 18S rRNA gene	Illumina MiSeq platform.	*Acanthamoeba* spp., *Entamoeba coli, E. dispar, E. moshkovskii, Naegleria fowleri, N. australiensis, N. clarki* and members of the *Hartmannellidae* family	Underestimated protozoan by metagenomic approach compared to bacteria and virus	(Rusiñol et al., 2020)
14.Urban sewage	Shotgun metagenomic sequencing	Illumina HiSeq (bacterial and	*Giardia* spp., *Plasmodium* spp., *Ascaris* spp., and *Blastocystis* spp.	Data obtained from urban sewage illustrated the potential for this	(Hendriksen et al., 2019)

(*continued on next page*)

Table 2 (*continued*)

Sample type	Metagenomic approach	Sequencing-platform	Protozoan parasite	Comments	Reference
		parasitic DNA) and MiSeq (DNA and RNA viruses)	were the most abundant parasites throughout the study period	method to be used for future public health disease surveillance	
15. Water samples	Shotgun metagenomic sequencing	MinION sequencing platform (Oxford Nanopore)	N/A	Bacteria were more abundant compared to waterborne human pathogenic Eukaryotes	(Hamner et al., 2019)
16.Ten single-cell genomes	Full-genome sequencing.	Illumina MiSeq	*Cryptosporidium*	demonstrated the power of applying single-cell genomics to dissect infectious disease caused by closely related parasite species or subtypes	(Troell et al., 2016)
17.Soil metazoa: microarthropods, enchytraeids, earthworms and nematodes	Amplicon sequencing-SSU rDNA—18S rDNA: V1–V3 region	Roche GS FLX 454	Amoebae, flagellates, ciliates	Ciliates Were generally over-represented in sequence numbers, while many amoeba and flagellate taxa were under-represented	(Geisen et al., 2015)
18. *Eimeria* oocysts	18S rRNA locus targeting Eimeria (hypervariable V4 region of the 18S rRNA locus)	Illumina MiSeq platform.	*Eimeria*	The study developed a data analysis pipeline for community analysis of eukaryotic organisms using *Eimeria* communities as a model. OTU assignment at at a lower threshold (95%) there was greater resolution between OTU consensus sequences	(Vermeulen et al., 2016)
19.Pre-diagnosed protozoan oocysts	Untargeted RNA metagenomics sequencing	Sequenced on the Ion Torrent S5XL platform	Protists and helminths	Demonstrated the applicability of untargeted RNA metagenomics for the parallel detection of parasites	(Wylezich et al., 2020)

(Hadziavdic et al., 2014). Analysis of water and wastewater samples has been conducted using 18S rRNA looking at different regions from V1-V9 within the taxonomic marker (Tanaka et al., 2014; Cooper et al., 2016; Popovic et al., 2018; Moreno et al., 2018). The commonly targeted regions of the 18S rRNA gene are the V4 (Moreno et al., 2018a, Stamps et al., 2018), V9 (Greay et al., 2018; Maritz et al., 2019), and V5 (Popovic et al., 2018) variable regions. However, the V4 region is the longest and the most conserved variable region within 18S rRNA, therefore it has been considered to have the highest resolution for protozoans (Hadziavdic et al., 2014; Pawlowski et al., 2016; Lear et al., 2018). Targeting of this region has led to the detection of sequences of *Toxoplasma gondii, Entamoeba histolytica, Cryptosporidium* spp. *Acanthamoeba castellanii, Giardia intestinalis, Blastocystis* spp. (Moreno et al., 2018). Several other studies looked at the same 18S rRNA region V4 to analyze parasites in water samples and detected *Entamoeba, Blastocystis, Acanthamoeba* spp., *Cryptosporidium parvum, Giardia intestinalis, Toxoplasma gondii, and Trichomonad* spp. (Bradley et al., 2016; Maritz et al., 2017; Stamps et al., 2018; Maritz et al., 2019).

Other studies have combined various regions within the 18S rRNA gene to achieve high species resolution. For example, water and wastewater-associated microbiome were investigated using the V9,V3 and V4 regions within the *Cryptosporidium* 18S rRNA gene (Greay et al., 2018). Results revealed that the eukaryotic V9 region had inadequate sensitivity for intestinal parasite detection in wastewater. Other studies analyzed water and wastewater protozoan parasite diversity by examining both V9 and V4 regions of the 18S rRNA to understand the protist pattern in sewage water (Maritz et al., 2019) and for characterization of the zoonotic and *Trichomonad* taxa (Maritz et al., 2017). The results revealed the mean relative abundance detected by both taxon to be 12.5% V4 and 15.4% V9. The V4 probe detected mostly common soil protist and V9 detecting kinetoplastids, additionally, intestinal parasites of vertebrates were present in low abundance. However, they further reported that the 18S rRNA regions selected did not provide a fine-scale zoonotic taxonomic resolution and could not define closely related species of *Trichomonas* spp. Bradley et al. (2016) investigated the effect of amplification biases on microbiome structure and diversity in freshwater, coastal, and wastewater samples using the V4 and V8-V9 regions. Results revealed V4 and V8-V9 regions had similar microbial community representations. Although the V9 section of the 18S rRNA gene is commonly used for the analysis of protists diversity (Ramirez et al., 2014), Lear et al. (2018) mentioned that this region tends to include organisms from other taxa. The combination of these variable regions of the 18S rRNA gene for the detection of protozoan parasites in soil and fecal samples has also been reported (Geisen et al., 2015; Cooper et al., 2016; Vermeulen et al., 2016).

3.3. DNA sequencing platforms used for metagenomic profiling of protozoan parasites

Next-Generation Sequencing systems facilitate deep amplicon sequencing and shotgun metagenomic studies by enabling massive parallel sequencing reactions and analysis of all DNA molecules in a sample. Different systems are employed by NGS including Roche 454, Pacific Biosciences, Ion Torrent, Illumina/Solexa, and Oxford Nanopore (Thomas et al., 2012). They all follow the same workflow of library preparation, sequencing, and raw data output (Escobar-Zepeda et al., 2015). The difference between the mentioned platforms is the sequencing methods listed in Table 3 which are: pyrosequencing, sequencing by synthesis, sequencing by ligation, and ion semiconductor sequencing (Kunin et al., 2008; Thomas et al., 2012; Ambardar et al., 2016; Goodwin et al., 2016). The selection of the appropriate NGS platform for sequencing depends on the metagenomic approach, questions being asked, costs, and genome length. Illumina platforms, for example, produce short read lengths, while PacBio and nanopore platforms produce longer read lengths, resulting in larger fragments, making bioinformatics easier (Shokralla et al., 2012; Ambardar et al., 2016). Therefore, PacBio and nanopore sequencing platforms can be advantageous when searching for larger genomes such as eukaryotes, however, Illumina could function best with species with smaller genomes, such as prokaryotes and short genome regions.

The Illumina MiSeq platform is also commonly used to sequence combinations of variable regions in the 18S rRNA gene targeting protozoans. For example, it was used to sequence the V4-V5 (Popovic et al., 2018) and V1-V3 hypervariable regions (Maritz et al., 2017; Moreno et al., 2018; Stamps et al., 2018; Greay et al., 2018; Zahedi et al., 2019). Illumina MiSeq was also used as a sequencing platform of choice for the development of a novel detection method for protozoans targeting the V9 region (200 bp) and analysis of the protozoan community in other

Table 3
High throughput sequencing platforms.

NGS methods	Systems	Advantages	Disadvantages	Read length (bp)	Reference
Pyrosequencing	Roche Gs Titanium x	-Long read length compared to Sanger. -Short run time	-Inaccurate homopolymer sequencing -High error rate - High cost	500–1000	(Shokralla et al., 2012, Miller et al., 2013, Ambardar et al., 2016)
Sequencing by synthesis	Illumina (HiSeq and MISeq)	-Overcome homopolymer due to terminator nucleotides/accurate sequencing of homopolymer regions. -High output /run compared to pyrosequencing. -relative short-read length because of optical signal decay and dephasing. -Low cost	-Increased error rate with increased length -Long run time -short read	36–250(HISeq), 150 (MiSeq)	(Shokralla et al., 2012, Miller et al., 2013, Ambardar et al., 2016, Raza and Ahmad, 2019)
	Pacific Biosciences SMRT DNA sequencer	-Non-PCR based-3rd generation. -Fast run time -Long read	-High DNA input -High error rate -expensive	2000–15,000	
	Oxford Nanopore-MinION	-Non-PCR based-3rd generation. -Fast run time -No sample preparation	N/A	48,000	
Sequencing by ligation	SOLiD sequencer Applied biosystem 5500 xl genetic analyzer.	-Overcome homopolymer due to terminator nucleotides/accurate sequencing of homopolymer regions -High output /run compared to pyrosequencing. -Oligonucleotides used instead of DNA polymerase	-Short reads -Increased error rate with increased length	N/A	(Huang et al., 2012, Liu et al., 2012)
Ion semiconductor	Life Technologies Ion Torrent	-Fast run time -Cost-effective and time-efficient.	-High error -Short read length	200–400	(Bragg and Tyson, 2014, Fujimoto et al., 2014)

environmental sample targeting the V4 and V5 regions (Audebert et al., 2016; Vermeulen et al., 2016; Hino et al., 2016; Kounosu et al., 2019). Target regions obtained were not more than the maximum read length for MiSeq of (2 × 300 bp), and total throughput of 1.5–2 Gb per run (Shokralla et al., 2012). Illumina NextSeq has a larger output and a read length (2x150bp) shorter than MiSeq (2x300bp) (Ambardar et al., 2016).

Illumina HiSeq platform is also used for shotgun metagenomics analysis, for example, it was used for whole community sequencing of Kenya urban sewage to monitor for the presence of protozoan pathogens (Hendriksen et al., 2019). The results indicated that pathogenic parasites had significantly higher and increasing read abundances over time. Furthermore, the HiSeq X has the highest throughput of 800-900GB, can sequence the whole genome within a day (Shokralla et al., 2012; Goodwin et al., 2016; Lokmer et al., 2019).

Ion Torrent platform employs the sequencing by detection of hydrogen ion and offers single read length-end of up to an average of 400 bp with 1–1.78% error rate (Zhang et al., 2015; Goodwin et al., 2016). Moreover, the system has a fast run time of 2-7 h (Glenn, 2011; Goodwin et al., 2016). This high throughput sequencing platform was used for comparative analysis of the complete water microbiome (Brumfield et al., 2020) and DNA of opportunistic plant and animal pathogens was identified including the protozoans, *Acanthamoeba mauritaniensis* and *Acanthamoeba palestinensis*.

MinION platform (produced by Oxford Nanopore technologies) is one of the real-time, third-generation sequencing technologies together with Pacific Bioscience and is commonly used for shotgun metagenomics. Moreover, they are non-PCR-based methods and generate long reads (Miller et al., 2013; Brown et al., 2017). MinION sequencer is one of the most advanced novel platforms and has been used for identification of pathogenic microbial contamination of waterways (Hamner et al., 2019), for sequencing of the protozoan parasite, *Trypanosoma cruzi* (Díaz-Viraqué et al., 2019), and demonstration of a novel diagnostic assay for malaria (Imai et al., 2017). MinION nanopore is the first portable sequencing device, thus it is applicable on the field, can generate up to 98 kb long reads, and is cheaper than conventional sequencers (Laver et al., 2015; Jain et al., 2016). Although it has a very fast run time with less complicated sample preparation (Miller et al., 2013), the error rate is extremely high, up to 38% (Ambardar et al., 2016).

Other sequencers that have not been commonly used for protozoan research include the first NGS real-time sequencing-by-synthesis pyrosequencing technology, Roche 454 Genome Sequencer FLX which was used for whole-genome sequencing in water (Kisand et al., 2012). The NGS: SOLiD 5500xl Lifescope Genomic (Hanevik et al., 2015) platform and Sanger sequencing were used for investigating the genetic diversity of *Cryptosporidium hominis* by whole genome sequencing (Gilchrist et al., 2018).

3.4. Bioinformatic analysis tools

Different bioinformatics tools are being used to analyze the sequencing data obtained using shotgun and amplicon sequencing. This includes QIIME (Kounosu et al., 2019), MetaPhlAn (Kibegwa et al., 2020), MetaPhyler (Liu et al., 2011; Beisser et al., 2017), and Mothur (Almeida and De Martinis, 2019), which are used for targeted metagenomics and map the fewest reads (Lindgreen et al., 2016). The tools such as CLAssifier, Genomic Origins Through Taxonomic, RIEMS tools (Scheuch et al., 2015), CHAllenge, Kraken, Genometa, MG-RAST, and MEGAN are used for analyzing all sequences present in each data set and commonly for untargeted metagenomics (Neves et al., 2017; Kibegwa et al., 2020). These bioinformatic tools rely on reference databases for analysis. Some of the reference databases for protozoans include SILVA (most used database for classification of 18S Illumina reads in QIIME) (Moreno et al., 2018), Eukaryotic Pathogen Genomics Database Resource (EuPathDB)(Aurrecoechea et al., 2017), PR2 protist ribosomal database (Guillou et al., 2013; Moreno et al., 2018), VEuPathDB.org, GeneDB.org and ProtVirDB (Ramana and Gupta, 2009).

Recently Moreno et al., 2018 reported the use of Software QIIME 1.9.1 and PR2 protist ribosomal database to analyze Illumina data and the results revealed the accuracy of the tool for taxonomic classification and identification of most waterborne protozoans down to species level. RIEMS is another software pipeline used for sensitive and detailed taxonomic classification of untargeted metagenomics dataset readings and was previously applied for parasites related sequencing. Their

results provided clear parasite species allocation and subtyping with reads related to *Blastocystis* spp. and *Entamoeba* spp. (Scheuch et al., 2015; Wylezich et al., 2020). Furthermore, a new bioinformatics pipeline, ContamFinder, for protozoan parasites was developed by (Borner and Burmester, 2017) and was able to identify apicomplexan contaminations in NGS sequencing data. Limitations of these different bioinformatic tools are discussed in the section below (section 3.6).

Presented in Fig. 2 is a flow of the common and recommended steps used in the metagenomic profiling of protozoan parasites in environmental samples.

3.5. General limitations

Despite the number of advantages mentioned for shotgun metagenomics, we noted common limiting factors associated with the shotgun metagenomics approach for protozoan biodiversity studies. This approach has been reported to result in limited depth coverage of protozoan parasites compared to other taxa present in environmental samples. For example, Lear et al. (2018) mentioned that this approach favors full genome coverage of small and less complex prokaryotes or viral genomes than large and complex eukaryotes. Protozoan parasites are reported to be poorly represented in reference databases due to fewer studies compared to prokaryotes, large eukaryote genome sizes, and non-coding regions found in eukaryotes genes (Bik et al., 2012; Marcelino et al., 2020). This is one of the frequently reported disadvantages which limits the application of the whole genome shotgun metagenomics.

Additionally, whole-genome shotgun sequencing results in a large amount of sequence data that is often time-consuming and challenging due to the complex bioinformatics analyses (Miller et al., 2013; Quince et al., 2017). Although the bioinformatics platforms have been developed for the analysis of generated data, they still require larger reference databases with complete sequences and larger genome sizes (Quince et al., 2017). Another challenge associated with amplification-free DNA library preparation is the requirement for high concentration of DNA (not less than 250-500 ng) (Thomas et al., 2012; Dulanto Chiang and Dekker, 2019). Certain type of samples yield a lower quantity of DNA which requires PCR amplification to increase the DNA concentration and this results in the introduction of biases such as over-amplification of some fragment over others, which may interfer with the accuracy of measured abundance and microbial diversity (Miller et al., 2013; Dulanto Chiang and Dekker, 2019).

Based on reviewed studies on the deep amplicon-based metagenomics approach (Table 2) , we noted that even though the approach offers higher species resolution, more target-specific analysis, and is less costly, several limitations were encountered. Firstly, this approach offers limited analysis and estimation of the protozoan parasite's diversity, it does not offer universal detection of protozoan parasites, mainly due to sequence length restrictions on available sequencing technologies (Hadziavdic et al., 2014). The use of different target regions instead of the whole taxonomic gene tends to exclude some protozoan parasites which may be of public health importance (Hadziavdic et al., 2014; Maritz et al., 2017; Maritz et al., 2019). Standard universal primers targeting all protozoans or eukaryotes are not yet available. The non-universal primers are subject to taxonomic biases during amplification, and inclusion of non-targeted domains such as bacteria or archaea, which further interfere with quantification accuracy or population structure (Hadziavdic et al., 2014). Pseudogenes and the presence of multiple copies of eukaryotic genes may also interfere with quantification. For instance, *Cryptosporidium parvum* has 5 copies in the 18S rRNA gene and *Entamoeba histolytica* has 200 copies (Zahedi et al., 2018). Therefore, this may lead to overestimation or underestimation of

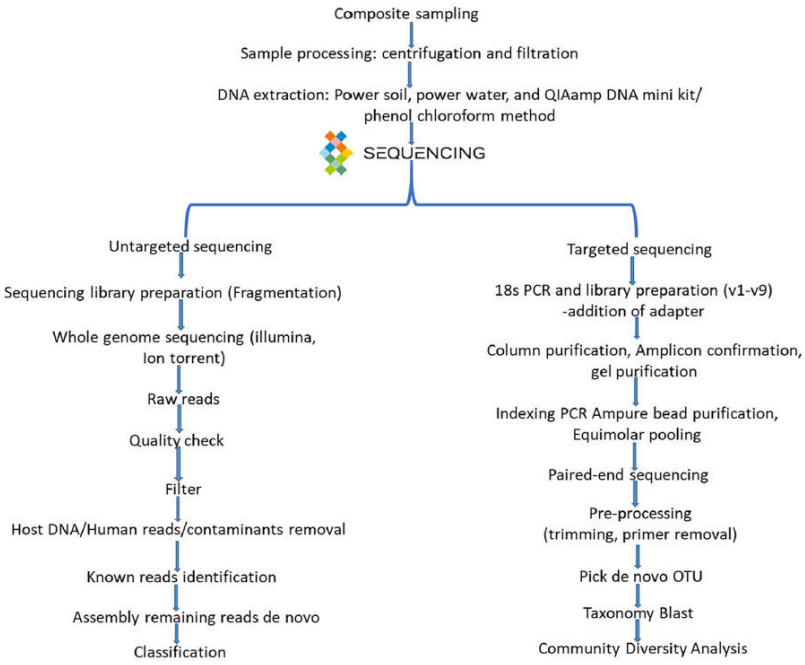

Fig. 2. Metagenomic workflow from sampling to bioinformatics.

microbes that are present as well as their proportions. At present, no standard primers are targeting the entire protozoan taxonomy or the entire eukaryotes domain. To avoid exclusion of some of the important pathogenic protozoans, we recommend the use of general eukaryote primers designed to exclude other taxa.We also recommend the use of combined variable regions of the 18S rRNA region to increase the chances of detecting more protozoans, for instance, V4 and V9 have been reported to be effective in most studies (Table 2).

