PREVALENCE OF DIARRHEA CAUSING BACTERIA, VIRUSES AND PARASITES IN WATER SOURCES IN THE RURAL COMMUNITIES IN THE VHEMBE DISTRICT

By

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DECLARATION

I, the undersigned **Simbarashe Karambwe (11613034)** hereby declare that this dissertation is my own original work and the literature has been acknowledged through referencing. This dissertation has not been submitted and will not be presented at any other University for similar or any other degree award.

..... 04/09/2017

Signature **Date**

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DEDICATION

To my family and my uncle Ovias Mutukura for their tireless support during my study.

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LIST OF ABBREVIATIONS

APHA – American Public Health Association

API 20E – analytical profile indexing for *Enterobacteriaceae*

BGMK– Buffalo Green Monkey Kidney

CA– cellulose acetate

Cfu- Colony forming units

CPE – Cytopathic effects

DAPI– 4',6-diamino-2 phenylindole

DIC– Differential Interference Contrast

DWAF – Department of Water and Forestry

EAEC- Enteroaggregative *E. coli*

EHEC- Enterohaemorrhagic *E. coli*

EIEC- Enteroinvasive *E. coli*

EPEC- Enteropathogenic *E. coli*

ETEC- Enterotoxigenic *E.coli*

FA – Fluorescent assay

IMS– Immunomagnetic separation

ISO- International Organization for Standardization

JMP– Joint Monitoring Programme

m-CP– membrane *Clostridium perfringens*

MDBK– Madin-Darby Bovine Kidney

MPN- Most probable number

MUG– 4-Methylumbelliferyl-β-D-glucuronide

ONPG– ortho-Nitrophenyl-β-galactoside

PCR– Polymerase Chain Reaction

RVS – Rappaport-Vassiliadis Soya

SANS– South African National Standards

spp. – species

S-S agar – Salmonella-Shigella agar

TCBS– Thiosulfate-citrate bile salts sucrose agar

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UNICEF– United Nations Children Educational Fund

USEPA- United States Environmental Protection Agency

WASH – Water, sanitation and hygiene

WGO– World Gastroenterology Organisation

WHO – World Health Organisation

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Abstract

Globally, several waterborne diseases and outbreaks have been linked to contaminated water. The objectiveof this study was to determine the prevalence of diarrhea causing bacteria, viruses and parasites in ten river water sources used by rural communities in the Vhembe District. Water quality was determined using indicator organisms (Total coliforms,*Escherichia coli* and*Clostridium perfringens*) and physical parameters (pH, Temperature, Total dissolved solids, Dissolved oxygen, Electrical conductivity and Salinity). In addition, this study determined the prevalence of pathogenic organisms such as *Salmonella*, *Shigella*, *Vibriocholerae*, *Cryptosporidium*, *Entamoeba*, *Giardia*, Rotaviruses, Noroviruses and Adenoviruses. Water was sampled once during the winter months (June-August 2016) and once during the summer months (October-December 2016) over a 12 months period. The Colilert Quanti-Tray®/2000 technique (IDEXX) was used for isolation of Total coliforms and *Escherichia coli*. Isolation of target bacteria was done using m-CP agar, Salmonella-Shigella agar and Thiosulfate-citrate bile salts sucrose agar. Presumptive isolates were confirmed by oxidase test, API 20E test, Gram staining and standardized published multiplex PCR protocols. Analysis of enteric viruses was done according to the South African National Standards for drinking water. The USEPA Method 1623.1 was employed for parasites. The results indicated that all the ten rivers were contaminated with Total coliforms and *Escherichia coli*;*Clostridium perfringens*bacteria were more prevalent in summer (90%) than winter (70%);*Vibrio cholerae*bacteria were more prevalent in summer (90%) than winter (50%); *Shigella*bacteria were not detected in any of the samples and *Salmonella* bacteria prevalence were similar for winter (90%) and summer (100%) months.Most of the viruses were detected in winter. Enteroviruses and Rotavirus were the most detected viruses. *Giardia* and *Cryptosporidium* were only detected in 20% and 10% of winter samples respectively. The results clearly indicate that raw surface water could be a potential health hazard if consumed without any treatment.

Key words: diarrhea, rural areas, Vhembe District, Water quality

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Chapter 1

INTRODUCTION

1.1 RATIONALE OF STUDY

Water problems in the last decade were a concern and at least 700 million people worldwide did not have access to clean water sources (WHO and UNICEF, 2012). In Africa, reports between 1990 and 2010 have shown that almost five times as many people in rural areas lack potable water sources relative to those living in urban areas (WHO and UNICEF, 2012). This lack of portable water sources forces people to supplement their water needs by using raw unsafe surface waters from rivers, dams, wells and streams which are vulnerable to faecal contamination (Chigor et al., 2012).

A significant proportion of the South African population relies on surface water as the main source of water for domestic purposes (Venter, 2001; Zamxaka, 2004). River water is mostly used for domestic purposes in rural communities of South Africa such as the Venda community (Obi et al., 2002; Bessong et al., 2009; Traore et al., 2016). The quality of surface water sources such as rivers is poor and hence a threat to human health if such water is consumed (Obi et al., 2003; Mpenyana-Monyatsi and Momba, 2012).

Waterborne diseases are those diseases that arise when microorganisms enter water sources mainly through faecal contamination and cause infections following ingestion of contaminated water (Leclerc et al., 2002; Momba et al., 2009; Yang et al., 2012).A wide spectrum of microorganisms such as bacteria, viruses, and parasites are important in causing waterborne outbreaks such as diarrhea (Wigle, 2003; Lewin et al., 2007; Obi et al., 2007; Samie et al., 2009; Batabyal et al., 2013). According to Bessong et al (2009), outbreaks of diarrhea and gastroenteritis in rural communities in South Africa are associated with the consumption of water of poor microbial quality.

The global distribution of outbreaks of water associated infectious diseases has seen waterborne diseases contributing more than 75% of the outbreaks (Yang et al., 2012). The aetiological agents dominating in waterborne outbreaks include *Vibrio*

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cholerae, which is mainly problematic in Africa, and the protozoan parasites, *Giardia* and *Cryptosporidium*, which are a concern in Europe (Yang et al., 2012). In addition, *Cryptosporidium* has enhanced endemic risks due to its frequent occurrence in surface water compared to *Giardia* (Leclerc et al., 2002). Enteric viruses such as Rotaviruses and Noroviruses are also a cause of concern in developing countries which see significant diarrheal outbreaks (Glass et al., 2009; Ayukekbong et al., 2015; Tate et al., 2016). Bacterial pathogens such as *Salmonella*, *Shigella*, *Campylobacter* and *E. coli* have been reportedly shown to cause significant outbreaks of diarrhea (Pegram et al., 1998; Hunter, 2003; Crump et al., 2004; Bessong et al., 2009)

Developing countries where resources are limited serves as hot spots for waterborne diseases due to lack of access to clean water and poor sanitation (Olajuyigbe et al., 2012). Cholera is a waterborne disease that readily leads to outbreaks following poor quality water, poor sanitation and poor hygiene and has been experienced at large in Africa (Rasam et al., 2014; WHO, 2016). According to WHO and UNICEF (2010), rural inhabitants lack improved drinking water sources and at least contribute 70% of the global population in need. The World Health Organisation (WHO) and UNICEF Joint Monitoring Programme for Water Supply and Sanitation reported that, about one third of the population of sub-Saharan Africa practices open defecation with another 23% of the population using unimproved sanitation facilities which do not ensure hygienic disposal of human excreta (WHO and UNICEF, 2008).

Regarding the limited reports on waterborne diarrhea in the rural communities in the Venda region, it is highly possible that some of the diarrhea cases go unreported. A possible link between diarrhea and water quality in the Venda region has been established (Bessong et al., 2009) and thus continuous monitoring of water quality is necessary to assess the possible risks associated with water sources used in the rural communities in the Venda region. Therefore, this study aimed at determining the prevalence of diarrhea causing bacteria, viruses, and parasites in river water sources used in the rural communities in the Venda region.

1.2. PROBLEM STATEMENT

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In a river catchment area, the quality of water provides an overview of the living habits of people, their attitudes, behavioural practices and their knowledge on environmental awareness. Rivers are an important source of water for man but the quality of water deteriorates due to various point and non-point pollution sources (Koshy and Nayar, 1999; Dreelin et al., 2014). According to Obi et al. (2004), human and animal activities in the Vhembe District negatively impacts the quality of water in various rivers.Contaminated water is a health hazard of concern because it is a vehicle for transmission of pathogenic bacteria, viruses and parasites to humans causing waterborne illnesses such as diarrhea (Lewin et al., 2007; Samie et al., 2009; Batabyal et al., 2013).

In a study done by Traore et al. (2016) on some rivers in the Venda region, activities such as animal grazing, laundry, bathing, diaper dumping (Figure 1.1), farming and drinking water collection were identified as the likely sources of pollution.



Figure 1.1: Human activities such as diaper dumping is a serious threat to water pollution

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Prevention of water pollution requires effective and continuous monitoring of physico-chemical and microbiological parameters (Chandra et al., 2006) in order to ascertain the possible risks associated with a water source. This is however not done regularly especially in developing countries due to limited resources. At the same time, some diarrheal outbreaks go unnoticed. This study forms part of a bigger study to investigate the prevalence of diarrhea causing bacteria, viruses, and parasites in the raw water sources used by the Venda communities.

1.3 OBJECTIVES

1.3.1 PRIMARY OBJECTIVE

To determine the prevalence of diarrhoea causing bacteria, viruses, and parasites in water sources in the rural communities in the Vhembe District, Limpopo Province, South Africa.

1.3.2 SECONDARY OBJECTIVES

- To determine the quality of ten selected river water sources in the Vhembe District using faecal indicator organisms (*E.coli,* Total coliforms, and *Clostridium* perfringens)
- To assess the prevalence of *E. coli* and other pathogenic bacteria (*V. cholerae*, *Salmonella* spp.and *Shigella* spp) using the Colilert Quanti-Tray®/2000 technique and membrane filtration method respectively.
- To confirm the identity of pathogenic bacteria (*E. coli*, *V. cholerae*, *Salmonella* spp., *Shigella* spp. and *C. perfringens*) using established multiplex PCR protocols (Water and Health Research Centre, University of Johannesburg).
- To determine the presence of protozoan parasites using the USEPA Method 1623.1 protocol (Rand Water Board).
- To determine the presence of enteric viruses using the cell culture and rt-RT PCR protocols (University of Pretoria).

1.4 HYPOTHESIS

- The microbial quality of river water used in rural areas is poor and hence a vehicle for transmission of diarrhea causing pathogens to humans.

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Chapter 2
LITERATURE REVIEW

2.1 INTRODUCTION

Water contaminated by pathogenic microorganisms increases the risk of acquiring waterborne infections such as diarrhea and gastroenteritis following consumption (Karanis et al., 2010). According to the World Health Organisation, WHO (1997), 33% of deaths in developing countries are due to the consumption of contaminated water. In South Africa, the majority of deaths due to diarrhea have been linked to unsafe water, sanitation, and hygiene (Lewin et al., 2007). Diarrheal incidence may be used as a call for the need to identify potential environmental hazards such as surface water sources (Obi et al., 2007; Wenhold and Faber, 2009).

Contamination of drinking water sources results from the introduction of faeces from humans, wild and/ or farm animals which are flushed into surface waters (Hunter and Thompson, 2005). In addition, sewage disposal and poorly managed waste treatment plants are also sources of water pollution (Dungeni and Momba, 2010). Human and animal activities such as laundry, diaper dumping, grazing around the water source, and farming have been shown to impact the quality of surface water sources in the Vhembe District (Traore et al., 2016).

South Africa is classified as a water-stressed country that is supplied by 9% from groundwater sources, 14% sourced from sewage and effluent purification and 77% from surface water such as dams and rivers. (Van Vuuren, 2009). In addition, nearly 80% of the population in South Africa relies on surface water as the main source of water and this figure alone imply that people still utilize raw surface water (untreated water) for domestic purposes (Venter, 2001).The urban population in South Africa enjoys well developed water infrastructure and thus utilizes potable water. However, in rural communities, it is unlikely so to have well established water infrastructure and may as well not exist. (Obi et al., 2007). In addition, settlements in rural areas are sparsely distributed due to the rough terrain and this hinders the capacity to provide a centralized drinking water system (Sibanda and Okoh, 2013). This is why rural populations obtain water on an individual or household basis from nearby surface and ground water sources where the microbial quality is often unknown

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(Peter-Varbanets et al., 2009). In the Vhembe District, Limpopo province, South Africa, the majority of the Venda community rely on river water sources which are devoid of treatment for their domestic water needs and the majority of river water is of poor microbiological quality and unsafe for consumption (Obi et al., 2002)

As a result of poor water quality in rural areas of South Africa, a considerable number of its residents are exposed to waterborne pathogens that cause enteric diseases such as diarrhoea. Thus microbial contamination is responsible for the most health related water quality problems (Smith et al., 2006). The lack of demographic statistics in rural communities poses difficulties in evaluating the impact of drinking water supply on the health of human beings and thus it is imperative to know the prevalence of pathogenic organisms and the incidences of diseases occurring in rural areas due to polluted water (Zamxaka et al., 2004). The effective monitoring of physical-chemical and microbiological parameters help mitigate the pollution of surface waters (Chandra et al., 2006).

2.2 MICROBIAL CONTAMINATION OF WATER SOURCES

Contamination of water is inevitable and yet water is essential to sustain life. Microbial hazards continue to be a threat in both developing and developed countries. According to Cabral (2010), the main risk to public health due to microbes in water is associated with the ingestion of water that is contaminated with human and animal excreta. Hurst et al. (2002) have linked animal faeces with the possible presence of various pathogens such as enteric bacteria including *Vibrio*, protozoa and enteric viruses.

Various sources have been documented in literature as contributing factors to surface water pollution. In a study thatwas done by Traore et al. (2016) in the Vhembe district, pollution sources such as human activities; laundry, baby diaper dumping, bathing, agriculture, car wash and animal grazing were identified in the various rivers as shown in Figure 2.1 to Figure 2.4. The same study observed that the presence of faecal matter from human, wild and domestic animals were the most probable source impacting water quality since they were amongst the top activities, whilst farming was the second highest activity.

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Figure 2.1: Laundry washing at the water source



Figure 2.2: Domestic animals grazing around the water source

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Figure 2.3: Surface run-off from agricultural lands



Figure 2.4: Dumping of diapers into water sources

According to Robin et al. (2012), enteric bacteria such as *E. coli*, *Salmonella spp* and *Shigellaspp*. defined as allochthonous in nature have been suggested to be introduced into thewater via agriculture and urban surface run-off, waste water discharges and domestic waste discharge.

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River water is an important reservoir for *V. cholerae,*whichisthe aetiological agent of cholera (Madoraba and Momba, 2010; Wang et al., 2010). *V. cholerae* has been isolated from surface water (Percival et al., 2004; Fraga et al., 2007) and the occurrence of *V. cholerae* in water sources can be linked to faecal pollution (Cox et al., 2005). In addition, *Salmonella* has been repeatedly detected in various types of natural waters such as rivers, lakes, coastal waters, estuarine as well as contaminated ground water (Moganedi et al., 2007; Haley et al., 2009; Wilkes et al., 2009; Levantesi et al., 2010). The presence of *Salmonella* in natural water resources has also been attributed to runoff from fields with animal husbandry and disposal of untreated sewage (Moganedi et al., 2007; Jenkins et al., 2008). A study byPotgieter et al. (2005) on surface water used as drinking water source in the Vhembe District of Limpopo Province in South Africa has shown that the water was positive for *Salmonella.*

The findings from previous studies detected *Shigella* in surface waters (Wose Kinge and Mbewe, 2010) which are an indication that surface waters can possibly transport *Shigella* strains. A study done by Traore et al. (2016) on some of the rivers in the Venda region detected the pathogenic strains of *E.coli* such as EIEC, ETEC, and EPEC which are strains known for causing diarrhea (Omar and Barnard, 2014).
E. coli is a member of faecal coliform bacteria which are inherent of the gastro-enteric tracts of humans. It is mostly associated with faeces and has been chosen as a prime indicator of faecal pollution. Its presence in water is predictive of other pathogenic microbes such as enteric bacteria, viruses, and parasites. Environmental monitoring of surface waters has employed *E. coli* on several occasions due to its easy detection by simple laboratory methods. However, *E. coli* is not reliable when predicting the presence of parasites and viruses. Other indicators include enterococci, *C. pefringens* and bacteriophages (Savichtcheva and Okabe, 2006). *C. perfringens* is an alternative indicator of faecal pollution and its detection in water signifies the possible presence of protozoan parasites as well as enteric viruses. It is also regarded as a conservative indicator of faecal pollution (Brookes et al., 2005). Bacteriophages indicate the possible presence of viruses in water.

