Isolation and characterization of bacteriophages specific to *Enterococcus faecalis* and *Enterococcus faecium* as potential markers to detect faecal contamination in water

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DECLARATION

I Mohomud Rashid Adem declare that, the dissertation entitled "Isolation and characterization of bacteriophages specific to *Entercoccus faecalis* and *Enterococcus faecium* as potential markers to detect faecal contamination in water", hereby submitted for the Degree of Master of Science in Biology (Molecular Microbiology) at the North-West University (Mafikeng Campus), has not been submitted by me for a degree at this or any other University. This is my own original work design and execution and that all material contained herein has been duly acknowledged.

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Signed at NWU on this 11th Day of October 2017

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DEDICATION

This work is dedicated to my parents Mr Rashid and Mrs Mariam Adem and to my siblings.

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ABSTRACT

The aim of the study was to isolate and identify *E. faecalis and E. faecium* from ground and surface water samples, and evaluate the potential of using *E. faecalis and E. faecium* specific phages as indicator organisms for determining faecal contamination in water. A total of 162 samples were collected from August 2015 to April 2016 and analysed for characteristics of *Enterococcus* species using Bile Esculin Agar (BEA) and 57 samples were positive for presumptive *Enterococcus* species. The samples were also analyzed to determine faecal coliform and total coliform bacterial counts and large proportion 90 (55%) of the samples were positive for either faecal or total coliform bacteria. A total of 119 presumptive isolates were picked from the 57 culture plates that were positive for *Enterococcus* species based on macroscopic characteristics. The isolates screened for the characteristics of *Enterococcus* (*E. faecalis* and *E. faecium*) using biochemical characterization and *E. faecalis* and *E. faecium* species specific PCR through amplification of the d-alanine: d-alanine ligase (*ddl*) gene sequences. All the isolates including the control strain were Gram positive cocci and were also catalase and oxidase negative. In addition, all the isolates grew in 6.5% (w/v) NaCl and this trait does not only identify the isolates as belonging to the genus *Enterococcus* but also differentiates them from organisms belonging to the genus *Streptococcus*. All the isolates were gamma haemolytic on sheep blood agar. A large proportion 50 (42%) of the isolates were confirmed as *E. faecium* while 16 (13%) were *E. faecalis*. All the isolates in this study were resistant to erythromycin while large proportions (62.1% to 97%) of these isolates were resistant to ampicillin, gentamycin, tetracycline and chloramphenicol. On the contrary, 27 (41%) of the isolates were resistant to vancomycin while only 12 (18.2%) were resistant to amoxycillin. The detection of vancomycin resistant enterococci was a cause for concern due to fact that these isolates may pose severe public health threats to humans. A total of 66 isolates comprising of 50 *E. faecium* and 16 *E. faecalis* were subjected to cluster analysis using Statistica version 10.0 (Statsoft, US). Out of the 66 isolates analysed two major clusters (Cluster 1 and Cluster 2) were generated. Cluster 1 was subdivided into two sub-clusters (Cluster 1A and Cluster 1B).

Furthermore, sub-clusters 1A and 1B, including Cluster 2, were analysed for patterns of association of isolates from different sources and/or locations. The largest sub-cluster (sub-cluster 1B1) contained isolates from three sampling areas. The largest proportion (58.8%) of isolates in this sub-cluster was derived from surface water samples in Mafikeng. In addition, small proportions (17.6% and 23.5%) of the isolates in this sub-cluster were obtained from surface water in Rustenburg and Zeerust respectively. The second largest sub-cluster (sub-cluster Cluster 1A1) contained 12 (18.2%) of the isolates analysed. The makeup of isolates in this cluster consisted of 3, 4 and 5 isolates from groundwater obtained in Rustenburg, Ventersdorp and Mafikeng respectively. Interestingly, all eight (8) isolates in sub-clusters 1A2 were obtained from groundwater in Mafikeng while all six (6) that were clustered in sub-cluster 1B2 were obtained from surface water in Coligny. These findings indicate that isolates that clustered together share similar antibiotic resistance profiles which may have resulted from similarities in the degree of exposure to these drugs. *E. faecalis* and *E. faecium* specific bacteriophages were detected.TEM data indicated that phages possessed morphologies that were similar to those of bacteriophages belonging to the *Siphoviridae* family. It was also revealed that *E. faecalis* and *E. faecium* specific bacteriophages can serve as reliable indicators of faecal pollution in water.

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LIST OF ACRONYMS AND ABBREVIATIONS

ATCC	: American Type Culture Collection
API	: Analytical Profile Index
BEA	: Bile Esculin Agar
Blast	: Basic Alignment Search Tool
CFU	: Colony forming unit
DNA	: deoxyribonucleic acid
E. coli	: *Escherichia coli*
FC	: faecal coliform
F-specific	: male-specific or F^+ coliphage
MNA	: Modified Nutrient Agar
NCBI	: National Centre for Biotechnology Information
PCR	: Polymerase Chain Reaction
PFU	: Plaque forming units
TEM	: Transmission Electron Microscope
VRE	: Vancomycin Resistant Enterococci
w/v	: weight per volume
WHO	: World Health Organization

DEFINITION OF CONCEPTS

Antibiotic resistance: The ability of a given bacterium to survive exposure to a defined concentration of an antimicrobial agent.

Bacteriophage: A virus that uses bacteria as its host; often called a phage.

Coliforms: Are a broad class of bacteria found in our environment, including the faeces of man and other warm-blooded animals.

Polymerase Chain Reaction:A molecular method that is used to amplify specific regions of DNA many times over using primers.

Primer: Is a short strand of RNA or DNA (generally about 18-22 bases) that serves as a starting point for DNA synthesis. It is required for DNA replication because the enzymes that catalyze this process, DNA polymerases, can only add new nucleotides to an existing strand of DNA.

Species: Collection of bacterial cells which share an overall similar pattern of traits in contrast other bacteria whose pattern differ significantly.

Strain: An isolate or group of isolates that can be distinguished from isolates of the same genus or other genus of the same species either by phenotypic characteristics and/or genotypic characteristics.

CHAPTER ONE

INTRODUCTION

CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1 Introduction

Water is a very important resource for life (WHO, 2003) and it is used for drinking, supporting plant life as well as used by humans in daily household activities. In many developing countries, including South Africa, human activities and industrialization are known to significantly affect the quality of water that is supplied to consumers (Martellini et al., 2005). The situation is even worse in some rural communities where individuals do not have access to potable water and most often obtain water from unprotected sources. Humans and animals are the normal hosts of microbial contaminants in water particularly bacterial species and these are released into the environment through excretion from the gastro-intestinal tract of their hosts (Cabral, 2010; Ateba and Maribeng, 2011). These pathogens may be released into water that is intended for human consumption or used for agricultural purposes. The consumption of contaminated water has serious health implications on humans by causing diseases that include diarrhoea, endocarditis, and bacteraemia (Nannini et al., 2005; Takeuchi et al., 2005). Pathogens that are associated with waterborne diseases include enteric viruses as well as bacterial species such *Enterococcus* species, *Escherichia coli* O157:H7 and protozoans such as *Cryptosporidium* and *Giardia* species (Ferguson et al., 2003).The complications associated with these pathogens is aggravated by the fact that some of these enteric organisms are able to cause illnesses in humans when only very few cells (1 to 10 particles) are consumed in contaminated water or food products (Hurst, 1996; Moe, 1996). This therefore increases the pathogenicity and clinical significance of these pathogens.The detection and identification of faecal microbial contaminants in water is essential from a public health view point (Kanyerere et al., 2012; Santiago-Rodriguez, 2013). Coliform bacterial species that include members belonging to the genus *Klebsiella*, *Enterobacter*, *Citrobacter* and particularly *Escherichia coli* have been used to assess the microbial quality of water based on the

premise that they are resident in the gastro intestinal tract of humans and animals (Pelczar et al., 1988; Willey et al., 2008; Santiago-Rodriguez et al., 2013). However, enterococci which are facultative anaerobic, Gram positive cocci also reside as normal flora in the gastrointestinal tract of humans and animals (Gambarotto et al., 2000; Mac et al., 2003) and have also been used as indicators of faecal pollution. The use of enterococci is based on the fact that they are usually found in the faeces of warm blooded animals and their presence in water has been reported to be comparable to most of the recommended bacterial indicator organisms (Wheeler et al., 2002).In addition, it has been shown that there is a direct relationship between the densities of enterococci and *E. coli* in water and the presence of these indicator organisms were linked to the occurrence of swimming-associated gastroenteritis in humans (USEPA, 1986a). With this in mind, both enterococci and *E. coli* have been used as bacterial indicators of water quality (USEPA, 1986b). *Enterococcus* species are able to grow in a wide range of temperatures (10 to 45°C), basic pH (9.6) and high salt content of 6.5% w/v NaCl and these characteristics are constantly used for preliminary identification and isolation.

It has been reported that the number of bacterial cells detected in water samples may be influenced by both climatic and seasonal conditions (Bruhn and Wolfson, 2007). Due to this variability the concentration of bacterial cells in water bodies cannot be predicted at any given time and the viable counts have been reported to increase significantly following a heavy storm, snow melt or other excessive runoff (Bruhn and Wolfson, 2007). In addition, indicator bacteria are usually higher in water samples during warmer months when compared to very cold temperatures such as in winter. Unfortunately, the absence of faecal indicators in water does not necessarily imply that other pathogenic microorganisms are absent (Jagals et al., 2006). This has therefore justified the need to search for other microbial agents that can be very reliable in determining the presence of faecal contaminants in water.

Bacteriophages are viruses that infect bacteria and these agents vary with respect to the strain they infect (Ogilvie et al., 2012). Given the challenges faced with using bacteria as suitable and reliable indicators of faecal contamination in water, bacteriophages and specifically coliphages are now widely utilised in water quality assessment studies. Somatic coliphages (Hilton and Stotzky, 1973; Kott et al., 1974; IAWPRC, 1991), F-specific bacteriophages (Havelaar and Hogeboom, 1984; Jofre et al., 1986) and bacteriophages infecting *Bacteroides fragilis* (Katzeneleson et al., 1974; Jofre et al., 1986; UNDP, 2004) are currently used as suitable indicators of faecal pollution particularly in drinking water systems. Protocols that utilise bacteriophages have several advantages over bacteriological procedures, as they are less expensive, results are obtained in less time and do not require lengthy confirmatory tests (Bonilla et al., 2010; Santiago-Rodríguez et al., 2010).In addition, bacteriophages are more resistant to the water purification processes than the currently utilized bacterial indicators (Katzeneleson et al., 1974; Payment et al., 1985; Payment et al., 1993). The present study is therefore designed to isolate and identify *E. faecalis and E. faecium* from water that is intended for human consumption in the North West Province, South Africa. Samples were also collected from randomly selected dams in the North West Province, South Africa that human access for various purposes. A further objective of the study was to evaluate the potential of using *E. faecalis* and *E. faecium* specific bacteriophages as indicator organisms for determining faecal contamination in water when compared to the usual bacterial indicators.

1.2 Problem Statement

Globally and particularly in South Africa the quality of water that is supplied or is available for human and or animal use or consumption is a public health concern (Kinge et al., 2010; Ateba and Maribeng, 2011; Ateba and Mbewe, 2014). Contaminated water jeopardizes the health of humans since the consumption of contaminated water has potentially serious health implications by causing diseases that include diarrhoea, endocarditis, and bacteraemia (Nannini et al., 2005; Takeuchi et al., 2005). Water

sources are usually contaminated through contact with faeces from humans and animals either as a result of rainfall runoffs or the uncontrolled disposal of faecal wastes (Hurst, 1996; Moe, 1996; Toranzos et al., 1996; Fong and Lipp, 2005). Contaminated water may contain pathogenic microorganisms such as *Salmonella*, *Campylobacter*, enteric viruses and pathogenic protozoa and these can be transmitted to consumers (Hurst, 1996; Moe, 1996; Toranzos et al., 1996; Fong and Lipp, 2005; Ateba and Maribeng, 2011; Santiago-Rodriguez, 2013; Murphy et al., 2016).

Coliform bacterial species include members belonging to the genus *Klebsiella*, *Enterobacter*, *Citrobacter* and particularly *Escherichia coli* have been used to assess the microbial quality of water sources based on the premise that they reside in the gastro intestinal tract of humans and animals (Pelczar et al., 1988; Willey et al., 2008; Santiago-Rodriguez et al., 2013). A number of studies conducted in the study area have revealed the presence of coliform bacteria in water, in animals and food products (Ateba et al., 2008; Phokela et al., 2011; Ateba and Mbewe, 2014). Given that the detection of faecal contaminants in water can be influenced by seasonal variations as well as the protocol used for assessment, there is a need to search for other microbial agents that can be more reliable for water quality monitoring. In the present study, previous investigations in the study area are expanded with a specific focus to evaluate the potential of using *E. faecalis and E. faecium* specific phages as reliable agents to detect faecal contamination in water when compared to indicator bacterial species.

1.3 Aim and objectives

1.3.1 Aim

The aim of the study was to isolate and identify *E. faecalis and E. faecium* from water that is intended for human consumption as well as from selected dams and rivers in the North West Province, South

Africa. A further objective was to evaluate the potential of using *E. faecalis and E. faecium* specific phages as indicator agents for determining faecal contamination in water.

1.3.2 Objectives

The specific objectives of the study were to:

i. isolate of *E. faecalis* and *E. faecium* from the water samples

ii. determine total coliforms and faecal coliform counts in water samples

iii. confirm the identities of *E. faecalis* and *E. faecium* using preliminary and confirmatory biochemical tests

iv. determine the antibiotic resistance profiles of the enterococcus isolates

v. isolate *E.faecalis* and *E.faecium* specific bacteriophages

vi. determine the morphology of isolated phages using electron microscopy as well as the virulence profiles of the phages against environmental *E. faecalis* and *E. faecium* strains

CHAPTER TWO

LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

2.1 Water contamination

Water is a very essential resource for life. The availability of clean drinking water is an important human necessity and therefore a simple right of every human being, as contaminated water endangers the health to consumers (WHO, 2003). Generally faecal contamination of water is associated with the presence of bacteria of faecal origin and these organisms have been reported to cause diseases that include diarrhoea, endocarditis, and bacteraemia (Nannini et al., 2005). In addition, pathogenic organisms such *Enterococcus* species, *Escherichia coli* O157:H7 and protozoans such as *Cryptosporidium* and *Giardia* species as well as enteric viruses that are also derived from faecal matter from humans and animals have been linked to waterborne diseases in most parts of the world (Ferguson et al., 2003). Taking into account the presence of thermotolerant coliforms, enterococci and bacteriophages in the environment as well as water bodies results from human and animal excreta has been used as indicators of faecal pollution in a number of studies (Moe, 1996; Toranzos et al., 1996). Although, results obtained when using these microbial indicators are sometimes questionable due to lack of reproducibility and hence reliability. This therefore justifies the need to develop more reliable water quality assessment schemes.

2.2Traditional Indicator bacteria

An indicator organism is one that can offer evidence about the microbial quality of a water body through the organism's occurrence, state, or numbers. Faecal indicator bacteria are found in the gastrointestinal tract of humans and animals and consist of coliform bacteria and members of the genus *Enterococci* (Anderson et al., 2005; Myers et al., 2007). Faecal indicator bacteria are used to evaluate the microbiological quality of water due to the fact that their presence usually correlates with that of several

waterborne disease-causing organisms (Myers et al., 2007). However, for these microorganisms to serve as affective agents for detecting faecal contamination in water they are expected to meet certain criteria which include the following: (i) they should be detected in water using simple laboratory methods, (ii) be present in faecally contaminated water bodies and be absent in clean, fresh and uncontaminated waters, (iii) be associated with the source of the contamination, (iv) possess a survival potential that is similar to that of the pathogen of concern and (v) be unable to replicate outside the host (Grabow, 1996; Fong and Lipp, 2005; WHO, 2008). Unfortunately not all organisms may fully satisfy these criteria which explains why there is a need to constantly evaluate the suitability of water assessment methods *viz* a *viz* the reliability of results obtained.

2.2.1 Total coliforms

Total coliforms are members of the family *Enterobacteriaceae*, and they are aerobic and facultative anaerobic, Gram negative, non-spore forming, oxidase negative, rod shaped bacteria that ferment lactose to acid and gas within 48 hours of incubation at 35°C (APHA, 1998). Typical genera amongst the total coliform group include *Escherichia, Enterobacter, Klebsiella* and *Citrobacter* and members of this family are part of the normal flora of the intestines of humans and other warm-blooded animals, and they represent only about 1% of the total population of bacteria in human faeces in concentrations ranging from 10^6 to 10^9 coliforms per gram of faeces (Brenner et al., 1982; Payment et al., 2003; Carrero-Colón et al., 2011). These organisms are found in the soil, surface water, plants and in human or animal waste (Rompré et al., 2002; Stevens et al., 2003). Bacterial contamination with total coliforms in surface water and groundwater originates from point sources such as toilets, septic tanks, cattle pens or stables, discharges of untreated sewage and rainfall runoff sand improper disposal of faecal matter(Herrero et al., 2000; Camargo and Alonso, 2007; Rebolledo-Martínez et al., 2011).

Regardless of the issues surrounding the use of these organisms as indicators of faecal contamination in water, total coliforms remain a water quality indicator in many countries and water quality assessment schemes and continue to be used to some degree as a monitoring parameter (Mesquita and Noble, 2013). This is based on the fact that even in situations where faecal contamination is present, total coliforms are usually more numerous than *E. coli*, which presents them to be a more sensitive indicator. Furthermore, some organisms that belong to the total coliform group are considerably more resistant to disinfection during the water treatment processes than *E. coli* and hence they are better indicators of inadequate disinfection (WHO, 2004; Fricker and Eldred, 2009). In addition, the extent to which these organisms are present in the water source can indicate the general quality of that water and the probability that the water is contaminated with faecal matter (USEPA, 2009).