The main challenge with bioinformatic analysis for protozoan studies is the insufficient reference sequences in available databases for identification/profiling (Lopes et al., 2017). Contaminations in the draft incomplete genomes (by fragments of sequences from other species) is another challenge in the bioinformatic analysis that often leads to errors in results (Borner and Burmester, 2017; Orosz, 2017; Fleetwood, 2018). Some studies reported that the SILVA database was limited and not broad enough to retrieve and identify waterborne protozoans in their sequence dataset (Tanaka et al., 2014; Moreno et al., 2018).

4. General conclusion and recommendation

We have reviewed current metagenomics approaches and a range of techniques that have been used to identify protozoan parasites from environmental matrices, from sample processing to sequencing and analysis. We observed that each step is crucial in attaining the final metagenomic data representing the original microbial community. From the available information, composite sampling is more suitable for metagenomics, as it provides and increases the chances of obtaining metadata instead of the snapshot offered by the grab sampling method. For sample preparation steps, centrifugation and filtration methods were found to be effective compared to other methods. . Similarly, a range of commercial DNA extraction kits such as Powersoil, Powerwater, and QIAamp DNA mini kit has been found useful for protozoan DNA extraction from environmental samples . Additionally, the phenol-chloroform protocol is also recommended as a cheaper alternative to these commercial kits.

Both targeted and non-targeted sequencing has been widely employed for protozoan profiling. Consistent gene regions were targeted in most protozoan studies, commonly targeting the V4, V5, and V9 regions of the 18S rRNA eukaryote gene. However, with the limitation and absence of universal primer for protozoans, more studies are required to design primers that will ensure proper representation of all parasite genomes in a sample if targeted sequencing is to be used. To investigate both known and unknown protozoan diversity, it is therefore recommended to use the shotgun whole-genome method.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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9.3 Appendix 3

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Development and evaluation of a molecular based protocol for detection and quantification of *Cryptosporidium* spp. in wastewater

N.P. Mthethwa [a,b], I.D. Amoah [a], P. Reddy [b], F. Bux [a], S. Kumari [a,*]

[a] Institute for Water and Wastewater Technology, Durban University of Technology, Durban, 4000, South Africa
[b] Department Community Health Studies, Faculty of Health Sciences, Durban University of Technology, Durban, 4000, South Africa

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ABSTRACT

Infections caused by protozoan parasites are a major public health concern globally. These infections are commonly diagnosed during water-borne outbreaks, necessitating accurate and highly sensitive detection procedures to assure public health protection. Current molecular techniques are challenged by several factors, such as low parasite concentration, inefficient DNA extraction methods, and inhibitors in environmental samples. This study focused on the development and validation of a molecular protocol for DNA extraction, efficient protozoan (oo)cyst recovery and quantification of protozoan parasites from wastewater using droplet digital polymerase chain reaction (ddPCR). Five DNA extraction methods, including commercial kits, custom phenol-chloroform, and in-house modified methods, were evaluated. The efficiency of each method was assessed via spectrophotometric analysis and ddPCR amplification using specific primers. Lastly, the developed protocol was evaluated for the detection and quantification of *Cryptosporidium parvum* in wastewater from different regions in South Africa. The conventional phenol-chloroform extraction method yielded the highest DNA concentration of 223 (±0.71) ng/μl and detected the highest number of *Cryptosporidium parvum* (1807 (±0.30) copies/ddPCR reaction) compared to other methods evaluated in this study. Additionally, the phenol-chloroform method demonstrated high sensitivity in extracting DNA from as few as one cyst/L of *Cryptosporidium parvum,* corresponding to 5.93 copies/ddPCR reaction. It was also observed that analysis of both the filtered supernatant and pellets after centrifugation improves the recovery efficiency of oocysts from wastewater by 10.5%, resulting in a total recovery of 64.1%. This optimized protocol was successfully applied to measure protozoan concentration in wastewater from different regions in South Africa. The improved DNA extraction and quantification method proposed in this study would be effective in monitoring protozoan concentration in the environment, which will help in instituting mitigation measures to reduce water-borne infections.

1. Introduction

Protozoan parasites are among the key waterborne pathogens globally and remain a major public health problem in developing countries (Fletcher et al., 2013; Kotloff et al., 2013; GBD, 2017). *Giardia duodenalis*, *Cryptosporidium* spp., *Cyclospora cayetanensis*, *Toxoplasma gondii,* and *Entamoeba histolytica* are the common human pathogenic protozoan parasites (Yaeger, 1996; Lane and Lloyd, 2002; Insulander et al., 2013; Omarova et al., 2018). These protozoan parasites have been detected in most surface waters in concentrations related to the level of fecal pollution (Hansen and Ongerth, 1991; Vermeulen et al., 2019). Their cystic forms are persistent in the environment and are resistant to the commonly used disinfection methods in wastewater and water treatment (Tomass and Kidane, 2012; Okojokwu et al., 2014). Furthermore, protozoan parasites have a low infectious dose, from 1 to 100 (oo) cyst or less (Steiner et al., 1997; Okhuysen et al., 1999; Fayer, 2004; Okojokwu et al., 2014).

Protozoan parasite *Cryptosporidium* is well documented as a major waterborne pathogen and the fourth leading cause of death from gastrointestinal diseases worldwide (Donnelly and Stentiford, 1997; Carmena, 2010; Hassan et al., 2020). In 2015, it accounted for roughly 12.1 percent of all deaths among children under the age of five worldwide (Khan et al., 2018; Hassan et al., 2020). *Cryptosporidium* spp. are distinguished from other protozoans by their ability to self-infect, their inherent resistance to disinfectants, and complex life cycle (Leitch and He, 2011; Ghazy et al., 2015). Human infections are caused by *C.*

* Corresponding author.
E-mail address: sheenak1@dut.ac.za (S. Kumari).

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andersoni, *C. muris*, *C. suis*, *C. wrairi*, and *C. felis*, with *C. hominis* and *C. parvum* accounting for roughly 90% of the infections (Helmy et al., 2013; Khan et al., 2018). Monitoring and detection of *Cryptosporidium* spp. in environmental samples such as water can be difficult due to their low numbers and the complexity of the environmental sample matrix (Bilung et al., 2017). Therefore, sensitive and reliable detection methods are critical.

Various techniques are available for the detection and quantification of protozoan parasites, these include microscopic examination (Abazaj et al., 2016; Ahmed and Karanis, 2018), antibody (IMS-IFA based), and enzyme-based analysis (Den Hartog et al., 2013), as well as molecular methods (Adeyemo et al., 2018). Molecular based methods such as polymerase chain reaction (PCR) (Ögren et al., 2020), restriction fragment length polymorphism (PCR-RFLP) (Azami et al., 2007; El-Alfy et al., 2019), real-time PCR (Bonilla et al., 2015) have become the most used methods in recent years due to their specificity and sensitivity. Another PCR-based technology that has recently attracted a lot of attention is the droplet digital PCR (ddPCR) (Xue et al., 2018; Mauvisseau et al., 2019). ddPCR is a relatively new method that uses water-oil emulsion droplet technology (Yang et al., 2014; Deprez et al., 2016). This method has shown higher sensitivity, precision, less sensitivity to inhibitors (Mauvisseau et al., 2019), and offers absolute quantification in molecular diagnosis (Basu, 2017). It has been used in diagnostic laboratories for viral, bacterial, and parasite analysis due to its advantages (Taylor et al., 2015; Maheshwari et al., 2017; Olmedillas-López et al., 2017; Cheng et al., 2019). While molecular-based methods can detect and quantify protozoans specifically, and sensibly, there is a lack of reliable and standardized preparation workflows.

The development of useful molecular diagnostic assays depends on the high precision of sample processing and extraction of high quality and quantity of DNA from various sample matrices. For this reason, the development of a molecular assay for the detection of protozoan parasites in environmental samples is very challenging, and results can be inconsistent due to several factors. These include (1) samples require a concentration step to get sufficient biomass containing (oo)cysts for DNA extraction, and (2) DNA extraction from protozoan parasites is often difficult due to the robust (oo)cysts cell wall that is not easy to lyse (Hawash, 2014; Shapiro et al., 2019), (3) lastly, environmental samples contain a higher concentration of inhibitors that can interfere with polymerase activity during PCR (Schrader et al., 2012; Hedman and Rådström, 2013). Therefore, optimization of sample processing procedures has been necessary before DNA extraction and amplification in PCR.

Recovery and concentration of (oo)cysts in water and wastewater is usually achieved through centrifugation and filtration (Moreno et al., 2018; Galvani et al., 2019). However, other less commonly used methods include immunomagnetic separation (De Jong, 2017), salt flotation (Kotian et al., 2014; Wells et al., 2016), glucose flotation (Norris et al., 2018), and formol-ether concentration (Parameshwarappa et al., 2012). Most of these methods are not specific for concentration of (oo)cysts, except the immunomagnetic separation method, therefore, it is possible to concentrate a large amount of foreign material of the same size, as the (oo)cysts, which could subsequently interfere with downstream molecular analysis. Additionally, the rigid cell wall of the (oo) cysts is a challenge for DNA extraction. Therefore, mechanical and chemical pretreatments such as freeze-thaw (Wells et al., 2016), sonication, and bead-beating have been applied as additional steps in commercial DNA extraction protocols to assist in (oo)cyst wall disruption (Psifidi et al., 2015; Felczykowska et al., 2015). Some of the commercial DNA extraction kits used for molecular analysis of protozoan parasites in environmental samples include DNeasy PowerSoil (Galvani et al., 2019), QIAamp Stool Mini Kit (Menu et al., 2018), MoBio PowerWater DNA Isolation kit (Djurhuus et al., 2017), DNeasy blood and tissue kit (Daniels et al., 2015) and YTA Stool DNA Isolation mini kit (Asgarian et al., 2018). Additionally, other studies have used modified or custom extraction procedures, such as phenol-chloroform (Djurhuus et al., 2017; Rosenbaum et al., 2019), salting-out method (Sun, 2010), UNEX protocol (Moreno et al., 2018), and MagnaPure 96 with mechanical grinding (Galvani et al., 2019). All these modifications are aimed at improving the efficiency of molecular methods in detecting protozoan parasites in environmental samples, such as wastewater or water. However, there are inconsistencies in their efficiency in extracting the DNA from oocysts.

This manuscript presents a workflow optimization to achieve maximum (oo)cysts recovery from wastewater samples, improve DNA extraction and successful quantification using the advanced ddPCR platform. This presents a protocol that can be adopted to improve the sensitivity and accuracy of molecular techniques for protozoan parasite detection in the water environment.

2. Methodology

Fig. 1 gives an overview of the experimental design of the study involving different phases. Phase 1 focused on optimization of droplet digital PCR (ddPCR) assay for the detection of *Cryptosporidium parvum* based on reference DNA (*C. parvum*). The second phase was to determine the best method for extraction of DNA from *C. parvum* using standard oocysts of known concentrations. Phase 3 focused on determination of the lowest number of oocysts that can be extracted using the best method determined in Phase 2. Phase 4 investigated the effects of sample centrifugation and filtration on oocyst recovery from wastewater. Finally, the last phase of the study applied the optimized methods to determine the presence of *Cryptosporidium* spp in different wastewater samples.

2.1. Optimization of droplet digital PCR (ddPCR) assay for the detection of Cryptosporidium spp.

The ddPCR amplification protocol and the detection limit were first optimized using synthetic DNA of *C. parvum* purchased from Microbiologics Inc. (1.1×10^7 copies/vial). The synthetic DNA was amplified using QX200 Droplet Digital PCR system (Bio-Rad). Each ddPCR assay mix was prepared in a final volume of 20 μL, containing 10 μL of 2 × evagreen ddPCR supermix, 0.5 μm forward and reverse primers (Table 1), and 1 μl of template DNA (4000 copies of synthetic DNA in a reaction volume). A no template control (NTC) was also included in the reaction. The generation of droplets was performed using QX200 Droplet Generator (Bio-Rad). PCR amplification was carried out on a T100 thermal cycler (Bio-Rad), using the conditions: 10 min at 95 °C, denaturation at 95 °C during 15 s and annealing at 56°C–60 °C for 1 min, 72 °C for 1 min, final extension at 72 °C for 10min, 50 cycles. The plates were analyzed using the QX200 Droplet Reader and QuantaSoft software (Bio-Rad). The threshold line was based on the negative and positive droplets separation. The synthetic DNA was further serially diluted using a 1:10 dilution factor to determine the detection limit of the ddPCR protocol.

2.1.1. Specificity of primers used

This study achieved the detection and amplification of the targets using the generic 18 S SSU rRNA and species-specific gp60 primer pair (Table 1). Primers were optimized and confirmed with gel electrophoresis to ensure specificity, and the results revealed single bands indicating specific amplification. Furthermore, the specificity of the SYBR green assay was also assessed with real time PCR, single specific melt curves were observed. The specificity of both primers using the ddPCR SYBR green assay was also confirmed by looking at good droplet separation of the negative and positive droplets (Fig. 4 appendix I), which is usually seen when there is nonspecific binding noise in between separation. Additionally, when testing environmental samples, a positive synthetic DNA was included to confirm the amplified product of interest and primer specificity. Following primer specificity analysis, only the gp60 primer assay was used to optimize the methodology in this study

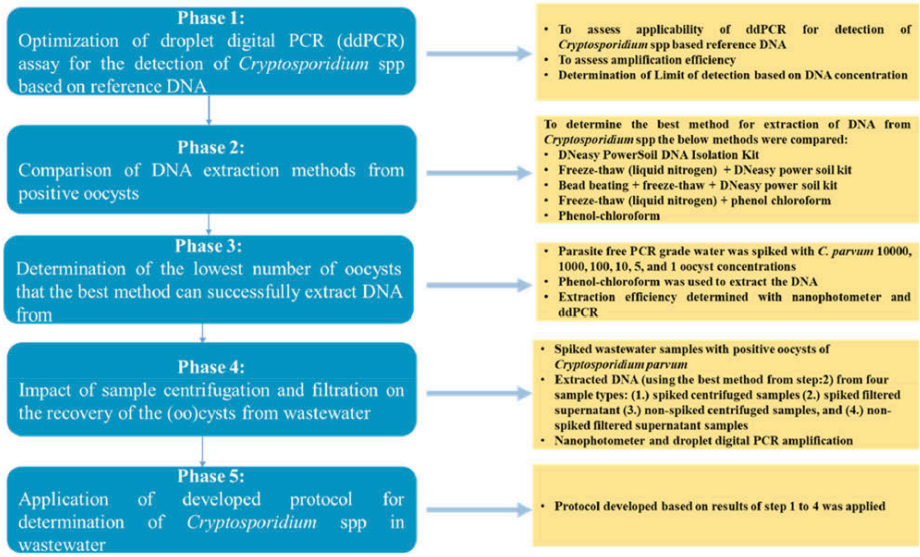

Fig. 1. Experimental design of the study.

Table 1
Primers used in the amplification of extracted DNA for the detection of *Cryptosporidium* spp.

Primers	Forward	Reverse	Size (bp)	Target	References
Cryptosporidium 18S rRNA	GTTTTCATTAATCAAGA ACGAAAGTTAGG	GAGTAAGGAACAACC TCCAATCTCTAG	100	All known *Cryptosporidium* species (21)	Burnet et al. (2013)
Cryptosporidium (gp60)	GCCGTTCCACTCAGAGGAAC	CCACATTACAAATGAAGTGCCGC	150	*C. parvum*	(Sánchez et al., 2018; Hunter et al., 2007)

(phase1 to phase 4). In phase 5, the generic 18s rRNA assay was performed and applied to see if the assay can target other *Cryptosporidium* species when needed.

2.1.2. Oocysts estimation based on the copy numbers per reaction volume

C. parvum oocyst concentrations were calculated directly from the ddPCR data, which has the advantage of providing more accurate and absolute quantification (Deprez et al., 2016). Briefly, the *C. parvum* gene copy numbers contained in individual samples were converted to oocyst number estimates, based on published evidence that there are five copies of the rDNA unit per haploid genome (Le Blancq et al., 1997), four haploid sporozoites per oocyst (Zahedi et al., 2018) and thus 20 18S rRNA gene copies of the *C. parvum* per oocyst.

The oocysts concentration was calculated using the formula (Equation (1)):

$$oocysts = \frac{x}{y} \quad (1)$$

where "x" is the copy number measured per 20 μl reaction volume and "y" is the number of copies of the gene per one oocyst (in this case 20).

2.2. Comparison of DNA extraction methods from standard C. parvum oocysts (Experiment 1)

Five different DNA extraction protocols were compared for maximum DNA recovery from oocysts. This includes a standard DNeasy Powersoil extraction kit (Qiagen, Norway), a conventional DNA extraction (phenol-chloroform) and modified protocols as described below. The PowerSoil extraction protocol was chosen based on previous literature that this extraction method is most suitable and gave the best results in terms of purity and speed when extracting DNA from parasites (Temesgen et al., 2020; Barbosa et al., 2017). The phenol-chloroform was chosen because it provides a cheaper alternative to the commercial kits. Moreover, it has been reported to be efficient in DNA extraction from protozoan (oo)cysts especially from water samples where protozoan parasites are mostly found in lower concentration (Rosenbaum et al., 2019).

The efficiency of these methods in extracting DNA from *C. parvum* oocysts was determined by spiking the 2.5×10^2 and 2.5×10^3 oocysts into 500 μl nuclease-free water. The parent stock of 1×10^6 *C. parvum* oocysts purchased from Waterborne Inc. was prepared and guaranteed 99% pure by the supplier. According to Waterborne Inc, they were purified by sucrose percoll gradient and water washes. Each suspension was then viewed microscopically. The stock volume was then calculated to give 2.5×10^2 and 2.5×10^3 of the oocysts and was spiked in the nuclease free water and was used as a starting material for the different DNA extraction methods. The purity of the extracted DNA was determined based on the OD260/OD280 and OD260/OD230 ratios measured using the Nanophotometer (Implen GmBH, NP80, Germany). The details of the extraction protocols are described in detail below.

2.2.1. Extraction method 1 (M1)- DNeasy PowerSoil kit

The first genomic DNA extraction method used was the DNeasy PowerSoil DNA Isolation Kit (Qiagen, Norway) as per the manufacturer's recommendation without any modifications. In this method, the oocysts were mechanically lysed using provided bead tubes in the kit with a lysis buffer solution followed by vortexing. The final DNA was suspended in a 50 µl elution buffer.

2.2.2. Extraction method 2 (M2)-freeze-thaw (liquid nitrogen) + DNeasy power soil kit

The samples were transferred into the bead tubes provided by DNeasy PowerSoil Kit. Lysis buffer was added to the tubes and briefly vortexed to mix. The samples were then set to freeze in liquid nitrogen for 5 min and thawed at 65 °C for 5 min. After 10 cycles of freeze-thaw (Wells et al., 2016), the remainder of the DNA isolation protocol was followed as per the DNeasy PowerSoil manufacturer instruction.