River water has been shown to be subject to contamination by protozoan parasites such as *Cryptosporidium* and *Giardia* due to point or non-point pollution sources

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(Dreelin et al., 2014). According to Robertson et al. (2006), both *Crytposporidium* and *Giardia* are associated with sewage and thus contamination of water sources by sewage threatens human health due to their low infectivity dose as low as 10 oocysts/ cysts (Dallingham etal., 2002; Wright, 2005). Outbreaks of cryptosporidiosis and giardiasis have been linked to the contamination of drinking water by sewage (Gaffga et al., 2007). Reports on parasitic organisms in wastewater are rare in South Africa and yet *Cryptosporidium* and *Giardia* are said to be the most commonly reported parasites in wastewater samples (Samie and Mashau, 2011).

Gastro-enteric viruses such as Rotaviruses and Noroviruses together with Human Adenoviruses are the most frequently detected human viruses in water samples (HAdV) (Calgua et al., 2013). Studies focused on river water have detected Noroviruses and Human adenoviruses in Llobregat River in Barcelona, Spain, Macacos and Fairas Timbo rivers in Rio de Janeiro, Brazil (Calgua et al., 2013). In Africa, Rotaviruses and Adenoviruses were detected in surface water in the rural areas of Benin, West Africa (Verheyen et al., 2009). In South Africa, a study conducted in the Tyme river in the Eastern Cape Province detected both Rotaviruses and Noroviruses and detection of the latter was sporadic (Sibanda and Okoh., 2013). Mans et al. (2013) also highlighted that Noroviruses are still detectable in sewage polluted rivers in South Africa. However, there is no data regarding detection of enteric viruses in surface waters in the Vhembe District except a study done by Obi et al. (2002) which detected somatic phages, a likely indicator of viral contamination of water (Grabow, 2001; Hot et al., 2003; Yousefi and Zazouli, 2008). Hence, little is known about the frequency and pattern of viral contamination of drinking water sources in resource poor settings such as the rural areas in the Vhembe District (Verheyen et al., 2009).

2.3 PHYSICO-CHEMICAL PARAMETERS IMPACTING WATER QUALITY

Physico-chemical and microbiological parameters are a direct measure of assessing pollution levels and thus an effective monitoring of these parameters may help mitigate pollution of surface waters (Chandra et al., 2006). The natural processes of pathogen reduction restrict the microbial density in water within limits. However,

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following pollution, the concentration of parameters changes and in most cases this expands the microbial density (Gerardi and Zimmerman, 2005).

Evaluation of the quality of potable water is mainly dependent on temperature which affects many phenomena such as the rate of chemical reactions in the water body, reduction in solubility of gases and amplifications of tastes and colours of water (Olajire and Imeokparia, 2001). Normal temperature range supportive of good surface water quality is between 0ºC and 30ºC (Chapman ed, 1996). Research has suggested that warmer temperatures during summer, go along with *Salmonella,Campylobacter* or *E.coli* infections (Kovats et al.,2004; Fleury et al.,2006) and low winter temperatures favour viruses such as Rotavirus and Noroviruses (Levy et al.,2009; Keller et al.,2010). High temperature has a negative impact on water quality by enhancing microbial growth (UNICEF, 2008). According to Bishankha et al (2013), anincrease in temperature increases the bacterial growth (P=0.002). Conversely, a negative correlation of temperature and enteric bacterial counts was established in India (Borade et al., 2015). However, it was argued that *E.coli*, *Salmonella* spp. and *Shigella* spp. are allochthonous in nature and hence they are introduced through agriculture, urban surface run-off, waste water discharges and from domestic waste (Fujioka, 2002; Robin et al., 2012).

The pH of most raw surface water sources ranges from 6.0 to 8.5 (WHO, 2002; Jordaan and Bezuidenhout, 2012). During thewet season, the pH of surface water is generally low around 7.0 and below and records above 7.0 when the season is dry (Oyhakilomeet al, 2012). This observation is also positively correlated to temperature (Frimpong et al., 2015). The relationship between pH and both *E.coli* and total coliforms has established that the bacterial counts are high in the pH range 6.0 to 7.0 and low counts above pH 7.0 (Oyhakilome et al, 2012; Frimpong et al., 2015). This observation is also supported by a negative correlation of pH and bacterial counts observed by Borade et al (2015). However, Bishankha et al (2013) observed no significant effect of pH on microbial growth (p=0.454).

Total dissolved solids (TDS) which is a quantitative measure of dissolved salts (Olawale, 2016) mainly assess the general water quality (DeZuane, 1977). Water sources gain the TDS concentration levels following sewage disposal, run-off from

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agricultural lands and industrial wastewater discharge (DWAF, 1996a; WHO, 2003). However, previous researches have associated wet seasons with low TDS concentrations due to dilution effects whilst dry seasons would see high levels of TDS (Oyhakilome et al., 2012; Olawale, 2016). Acceptable limits for water quality standards based on TDS is 1000 mg/L, however, there is no data regarding the health effects associated with consumption of such water though high TDS levels negatively impact the aesthetic value of water as well as taste (DWAF, 1996a; WHO, 2003). The freshness of a water source relies on its conductivity (Aiyesanmi et al., 2006) and there is an established direct relationship between conductivity and TDS since conductivity depends on the dissolved salts in water (Arimieari et al., 2014). According to McKelvie (2004), the freshness and potability of a water source based on conductivity are only credible within 0-325 µS/cm range. However, the South African Guidelines on water quality for domestic use have no guideline stated (DWAF, 1996a).

The levels of dissolved oxygen (DO) in water impacts the freshness of water as well as aquatic life (Arimieari et al., 2014). According to Olawale (2016), a community water supply with high levels of DO provides water of good taste. The factors impacting on DO levels in surface water sources such as rivers includes domestic, agricultural, industrial effluent and waste discharges (Olawale, 2016). Aiyesanmi et al (2006) highlighted that, DO levels >6 mg/L in surface water have acceptable quality whilst DO levels <4 mg/L signifies pollution and thus poor water quality.

2.4 SUMMARY

Waterborne diseases are problematic especially in areas with poorly managed water systems. Contaminated water is the key player in waterborne diseases. Surface water sources such as rivers, dams, and streams are vulnerable to contamination by faeces due to various human and animal activities. The various sources of contamination such as domestic sewage disposal, industrial effluent discharge, and surface run-off among others, impact changes in the quality of water. Physico-chemical and microbial parameters are the key measures in determining thewater quality of surface water sources and requires continuous monitoring. Physico-chemical parameters directly or indirectly influence the microbial composition of water. Microbial contamination of water is a threat to human health since pathogens

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such as enteric bacteria, viruses and parasites are well known causes of gastroenteritis and diarrhea in different parts of the world. Continuous monitoring of water sources is a necessity in mapping environmental awareness in order to mitigate the potential occurrence of waterborne outbreaks. Therefore this study aims to determine the prevalence of diarrhea causing bacteria, viruses and parasites in water sources used by the rural communities in the Vhembe District. In addition, this is the first study to address on the prevalence of enteric viruses in rivers around the Vhembe District.

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Chapter 3

MATERIALS AND METHODS

3.1 SCOPE OF STUDY AND STUDY AREA

The Vhembe district is largely rural and the area is faced with infrastructural backlogs for water, sanitation, and electricity which impact negatively on the health of these communities. The Vhembe district is bounded on the north by the Limpopo River, on the west by Sand River, on the south and east by the Luvuvhu River (Samie et al., 2011b). The district is served by four local municipalities, namely, Makhado, Musina, Mutale and Thulamela.

Rivers were identified as a prime source utilised by people in rural areas as an alternative means of meeting their demands in terms of water supply (Obi et al., 2002). This study was focused on ten selected rivers which were identified prior to the commencement of sampling based on proximity to human communities and Public Health Care clinics. The water abstraction points were chosen as sampling points.

- In the Thulamela catchment, the Tshinane, Mutshundudi, Sambandou, Luvuvhu (Mutoti site), Luvuvhu (Mhinga site), Dzindi and Madadzhe rivers were assessed.
- In the Mutale catchment, the Mutale River was assessed.
- In the Makhado catchment, the Nzhelele and Luvuvhu (Tshino site) rivers were assessed.

Figure 3.1 indicates some of the rivers included in this study. The rivers were selected based on the availability of water throughout the year since the sampling was intended to cover two seasons. The collection of samples covered winter months (June – August 2016) and summer months (October-December 2016). The various activities taking place around the river sources were also noted.

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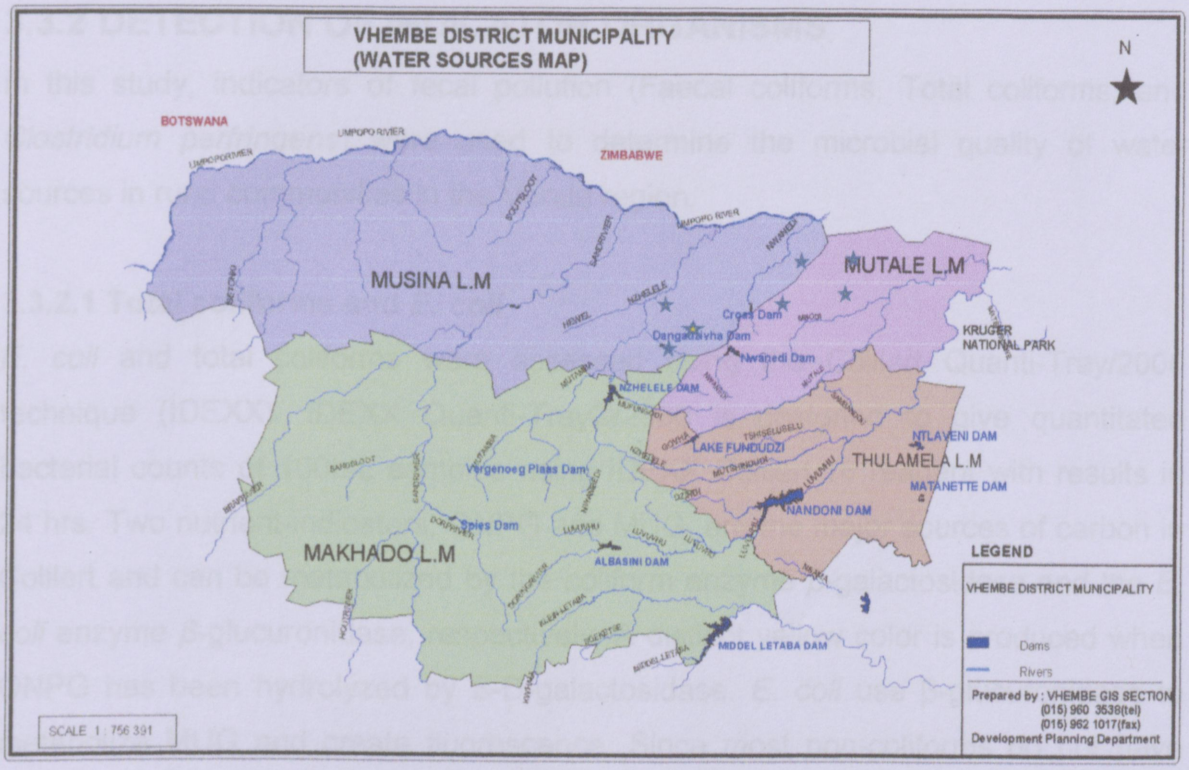


Figure 3.1 River water sources used in the study was the Vhembe District(adapted from Mutale local municipality IDP draft review for 2014/15)

3.2 COLLECTION OF WATER SAMPLES AND OBSERVATIONS

Water samples were collected in sterile plastic 500ml Nalgene containers (for bacterial analysis) from river water sources. Sterile 10L containers were used to collect river water for analysis of parasites and viruses. The samples were kept on ice at 4ºC during transportation to the University of Venda as well as to Rand Water Board (for parasite analysis) and the Enteric Virus and Environmental Research Group, the University of Pretoria (for analysis of viruses).

3.3 ANALYSIS OF WATER SAMPLES

3.3.1 PHYSICAL PARAMETERS

Physical parameters included pH, temperature, salinity, electrical conductivity, total dissolved solids and dissolved oxygen which were measured on site using a Portable Multi-meter, Bante900P model (Shanghai, China).

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3.3.2 DETECTION OF INDICATOR ORGANISMS

In this study, indicators of fecal pollution (Faecal coliforms, Total coliforms, and *Clostridium perfringens*) were used to determine the microbial quality of water sources in rural communities in the Venda region.

3.3.2.1 Total coliforms and *E. coli*

E. coli and total coliforms were assessed using the Colilert Quanti-Tray/2000 technique (IDEXX). IDEXX Quanti-Tray®/2000 is designed to give quantitated bacterial counts of 100mL samples using IDEXX Colilert-18 reagent with results in 24 hrs. Two nutrient-indicators, ONPG and MUG, are the major sources of carbon in Colilert and can be metabolized by the coliform enzyme *β*-galactosidase and the *E. coli* enzyme *β*-glucuronidase, respectively. A distinct yellow color is produced when ONPG has been hydrolyzed by ß-D-galactosidase. *E. coli* use β-glucuronidase to metabolize MUG and create fluorescence. Since most non-coliforms do not have these enzymes, they are unable to grow and interfere. The few non-coliforms that do have these enzymes are selectively suppressed by the Colilert test's specifically formulated matrix. The number of positive large and small wells (yellow for total coliforms and fluorescent for *E. coli*) is counted and the MPN/100 ml is determined using the IDEXX MPN Table.A volume of 100 ml each water sample was poured into a graduated 120ml IDEXX vessel followed by addition of the contents of one packet of the Colilert-18 reagent. The vessel was capped and the mixture was shaken until all the reagent had dissolved. This reagent mixture was then poured into a Quanti-Tray and was sealed in an IDEXX Quanti-Tray® Sealer. The trays were then incubated at 35.5^{0}C and the results were recorded after 24 hrs. The Spectroline Model CM-10 Fluorescent Analysis Cabinet was used to visualize the presence of *E. coli* by fluorescence.

3.3.2.2*Clostridium perfringens*

The water samples were analysed by membrane filtration method followed by culturing of the filters on m-CP agar plates. The m-CP agar is a selective and chromogenic medium for rapid and presumptive identification of *Clostridium perfringens* from water samples. Bisson and Cabelli (1979) were the first to hint on the quantitation of *Clostridium perfringens* in various water samples using m-CP agar

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medium. Mueller-Spitz et al. (2009) demonstrated that the m-CP method can correctly identify the majority of culturable *Clostridium perfringens* from diverse pollution sources. In this study the method described by Bisson and Cabelli (1979) was used, Figure 3.2 indicates the flow chart of the method.

According to the published method (EPA, 1996; Appendix 10c), for clean surface water, 10ml, 30ml, and 50ml may be filtered. In the case of turbid surface water, 3ml, 10ml, and 30ml may be filtered. In this study, the latter volumes were considered and 0.45 µm, 47 mm CA membrane filters (Sartorius Biolab Products) were used. The filters with the trapped microorganisms were overlaid on m-CP agar (Oxoid CM0992, pH 7.6) using sterile forceps. The plates were incubated anaerobically at 42^{0}C (Bonde, 1963) for 24 hrs in a jar containing anaerogen sachets (Oxoid). Yellow colonies that turned pink on exposure to ammonium hydroxide fumes were considered presumptive and were then Gram-stained following the protocol described by Prescott (2002) and Willey et al. (2014).