2.2.2 Faecal coliforms

Faecal coliforms are also called thermo-tolerant coliforms and these organisms are able to ferment lactose when they are at a temperature of 44.5°C for 24 hours. Faecal coliforms are also used as indicators to determine faecal pollution in wastewater effluents, rivers, marine environments, recreational and untreated water sources (Geldreich, 1978; Kinzelman et al., 2003; Johnston and Jaykus, 2004). Despite the fact that organisms belonging to the genera *Citrobacter, Enterobacter* and *Klebsiella* are also included in this group *Escherichia* species are known to be the dominant among faecal coliforms (Fujioka et al., 1999; Carrero-Colón et al., 2011) . The faecal coliform group comprises organisms that originate from the intestinal tract of humans and warm-blooded animals (Thomann and Mueller, 1987) and their presence in environmental water bodies may be as a result of the overflow of domestic sewage or non-point sources of human or animal waste into water bodies (McMath et al., 1999).

2.2.3 Escherichia coli

E. coli is a member of the thermo-tolerant coliforms that produce indole from tryptophan and they are enzymatically distinct from the other members of the family *Enterobacteriaceae* due to the fact that the organism does not possess urease but produces β-galactosidase and β-glucoronidase (Leclerc et al., 2001; Payment et al., 2003). *E. coli*is a faecal coliform bacterium, it is regarded as the most sensitive and reliable indicator of faecal pollution in water (Edberg et al., 2000). *E. coli* is able to survive in drinking water for up to twelve weeks and this explains why it is able to withstand a wide range of environmental conditions. In addition, *E. coli* strains are found in faeces of humans and warm-blooded animals at concentrations of 10^9 CFU/1 g (Edberg et al., 2000). These characteristics make *E. coli* a suitable indicator of water pollution and hence have been utilized in a number of studies to assess water quality (Scott et al., 2002; Odonkor and Ampofo, 2013).

2.3 Enterococci

Organisms belonging to the genus *Enterococcus* are important indicators of faecal pollution in water. Enterococci are non-spore-forming catalase negative cocci that live as normal flora in the gastrointestinal tract of humans and animals (Mac et al., 2003). The concentrations of *Enterococcus* species in the intestines of humans range from $10^5$to 10^8 CFU/g of faeces and *Enterococcus faecalis* and *Enterococcus faecium* are the species that are frequently isolated in water quality assessment surveys (Ruoff et al., 1990; Manero et al., 2002; Gelsomino et al., 2003). Despite the fact that *E. coli* are the most widely utilized indicator bacteria, a number of studies have indicated that enterococci are also suitable for use in water quality assessment mainly due to the fact that their concentrations in faeces of humans and animals is proportionate to those of *E. coli* (USEPA, 1986a). In addition, enterococci can survive very harsh environments including extreme alkaline pH (9.6) and high salt concentrations (6.5% NaCl). They are also able to resist bile salts, detergents, heavy metals, ethanol, azide, and desiccation (Rivera et al., 1988; Mascini et al., 2006). *Enterococcus* species can grow in

temperatures ranging from 10°C to 45°C and also survive in high temperatures of 60°C for 30 minutes (Mascini et al., 2006; Fisher and Phillips, 2009).

2.3.1 Classification of organisms in the Genus Enterococcus

The enterococci were formerly classified as a major group of organisms within the genus *Streptococcus*. This classification was based on the fact that they show high resistance to chemicals and physical agents as well as the fact that they agglutinate when reacted with serological group D streptococci antiserum (Teixeira and Merquior, 2013). However, these organisms have undergone significant changes in their nomenclature partly due to the introduction of molecular identification and typing based methods that have resulted in the organisms being classified as a separate genus now called *Enterococcus* (Schleifer and Kilpper-Bälz, 1984). *Streptococcus faecalis* and *Streptococcus faecium* were the first species to be reassigned to the new genus and were renamed *Enterococcus faecalis* and *Enterococcus faecium*, respectively(Teixeira and Merquior, 2013). The continuous use of molecular identification tools has provided opportunities for major advances in the classification of enterococci (Euzéby, 1997; Facklam et al., 2002; Teixeira et al., 2007).

2.3.2 Clinical Significance and Epidemiology of Enterococci

Enterococci are opportunistic pathogens and these organisms are able to cause diseasesin their hosts only when the immune system has been suppressed (Fisher and Phillips, 2009). Nosocomial *Enterococcus* infections are also common in debilitated patients who have been exposed to broad-spectrum antibiotics (Grabow and Coubrough, 1986; Mascini et al., 2006). *Enterococcus faecalis* and *Enterococcus faecium* are the most common species that are frequently isolated from human clinical samples and hospital environments and therefore account for more than 90% of clinical isolates(Teixeira et al., 2007). Against this background, *E. faecalis* and *E. faecium* are among the leading cause of nosocomial infections including urinary tract infections, abdominal and wound

infections, endocarditis, and bacteraemia in humans (Murray, 1990; Jett et al., 1994; Murray and Weinstock, 1999; Arias et al., 2009; Willems and Van Schaik, 2009). Enterococci are the third most common cause of bacteraemia even in countries such as the United States of America that has more advanced and strict public health policies (Chavers et al., 2003; Thouverez and Talon, 2004). This therefore justifies the need to constantly assess contamination with these organisms.

2.3.3 Antibiotic resistance

Enterococcus species have displayed resistance to a large proportion of drugs that are usedin both human and animal medicine (Hayes et al., 2004; Kilonzo-Nthenge et al., 2015). However, the emergence of these organisms as important nosocomial pathogens coupled with the fact that they are now known to express increased resistance to a number of antimicrobial agents, there is therefore a need to constantly monitor the resistance profiles of isolates in a given area (Hollenbeck and Rice, 2012). *Enterococcus* species are able to display intrinsic and/or acquired resistance to several clinically relevant antimicrobial agents (Gold and Moellering Jr, 1996). The different types of antibiotics to which both intrinsic and acquired resistance mechanisms(Jean et al., 2001)are used by enterococci to evade destruction are shown in Table 2.1. The incidence of acquired traits leading to high-level resistance to aminoglycosides and to glycopeptides particularly to vancomycin, is of specific clinical significance due to the impact of these organisms in susceptible patients as well as the difficulty in treating infections caused by *Enterococcus* species.

Infections with vancomycin-resistant enterococci (VRE) are characterised by increased illness, high mortality rates and increase hospital costs (Chou et al., 2008). Resistance to antibiotics result from the presence of antibiotic resistance genes and notably resistance to vancomycin is associated with the presence of *van*A, *van*B, and *van*C genes. The *vanA* and *van*B genes are usually associated with *E. faecium* and *E. faecalis* isolates, and are easily transmitted between isolates through plasmids and are

known to account for increased resistance among enterococci (Murray, 1998). In addition, the vanA and vanB genes have also been identified in vancomycin resistant *Staphylococcus aureus* (Weigel et al., 2003). Given that VRE can be transmitted from person to person through contact these organisms have been reported to cause severe life threatening complications in hospitals and chronic-care facilities. This explains why antimicrobial resistant organisms particular VRE pose severe challenges to the medical profession even in countries with advance health care systems (Deshpande et al., 2007; Simner et al., 2015).

Table 2.1: Intrinsic and acquired antimicrobial drug resistance in enterococci

Type of resistance, antimicrobial drug	
Intrinsic	**Acquired**
β-Lactams (particularly cephalosporins and penicillinase resistant penicillins)	High concentrations of β-lactams (via penicillin-binding proteins or β -lactamase)
Low concentrations of aminoglycosides	High concentrations of aminoglycosides
Clindamycin	Glycopeptides (vancomycin and teicoplanin)
Fluoroquinolones	Tetracycline, Erythromycin, Fluoroquinolones, Rifampin, Chloramphenicol
Trimethoprim-sulfamethoxazole (in-vivo)	Fusidic acid, Nitrofurantoin

2.4 Treatment

Enterococcus species have the ability to adjust to their surroundings and acquire antibiotic resistance determinants (Miller et al., 2014). However, the treatment of bacterial infections including severe enterococcal infections such as endocarditis requires a cell-wall active antimicrobial agent (Murray, 2000). Antibiotics such as ampicillin, penicillin and vancomycin including an aminoglycoside such as gentamicin or streptomycin have been used to treat enterococcal infections and success rate has been very impressive. However, infections that are not very serious such as urinary tract infections are treated with a single agent, such as ampicillin or nitrofurantoin (Murray, 2000).

Unfortunately, *Enterococcus* species have developed increased resistance to ampicillin, vancomycin and also display high-level resistance to aminoglycosides. This has significantly reduced treatment options and has therefore resulted in renewed efforts for the search of more effective antimicrobial agents. Newer agents such as linezolid, quinupristin-dalfopristin and sultamicillin are now used to treat infections caused by vancomycin-resistant enterococci (VRE) (Mascini *et al.*, 2006; Arias and Murray, 2012).

2.5 Preliminary biochemical tests for *Enterococcus* species

Bile-esculin test is widely used to differentiate enterococci and group D streptococci, which are bile tolerant and can hydrolyze esculin to esculetin, from non-group D viridans group streptococci, which grow poorly on bile (Facklam, 1973; Mascini et al., 2006). Esculetin combines with ferric ions to produce a black complex visible as black zones around colonies. It is a low cost, rapid test with good sensitivity and specificity (>90%). Thus, *Enterococcus faecalis* and *Enterococcus faecium* can be selectively cultured on Bile Esculin Agar, and can also be confirmed by morphology through the use of a microscope. *E. faecalis* and *E. faecium* are gram-positive cocci, which can be either in clusters; short chains, diplococci or single cocci. It can also be diagnosed by serological tests using Latex Agglutination Test for Lancefield group D streptococci, through antibody-antigen reaction (Wheeler et al., 2002; Groth and Calos, 2004). Preliminary biochemical tests for enterococci include; catalase test, growth on Sodium chloride (NaCl) broth and haemolysis on blood agar.

2.6 Bacteriophages as faecal indicators

Bacteriophages are viruses that infect bacteria. They were discovered independently first in England by Frederick William Twort, in 1915, and then in Paris by Félix Hubert d'Hérelle in 1917 (Duckworth, 1976).

Twort did not follow on his findings, whereas d'Hérelle thoroughly examined the nature of bacteriophages and explored their role to function as therapeutic agents (Pelczar et al., 1988). During World War I, phages were the last class of the three major classes of viruses to be discovered. The other two viruses that were discovered first were the plant viruses and animal viruses (Grabow et al., 1993).

Since the 1970's they have been specifically suggested and used as indicators to determine the viral quality of water (Hilton and Stotzky, 1973). Procedures that use bacteriophages have several advantages over bacteriological procedures, as they are less costly, results are obtained in a shorter period and do not require prolonged confirmatory tests (Bonilla et al., 2010; Santiago-Rodríguez et al., 2010).In addition, bacteriophages are more resistant to the water purification processes than the currently applied bacterial indicators (Katzeneleson et al., 1974; Payment et al., 1985; Payment et al., 1993). Bacteriophages are widely distributed and the most prevalent entities on earth, and can be found in all environments populated by bacterial hosts, such as soil, water and within other organisms (Clokie et al., 2011; Dutilh et al., 2014; Díaz-Muñoz and Koskella, 2014). However, they can only reproduce inside susceptible and metabolizing host bacteria (Grabow, 2001; Brüssow et al., 2004; Jończyk et al., 2011).

Phages have many life cycles and the two main cycles are the lytic and the lysogenic cycles. The lytic cycle (virulent phage), redirects the host's metabolisms towards the production of new phages in the end resulting in the lysis of the host cell. While in the lysogenic cycle, also called the temperate phage, usually remains in the host in a dormant state (prophage) and replicates alongside the host, until the lytic cycle is induced (Weinbauer, 2004). The lytic cycle is illustrated in Figure 2.1, and involves the stages of adsorption, infection, and the lysis of the host cell and release of progeny phages.

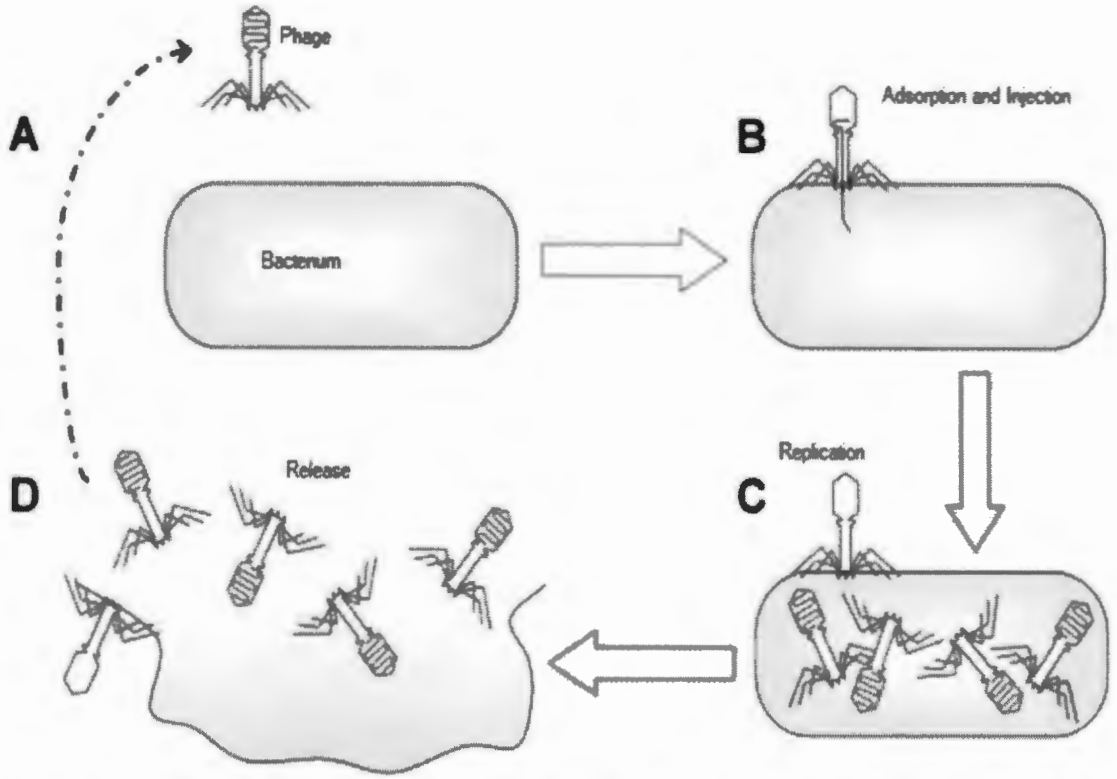

Figure 2.1: The life cycle of a typical lytic bacteriophage. (A) A single infective phage particle with its bacterial host cell. (B) The phage attaches itself to a host cell (adsorption) and injects its genome. (C) The phage genome is copied, structural proteins are synthesised, and then is assembled. (D) The host cell lyses, releasing the new progeny phages. (Kerr et al., 2008)

2.7 Bacteriophages Characteristics

The structure and morphology of bacteriophages practically comprise of a nucleic acid molecule (genome) enclosed by a protein coat (capsid). The capsid is made up of morphological subunits called capsomeres, and consists of a number of protein subunits or molecules called protomers. Some phages also contain lipid and additional structures such as tails and spikes (Grabow, 2001). They are categorized morphologically into tailed and non-tailed phages. More than 96% of all characterised phage contain dsDNA and are tailed (Ackermann, 2009). The tailed bacteriophages consist of *Myoviridae*, *Siphoviridae*, and *Podoviridae* families. The non-tailed bacteriophages include filamentous (e.g. Inoviridae), polyhedral (e.g. *Leviviridae*) and pleomorphic (*Guttaviridae* and *Fuselloviridae*) morphologies (Ackermann, 2003; Ackermann, 2007).

2.8 Phage classification

The International Committee on Taxonomy of Viruses (ICTV) classifies bacteriophages into 1 order of 14 families and 37 genera according to their type of nucleic acid and morphology (Lavigne et al., 2008). The genetic material of bacteriophages can be composed of a single-stranded (ss) or double-stranded (ds) DNA or RNA (Frost et al., 2005), Figure 2.2 demonstrates the main phage groups. Goyal et al. (1987) categorized phages into three groups according to position of receptor sites on the following parts of host bacteria:

- Appendages
- Outer layer
- Cell wall

Phage identification by morphological criteria using electron microscopy is simple, quick and cost-effective. More than 5500 phages have been examined under the electron microscope (Ackermann, 2007).

Furthermore, there are a number of other families like the following

- Corticoviridae (icosahedral capsid with circular double-stranded DNA)
- Lipothrixviridae (rod-shaped enveloped capsid with linear double-stranded DNA)
- Tectiviridae (double icosahedral capsid with linear double-stranded DNA)
- Cystoviridae (icosahedral capsid, enveloped, with double-stranded RNA)
- Rudiviridae (rodshaped capsid non-enveloped with linear double-stranded DNA)
- Fuselloviridae (nonenveloped with circular double-stranded DNA)
- Plasmaviridae (pleomorphic capsid enveloped with circular double-stranded DNA)

Most of the families of phages have been identified in a wide range of water environments(Pedros and Martins, 1995; Grabow et al., 1998).