2.2.3. Extraction method 3 (M3)- bead beating + freeze-thaw + DNeasy power soil kit

The samples were subjected to the DNA extraction protocol as described in Method 2 (M2) with an additional mechanical lysis step. Briefly, the bead tubes containing the cysts and the lysis buffer provided by the manufacturer were subjected to bead beating at high speed for 60s (two cycles). To achieve higher lysis efficiency, the bead beating step in the extraction kit was replaced with a stronger homogenization (higher velocity) with Bead Rupture 12 Homogenizer (Omni International, USA). After bead beating, the samples were subjected to freeze-thaw as described in (M2), followed by the DNeasy PowerSoil extraction kit following the manufacturer's instructions.

2.2.4. Extraction method 4 (M4)-freeze-thaw (liquid nitrogen) + phenol-chloroform

DNA was extracted using the phenol-chloroform method and freeze-thaw (liquid nitrogen) pretreatment. Briefly, the oocyst of *C. parvum* (2.5×10^2 and 2.5×10^3) were suspended in 500 µl of lysis buffer (10 mmol/L Tris/HCl pH 8.0, 10 mmol/L EDTA, 0.1 mol/L NaCl, 2% SDS pH 8.0) and 100 µl protease K) followed by incubation for 2 h at 55 °C - 60 °C in a water bath (Djurhuus et al., 2017; Awolusi, 2016). The samples were subjected to pretreatment using 10 cycles of freeze-thaw steps (Wells et al., 2015, 2016; Babaei et al., 2011) as described in M2. After the pretreatment the proteins and other impurities were removed by adding phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v), followed by phenol removal by adding an equal volume of chloroform. The gDNA was then precipitated by adding 0.6 X volume of isopropyl alcohol and incubated overnight at −20 °C (Javadi et al., 2014; Butler, 2012; Djurhuus et al., 2017). The DNA was then washed with 70% ethanol and suspended in 1X Tris–EDTA buffer.

2.2.5. Extraction method 5 (M5)- phenol-chloroform

The total gDNA was extracted from samples as described in method 4 (M4) with modifications. In this protocol the additional pretreatment step was avoided and followed the standard phenol-chloroform methods described by Djurhuus et al. (2017) and Awolusi (2016).

2.3. Determination of the lowest number of oocysts required for DNA extraction, detection and quantification (Experiment 2)

The limit of detection was determined to ascertain the lowest concentration of *C. parvum* oocysts that the optimized phenol-chloroform protocol can successfully extract DNA from. The phenol-chloroform protocol was chosen based on the results obtained during the method comparison phase. The protocol was applied to triplicate nuclease free water samples spiked with *C. parvum* oocysts (Fig. 2). The concentration of *C. parvum* oocysts used were 10 000, 1000, 100, 10, 5, and 1 oocyst, spiked into 500 µl nuclease-free water. All spiked samples and negative control (without spiked oocysts) were subjected to the optimized phenol-chloroform DNA extraction protocol and the extracted DNA was analyzed using both nanophotometer and ddPCR amplification.

2.4. Impact of sample centrifugation and filtration on the recovery of the (oo)cyst from wastewater (Experiment 3)

To ascertain the impact of filtration and centrifugation steps on the recovery of (oo)cysts from wastewater, the optimized DNA extraction protocol was applied on influent wastewater samples. Wastewater samples were obtained from the treatment plants in different cities within South Africa. Approximately, 2 L of the samples (raw wastewater) were taken from the head of works in sterilized sample bottles and kept at room temperature and transported to the laboratory for analysis. In the laboratory (Fig. 2), the samples were split into two (1 L each), the first potion was spiked with 12 500 oocysts of *C. parvum*. The spiked samples were concentrated by centrifugation (Hermle

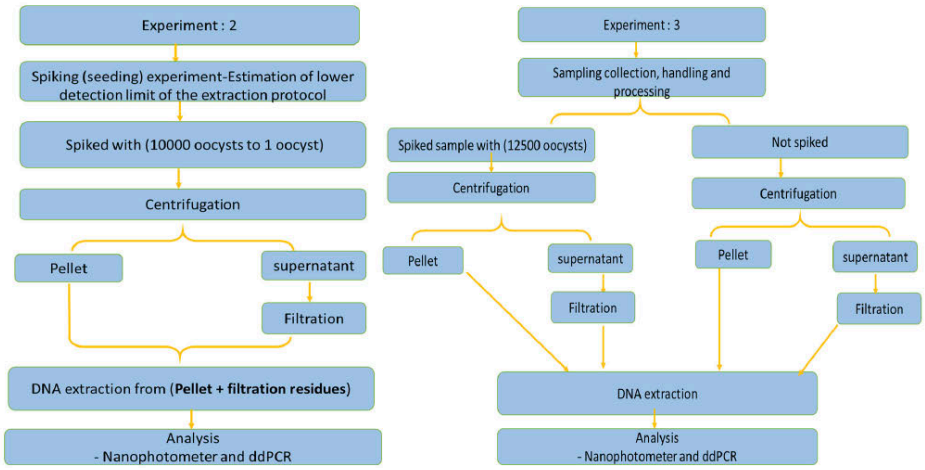

Fig. 2. Methodological flow for determination of limit of detection and sample handling experiment.

Labortechnik GmbH) at 3500 rpm (max 4 × 340 g) for 10 min and the total DNA was extracted from the pellet using the phenol-chloroform method as described above (M5). In addition, the supernatant after the initial centrifugation step was also filtered using 0.1 μm pore size cellulose acetate filter paper. The membrane filter paper was placed into a centrifuge tube, eluted using PBS as in the USEPA 1623 method. The DNA was extracted from residual material on the filter paper using the same method as above. This was done to see if there are any residual (oo) cysts in the supernatant. Several past studies have either centrifuged (Moreno et al., 2018; Hendriksen et al., 2019) or filtered (Galvani et al., 2019; Zahedi et al., 2019) their samples, but not both.

The procedure described above was then repeated with non-spiked wastewater samples. Therefore, in total DNA was extracted from four types of samples (in triplicate): (1.) spiked centrifuged samples (2.) spiked filtered supernatant (3.) non-spiked centrifuged samples, and (4.) non-spiked filtered supernatant samples. The extracted DNA from the set of spiked and non-spiked samples were analyzed for DNA quality and quantity and subsequently amplified using ddPCR targeting *C. parvum* gp60 gene. Therefore, the percentage recovery of spiked oocysts (12 500 oocysts) was determined using the formula (equation (2)):

Equation (2) (oocysts % recovery)

$$P\% = \frac{y}{x} \times 100 \quad (2)$$

where "x" is the total number of spiked oocysts and "y" is the total oocyst recovered in the spiked sample minus the total oocysts recovered in the non-spiked sample.

2.5. Application of developed protocol for detection and quantification of Cryptosporidium spp in wastewater

The outcomes of the three experiments above were evaluated for the detection and quantification of *Cryptosporidium* spp using the 18S rRNA assay in wastewater from three geographical regions in South Africa. The extracted DNA was quantified using ddPCR following the protocol described in section 1 using the primers listed in Table 1.

3. Results

3.1. Optimization of droplet digital PCR for the detection of Cryptosporidium parvum based on reference DNA

The ddPCR assay was successfully optimized for detection and quantification of *C. parvum* using primers in Table 1. The copy number of *C. parvum* measured by ddPCR was 3342.39 copies/20 μl reaction volume with clear separation of positive and negative droplets, and without any sign of inhibitors or nonspecific binding. When diluted through the 1:10 dilution factor, the decreasing droplet concentration was observed (Fig. 4 appendix). Based on the obtained results (Table 2), the limit of detection of the ddPCR assay for *C. parvum* is 0.07 copies/μl (1.32 copies in 20 μl reaction). Further dilutions indicated a "no-call" or undetectable copies.

Table 2
Limit of detection for the droplet digital PCR assay using reference DNA.

Dilution	Copies detected in ddPCR (20 μl reaction volume)	Detection rate (X detected/Y (2) replicates)
10^{-1}	3342.39	2/2
10^{-2}	270.5	2/2
10^{-3}	29.39	2/2
10^{-4}	6.03	2/2
10^{-5}	3.09	2/2
10^{-6}	2.77	2/2
10^{-7}	1.32	2/2
10^{-8}	0	0/2

3.2. Comparison of DNA extraction methods from positive oocysts

Genomic DNA extracted from positive *C. parvum* oocysts using all methods as shown in Table 3 had a detectable nucleic acid concentration using the nanophotometer measurement, except for the DNeasy PowerSoil kit (M1) and the Bead beating + freeze-thaw + PowerSoil kit (M3). The phenol-chloroform extraction protocol showed the best performances with an average DNA concentration of 223 (±0.7) ng/μl (Table 2). The other methods tested (Table 3) in this study displayed varying detectable DNA concentrations, ranging from 0.275 to 100 (±0.01- ±0.07) ng/μl. Furthermore, the purity of the detectable DNA concentrations ranged between 1.5 and 2 (OD260/OD280) and 1.5–2.2 (OD260/OD230). However, the best purity of 1.8 (260/280 ratio) and 1.9–2 (260/230 ratio) was achieved with both the DNeasy PowerSoil extraction kit and phenol-chloroform extraction kit.

The *C. parvum* (GP60) gene was detected from all the samples irrespective of the extraction protocol used, including those samples where DNA was not detectable using the nanophotometer (Table 3). For instance, the PowerSoil isolation kit resulted in an undetected DNA concentration based on the nanophotometer reading (Table 3). However, 72.38 (±0.18) copies/reaction volume of the *C. parvum* gene were detected using the ddPCR (Table 3). Phenol-chloroform method without any pretreatment resulted in the highest copies of 1807.15 (±0.30) copies/reaction based on the ddPCR analysis. Therefore, the phenol-chloroform method was chosen as the best performing method for further experiments.

3.3. Determination of a lower detection limit (LOD) of the phenol-chloroform extraction protocol for C. parvum oocysts in nuclease-free water

The results of the LOD assessment indicated high sensitivity of the

Table 3
DNA concentration and purity based on the nanophotometer and ddPCR measurements.

DNA extraction methods	ng/μl (±SD)	*Cryptosporidium parvum* copies/20 μl reaction (ddPCR) (±SD)	260/280 ratio	260/230 ratio
(M1) 2.50 × 10^3 PowerSoil kit	–	71.57 (±0.01)	–	–
(M2) 2.50× 10^2 (cyst) liquid nitrogen + PowerSoil kit	1.25 (±0,01)	34.52 (±0.38)		
(M2.1) 2.50 × 10^3 (cysts) liquid nitrogen + PowerSoil kit	0.275 (±0.01)	103.08 (±0.42)	–	–
(M3) 2.50× 10^2 (cysts)- Bead beating + freeze-thaw + PowerSoil kit	–	72.38 (±0.18)	–	–
(M3.1) 2.50 × 10^3 Bead beating + freeze-thaw + PowerSoil kit	0.45 (±0.02)	127.41 (±0.33)	–	–
(M4) 2.50× 10^2 (cyst) liquid nitrogen + phenol chloroform	3.05 (±0.01)	184.84 (±0.79)	1.8	1.9
(M4.1) 2.50× 10^3 (cysts) liquid nitrogen + phenol chloroform	4.5 (±0.04)	1310.73 (±0.26)	1.8	1.9
(M5) 2.50× 10^2 (cyst) phenol chloroform	100 (±0)	465.80 (±0.18)	1.8	2
(M5.1) 2.50× 10^3 (cyst) phenol chloroform	223 (±0.71)	1807.15 (±0.30)	1.8	2

— Undetected.

phenol-chloroform extraction protocol with successful DNA extraction from all spiked oocysts concentration (10 000–1 oocyst). DNA measured using a nanophotometer indicated a concentration range from 31 (±0.7) to 230 (±0.35) ng/μl. Furthermore, DNA extracted from the lowest spiked oocysts (1 oocyst of *C. parvum*) was subsequently detected using ddPCR assay obtaining 5.93 copies/reaction volume (Table 4).

3.4. Impact of sample centrifugation and filtration on the recovery of the (oo)cysts from wastewater

Total extracted DNA and *C. parvum* copies were found in significant concentrations from both samples pellets and filtered supernatant after centrifugation. For instance, in the non-spiked wastewater samples, that were centrifuged, 281.32 (±0.31) ng/μl of DNA was extracted, when spiked, the concentration of DNA extracted was 669.47 (±0.81) ng/μl. However, when the supernatant was further filtered, 52.33 (±0.71) and 281,32 (±0,31) of DNA were extracted in the non-spiked and spiked samples, respectively. *C. parvum* gene detected via the ddPCR analysis indicated a similar trend of 167592.4 (±1.2) gene copies/L of wastewater in the spiked sample and 33528.56 (±0.8) gene copies/L of wastewater in the non-spiked sample. Corresponding to a recovery of approximately 8379 of oocysts per liter of wastewater for the spiked samples and approximately 1676 oocysts per liter of wastewater for the non-spiked samples (Table 5). Further filtration of the supernatant after centrifugation achieved 31392.20 (±0.63) gene copies/L of *C. parvum* in the spiked samples and 5136.45 (±0.22) gene copies/L of wastewater for the non-spiked wastewater samples (Table 5). Based on these measurements, we were able to determine that further filtration of the supernatant after centrifugation improves recovery of the oocysts by 10.5% (Table 5).

3.5. Evaluation of a molecular protocol for detection of Cryptosporidium spp in wastewater samples

All wastewater samples (9) from different regions of South Africa showed positive amplification when 1 ng of DNA was used as template DNA concentration for ddPCR (Table 1). The mean (±SD) concentrations measured were 5375.78 (±6.38) gene copies/reaction (Cape town), 196.054 (±0.44) gene copies/reaction (Johannesburg) and 54,58 (±2.59) gene copies/reaction (Durban). This translates to an estimated *C. parvum* oocysts concentration of approximately 268.79, 9.80 and 2.73 oocysts, respectively.

Briefly, the workflow established (Fig. 3) begins with composite sampling for a complete representation of the microbial population from source water, followed by application of both centrifugation and filtration of supernatant to increase oocysts recovery. DNA extraction using the phenol-chloroform protocol and lastly amplification with ddPCR (Fig. 3).

4. Discussion

Molecular detection of microorganisms in environmental samples is largely dependent on the extraction of the nucleic acid material and other factors (sample collection and preparation). In this study, the phenol-chloroform DNA extraction method comprised of the lysis step (500 μl cell lysis buffer (10 mmol/L Tris/HCl pH 8.0, 10 mmol/L EDTA, 0.1 mol/L NaCl, 2% SDS pH 8.0) (Djurhuus et al., 2017; Awolusi, 2016) was determined to be the best method for the extraction of the protozoan parasite's DNA. The findings indicated consistency in DNA yield from *Cryptosporidium parvum* oocysts, with the highest concentration of 223 ng/μl and high purity of 1.8 (260/280) and 2 (260/230) (Table 3). The DNA extracted with phenol-chloroform proved to be compatible with ddPCR, showing good amplification with high copy numbers and no sign of inhibition. The high DNA yield achieved by the phenol-chloroform extraction method could be linked to the lysis step being effective in

Table 4
LOD phenol-chloroform DNA extraction.

Spiked oocysts	Copies of *Cryptosporidium parvum*/20 μl reaction volume
10 000	1169.81
1000	529.31
100	320.00
10	35.80
5	17.16
1	5.93

Table 5
Measured *C. parvum* copies (ddPCR) in both centrifuged only samples and filtered supernatant after centrifugation.

Samples	Mean copies gene/L	SD	Total recovered oocysts/L	Difference	% Oocysts recovery
Spiked centrifuged sample	167592.40	±1.2	8379.62		
Non-spiked, centrifuged sample	33528.56	±0.8	1676.43	6703.19	53.63
Spiked filtered supernatant sample	31392.20	±0.63	1569.61		
Non-spiked filtered supernatant sample	5136.45	±0.22	256.82	1312.79	10.5

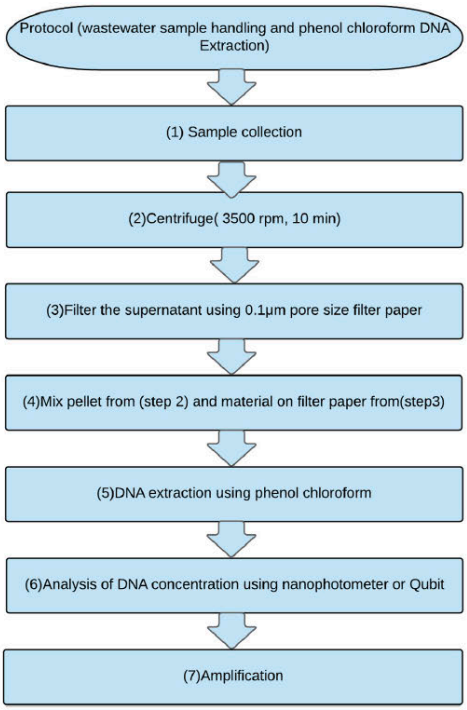

Fig. 3. Wastewater processing, DNA extraction, and amplification using ddPCR.

breaking the oocyst shell and releasing enough genetic material. This could be due to the SDS and proteinase K added to the lysis buffer, SDS assists in cell membrane digestion, facilitate the solubilization of nucleic acid complexes, and leads to improved precipitation of the nucleic acids (Djurhuus et al., 2017; Barbier et al., 2019). Additionally, the protease K breaks down the proteins that protect the DNA molecules while they are in chromosomes (Mckiernan and Danielson, 2017). The phenol-chloroform method has been previously recommended for recalcitrant species such as the robust protozoan oocysts (Butler, 2012; Barbier et al., 2019).

It was also observed that removing the freeze-thaw (liquid nitrogen) modification step from the phenol-chloroform method (Table 3 method: liquid nitrogen + phenol chloroform) improved the results as the DNA concentration increased (Table 3 method: Phenol chloroform). This implies that the method was too harsh for the cells and contributed to gDNA degradation. A similar study found that freeze-thaw during extraction caused damage to *Giardia* cysts, resulting in a low gDNA yield (Babaei et al., 2011).

The phenol-chloroform extraction protocol used in this study further showed high sensitivity with successful DNA extraction from as low as 1 oocyst of *C. parvum*. Additionally, the DNA extracted directly from 1 oocyst spiked into 500 μl of nuclease-free water was subsequently detected by ddPCR. This demonstrates the ddPCR's sensitivity in detecting such a small starting quantity, which is essential in water quality monitoring of protozoan parasites since the infectious dose of protozoans could be as low as 10 (oo)cysts (Zacharia et al., 2019). The lowest detection limit of the ddPCR with the extraction protocol was 5.93 copies equivalent to ≤1 oocyst per reaction. The number of oocysts that can be detected (LOD) in wastewater, surface water, or drinking water is imperative for public health protection. The operating water quality standards for *Cryptosporidium* spp and *Giardia* spp in drinking water, for example, are less than 1 (oo)cyst/10 L and 1 cyst/10 L, respectively, according to the South African National Standard (SANS) 241–1 (Sigudu et al., 2014). According to WHO Guidelines, the operational water quality value for (oo)cysts in raw water are 10 oocysts/L and in wastewater, the 8.89 log reduction value is required (WHO, 2017). Hawash (2014) reported the LOD with the Qiagen mini stool extraction protocol to be 2 oocysts per reaction. Their LOD is similar to our study, however, the difference is that their extraction was directly from feces. Another study reported *C. parvum* DNA extraction from stool samples with less sensitivity, with LODs of 10 and 50 oocysts for a real-time PCR assay, and Quick DNA Fecal/Soil Microbe-Miniprep kit, respectively (Valeix et al., 2020).