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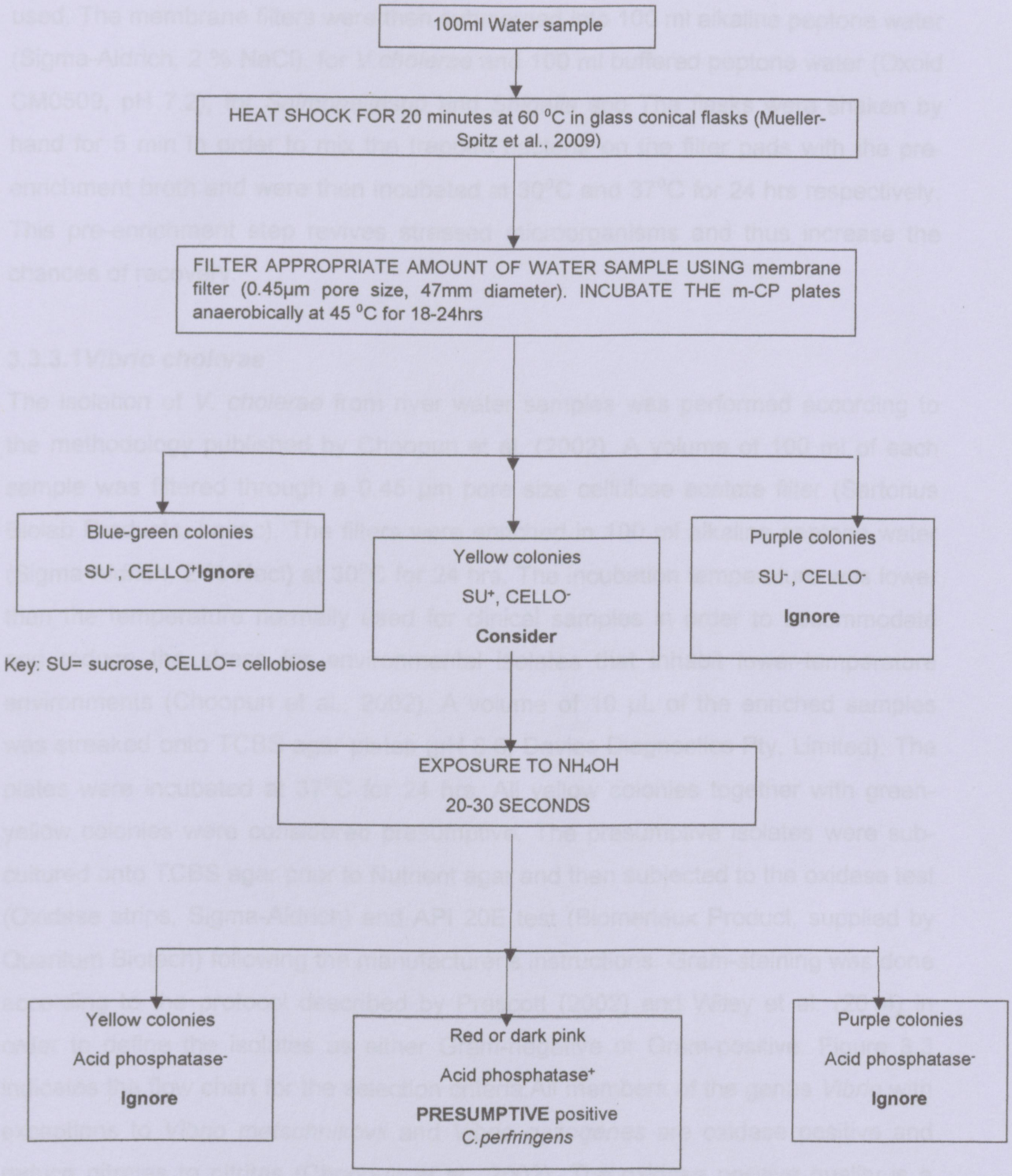


Figure 3.2: Flow scheme for m-CP agar Protocol (Bisson and Cabelli, 1979)

3.3.3. ISOLATION OF PATHOGENIC BACTERIA

Water samples (100 ml) were concentrated using membrane filtration technique in order to increase the chances of recovering *V. cholerae*, *Salmonella*spp,and *Shigella* spp.Membrane filters (0.45 µm, 47 mm CA, Sartorius Biolab Products, Lasec) were

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used. The membrane filters were then submerged into 100 ml alkaline peptone water (Sigma-Aldrich, 2 % NaCl), for *V.cholerae* and 100 ml buffered peptone water (Oxoid CM0509, pH 7.2), for *Salmonella*spp and *Shigella* spp The flasks were shaken by hand for 5 min in order to mix the trapped bacteria on the filter pads with the pre-enrichment broth and were then incubated at 30^{0}C and 37^{0}C for 24 hrs respectively. This pre-enrichment step revives stressed microorganisms and thus increase the chances of recovery.

3.3.3.1 *Vibrio cholerae*

The isolation of *V. cholerae* from river water samples was performed according to the methodology published by Choopun et al. (2002). A volume of 100 ml of each sample was filtered through a 0.45 µm pore size cellulose acetate filter (Sartorius Biolab Products, Lasec). The filters were enriched in 100 ml alkaline peptone water (Sigma-Aldrich, 2 % Nacl) at 30^{0}C for 24 hrs. The incubation temperature was lower than the temperature normally used for clinical samples in order to accommodate and reduce the stress for environmental isolates that inhabit lower-temperature environments (Choopun et al., 2002). A volume of 10 µL of the enriched samples was streaked onto TCBS agar plates (pH 8.6, Davies Diagnostics Pty, Limited). The plates were incubated at 37^{0}C for 24 hrs. All yellow colonies together with green-yellow colonies were considered presumptive. The presumptive isolates were sub-cultured onto TCBS agar prior to Nutrient agar and then subjected to the oxidase test (Oxidase strips, Sigma-Aldrich) and API 20E test (Biomerieux Product, supplied by Quantum Biotech) following the manufacturer's instructions. Gram-staining was done according to the protocol described by Prescott (2002) and Wiley et al. (2014) in order to define the isolates as either Gram-negative or Gram-positive. Figure 3.3 indicates the flow chart for the selection criteria.All members of the genus *Vibrio* with exceptions to *Vibrio metschnikovii* and *Vibrio gazogenes* are oxidase positive and reduce nitrates to nitrites (Choopun et al., 2002). The oxidase positive quality is a primary characteristic used to differentiate *V. cholerae* from *Enterobacteriaceae,* however, false negative oxidase test results have been obtained with colonies from TCBS agar medium (Vila et al., 1992).The isolates that showed consistency with the expected results of oxidase test, Gram-staining and API 20 E were preserved in nutrient broth and stored at -20^{0}C until further analysis (PCR).

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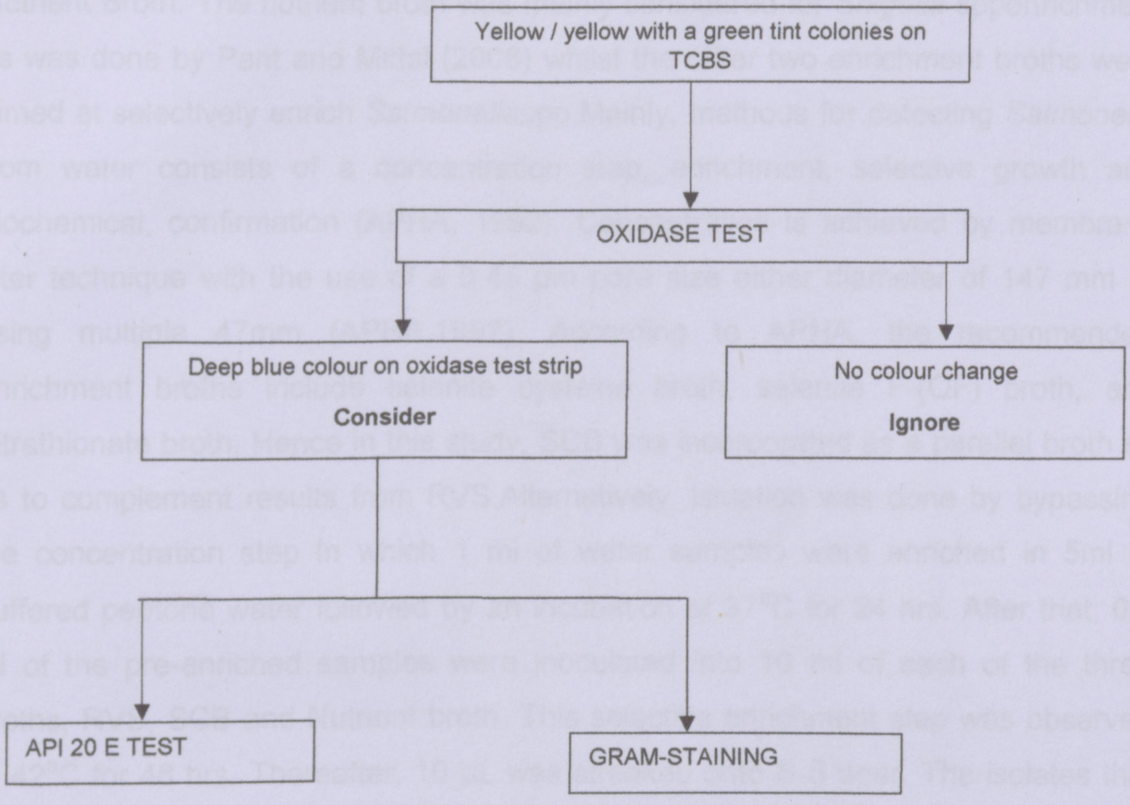


Figure 3.3: Criteria for *V. cholerae* isolation as used in this study.

3.3.3.2 *Salmonella* and *Shigella*

A successive selective enrichment step in Rappaport-Vassiliadis Soya Peptone Broth (RVS) (Oxoid CM0866, pH 5.2), Nutrient broth (Sigma-Aldrich pH 7.5) and Selenite cysteine broth (SCB) (Difco, BD Product pH 7.0) accompanied by incubation at 42^{0}C for 48 hrs was observed.

Thereafter, a loopful of the enriched samples was then streaked on selective media; S-S agar (Difco, BD Product pH 7.0), a selective media for *Salmonella* and *Shigella* spp and the plates were incubated at 37^{0}C for 24 hrs. Presumptive isolates were sub-cultured onto S-S agar prior to sub-culturing onto Nutrient agar and then subjected to biochemical tests; oxidase test following the manufacturer's instructions (Oxidase strips, Sigma-Aldrich) and API 20E test following the manufacturer's instructions (Biomerieux Product, supplied by Quantum Biotech). Gram-staining was also performed in order to define the isolates as either Gram negative or Gram positive (Prescott, 2002; Willey et al., 2014).The isolation of *Salmonella* sppand

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*Shigella*spp incorporated the use of three selective broth, that is, RVS, SCB and Nutrient Broth. The nutrient broth was mainly considered for *Shigella* sppenrichment as was done by Pant and Mittal (2006) whilst the other two enrichment broths were aimed at selectively enrich *Salmonella*spp.Mainly, methods for detecting *Salmonella* from water consists of a concentration step, enrichment, selective growth and biochemical, confirmation (APHA, 1992). Concentration is achieved by membrane filter technique with the use of a 0.45 µm pore size either diameter of 147 mm or using multiple 47mm (APHA,1992). According to APHA, the recommended enrichment broths include selenite cysteine broth, selenite F-(OF) broth, and tetrathionate broth. Hence in this study, SCB was incorporated as a parallel broth so as to complement results from RVS.Alternatively, isolation was done by bypassing the concentration step in which 1 ml of water samples were enriched in 5ml of buffered peptone water followed by an incubation at 37^{0}C for 24 hrs. After that, 0.1 ml of the pre-enriched samples were inoculated into 10 ml of each of the three broths, RVS, SCB and Nutrient broth. This selective enrichment step was observed at 42^{0}C for 48 hrs. Thereafter, 10 µL was streaked onto S-S agar. The isolates that showed some consistency with expected results of oxidase, Gram- staining and API 20 E were preserved in nutrient broth and then stored at -20 ^{0}C until further analysis (PCR).

3.3.4 MULTIPLEX PCR (m-PCR) FOR PATHOGENIC BACTERIA

3.3.4.1 *E. coli* strains

The samples were analysed by the Water and Health Research Centre (WHRC) at the University of Johannesburg. Briefly this method was performed as described by Omar et al. (2010) and Traore et al. (2016). A volume of 2 ml was extracted from randomly selected 10 positive wells of the Colilert Quanti-Tray using a 1 ml disposable syringe and then aliquoted into 2 ml Eppendorf tubes. The tubes were stored at -20^{0}C until further analysis.

The extraction of DNA and m-PCR reactions were performed according to the methodology described by Omar and Barnard (2014). The extracted DNA was used as a template in all PCR reactions. All the PCR reactions proceeded in a Biorad Mycycler™ Thermal cycler in a total volume of 20 µL. The targeted genes for the pathogenic *E. coli* strains and their primers are indicated in Table 3.1.

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Table 3.1 Primers used in the molecular detection of pathogenic *E. coli* strains (Omar and Barnard, 2014)

E. coli strain	Target gene	Primer (5'-3')	Primer concentration (µM)	Size (bp)	Reference
E. coli	*Mdh*	(F) GGT ATG GAT CGT TCC GAC CT (R) GGC AGA ATG GTA ACA CCA GAG T	0.1	304	Tarr et al. (2002)
EIEC	*Ial*	(F) GGT ATG ATG ATG ATG AGT CCA (R) GGA GGC CAA CAA TTA TTT CC	0.2	650	Lo´pez-Saucedo et al (2003)
EHEC/atyp EPEC	*eaeA*	(F) CTG AAC GGC GAT TAC GCG AA (R) CCA GAC GAT ACG ATC CAG	0.3	917	Aranda et al. (2004)
Typ EPEC	*Bfp*	(F) AAT GGT GCT TGC GCT TGC TGC (R) TAT TAA CAC CGT AGC CTT TCG CTG AAG TAC CT	0.2	410	Aranda et al. (2004)
EAEC	*Eagg*	(F) AGA CTC TGG CGA AAG ACT GTA TC (R) ATG GCT GTC TGT AAT AGA TGA GAA C	0.2	194	Pass et al. (2000)
EHEC	*stx1*	(F) ACA CTG GAT GAT CTC AGT GG (R) CTG AAT CCC CCT CCA TTA TG	0.5	614	Moses et al. (2006)
	stx2	(F) CCA TGA CAA CGG ACA GCA GTT (R) CCT GTC AAC TGA GCA CTT TG	0.3	779	Moses et al. (2006)
ETEC	*Lt*	(F) GGC GAC AGA TTA TAC CGT GC (R) CGG TCT CTA TAT TCC CTG TT	0.1	360	Pass et al. (2000)
	St	(F) TTT CCC CTC TTT TAG TCA GTC AAC TG (R) GGC AGG ATT ACA ACA AAG TTC ACA	0.5	160	Pass et al. (2000)
E.coli toxin	*astA*	(F) GCC ATC AAC ACA GTA TAT CC (R) GAG TGA CGG CTT TGT AGT C	0.2	106	Kimata et al. (2005)
External control	*Gapdh*	(F) GAG TCA ACG GAT TTG GTC GT (R) TTG ATT TTG GAG GGA TCT CG	0.2	238	Mbene et al. (2009)

KEY: F- forward primer; R- reverse primer

3.3.4.2 *Vibrio*spp, *Salmonella*spp and *Shigella* sppconfirmation.

The samples were analysed by the Water and Health Research Centre (WHRC) at the University of Johannesburg.The analysis was performed according to the method described by Mieta et al. (2010). The m-PCR targeted the *ial* gene for EIEC and *Shigella*spp, *ipa*B gene for *Salmonella*spp, *ipa*H gene for *Shigella* sppand the *sod*B gene for *Vibrio*spp. The primers used for the m-PCR are shown in Table 3.2.The Biorad Mycycler Thermal cycler was used for all the PCR reactions which were in a total volume of 20 µL. A positive control was a 2 µL mixture of *V. cholera* O1, EIEC,

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S. dysenteriae and *S. typhimurium* DNA. The negative control excluded template DNA.

Table 3.2 Primers used for the multiplex PCR(Mieta et al., 2010)

Primer and target gene	Sequence (5'-3')	Size	Reference
ial EIEC and *Shigella*	(F): GGTATGATGATGATGAGTGGC	630	Paton and Paton (1998)
	(R): GGAGGCCAACAATTATTTCC		
*ipa*H *Shigella*	(F) CCTTGACCGCCTTTCCGATA	606	Kong et al. (2002)
	R): CAGCCACCCTCTGAGGTACT		
*ipa*B *Salmonella*	(F): GGACTTTTTAAAAGCGGCGG	314	Kong et al. (2002)
	(R): GCCTCTCCCAGAGCCGTCTGG		
*sod*B *Vibrio*	(F): AAGACCTCAACTGGCGGTA	248	Tarr et al. (2007)
	(R):GAAGTGTTAGTGATCGCCAGAGT		

3.3.5 DETECTION OF PROTOZOAN PARASITES

The samples were analysed by the Rand water, an SANS accredited laboratory in compliance with ISO/IEC 17025. Method 1623.1 as described by the United States Environmental Protection Agency (USEPA) was employed. This method is based on concentration (filtration), purification (IMS) and detection (FA) in which the target parasites, *Cryptosporidium*oocysts, and *Giardia* cysts are identified only at the genus level. Water samples (10L) were filtered by use of the PALL system employing Environchek capsules. The trapped oocysts and cysts together with extraneous materials were eluted using an elution buffer solution. The eluate was centrifuged at 1500 x g to pellet the oocysts and cysts. The supernatant was aspirated. The oocysts and cysts were then separated from the extraneous materials by immunomagnetic separation technique employing paramagnetic beads, (Dynabeads® GC-Combo) conjugated to anti- *Cryptosporidium* and anti-*Giardia* antibodies. In the presence of oocysts or cysts, a paramagnetic complex is formed which is then attracted by a magnet and thus separating the oocysts/ cysts from the extraneous materials. The beads were then detached from the oocysts/cysts. The oocysts and cysts were then stained on well slides with fluorescently labelled monoclonal antibodies and DAPI, a fluorescent stain that binds strongly to A-T rich regions in DNA. The stained samples were examined using fluorescence DIC microscopy.Qualitative analysis was performed by scanning each slide well for

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objects that met the size, shape and fluoresce characteristics of *Cryptosporidium* oocysts or *Giardia* cysts as shown in Figure 3.4.Quantitative analysis was performed by counting the total number of objects on the slides that met FA, DAPI and DIC criteria for oocysts or cysts(Figure 3.4).