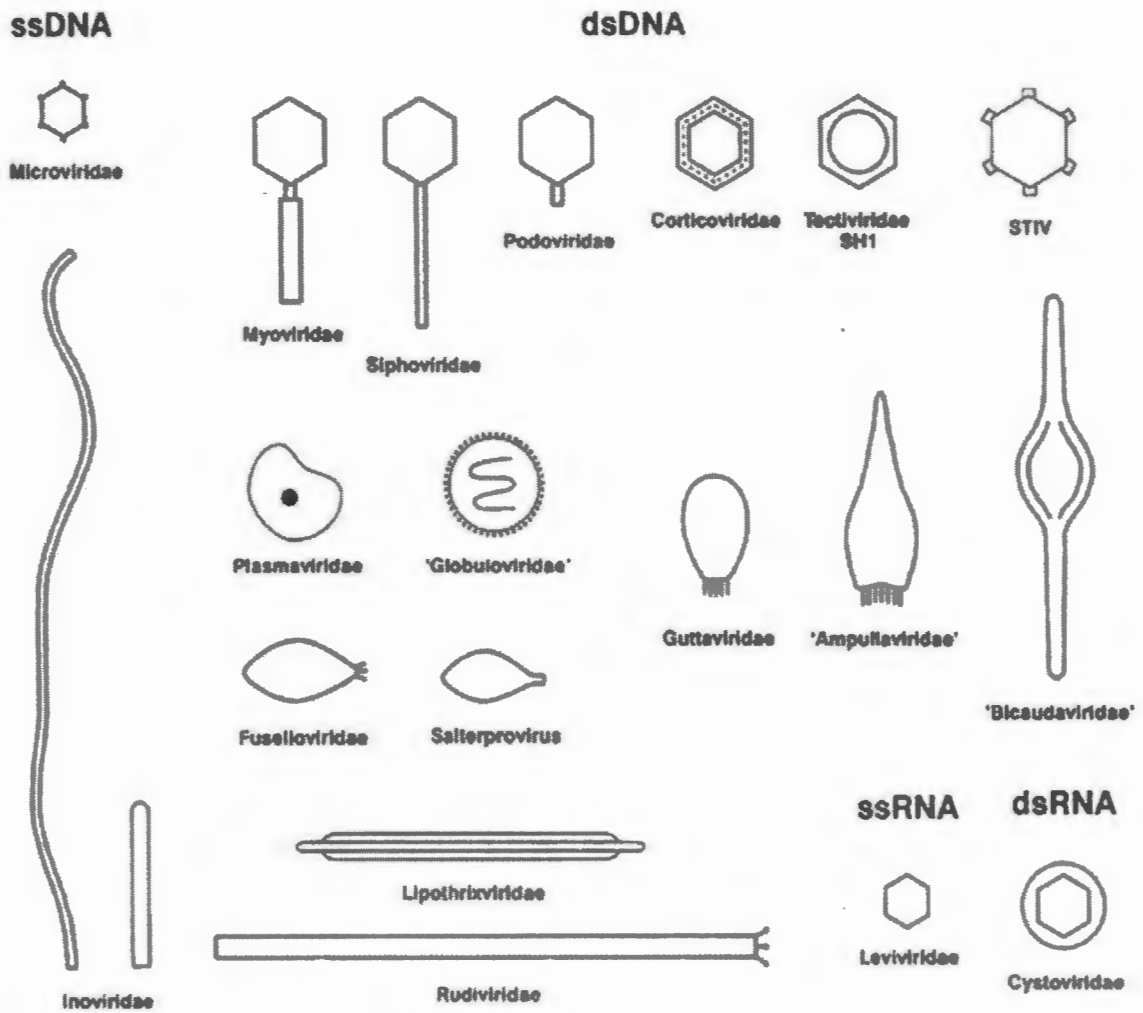

Figure 2.2: Morphotypes of bacteriophages (Ackermann, 2009)

Water quality assessments using phages are presently categorised into six families (Table 2.2), mainly according to nucleic acid content and morphology (Pelczar et al., 1988; Maniloff and Ackermann, 1998):

Table 2.2: Phage families according nucleic acid content and morphology

Family	Structure/ Nucleic acid	Structure of tail
Myoviridae	Elongated (Cubic capsid), linear double-stranded	Long contractile tail.
Siphoviridae	Icosahedral capsid, linear double-stranded DNA	Long non-contractile tail
Podoviridae	Icosahedral capsid, linear double-stranded DNA	Short non-contractile tail
Microviridae	Icosahedral capsid, circular single-strand DNA	No tail
Inoviridae	Rod-shaped, circular single-strand DNA	No tail
Leviviridae	Icosahedral capsid, linear single-strand RNA	No tail

Source:

2.9 Enterococcus phages

Enterococcus phages have recently been considered as indicators for faecal and viral contamination in recreational waters and as tools for microbial source tracking (Bonilla et al., 2010; Santiago-Rodríguez et al., 2010; Purnell et al., 2011; Santiago-Rodriguez et al., 2013). Although the main categories of phages that have been suggested as indicators of faecal contamination are those that infect coliphages (coliform bacteria). The amount of enterococcal phages that have been examined by electron microscope are moderate, most of these phages belonged to tailed phage category (Family Siphoviridae) (Ackermann, 2007).

Based on the aforementioned, somatic coliphages (Hilton and Stotzky, 1973; Kott et al., 1974; IAWPRC, 1991), F-specific bacteriophages (Havelaar and Hogeboom, 1984; Jofre et al., 1986) and bacteriophages infecting *Bacteroides fragilis* (Katzeneleson et al., 1974; Jofre et al., 1986; UNDP, 2004) are currently used as suitable indicators of faecal pollution particularly in drinking water systems.

2.9.1 Somatic coliphages

Somatic coliphages are numerous and the most easily found phage group in the environment. In addition they are existing in greater amounts than phage infecting *Bacteroides* species, largely because *E. coli* are facultative anaerobes, however they cannot be used to establish sources of contamination as they do not validate host specificity (Mocé-Llivina et al., 2005; Lee and Sobsey, 2011). Somatic coliphages may have advantages to F+ coliphages, founded on their perseverance, as more environmentally persistent indicators of enteric viruses in water (Lee and Sobsey, 2011).

It is a heterogeneous group of bacteriophages that are able to infect *E. coli*, and other members of the *Enterobacteriaceae* family. Their members belong to the *Myoviridae*, *Siphoviridae*, *Podoviridae* and *Microviridae* families (Hayes, 1968; Grabow, 2001). They are present in human faeces in low numbers but numerous in untreated domestic sewage and in animal faeces (Havelaar, 1986). They are detectable by reasonably simple, low-cost and quick plaque assays (Green et al., 2000).

2.9.2 F-RNA bacteriophages

F or male specific RNA bacteriophages belong to the *Leviviridae* family and its two genera, *Levivirus* and *Allolevivirus*. They infect bacteria that have an F plasmid, which codes for an F or sex, pilus. These phages have been broken down into four genogroups GI, GII, GIII, and GIV in reference to their serological and physicochemical properties (Leclerc et al., 2000; Grabow, 2001; Scott et al., 2002; Vinjé et al., 2004; Ackermann, 2009). In addition genogroups I and IV are generally found in animal wastes, while subgroups II and III are associated to human faecal pollution (Cole et al., 2003; Vinjé et al., 2004).

Serotyping or genotyping is used on these phages in order to differentiate between human and non-human microbial sources.

F-RNA bacteriophages have been broadly researched due to their comparison (in size, shape, morphology and composition) to several pathogenic human enteric viruses (Jofre et al., 2011). Further useful attributes of these phages resembling those of human viruses include their failure to multiply in water environments. In addition their presence in high numbers in wastewater and their resistance to unfavourable conditions and disinfection processes (chlorination) contribute to their usefulness as process indicators, indices of sewage pollution, and conservative models of human viruses in water and shellfish (Grabow, 1990; Havelaar, 1993; Love and Sobsey, 2007). The finding of F-RNA coliphages is not easy and simple by plaque assays as in the case of somatic coliphages (Grabow et al., 1998).

2.9.3 Bacteriophages of Bacteroides fragilis

Bacteriophages of *Bacteroides fragilis* and other *Bacteroides* species rank third in abundance in natural waters, and have been suggested as potential indicators of human viruses in the environment. Their host *Bacteroides fragilis* is an obligate anaerobe, and is abundant in human faeces (Tartera and Jofre, 1987). Phages infecting *B. fragilis* belong to the family *Siphoviridae*, and contain a double stranded DNA (Lasobras et al., 1997; Queralt et al., 2003; Diston et al., 2012). Studies of inactivation have shown phages of *B. fragilis* to be more resistant to drinking water treatments than either F-specific RNA coliphages or somatic coliphages (Jofre et al., 1995; Duran et al., 2003).

2.10 Detection techniques

Phages can be recovered or detected by using many techniques and approaches. These include culture-based methods and rapid methods which include immunology- and molecular-based methods. Each type of method has advantages and disadvantages. Despite the above, more research is still in progress that is aimed on improving these methods or techniques. The most often used technique for

detecting, isolating and enumeration of *Enterococcus* phages is the Plaque assay (Santiago-Rodriguez, 2010). Plaque assays are a typical culture-based technique used for enumerating infectious virus particles (Grabow, 2001; Rodríguez et al., 2012). Plaque assay for somatic coliphages is simple, inexpensive, less labour-intensive and less time-consuming when compared to that of *Bacteroidesfragilis* phages and F-RNA coliphages (Grabow, 2001). Complex growth media supplemented with antibiotics is required for culturing and plates have to be incubated anaerobically when detecting *Bacteroides fragilis* phages. Theoretically, waste waters may be expected to contain higher levels of *B. fragilis* than those on record (Fong and Lipp, 2005). The reason for the discrepancy may be due to failure in maintaining high levels of sufficient and suitable conditions when performing plaque assay.

2.11 Transmission

It has previously been reported that phages are host specific, meaning that they only infect their target host. On the other hand, the bacterial host of different phages like *Enterococcus faecalis* and *Enterococcus faecium* does cause certain human infections (Puig et al., 2000). The phages are mostly present in the same habitats as their hosts. They cause infections when displaced into the bloodstream (by either being transported into circulation or entering through the intestines when there is a severe damage to the wall of the intestines) or surrounding tissue following surgery, disease, or trauma (Mascini et al., 2006). Transmission can also occur from one person to the other through ingestion and consumption of water or food contaminated with human faeces. Under normal circumstances the host bacteria are found in the gastrointestinal tract as normal flora. The phages move from the gastro-intestines to the waste water or sewage systems through fecal materials.

2.12 Survival

The ability of bacteriophages to survive under unfavourable conditions is highly diversified. Aspects which influence the amount and behaviour of phages in water environments include: the densities of both host bacteria and phages; the association of phages and bacteria with solids; the presence of organic matter, especially organic matter that influences the metabolic activity of the host bacteria; ultraviolet and visible light; temperature; pH; the concentration and type of ions; and the metabolic activities of micro-organisms other than the host bacteria (Sobsey and Hickey, 1985; Gantzer et al., 2001; Feng et al., 2003; Lu et al., 2003; Ackermann et al., 2004; Jepson and March, 2004; Olson et al., 2004; Tey et al., 2009; Jończyk et al., 2011; Yang and Griffiths, 2013).Temperature is a crucial factor for the survival of somatic and F-RNA bacteriophages in water and other environments such as the soil (Gantzer et al., 2001; Olson et al., 2004; Tey et al., 2009). Temperature plays a major role in the attachment, penetration, multiplication and the length of the latent period of bacteriophages. The effects of several of these variables on phage survival have been studied under laboratory conditions (Tartera et al., 1989). Organic compounds which affect phage survival in water environments include humic and fulvic acids, which may influence the attachment of phages to sediments and other solids, and possibly also to their host bacteria (Sobsey and Hickey, 1985). As a result of the many variables concerned which affect the survival and replication of phages, it is generally difficult to predict the incidence and behaviour of phages in water environments.

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

3.1 Area of the study

This study was conducted in the Molecular Microbiology Laboratory of the Department of Biological Sciences at North-West University-Mafikeng Campus, South Africa.

3.2 Sample collection

3.2.1 Sample size determination

The number of samples collected during the study was determined using the formula outlined below

$$\text{Sample (n)} = \frac{Z_{1-\alpha/2}^2 P (1-P)}{d^2}$$

$Z_{1-\alpha/2}$ = is standard normal variate (at 5% type I error ($P< 0.05$) it is 0.05)

P = Expected prevalence in population based on previous studies

d = Absolute error or precision (which is 5%)

In order to determine the prevalence of vancomycin resistant enterococci, the sample size for this study was determined using a prevalence of 88% obtained in a study that was conducted in Australia. An expected prevalence at 95% confidence level and desired precision of 5% were considered in the calculations (Sidhu et al., 2014). The formula described by Charan and Biswas (2013) was used and the minimum sample size required for the present study was162 water samples.

3.2.2 Sampling technique

Random sampling was used to collect water samples from different selected areas in the North West Province based on availability. Groundwater samples were collected from borehole taps in both urban and rural communities, surface water samples were collected from dams and rivers using sterile 500mL

Duran Schott bottles. The samples were appropriately labeled and transported on ice to the laboratory for analysis. Upon arrival in the laboratory, the water samples were immediately analyzed for the presence of *Enterococcus* species. The numbers of water samples collected from the different areas are shown in Table 3.1.

Table 3.1: Areas from which samples were collected

Districts	Sampling Area/Station	Type of samples	Number of samples
Bojanala Platinum	Rustenburg	Ground water = 19 Surface water = 6	28
	Swartruggens	Ground water = 3	
Dr Kenneth Kaunda	Potchefstroom	Groundwater = 8 Surface water = 12	20
	Ventersdorp	Ground water = 6	6
Dr Ruth Segomotsi Mompati	Taung	Groundwater = 9 Surface water = 3	12
Ngaka Modiri Molema	Lichtenburg	Groundwater = 3	3
	Coligny	Groundwater = 6 Surface water = 6	12
	Mafikeng	Groundwater = 41 Surface water = 18	59
	Setlagole	Ground water = 3	3
	Zeerust	Groundwater = 7 Surface water = 12	19
Total			**162**

3.3 Sample analysis

3.3.1 Selective isolation of Enterococci species

Water samples were analysed for the presence of *Enterococcus* species immediately upon arrival in the laboratory using standard methods (APHA, 1998). An aliquot of 100 mL from each sample was filtered through a 0.45µm filter paper (Whatman®Glass Microfiber GS filter paper) on a water pump machine (model Sartorius 16824). A sterile forceps was used to remove the membrane filters from the machine

and the filter papers were placed, grid side up, on Bile Esculin Agar (BEA) plates. The plates were incubated aerobically at 37°C for 24 h. After incubation typical black colonies on the BEA agar were considered as presumptive *Enterococcus* species and these isolates were purified by sub-culturing on BEA. Pure colonies were preserved for further microbiological identification tests.

3.3.2 Detection of total and faecal coliforms

The presence of faecal and total coliform bacteria in the water samples was determined using standard methods (APHA, 1998). Aliquots of 100 mL from each sample were filtered through a 0.45 µm filter paper (Whatman®Glass Microfiber GS filter paper) on a water pump machine (model Sartorius 16824). A sterile forceps was used to remove the membrane filters from the machine and the filter papers were placed on mFC and m-Endo agar to detect faecal and total coliform bacteria respectively. The mFC and m-ENDO plates were incubated aerobically at 45°C and 37°C respectively for 24 h. Metallic-sheen and typical blue colonies on m-ENDO and mFC agar plates were regarded as potential total and faecal coliforms respectively. The number of colony forming units (CFU) /per 100mL of water was counted and recorded.

3.3.3 Control strains

E. faecalis (ATCC 29212) and *E. faecium* (ATCC 6569) were used as positive control strains during the experiments.

3.4 Bacterial identification

Presumptive isolates that were black colonies on BEA were identified using the following criteria:

3.4.1 Cellular morphology

The isolates were Gram stained using a standard method (Cruickshank et al., 1975) and Gram-positive cocci were subjected to both preliminary and confirmatory identification tests that are specific for *Enterococcus* species.

3.5 Preliminary biochemical identification tests for enterococci

3.5.1 Oxidase test

The oxidase test was performed using the Microbact test strips which were impregnated with NNN'N' tetramethyl -p- phenylene-diamine dihydrochloride to facilitate detection of bacterial cytochrome oxidase enzyme. The test was performed as instructed by the manufacturer (Oxoid - United Kingdom). A sterile toothpick was used to transfer pure colonies of the test isolates onto the Microbact test strips and the results were observed within 30 sec. The formation of a purple colour indicated a positive result for the test and vice versa. *Enterococcus* species are oxidase negative therefore all isolates that satisfied this identification criterion were retained.

3.5.2 Catalase test

Catalase is an enzyme that breaks down hydrogen peroxide, which is toxic to the cell, into oxygen and water and therefore the catalase test is used to detect the presence of catalase enzyme in bacteria strains.To perform the test a sterile plastic loop was used to transfer a bacterial colony to a glass slide and a drop of 2% (v/v) hydrogen peroxide was added to the culture. The formation of bubbles was considered a positive reaction and vice versa. Enterococci are catalase negative thus isolates that satisfied this preliminary identification test was recorded.

3.5.3 Growth on Sodium chloride (NaCl)

Enterococci are differentiated from other streptococci based on their ability to grow on 6.5% (w/v) sodium chloride (Eaton et al., 1998). Presumptive isolates were inoculated in 10 mL of 6.5% sodium chloride and incubated aerobically at 37°C for 24 h. Two tubes that was inoculated with cultures of *E. faecalis* (ATCC 29212) and *E. faecium* (ATCC 6569) respectively in 6.5% NaCl and one without any bacteria culture were used as positive and negative controls respectively. Turbidity indicated bacterial growth which was measured using a spectrophotometer.

3.5.4 Haemolysis on blood agar

Blood agar was used to determine the haemolytic patterns of isolates (Rogers, 2008). Plates were spot inoculated with enterococci and incubated aerobically at 37°C for 24 h. After incubation plates were observed for the presence of haemolysis or degree to which red blood cells were broken down. Enterococci produce gamma haemolytic patterns indicating they do not breakdown erythrocytes.

3.6 Confirmatory identification tests for *Enterococcus* species

3.6.1 Serotyping

A SLIDEX® Strepto Plus Latex agglutination test kit obtained from BioMérieux (France) was used for serological identification of *Enterococcus* species based on the Lancefield grouping. The test was performed by reacting a cell wall specific carbohydrate extract of isolates with a suspension of polystyrene microparticles sensitized with rabbit anti-group D streptococcal antibodies.The development of an agglutination pattern displaying visible clumping of the latex particles in approximately 2 min andpositive results were recorded.