The phenol-chloroform method has been utilized for many years and is reported to remain one of the most effective, reliable, and efficient DNA extraction methods, especially for high molecular weight DNA or eukaryotes such as protozoans (Butler, 2012; Sessions, 2013, Mckiernan and Danielson, 2017). For instance, Rosenbaum et al. (2019) reported that phenol-chloroform extraction method showed a better performance compared to eight other DNA extraction techniques tested. However, the phenol-chloroform method is time-consuming (Mckiernan and Danielson, 2017), and the phenol used is hazardous (Hawash, 2014). Therefore, further studies are required to optimize this approach for a shorter time and proper phenol handling to reduce the risks of exposure.

Before DNA extraction, one of the most critical steps in the detection of protozoan parasites in wastewater is the concentration of the (oo) cysts. (Oo)cyst losses are known to occur at the sample handling and processing stages of filtration, regular centrifugation, and density gradient centrifugation (Al-Sabi et al., 2015; Razakandrainibe et al., 2020). Alternative methods such as alkaline and acid flocculation (Sammarro Silva and Sabogal-Paz, 2020), new filtration units (Al-Sabi et al., 2015), and centrifugation at 1550 rcf for 10 min (Razakandrainibe et al., 2020) have been suggested and assessed. However, they are time-consuming and reduce recovery rate, thus there is still no standard oocysts recovery protocol applicable before DNA extraction. We observed that further filtering of the supernatant after centrifugation resulted in additional oocysts recovery (Table 5). For instance, 8379.62 oocysts were recovered from spiked samples that were only centrifuged and a total of 1569.61 oocysts were recovered from the filtered supernatant (Table 5). This could mean that when the samples were concentrated with centrifugation alone at 3500 rpm (max 4 × 340 g) for 10 min, some oocysts with lower density remained in the supernatant. The recovery of oocysts in the supernatant through filtration could be due to the filter paper pore size of 0.1 μm used for filtration able to capture all oocysts left in the supernatant after centrifugation. Therefore, centrifugation alone may lead to an underestimation of oocysts concentration; thus our approach is necessary for improving oocysts recovery in water samples.

Finally, the established workflow (Fig. 3) was applied to wastewater samples from different areas. *Cryptosporidium* spp were detected in all wastewater samples from the different parts of South Africa with varying concentrations (section 3.5). The copy number concentrations were slightly higher than previously detected *Cryptosporidium parvum* copies (3084 copies/μl) from environmental water samples analyzed using the qPCR Genesig kit (Lombard, 2016). The findings show that the new protocol (Fig. 3) when applied to wastewater samples is applicable for monitoring protozoan parasites like *Cryptosporidium* spp in the environment, especially wastewater. This could be attributed to the improved oocysts recovery via the use of both centrifugation and filtration of the supernatant, DNA extraction with the phenol-chloroform method which has been shown to improve DNA extraction and finally amplification with the ddPCR.

5. Conclusions

This study successfully developed and evaluated a protocol for protozoan parasite detection in wastewater using ddPCR. The use of ddPCR to detect *Cryptosporidium parvum* demonstrated high sensitivity, with a limit of detection of 1.32 copies/20 μl reaction volume.

The workflow approach proved that filtration of the supernatant after centrifugation leads to enhanced (oo)cysts recovery by 10.5%, bringing the total recovery to approximately 64.1%. This means that relying on only centrifugation could potentially lead to underestimation of protozoan concentration in wastewater and other water matrices. The use of phenol-chloroform DNA extraction protocol without any prior pretreatments achieved efficient high DNA yield with a sensitive LOD of as low as 1 (oo)cyst per DNA extraction. Therefore, the optimized method in this study could be very important in water safety monitoring, which is critical for the design and implementation of effective measures aimed at reducing waterborne protozoan infections.

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Appendix I

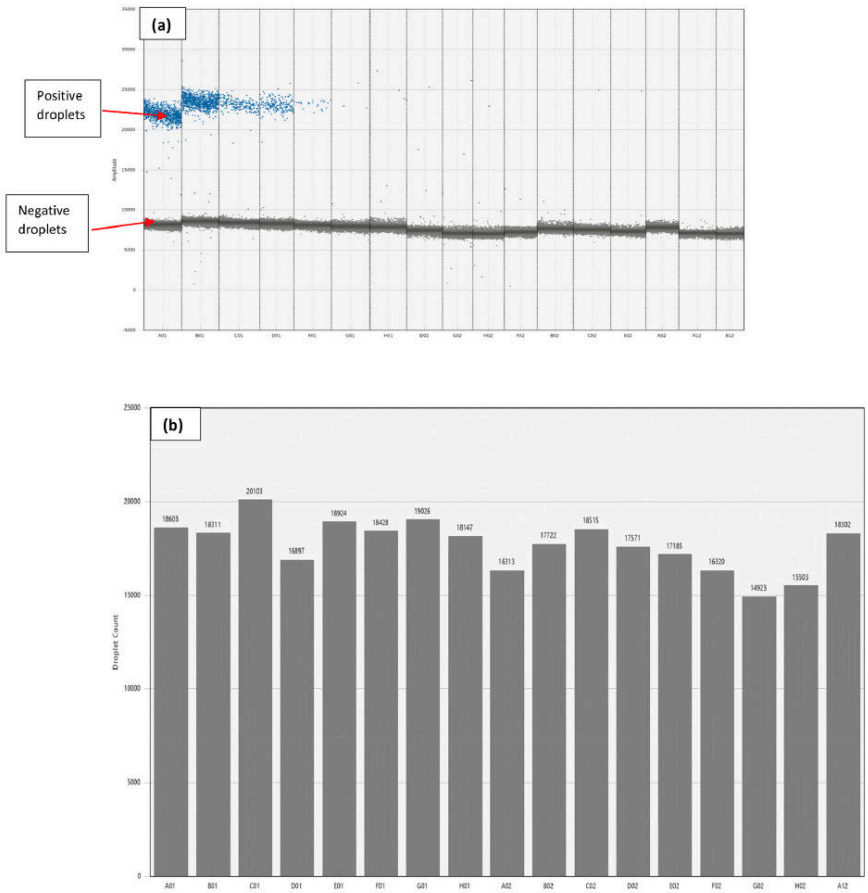

Fig. 4. Droplet digital PCR, Phase 1 results showing limit of detection for the GP60 assay. (a) Decreasing copy number concentration. (b)The total number of accepted droplets. Y-axis: shows amplitude/droplet count. X-axis: shows sample wells.

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9.4 Appendix 4

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Profiling pathogenic protozoan and their functional pathways in wastewater using 18S rRNA and shotgun metagenomics

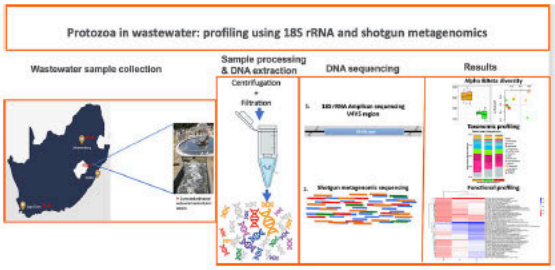
Nonsikelelo P. Mthethwa-Hlongwa [a,b], Isaac D. Amoah [a,d], Andres Gomez [c], Sam Davison [c], Poovendhree Reddy [b], Faizal Bux [a], Sheena Kumari [a,*]

[a] Institute for Water and Wastewater Technology, Durban University of Technology, Durban 4000, South Africa
[b] Department Community Health Studies, Faculty of Health Sciences, Durban University of Technology, Durban 4000, South Africa
[c] Department of Animal Science, University of Minnesota, St. Paul, MN, USA
[d] Department of Environmental Science, The University of Arizona, Shantz Building Rm 4291177 E 4th St., Tucson, AZ 85721, USA

HIGHLIGHTS

- Protozoans in selected South African WWTPs were surveyed via 18S rRNA and shotgun sequencing.
- Protozoan diversity was mainly affected by water treatment, but also by location.
- Pathogenic protozoa and their virulence factors abounded in untreated wastewater.
- High-throughput sequencing is effective for surveilling protozoan pathogens in WWTPs.

GRAPHICAL ABSTRACT



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ABSTRACT

Despite extensive research, little is known about the composition of eukaryotic protists in environmental samples. This is due to low parasite concentrations, the complexity of parasite diversity, and a lack of suitable reference databases and standardized protocols. To bridge this knowledge gap, this study used 18S rRNA short amplicon and shotgun metagenomic sequencing approaches to profile protozoan microbial communities as well as their functional pathways in treated and untreated wastewater samples collected from different regions of South Africa. Results demonstrated that protozoan diversity (Shannon index *P*-value = 0.03) and taxonomic composition (PERMANOVA, *P*-value = 0.02) was mainly driven by the type of wastewater samples (treated & untreated) and geographic location. However, these WWTPs were also found to contain a core community of protozoan parasites. The untreated wastewater samples revealed a predominant presence of free-living, parasitic, and potentially pathogenic protists typically found in humans and animals, ranging from Alveolata (27 %) phylum (Apicomplexa and Ciliophora) to Excavata (3.88 %) (Discoba and Parasalia) and Amoebozoa (2.84 %) (*Entamoeba* and *Acanthamoeba*). Shotgun metagenomics analyses in a subset of the untreated wastewater samples confirmed the presence of public health-importance protozoa, including *Cryptosporidium* species (3.48 %), *Entamoeba hystolitica* (6.58 %), *Blastocystis hominis* (2.91 %), *Naegleria gruberi* (2.37 %), *Toxoplasma gondii* (1.98

* Corresponding author.
E-mail address: sheenak1@dut.ac.za (S. Kumari).

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%), *Cyclospora cayetanensis* (1.30 %), and *Giardia intestinalis* (0.31 %). Virulent gene families linked to pathogenic protozoa, such as serine/threonine protein phosphatase and mucin-desulfating sulfatase were identified. Additionally, enriched pathways included thiamine diphosphate biosynthesis III, heme biosynthesis, Methylerythritol 4-Phosphate Pathway, methyl erythritol phosphate (MEP), and pentose phosphate pathways. These findings suggest that protozoan pathogens may possess metabolic and growth potential within WWTPs, posing a severe risk of transmission to humans and animals if inadequately disinfected before release. This study provides a baseline for the future investigation of diverse protozoal communities in wastewater, which are of public health importance.

1. Introduction

Infectious disease accounts for a large proportion of death and disability worldwide, and in certain regions, it remains the most serious cause of ill health (WHO, 2020). Traditional public health and sanitation measures have long been effective in limiting many pathogens that spread through contaminated water or vectors (Maritz et al., 2019; Zacharia et al., 2018). However, there is a need for more effective surveillance to reduce the disease burden, and spread of infectious disease and mitigate health risks based on analyses of microbial diversity (Garcia et al., 2017). Determining the genetic diversity and geographical distribution of microbes is important for tracking pathogen adaptation and evolution. In addition, it is critical to understand the relationship between environmental factors and microbial community structure (Wu et al., 2019; Feng et al., 2018).

Recent advances in metagenomic sequencing have revolutionized the understanding of complex microbial communities, including analyses of the human gut, and environmental (soil-water) systems. Nevertheless, there are few available data on the diversity and biogeography of eukaryotic microbial communities in wastewater treatment plants. Several studies have indicated that wastewater treatment plants are a significant reservoir of human and animal pathogens, posing a severe threat to public health and the environment (Yashas and Udayashankara, 2017). The relative abundance of these pathogens in untreated wastewater may also vary depending on the location and source (Ahmed et al., 2018; Omarova et al., 2018). However, compared to bacteria, the genetic diversity and distribution of protozoa in wastewater have received little attention, especially in African countries with high disease burdens. Enteric pathogenic protozoa found in water are a major cause of severe human disease, causing epidemics and endemics in developed and developing countries (Roth et al., 2018). Cryptosporidiosis, Giardiasis, Toxoplasmosis, Cystoisosporiasis, intestinal amoebiasis, cyanosporiasis, Blastocystosis, Balantidiasis, and Granulomatous Encephalitis (GAE) are among the diseases caused by waterborne protozoans that are of public health concern (Bridle, 2014; Garcia et al., 2017; Griffiths, 2017; Xiao et al., 2018a, 2018b; Ajonina et al., 2018). These pathogenic infections are spread directly through the fecal-oral route or indirectly through contaminated water or food (Omarova et al., 2018). These pathogens are also resistant to disinfectants like chlorine, and are environmentally robust, with some being small enough (1–17 μm) to avoid water treatment (Widmer and Sullivan, 2012). Therefore, a full understanding of the diversity of these parasites in the environment will provide insights into their infection patterns and potential measures to reduce transmission.

A reliable detection and identification method is essential for determining the prevalence and distribution of protists in sewage samples. The current advanced methods for profiling eukaryotic diversity in complex environmental samples rely on sequencing the 18S rRNA eukaryotic gene and shotgun metagenomic analysis. The 18S rRNA short amplicon-based sequencing method amplifies and sequences a specific variable region of the 18S rRNA gene in wastewater microbial DNA. The 18S rRNA gene is conserved in all eukaryotic organisms, including protozoans. Pathogenic protozoans can therefore be identified by comparing wastewater sequences to a database (Miller et al., 2013; Maritz et al., 2019). On the other hand, shotgun metagenomic sequencing sequences all the DNA fragments in a wastewater sample without prior knowledge of the microorganism present (Quince et al., 2017). Both techniques have potential advantages, however, their applications are limited, especially when it comes to identifying protozoan parasites, which are found in low numbers in wastewater samples (Wang et al., 2014; Maritz et al., 2017; Marzano et al., 2017; Maritz et al., 2019). The lack of metagenomic studies on protozoan parasites has been attributed to the complexity of eukaryotic genomes, their large genome size, higher variability of the genome, limited reference databases, computational challenges, and low abundance compared to prokaryotes (Castellanos-Gonzalez et al., 2018; Zahedi et al., 2019; Zahedi et al., 2018; Huang et al., 2016). Therefore, fewer studies and limited complete genome sequences are available in reference databases for environmental samples. It is necessary to optimize methods and advance sequencing technology, reference databases, and computational tools in order to meet these challenges.

This study focused on profiling protozoan parasites from treated and untreated sewage collected at ten area points across three major South African cities: Cape Town, Durban, and Johannesburg. These regions are high-risk areas due to their popularity as tourist destinations, large populations living in informal settlements, and inadequate access to safe water, sanitation, and healthcare (Corburn and Sverdlik, 2019). Additionally, protozoan infections are common as a large part of the population is immunocompromised e.g., HIV positive (De Jong, 2017). To monitor the prevalence of infection risks associated with protozoan pathogens in these different areas, the genetic profiles of important human and non-human pathogenic protozoans in wastewater treatment plant samples were evaluated using 18S rRNA short amplicon sequencing and shotgun metagenomic sequencing approaches.

2. Materials and methods

2.1. Description of Wastewater Treatment Plants (WWTPs) and sample collection

Wastewater samples ($n = 10$) were collected from three of South Africa's largest and most populated cities, namely, Cape Town (CPT, $n = 4$), Durban (DBN, $n = 4$), and Johannesburg (JHB, $n = 2$) (Fig. 1(a-b)). The study limited the sample size to only domestic wastewater treatment plants (WWTPs) in order to minimize the variability in untreated wastewater composition. Two WWTPs, close to each other, were selected from each city. Both treated ($n = 4$) and untreated ($n = 6$) wastewater samples were collected within each WWTP. A pooled composite sample of approximately 2 L was taken from each sampling point within the wastewater treatment plant at different time intervals of 15/120 min. The samples from different locations were then transported overnight (in a cooler box) to the laboratory for analysis. Sample details are provided in Table S1 (supplementary).

2.2. Sample preparation and DNA extraction

The DNA was extracted in accordance with our previous study (Mthethwa et al., 2022). Briefly, the wastewater samples were subjected to centrifugation at 3500 rpm (max 4 × 340 g) for 10 min, followed by filtration of the supernatant using a 0.1 μm pore size cellulose acetate

filter paper. Total DNA was extracted from both the pellets obtained after centrifugation and the residual material on filter paper following the phenol-chloroform method (Mthethwa et al., 2022; Awolusi, 2016). A suspension of the pellet as well as residual material on the filter paper was prepared in lysis buffer (10 mmol/L Tris/HCl pH 8.0, 10 mmol/L EDTA, 0.1 mol/L NaCl, 2 % SDS, pH 8.0) and then treated with protease K. The mixture was incubated at 55 to 60 °C in a water bath for two hours (Mckiernan and Danielson, 2017; Awolusi, 2016), followed by five consecutive freeze-thaw cycles. After that, Tris-saturated Phenol-Chloroform-Isoamyl alcohol (25:24:1) was added, mixed well, and centrifuged for two minutes at 12000 rpm (Awolusi, 2016; Wells et al., 2016; Babaei et al., 2011). An equal volume of Chloroform was then added to the supernatant, followed by centrifugation at 12000 rpm for two minutes. The supernatant was transferred to a new tube and precipitated with isopropyl alcohol following centrifugation at 3000 rpm for 2 min. This was followed by an ethanol (70 %) wash. The final product, DNA, was air-dried and stored in TE buffer at −20 °C for further analysis.

2.3. 18S rRNA gene amplification and Illumina sequencing

The extracted DNA was quantified using a Nanophotometer (Implem, United Scientific, South Africa). Thereafter the DNA was sent to a commercial laboratory (Inqaba Biotech, South Africa) for high-throughput sequencing and analysis. Briefly, for amplicon sequencing, two published primers (Hadziavdic et al., 2014) were used to target the largest hypervariable (V4-V5) region of the Eukaryotes 18S rRNA gene. The forward (5'-CAGCAGCCGCGGTAATTCC-3') and reverse (5'-CCCGTGTTGAGTCAAATTAAGC-3') primers with adapter amplicon lengths compatible with Illumina MiSeq specifications (2 × 300 nt pair-end reads) were synthesized and used for amplification. The PCR reactions were carried out under the following conditions: initial denaturation at 95 °C for 15 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 1 min. The amplification was completed with a final extension step at 72 °C for 10 min. The resulting PCR products were purified using a column-based PCR purification kit (Thermo Fisher Scientific, South Africa), separated on gel electrophoresis to verify the size and quality, and the PCR products were quantified using the Qubit (Thermo Fisher Scientific, South Africa) instrument. Indexing PCR, Ampure bead purification, Equimolar pooling, and Sequencing on Illumina Miseq was performed at the commercial lab (Inqaba Biotech, South Africa).