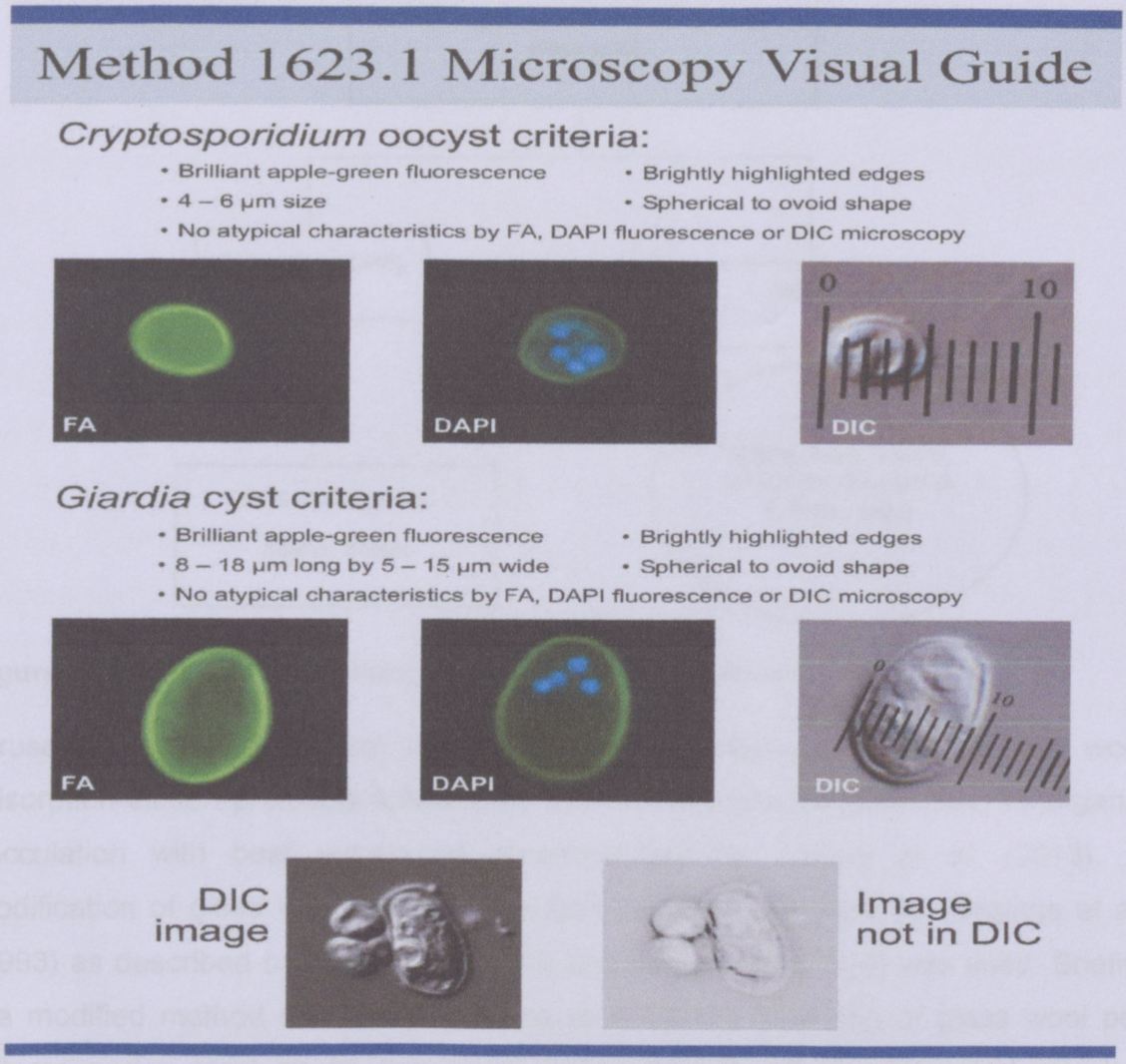


Figure 3.4: Microscopy visual guide for method 1623.1, EPA (2012).

3.3.6 DETECTION OF ENTERIC VIRUSES

Analysis of viruses was achieved by employing the conventional cell culture propagation technique, integrated PCR-cell culture technique,and direct PCR. This was performed by the Enteric Virus and Environmental Virology Research Lab at the University of Pretoria, according to SANS 241-1 (2015) Ed 2 for drinking water. Figure 3.5 highlights the flowchart for the analysis of viruses.

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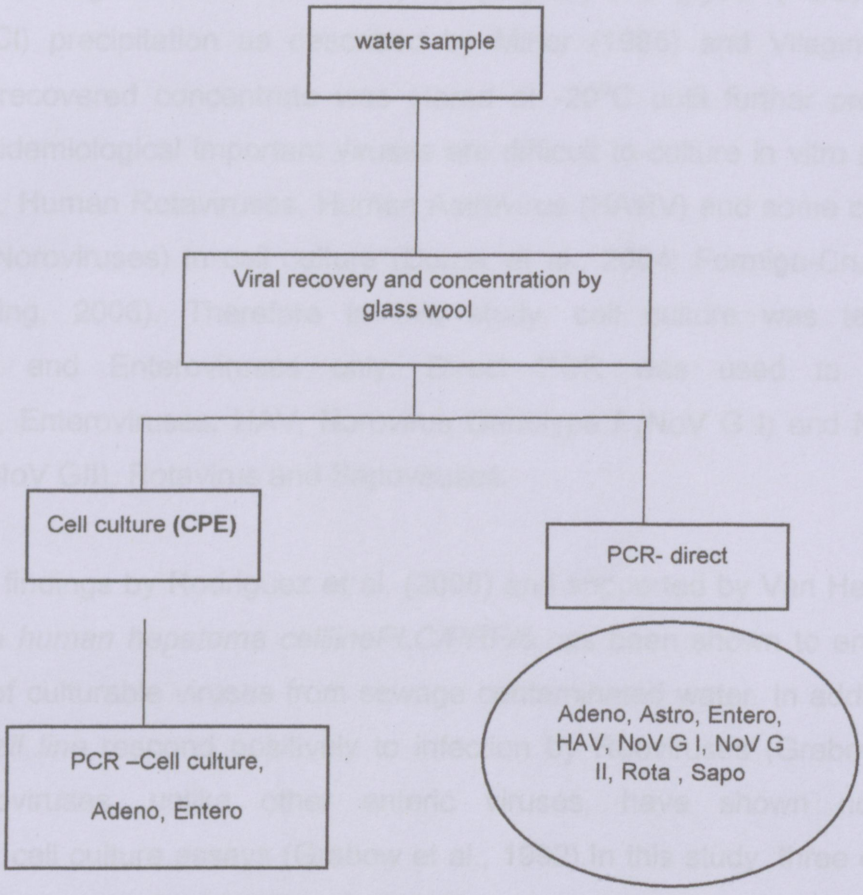


Figure 3.5: Flowchart summarising the analysis of enteric viruses

Viruses were recovered from 10 L surface water samples by standard glass wool adsorption-elution protocols followed by a secondary concentration step by organic flocculation with beef extract as recommended by Calgua et al. (2013). A modification of glass wool adsorption-elution method developed by Vilagines et al. (1993) as described by Mans et al (2013) and Kiulia et al. (2014) was used. Briefly, the modified method described by Mans et al (2013) used 15g of glass wool per column and a steel gauze grid (pore size = 1 mm^2, 30 mm diameter) inserted between each of the three 5g portions of the glass wool. Positively charged glass wool columns were used in order to capture the viruses which are generally negatively charged (Kiula et al., 2010).The negatively charged viruses, which had adsorbed to the glass wool were eluted with 100 ml of sterile glycine-beef-extract buffer of pH 9.5 (0.3754 % w/v glycine, 0.5 % beef extract) which reverses the ionic charges of the viruses and releases them from glass wool. The pH of the eluate was adjusted to neutral with 1 M HCl.In the secondary concentration step, the 100 ml eluate was concentrated to a final volume of 20 ml in sterile phosphate buffered

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saline (pH 7.4, Sigma-Aldrich Co.,USA) by polyethylene glycol (PEG)/ sodium chloride (NaCl) precipitation as described by Minor (1985) and Vilagines et al. (1997). The recovered concentrate was stored at -20^{0}C until further processing. Generally, epidemiological important viruses are difficult to culture in vitro (Hepatitis AVirus (HAV), Human Rotaviruses, Human Astrovirus (HAstV) and some cannot be propagated (Noroviruses) in cell culture (Duizer et al., 2004; Formiga-Crutz et al., 2005; Greening, 2006). Therefore in this study, cell culture was tested for Adenoviruses and Enteroviruses only. Direct PCR was used to test for Adenoviruses, Enteroviruses, HAV, Norovirus Genotype I (NoV G I) and Norovirus Genotype II (NoV GII), Rotavirus and Sapoviruses.

Based on the findings by Rodriguez et al. (2008) and supported by Van Heerden et al. (2003), the *human hepatoma celllinePLC/PRF/5* has been shown to enable the enumeration of culturable viruses from sewage contaminated water. In addition, the *PLC/PRF/5 cell line* respond positively to infection by Reoviruses (Grabow et al., 1999). Adenoviruses, unlike other enteric viruses, have shown noticeable propagation in cell culture assays (Grabow et al., 1992).In this study, three cell lines were used namely, the *PLC/PRF/5cell line* (European Collection of Cell Cultures, (ECACC 85061113), Salisbury, UK (Alexander et al., 1976), the *BGM African Green Monkey Kidney cell line*, (ECACC 90092601), (Dahling et al., 1974) and the *Vero African Green Monkey Kidney cell line* (ECACC 84113001). All the cell lines were propagated, maintained and infected using standard cell culture techniques as described by Grabow et al (1999), Vivier et al (2004) and Mans et al (2013). Briefly, the confluent monolayers of the *PLC/PRF/5 cell line* and BGM African Green Monkey cell line prepared in 25 cm^2 plastic flasks were infected with 1 ml of the antibiotic and anti-mycotic treated viral concentrate (Vivier et al., 2004). The infected cells were incubated for 14 days inclusive of a blind passage at day 7 after infection. The infected cells were harvested for molecular analysis as well as for blind passage onto monolayers of Vero cells in cell culture tubes with flying coverslips. After further incubation for 7 days at 37 ^{0}C, the infected cell cultures were examined for cytopathic effects (CPE), stained with haematoxylin and eosin and examined for virus-specific inclusion bodies (Malherbe and Strickland-Cholmley, 1980).

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3.3.6.1 Virus detection by molecular-based assays

The assay was performed by the Enteric Virus and Environmental Virology Research Lab at the University of Pretoria.Recovered viral concentrate (1 ml) was seeded with 10 µL of mengovirus (5 x 10^5 copies) as an extraction control. The extracted nucleic acid were eluted in 100 µL, aliquoted into small volumes and stored at -70^{0}C. The harvested cell culture suspensions (500 µL) were subjected to three cycles of freezing and thawing prior to nucleic acid extraction. Nucleic acids were extracted from 200 µL of the harvested cell culture extracts using the NucliSENS®EasyMAG® instrument (Biomerieux, Marcy l'Etoile, France) according to the manufacturer's instructions. The extracted nucleic acids were eluted in 50 µL, aliquoted in smaller volumes and stored at -70^{0}C.

Commercial real-time RT-PCR assays using TaqMan® technology were applied to the direct detection of selected human pathogenic enteric viruses in the recovered virus concentrate, namely Adenoviruses, Astroviruses, Enteroviruses, Hepatitis A viruses (HAV), Mengovirus, Norovirus GI, Norovirus GII, Rotaviruses, and Sapoviruses.

The TaqMan® Environmental Master Mix 2.0 kit (Applied Biosystems, Foster City, CA) was used for Adenoviruses. The Transcriptor First Strand cDNA synthesis kit (Roche) in conjunction with the LightCycler® Taqman® master kit (Roche) was used for molecular amplification of and real-time detection of Sapoviruses. For Astroviruses, Enteroviruses, Hepatitis A virus, Mengovirus, Norovirus GI, Norovirus GII and Rotaviruses, the ceeramTools® using the Taqman® technology was used for the analysis of all samples (Table 3.5). Artificially seeded samples were used for analysis of Mengoviruses. The primers and probes used for the direct analysis of viruses are shown in Table 3.6. For the integrated cell culture-molecular based assay, entroviruses were detected with a one-step real-time RT-PCR assay using the Quantitect Probe RT-PCR Kit (Qiagen, Hilden, Germany) and primers and a hydrolysis probe as previously described by Fuhrman et al (2005) and the Adenoviruses using primers and probes as described by Heim et al (2003) in the TaqMan® Environmental Master Mix 2.0 kit.

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Table 3.3 The commercial rt RT-PCR assay

Virus	rt RT-PCR assay
Astroviruses	KASV: ceeramTools®, CEERAM S.A.S., La Chapelle Sur Erdre, France
Enteroviruses	KENV: ceeramTools®
Hepatitis A virus	KHAV: ceeramTools®
Mengovirus	KMG: ceeramTools®
Norovirus GI	KNVGI: ceeramTools®
Norovirus GII	KNVGII: ceeramTools®
Rotaviruses	KRV: ceeramTools®

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Table 3.4 Primers and Probes used in real-time RT-PCR

Virus	Primer and Sequence (5'->3')	Reference
Adenovirus	50-GCC-ACG-GTG-GGG-TTT-CTA-AAC-TT-30, Adenoquant 1 (AQ1) 50-GCC-CCA-GTG-GTC-TTA-CATGCA- CAT-C-30, Adenoquant 2 (AQ2). 50-TGC-ACC-AGA-CCC-GGG-CTC-AGGTAC- TCC-GA-30 (Adenoprobe, AP) FAM labelled	Heim et al 2003
HAV	**R:** HAV240 (5'-GGAGAGCCCTGGAAGAAA G-3'), **F**: HAV68 (5'-TCACCGCCGTTTGCCTAG-3'), **Probe:** HAV150 (5'-TTAATTCCTGCAGGTTCAGG-3'	Costafreda et al 2006
Mengovirus	**R:Mengo209** (5'-GAAGTAACATATAGACAGACGCACAC-3'), **F:Mengo110** (5'-GCGGGTCCTGCCGAAAGT-3'), **FAM-MGB probe Mengo147** (5'-ATCACATTACTGGCCGAAGC-3').	Pinto et al 2007
Norovirus GI	**F**: QNIF4 (CGCTGGATGCGNT TCCAT), **R**: NV1LCR (CCTTAGACGCCATCATCATTTAC) (33) Probe: NV1LCpr(6-carboxyfluorescein–TGGACAGGAGAYCGCRA TCT–6-carboxytetramethylrhodamine)	Da Silva et al 2007; Svraka et al 2007
Norovirus GII	COG2F:CARGARBCNATGTTYAGRTGGATGAG (+)5003c COG2R:TCGACGCCATCTTCATTCACA (-)5100c Probe:RING2-TPFAM-TGGGAGGGCGATCGCAATCT-TAMRA (+)5048c	Kageyama et al 2003; Loisy et al 2005
Sapovirus	CU-SV-**F1** GAC CAG GCT CTC GCY ACC TAC 5074–5094a CU-SV-**F2** TTG GCC CTC GCC ACC TAC 786–803b CU-SV-**R** CCC TCC ATY TCA AAC ACT AWT TTG 5177–5154a CU-SV-**Probec** TGG TTY ATA GGY GGT AC 5101–5117a	Chan et al 2006
Enterovirus	R:Ev1[5-GATTGTCACCATAAGCAGC-3 F:Ev2 5-CCCCTGAATGCGGCTAATC-3 Ev-probe (5-FAM-CGGAACCGACTACTTTGGGTGTCCGT-BHQ-Phosphor-3	Fuhrman et al 2005

a GenBank accession number NC 006269; **b** GenBank accession number U95644.

c Corresponding nucleotide position of Camberwell virus (accession no. AF145896) of the 5end.