3.6.2 Genomic DNA extraction

Bacterial isolates were sub-cultured on Bile Esculin agar and incubated at 37°C for 24 h and the fresh culture was used for DNA extraction. Chromosomal DNA was extracted using ZR Genomic DNA™-Tissue MiniPrep (Zymo Research) extraction kit based on the manufacturer's instruction. Aliquots of the bacterial culture were transferred to a microcentrifuge and 95 µL of 2 × Digestion Buffer as well as 5 µL of Proteinase K were added to the tube. The contents in the tube were mixed thoroughly and incubated at 55°C for 20 min. Seven hundred microliters of Genomic Lysis Buffer was added to each tube and mixed thoroughly by vortexing. The mixture in each tube was transferred to a Zymo-Spin™ IIC column in a collection tube, and centrifuged at 10,000 × *g* for 1 min. The Zymo-Spin™ IIC column was placed in a new collection tube and 200 µL of DNA Pre-Wash Buffer was added to the tube. The tubes were centrifuged at 10,000 × *g* for 1 min. After centrifugation, 400 µL of g-DNA Wash Buffer was added to the Zymo-Spin™ IIC column and centrifuged at 10,000 ×*g* for 1 min. The spin column was transferred to a clean micro-centrifuge tube and 100 µL of DNA Elution Buffer was added, and centrifuged at 10,000 × *g* for 30 sec to elute the DNA.

3.6.3 DNA quality and quantity determination

The quality and quantity of genomic DNA extracted from the isolates was determined using spectrophotometric analysis by measuring the optical density at 260nm and 280nm (Sambrook et al., 1989). A WPA light wave UV/visible spectrophotometer (WPA, UK) was used.

3.6.4 PCR analysis for the identification of E. faecalis and E. faecium

The identities of the target organisms was determined using simplex PCR assays that were designed to amplify *E. faecalis* and *E. faecium* d-alanine:d-alanine ligase (*ddl*) species specific gene fragments using specific primer sequences (Kariyama et al., 2000). The primer sequences that were used are shown in Table 3.2. PCR reaction mixtures comprised 1× PCR buffer, 2mM $MgCl_2$, 0.2mM of each

dNTPs, 0.3μM each of the *ddl* primer, 0.6 units of *Taq* DNA polymerase in a final volume of 25 μL. PCR cycling involved an initial denaturing step of 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for1 min, extension at 72°C for 1 min and then a final extension at 72°C for 7 min. Genomic DNA from *E. faecalis* (ATCC 29212) and *E. faecium* (ATCC 6569) were used as positive controls while a no DNA template reaction was included as a negative control.

Table 3.2: Primer sequences that were used to detect *E. faecalis* and *E. faecium* by PCR analysis

Primer Name		Sequence (5' – 3')	Amplicon size (bp)
ddl E. faecalis	F	ATCAAGTACAGTTAGTCTTTATTAG	941
	R	ACGATTCAAAGCTAACTGAATCAGT	
ddl E. faecium	F	TTGAGGCAGACCAGATTGACG	658
	R	TATGACAGCGACTCCGATTCC	

3.6.5 Sequence analysis of PCR amplicons

The PCR amplicons were cleaned using a GeneJet PCR Purification kit (Lot 00227048, Thermo Scientific, Lithuania) and sequenced at Inqaba Biotec – Pretoria. The identities of the isolates were confirmed through a Blast Search with the NCBI Search Tool: (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.6.6 Antimicrobial susceptibility test

The antibiotic resistant profiles of the isolates were determined using the Kirby-Bauer disc diffusion technique (Kirby et al., 1966). Before antibiotic sensitivity testing, the isolates were revived by sub-culturing onto BEA plates. The plates were incubated aerobically at 37°C for 24 h. Bacterial suspensions were prepared using pure isolates and aliquots of 100μL from these suspensions were spread-plated on Mueller Hinton agar (Biolab, Merck, South Africa). The antibiotic susceptibility of the isolates was determined against a panel of seven different antibiotic discs obtained from Mast

Diagnostics – South Africa. The antibiotic discs were gently pressed onto inoculated Mueller Hinton agar to ensure intimate contact with the surface and the plates were incubated aerobically at 37°C for 24 h (NCCLS, 1999). The antibiotic inhibition zone diameters were measured and the results obtained were used to classify isolates as being resistant, intermediate resistant or susceptible to a particular antibiotic based on standard reference values (NCCLS, 1999).Table 3.3 indicates the details of antibiotics that were used in the study.

Table 3.3: Details of the antibiotics that were used in the study

Group	Antibiotic	Abbreviation	Disc conc. (µg)	Inhibition zone (mm)		
				S	I	R
Penicillins	Ampicillin	AMP	10	≥ 17	–	≤16
	Amoxycillin	AMO	10	≥ 17	–	≤16
Macrolides	Erythromycin	ERY	15	≥23	14–22	≤13
Tetracyclines	Tetracycline	TET	30	≥19	15–18	≤14
Glycopeptides	Vancomycin	VAN	30	≥ 17	15–16	≤14
Phenicols	Chloramphenicol	CHL	30	≥ 18	13–17	≤12
Aminoglycosides	Gentamicin	GEN	10	≥ 15	13–14	≤12

Legend: R= resistant, I= intermediate resistant and S= susceptible

3.7 Cluster analysis of *E. faecium* and *E. faecalis* using antibiotic inhibition zone diameter data

Cluster analysis of antibiotic susceptibility data for *E. faecium* and *E. faecalis* isolated from the different stations was determined using Wards algorithm and Euclidean distances on Statistica version 10.0 (Statsoft, US).

3.8 Bacteriophage isolation

: Bacteriophages were isolated from the water samples using the following criteria:

3.8.1 Preparation of Modified Nutrient Agar (MNA) plates and samples

Modified Nutrient Agar (MNA) [Nutrient broth (20g), NACl (8.5g), Agar (10g), $CaCl_2$ (8.325mg/L), $FeCl_3$ (1.1mg/L), $MgSO_4.7H_2O$ (0.5g), 30% (w/v) Glucose (10 mL)] was prepared based on standard protocols and the plates were stored at 4°C. Before analysis plates were kept overnight at room temperature in order to dry.

3.8.2 Preparation of host strain for detection of bacteriophages

Aliquots of 10 mL of sterilized Tryptic soy broth (TSB) were inoculated with a fresh colony of *E. faecium* (ATCC 6569), *E. faecalis* (ATCC 29212) andenvironmental strains that were confirmed as enterococci by PCR analysis. Tubes were incubated at 37°Cfor 18 h without shaking. The optical density of the culture was monitored and the cells were considered competent when the OD ranged from 0.5 to 0.6. The competent cells were used as potential hosts for isolation of bacteriophages from water samples.

3.8.3 Preparation of water samples for phage detection

Water samples (10 mL) were transferred into 15mL polypropylene conical Falcon tubes and centrifuged at 11000 *g* for 10 min in order to sediment debris and bacterial cells. The supernatant was filtered

through 0.22 µm Acrodisc syringe filter (Pall Corporation, Ann Arbor, MI, USA) and the resulting filtrate was used to prepare phage dilutions.

3.8.4 Serial dilutions of phage lysates

Tenfold serial dilution of the phages was prepared by adding 50 µL of the filtrate (phage lysates) to 450 µL of Lambda diluent [(10 mL 1 M Tris HCl (pH 7.5); 2g of $MgSO_4.7H_2O$; 990 mL of deionized water)]. Serial dilutions were prepared up to 10^{-4} for each sample and only 10^0, 10^{-1} and 10^{-4} were used for enumeration of phages.

3.8.5 Double layer agar assay and plating dilutions

Phages infecting *Enterococcus* strains were isolated from samples using standard protocol (Sambrook and Russell, 2001). Aliquots of 100 µL from the selected phage dilutions (10^0, 10^{-1} and 10^{-4}) were added to 100 µL of the host culture in 15mL polypropylene conical Falcon tubes. For the blank, 200 µL of Lambda diluent was added to the polypropylene tube. For the negative control A, 100 µL of Lambda diluent was added to 100 µL of host culture while the negative control B contained 200 µL of the host culture. Both the test and control reaction tubes were allowed to stand at room temperature for 2 minutes to facilitate attachment of the potential phages to the host bacteria cells. Three milliliters of molten 0.3% (w/v) Bioline Molecular Grade agarose (Cat No: BIO-41025) that contained 10 mM $MgSO_4$ (kept at 50°C in a water bath) was added to each tube, mixed by inverting 2 times and quickly poured onto pre-hardened MNA plates.

The bacteria-phage-agarose mixture was swirled gently to ensure it is evenly distributed over the MNA plate. The overlays were allowed to solidify and the plates be incubated aerobically in inverted positions at 37°C for 24 h (Sambrook and Russell, 2001). After incubation the plates were observed for the presence of plaques (areas of clear zones) and these were enumerated and recorded. For quality

control purposes results were considered appropriate when no bacterial colonies (NC) were present on the plates with agar only and no plaques (NP) were present in the negative control plates A and B.

3.9 Phage purification

A single isolated plaque was picked and placed in 1 mL of Lambda diluent in a 1.5 mL Eppendorf tube. The tubes were kept at 4°C overnight and later centrifuged at 10000 *g* for 10 min. The supernatant was filtered through a 0.22 µm syringe filter (Pall Life Science, Mississauga, ON, Canada) to remove any agar and bacterial cells (Niu et al., 2012). Lysates were mixed with bacterial cultures and 3 mL of 0.3% (w/v) agarose. The contents in the tubes were mixed by inverting 2 times and quickly poured onto pre-hardened MNA plates.

3.10 Phenotypic characterization of phages

3.10.1 Morphological characterization using electron microscopy

Phages were purified using PEG (polyethylene glycol) according to the procedure described by Sambrook and Russell (2001). Phage suspensions were centrifuged at 25 000 *g* for 60 min and then they were washed twice in ammonium acetate (0.1 M, pH 7.0). Sediments were deposited on copper grids with carbon-coated Formvar film, and stained with 2% (w/v) uranyl acetate (pH 4.2). The morphologies of the phages were examined under a transmission electron microscope an accelerating voltage of 60 kv and 10-25000x instrumental magnification.

3.10.2 Evaluation of the lytic capabilities of phages against environmental Enterococcus strains (Phage Virulence Assay)

Bacterial host strains were prepared for phage lysis using *Enterococcus* species isolated from groundwater and surface water samples. The environmental host cultures were prepared 5 hours earlier in a Tryptic soy broth. Host range and lytic capability of the phages were assessed using a

microplate phage virulence assay (Niu et al., 2009). The phage stocks with high titres were serially diluted in 96-well microplates by adding an aliquot of 20 µL of the phage lysate to 180 µL of TSB broth. Twenty microliters of log phase cultures of environmental *Enterococcus* strains isolated in this study were added to the phage dilutions and microtitre plates were incubated for 5 h at 37°C overnight. After incubation, wells were examined visually for turbidity and the highest dilution that resulted in complete lysis of bacteria was recorded.

CHAPTER FOUR

RESULTS

CHAPTER 4

RESULTS

4.1 Detection of *Enterococcus* isolates and coliform bacteria (Faecal, Total) from ground and surface water samples

A total of 162 samples were collected from August 2015 to April 2016 and analysed for characteristics of *Enterococcus* species using Bile Esculin Agar (BEA). A total of 57 samples were positive for presumptive *Enterococcus* species. The samples were also analyzed to determine faecal coliform and total coliform bacterial counts and large proportion 90 (55%) of the samples were positive for either faecal or total coliform bacteria. Results obtained were recorded as number of colony forming units (CFU) per 100 mL of water sample and are shown in Table 4.1. A total of 119 presumptive isolates were selected from the 57 culture plates that were positive for *Enterococcus* species based on macroscopic characteristics. These isolates were subjected to both preliminary and confirmatory biochemical tests specific for enterococci.

4.2 Identification of isolates based on preliminary and confirmatory biochemical tests

A total of 119 isolates obtained from water samples were processed for *Enterococcus* specific biochemical tests to confirm their identities and the results are shown in Table 4.2. All the isolates including the control strain were Gram positive cocci and were also catalase and oxidase negative. In addition, all the isolates grew on 6.5% (w/v) NaCl and this trait does not only identify the isolates as belonging to the genus *Enterococcus* but also differentiates them from organisms belonging to the genus *Streptococcus*. All the isolates were gamma haemolytic on sheep blood agar. The isolates that satisfied these preliminary identification tests were further processed with *Enterococcus* specific confirmatory tests that included serotyping, PCR analysis and *ddl E. faecalis* as well as *ddl E. faecium* sequence analysis.

Table 4.1: The total number of bacterial counts from ground and surface water samples

Sample	FCC	TCC	Sample	FCC	TCC	Sample	FCC	TCC
SEWW1	0	88	SETD4	6	144	PELD3	2	146
SEWW3	0	166	SETD5	4	43	PELD4	4	134
SEWW4	0	42	SETD6	0	24	PELD5	0	86
SEWW5	2	152	LOTD1	16	68	PELD6	4	116
SEWW6	0	4	LOTD2	22	136	SWTW1	0	18
SEWW7	0	22	LOTD3	0	12	COLD1	137	218
SEWW8	0	12	LOTD4	6	38	COLD2	152	163
MOTW2	0	48	LOTD5	4	33	COLD3	16	72
MOTW3	2	190	LOTD6	8	176	COLD4	8	34
MOTW7	8	28	STGW1	13	24	COLD5	0	21
MOTW8	2	46	STGW2	17	32	COLD6	4	33
MOTW9	0	84	MABD1	0	126	VNTW1	0	41
DISW3	0	31	MABD2	0	396	VNTW4	0	26
STLW1	0	4	MABD3	6	131	VYFW2	0	11
STLW3	0	14	MABD4	16	288	MOIR1	8	246
STLW5	0	2	MABD5	0	119	MOIR2	4	167
STLW6	0	6	MABD6	0	123	MOIR3	4	182
SIGW3	159	352	KLMD1	0	132	MOIR4	0	103
DIBW1	6	28	KLMD2	12	148	MOIR5	2	98
DIBW2	6	34	KLMD3	4	108	MOIR6	0	111
DIBW6	0	32	KSPW1	24	16	PORD1	0	144

DISD1	4	71	DKSW1	8	124	PORD2	0	76
DISD2	14	312	PELW1	8	6	PORD3	0	63
DISD3	0	36	PELW4	28	216	POTD1	104	108
DISD4	6	24	PELW5	0	4	POTD2	2	66
DISD5	4	16	SWKW5	0	22	POTD3	4	81
DISD6	0	44	SWKW6	4	2	MRTW2	0	6
SETD1	8	125	SWKW7	0	9	HRTR1	0	191
SETD2	10	168	PELD1	0	159	HRTR2	0	174
SETD3	0	92	PELD2	0	111	HRTR3	0	113

Legend: SEWW = Seweding water; MOTW = Motlhabeng water; DISW = Disaneng water; STLW = Setlopo water; SIGW = Signal Hill water; DIBW = Dibate water; DISD = Disaneng Dam; SETD = Setumo Dam; LOTD = Lotlamoreng Dam; STGW = Setlagole water; MABD = Marico-Bosveld Dam; KLMD = Klein-Maricopoort Dam; KSPW = Kareespruit water; DKSW = Dinokana Spring water; PELW = Pella water; SWKW = Silwerkrans water; PELD = Pella Dam; SWTW = Swartruggens water; COLD = Coligny Dam; VNTW = Ventersdorp water; MOIR = Mooi River; PORD = Poortjie Dam; POTD = Potchefstroom Dam; MRTW = Moretele water; HRTR = Harts River; FCC = Faecal coliform count (CFU/100 mL); TCC = Total coliform count (CFU/100 mL)

Table 4.2: Biochemical and confirmatory identification tests of the environmental isolates

Sample	Gram staining (+ve coccus)	Oxidase test (-ve)	Catalase test (-ve)	Growth on 6.5% NaCl Broth	Haemolysis on Blood Agar (γ)	Serotyping (No. +ve)
Seweding (NT=6)	6	6	6	6	6	6
Motlhabeng (NT=7)	7	7	7	7	7	7
Setlopo (NT=7)	7	7	7	7	7	7
Dibate (NT=5)	5	5	5	5	5	5
Disaneng Dam (NT=6)	6	6	6	6	6	6
Setumo Dam (NT=6)	6	6	6	6	6	6
Lotlamoreng Dam (NT=6)	6	6	6	6	6	6
Setlagole (NT=4)	4	4	4	4	4	4
Marico-Bosveld Dam (NT=6)	6	6	6	6	6	6
Klein-Maricopoort Dam (NT=3)	3	3	3	3	3	3
Kareespruit (NT=2)	2	2	2	2	2	2
Dinokana (NT=6)	6	6	6	6	6	6
Dinokana Spring (NT=3)	3	3	3	3	3	3
Pella (NT=3)	3	3	3	3	3	3
Silwerkrans (NT=3)	3	3	3	3	3	3
Pella Dam (NT=6)	6	6	6	6	6	6
Swartruggens (NT=3)	3	3	3	3	3	3
Coligny (NT=7)	7	7	7	7	7	7
Coligny (NT=6)	6	6	6	6	6	6
Ventersdorp (NT=4)	4	4	4	4	4	4
Vyfhoek (NT=3)	3	3	3	3	3	3
Potchefstroom (NT=1)	1	1	1	1	1	1
Potchefstroom Dam (NT=3)	3	3	3	3	3	3
Mooi River (NT=6)	6	6	6	6	6	6
Poortjie Dam (NT=3)	3	3	3	3	3	3
Moretele (NT=1)	1	1	1	1	1	1

Harts River (NT=3)	3	3	3	3	3	3
Total No of environmental strains positive for test	119	119	119	119	119	119
***E. faecium* (+ve Control)**	1	1	1	1	1	1
***E. faecalis* (+ve Control)**	1	1	1	1	1	1

Legend: NT= Number tested

4.3 DNA extracted from *Enterococcus* isolates

DNA was extracted from all the 119 isolates including *E. faecalis* (ATCC 29212) and *E. faecium* (ATCC 6569) control strains as described in Materials and Methods (Section 3.3.3). The presence of DNA was confirmed by electrophoresis and Figure 4.1 indicates a 1% (w/v) agarose gel of DNA extracted from the isolates as well as the control strains.