2.4. Shotgun metagenomic sequencing

Shotgun metagenomic sequencing for all untreated samples was performed in parallel with 18S rRNA short amplicon sequencing. This was done to increase the taxonomic resolution of each organism detected down to species and strain level. Additionally, the shotgun procedure was used to obtain functional profiles of the Eukaryotic microbial community present in the samples. The metagenomic libraries were constructed at the Inqaba Biotech company in South Africa using a Library Prep kit (gDNA, amplicon, plasmids). High-throughput DNA sequencing was performed using the Illumina NexSeq platform.

2.5. Data analysis

2.5.1. Bioinformatics processing

The 18S rRNA raw paired-end reads were subjected to quality control, which included trimming of primers, sequencing adapter, and elimination of low quality (Q = 30) and short length reads using cutadapt (Zamora-Terol et al., 2020). Paired-end reads were merged using BBMap (Brandt et al., 2021). High-quality reads were considered for downstream analysis using the DADA2 plunging within QIIME2 (qiime2.org) to produce unique amplicon sequence variants (ASV's). Taxonomic assignment of the ASVs was carried out by first training a SILVA reference database classifier (clustered at 99 % sequence identity) according to the primer used and was then applied to generate a taxonomy frequency and abundance table. Relative taxonomic abundances were calculated for all wastewater samples and displayed in the stacked bar plot for phylum, class, and genera distributions. Additionally, the percentage distribution of phylum, class, and genera across all samples are also represented using pie charts in the supplementary section. The relative abundance of the Eukaryote taxa in different samples at the phylum, class, and genus level (ASVs) was calculated using indicator species analyses (Dufrêne and Legendre, 1997) and visualized using boxplots.

Shotgun metagenomic bioinformatics processing was done by removing reads mapping to the human genome using kneaddata (Langmead and Salzberg, 2012). Basic quality control was performed by trimming reads with quality scores below 20, and shorter reads (90 bp) using Trimmomatics (Bolger et al., 2014). Taxonomic classification and

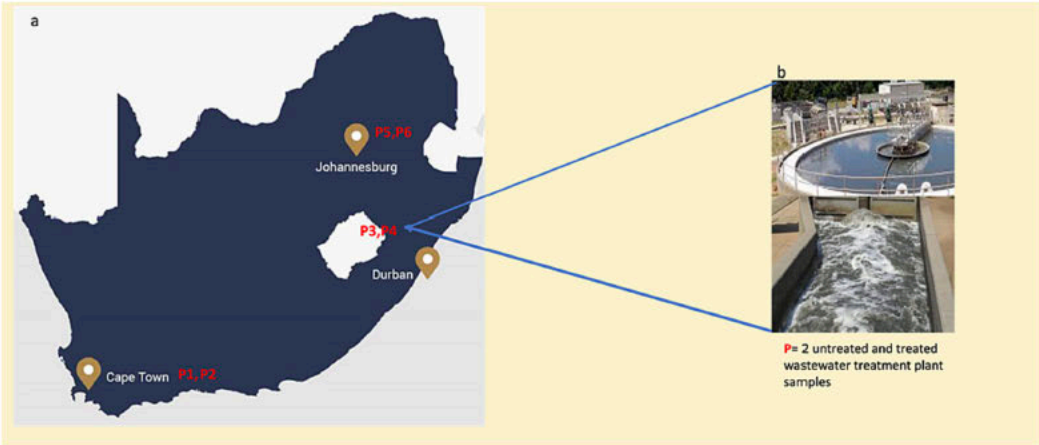

Fig. 1. (a-b). Geographical location of the samples collected. Samples were collected from two wastewater treatment plants in each location, both untreated and treated. A total of 12 samples were collected (2 L each).

relative abundance estimation were done using Kraken 2 (Wood et al., 2019). Lastly, HUMAnN 3.0 was used to profile microbial pathways and virulent gene families. The databases used included Metaphlan4, Uniref 90 full, and Chocophlan (Maritz et al., 2019). Each pathway derived from the shotgun approach was screened and only those pertaining exclusively to eukaryotes and protozoans were discussed. All bioinformatics analyses were conducted using resources from the University of Minnesota's Supercomputer Institute (MSI).

2.5.2. Statistical analyses

All microbial community analyses were carried out using the R statistical interface (Maritz et al., 2019). The R vegan package was used to calculate alpha diversity (Shannon, and richness) and Beta diversity (Bray Curtis distances) (Jin et al., 2017; Liu et al., 2021). Weighted and unweighted Bray Curtis Principal Coordinate Analysis (PCoA) distances were computed. The permutational multivariate analyses of variance (PERMANOVA) test was used to determine statistically significant differences in microbial community composition. False discovery rate (FDR)-adjusted Kruskal-Wallis tests for multiple comparisons (q 0.05) and species indicator analysis (indicator values) implemented in the labdsv R package (Maritz et al., 2019) were used in tandem to detect taxa that were differentially abundant at different geographical locations and between treated and untreated samples. The stats, vegan, heatmap, and ggplots2 R packages were used to create the graphs (Jin et al., 2017, Liu et al., 2021). Venn diagrams were generated to identify sample similarities(core) and unique species (Chen et al., 2021).

3. Results and discussion

A total of 669,617 (1,339,234 PE) 18S rRNA paired-end raw amplicon reads were obtained from 10 wastewater samples, resulting in 337,249 high-quality reads mapped in the SILVA reference database (Table S1 supplementary). The mean sequencing depth was 33,724.9 reads/sample, identifying a total of 438 species. Shotgun metagenomic sequencing of untreated wastewater samples from selected locations produced 23,826,052 paired-end raw reads. Using a Kraken2 standard plus Eukaryotes reference database, 19,493,187 (78.8 % - 84.45 %) high-quality reads were mapped. Of the 82.11 % averaged mapped reads, only 1.02 % average reads were of Eukaryotes with a mean sequencing depth of 66,816 reads/sample (Table S2) (supplementary).

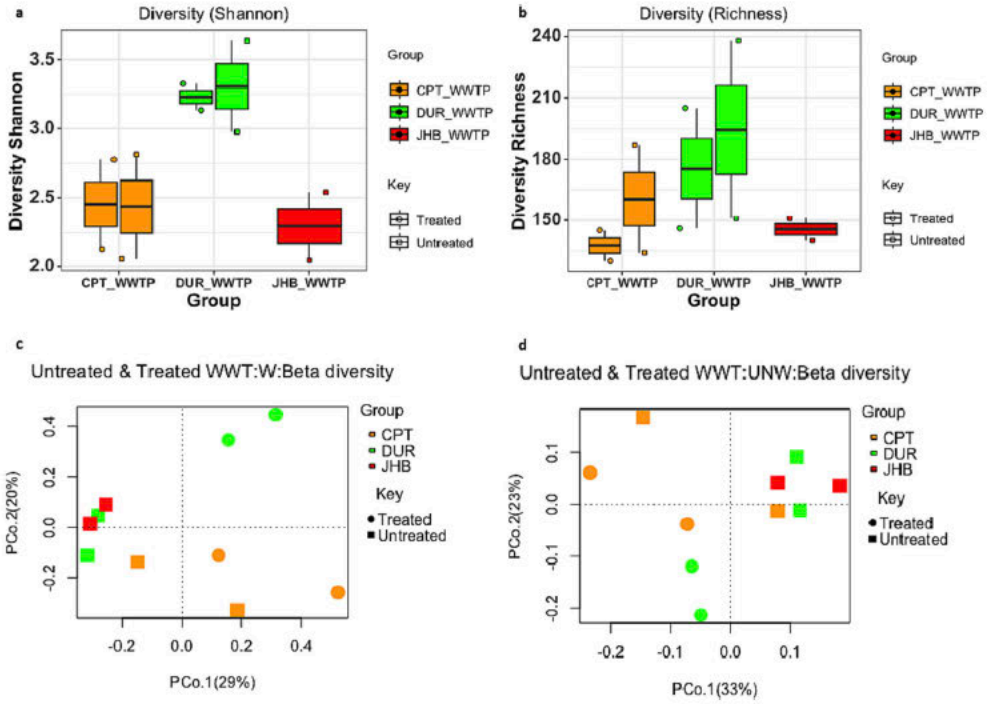

Fig. 2. (a-d). Eukaryotes diversity showing treated wastewater and untreated wastewater sampled from CTP_WWTP = Cape town wastewater treatment plants, DUR_WWTP=Durban wastewater treatment plants and JHB_WWTP = Johannesburg wastewater treatment plants. A Kruskal-Wallis rank sum test was used for testing the box plot distributions with *P*-value adjustment method: Benjamini-Hochberg (BH). (a)Boxplot of alpha diversity as measured by Shannon diversity index, showing higher diversity in samples from Durban compared to CPT and JHB geographical locations (*P*-value 0.03). (b) Alpha diversity showing higher eukaryotes species richness in DBN location with no significant difference between all three sampled locations (*P*-value = 0.17). The pairwise-Wilcox test showed no significant differences between both location groups nor type of wastewater (treated or untreated). (c-d). Principal coordinates analysis (PCoA) plot of Beta diversity as measured by Bray Curtis dissimilarity matrices is reported and the proportion of variation explained by each axis is listed in parentheses. (c) Weighted PCoA showing significant difference in microbiome composition across water types and the geographical locations ($R^2 = 0.28$, pseudo-F = 1.82, $P < 0.022$) and across the type (Key) of a wastewater ($R^2 = 0.21$, pseudo-F = 2.27, $P < 0.003$) according to PERMANOVA tests and ANOSIM ($R = 0.17$, $P < 0.041$). (d) Unweighted Beta diversity showing significant difference in microbiome composition across water types and geographical locations ($R^2 = 0.32$ pseudo-F = 1.82, $P < 0.040$) and type of wastewater ($R^2 = 0.181$, pseudo-F = 2.10, $P < 0.035$) according to PERMANOVA and ANOSIM ($R = 0.26$, $P < 0.011$).

3.1. Alpha diversity of protist communities in different wastewater samples

Alpha diversity analyses (Shannon index) of the 18S rRNA gene sequencing results revealed significantly higher Eukaryote diversity in both untreated and treated wastewater samples from DBN compared to CPT and JHB samples (Kruskal-Wallis test, P-value = 0.03) (Fig. 2a). Microbial richness (Fig. 2b), which only considers the presence or absence of taxonomic groups, revealed a pattern similar to that in Fig. 2a, but no statistical significance was observed (Kruskal-Wallis test, P-value = 0.17). Due to the limited number of samples collected for each type of wastewater (treated or untreated), statistical differences were not determined between the types of wastewater Despite this, some potential differences were visually apparent. Fig. 2b, for example, illustrates that untreated samples may exhibit greater microbial diversity. Rarefaction curves (Fig. S1a) supported the diversity patterns observed in Fig. 2a-b, accounting for sequencing depth. Notably, DBN samples exhibited higher microbial richness, whereas CPT samples showed lower richness, but relatively better coverage and sequencing depth. Analysis of shotgun metagenomic data for alpha diversity revealed no significant differences in community richness and diversity between samples (Fig. S2 (a-b)). Rarefaction curves (Fig. S2c) suggested that metagenomic sequencing depth adequately captured diversity, reaching asymptotic values as the number of sequences increased. Results from our study indicate that different factors could influence protozoan diversity within WWTPs, including wastewater source, sewage infrastructure, geographical location, environmental factors, and microbial adaptations as demonstrated by various other authors (Xu et al., 2018; Goux et al., 2015; Mclellan and Roguet, 2019; Manaia et al., 2018).

3.2. Beta diversity analyses across different types of wastewater

Principal coordinates analysis (PCoA) of Beta diversity as measured by weighted and unweighted Bray Curtis dissimilarity matrices for the 18S rRNA gene sequencing approach is shown in Fig. 2 (c-d). These analyses revealed noticeable similarities and distinctions in the microbial community patterns influenced mainly by the type of wastewater (Fig. 2c-d). Treated, and untreated wastewater samples were clearly separated, with dissimilarities being more prominent after treatment. For instance, the treated samples from CPT and DBN formed distinct clusters, separated from the untreated cluster, while also being positioned apart from each other along axis 2 (Fig. 2c-d). The untreated wastewater samples also showed a significantly different composition from the treated samples along axis 1 (Fig. 2c-d). Axis one of this PCoA model explained 29 % and 38 % of the variation in the data set for both weighted and unweighted PCoA, respectively (Fig. 2c and d). Permutational multivariance analysis of variance (PERMANOVA) using the adonis2 function in R (vegan package) corroborated statistical differences in microbial composition in different sample types, regardless of the location ($R^2 = 0.21$, pseudo-F = 2.27, $P < 0.003$ and: $R^2 = 0.181$, pseudo-F = 2.10, $P < 0.035$) for weighted and unweighted Bray-Curtis distances, respectively. The analysis of similarities (ANOSIM) also confirmed significant differences in microbial communities between sample types (R: 0.52, $P < 0.02$) and (R: 0.44, $P < 0.02$) for unweighted and weighted distances, respectively. Various factors such as the treatment process used, the source of influence, and the geographic location may contribute to the specific microbiome profiles of treated wastewater samples (Oluseyi Osunmakinde et al., 2019; Thobejane et al., 2023; Giwa et al., 2020; Verburg et al., 2021). In a previous study, sewage microbiome patterns were examined at various stages (influent, activated sludge, and effluent) in Hong Kong's largest wastewater treatment plant, and their PCoA profiles also revealed a dispersed pattern among effluent samples (treated) and overlapping influent samples (untreated), as well as a distinct separation between treated and untreated samples. (Cai et al., 2014). The study also observed that all untreated samples from the wastewater treatment plants were clustered together, with the exception of one sample. The increased similarity in the microbiome patterns observed among the analyzed untreated samples may be attributed to consistent influent (untreated) wastewater composition, shared microbial sources from human and environmental origins, limited treatment efficiency, and resilient microbial populations (Cai et al., 2014; Fletcher et al., 2012).

3.3. Differentially abundant taxa in untreated and treated wastewater sample type

Indicator species analysis (Indval) identified particular taxa distinguishing untreated and treated samples (Fig. 3a-f) (Table S3). Untreated wastewater samples specifically exhibited a significant presence of phyla Alveolata, Stramenopiles, Apusomonadidae, and Nucletmycea (Fig. 3d-e). On the other hand, the phyla Tubulinea, Rhizaria, and Discoba were largely detected in the treated water samples (Fig. 3f-h), whereas Chloroplastida and Holozoa appeared in both treated and untreated samples (Table S3). The abundance of Holozoa and Tubulinea in the treated samples may be attributed to their feeding habits, as they consume bacteria or act as hosts for prokaryotic symbionts (Betat et al., 2015). Other studies have also reported increased viruses and certain eukaryotes in treated wastewater samples compared to untreated samples (Azli et al., 2022; Yasir, 2020). According to those studies, these microorganisms displayed high resistance to sedimentation and inactivation throughout the entire treatment process. As a result, even after being discharged, the remaining microorganisms, such as viruses and larger eukaryotes, can continue to reproduce, grow, and thrive in the treated water (Azli et al., 2022).

3.4. Similarities and variations observed in different geographical locations

While the type of wastewater was primarily responsible for patterns of differentiation in microbial communities, similarities and variations across geographical regions were also evident (Fig. 2c-d & Fig. S2 (d-e)). Microbial analysis of samples from Johannesburg (JHB), Durban (DBN), and Cape Town (CPT) revealed distinct patterns, with JHB and DBN showing less variation and clustering, while one CPT sample differed significantly (Fig. 2c-d). Additional shotgun sequencing data further confirmed unique microbial compositions in each city (Fig. S2(d-e)). Despite the limitation of one representative sample per site, principal coordinate analysis (PCoA) suggested greater variation in the CPT sample along PCo axis 1 (95 % and 75 % variation) (Fig. S2(d-e). These findings could suggest that the geographical proximity between human communities plays a role in shaping the microbial composition of wastewater (Kim et al., 2021). These findings agree with a previous study where activated sludge community similarity decreased as a result of the distance decay relationship (Kim et al., 2021). PERMANOVA and ANOSIM analyses based on location show differences in environmental microbial communities across the cities, with CPT samples showing distant microbial profiles from those in JHB and DBN samples (test $R^2 = 0.28$, pseudo-F = 1.82, $P < 0.022$ and ANOSIM's $R = 0.17$, $P < 0.05$ for weighted analyses and $R^2 = 0.32$ pseudo-F = 1.82, $P < 0.040$ and ANOSIM's $R = 0.18$, $P < 0.02$ for unweighted distances). The interaction between the location group samples and the type of wastewater showed no statistical significance according to the weighted ($R^2 = 0.11$ pseudo-F = 1.42, $P < 0.16$) and unweighted PCoA PERMANOVA test ($R^2 = 0.08$ pseudo-F = 0.88, $P < 0.58$). However, in this study, further research is recommended with larger sample sizes to confirm these observations.