3.4 DATA ANALYSIS

Microsoft Excel 2013 was used for analysing data by way of graphs

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Chapter 4

RESULTS AND DISCUSSION

The objective of this study was to determine the prevalence of diarrhoea causing bacteria, viruses, and parasites in river water sources in the rural communities in the Vhembe District, Limpopo Province, South Africa. To accomplish this, indicator organisms including Total coliforms, *E. coli* and *Clostridium perfringens* were used to predict the presence of pathogenic bacteria, viruses and parasites respectively and provide an indication of the water quality. The targeted pathogenic bacteria included pathogenic *E. coli* strains, *Salmonella* spp, *Shigella* spp and *Vibrio* spp. The target parasites included *Cryptosporidium*, *Entamoeba* and *Giardia*. The target viruses included Rotaviruses, Noroviruses and Adenoviruses. The prevalence of these organismswas assessed in two seasons namely winter and summer in the year of sampling. Physical parameters such as pH, temperature, Electrical conductivity (EC), Tota dissolved solids (TDS), dissolved oxygen (DO), and salinity were measured in order to complement indicator organisms in assessing the water quality. The various activities taking place around the rivers were listed in Table 4.1.

Table 4.1: Activities seen around the selected river water source areas

Sampling site	Description of observed activities
Mutale	Laundry, bathing, car wash, cattle drink from the river
Sambandou	Laundry, cattles and donkeys drink from the river, animal grazing, people fetch water for construction purposes and other household duties, animal faecal matter
Tshinane	Laundry, bathing, dumping of chicken feathers
Mutshundudi	Laundry, bathing, littering
Madadzhe	Agriculture, domestic sewage disposal, car wash
Luvuvhu (Mhinga)	Laundry, car wash, swimming, bathing, fishing
Luvuvhu (Mutoti)	Laundry, bathing, fishing, car wash, cattle drink from the river, people fetch water for construction purposes
Nzhelele	Car wash, people fetch water, swimming, agriculture
Luvuvhu (Tshino)	Cattle grazing, fishing,
Dzindi	Agriculture

The results in Table 4.1 agree with the findings by Nevondo and Cloete (1999) and Traore et al (2016) that the majority of rivers in the rural areas of South Africa are

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subject to pollution through activities such as washing clothes, bathing, and animal activities.

4.1 PHYSICAL PARAMETERS

4.1.1 pH

Low pH or high pH in water sources negatively impacts aquatic life, however a narrow range between slightly acidic pH and slightly alkaline pH is favourable for microbial survival in water (Oyhakilome et al, 2012; Frimpong et al., 2015). According to the South African Water Quality Guidelines, the target water quality range for pH of raw surface water sources must be between 6.5 and 8.5 (DWAF, 1996a). The acceptable pH range for surface water in accordance withthe World Health Organisation (WHO) guidelines lie within the 6.0-8.5 range (WHO, 2002; Jordaan and Bezuidenhout, 2012). In this study, there was not much difference between pH for both winter (7.2-8.4) and summer (6.8-8.2) readings as shown in Figure 4.1 and the recordings were all within the acceptable range of 6.5-8.5. Other studies which assessed the water quality of dam water in Nigeria, stream water in Ghana and river water in Vhembe District South Africa, also reported similar pH results in the range 6.0-9.50 (Oyhakilomeet al., 2012; Frimpong et al., 2015; Traore et al., 2016) respectively.

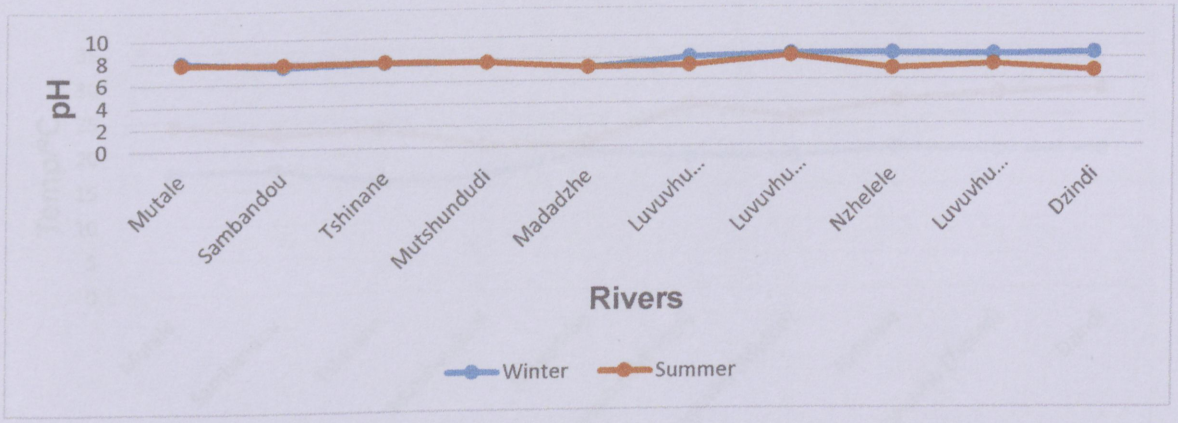


Figure 4.1: pH assessment in various river water sources

4.1.2 TEMPERATURE

Temperature is a key determinant of growth and survival of microbes in water and plays an important role in their survival. (UNICEF, 2008; Bishankha et al., 2013). Growth increases with temperature, and this may increase the problems related to taste, odour and colour of water (WHO, 2011). Too high temperatures may result in some microorganisms being inactivated (Krauss and Griebler, 2011). According to

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DWAF (1996), the recommended guideline for temperature for surface water is between 18 and 24ºC. The WHO does not specify the guideline for temperature, but rather emphasises that, cool water is more palatable than warm water (WHO, 2011). In this study, as shown in Figure 4.2, the minimum temperature recorded was 16.0ºC (winter) and 21.1ºC (summer) whilst the maximum recorded temperature was 20.3ºC (winter) and 28.8ºC (summer). Most of the winter temperatures in this study were within the South African guideline range of 18-24ºC whilst most summer temperatures were outside the acceptable range. The results of this study were similar to a study done in the Vhembe District on river water and reported a temperature range between 18 and 30ºC (Traore et al., 2016). Another study in Ghana on stream water recorded summer temperatures between 24 and 28ºC (Frimpong et al., 2015) which is in agreement with the summer recorded temperature in this study. According to literature, warmer temperatures during summer, go along with *Salmonella,Campylobacter* or *E. coli* infections (Kovats et al.,2004; Fleury et al.,2006) and low winter temperatures favours viruses such as Rotavirus and Noroviruses (Levy et al.,2009; Keller et al.,2010).

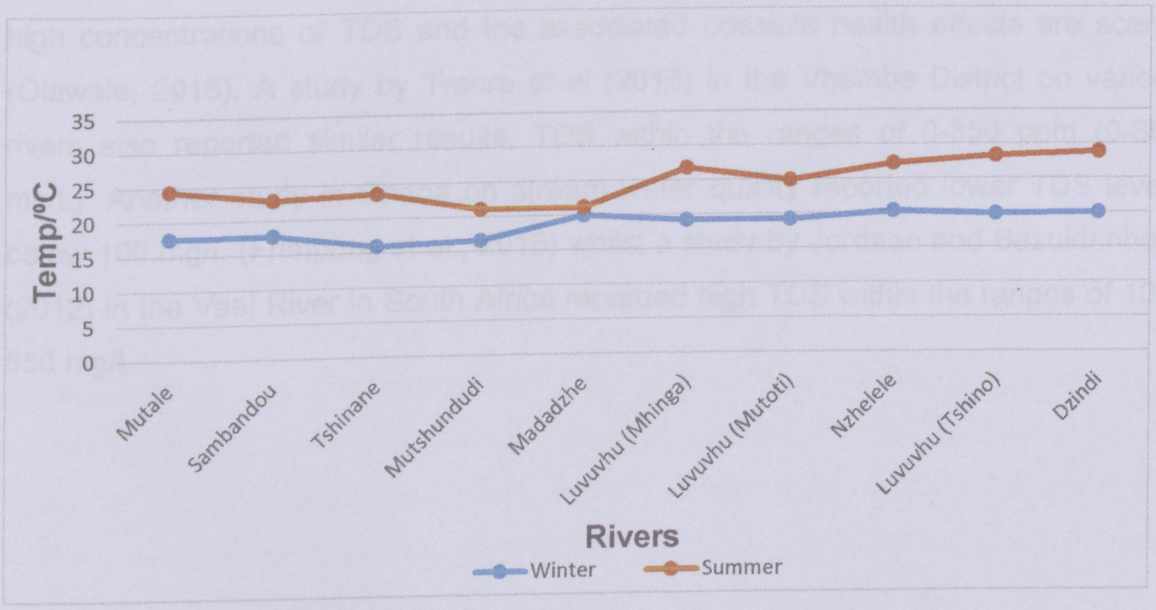


Figure 4.2: Temperature assessment in various river water sources

4.1.3 TOTAL DISSOLVED SOLIDS

Total dissolved solids (TDS) measures the amount of dissolved ions in water which stipulates the guidelines for TDS to be based on aesthetic and human health effects as well as economic impacts (DWAF, 1996a). The South African Water Quality

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Guidelines for TDS favourable for domestic water use ranges from 0-450 mg/L (DWAF, 1996a), however, TDS up to 1000 mg/L has been tolerable (SABS, 2011). The levels of TDS influence the palatability of drinking water (WHO, 2011). According to WHO guidelines for drinking water quality, TDS levels below 600 mg/L are considered good for drinking water. In this study, Nzhelele recorded the highest TDS amongst the ten rivers as shown in Figure 4.3. The minimum TDS was recorded in Mutale River (29.9 ppm or 29.9 mg/L) whilst the maximum recorded TDS was noted in Nzhelele River (348.5 ppm or 348.5 mg/L). The winter TDS ranged from 25-350 ppm (25-350 mg/L) (Fischer and Davidson, 2007) whilst the summer TDS was within the range of 30-150 ppm (30-150 mg/L). According to the South African Water Guideline, the results from this study shows that all the rivers were within the target water quality range for TDS tolerable for domestic water use (0-450 mg/L) and also the results were within the WHO acceptable limits (less than 600 mg/L). In addition, no health effects would be associated with water of TDS < 450 mg/L and no health effects are likely to occur if TDS fall in the range 450-1000 mg/L (DWAF, 1996a). Increasing levels of TDS above 1000 mg/L renders drinking water unpalatable (WHO, 2008; WHO, 2011). Reports linking the ingestion of water with high concentrations of TDS and the associated possible health effects are scanty (Olawale, 2016). A study by Traore et al (2016) in the Vhembe District on various rivers also reported similar results, TDS within the ranges of 0-350 ppm (0-350 mg/L). Another study in Ghana on stream water quality reported lower TDS levels below 100 mg/L (Frimpong et al., 2015) whilst a study by Jordaan and Bezuidenhout (2012) in the Vaal River in South Africa recorded high TDS within the ranges of 100-550 mg/L.

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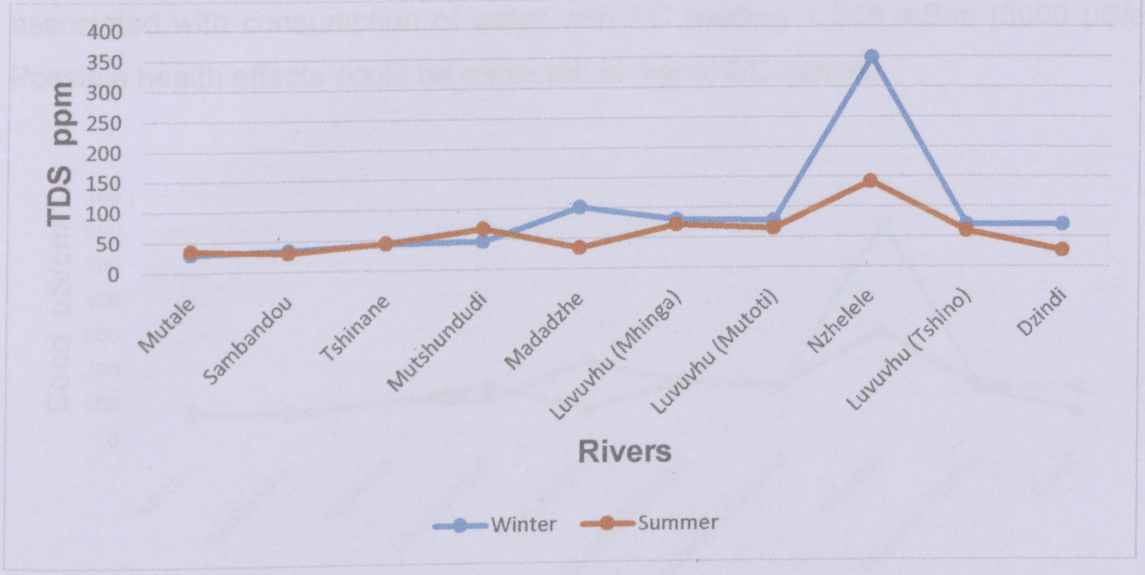


Figure 4.3: TDS assessment in various river water sources

4.1.4 ELECTRICAL CONDUCTIVITY

Electrical conductivity (EC) is a measure of the ability of water to conduct electricity and this is directly dependent on the concentration of dissolved ions which establishes a direct relationship between EC and TDS (DWAF, 1996a). According to the DWAF (1996a), the target water quality guideline range for domestic use based on conductivity is 0-70 mS/m (0-700 µS/cm). The latest updates by SANS 241:2015 has specified the standard limits based on EC to be <170 mS/m (1700 µS/cm). In this study, the minimum conductivity was 53.3 µS/cm (Dzindi) whilst the maximum was 601 µS/cm (Nzhelele). The EC ranged between 63.3 and 601 µS/cm in winter whilst the readings in summer ranged between 53.3 and 285 µS/cm as shown in Figure 4.4. The low readings for EC in summer might have been due to dilution effects because of the rainy season (DWAF, 1996a). The EC readings in this study shows that all the rivers were within the recommended standard guideline limits. A study by Traore et al (2016) in the Vhembe District also reported EC within the standard limits similar to the results from the present study. A study by Jordaan and Bezuidenhout (2012) assessing the impact of physico-chemical water quality parameters on bacterial diversity in the Vaal River, South Africa also recorded EC readings within the standard limits, however, the only difference with the present study is that, the former study had minimum readings for both winter and summer above 150 µS/cm. According to DWAF (1996a), there are no health effects

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associated with consumption of water with EC reading < 300 mS/m (3000 µS/cm). Possible health effects could be expected at higher EC values.

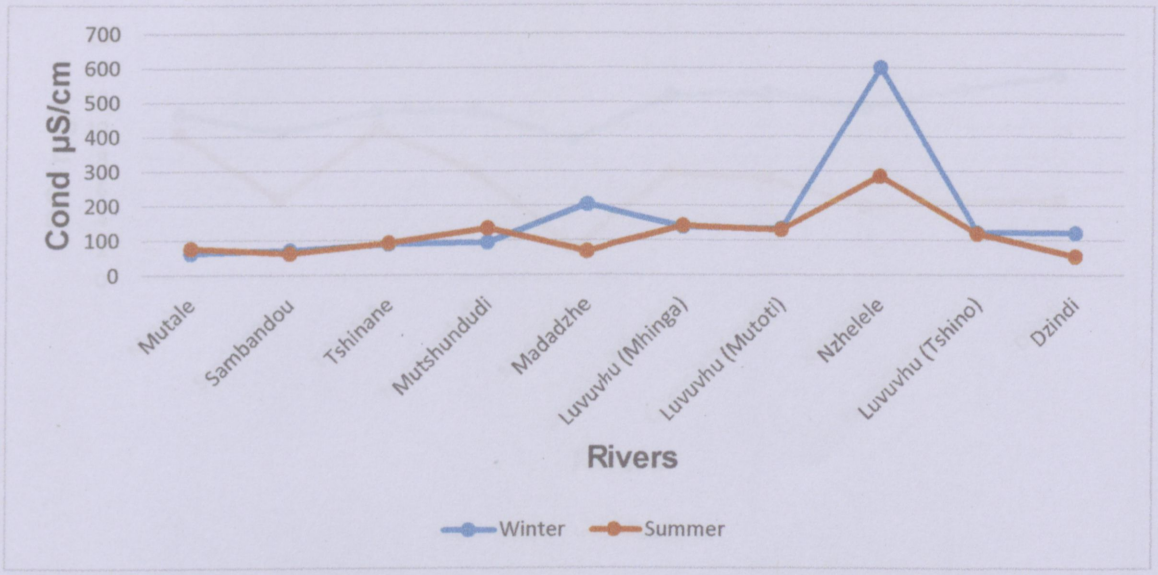


Figure 4.4: Conductivity assessment in various river water sources

4.1.5 DISSOLVED OXYGEN

Dissolved oxygen (DO) characterises the freshness of surface water which is directly related to the amount of oxygen dissolved. This is supported by a positive correlation between biological oxygen demand (BOD) and bacterial counts that were observed by Borade et al (2015). According to DWAF (1996a), there are no specific guidelines in place for DO. Also, the WHO does not have a guideline for DO (WHO, 2008; WHO, 2011). Aiyesanmi et al (2006) classified water of DO within 6.0-8.0 mg/L as acceptable quality whilst DO <6.0 mg/L as polluted water. Rao (2005) commented that dissolved oxygen concentration in unpolluted water normally ranges between 8 and 10 mg/L and concentration less than 5 mg/L adversely affect aquatic life. In the present study, dissolved oxygen was higher in winter than in summer as shown in Figure 4.5. The minimum recorded DO was 9.16 mg/L in winter whilst 2.04 mg/L in summer and the maximum DO was 13.43 mg/L and 9.88 mg/L in winter and summer respectively. In addition, the DO range in winter was within 9.0-14.0 mg/L whilst in summer it was 2.0-10.0 mg/L. According to literature as highlighted above, in the present study, most of the rivers were generally polluted since very few rivers recorded DO within 6-10 mg/L. In summer, 50% of the rivers had DO within the range 6-10 mg/L whilst only 20% of the rivers in winter had readings in that range

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(Figure 4.5). The low DO in summer and high DO in winter may have been due to water temperature which is inversely related to DO (EPA, 2001).