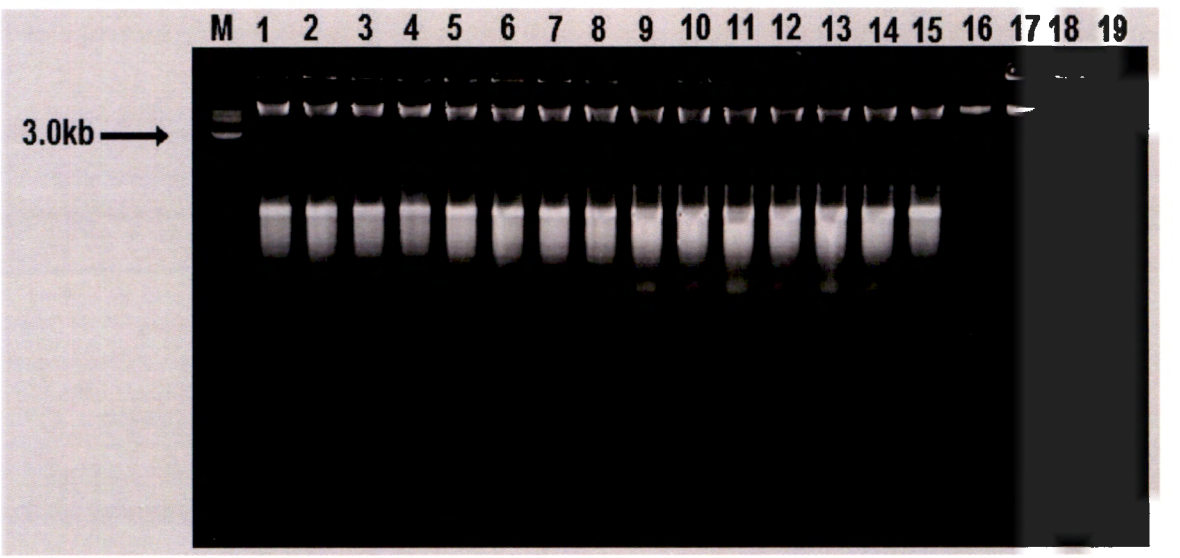

Figure 4.1: Agarose gel (1% w/v) image showing DNA extracted from Enterococcal isolates and control strains. Lane M= DNA marker (O'GeneRuler 1kb DNA ladder), lane (1, 2) = DNA extracted from (*E. faecalis* ATCC 29212 and *E. faecium* ATCC 6569, and lane 3-19= DNA extracted from Enterococcal isolates.

4.4 PCR analysis for confirming the identities of *Enterococcus* species

PCR analysis using *Enterococcus* species-specific PCR primers for *E. faecalis* and *E. faecium* designed to amplify the *ddl* genes encoding d-alanine:d-alanine ligases was carried out on all the 119 presumptive enterococcal isolates. A total of 16(13.4%) of the isolates were positively identified as *E. Faecalis* while a larger proportion 50 (42%) were *E. faecium* isolates. Figures 4.2 and 4.3 indicate the *ddl E. faecium* (650bp) and the *ddl E. faecalis* (900bp) gene fragments respectively that were amplified from the isolates.

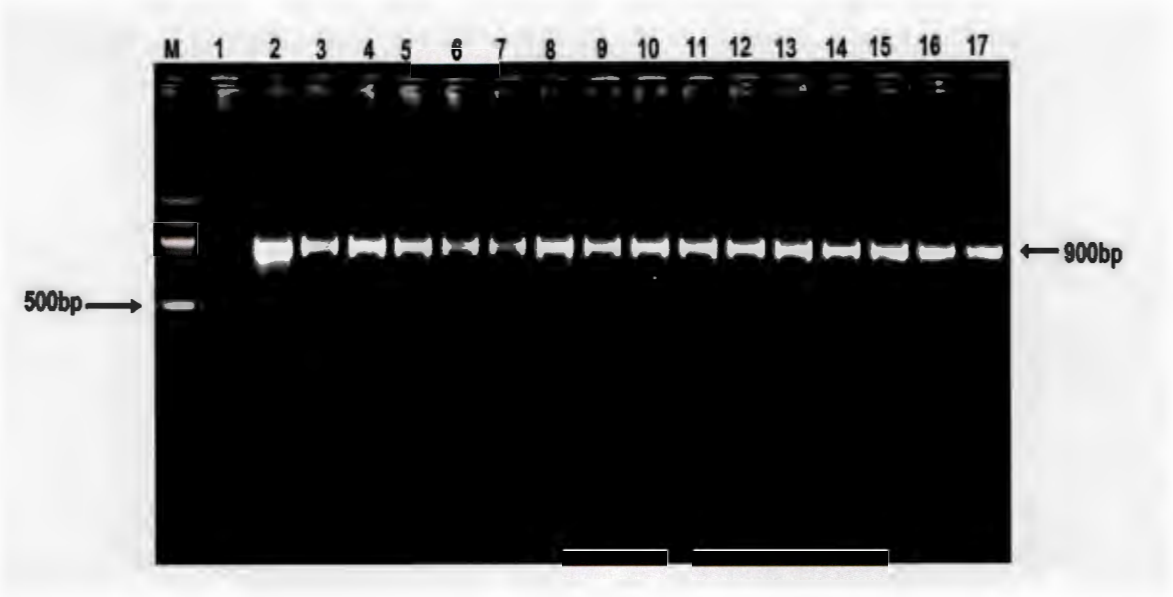

Figure 4.2: Agarose Gel Electrophoresis of PCR products for the detection of *ddlE E. faecalis* gene in enterococcal isolates. Lane M=100 bp DNA Ladder (Molecular marker), lane 1= negative control, lane2= positive control, and lanes 3-18 refer to the enterococcal isolates' number

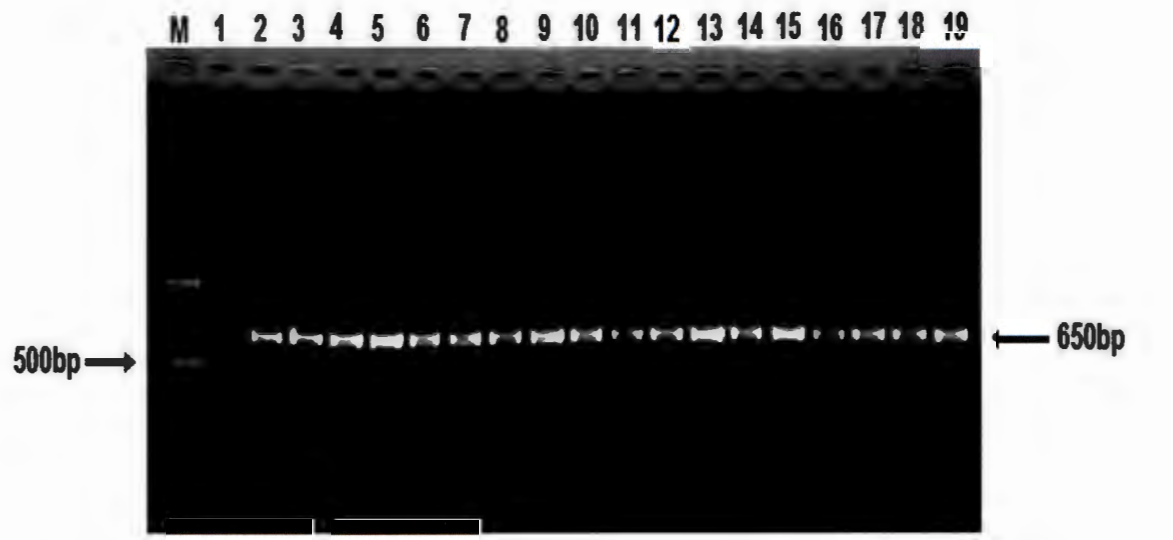

Figure 4.3: Agarose Gel Electrophoresis of PCR products for the detection of *ddlE E. faecium* gene in enterococcal isolates. Lane M=100 bp DNA Ladder (molecular marker), lane 1= negative control, lane2= positive control, and lanes 3-19 refer to the enterococcal isolates' number.

4.5 Enterococcus species specific ddl sequence analysis

The *E. faecium*and *E. faecalis ddl* gene fragments were sequenced and blast searched. The search revealed that the isolates were correctly identified as *E. faecium* with percentage similarities of 83 to 99% while *E. faecalis* isolates had percentage similarities of 98% with sequences previously deposited in GeneBank. Table 4.3 indicates the identities, accession numbers and percentage similarities of representative isolates that were sequenced.

Table 4.3: Genbank nucleotide sequence analysis

Isolate ID	Description	Accession Number	% similarity
SWEFAE3B	*Enterococcus faecium*	NC 017960.1	99%
PLEFAE5A	*Enterococcus faecium*	NC 017960.1	94%
DBEFAE1B	*Enterococcus faecium*	NC 017960.1	99%
DDEFAED	*Enterococcus faecium*	NC 017960.1	83%
CDEFAEB	*Enterococcus faecium*	NC 017960.1	99%
STEFEC1B	*Enterococcus faecalis*	NC 004668.1	98%
STEFEC2B	*Enterococcus faecalis*	NC 004668.1	98%
STEFEC3B	*Enterococcus faecalis*	NC 004668.1	98%
MDEFECB	*Enterococcus faecalis*	NC 004668.1	98%
SDEFECB	*Enterococcus faecalis*	NC 004668.1	98%

4.6 Antibiotic susceptibility profiles of isolates

The antibiotic susceptibility profiles of 66 confirmed *Enterococcus* isolates was determined using the disc diffusion assay (Kirby et al., 1966). All the isolates in this study were resistant to erythromycin while large proportions (62.1% to 97%) of these isolates were resistant to ampicillin, gentamycin, tetracycline and chloramphenicol (Table 4.4). On the contrary, a small proportion 27 (41%) of the isolates were resistant to vancomycin while only 12 (18.2%) were resistant to amoxycillin.

Table 4.4: Isolates from the different stations that were resistant to the antibiotics tested

Source	Sampling station	Ampicillin (10µg)	Amoxycillin (10µg)	Erythromycin (15µg)	Tetracycline (30µg)	Vancomycin (30µg)	Chloramphenicol (30µg)	Gentamycin (10µg)
Ground water	**Seweding (NT=6)**	**6**	**0**	**6**	**6**	**1**	**6**	**6**
	Motlhabeng (NT=1)	1	0	1	1	0	1	1
	Dibate (NT=5)	**5**	**0**	**5**	**5**	**1**	**5**	**2**
	Setlopo (NT=7)	0	0	7	3	3	7	0
	Pella (NT=1)	**1**	**0**	**1**	**1**	**0**	**1**	**1**
	Setlagole (NT=3)	3	0	3	3	1	3	3
	Silwerkrans (NT=3)	**2**	**0**	**2**	**2**	**1**	**2**	**2**
	Moretele (NT=1)	1	1	1	0	0	1	0
	Ventersdorp (NT=4)	**4**	**3**	**4**	**4**	**2**	**2**	**2**
Surface water	Disaneng Dam (NT=5)	0	0	5	5	3	5	5
	Setumo Dam (NT=2)	**0**	**0**	**2**	**0**	**0**	**2**	**0**
	Lothlamoreng Dam (NT=5)	3	1	5	5	4	5	5
	Klein-Maricopoort Dam (NT=3)	**2**	**0**	**3**	**3**	**0**	**3**	**3**
	Marico-Bosveld Dam (NT=6)	0	0	6	3	0	6	0
	Pella Dam (NT=4)	**3**	**0**	**4**	**4**	**0**	**4**	**4**
	Coligny Dam (NT=6)	6	5	6	6	6	6	6
	Mooi River (NT=3)	**3**	**1**	**3**	**3**	**3**	**3**	**3**
	Poortjies Dam (NT=1)	0	0	1	0	1	1	0
	Harts River (NT=1)	**1**	**1**	**1**	**1**	**1**	**1**	**1**
	Total (NT=66)	41 (62.1%)	12 (18.2%)	66 (100%)	55 (83.3%)	27 (41%)	64 (97%)	44 (67%)

4.7Cluster analysis of *E. faecium* and *E. faecalis* based on antibiotic inhibition zone diameter data

A total of 66 isolates comprising of 50 *E. faecium* and 16 *E. faecalis* based on antibiotic inhibition zones had cluster analysis performed on them using Statistica version 10.0 (Statsoft, US).Out of the 66 isolates analysed two major clusters (Cluster 1 and Cluster 2) were generated (Figure 4.4). Cluster 1 was subdivided into two sub-clusters (Cluster 1A and Cluster 1B). Furthermore, sub-clusters 1A and 1B, including Cluster 2, were analysed for patterns of association of isolates from different sources and/or locations (Table 4.5).

As indicated in Figure 4.4, the largest sub-cluster (sub-cluster 1B1) contained isolates from three sampling areas. The largest proportion (58.8%) of isolates in this sub-cluster was derived from surface water samples in Mafikeng. In addition, small proportions (17.6% and 23.5%) of the isolates in this sub-cluster were obtained from surface water in Rustenburg and Zeerust respectively. The second largest sub-cluster (sub-cluster Cluster 1A1) contained 12 (18.2%) of the isolates analysed. The makeup of isolates in this cluster consisted of 3, 4 and 5 isolates from groundwater obtained in Rustenburg, Ventersdorp and Mafikeng respectively. Interestingly, all (8) the isolates in sub-clusters 1A2 were obtained from groundwater in Mafikeng while all (6) clustered in sub-cluster 1B2 were obtained from surface water in Coligny. These findings indicated that the isolates that clustered together shared similar antibiotic resistance profiles which may have resulted from similarities in the degree of exposure to these drugs. However, similarities in the antibiotic resistance profiles may have also resulted through cross contamination.

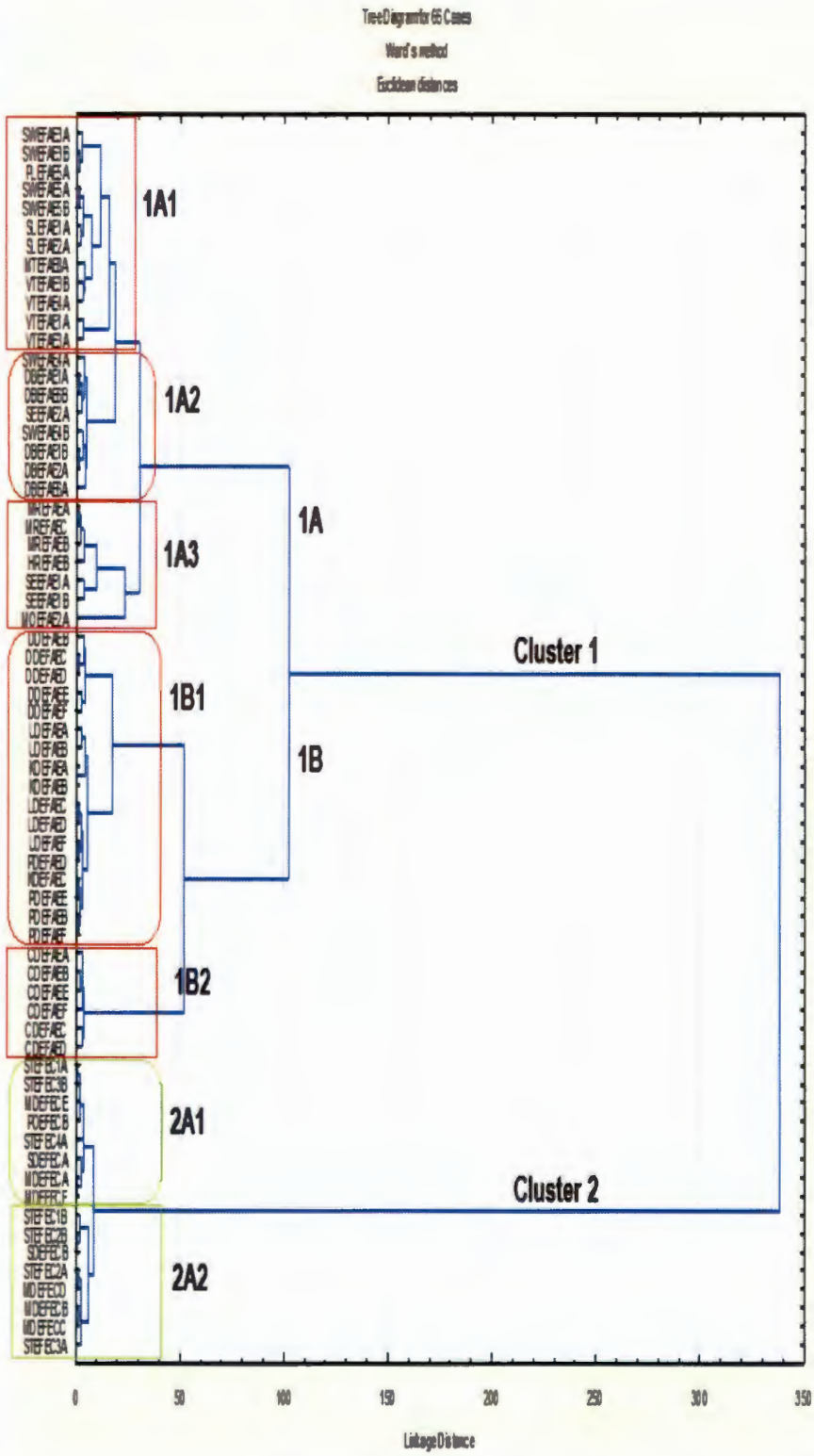

Figure 4.4: Dendrogram showing the relationship between isolates obtained from ground and surface water samples based on antimicrobial inhibition zone diameter data.