3.5. Core eukaryote community

A core eukaryote community was evident across all wastewater treatment plants sites in South Africa, comprising of 40 different species of the 18S rRNA short amplicon data (Fig. 3a) and 116 species of the shotgun metagenomics data (Fig. 3b) (Table S4). This included both

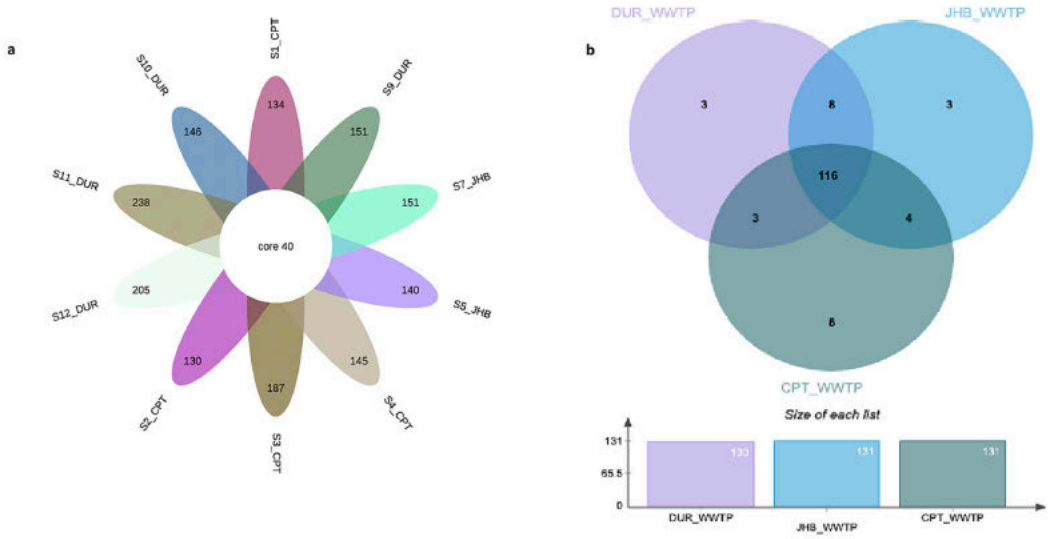

Fig. 3. (a) 18S rRNA short amplicon sequencing: Venn flower diagram showing core and unique species across all wastewater treatment plant samples. The central circle shows the number of species common to all samples while the petals show the number of species in addition to the core set unique. Abbreviated sample names (S1-S2) are given outside each petal, and details are given in Table S1 (supplementary). (b) Shotgun metagenomic: Venn diagram depicting the number of shared and unique number of species from the geographical locations.

disease-causing and non-disease-causing organisms. Some of the 116 core (common) pathogenic species as presented in Table S4 included *Cryptosporidium hominis* TU502, *Trypanosoma brucei gambiense* dal972, *Leshmania donovani*, *Cryptosporisdium parvium lowa* II and *Girdia intestinalis*. Several factors may contribute to the core pathogenic community, including travelers, shared facilities, as well as similar environmental and socio-economic conditions between different regions within the country (Fletcher et al., 2012). For instance, DBN and CPT are two of the most populated, busiest, and premier South African tourist destinations. Infection transmission/carryover between the two cities could be caused

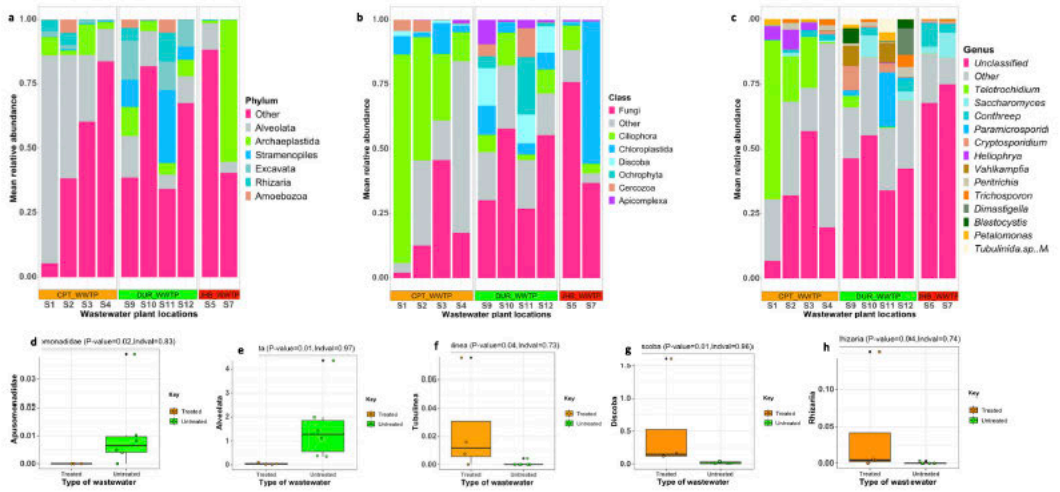

Fig. 4. (a-f). 18S rRNA generated Eukaryote relative abundance of individual wastewater samples (S1 to S12) from different locations. (a) Phylum-level Relative abundance of individual samples. (b) Relative abundance of the Eukaryote at the class level per location per sample. (c) Relative abundance of the Eukaryote at genus level per location per sample. (d-h)Differential relative abundance analysis of the eukaryote taxa associated with type of wastewater samples (untreated and treated) across different geographical locations. Taxa identified as discriminating based on indicator value analysis-are shown in box plots depicting the most abundant taxa associated with each group. (d-e) Show Apusomonadidae and Alveolata significantly abundant in untreated samples (f-h) Show Tubulinea, Discoba, and Rhizaria significantly abundant in treated samples.

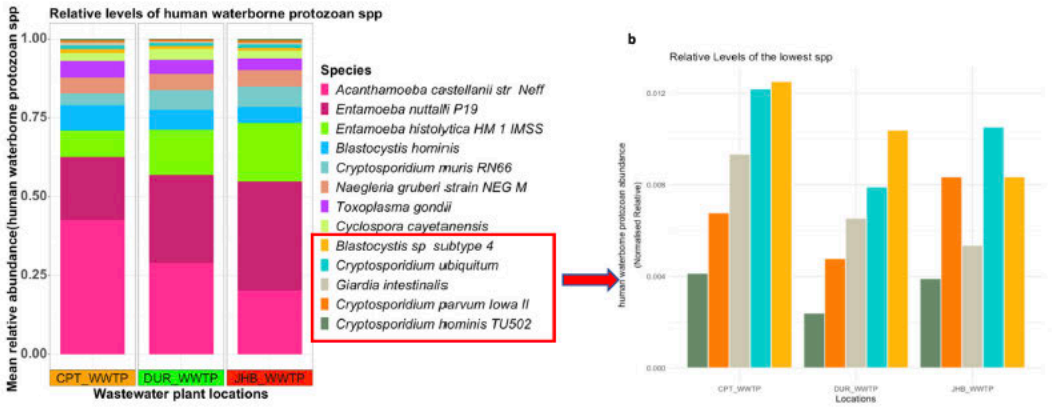

Fig. 6. (a-b). The relative abundance of the most important human waterborne protozoa in each city is shown, as per WHO and CDC guidelines/priority.

spread by infected water sources, food, animals, or person-to-person contact (Ma et al., 2022; Gerba, 2015). Thus, underlines the necessity of effective wastewater treatment and suitable disposal methods as well as the need for monitoring their prevalence in treated wastewater. The prevalence of pathogenic protozoa, including *Acanthamoeba* spp., which are also associated with *Acanthamoeba keratitis* and severe central nervous system infections, raises concerns for public health (Yoder et al., 2012, Chalmers, 2014, CDC, 2021). These pathogens are difficult to treat and can survive in various environmental conditions, their presence in wastewater is a potential health concern (Omarova et al., 2018). They have been found in river waters, soils, and untreated surface irrigation water mixed with wastewater (Mahmoudi et al., 2015; Moreno et al., 2018). They are the third leading cause of waterborne protozoal contamination across ten African countries (Uganda, Tunisia, Sudan, South Africa, Namibia, Guinea Bissau, Ethiopia, Egypt, Central African Republic, and Benin), accounting for more than a third of all documented cases (Ben Ayed et al., 2019). Additionally, the identified Amoebozoa can act as a host and carrier for several other bacterial pathogens such as *Salmonella enterica*, *Pseudomonas* spp., *Mycobacterium*, *Vibrio*, and *Legionella pneumophila* (Juárez et al., 2018; He et al., 2021). Additionally, these pathogens exhibit resistance to common disinfection methods such as chlorine treatment (He et al., 2021).

Regional variations were observed, with certain pathogenic species more abundant in specific areas. For example, *C. parvum* was more prevalent in JHB, while *Blastocystis* spp., *C. ubiquitum*, *G. intestinalis*, and *C. hominis* were more abundant in CPT. This observation could reflect the high human and zoonotic-related disease burden in this region. These pathogens have been shown to cause a significant proportion of reported cases of waterborne disease, with cryptosporidiosis being the leading cause of worldwide waterborne protozoan outbreaks (WHO, 2020; Garcia et al., 2017; Ma et al., 2022). These findings are consistent with previous findings in South Africa (Omoruyi et al., 2011) and other developing countries such as Kenya (Delahoy et al., 2018), Ghana (Alum et al., 2014; Sampson et al., 2017), and Nigeria (Jombo et al., 2010), where diarrheal infections are primarily linked to *Cryptosporidium* spp. De Jong (2017) investigated three WWTP in Durban, KwaZulu Natal, and found that *Cryptosporidium* spp. dominated all of them. Furthermore, Abu Samra et al. (2013) discovered the presence of *Cryptosporidium* in four other South African regions (Gauteng, KwaZulu Natal, Mpumalanga, and Northwest). This study analyzed clinical stool samples from hospitals, indicating their prevalence among the populations in the area (Abu Samra et al., 2013).

Additionally, all WWTP locations showed the least abundance of other protozoans of public importance, including *Blastocystis hominis*, *Naegleria gruberi*, *Toxoplasma gondii*, *Cyclospora cayetanensis*, and *Giardia intestinalis* (Fig. 6a-b). The highest counts were found in CPT and JHB untreated WWTPs, likely reflecting public health patterns in the surrounding community at the time of sampling.

3.7. Functional pathways and virulent gene families distinguishing WWTPs microbiome

Virulence gene families and enriched functional pathways linked to protozoa have been identified in wastewater samples collected from diverse locations (Fig. 7). These findings indicate the widespread presence of protozoan parasite and corroborate the above taxonomic profile results, as illustrated in Fig. 7a and b. Specific observations include an increased abundance of certain genes in JHB, such as serine/threonine protein phosphatase and mucin-desulfating sulphatase, and elevated levels of TRAP Transporter in DBN (Fig. 7a). The exclusive presence of specific gene families, such as Trypsin-Like Serine and Cysteine Protease in JHB highlights potential regional distinctions in protozoan parasite-associated virulence factors. Previous studies have identified potential virulence factors linked to *Cryptosporidium* spp., and other protozoa and elucidated the genes and proteins crucial for parasite survival, replication, and potential host infection. These studies have highlighted serine protease, aminopeptidase, CSL, gp900, gp60, and a sporozoite and merozoite cell surface protein complex (gp15/40/60), which may be involved in excystation, adhesion, and invasion (Bouzid et al., 2013; Audebert et al., 2020; Wang et al., 2022; Xu et al., 2020; David Sibley, 2011).

The results of shotgun metagenomic analysis further confirmed the presence of functional pathways associated with pathogenic waterborne protozoa in all wastewater treatment plants (WWTPs) (Fig. 7b). These pathways include amino acid biosynthesis, peptidoglycan maturation, adenosine and branched-chain amino acid biosynthesis, and the Calvin-Benson-Bassham cycle. Notably, protozoa such as *Cryptosporidium parvum*, *Toxoplasma gondii*, and *Giardia intestinalis* rely on various amino acids for energy and protein synthesis (Bouzid et al., 2013; Krishnan and Soldati-Favre, 2021). Polyamine pathways (spermidine biosynthesis) are important compounds for the growth and replication of pathogenic protozoa such as *Leishmania donovani* and *Trypanosoma cruzi* (Verdaguer et al., 2019). Additionally, essential pathways for eukaryotes such as heme B biosynthesis, glycolysis and chitin were also found. While heme B biosynthesis pathways are actively involved in the degradation of organic matter, some waterborne protozoan parasites rely on heme for their survival and growth (Verdaguer et al., 2019). Pathogenic protozoa such as *Entamoeba histolytica* and *Trichomonas vaginalis* use glycolysis for

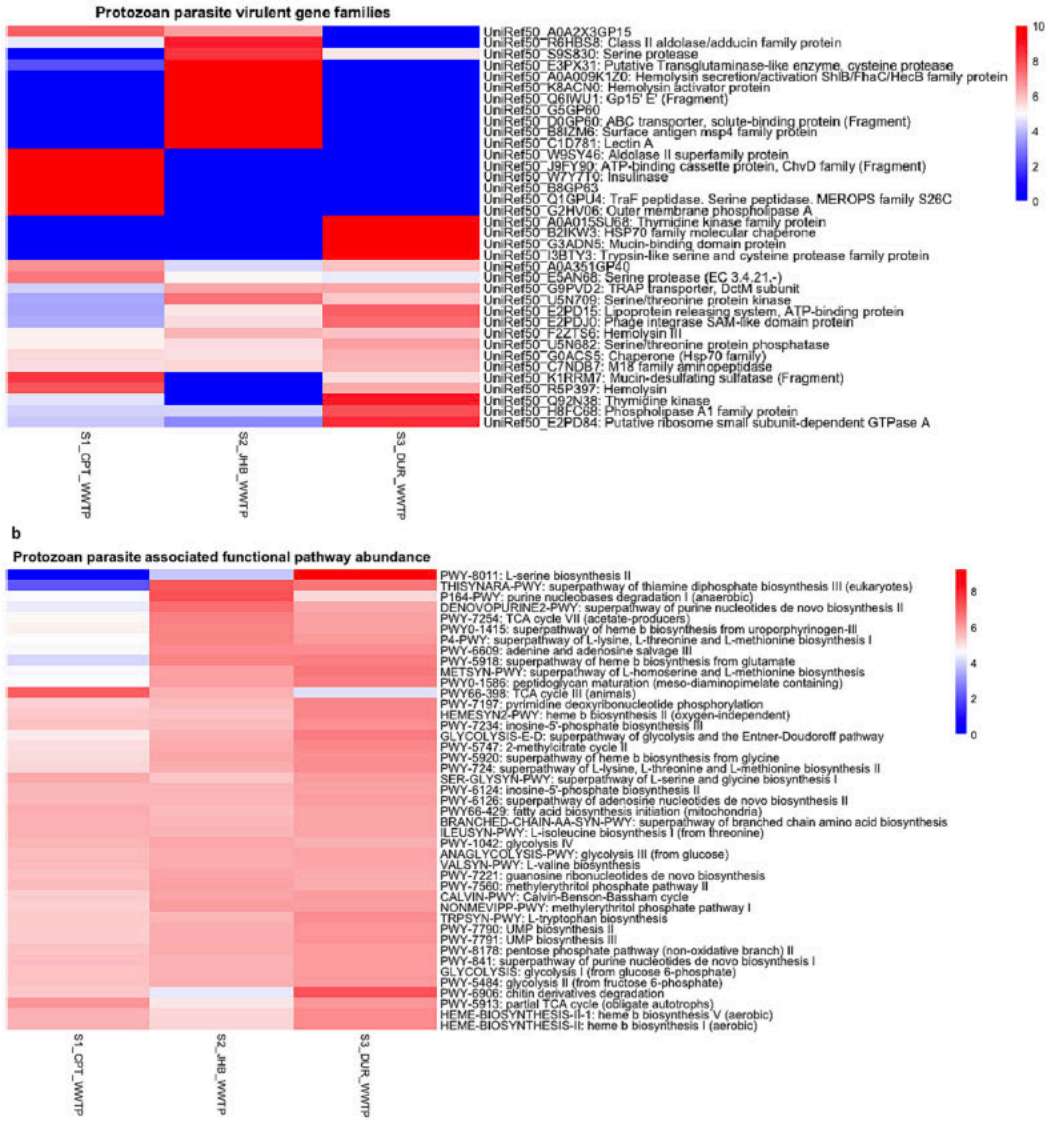

Fig. 7. Heatmaps showing (a) Protozoan parasite virulent gene families and (b) Functional pathways abundance of the influent (untreated) metagenomes in each city.

their energy metabolism (Verdaguer et al., 2019) and were observed in all WWTP in this study. Chitin is also a major component of the cyst walls of the protozoan parasites *A. Castellani* and *E. histolitica*, which utilize chitin for their survival and virulence (Michael, 2016; Verdaguer et al., 2019). Lastly, pentose phosphate pathway (non-oxidative branch) II, specifically linked to *Entamoeba hystolitica*, was found in all WWTP. This suggests that the protozoan pathogens may show metabolizing and growing functional potential within the WWTPs. This could lead to an increased risk of virulence and transmission to humans and animals if the treated wastewater is not properly disinfected before being released into the environment. Accordingly, this study emphasizes the need to regularly monitor WWTPs for the presence of pathogenic protozoa and to take measures to ensure that treated wastewater can be discharged without risk.

4. Conclusions

Using 18S rRNA gene amplicon sequencing and shotgun

metagenomic profiling, this study explored the genetic diversity of human and non-human protozoan parasites in South African wastewater treatment plants (WWTPs). There was a significant difference in the microbial diversity structure depending on the type of wastewater (treated or untreated) as well as the treatment process used at each geographical location. In addition, all WWTPs shared a core community. Several protozoa of public health importance were found in the untreated wastewater samples, including *Cryptosporidium* spp., *Entamoeba histolitica*, *Blastocystis hominis*, *Naegleria gruberi*, *Toxoplasma gondii*, *Cyclospora cayetanensis*, and *Giardia intestinalis*. Thus, these findings contribute to the understanding of the incidence of recognized and emerging protozoan pathogenic species with specific relevance to South African environments. Although this study provides an overview of possible protozoan pathogens in the region, further research is still necessary to better understand their prevalence. For a more comprehensive understanding of the protozoan species in wastewater samples and the impact of different factors on their prevalence, additional longitudinal research based on next generation approaches should be conducted, including a larger sample size within an extended geographical scope. This approach could aid in the development of intervention approaches to address specific protozoans of public health importance and reduce the spread of waterborne diseases in countries with similar public health challenges.

CRediT authorship contribution statement

Nonsikelelo P. Mthethwa-Hlongwa: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Isaac D. Amoah:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Andres Gomez:** Formal analysis, Resources, Supervision, Visualization, Writing – review & editing, Validation. **Sam Davison:** Formal analysis. **Poovendhree Reddy:** Supervision, Validation, Writing – review & editing. **Faizal Bux:** Funding acquisition, Supervision, Validation, Writing – review & editing. **Sheena Kumari:** Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.169602.

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9.5 Appendix 5

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Fluorescence and colorimetric LAMP-based real-time detection of human pathogenic *Cryptosporidium* spp. from environmental samples

Nonsikelelo P. Mthethwa [a,b], Isaac D. Amoah [a], Poovendhree Reddy [b], Faizal Bux [a], Sheena Kumari [a,*]

[a] Institute for Water and Wastewater Technology, Durban University of Technology, Durban 4000, South Africa
[b] Department Community Health Studies, Faculty of Health Sciences, Durban University of Technology, Durban 4000, South Africa

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ABSTRACT

Public health concerns related to pathogenic protozoa are widespread, causing significant morbidity and mortality worldwide. Due to the lack of rapid and cost-effective diagnostic methods, timely treatment and control interventions are hindered. In this study, loop mediated isothermal amplification (LAMP) methods were optimised and evaluated for the rapid detection of human pathogenic *Cryptosporidium* species in environmental samples. Real-time fluorescence and colorimetric detection were tested simultaneously. As a reference method, the results were compared to the well-established droplet digital polymerase chain reaction (ddPCR) method. Both LAMP-based methods successfully detected the *Cryptosporidium parvum* (*GP60* gene) and the entire *Cryptosporidium* genus (*SAM* gene) from environmental samples with 100% specificity and no cross-reactivity. Furthermore, both colorimetric and fluorescent methods demonstrated a high sensitivity, with the same limit of detection (LOD) of 1.1 copies of *C. parvum* per 25 µl reaction (0.02 ng/µl). For real-time fluorescence monitoring, the LAMP reaction can be completed within 28 min, and for visual detection, within 30 min. In addition, both fluorescent and colorimetric LAMP methods showed substantial agreement with the reference method (ddPCR) used. The developed protocols were able to detect *C. parvum* and *Cryptosporidium* spp. in 50–85% (n = 60) of environmental samples (treated and untreated wastewater, sludge, and surface water) compared to 58–98% (n = 60) detected by ddPCR. The results further demonstrate that LAMP is an efficient technique for detecting *Cryptosporidium* spp. in environmental samples due to its simplicity, low cost, sensitivity, and specificity. Therefore, it has great potential as a useful diagnostic tool for disease control and public health protection.