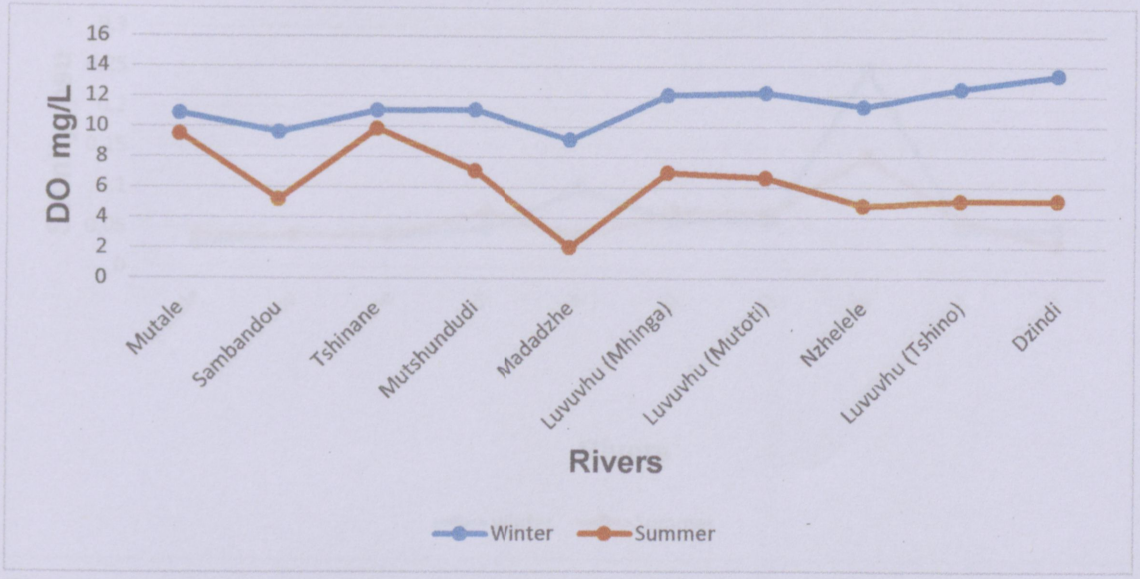


Figure 4.5: DO assessment in various river water sources

4.1.6 SALINITY

Salinity measures tha amount of salts dissolved in water and this affects many apsects such as taste, odour, the chemistry and biological processes within a water source (EPA, 2001). High salt content may render a water source unsuitable for domestic, industrial or agricultural use (EPA, 2001). In addition, high salinity levels are associated with deleterious effects on bacterial survival in water (Bordalo et al., 2002). The Department of Water Affairs of South Africa (1996a) and together with the WHO have no standard guideline for salinity. In the present study, the results of salinity did not show many differences in most of the rivers. The differences were within 0.01 psu units, however, Madadzhe and Nzhelele showed a greater deviation, both rivers recorded 0.10 psu and 0.25 psu respectively. The winter salinity ranged from 0.03-0.25 psu whilst the summer salinity range was 0.03-0.2 psu. Generally, salinity was consistent throughout the seasons. A study done by Pereira et al (2010) in Brazil on river estuary recorded salinity range between 0-0.6 psu during the rainy season which was slightly higher compared to the results of the present study. The occurrence of *V. cholerae* in the aquatic environment is primarily favoured by temperature and salinity (Singleton et al., 1982b). However, salinity favourable for *V.*

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cholerae growths are found in inland coastal areas and estuaries and the optimum salinity for *V. cholerae* growth are between 5 and 25 psu (Sedas, 2007).

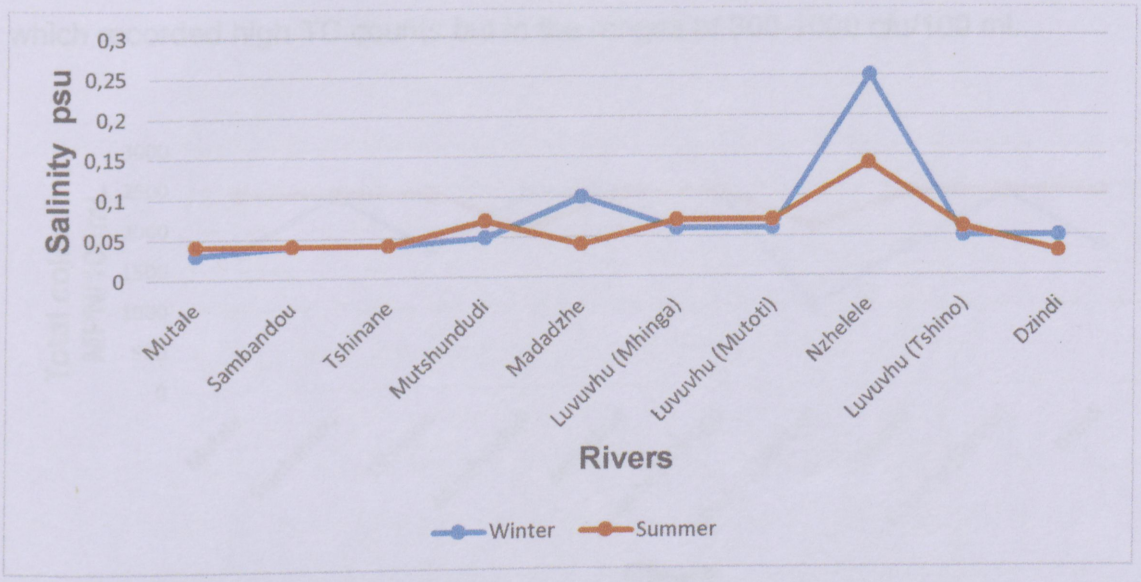


Figure 4.6: Salinity assessment in various river water sources

4.2 INDICATOR ORGANISMS

4.2.1 TOTAL COLIFORMS

Total coliforms (TC) are considered to be of faecal origin and include bacteria from the genera *Escherichia*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Serratia* and *Rahnella*, however some bacteria in this group are not of faecal origin (DWAF, 1996a; WHO, 1996). The significance of TC in water quality studies is that they generally give the overview on the hygienic quality of water (DWAF, 1996a). According to DWAF (1996a), the target water quality guideline range for TC is 0-5 counts/ 100 ml which have anegligible risk of infection whilst counts greater than 100 counts per 100 ml which have a significant threat to human health. In the present study, all the rivers had high TC counts above 1700 MPN/100 ml throughout the seasons. The winter recordings for TC ranged within 1700-2500 MPN/100 ml whilst the range for summer was within 1900-2500 MPN/ 100 ml as shown in Figure 4.7. The present results showthat the river water is not safe for consumption since the presence of TC may also indicate the possible presence of enteric bacterial pathogens such as *Salmonella*spp, *Shigella*spp, and *Vibrio* spp(DWAF, 1996a). A study by Traore et al (2016) on rivers in the Vhembe District also reported high counts of TC above 1000 MPN/100 ml similar to the present study. A study by Potgieter et al (2009) on river

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water and communal taps in the Vhembe District reported TC counts below 1000 cfu/100 ml. The present study also differs from studies done in Nigeria on dam water (Oyhakilome et al., 2012) and in Ghana on stream water (Frimpong et al., 2015) which recorded high TC counts but in the ranges of 300-1000 cfu/100 ml.

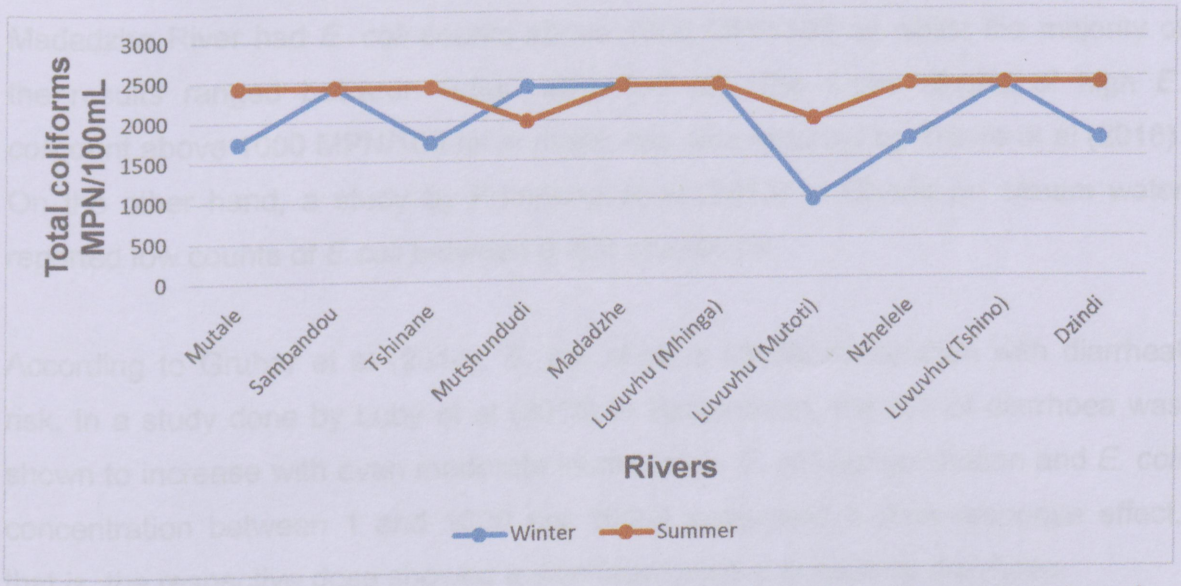


Figure 4.7: Assessment of microbial water quality from various river water sources based on Total coliforms

4.2.2 *Escherichia coli (E. coli)*

E. coli is a member of the faecal coliforms and are regarded as indicators of faecal pollution and generally indicates the possible presence of pathogenic bacteria such as *Salmonella* spp., *Shigella* spp. *Vibrio cholerae*, *Campylobacter jejuni*, *Campylobacter coli, Yersinia enterocolitica* and pathogenic *E. coli* (DWAF, 1996a). The South African Water Quality Guidelines for potable water requires 0 MPN/100ml of *E. coli* and counts above 20 MPN/100 ml pose significant health risks on continuous exposure (DWAF, 1996a). The WHO risk assessment criterion suggests that, counts less than 1 MPN/100ml presents no risk, at counts between 1 and 10 MPN/100ml, there is low risk, intermediate risk is reported on counts between 11 and 100 MPN/100ml whilst a very high risk is posed when counts are above 100MPN/100ml (WHO, 2012). In this study, all the ten rivers were contaminated with *E. coli* and the counts exceeded the quality standards for potable water. This may pose a risk to people who would consume the river water without any form of treatment. For both winter and summer, none of the ten rivers were within the low

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risk criterion and at least 80% of the rivers were marked by high risk in winter and summer seasons (Figure 4.9). A study by Traore et al (2016) on rivers in the Vhembe District found similar results whereby all the rivers were detected as having a very high risk score. The *E. coli* counts ranged between 10-2500 MPN/100 ml in winter and between 50-1300 MPN/100 ml in summer as shown in Figure 4.8. Only Madadzhe River had *E. coli* counts above 1000 MPN/100 ml whilst the majority of the results ranged between 0-500 MPN/100 ml. The same results of high *E. coli*count above 1000 MPN/100 ml in rivers was also reported by Traore et al (2016). On the other hand, a study by Frimpong et al (2015) in Ghana on stream water reported low counts of *E.coli* between 0-400 cfu/100 ml.

According to Gruber et al (2014), *E. coli* show a strong association with diarrheal risk. In a study done by Luby et al (2015) in Bangladesh, the risk of diarrhoea was shown to increase with even moderate increases in *E. coli* contamination and *E. coli* concentration between 1 and 1000 per 100ml suggested a dose-response effect, that is, the respective dose showed a significant impact in causing diarrhoea.
The Madadzhe River is generally subject to domestic sewage contamination as it flows through the residential areas. This is evident from the results indicated in Figure 4.8 that the highest *E. coli* count above 2400 MPN/100ml was recorded in winter and a count of about 1300 MPN/100ml was recorded in summer. A study by Traore et al (2016) on Madadzhe River found similar results to the present study. This river is mainly utilised for irrigation purposes by the people around the catchment. Literature has highlighted the role of sewage contaminated irrigation water in the incidence of diarrheal diseases due to consumption of raw fresh produce (Cifuentes, 1998; Watchel et al., 2002; Allende and Monaghan, 2015). In Sweden, lettuce irrigated by water from a small stream was incriminated in the outbreak due to infection by verotoxin-producing *E. coli* O157 which was also identical to the isolates from cattle at a farm upstream from the irrigation point (So¨ derstro¨m et al., 2008).

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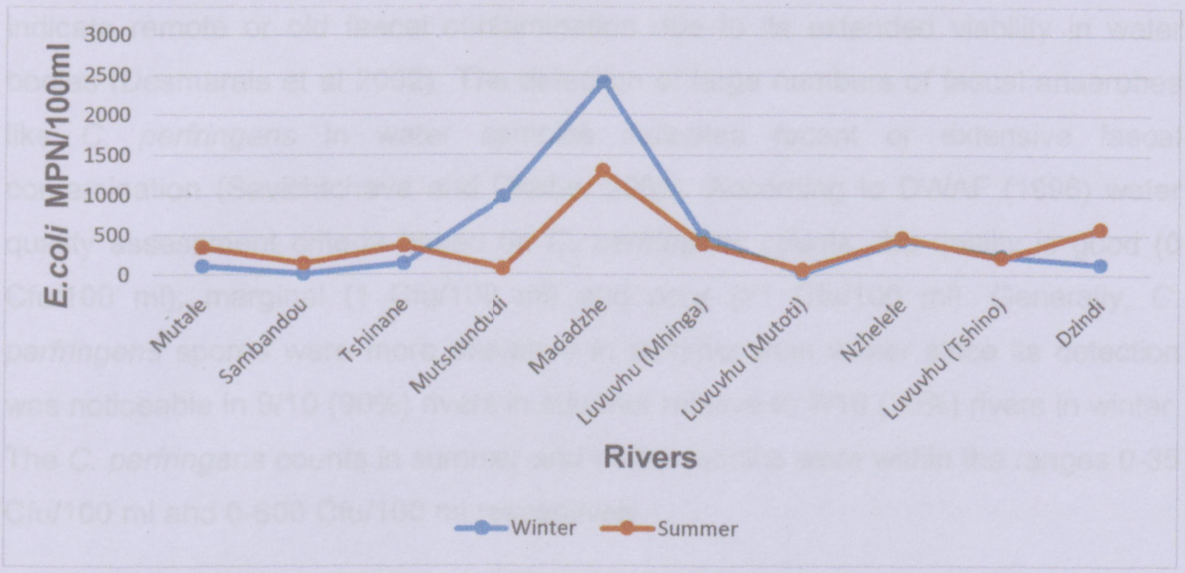


Figure 4.8: Assessment of microbial water quality from various river water sources based on *E. coli*