Table 4.5: Percentage representation of *E. faecium* and *E. faecalis* isolated from various areas and/or sources within different clusters

Sampling Area/Station	Type of samples	Cluster 1A1 *n*=12	Cluster 1A2 *n*=8	Cluster 1A3 *n*=7	Cluster 1B1 *n*=17	Cluster 1B2 *n*=6	Cluster 2A1 *n*=8	Cluster 2A2 *n*=8
Rustenburg	Ground water	**3**	**0**	**0**	**0**	**0**	**0**	
	Surface water	0	0	0	**4**	0	0	
Swartruggens	Ground water	**0**	**0**	**0**	**0**	**0**	**0**	
Potchefstroom	Ground water	0	0	0	0	0	0	
	Surface water	0	0	**3**	0	0	**1**	
Ventersdorp	Ground water	**4**	0	0	0	0	0	
Taung	Ground water	**0**	**0**	**1**	**0**	**0**	**0**	
	Surface water	0	0	**1**	0	0	0	
Lichtenburg	Ground water	**0**	**0**	**0**	**0**	**0**	**0**	
Coligny	Ground water	0	0	0	0	0	0	
	Surface water	0	0	0	0	**6**	0	
Mafikeng	Ground water	**5**	**8**	**2**	0	0	**3**	**4**
	Surface water	**0**	**0**	**0**	**10**	**0**	**1**	**1**
Setlagole	Ground water	0	0	0	0	0	0	
Zeerust	Ground water	**0**	**0**	**0**	**0**	**0**	**0**	
	Surface water	0	0	0	**3**	0	**3**	**3**

4.8 Morphological characterization of phages

Lytic bacteriophages were isolated from both ground and surface water samples analysed using environmental *Enterococcus* strains obtained in the study as well as the *Enterococcus faecalis* (ATCC 29212) and *Enterococcus faecium* (ATCC 6569) as the host strains. Plaques were detected on the agar and they possessed distinct morphologies that ranged from small to large. Figure 4.5A shows plaques with complete lysis producing larger plaques while Figures 4.5B has numerous plaques with uniform 'pin-hole' morphologies. Despite the fact that the plaques were generally small, they were clear and readable. In some samples, the phages were larger (usually greater than 1 mm in diameter) producing a halo formation around the plaque. The numbers of plaques obtained from the different samples are shown in Table 4.6. Despite the fact that phages were detected in all the samples analysed, water samples obtained from Lotlamoreng Dam and Marico-Bosveld Dam B had very high concentrations of the phages when compared to water from boreholes. This was not surprising since water from the dams is usually more contaminated and hence phages are known to be present where potential bacterial host strains are present. It was also identified that *Enterococcus* species were isolated in all these samples.

4.9 Characterization of phages using transmission electron microscopy

In order to determine the morphological diversity of the phages that infected the environmental *Enterococcus* strains obtained in the study as well as *Enterococcus faecalis* (ATCC 29212) and *Enterococcus faecium* (ATCC 6569) control strains a total of nine phage lysates were observed using transmission electron microscopy (TEM). The TEM phage micrographs of the three distinct phage morphologies were obtained in the study (Figure 4.6). The TEM data revealed that a large proportion of the phages had helical tails which may be classified as belonging to the order *Caudovirales* that consist of three families (*Myoviridae*, *Siphoviridae* and *Podoviridae*). However, some of the phages did not possess tails. Despite this it was observed in some micrographs that the heads of the phages had

disintegrated from the rest of the structure. In addition, capsid morphologies of the phages were icosahedral in shape.

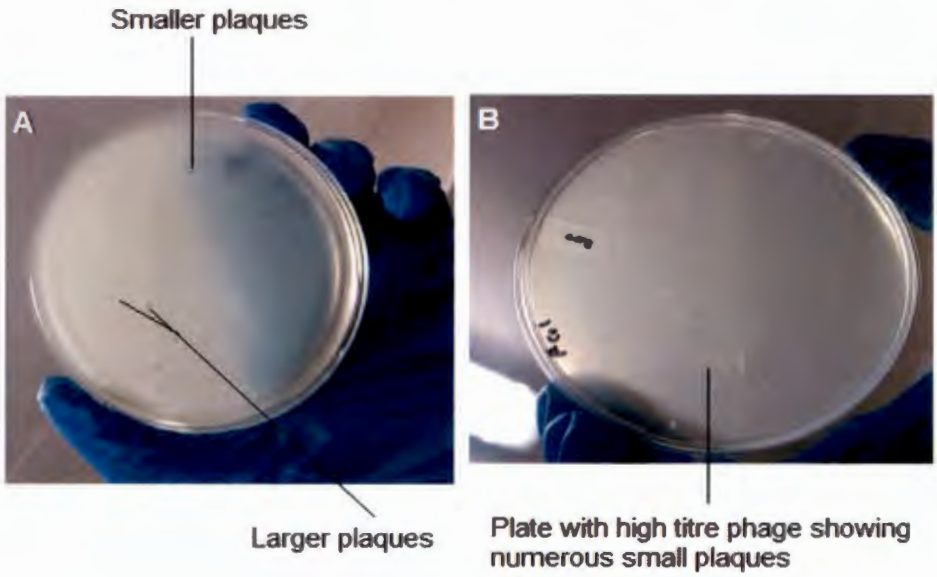

Figure 4.5: Plaques with diverse morphologies on Modified Nutrient agar plates.

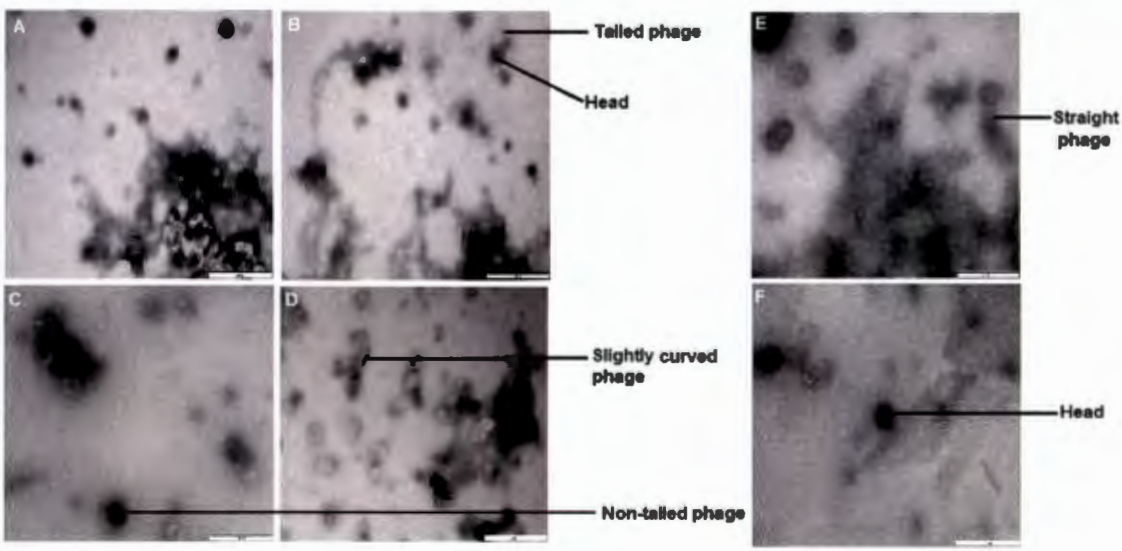

Figure 4.6: Micrographs of stained *Enterococcus* specific phages showing tailed and non-tailed morphologies (bar=200 nm).

Table 4.6: Plaque assay results for *Enterococcus* species

Samples	Results on Plaque Assay (Average number of plaques on the plates)			Total number of plaques per sample
	10^0	10^{-1}	10^{-3}	
Seweding 1	17	11	4	32
Seweding 2	19	10	6	35
Motlhabeng	20	14	5	39
Silverkraans	13	9	6	28
Lotlamoreng Dam	86	24	9	**119**
Setlopo 1	22	8	7	37
Setlopo 2	17	12	5	34
Marico-Bosveld Dam A	34	15	5	54
Marico-Bosveld Dam B	46	19	7	**72**
Negative Controls (A & B, Blank)	0 (NP) 0 (NP)	0 (NP) 0 (NP)	0 (NP) 0 (NP)	0

4.10Host range and lytic capabilityphages using virulence assay

Two lytic phages were subjected to the phage virulence assay. The results were recorded in Tables 4.7 and 4.8.The phages lysed the *Enterococcus* species that were used in the analysis. The positive result of this was represented by the fact that the mixture of *Enterococcus* species-*Enterococcus* specific phages in a TSB broth did become clear as the normal broth after 5 hours of incubation under 37°C aerobically.

Table 4.7: Virulence assay result for *E. faecium* phage stock filtrates

	Seweding1	Seweding2	Motlhabeng	Silverkraans	Lotlamoreng Dam	Blank
A	S	S	S	S	S	-
B	S	S	S	S	S	-
C	S	S	S	S	S	-
D	S	S	S	R	S	-
E	S	S	S	R	S	-
F	S	S	S	R	S	-
G	S	S	S	R	S	-
H	S	S	S	R	S	-

Legend: - = Blank (No bacteria was added); S= bacteria was lysed; R=bacteria was not lysed

Table 4.8: Virulence assay result for *E. faecalis* phage stock filtrates

	Setlopo1	Setlopo2	Marico-BosVeld Dam A	Marico-BosVeld Dam B	Blank
A	S	S	S	S	-
B	S	S	S	S	-
C	S	S	S	S	-
D	S	S	S	S	-
E	S	S	S	S	-
F	S	S	S	S	-
G	S	S	S	S	-
H	S	S	S	S	-

Legend: - = Blank (No bacteria was added); S = bacteria was lysed; R=bacteria was not lysed

CHAPTER FIVE

DICUSSION AND CONCLUSION

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

The primary objective of the study was to isolate and identify the phages specific to *Enterococcus faecalis* and *Enterococcus faecium* from ground water and surface water samples collected from randomly selected areas in the North West Province. This was motivated by the fact that previous baseline investigations in the study area revealed the presence of enterococci in groundwater that was intended for human consumption (Ateba and Maribeng, 2011; Ateba et al., 2013) and leafy vegetables (Mohapi and Ateba, 2013). In addition, a number of studies have indicated that enterococci may be used as microbial indicators of faecal pollution in water (Wheeler et al., 2002; Byappanahalli et al., 2012). In the present study *E. faecium* and *E. faecalis* were successfully isolated from both ground and surface water. A total of 57 out of 162 samples were positive for enterococci based colonial morphologies while large proportions (55%) possessed coliform bacteria. Presumptive identification of the isolates was achieved using Gram staining, catalase test, oxidase test, growth on 6.5% (w/v) NaCl and the production of gamma haemolytic patterns on sheep blood agar. Similar to the findings of this study, a number of studies have revealed that *Enterococcus* species can provisionally be identified on the basis of the Gram reaction, cell arrangement, catalase test, the presence of group D antigen by serological assays and a positive reaction (black colonies) on bile esculin agar (Facklam and Sahm, 1995; Facklam et al., 2002).

Despite this it has been reported that the identification and differentiation of enterococci belonging to different species such as *E. faecium*, *E. gallinarum*, *E. durans*, *E. avium*, *E. raffinosus*, *E. hirae*, and *E. mundtii* was limiting and may not produce reliable results (Willey et al., 1999; d'Azevedo et al., 2001). In addition, most biochemical identification assays are laborious, time consuming and have serious limitations when used on environmental isolates. Against this background, molecular based techniques

that involve the amplification of species specific sequences in bacterial DNA are considered gold standard approaches for confirming the identities of isolates (Mannu and Paba, 2002; Marino et al., 2003; Delgado and Mayo, 2004) .In the present study, *E. faecalis* and *E. faecium* species specific primers designed to amplify the *ddl* genes encoding d-alanine:d-alanine ligases in enterococci were used and a total of 16(13.4%) and 50 (42%) *E. faecalis* and *E. faecium* isolates respectively were detected. Similar observations have been reported (Angeletti et al., 2001).

Another objective of the study was to determine the antimicrobial resistance profiles of enterococci obtained in the study. All the isolates in this study were resistant to erythromycin. These findings are different from those obtained in previous studies in which only small proportions 9 (15.3%) and 6 (15%) of *E. faecalis* and *E. faecium* respectively were resistant to erythromycin (Santos et al., 2013). Moreover, in this study *ermB* gene that codes for resistance to erythromycin was frequently detected in the isolates (Barreto et al., 2009). On the contrary, large proportions (93.7% to 100%) of the isolates were resistant to erythromycin (Ateba and Maribeng, 2011). Given that there are wide variety of genes that code for resistance to these antibiotic as well as different mechanisms of resistance to an antimicrobial agent there is a need for continuous monitoring. Large proportions (62.1% to 97%) of these isolates were resistant to ampicillin, gentamycin, tetracycline and chloramphenicol. Results obtained in the present study contradict those of a previous study in which very small proportions (0% - 2.8%)of *E. faecalis* and *E. faecium* isolated from water were resistant to ampicillin (Castillo-Rojas et al., 2013). In addition, 17.2% and 25% of *E. faecalis* and *E. faecium* respectively were resistant to tetracycline (Castillo-Rojas et al., 2013). On the contrary the isolates obtained from clinical samples in the study by Castillo-Rojas et al. (2013) were highly resistant (57.1% - 78.6%) to ampicillin and tetracycline. However, in a previous study that was conducted in the Mafkeng area very small proportions (9.2% to 18.7%) of the isolates were resistant to ampicillin while large proportions (93.7) of these isolates were resistant to tetracycline (Ateba and Maribeng, 2011).

Small proportions (18.2% - 41%) of the isolates obtained in the present study were resistant to vancomycin and amoxycillin. This was contrary to the findings of a previous study in which up to 75% and 100% of the isolates from groundwater were resistant to vancomycin and amoxycillin respectively (Ateba and Maribeng, 2011). Similarly, very large proportions (77.8% to 100%) of the isolates obtained from groundwater samples in another study were resistant to vancomycin and amoxycillin (Ateba et al., 2013). The detection of vancomycin resistant enterococci was a cause for concern since these isolates may pose severe public health threats in humans.

A further objective of the study was to isolate and determine the morphologies of *E. faecalis* and *E. faecium* specific bacteriophages from ground and surface water samples. Over the years, coliform bacterial species and members belonging to the genus *Enterococcus* have been used as indicators of faecal contaminants of water (Cabelli et al., 1982; Scott et al., 2002; Byappanahalli et al., 2012). However, results obtained when bacteria are used may be unreliable and thus generated renewed interest in the search for more reliable indicators of faecal pollution in water. This is based on the fact that all bacteria species that are used to detect faecal contamination in water fail to fulfill the criteria of an ideal microbial indicator (Baele et al., 2002; Cox and Gilmore, 2007; Bonilla et al., 2010). In addition bacterial indicator organisms that are currently utilized in assessing water quality have been found to be highly susceptible to chlorine and hence may provide false negative results (Tavakoli et al., 2005). Against this background, a number of studies have assessed the quality of water using bacteriophages as indicators organisms (Grabow et al., 1993; Moe, 1996; Puig et al., 1999; Wheeler et al., 2002).

Enteric viruses resist most water treatment procedures including chlorination and are therefore considered to be a new and reliable group of indicator organisms that can be used to assess human faecal contamination in water (Keswick et al., 1985; Mocé-Llivina et al., 2005). Bacteriophages that

specifically infect *Enterococcus* species particularly *Enterococcus faecalis* are termed enterophages and are more frequently isolated from recreational waters. The morphology of the enterophages isolated in the current study is very similar to viruses belonging to the family *Siphoviridae* which are phages with tails but possessing icosahedral capsids. On the contrary, phages with bigger capsids and longer tails have also been isolates using *Enterococcus* species and characterised to be members of the family *Siphoviridae* (Bonilla et al., 2010).

In the present study, faecal and total coliform bacteria, *Enterococcus* species as well as *Enterococcus* specific bacteriophages were isolated from water samples. It was identified that samples that were positive for *Enterococcus* species specific phages also contained both faecal and total coliforms. From these results it was suggested that *Enterococcus* species specific phages may be very suitable and more reliable for use as indicators of the presence of faecal microbial contaminants in water. *Enterococcus* species and Enterophages have also been reported to serve as markers of human faecal contamination in water systems (Simkova and Cervenka, 1981; Grabow and Coubrough, 1986; Toranzos et al., 1996; Cole et al., 2003; Santiago-Rodriguez, 2013). Given the public health issues associated with water quality assessments and the need to have suitable indicators that will produce reliable results more studies that are aimed at evaluating the potential of bacteriophages as indicators of water quality assessment schemes should be conducted.

TEM data obtained for the phages during the study indicated that the majority of the phages showed long simple non-contractile tails and may belong to the family *Siphoviridae*. Similar findings had been reported in which *Enterococcus* specific phages most often belong to the *Siphoviridae* family were detected (Ackermann, 2007). Despite the fact that *Enterococcus* specific phages obtained during the present study were not identified using molecular assays, the plaque assay was able to detect the presence of enterophages in the water samples in other studies (Grabow, 2001; Bonilla et al., 2010). It

is therefore suggested that these phages be characterized fully later using genotypic/molecular techniques.

5.2 Conclusion and Recommendations

The present study was designed to isolate *Enterococcus faecalis* and *Enterococcus faecium* species as well as their specific phages and assess their potential for use as indicators for detecting the presence of faecal microbial contaminants in water. *Enterococcus* species and *Enterococcus* species specific bacteriophages were successfully isolated from the ground and surface water samples analyzed in the study. In addition, the antibiotic resistance profiles of the bacterial isolates indicated that a large proportion were multi-drug resistant to the antibiotics analyzed. A cause for concern was the presence of isolates that were resistant to vancomycin despite the fact that this drug is not used in both human and animal medicine. The morphologies of the bacteriophages were determined and phages were very similar to those belonging to the family *Siphoviridae*. In addition, the phages were able to lyse multiple antibiotic resistant *Enterococcus* species isolated during the study. From these findings it is recommended that the phages obtained in this study be characterized further using molecular typing tools.

REFERENCES

Ackermann, H.W. 2003. Bacteriophage observations and evolution. *Research in Microbiology,* 154, 245-251.

Ackermann, H.W. 2007. 5500 Phages examined in the electron microscope. *Archives of Virology,* 152, 227-243.

Ackermann, H.W. 2009. Phage classification and characterization. Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions. 127-140.