1. Introduction

Infections caused by *Cryptosporidium* spp. are a serious public health issue that can be transmitted by contaminated food, water, and wastewater (Hassan et al., 2020). The parasites have been linked to several large outbreaks of water-borne disease, including the largest known outbreak in 1993, which affected more than 400,000 people (World Health Organization WHO, 2019). High rates of *Cryptosporidium* spp. infections occur in developing countries, where sanitation is inadequate and safe drinking water is scarce (Kurenzvi et al., 2020). Reports indicate that 75% and 88% of global *Cryptosporidium* spp. infections and deaths occur in Africa, with Nigeria and the Democratic Republic of Congo (DRC) contributing the greatest number of cases (Wanyiri et al., 2014; Kombo Mpindou et al., 2021). The resistance of *Cryptosporidium* spp. to commonly used water disinfection methods such as chlorination, exacerbates the public health concern (Omarova et al., 2018). Their infective oocysts can survive in harsh environmental conditions for months, and the dose required to infect humans (1–10 oocysts) is very low (World Health Organization WHO, 2017). Thus, water treatments are governed by a set of published guidelines issued by environmental protection agencies, such as the United States Environmental Protection Agency (EPA), the World Health Organization (WHO), and provincial regulators, in order to prevent disease-causing organisms from entering water supplies at levels that are safe for the environment and public health (Feng et al., 2021; World Health Organization WHO, 2019).

In order to monitor the reduction of potential infections or oocyst concentrations to health standards, an efficient and rapid method for the detection and quantification of *Cryptosporidium* spp. oocysts is essential. Nevertheless, due to their low numbers and the complexity of the sample matrix, their detection in environmental samples such as wastewater can

* Corresponding author.
E-mail address: sheenak1@dut.ac.za (S. Kumari).

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be challenging (Bilung et al., 2017). The current diagnosis of *Cryptosporidium* spp. relies on microscopic, immunological, and advanced molecular methods for quantitative and qualitative testing (Adeyemo et al., 2018). However, these methods have certain limitations. For example, the microscope technique is time-consuming, requiring extensive experience and highly skilled personnel, and does not provide specific identification information (Hassan et al., 2020; Ryan et al., 2017). While immunological techniques such as immunochromatography and enzyme-linked immunosorbent assay (ELISA) are rapid, they possess limited specificity and sensitivity (Ryan et al., 2017). Although molecular techniques such as polymerase chain reaction (PCR), real-time PCR (RT-PCR), and droplet digital PCR (ddPCR) overcome most of the constraints mentioned above, they are expensive, and they are not readily available, especially in low-income countries where disease burden is high (Keikha, 2018; Silva et al., 2020; Ryan et al., 2017). Therefore, methods with increased speed, sensitivity, specificity, reproducibility, automation, and low cost are required to facilitate the detection and monitoring of infections. A promising candidate is the isothermal amplification technique since it allows detection of a nucleic acid target sequence without the use of thermal cyclers and high temperatures required to denature double-stranded DNA (Liu et al., 2019; Rahimi Esboei et al., 2022).

These methods use a strand displacement polymerase to enable primer binding and initiation of amplification reaction at a single constant temperature (25–65 °C) (Wang et al., 2015). Examples of isothermal amplification methods are: loop mediated isothermal amplification (LAMP), whole genome amplification (WGA), strand displacement (SDA), helicase dependant amplification (HAD), recombinase polymerase amplification (RPA) and nucleic acid sequences based amplification (NASBA) (Ahmad and Hashsham, 2012). All these methods have gained significant attention in molecular diagnosis, and some have been used to provide point-of-care diagnosis in resource-limited settings (Wilisiani et al., 2019). The LAMP is a promising candidate for the detection of protozoan parasites, owing to several good features discussed below. The LAMP method was developed by Notomi et al. (2000) and is both simple and fast: under ideal conditions, the reaction can be completed in less than an hour (Notomi et al., 2000; Lu et al., 2020; Keikha, 2018; Fallahi et al., 2020). This method is considered sensitive, because it can detect DNA at very low concentrations and it is highly specific: six distinct regions/sequences on the target gene are recognized by four specific primers (Liu et al., 2019; Tavares et al., 2011). Moreover, one of the key advantages of LAMP is its ability to process complex samples such as water or wastewater with minimal effort as the *Bst* polymerase is reported to be highly resistant to inhibitor molecules (Keikha, 2018). Additionally, it allows the use of regular water baths to regulate the temperature of amplification reactions without the need for costly thermal cyclers (Ahmad and Hashsham, 2012; Silva et al., 2020). LAMP efficient amplification has been widely applied for rapid diagnosis of different plant pathogens (Bühlmann et al., 2013; Lu et al., 2015), pathogenic parasites (Fallahi et al., 2015, 2018; Gallas-Lindemann et al., 2013), malaria causing pathogen (Picot et al., 2020), *E.coli* (Liu et al., 2019), *Mycobacterium* spp. (Yashiki et al., 2019) and recently SARS-CoV-2 (Lu et al., 2020; Zhang et al., 2020; Park et al., 2020; Amoah et al., 2021). A prior study demonstrated the utilization of the traditional LAMP approach, using magnesium pyrophosphate and UV light for results analysis, in detecting waterborne protozoan parasites (*Cryptosporidium* spp., *Giardia* spp., *and Toxoplasma* spp.) in environmental samples (Sotiriadou, 2012).

Using a combination of calorimetric and real-time fluorescence methods, this study sought to develop and evaluate rapid, sensitive, and specific LAMP methods for the detection of *Cryptosporidium* spp. from environmental matrices. The first assay was based on the colorimetric LAMP method, which relies on instant visual detection of amplification results, eliminating the need for further gel electrophoresis and the use of hazardous ethidium bromide. It has the potential to reduce workflow time and costs, enabling it to be used at the point-of-care or on the field. The second method used a real-time LAMP assay that enabled the monitoring of results in real time. With this method, a fluorescent dye (SYBR green) was added to the reaction, which allowed results to be monitored in a qPCR thermocycler machine or on a connected fluorometer screen. All tests were conducted alongside established Droplet digital PCR as a reference method, for method evaluation and validation.

2. Methodology

2.1. Reference oocysts and DNA

The LAMP assays were optimized by using non-viable *Cryptosporidium* spp. (*Cryptosporidium parvum* and *Cryptosporidium muris)* oocysts obtained from Waterborne Inc. (New Orleans, USA). A stock of 1 x 10^6 oocysts of *Cryptosporidium* spp. were prepared and certified to be 99% pure by the supplier. DNA was extracted from the reference oocysts according to a modified Phenol-chloroform extraction method (Mthethwa et al., 2022). LAMP type-specific primer sets that target the *SAM* gene (GenBank accession number: AY161084) and *GP60* gene (GenBank accession number: AB237136) found in *C. parvum*, *Cryptosporidium hominis* and *Cryptosporidium meleagridis* were used for optimization of both LAMP and ddPCR method (Table 1). Melting temperature value, primer length, primer dimers, and specificity of the primers were first assessed using the Basic Local Alignment Search Tool (BLAST) for sequence comparison from the National Centre for Biotechnology Information (NCBI). A synthetic DNA of *C. parvum* purchased from Microbiologics Inc. (1.1x10^7 copies/1.5 ml) was also used as a positive control for all reactions.

2.2. Optimisation of LAMP methods for detection of Cryptosporidium spp

LAMP methods were first developed using the colorimetric LAMP technique (cLAMP), which allows for visual interpretation of the results through the changes in color. The second was the fluorescent LAMP (fLAMP), which utilizes a DNA double strand intercalating fluorescent dye for the detection and semi-quantification of positive amplifications. The following sections provide more details about these two methods.

2.2.1. Colorimetric/visual LAMP method (phenol red indicator)

The methods were carried out at 60 °C, and 65 °C to determine the

Table 1
Primer sequences used in LAMP amplification experiments.

***Cryptosporidium parvum GP60* gene**[□]	
Primer type	**Sequence 5'-3'**
F3,	TCG CAC CAG CAA ATA AGG C
B3	GCC GCA TTC TTC TIT TGG AG
FIP	ACC CTG GCT ACC AGA AGC TTC AGA ACT GGA GAC GCA GAA
BIP	GGC CAA ACT AGT GCT GCT TCC CGT TTC GGT AGT TGC GCC TT
LF (loop primer) LF1	GTACCACTAGAATCTTGACTGCC
LB (loop primer) LB1	AACCCACTACTCCAGCTCAAAGT
***Cryptosporidium* spp. *SAM* gene**[□]	
F3,	ATTTGATRGACAAAGAAACTAG
B3	CGATTGACTTTGCAACAAG
FIP	TTGCGCCCTGTTAATCCAGCATTAATTAATCCATCTGGCAGRTTT
BIP	TTGTAGATACATACGGAGGATGGGTCTACTTTAGTTGCATCTTTCC
LF (loop primer) LF1	CTGCTGGCCCMCCAATTG
LB (loop primer) LB1	CATGGRGGTGGTGCATTTAG

□ (Sotiriadou, 2012).

optimal reaction temperature of the primers. These temperatures were chosen based on the melting temperature of the primer and previous literature recommendations. On the basis of specific binding, brightness, and sharpness of the ladder-like pattern of the LAMP products in gel electrophoresis, the concentrations of different LAMP reagents such as mixture of dNTPs, MgSO4, and betaine were optimized. The assays were also run for various time intervals ranging from 15 to 60 min, with results taken every 5 min, to determine the best time for amplification. Following optimization, the reaction was carried out at 65 °C using the heating block and the mixture consisted of the following as per individual method:

The cLAMP was performed in a 25 µl total reaction volume which consisted of 12.5 µl WarmStart cLAMP 2X Master Mix (WarmStart isothermal amplification buffer, Bst 2.0 Warm- Start DNA Polymerase) (New England Biolab, USA), Phenol red indicator (for visual colour), LAMP Primer Mix (10X) (2 µM F3 primer, 2Mm B3 primer,16 µM FIP, 16 µM BIP, 8 µM LoopF and 8 µM LoopB), 2 µl template DNA, and 8 µl of nuclease-free water was added. The phenol red indicator used for analysis allows for a clear visual detection of amplification. It relies on the production of protons and a decrease in pH that occurs due to extensive DNA polymerase activity in a LAMP reaction, resulting in a color change from red/pink to yellow. A visual inspection of the results was immediately conducted following removal of the tubes from the heating block. The preliminary results were confirmed by gel electrophoresis. In order to determine the minimum time required for a positive reaction to occur, results were taken every five minutes during the optimization phase.

2.2.2. Fluorescent real-time LAMP (fluorescent dye)

The fluorescent real-time LAMP (fLAMP) reactions were performed in a QuantStudio™ 3 Real-Time PCR System (Thermofisher, USA). The 25 µl fLAMP reaction mixture contained 12.5 µl of WarmStart amplification kit which contained the same reaction mix components and conditions as described in the colorimetric/visual LAMP method above (section a). The only difference was the replacement of the phenol red indicator with 50X SYBR green (fluorescent dye), which binds to dsDNA for real-time detection of LAMP. Fluorescence intensity was measured every minute, and the time it took for a sample (reaction) to cross the 400,000 threshold was recorded to estimate the time for positive amplification. The melting analysis was then performed, enabling the generation of derivative melting curves. The LAMP tests were performed in triplicate and negative (no template) controls were included.

2.3. Specificity and sensitivity of both cLAMP and real time fLAMP

The specificity of optimised cLAMP and fLAMP *C. parvum* methods was evaluated using closely related species of the same genus (*C. muris*), other waterborne protozoan parasites (*Giardia lamblia and Giardia muris*), and a group of bacteria commonly found in the environment, such as soil and water (*Escherichia coli,* and *Aeromonas* spp.). The limit of detection was determined by serial dilutions of *C. parvum* DNA template up to 0.004 ng/µl, starting at 20 ng = 1132copies/µl for both cLAMP and fLAMP. For fLAMP, after setting the threshold line to 400,000, the assay was then tested for the lowest concentration of template DNA that could be detected without false positive amplification.

2.4. Confirmation of cLAMP and fLAMP methods

After amplification and electrophoresis on agarose gel, LAMP products were confirmed by cutting the lowest band from the 2 % agarose gel of LAMP reaction and purified using QIAquick Gel Extraction Kit (Quiagen, Hilden, Germany). The pure amplicons were then cloned and sequenced by a commercial lab (Inqaba Biotec, SA) (Bakheit et al., 2008; Bilgiç et al., 2017; Choi et al., 2018). The Sequencing Analysis 5.2.0 program (Applied Biosystems, CA, USA) was used to convert the raw sequencing data to chromatograms. The Bioedit (version 7.2.5.0) and NCBI database were used for further analysis and identification.

2.5. Droplet digital PCR (ddPCR) method

The ddPCR reactions were performed on all samples using the QX200 Droplet Digital PCR system (Bio-Rad). Each assay mix was prepared in a final volume of 20 µl, containing 2× evagreen ddPCR Supermix (10 µl), 0.5 µm forward (1µl) and reverse primers (1 µl) (Table 2) and template DNA (2µl). Generation of droplets was performed by the QX200 Droplet Generator (Bio-Rad). PCR amplification was carried out on a T100 thermal cycler (Bio-Rad), using the conditions: 10 min at 95 °C, followed by 50 cycles of denaturation at 95 °C for 15 s, annealing at 56–60 °C for 1 min, and extension at 72 °C for 1 min, and a final step of extension at 72 °C for 10 min. After amplification in thermal cycler the samples were analysed using the QX200 Droplet Reader and QuantaSoft software (Bio-Rad). Based on the No Template Control (NTC) and positive control, a threshold line was drawn.

2.6. Detection of Cryptosporidium parvum and Cryptosporidium spp. from environmental samples

We further evaluated the efficacy of the optimized fLAMP method using environmental samples for the identification of *C. parvum* and *Cryptosporidium* spp. using the *GP60* and *SAM* genes. The fLAMP method was chosen because of its sensitivity, high speed, and real-time analysis of results. A total of 60 samples were collected from the province of KwaZulu-Natal in South Africa for this study, comprising untreated wastewater (15), treated wastewater (15), surface water (15), and sludge (15) (Fig. 1 Supplementary material). Sample collection, processing, and DNA extraction were done in accordance with our previously optimized protocol (Mthethwa et al., 2022). In brief, 2L of samples from different matrices (untreated and treated wastewaters, sludges, and surface waters) were collected at different time interval of 15/120 min using the composite sampling technique. This was done in order to obtain a complete representation of the microbial community in the studied source water. The samples were kept on ice box in sterile sampling bottles before being transported to the laboratory for analysis. In the laboratory, the samples were concentrated by centrifugation (Hermle Labortechnik GmbH) at 3500 rpm (max 4 x 340 g) for 10 min plus filtration of supernatant for the maximum recovery as optimised in our previous study (Mthethwa et al., 2022). The supernatant was filtered using 0.1µm pore size cellulose acetate filter paper. The total DNA was then extracted from both the pellets and the residual material on the filter paper mixed using the phenol-chloroform method (Djurhuus et al., 2017; Awolusi, 2016). The extracted DNA was subsequently amplified using fLAMP as described in Section 2.2(b). The ddPCR method described in Section 2.6 was applied to the same samples as a reference method. The primers listed in Tables 1 and 2 were used.

3. Results

3.1. Optimization of cLAMP method for the detection of Cryptosporidium spp

The cLAMP method was successfully carried out at 65 °C with both *Cryptosporidium* spp. and *C. parvum* using species-specific primers. Positive reaction results were characterized by a change from red/pink to yellow, whereas negative controls remained pink (Table 3). Moreover, agarose gel electrophoresis (Table 3) indicated a ladderlike pattern that indicated stem loops with inverted repeats, confirming the positive LAMP product. As indicated by a change in colour, the amplification of DNA template samples began within 15 min of incubation, and the efficiency declined after 45 min with one false positive NTC. Furthermore, a non-specific amplification or false positive was detected in one of the three NTC reaction tubes after an increase in reaction time beyond 45 min (Table 3). Consequently, the incubation time and input DNA

Table 2
Primer sequences used in ddPCR amplification experiments.

Primers	Forward 5'-3'	Reverse 5'-3'	Size (bp)	Refs.
Cryptosporidium spp. (*SAM* gene)	GTTTTCATTAATCAAGA ACGAAAGTTAGG	GAGTAAGGAACAACC TCCAATCTCTAG	100	Burnet et al. (2013)
C. parvum (*GP60*)	GCCGTTCCACTCAGAGGAAC	CCACATTACAAATGAAGTGCCGC	150	Wu et al. (2000)

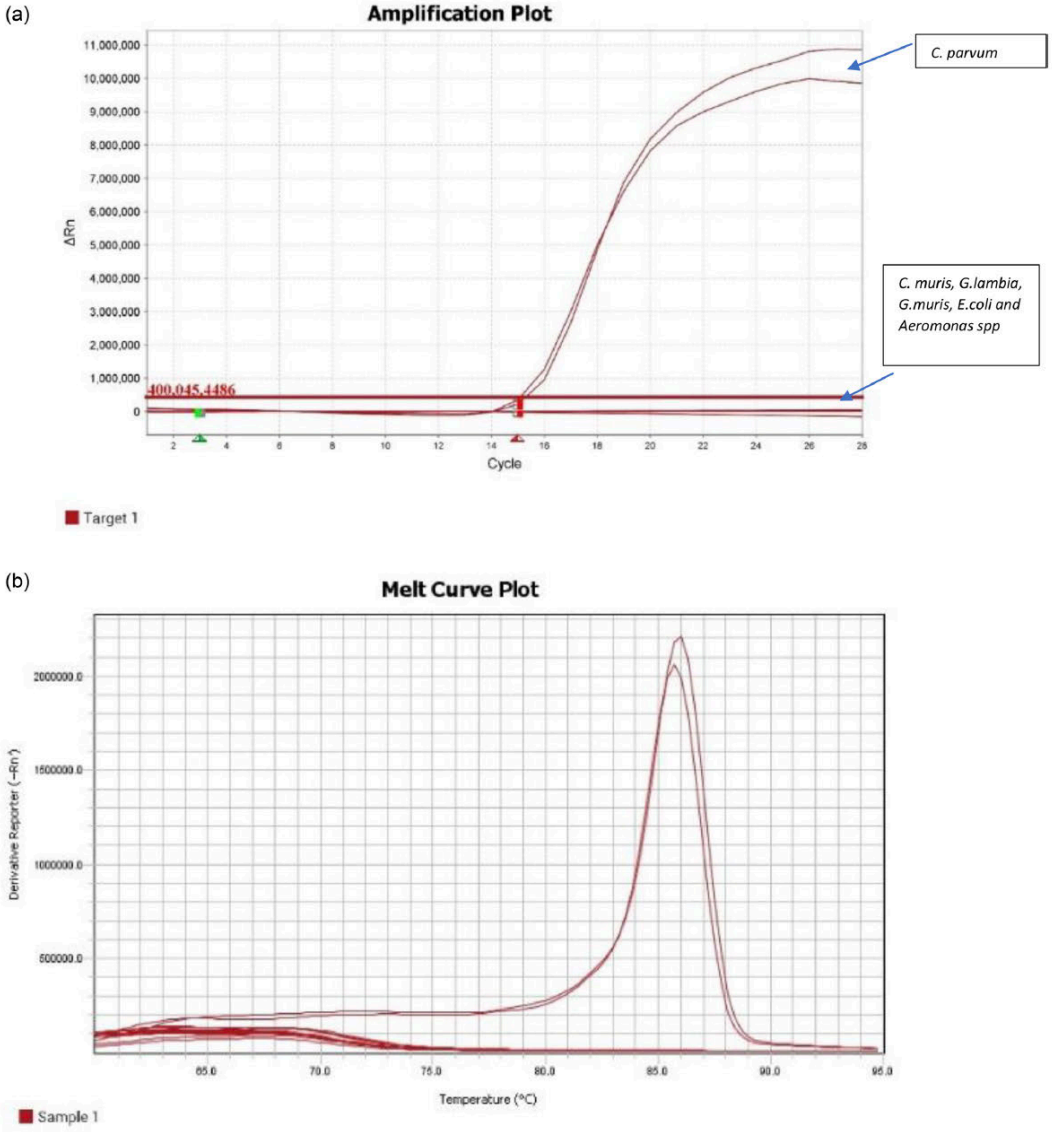


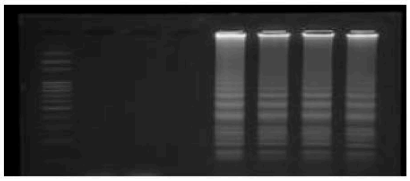
Fig. 1. Results demonstrating the specificity of the *GP60* gene against pathogens found in the soil/water environment using *C. parvum*. (a) showing amplification curves and (b) showing melting curves.