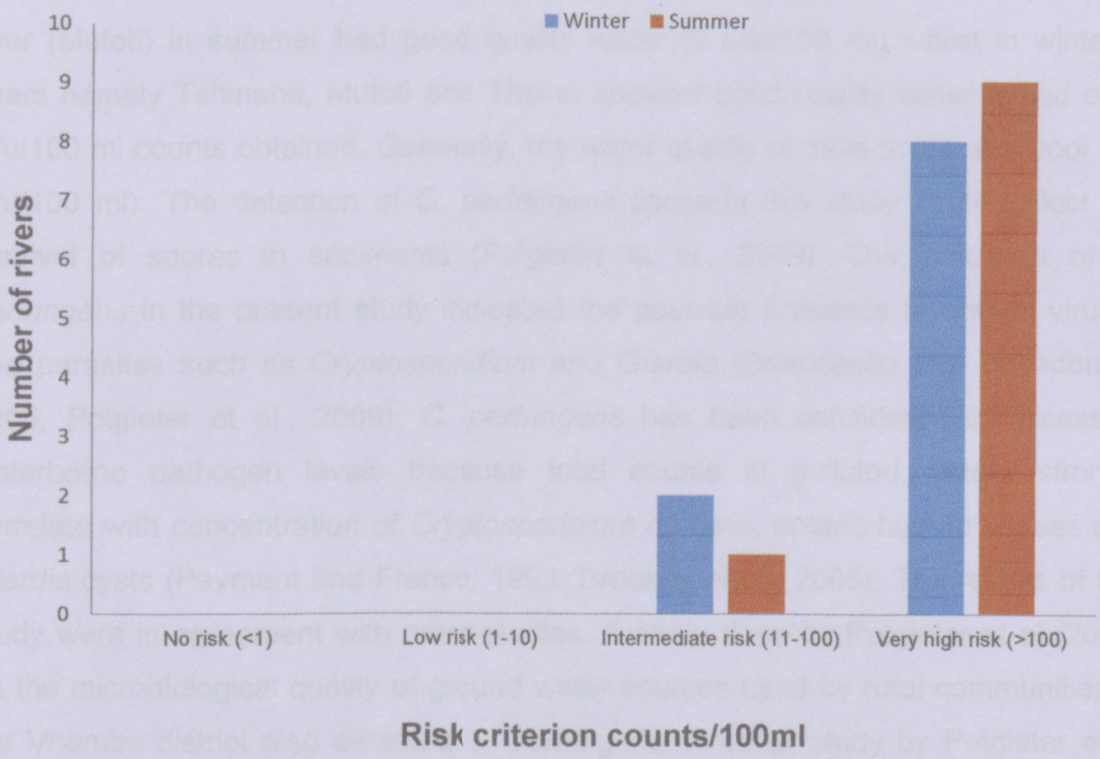


Figure 4.9: Risk assessment based on WHO (2012) criterion for *E. coli* indicator

4.2.3 *Clostridium perfringens*

Clostridium bacteria are used worldwide as indicators of faecal contamination and also for the presence of parasitic protozoans like *Giardia* and *Cryptosporidium* oocysts (Briancesco and Bonadonna, 2005). *Clostridium.perfringens* normally

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indicate remote or old faecal contamination due to its extended viability in water bodies (Desmarais et al 2002). The detection of large numbers of faecal anaerobes like *C. perfringens* in water samples indicates recent or extensive faecal contamination (Savichtcheva and Okabe, 2006). According to DWAF (1996) water quality assessment criteria based on *C. perfringens* counts, the quality is good (0 Cfu/100 ml); marginal (1 Cfu/100 ml) and poor (>1 Cfu/100 ml). Generally, *C. perfringens* spores were more prevalent in summer than winter since its detection was noticeable in 9/10 (90%) rivers in summer relative to 7/10 (70%) rivers in winter. The *C. perfringens* counts in summer and winter months were within the ranges 0-35 Cfu/100 ml and 0-600 Cfu/100 ml respectively.

The winter results in Figure 4.10 would suggest that Madadzhe and Mutshundudi Rivers had extensive faecal contamination (Savichtcheva and Okabe, 2006). Only 1 river (Mutoti) in summer had good quality water (0 Cfu/100 ml) whilst in winter 3 rivers namely Tshinane, Mutoti and Thsino showed good quality water based on 0 Cfu/100 ml counts obtained. Generally, the water quality of most rivers was poor (>1 Cfu/100 ml). The detection of *C. perfringens* sporesin this study might reflect the survival of spores in sediments (Potgieter et al., 2009). The presence of *C. perfringens* in the present study indicated the possible presence of enteric viruses and parasites such as *Cryptosporidium* and *Giardia* (Briancesco and Bonadonna, 2005; Potgieter et al., 2009). *C. perfringens* has been considered in assessing waterborne pathogen levels because total counts in polluted waters strongly correlate with concentration of *Cryptosporidium* oocysts, enteric human viruses and *Giardia* cysts (Payment and Franco, 1993; Brookes et al., 2005). The results of this study were in agreement with other studies. A study done by Potgieter et al (2006) on the microbiological quality of ground water sources used by rural communities in the Vhembe district also detected *C. perfringens*. Another study by Potgieter et al (2009) in the Vhembe District also detected *C. perfringens* in river water.

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Figure 4.10: Assessment of microbial water quality from various river water sources based on *C. perfringens*

4.3. ISOLATION OF SELECTED PATHOGENS IN RIVER WATER SOURCE

4.3.1 *E. coli*

The characterisation of pathogenic *E. coli* strains has been successively achieved through the use of multiplex PCR (m-PCR) (Omar and Barnard, 2014; Traore et al., 2016). Table 4.1 shows the results of the present study for the various pathogenic *E. coli* strains identified.

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Table 4.2 Distribution of *E. coli* pathotypes in winter and summer

River	Winter		Summer	
	Pathotype	Detected genes	Pathotype	Detected genes
Sambandou	EPEC (atyp)	*mdh, eaeA, astA, gapdh*	EPEC (atyp), EIEC, ETEC	*mdh, eaeA, ial, lt, gapdh, astA*
Mutale	EPEC (atyp), EAEC	*mdh, eaeA, eagg, astA*	EPEC (atyp), EHEC, EAEC	*mdh, eaeA, stx1, stx2, eagg, gapdh, astA*
Tshinane	EPEC (atyp), EAEC	*mdh, eaeA, eagg, astA*	EPEC (atyp), EPEC (typ), EHEC	*mdh, eaeA, bfp, stx1, astA*
Mutshundudi	EPEC (atyp), ETEC, EAEC	*mdh, eaeA, st, eagg, astA*	EPEC (atyp), EPEC (typ), ETEC, EAEC	*mdh, eaeA, bfp, lt, eagg, gapdh, astA*
Madadzhe	EHEC, EIEC, EAEC	*mdh, stx1, stx2, ial, eagg, astA*	EPEC (atyp), ETEC, EAEC	*mdh, eaeA, lt, eagg, gapdh, astA*
Luvuvhu (Mhinga)	COM	*mdh*	EPEC (atyp), EHEC, EIEC, ETEC, EAEC	*mdh, eaeA, stx1, ial, lt, st, eagg, astA*
Luvuvhu (Mutoti)	COM	*mdh*	EHEC, EPEC (atyp)	*mdh, stx2, eaeA,*
Nzhelele	EPEC (atyp), EAEC	*mdh, eaeA, eagg, astA*	EPEC (atyp), EPEC (typ), EHEC, EAEC	*mdh, eaeA, bfp, stx1, eagg, gapdh,astA*
Luvuvhu (Tshino)	EPEC (atyp), EPEC (typ), EAEC	*mdh, eaeA, bfp, eagg, astA*	EPEC (atyp), EPEC (typ), EIEC, ETEC	*mdh, eagg, bfp, ial, lt, st, astA, gapdh*
Dzindi	EPEC (atyp), ETEC	*mdh, eaeA, lt, gapdh, astA*	EPEC (atyp), EPEC (typ), EHEC, ETEC, EAEC	*mdh, eaeA, bfp, stx1, stx2, lt, eagg, gapdh, astA*

Com = commensal, EPEC (atyp) = atypical Enteropathogenic *E. coli*, EPEC (typ) = typical Enteropathogenic *E. coli*, EAEC = Enteroaggregative *E. coli*, EHEC = Enterohaemorrhagic *E. coli*, EIEC = Enteroinvasive *E. coli*, ETEC = Enterotoxigenic *E. coli*

The *mdh* gene was 100% detected in all the samples. This gene serves to confirm the presence of *E. coli* in the case of zero detection of tested pathogenic genes (Omar and Barnard, 2014). The *eaeA*, *eagg* and *astA* genes were frequently detected. As shown in Table 4.1. Generally, all the five pathotypes were detected in this study. The current results are similar to results obtained in the study done by Traore et al. (2016) in the Venda region on the rivers around the rural areas which detected all the five pathotypes. Another study by Ndlovu et al (2015) in Western

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Cape, South Africa on river water also detected pathogenic strains of *E. coli*such as EAEC, EIEC and EHEC. The latter study differs from the present study in that, the *ipa*H gene was used for EIEC whilst in the present study, the *ial* gene was used for EIEC. In another study in Western-Nigeria by Titilawo et al (2015) on river water, also detected pathogenic strains of *E. coli* such as ETEC, EPEC and EHEC and no *ipa*H gene was detected. However, a study done by Sidhu et al (2013) in Austria on river water detected the *ipa*H gene for EIEC. Also the latter study detected *lt* and *st* genes infrequently which is similar to this study.

Commensal *E. coli* was detected in only 2 rivers, Mhinga and Mutoti during winter as shown in Table 4.1. However, summer results for the same two rivers were infested with pathogenic *E. coli* strains such as EPEC, ETEC, EAEC, EHEC, and EIEC. This would probably mean that people are carriers of the pathogenic strains of *E. coli* and. according to literature, warmer temperatures during summer, go along with *E. coli* infections (Kovats et al., 2004; Fleury et al., 2006).

The high prevalence of EHEC and other pathotypes in summer (60%) compared to winter (10%) would suggest that both humans and animals were major contributors of faecal contamination (Titilawo et al., 2015). EHEC is well known for causing bloody diarrhea and its presence in water would pose high risk of bloody diarrhea. According to Isaacson (1993), the consumption of surface water in South Africa and Swaziland saw a large outbreak of bloody diarrhea. In this study both EPEC and EAEC were detected more frequently in summer as well as winter. A study done in India on surface water from shallow wells reflected that the consumption of water contaminated with EAEC was significant in causing diarrhea (Hunter, 2003).

4.3.2*Vibrio* spp.

Table 4.3, indicates the isolates for *Vibrio*species detected in summer (19) and winter (11).

Table 4.3 Prevalence of *Vibrio* isolates confirmed by PCR

Season	***Vibrio***
Winter	7/11 (64%)
Summer	14/19 (74%)

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The outcomes from API 20 E tests did not identify *Vibrio cholerae* (*V. cholerae*), although a doubtful presence of *V. cholerae* was confirmed in Luvuvhu, which turned out to be a related species such as *Vibrio fluvialis, Vibrio parahaemolyticus, Vibrio alginolyticus.* Using PCR (Table 4.3) the results revealed the presence of *V. cholerae* through the detection of the *sodB* gene (Ntema et al., 2010). This gene was detected in 7/11 (64%) of the winter isolates and in 14/19 (74%) of the summer isolates (Table 4.2). PCR was more specific in identifying *V. cholerae* unlike API 20 E that assumed possibilities of *V. fluvialis* as well as other enteric bacteria listed in Table 4.3. Generally, *V. cholerae* was more prevalent in summer than winter since it was detected in 9/10 (90%) and in 5/10 (50%) of the sources respectively (Table 4.3).

A study done by Ntema et al. (2010) in the Nwanedi catchment in the Vhembe district showed the occurrence of *V. cholerae* in surface water which support the findings from the present study. According to Fraga et al. (2007), *V. cholerae* O1 and O139 have been the cause of cholera epidemics. The results of this study clearly show that *V. cholerae* is circulating in the region and thus a public health threat especially to communities lacking potable water supply. No O1 or O139 strains were detected in this study.

Table 4.4 *Vibrio* spp recovered from water sources used in rural communities of Venda

Source	Winter		Summer	
	API 20E Possibilities	PCR (*sod*B)	API 20E Possibilities	PCR (*Sod*B)
Mutale	*V. fluvialis, Aeromonas hydrophila,*	*V. cholerae*	*V. fluvialis, V. alginolyticus*	*V. cholerae*
Sambandou	-	-	*Providencia rettgeri*	-
Tshinane	*V. fluvialis, Biberstenia trehalosi*	-	*V. fluvialis*	*V. cholerae*
Mutshundudi	*V. fluvialis, Biberstenia trehalosi*	*V. cholerae*	*V. fluvialis, V. parahemolyticus*	*V. cholerae*
Madadzhe	*V. fluvialis*	*V. cholerae*	*V. fluvialis, Proteus mirabilis*	*V. cholerae*
Luvuvhu (Mhinga)	-	-	*V. cholerae, V. parahemolyticus, V. fluvialis*	*V. cholerae*
Luvuvhu (Mutoti)	-	-	*V. fluvialis*	*V. cholerae*
Nzhelele	*V. fluvialis, V. alginolyticus, V.*	*V. cholerae*	*V. fluvialis, Biberstenia trehalosi*	*V. cholerae*

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	parahemolyticus, Biberstenia trehalosi			
Luvuvhu (Tshino)	*V. fluvialis, Biberstenia trehalosi*	*V. cholerae*	*V. fluvialis*	*V. cholerae*
Dzindi	-	-	*V. fluvialis, Biberstenia trehalosi*	*V. cholerae*

4.3.3 *Salmonella* spp. and *Shigella* spp.

*Shigella*spp were not detected in any of the samples tested (Table 4.4). However, the water samples from Mutale were positive for *Pleisiomonas shigelloides* which are also an important enteric bacteria and shares similarities with some serotypes of *Shigella* spp, specifically, *S. flexenneri,S. sonnei* and *S. dysenteriae*, (Shimada and Sakazaki, 1978; Albert et al., 1993). API 20E confirmed most of the presumptive positives to be members of the Total coliforms such as *Citrobacter spp* and *Enterobacter spp.* PCRconfirmed the very same presumptives to be *Salmonella*spp and was detected more frequently (Table 4.5).Generally, the prevalence of *Salmonella*spp was high in both summer (100%) and winter (90%). The high prevalence in summer might have been due to the surface run-off since it rained more frequently in summer (Jenkins et al., 2008; Robin et al., 2012). In addition, warmer temperatures in summer are favourable for *Salmonella* spp(Fleury et al., 2006).

The results on *Salmonella*spp are in agreement with other findings. In Limpopo province, Potgieter et al. (2005) studies on surface water used as drinking water source also detected *Salmonella* spp. According to Smith et al. (2011), typhoid in South Africa remains endemic in some rural areas lacking potable water supply.

Table 4.5 Prevalence of *Salmonella*spp and *Shigella*spp isolates confirmed by PCR

Season	***Shigella*spp**	***Salmonella*spp**
Winter	-	18/20 (90%)
Summer	-	38/38 (100%)

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Table 4.6 *Salmonella* sppprecovered from water sources used in rural communities of Venda

Source	Winter		Summer	
	API 20E	PCR (*ipa*B)	API 20E	PCR
Mutale	*Pleisiomonas shielloides*	*Salmonella*spp	*Salmonella spp., Salmonella enterica spp arizonae*	*Salmonella*spp
Sambandou	*Enterobacter cloaceae*	*Salmonella*spp	*Salmonella spp., S. enterica spp arizonae*	*Salmonella*spp
Tshinane	-	-	*Salmonella spp., S. enterica spp arizonae, Providencia rettgeri, E. hoshinae*	*Salmonella*spp
Mutshundudi	*C. freundii*	*Salmonella*spp	*E. hoshinae, Salmonella spp.*	*Salmonella*spp
Madadzhe	*P. mirabilis, Serratia liquefaciens*	*Salmonella*spp	*Salmonella spp., S. enterica spp arizonae, Providencia rettgeri*	*Salmonella*spp
Luvuvhu (Mhinga)	*E. hoshinae, P. mirabilis*	*Salmonella*spp	*Salmonella spp., S. enterica spp arizonae, Proteus mirabilis*	*Salmonella*spp
Luvuvhu (Mutoti)	*E. cloacae, C. fruendi*	*Salmonella*spp	*Edwardsiella hoshinae*	*Salmonella*spp
Nzhelele	*E. cloacae, C. fruendi, Salmonella spp, S. enterica spp arizonae*	*Salmonella*spp	*Salmonella spp, S. enterica spp arizonae, Pseudomonas aeruginosa, C. freundii*	*Salmonella*spp
Luvuvhu (Tshino)	*C. freundii*	*Salmonella*spp	*Salmonella spp, S. enterica spp arizonae, E. coli 2*	*Salmonella*spp
Dzindi	*E. cloaceae, Yersinia enterolitica*	*Salmonella*spp	*Salmonella spp, S. enterica spp*	*Salmonella*spp

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4.4 VIRUSES

Enteric viruses are transmissible via the fecal-oral route through ingestion of contaminated water or food (DWAF, 1996a). The Total Culturable Virus Assay (TCVA) is generally recommended by the United States Environmental Protection Agency (USEPA) for monitoring viruses in surface water and the presence of viruses is registered as cytopathic effects (CPE) in specific host cells (Chapron et al., 2000). Generally, epidemiologically important viruses are not detected by the TCVA method, specifically, wild type strains of Hepatitis A Viruses (HAV), human Rotaviruses, human Astroviruses and Noroviruses are difficult to culture in vitro (Formiga-Cruz et al.,2005; Greening, 2006).