Ackermann, H.W., Tremblay, D., Moineau, S. 2004. Long-term bacteriophage preservation. *WFCC Newsletter,* 38, 35-40.

Anderson, K.L., Whitlock, J.E., Harwood, V.J. 2005. Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. *Applied and Environmental Microbiology,* 71, 3041-3048.

Angeletti, S., Lorino, G., Gherardi, G., Battistoni, F., De Cesaris, M., Dicuonzo, G. 2001. Routine molecular identification of enterococci by gene-specific PCR and 16S ribosomal DNA sequencing. *Journal of Clinical Microbiology,* 39, 794-797.

APHA 1998. Standard Methods for the Examination of Water and Wastewater, 20th Edition, Washington, D.C. American Public Health Association.

Arias, C.A., Panesso, D., Singh, K.V., Rice, L.B., Murray, B.E. 2009. Cotransfer of antibiotic resistance genes and a hylEfm-containing virulence plasmid in Enterococcus faecium. *Antimicrobial Agents and Chemotherapy,* 53, 4240-4246.

Ateba, C., Mbewe, M., Bezuidenhout, C. 2008. Prevalence of *Escherichia coli* O157 strains in cattle, pigs and humans in North West province, South Africa. *South African Journal of Science,* 104, 7-8.

Ateba, C.N., Lekoma, K.P., Kawadza, D.T. 2013. Detection of *vanA* and *vanB* genes in vancomycin-resistant enterococci (VRE) from groundwater using multiplex PCR analysis. *Journal of Water and Health,* 11, 684-691.

Ateba, C.N., Maribeng, M.D. 2011. Detection of *Enterococcus* species in groundwater from some rural communities in the Mmabatho area, South Africa: A risk analysis. *African Journal of Microbiology Research,* 5, 3930-3935.

Ateba, C.N., Mbewe, M. 2014. Genotypic Characterization of *Escherichia coli* O157: H7 Isolates from Different Sources in the North-West Province, South Africa, Using Enterobacterial Repetitive Intergenic Consensus PCR Analysis. *International Journal of Molecular Sciences,* 15, 9735-9747.

Baele, M., Devriese, L., Butaye, P., Haesebrouck, F. 2002. Composition of enterococcal and streptococcal flora from pigeon intestines. *Journal of Applied Microbiology,* 92, 348-351.

Barreto, Â., Guimarães, B., Radhouani, H., Araújo, C., Gonçalves, A., Gaspar, E., Rodrigues, J., Igrejas, G., Poeta, P. 2009. Detection of antibiotic resistant *Escherichia coli* and Enterococcus species in stool of healthy growing children in Portugal. *Journal of Basic Microbiology,* 49, 503-512.

Bonilla, N., Santiago, T., Marcos, P., Urdaneta, M., Domingo, J.S., Toranzos, G. 2010. Enterophages, a group of phages infecting *Enterococcus faecalis*, and their potential as alternate indicators of human faecal contamination. *Water Science and Technology,* 61, 293-300.

Brenner, D., Davis, B., Steigerwalt, A., Riddle, C., McWhorter, A., Allen, S., Farmer, J., Saitoh, Y., Fanning, G. 1982. Atypical biogroups of *Escherichia coli* found in clinical specimens and description of *Escherichia hermannii* sp. nov. *Journal of Clinical Microbiology,* 15, 703-713.

Bruhn, L., Wolfson, L. 2007. Citizens Monitoring Bacteria: A Training Manual for Monitoring *Escherichia coli*.

Brüssow, H., Canchaya, C., Hardt, W.-D. 2004. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiology and Molecular Biology Reviews,* 68, 560-602.

Byappanahalli, M.N., Nevers, M.B., Korajkic, A., Staley, Z.R., Harwood, V.J. 2012. Enterococci in the environment. *Microbiology and Molecular Biology Reviews,* 76, 685-706.

Cabelli, V.J., Dufour, A.P., McCabe, L., Levin, M. 1982. Swimming-associated gastoenteritis and water quality1. *American Journal of Epidemiology,* 115, 606-616.

Cabral, J.P. 2010. Water microbiology. Bacterial pathogens and water. *International Journal of Environmental Research and Public Health,* 7, 3657-3703.

Camargo, J., Alonso, A. 2007. Contaminación por nitrógeno inorgánico en los ecosistemas acuáticos: problemas medioambientales, criterios de calidad del agua, e implicaciones del cambio climático. *Revista Ecosistemas,* 16, 98-110.

Carrero-Colón, M., Wickham, G.S., Turco, R.F. 2011. Taxonomy, phylogeny, and physiology of fecal indicator bacteria. The Fecal Bacteria. *American Society of Microbiology*, 23-38.

Castillo-Rojas, G., Mazari-Hiríart, M., de León, S.P., Amieva-Fernández, R.I., Agis-Juárez, R.A., Huebner, J., López-Vidal, Y. 2013. Comparison of *Enterococcus faecium* and *Enterococcus faecalis* strains isolated from water and clinical samples: Antimicrobial susceptibility and genetic relationships. *PloS One,* 8, e59491.

Charan, J., Biswas, T. 2013. How to calculate sample size for different study designs in medical research? *Indian Journal of Psychological Medicine,* 35, 121.

Chavers, L., Moser, S., Benjamin, W., Banks, S., Steinhauer, J., Smith, A., Johnson, C., Funkhouser, E., Chavers, L., Stamm, A. 2003. Vancomycin-resistant enterococci: 15 years and counting. *Journal of Hospital Infection,* 53, 159-171.

Chou, Y.Y., Lin, T.Y., Lin, J.C., Wang, N.C., Peng, M.Y., Chang, F.Y. 2008. Vancomycin-resistant enterococcal bacteremia: comparison of clinical features and outcome between *Enterococcus*

faecium and *Enterococcus faecalis*. *Journal of Microbiology, Immunology and Infection,* 41, 124-129.

Clokie, M.R., Millard, A.D., Letarov, A.V., Heaphy, S. 2011. Phages in nature. *Bacteriophage,* 1, 31-45.

Cole, D., Long, S.C., Sobsey, M.D. 2003. Evaluation of F+ RNA and DNA coliphages as source-specific indicators of fecal contamination in surface waters. *Applied and Environmental Microbiology,* 69, 6507-6514.

Cox, C.R., Gilmore, M.S. 2007. Native microbial colonization of Drosophila melanogaster and its use as a model of *Enterococcus faecalis* pathogenesis. *Infection and Immunity,* 75, 1565-1576.

Cruickshank, R., Duguid, J., Marmoin, B., Swain, R. 1975. Medical Microbiology, 12th Edition, New York. Longman Group Limited 2, 34.

d'Azevedo, P.A., Dias, C.c.A., Gonçalves, A.L.S., Rowe, F., Teixeira, L.M. 2001. Evaluation of an automated system for the identification and antimicrobial susceptibility testing of enterococci. *Diagnostic Microbiology and Infectious Disease,* 40, 157-161.

Delgado, S., Mayo, B. 2004. Phenotypic and genetic diversity of *Lactococcus lactis* and *Enterococcus* species strains isolated from Northern Spain starter-free farmhouse cheeses. *International Journal of Food Microbiology,* 90, 309-319.

Deshpande, L.M., Fritsche, T.R., Moet, G.J., Biedenbach, D.J., Jones, R.N. 2007. Antimicrobial resistance and molecular epidemiology of vancomycin-resistant enterococci from North America and Europe: a report from the SENTRY antimicrobial surveillance program. *Diagnostic Microbiology and Infectious Disease,* 58, 163-170.

Díaz-Muñoz, S.L., Koskella, B. 2014. Bacteria-phage interactions in natural environments. *Advances in Applied Microbiology,* 89, 135-83.

Diston, D., Ebdon, J., Taylor, H. 2012. The effect of UV-C radiation (254 nm) on candidate microbial source tracking phages infecting a human-specific strain of Bacteroides fragilis (GB-124). *Journal of Water and Health,* 10, 262-270.

Duckworth, D.H. 1976. " Who discovered bacteriophage?". *Bacteriological Reviews,* 40, 793-802.

Duran, A., Muniesa, M., Mocé-Llivina, L., Campos, C., Jofre, J., Lucena, F. 2003. Usefulness of different groups of bacteriophages as model micro-organisms for evaluating chlorination. *Journal of Applied Microbiology,* 95, 29-37.

Dutilh, B.E., Cassman, N., McNair, K., Sanchez, S.E., Silva, G.G., Boling, L., Barr, J.J., Speth, D.R., Seguritan, V., Aziz, R.K. 2014. A highly abundant bacteriophage discovered in the unknown sequences of human faecal metagenomes. *Nature Communications,* 5.

Eaton, A., Clesceri, L., Greenberg, A. 1998. Standard methods for the examination of water and wastewater, 20th Edition, Washington, DC, American Public Health Association.

Edberg, S., Rice, E., Karlin, R., Allen, M. 2000. *Escherichia coli*: the best biological drinking water indicator for public health protection. *Journal of Applied Microbiology,* 88, 106S-116S.

Euzéby, J.P. 1997. List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. *International Journal of Systematic and Evolutionary Microbiology,* 47, 590-592.

Facklam, R. 1973. Comparison of several laboratory media for presumptive identification of enterococci and group D streptococci. *Applied Microbiology,* 26, 138-145.

Facklam, R.R., Maria da Gloria, S.C., Teixeira, L.M. 2002. History, taxonomy, biochemical characteristics, and antibiotic susceptibility testing of enterococci. *The Enterococci.* American Society of Microbiology.

Facklam, R.R., Sahm, D.F. 1995. *Enterococcus.* In Manual of Clinical Microbiology, 6th Edition. eds Murray, P.R., Baron, E.J., Pfaller, M.A., Tenover, F.C. and Yolken, R.H. Washington DC: ASM Press. 308-314.

Feng, Y.Y., Ong, S.L., Hu, J.Y., Tan, X.L., Ng, W.J. 2003. Effects of pH and temperature on the survival of coliphages MS2 and Qβ. *Journal of Industrial Microbiology and Biotechnology,* 30, 549-552.

Ferguson, C., Husman, A.M.d.R., Altavilla, N., Deere, D., Ashbolt, N. 2003. Fate and transport of surface water pathogens in watersheds. *Critical Reviews in Environmental Science and Technology,* 33, 299-361.

Fisher, K., Phillips, C. 2009. The ecology, epidemiology and virulence of *Enterococcus. Microbiology,* 155, 1749-1757.

Fong, T.-T., Lipp, E.K. 2005. Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. *Microbiology and Molecular Biology Reviews,* 69, 357-371.

Fricker, C., Eldred, B. 2009. Identification of coliform genera recovered from water using different technologies. *Letters in Applied Microbiology,* 49, 685-688.

Frost, L.S., Leplae, R., Summers, A.O., Toussaint, A. 2005. Mobile genetic elements: the agents of open source evolution. *Nature Reviews Microbiology,* 3, 722-732.

Fujioka, R., Sian-Denton, C., Borja, M., Castro, J., Morphew, K. 1999. Soil: the environmental source of *Escherichia coli* and enterococci in Guam's streams. *Journal of Applied Microbiology,* 85, 83S-89S.

Gambarotto, K., Ploy, M.-C., Turlure, P., Grélaud, C., Martin, C., Bordessoule, D., Denis, F. 2000. Prevalence of vancomycin-resistant enterococci in fecal samples from hospitalized patients and nonhospitalized controls in a cattle-rearing area of France. *Journal of Clinical Microbiology,* 38, 620-624.

Gantzer, C., Gillerman, L., Kuznetsov, M., Oron, G. 2001. Adsorption and survival of faecal coliforms, somatic coliphages and F-specific RNA phages in soil irrigated with wastewater. *Water Science and Technology,* 43, 117-124.

Geldreich, E.E. 1978. Bacterial populations and indicator concepts in feces, sewage, stormwater and solid wastes. *Indicators of Viruses in Water and Food,* 51-97.

Gelsomino, R., Vancanneyt, M., Cogan, T.M., Swings, J. 2003. Effect of raw-milk cheese consumption on the enterococcal flora of human feces. *Applied and Environmental Microbiology,* 69, 312-319.

Gold, H.S., Moellering Jr, R.C. 1996. Antimicrobial-drug resistance. *New England Journal of Medicine,* 335, 1445-1453.

Goyal, S.M., Gerba, C.P., Bitton, G. 1987. Phage Ecology. John Wiley and Sons, New York. 321.

Grabow, W. 1990. Microbiology of drinking water treatment: reclaimed wastewater. In: McFeters GA (Edition.) Drinking Water Microbiology - Progress and Recent Developments. Springer Verlag, New York, 185-203.

Grabow, W. 1996. Waterborne diseases: Update on water quality assessment and control. *Water SA,* 22, 193-202.

Grabow, W. 2001. Bacteriophages: update on application as models for viruses in water. *Water SA,* 27, 251-268.

Grabow, W., Coubrough, P. 1986. Practical direct plaque assay for coliphages in 100-ml samples of drinking water. *Applied and Environmental Microbiology,* 52, 430-433.

Grabow, W., Holtzhausen, C.S., De Villiers, J.C. 1993. Research on Bacteriophages as Indicators of Water Quality, 1 January 1990 to 31 December 1992: Final Report, Water Research Commission, 147.

Grabow, W., Vrey, A., Uys, M., De Villiers, J. 1998. Evaluation of the Application of Bacteriophages as Indicators of Water Quality. WRC Report No 540/1/98. Water Research Commission, Pretoria.

Green, J., Bruce, K., Conner, C., Mutesi, R. 2000. The APHA standard method for the enumeration of somatic coliphages in water has low efficiency of plating. *Water Research,* 34, 759-762.

Groth, A.C., Calos, M.P. 2004. Phage integrases: biology and applications. *Journal of Molecular Biology,* 335, 667-678.

Havelaar, A. 1993. Bacteriophages as models of enteric viruses in water treatment processes. *American Society for Microbiology News,* 59, 614-619.

Havelaar, A., Hogeboom, W. 1984. A method for the enumeration of male-specific bacteriophages in sewage. *Journal of Applied Bacteriology,* 56, 439-447.

Havelaar, A.H. 1986. *F-specific RNA bacteriophages as model viruses in water treatment processes.*

Hayes, J.R., English, L.L., Carr, L.E., Wagner, D.D., Joseph, S.W. 2004. Multiple-antibiotic resistance of *Enterococcus* species isolated from commercial poultry production environments. *Applied and Environmental Microbiology,* 70, 6005-6011.

Hayes, W. 1968. The Genetics of Bacteria and their Viruses (2nd Edition), Blackwell Scientific Publications: Oxford, UK.

Herrero, M., Maldonado May, V., Sardi, G., FLORES, F., Orlando, A., Carbó, L. 2000. Distribución de la calidad de agua subterránea en sistemas de producción agropecuarios bonaerenses, 2-Condiciones de manejo y grado de contaminación. *Prod. Animal,* 20, 237-245.

Hilton, M.C., Stotzky, G. 1973. Use of coliphages as indicators of water pollution. *Canadian Journal of Microbiology,* 19, 747-751.

Hollenbeck, B.L., Rice, L.B. 2012. Intrinsic and acquired resistance mechanisms in *Enterococcus*. *Virulence,* 3, 421-569.

Hurst, C.J. 1996. Overview of water microbiology as it relates to public health. In: Crawford, R.L. Editor. *Manual of Environmental Microbiology*, 219-222.

IAWPRC 1991. Bacteriophages as model viruses in water quality control. *Water Research* 25, 529-545.

Jagals, P., Traore, H.N., Barnard, T.G. 2006. *Inflammatory potential measurement as a supplement to health-related microbial water-quality assessment; Report No. TT1444/1/06*, Water Research Commission: Pretoria, South Africa.

Jean, S., Fang, C.T., Wang, H.K., Hsue, P., Chang, S.C., Luh, K.T. 2001. Invasive infections due to vancomycin-resistant enterococci in adult patients. *Journal of Microbiology, Immunology, and Infection= Wei mian yu gan ran za zhi,* 34, 281-286.

Jepson, C.D., March, J.B. 2004. Bacteriophage lambda is a highly stable DNA vaccine delivery vehicle. *Vaccine,* 22, 2413-2419.

Jett, B.D., Huycke, M.M., Gilmore, M.S. 1994. Virulence of enterococci. *Clinical Microbiology Reviews,* 7, 462-478.

Jofre, J., Bosch, A., Lucena, F., Girones, R., Tartera, C. 1986. Evaluation of Bacteroides fragilis bacteriophages as indicators of the virological quality of water. *Water Science and Technology,* 18, 167-173.

Jofre, J., Olle, E., Ribas, F., Vidal, A., Lucena, F. 1995. Potential usefulness of bacteriophages that infect Bacteroides fragilis as model organisms for monitoring virus removal in drinking water treatment plants. *Applied and Environmental Microbiology,* 61, 3227-3231.

Jofre, J., Stewart, J.R., Grabow, W. 2011. Phage methods. *Microbial Source Tracking: Methods, Applications, and Case Studies. Springer, New York, 137-156.*

Johnston, L.M., Jaykus, L.-A. 2004. Antimicrobial resistance of *Enterococcus* species isolated from produce. *Applied and Environmental Microbiology,* 70, 3133-3137.

Jończyk, E., Kłak, M., Międzybrodzki, R., Górski, A. 2011. The influence of external factors on bacteriophages—review. *Folia Microbiologica,* 56, 191-200.

Kanyerere, T., Levy, J., Xu, Y., Saka, J. 2012. Assessment of microbial contamination of groundwater in upper Limphasa River catchment, located in a rural area of northern Malawi. *Water SA,* 38, 581-596.

Kariyama, R., Mitsuhata, R., Chow, J.W., Clewell, D.B., Kumon, H. 2000. Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. *Journal of Clinical Microbiology,* 38, 3092-3095.

Katzeneleson, E., Lketter, B., Schechter, H., Shuval, H.I. 1974. Inactivation of viruses and bacteria by ozone. *In Chemistry of Water Supply, Treatment and Distribution; Rubin, A.J., Ed, Ann Arbor Science: Ann Arbor Michigan*, 409–421.