Table 3
Results of colorimetric methods after amplification and electrophoresis on 2% agarose gel*.

Detection of *Cryptosporidium* spp. using the *SAM* gene

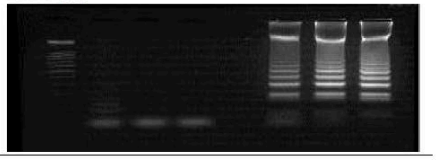
Colorimetric LAMP products
P P P P P P N N N

Gel electrophoresis of colorimetric LAMP products
L N N P P P P



Detection of *C. parvum* using the *GP60* gene
N N N N P P P

N N N N P P P



* N = No Template Control (NTC) and P = DNA template samples (positive amplification), L = DNA ladder.

Table 4
Limit of detection based on input *Cryptosporidium parvum* DNA copy number for 30 min incubation at 65 °C.

DNA copies /25 µl reaction	1132	226.4	45.28	9.05	4.5	2.3	1.132	0.6	0.2
Positive reaction	100%	100%	100%	100%	75%	75%	75%	0%	0%

concentration were optimized in order to eliminate false positives results. Table 4 shows that all DNA template positive reaction samples displayed 100% amplification and no amplification in NTC after 25 to 30 min of incubation when the template concentration was 20 ng/µl (1132 copies of *C. parvum*). Thus, in this study, 30 min was chosen as the maximum reaction time without any false positives/non-specific binding, that is, the cut-off time.

3.2. Detection limits and specificity of the cLAMP method

The cLAMP method was further evaluated for its limit of detection on the basis of *C. parvum* copy number and input concentration of DNA template for 30 min incubation at 65 °C. A positive result was considered to be obtained from at least two of the three replicates examined (75%) for each sample. In Table 4, it is shown that the *C. parvum* LOD is 1.1 copies per 25 µl reaction (0.02 ng/µl), which is the lowest input amount that was detected with 75% efficiency. The results also revealed that 9 copies/25 µl reaction (0.16 ng/µl) was the lowest input DNA template concentration at which 100% efficiency was obtained. Based on the results presented in Table 4, template DNA at 0.6 copies/25 µl reaction and lower failed to amplify.

Furthermore, the colorimetric LAMP method for *C. parvum* was noted to be highly specific, with no cross reactivity observed for other species lacking the *GP60* gene. DNA from closely related species and other pathogens such as *C. muris, G. lamblia, G. muris, E. coli*, and Aeromonas spp. were not detected by the assay (Supplementary Table 3).

3.3. Optimization of the fLAMP method and the limit of detection and specificity of the fLAMP method

Using the real-time fLAMP results shown in Fig. 1 of Appendix 1, it was demonstrated that all samples tested had been successfully amplified in 28 min at 65°C for a particular sample. The amplification curves appeared and crossed the threshold line of 400,000 within 26 min and reached the plateau phase within 28 min of the limits of detection for fLAMP were further tested. Table 5 shows that the lowest concentration of *C. parvum*/25 µl reaction that crossed the threshold within 28 min was 1.10 target copies (0.0175 ng/µl). At a concentration of 0.035 ng/µl, the detection efficiency dropped below 100%, with some replicate samples crossing the threshold after 28 min. In *C. parvum*, the time required for positive amplification was dependent upon the number of target copies (Table 5); the higher the target copies, the shorter the time required for positive amplification specificity tests (Fig. 1) demonstrated that no amplification curves were observed for other *Cryptosporidium* species or pathogens lacking the *GP60* gene. Fig. 1b illustrates the melting curve plot which indicates the presence of only one product and high specificity of the method (Fig. 1b).

Table 5
Limit of detection for the real time fLAMP (based on *C. parvum* copy number/input concentration of DNA template) incubation at 65 °C.

DNA copies /25 µlreaction	1132	566	283	141	70.75	35	17	8	4	2	1.10	0.55
DNA concentration in ng/µl	20	10	5	2.5	1.25	0.625	0.31	0.156	0.07	0.035	0.0175	0.008
Positive reactions	100%	100%	100%	100%	100%	100%	100%	100%	100%	75%	75%	0%
Obtained LAMP CT values	14,97 (± 0)	16,335(± 0.01)	16,804 (±0.09)	1765(± 0.3)	1804(± 0.05)	18,7 (± 0)	1935(± 0.3)	2197(± 1.07)	22,1(± 2.11)	2667 (±0.31)	27833 (± 3.35)	329 (± 3.31)

3.4. Evaluation and confirmation of the optimised LAMP method for the detection of C. parvum and Cryptosporidium spp. in wastewater samples

In further tests, the optimized method was applied to different environmental samples and was found to be successful in detecting *Cryptosporidium* spp. up to 50% to 60% of all samples tested with a concentration of 1ng/µl template DNA (Table 6). The high prevalence of *Cryptosporidium* spp. was observed in untreated wastewater samples with 67-100%. However, surface water had the lowest prevalence of 13% (2/15). Results also indicated that when fLAMP was applied in different water matrices, it achieved a total detection rate of 85% for primer 1 and 52 % for primer 2. While the reference method, ddPCR achieved a total detection rate of 98% for primer 1 and 58% for primer 2 (Table 6). Correspondence in detection was not statistically significant, with a p value of 0.56 between ddPCR and fLAMP method. Droplet digital PCR successfully detected *Cryptosporidium* spp. in all wastewater sample types with slightly better sensitivity than fLAMP (Table 6) ranging from 03/15 to 15/15 (number of positive amplification/tested samples). Surface water samples also showed the lowest prevalence for ddPCR when primer 2 was used, which was consistent with the results obtained in the fLAMP analysis noted earlier.

Table 7 summarizes the comparison of the requirement of LAMP (fLAMP and cLAMP) and ddPCR. For each run (experimental run), 8 wells in a 96 well reaction plate are required (this includes unknown sample in triplicate, NTCs and positive control). Costs and time were considered in the comparison. For instance, a ddPCR system, cost over $125,000 compared to cLAMP and fLAMP requiring only heating block, which cost around $600 depending on the type. Additional costs for the fLAMP include a fluorometer, which is estimated to cost $3135 However, for this study, a real-time PCR was used, for the fLAMP analysis, which is estimated to cost $ 15,000. Furthermore, the ddPCR consumables and reagents are estimated to cost more than $ 29/ 8 well reaction, whereas the LAMP costs only more than $8/8 well reaction. In respect to time, the turnaround time for ddPCR was also longer than for LAMP methods. For example, for a single run, it took approximately 3 h to prepare, generate droplets, run the thermal cycler, read the droplets, and analyse results. Whereas LAMP required a one-hour turnaround time. Additionally, training for ddPCR took at least two days, while LAMP took less than four hours to train on the use of the protocol for the first time by following simple instructions (Table 7).

4. Discussion

Cryptosporidiosis is one of the most important emerging and commonly recognised waterborne infections, which affect humans worldwide. Monitoring of wastewater for reuse or discharge into surface water bodies may contribute significantly to reducing *Cryptosporidium* spp. related diarrheal infections. As a result, the development of accurate, sensitive, and cost-effective methods could contribute to achieving this goal.

The cLAMP and fLAMP technologies have been utilized successfully for the detection and identification of viral (Quoc et al., 2018; Amoah et al., 2021) and microbial infections (Huang et al., 2018; Dea-Ayuela et al., 2018). In this study, both the cLAMP and real time fLAMP assays proved to be effective in detecting *Cryptosporidium* spp. and yielded results in a shorter period of time compared to PCR. The extra two forward and backward loop primers used in the assays are known as amplification reaction accelerators, which reduces the reaction time required for LAMP (Nzelu et al., 2019). However, when the LAMP reactions were incubated for more than 45 min during optimisation stage, nonspecific products or false positives were observed in one of the no templates controls (NTC) for both cLAMP and fLAMP products. In this context, it is possible that when the reaction is incubated for too long or the conditions are not fully optimized, an uneven reagent ratio, primer dimers, or nonspecific amplifications occur, resulting in false positives (Aoki et al., 2021). Similar results were obtained in previous studies when the Lateral flow Loop-Mediated Isothermal Amplification test reaction was left for 70 min resulting in the formation of primer dimers as well as the binding of the intercalating dye (SYBR green 1) to both products and primer dimers (Mamba et al., 2018).

The fLAMP method was found to be slightly faster than the colorimetric method in this study. This may be due to the intercalating fluorescent dye used in fLAMP, which only binds to double-strand DNA and allows the results to be seen in real time (Quyen et al., 2019). The colorimetric assay on the other hand relies on time for the phenol red indicator to change color for positive results, which is dependent on the release of enough protons for the pH to change. Similar observations were previously reported, where real-time LAMP positive amplification of *Xanthomonas gardneri* DNA was first observed after 15 min, but amplification of the same DNA was visible in the colorimetric assay after 30 min (Stehlíková et al., 2020). A recent study by Amoah et al. (2021) has reported a similar amplification time of 35 min for the Reverse Transcription cLAMP method for detection of SARS-CoV-2 in wastewater (Amoah et al., 2021). Another study used LAMP with a cut-off time of 40 min to identify SARS-CoV-2 virus in clinical samples (Lu et al., 2020), further demonstrating the rapidity of the LAMP assay in detecting pathogens from environmental samples.

The cLAMP and fLAMP methods were highly sensitive and specific for detecting *Cryptosporidium* species with a limit of detection of 1.1 copies/25 µl reaction (0.02 ng/µl) with 75% efficiency. This indicates that the developed assays can be applied to environmental samples that contain pathogens, even at low concentrations such as diluted samples. However, for 100% detection efficiency, the concentration of template DNA must be at a high level, or a confirmation method, such as DNA sequencing or real-time PCR, must be used. We also observed a decrease in cLAMP efficiency as the number of copies was reduced, which is in agreement with previous findings that observed fading changes in colour when fewer DNA/RNA template copies were used (Lu et al., 2020). It should be noted that, as demonstrated in this study, all reactions should be performed in triplicate or more for accuracy. Previous research has shown that the LAMP is sensitive and capable of starting amplification with a small amount of DNA (10–100 copies) when used for library preparation prior to sequencing (Imai et al., 2017). The LOD observed in this study for *Mycobacterium* spp is consistent with previous

Table 6
Detection of *Cryptosporidium* spp. in environmental samples using both fLAMP and ddPCR.

	Number of detected positive reactions /total number of tested samples			
	Primer 1(*Cryptosporidium 18s rRNA*)		Primer 2 (*C. parvum*)	
Sample type	**fLAMP**	**ddPCR**	**fLAMP**	**ddPCR**
Untreated wastewater	15/15	15/15	10/15	13/15
Treated wastewater	13/15	15/15	09/15	10/15
Sludge	13/15	14/15	10/15	09/15
Surface water	10/15	15/15	02/15	03/15
Total detection rate	85%	98%	52%	58%

Table 7
Comparison of ddPCR and LAMP methods (cLAMP and fLAMP).

Requirement/ specification	ddPCR	fLAMP	cLAMP
Equipment cost/unit cost ($)	> 125,000	> 1,500,000 (qPCR thermal cycler/ $3135.00 fluorometer	> 600 (heating block)
Consumables and reagents required/ 8well run ($)	> 29	> 8	> 8
Training required (h)	< 1 day training	< 4 h	< 4 h
Turnaround time (h)	> 3	> 1	> 1

research that indicated LOD for *Mycobacterium avium* LAMP tests to be 0.4 pg of genomic DNA per reaction, which is 100–1000 times lower than the LOD for PCR testing (Yashiki et al., 2019). Furthermore, Daskou et al. (2019) reported that cLAMP had a limit of detection of 10 copies of *HPV16*, whereas PCR had a limit of detection of 100 copies, indicating higher and sometimes similar sensitivity than some conventional molecular methods.

The optimized LAMP methods (fLAMP and cLAMP) in this study also demonstrated high specificity for *Cryptosporidium* specific primers (Fig. 1 and Table 3 of supplementary file), with no positive reactions for other pathogens (*E. coli and Aeromonas* spp.) commonly found in soil and water, indicating that the method is reliable and accurate for the detection of protozoa parasites from environmental samples. The specificity of the methods relies on the use of four primers, which recognizes six distinct sequences of target DNA. The fLAMP assay specificity was also assessed using melting curve analysis of amplified products (Fig. 1b), and only peaks of the same melting temperature observed for C. parvum. This provided an advantage over colorimetric assay as it ensures the assay's accuracy and reliability by allowing for easy discrimination of non-specificity through the observation of melting curves. Furthermore, the amplicons for both assays were subjected to DNA sequencing and analysis, which confirmed the specificity by indicating the presence of only the genes of interest.

The detection of C. parvum and Cryptosporidium spp. in different environmental matrices using fLAMP demonstrated great potential applicability in complex environmental samples, with only a slight difference of 13% (SAM gene) and 6% (GP60 gene) detection rate when compared to ddPCR (Table 6). The obtained higher detection percentage in ddPCR could be attributed to improved LOD and reduced inhibitor impact because of partitioning micro-reactions via droplet formation (Dingle et al., 2013). Previous study also demonstrated higher prevalence of SARS-CoV-2 in wastewater with ddPCR technique compared to RT-LAMP (Amoah et al., 2021). Influent wastewater samples tested for *C. parvum* and other *Cryptosporidium* spp. using both fLAMP and ddPCR showed the greatest prevalence of all screened samples. This may be due to their abundance in influent wastewater samples as a result of the high disease burden in the community around them. In contrast, both fLAMP and ddPCR revealed a very low prevalence of *C. parvum* in surface water compared to primer one (*SAM* gene) which targets a wide range of *Cryptosporidium* spp. This could also be attributed to lower copy numbers of *C. parvum* in extracted DNA resulted from less concentrated surface water volume and lower biomass. Detection of *Cryptosporidium* spp. in surface water has been reported to be challenged by very low oocysts concentrations which requires large volumes of water collection (> 10 L) for concentration step (Hassan et al., 2020).

4.1. The significance of the findings for public health protection

The two LAMP methods optimised in this study demonstrates a great potential as an environmental and laboratory surveillance tool with the following reasons. First, both fluorescent and colorimetric LAMP protocol used proved to be very sensitive in detecting as few as 1.10 target copies of *C. parvum* equivalent to one oocyst, which is well within the water quality guidelines discussed above as well as published acceptable range of (10-100 oocyst) for infection monitoring and prevention (Dixon et al., 2011; Zacharia et al., 2019). The use of highly sensitive detection tools for pathogens in water quality monitoring is one of the key fundamental principles for decision-making regarding water distribution system infrastructure, the best water treatment, and prevention of waterborne outbreaks (Ramírez-Castillo et al., 2015). Second, the developed LAMP assays can be performed at a single constant temperature of 65 °C for less than 60 min. According to Caliendo et al., (2013) new tests should be simple to use and provide a quick result (ideally within an hour) to have a positive impact on infection care, pathogen detection, pathogen discovery, and disease surveillance (Caliendo et al., 2013).

The optimized protocol in this study provides an even faster time as well as other excellent features that are critical in public health protection. This includes the easier visual interpretation of results with fast turnaround time due to elimination of additional time needed for gel electrophoresis or the use of carcinogenic ethidium bromide or UV exposure. Furthermore, when using either colorimetric or fluorescent LAMP, the use of constant temperature (65 °C) for amplification eliminates the need for expensive thermal cycling equipment; the instrument required is a heating block, water bath or fluorometer for fLAMP, both of which are portable and commonly available in most laboratories. As a result, the cost is reduced even further, allowing it to be used in resource-limited countries and as a portable field device (Table 7). Therefore, the adoption of these assays could play significant roles in public health protection.

5. Conclusions and recommendations

Despite ongoing efforts to ensure water safety, outbreaks of waterborne diseases continue to occur. The use of test methods that adhere to the World Health Organization's (WHO) guidelines for developing countries, specifically ASSURED (Affordable, Specific, User-Friendly, Rapid, Robust, Equipment-Free, and Deliverable to End Users), are crucial for reducing disease burdens and protecting public health. This study was able to successfully develop and evaluate a highly specific, sensitive, and rapid LAMP assay for the detection of human pathogenic *Cryptosporidium* species. The method has been validated by its application to environmental samples and comparison with the more advanced ddPCR. With the following findings:

The methods optimised can be carried out in a heating block, water bath, or thermal cycler at constant temperature of 65 °C for 30 min for colorimetric LAMP and 28 min for the real time fluorescent LAMP. The results can easily be interpreted in real time by colour change from red/pink to yellow, or by use of fluorescent dye. A limit of detection (LOD) of 1.1 copies per 25 µl reaction volume input template DNA was obtained.

Future research should focus on the optimization and development of direct LAMP, which eliminates the need for a separate DNA extraction step. This will further reduce the time required to achieve the desired results as well as the cost involved. As a precautionary measure, we recommend that further research be conducted to improve the LAMP quantification method. The overall outcome of this study contributes to and addresses Sustainable Development Goals (SDGs) 3 and 6 by providing *Cryptosporidium* LAMP methods as a tool for promoting better health through effective water management, water-related disease surveillance, and outbreak management related to drinking water supply.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2022.106606.

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