The South African standard guidelines for viruses in water based on the TID_{50}/10L of sample stipulates that no viruses be found in water which is the target water quality range and viral doses greater than 10 pose very high risk (DWAF, 1996a). Also, the SANS 241-1: 2011 Standards imply that cytopathogenic viruses not be detected in water otherwise at least a viral dose of 2 poses human health risk. Table 4.6 shows the results obtained from cell culture for cytopathogenic viruses, direct PCR for enteric viruses and PCR-cell culture for enteric viruses.

In this study, the cell culture results revealed only Reovirus that showed a positive result on cytopathogenicity and this was detected only in Madadzhe and Nzhelele sites in winter and summer respectively (Table 4.6). The findings from this study on Reovirus are in line with the findings by Matsuura et al (1984) that, Reoviruses were the most commonly isolated viruses in CPE-positive samples. Reoviruses exhibit moderate clinical significance and studies regarding their detection in environmental water samples are still limited (Spinner and Giovanni, 2001).

Despite negative results under TCVA, the results could be expected since most viruses do not show cytopathogenic effects. According to Lee et al (2005), analysis of enteric viruses by cell culture alone might underestimate the concentration of viruses in environmental samples. Hence, an integration between TCVA and PCR guarantees conclusive results since more viral particles could be detected (Grabow et al., 2004; Lee et al., 2005).

The integrated cell culture-PCR results for the present study revealed that both Enteroviruses and Adenoviruses do not show cytopathogenic effects in cell cultures

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is used as an irrigation source by individuals to water their gardens and this could possibly contribute to the transmission of viruses to consumers of the fresh produce from the gardens.

Table 4.7 Virological assessment of water quality from various river water sources used in rural communities of Venda

River sites	Winter months				Summer months			
	Rota	Noro	Adeno	Other	Rota	Noro	Adeno	Other
Mutale	ND	ND	ND	Entero[3]	ND	ND	ND	ND
Sambandou	ND	ND	ND	ND	ND	ND	ND	ND
Tshinane	Rota[2]	ND	ND	ND	ND	ND	ND	ND
Mutshundudi	Rota[2]	ND	ND	Entero[2]	ND	ND	ND	ND
Madadzhe	ND	Noro GI[2], Noro GII[2]	Adeno[3]	Reo[1], HAV[2], Entero[2,3] Astro[2], Sapo[2]	ND	ND	Adeno[2]	ND
Luvuvhu (Mhinga)	ND	ND	ND	Astro[2]	ND	ND	ND	ND
Luvuvhu (Mutoti)	Rota[2]	ND	ND	ND	ND	ND	ND	ND
Nzhelele	ND	ND	ND	ND	ND	ND	ND	Reo[1]
Luvuvhu (Tshino)	ND	ND	ND	Entero[2]	ND	ND	ND	ND
Dzindi	Rota[2]	ND	ND	ND	ND	ND	ND	ND

ND- Not detected, 1- Cell culture, 2- PCR (direct), 3- PCR (cell culture), Rota-Rotaviruses, Adeno- Adenoviruses, Entero-Enteroviruses, Sapo-Sapoviruses, Noro G I-Norovirus Genotype I, Noro G II-Norovirus Genotype II, Astro-Astroviruses, Reo-Reoviruses, HAV- Hepatitis A Virus

4.5 PARASITES

The number of parasites required to induce infection is relatively low and the infectious dose (ID_{50}) has been estimated to be as low as 83-123 oocysts for *Cryptosporidium* (Chappell et al., 2006) and 19-50 cysts for *Giardia deudenalis* (Adam, 2001). On the other hand, extrapolations from the dose-response data analysed from the Milwaukee Cryptosporidiosis Outbreak indicated that, ingestion of 1-10 oocysts gives a discrete probability of infection for some individuals (Eisenberg et al., 1998).

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According to the South African Guidelines for water quality, the target water quality range for cysts (*Giardia*) or oocysts (*Cryptosporidium*) in water for domestic use is 0/10L and doses greater than 1 may be a risk of parasite infection (DWAF, 1996a). In this study, parasites were detected in 30% of winter samples and 10% of summer samples (Table 4.7). This suggests that the quality of water from the four rivers; Sambandou, Madadzhe, Mutshundudi and Tshinane, is more likely a health hazard of concern if the water is consumed continuously without treatment (DWAF, 1996a). The result of 2 cysts and oocysts per 10L found in this study are in agreement with results found in one of the rivers studied by Sigudu et al (2014) in Gauteng Province, South Africa. Nevertheless, the negative result recorded for most rivers could be regarded tentative since the chance of detecting protozoan parasites might be directly proportional to the volume of water filtered.

C. perfringens results predicted that Mutshundudi and Madadzhe, could have have parasites present and actually, *Giardia* cysts were detected only in these two rivers. This result clearly agrees with the theory that *C. perfringens* could be used as an indicator for the presence of parasites (Savichtcheva and Okabe, 2006). Table 4.7 shows the results obtained for parasites

Table 4.8 Parasitic assessment of water quality from various river water sources in rural communities of Venda

Sources	Winter		Summer	
	Cryptosporidium oocysts/10L	Giardia cysts/10L	Cryptosporidium oocysts/10L	Giardia cysts/10L
Mutale	ND	ND	ND	ND
Sambandou	ND	ND	ND	1
Tshinane	2	ND	ND	ND
Mutshundudi	ND	2	ND	ND
Madadzhe	ND	62	ND	ND
Luvuvhu (Mhinga)	ND	ND	ND	ND
Luvuvhu (Mutoti)	ND	ND	ND	ND
Nzhelele	ND	ND	ND	ND
Luvuhvu (Tshino)	ND	ND	ND	ND
Dzindi	ND	ND	ND	ND

ND- Not detected

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Chapter 5
CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

The primary objectiveof this study was to determine the prevalence of diarrhoea causing bacteria, viruses, and parasites in water sources used by the rural communities in the Vhembe District, Limpopo Province, South Africa. To achieve this, ten selected rivers used by the communities in the Vhembe District to supplement their domestic water needs were studied for their water quality using indicator organisms (Total coliforms, *Escherichia coli* and *Clostridium perfringens*). Figures 4.7, 4.8 and 4.10 shows the results achieved respectively. The second objective was to assess the prevalence of pathogenic bacteria (pathogenic *E. coli* strains, *Salmonella* spp, *Shigella* spp, and *Vibrio* spp). This was done using culture methods and multiplex-PCR technique (Mieta et al, 2010; Omar and Barnard, 2014, WHRC University of Johannesburg). Table 4.1 (*E. coli* strains), Tables 4.2 and 4.3 (*Vibrio* spp) and Tables 4.4 and 4.5 (*Salmonella*spp and *Shigella* spp) shows the achieved results. The third objective was to determine the prevalence of protozoan parasites in river water and this was done using *Cryptosporidium* and *Giardia* (USEPA Method 1623.1 protocol, Rand Water). Table 4.7 shows the results achieved. The last objective was to determine the prevalence of enteric viruses in river water and this was done using the SANS 241-1 (2015) protocol (University of Pretoria). Table 4.6 shows the results achieved. The results indicated that bacterial pathogens were detected in most of the rivers in both winter and summer unlike parasites and viruses which were more prevalent in winter only. The presence of pathogenic *E. coli* strains in water could be a concern for potential health risks since strains like EHEC associated with bloody diarrhea have potential for causing outbreaks (Isaacson, 1993; Anon, 2000). Although this study did not determine the pathogenic nature of *Salmonella*spp and *Vibrio*spp, the results of this study poses a concern since evidence of diarrhea outbreaks have been reported in the Vhembe region (Bessong et al., 2009). This study is the first to assess enteric viruses in river water sources in the Vhembe District after Obi et al (2002) study that detected somatic phages. The presence of enteric viruses in water as shown in this study could be a cause of concern since water contaminated with low concentration of viruses poses significant health risks (Perez-Mendez, 2014). The prevalence of

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parasites was very low, however despite the lack of cryptosporidiosis and giardiasis epidemiological data in South Africa (Sigudu et al, 2014), the presence of parasites in water shown in this study could be evident that people are carriers of the disease (Samie et al., 2006; Samie et al., 2009). In conclusion, the results of this study has shown that the microbial quality of river water used by the rural communities in the Vhembe District was poor and hence a potential vehicle for transmission of diarrhea causing pathogens to humans.

5.2 RECOMMENDATION

The indicators of water quality were detected above the standard limit for no risk in all the rivers. So it is imperative to continuously monitor the water quality of rivers since the concentration of *E. coli* in water measures the public health risks associated with the domestic use of such water (WHO, 2012). Although *C. perfringens*is a reliable indicator for parasites, the low prevalence of parasites could have been due to cysts or oocysts stuck in sediments. This study did not assess sediment samples which are mostly associated with the parasites, as well as the *C. perfrigens* spores (Muller-Spitz et al., 2009) and thus more studies, are needed to assess the prevalence of both the parasites and *C. perfringens* in sediment samples. It is possible that the chances of recovery of parasites would increase if larger volumes of water samples beyond 10L are filtered. The results of this study highlighted the occurrence of enteric pathogens in rivers used by the local communities.This would be informative and help bring awareness to the people as well as help intercept the possibilities of waterborne outbreaks.

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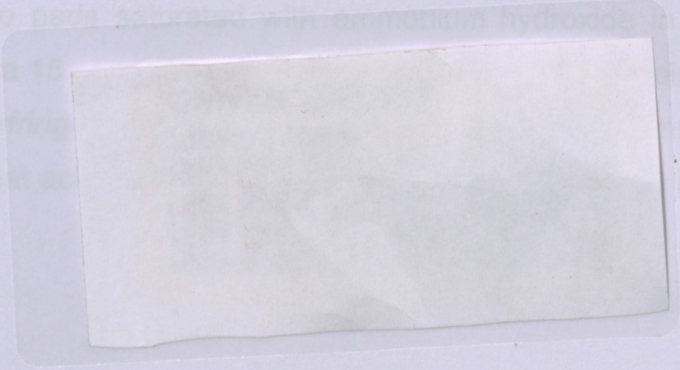
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APPENDIX

APPENDIX C10

OHIO WATER MICROBIOLOGY LABORATORY

MCP AGAR METHOD FOR *CLOSTRIDIUM PERFRINGENS*

Modified from U.S. Environmental Protection Agency (1996)

Updated March 2013

The mCP agar method is a two-step membrane-filtration technique for the detection of *Clostridium perfringens* (*C. perfringens*) vegetative cells and spores in environmental waters. This method cannot be performed in the field; it must be completed in a laboratory, due to the need for colony exposure to ammonium hydroxide in a chemical fume hood.

THEORY: First, the sample is filtered through a 0.45 □m membrane and the filter is transferred to an mCP agar plate. The plates are incubated anaerobically (either in an anaerobic jar or bag) at 44.5˚C for 24 hours. The mCP agar contains differential and selective agents. D-cycloserine and polymyxin B sulfate are antibiotics that inhibit background growth. Bromocresol purple identifies colonies, including *C. perfringens* that can ferment sucrose. Phenolphthalein diphosphate identifies colonies that produce acid phosphatase, such as *C. perfringens*.

After incubation on mCP, filter membranes containing yellow straw-colored colonies are transferred to pads saturated with ammonium hydroxide in a chemical fume hood. Following a 15 second exposure, colonies that turn dark pink to magenta are counted as *C. perfringens*. The color change upon exposure to ammonium hydroxide is an indication that acid phosphatase has cleaved the phosphate in phenolphthalein diphosphate.

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USE: The mCP method can be used for monitoring all types of waters. *C. perfringens* is present in large numbers in human and animal wastes, and its spores are resistant to wastewater-treatment practices, extremes in temperature, and environmental stress. The method has been recommended for use in the examination of (1) chlorinated waters and untreated water containing industrial wastes lethal to non-sporeforming bacteria, (2) sewage sludge, and (3) situations in which the detection of remote, as well as recent, contamination is desirable.

To quantify the number of *C. perfringens* spores present in a sample, the above procedure is modified. Prior to filtration, the samples are heat treated at 60 °C for 15 min to kill vegetative cells. Although *Clostridium perfringens* spores are not an official surrogate of contamination, some researchers have proposed their use as an indicator for viral and parasite removal in drinking water. This adaptation of the procedure is for research purposes, and therefore does not have a parameter code.

MEDIA SOURCES: The mCP basal medium can be bought (Oxoid CM0992) or made by combining individual chemicals. The Ohio Water Microbiology Laboratory (OWML) prepares the medium in 100-mL quantities. The agar medium may be stored at 4°C for up to 6 months. Before agar plates are poured, five reagents must be added to the tempered medium. Once poured, the plates can be stored at 4°C for no more than one month. Anaerobic GasPack bags can be purchased from Fisher (B260683).

Use phosphate buffered dilution water and 0.45 □m membrane filters. Buffer can be purchased from Hardy Diagnostics (800/346-2766, Cat D699 (99mL) or Cat U193 (500mL)). See buffer preparation (Appendix A2).

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MEDIA PREPARATION

1. Prepare mCP basal agar as follows (or order Oxoid CM0992):

   1 L reagent water

   30.0 g tryptose

   20.0 g yeast extract

   5.0 g sucrose

   1.0 g L-cysteine hydrochloride

   0.1 g $MgSO_4 \cdot 7H_2O$

   0.04 g bromcresol purple

   15.0 g Bacto agar

2. Adjust the pH of the agar to 7.6 (with 1 N NaOH)
3. Dispense 100-mL aliquots into dilution bottles and autoclave for 15 minutes at 121°C.
4. Store bottles in the refrigerator for up to 6 months.
5. To prepare plates, melt the agar using a beaker with water on a hot plate **OR** by placing in the autoclave for a 5-minute cycle. After the agar is tempered and before pouring plates, add the following ingredients to each 100-mL bottle of agar:

   0.04 g D-cycloserine

   0.0025 g polymyxin-B sulfate

   0.2 mL 4.5% $FeCl_3 \cdot 6H_2O$ solution

   (filter-sterilize 0.45 g $FeCl_3$ in 10 mL reagent water, store at 4°C for up to 2 months)

   2.0 mL 0.5% Phenolphthalein diphosphate solution*

   (filter-sterilize 0.5 g phenolphthalein diphosphate in 100 mL reagent water, store at 4°C)

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8.0 mL 0.075% indoxyl □-D glucoside solution**

(dissolve 0.006 g indoxyl □-D glucoside in 8.0 mL sterile reagent water)

*Alternatively, 0.01 g phenolphthalein diphosphate can be added directly without making the solution.

**Alternatively, 0.006 g indoxyl □-D glucoside can be added directly without making the solution.

6. To make a large batch of agar, combine the 100-mL bottles into a sterile flask, calculate the amounts of additives for the total volume of agar, and add each additive in one batch.
7. Store plates inverted in the refrigerator for up to 1 month.
8. Record results on a bench sheet form (an example is attached).

ANALYZING SAMPLES:

1. Analyze samples by membrane filtration using appropriate volumes. For turbid surface water, use 3-, 10-, and 30-mL volumes. For clean surface water, use 10-, 30-, and 50-mL volumes. Record media batch and sub-batch numbers on the Results Worksheet.

Note: To quantify the concentration of spores only, heat treat the sample at 60°C for 15 min prior to filtration.

2. Run a filter blank using buffer water before filtering each sample.

3. Plate a positive control (10 and 30 mL of 10^{-2} dilution raw sewage) for every 20th sample or when a new analyst is processing or reading plates.

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4. Incubate plates anaerobically in a GasPak bag or chamber at 44.5°C for 24 hours for the analysis of vegetative bacteria or at 41°C for 24 hours for the analysis of spores.

5. In the fume hood using protective gloves and forceps, place membrane filters with straw-yellow colonies onto cellulose pads saturated with NH_4OH. Wait for approximately 15 seconds, then examine for positive colonies.

6. Magenta colonies that are approximately 1 to 2 mm in diameter are enumerated as *C. perfringens*. New analysts may compare size and color of colonies to those in the positive control.

7. Calculate the number of colony-forming units per 100 mL (CFU/100 mL) sample using the following equation:

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NWIS CODES:

C. perfringens, mCP membrane-filtration method, colony-forming units per 100 mL

Parameter code: 90915

Method code: BAC04

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