Kerr, B., West, J., Bohannan, B. 2008. Bacteriophages: models for exploring basic principles of ecology. *Bacteriophage ecology*, 31-63.

Keswick, B.H., Satterwhite, T.K., Johnson, P.C., DuPont, H.L., Secor, S.L., Bitsura, J.A., Gary, G.W., Hoff, J.C. 1985. Inactivation of Norwalk virus in drinking water by chlorine. *Applied and Environmental Microbiology,* 50, 261-264.

Kilonzo-Nthenge, A., Brown, A., Nahashon, S., Long, D. 2015. Occurrence and antimicrobial resistance of enterococci isolated from organic and conventional retail chicken. *Journal of Food Protection,* 78, 760-766.

Kinge, C.N.W., Ateba, C.N., Kawadza, D.T. 2010. Antibiotic resistance profiles of *Escherichia coli* isolated from different water sources in the Mmabatho locality, north-west province, South Africa. *South African Journal of Science,* 106, 44-49.

Kinzelman, J., Ng, C., Jackson, E., Gradus, S., Bagley, R. 2003. Enterococci as indicators of Lake Michigan recreational water quality: comparison of two methodologies and their impacts on public health regulatory events. *Applied and Environmental Microbiology,* 69, 92-96.

Kirby, W.M.M., Bauer, A.W., Sherris, J.C., Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology,* 45, 493.

Kott, Y., Roze, N., Sperber, S., Betzer, N. 1974. Bacteriophages as viral pollution indicators. *Water Research,* 8, 165-171.

Lasobras, J., Muniesa, M., Frias, J., Lucena, F., Jofre, J. 1997. Relationship between the morphology of bacteriophages and their persistence in the environment. *Water Science and Technology,* 35, 129-132.

Lavigne, R., Seto, D., Mahadevan, P., Ackermann, H.-W., Kropinski, A.M. 2008. Unifying classical and molecular taxonomic classification: analysis of the Podoviridae using BLASTP-based tools. *Research in Microbiology,* 159, 406-414.

Leclerc, H., Edberg, S., Pierzo, V., Delattre, J. 2000. Bacteriophages as indicators of enteric viruses and public health risk in groundwaters. *Journal of Applied Microbiology,* 88, 5-21.

Leclerc, H., Mossel, D., Edberg, S., Struijk, C. 2001. Advances in the bacteriology of the coliform group: their suitability as markers of microbial water safety. *Annual Reviews in Microbiology,* 55, 201-234.

Lee, H.S., Sobsey, M.D. 2011. Survival of prototype strains of somatic coliphage families in environmental waters and when exposed to UV low-pressure monochromatic radiation or heat. *Water Research,* 45, 3723-3734.

Love, D.C., Sobsey, M.D. 2007. Simple and rapid F+ coliphage culture, latex agglutination, and typing assay to detect and source track fecal contamination. *Applied and Environmental Microbiology,* 73, 4110-4118.

Lu, Z., Breidt, F., Plengvidhya, V., Fleming, H. 2003. Bacteriophage ecology in commercial sauerkraut fermentations. *Applied and Environmental Microbiology,* 69, 3192-3202.

Mac, K., Wichmann-Schauer, H., Peters, J., Ellerbroek, L. 2003. Species identification and detection of vancomycin resistance genes in enterococci of animal origin by multiplex PCR. *International Journal of Food Microbiology,* 88, 305-309.

Manero, A., Vilanova, X., Cerdà-Cuéllar, M., Blanch, A.R. 2002. Characterization of sewage waters by biochemical fingerprinting of Enterococci. *Water Research,* 36, 2831-2835.

Maniloff, J., Ackermann, H.-W. 1998. Taxonomy of bacterial viruses: establishment of tailed virus genera and the other Caudovirales. *Archives of Virology,* 143, 2051-2063.

Mannu, L., Paba, A. 2002. Genetic diversity of lactococci and enterococci isolated from home-made Pecorino Sardo ewes' milk cheese. *Journal of Applied Microbiology,* 92, 55-62.

Marino, M., Maifreni, M., Rondinini, G. 2003. Microbiological characterization of artisanal Montasio cheese: analysis of its indigenous lactic acid bacteria. *FEMS Microbiology Letters,* 229, 133-140.

Martellini, A., Payment, P., Villemur, R. 2005. Use of eukaryotic mitochondrial DNA to differentiate human, bovine, porcine and ovine sources in fecally contaminated surface water. *Water Research,* 39, 541-548.

Mascini, E., Troelstra, A., Beitsma, M., Blok, H., Jalink, K., Hopmans, T., Fluit, A., Hene, R., Willems, R., Verhoef, J. 2006. Genotyping and preemptive isolation to control an outbreak of vancomycin-resistant *Enterococcus faecium. Clinical Infectious Diseases,* 42, 739-746.

McMath, S., Sumpter, C., Holt, D., Delanoue, A., Chamberlain, A. 1999. The fate of environmental coliforms in a model water distribution system. *Letters in Applied Microbiology,* 28, 93-97.

Mesquita, S., Noble, R.T. 2013. Recent developments in monitoring of microbiological indicators of water quality across a range of water types. *Water Resources Planning, Development and Management*, 29-53.

Miller, W.R., Munita, J.M., Arias, C.A. 2014. Mechanisms of antibiotic resistance in enterococci. *Expert Review of Anti-Infective Therapy,* 12, 1221-1236.

Mocé-Llivina, L., Lucena, F., Jofre, J. 2005. Enteroviruses and bacteriophages in bathing waters. *Applied and Environmental Microbiology,* 71, 6838-6844.

Moe, C.L. 1996. Waterborne Transmission of Infectious Agents In: Crawford, R.L. Editor. *Manual of Environmental Microbiology*, 222-240.

Mohapi, I.M., Ateba, C.N. 2013. Isolation of vancomycin resistant enterococci isolated from leafy vegetables (lettuce) from North West Province. *Life Science Journal,* 10.

Murphy, H., Thomas, M., Schmidt, P., Medeiros, D., McFadyen, S., Pintar, K. 2016. Estimating the burden of acute gastrointestinal illness due to *Giardia*, *Cryptosporidium*, *Campylobacter*,

Escherichia coli O157 and norovirus associated with private wells and small water systems in Canada. *Epidemiology and Infection,* 144, 1355-1370.

Murray, B.E. 1990. The life and times of the *Enterococcus*. *Clinical Microbiology Reviews,* 3, 46-65.

Murray, B.E. 1998. Diversity among multidrug-resistant enterococci. *Emerging Infectious Diseases,* 4, 37.

Murray, B.E. 2000. Vancomycin-resistant enterococcal infections. *New England Journal of Medicine,* 342, 710-721.

Murray, B.E., Weinstock, G.M. 1999. Enterococci: new aspects of an old organism. *Proceedings of the Association of American Physicians,* 111, 328-334.

Myers, D., Stoeckel, D., Bushon, R., Francy, D., Brady, A. 2007. Fecal indicator bacteria (ver. 2.0): US Geological Survey Techniques of Water-Resources Investigations, book 9, chap. *A7, section,* 7.

Nannini, E.C., Teng, F., Singh, K.V., Murray, B.E. 2005. Decreased virulence of a gls24 mutant of *Enterococcus faecalis* OG1RF in an experimental endocarditis model. *Infection and Immunity,* 73, 7772-7774.

NCCLS 1999. Performance standards for antimicrobial disc and dilution susceptibility test for bacteria isolated from animals. National Committee for Clinical Laboratory Standards, Wayne, Pennsylvania, Approved Standards M 31-A19, p. 11.

Niu, Y., Johnson, R., Xu, Y., McAllister, T., Sharma, R., Louie, M., Stanford, K. 2009. Host range and lytic capability of four bacteriophages against bovine and clinical human isolates of Shiga toxin-producing *Escherichia coli* O157: H7. *Journal of Applied Microbiology,* 107, 646-656.

Niu, Y.D., Stanford, K., Ackermann, H.-W., McAllister, T.A. 2012. Characterization of 4 T1-like lytic bacteriophages that lyse Shiga-toxin *Escherichia coli* O157: H7. *Canadian Journal of Microbiology,* 58, 923-927.

Odonkor, S.T., Ampofo, J.K. 2013. *Escherichia coli* as an indicator of bacteriological quality of water: an overview. *Microbiology Research,* 4, 2.

Ogilvie, L.A., Caplin, J., Dedi, C., Diston, D., Cheek, E., Bowler, L., Taylor, H., Ebdon, J., Jones, B.V. 2012. Comparative (meta) genomic analysis and ecological profiling of human gut-specific bacteriophage phiB124-14. *PloS one,* 7, e35053.

Olson, M.R., Axler, R.P., Hicks, R.E. 2004. Effects of freezing and storage temperature on MS2 viability. *Journal of Virological Methods,* 122, 147-152.

Payment, P., Franco, E., Siemiatycki, J. 1993. Absence of relationship between health effects due to tap water consumption and drinking water quality parameters. *Water Science and Technology,* 27, 137-143.

Payment, P., Trudel, M., Plante, R. 1985. Elimination of viruses and indicator bacteria at each step of treatment during preparation of drinking water at seven water treatment plants. *Applied and Environmental Microbiology,* 49, 1418-1428.

Payment, P., Waite, M., Dufour, A. 2003. Introducing parameters for the assessment of drinking water quality. *Assessing Microbial Safety of Drinking Water,* 4, 47-77.

Pedroso, D., Martins, M. 1995. Ultra-morphology of coliphages isolated from water. *Water Research,* 29, 1199-1202.

Pelczar, M.J., Chan, E.C.S., Krieg, N. 1988. Microbiology (5th Edition) McGraw-Hill Book Company, Singapore, 416.

Phokela, P.T., Ateba, C.N., Kawadza, D.T. 2011. Assessing antibiotic resistance profiles in *Escherichia coli* and Salmonella species from groundwater in the Mafikeng area, South Africa. *African Journal of Microbiology Research,* 5, 5902-5909.

Puig, A., Queralt, N., Jofre, J., Araujo, R. 1999. Diversity of Bacteroides fragilisStrains in Their Capacity To Recover Phages from Human and Animal Wastes and from Fecally Polluted Wastewater. *Applied and Environmental Microbiology,* 65, 1772-1776.

Puig, M., Pina, S., Lucena, F., Jofre, J., Girones, R. 2000. Description of a DNA amplification procedure for the detection of bacteriophages of Bacteroides fragilis HSP40 in environmental samples. *Journal of Virological Methods,* 89, 159-166.

Purnell, S.E., Ebdon, J.E., Taylor, H.D. 2011. Bacteriophage lysis of *Enterococcus* host strains: a tool for microbial source tracking? *Environmental Science and Technology,* 45, 10699-10705.

Queralt, N., Jofre, J., Araujo, R., Muniesa, M. 2003. Homogeneity of the morphological groups of bacteriophages infecting Bacteroides fragilis strain HSP40 and strain RYC2056. *Current Microbiology,* 46, 0163-0168.

Rebolledo-Martínez, A., del Ángel-Pérez, A.L., Megchún-García, J.V., García, J.A., Nataren-Velázquez, J., Capetillo-Burela, Á. 2011. Coberteras vivas para el manejo de malezas en mango (Mangifera indica L.) cv. Manila. *Tropical and Subtropical Agroecosystems,* 13, 327-338.

Rivera, S.C., Hazen, T.C., Toranzos, G.A. 1988. Isolation of fecal coliforms from pristine sites in a tropical rain forest. *Applied and Environmental Microbiology,* 54, 513-517.

Rodríguez, R.A., Love, D.C., Stewart, J.R., Tajuba, J., Knee, J., Dickerson, J.W., Webster, L.F., Sobsey, M.D. 2012. Comparison of methods for the detection of coliphages in recreational water at two California, United States beaches. *Journal of Virological Methods,* 181, 73-79.

Rogers, A.H. 2008. Molecular Oral Microbiology. Caister Academic Press. ISBN 978-1-904455-24-0.

Rompré, A., Servais, P., Baudart, J., de-Roubin, M.-R., Laurent, P. 2002. Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. *Journal of Microbiological Methods,* 49, 31-54.

Ruoff, K.L., De La Maza, L., Murtagh, M.J., Spargo, J.D., Ferraro, M.J. 1990. Species identities of enterococci isolated from clinical specimens. *Journal of Clinical Microbiology,* 28, 435-437.

Sambrook, J., Fritsch, E.F., Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sambrook, J., Russell, D. 2001. Molecular Cloning: A Laboratory Manual, 3rd Edition. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.

Santiago-Rodriguez, T.M. 2013. *Enterococcus faecalis-infecting phages (enterophages) as markers of human fecal pollution and as reservoirs of antibiotic-resistance and virulence genes.* University of Puerto Rico.

Santiago-Rodríguez, T.M., Dávila, C., González, J., Bonilla, N., Marcos, P., Urdaneta, M., Cadete, M., Monteiro, S., Santos, R., Santo Domingo, J. 2010. Characterization of *Enterococcus faecalis*-infecting phages (enterophages) as markers of human fecal pollution in recreational waters. *Water Research,* 44, 4716-4725.

Santiago-Rodriguez, T.M., Marcos, P., Monteiro, S., Urdaneta, M., Santos, R., Toranzos, G.A. 2013. Evaluation of enterococcus-infecting phages as indices of fecal pollution. *Journal of Water and Health,* 11, 51-63.

Santos, T., Silva, N., Igrejas, G., Rodrigues, P., Micael, J., Rodrigues, T., Resendes, R., Gonçalves, A., Marinho, C., Gonçalves, D. 2013. Dissemination of antibiotic resistant *Enterococcus* species and *Escherichia coli* from wild birds of Azores Archipelago. *Anaerobe,* 24, 25-31.

Schleifer, K.H., Kilpper-Bälz, R. 1984. Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the Genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. *International Journal of Systematic and Evolutionary Microbiology,* 34, 31-34.

Scott, T.M., Rose, J.B., Jenkins, T.M., Farrah, S.R., Lukasik, J. 2002. Microbial source tracking: current methodology and future directions. *Applied and Environmental Microbiology,* 68, 5796-5803.

Sidhu, J., Skelly, E., Hodgers, L., Ahmed, W., Li, Y., Toze, S. 2014. Prevalence of *Enterococcus* Species and Their Virulence Genes in Fresh Water Prior to and after Storm Events. *Environmental Science and Technology,* 48, 2979-2988.

Simkova, A., Cervenka, J. 1981. Coliphages as ecological indicators of enteroviruses in various water systems. *Bulletin of the World Health Organization,* 59, 611.

Simner, P., Adam, H., Baxter, M., McCracken, M., Golding, G., Karlowsky, J., Nichol, K., Lagacé-Wiens, P., Gilmour, M., Hoban, D. 2015. Epidemiology of vancomycin-resistant enterococci in Canadian hospitals (CANWARD study, 2007 to 2013). *Antimicrobial Agents and Chemotherapy,* 59, 4315-4317.

Sobsey, M.D., Hickey, A. 1985. Effects of humic and fulvic acids on poliovirus concentration from water by microporous filtration. *Applied and Environmental Microbiology,* 49, 259-264.

Stevens, M., Ashbolt, N., Cunliffe, D. 2003. Review of Coliforms as Microbial Indicators of Drinking Water Quality-Recommendations to change the use of Coliforms as Microbial indicators of drinking water quality. NHMRC, Biotext Pty Ltd, Canberra, Australia, 1-42.

Takeuchi, K., Tomita, H., Fujimoto, S., Kudo, M., Kuwano, H., Ike, Y. 2005. Drug resistance of *Enterococcus faecium* clinical isolates and the conjugative transfer of gentamicin and erythromycin resistance traits. *FEMS Microbiology Letters,* 243, 347-354.

Tartera, C., Jofre, J. 1987. Bacteriophages active against Bacteroides fragilis in sewage-polluted waters. *Applied and Environmental Microbiology,* 53, 1632-1637.

Tartera, C., Lucena, F., Jofre, J. 1989. Human origin of Bacteroides fragilis bacteriophages present in the environment. *Applied and Environmental Microbiology,* 55, 2696-2701.

Tavakoli, A., Yazdani, R., Shahmansouri, M., Isfahani, B. 2005. Chlorine residual efficiency in inactivating bacteria from secondary contamination in Isfahan, *Eastern Mediterranean Health Journal*, 425-434.

Teixeira, L.M., Carvalho, M.G., Facklam, R.R. 2007. *Enterococcus* . In: Murray BE, Baron EJ, Jorgensen JH (eds) Manual of clinical microbiology, 9th Edition. ASM, Washington, DC.

Teixeira, L.M., Merquior, V.L.C. 2013. *Enterococcus. Molecular Typing in Bacterial Infections.* Springer.

Tey, B.T., Ooi, S.T., Yong, K.C., Ng, M.Y.T., Ling, T.C., Tan, W.S. 2009. Production of fusion m13 phage bearing the di-sulphide constrained peptide sequence (C-WSFFSNI-C) that interacts with hepatitis B core antigen. *African Journal of Biotechnology,* 8, 268.

Thomann, R.V., Mueller, J.A. 1987. Principles of surface water quality modeling and control. Harper and Row, New York.

Thouverez, M., Talon, D. 2004. Microbiological and epidemiological studies of *Enterococcus faecium* resistant to amoxycillin in a university hospital in eastern France. *Clinical Microbiology and Infection,* 10, 441-447.

Toranzos, G., McFeters, G., Borrego, J., Savill, M. 1996. Detection of microorganisms in environmental freshwaters and drinking waters. In: Crawford, R.L. Editor. *Manual of Environmental Microbiology*, ASM, 249-260.

UNDP 2004. South African Human Development Report 2003; Oxford University Press: Cape Town, Southern Africa.

USEPA 1986a. Ambient Water Quality Criteria for Bacteria-1986. Office of Water Regulations and Standards, Criteria and Standards Division, Washington, DC. EPA-440/5-84/002.

USEPA 1986b. Bacteriological ambient water quality criteria; availability. Federal Register 51, 8012-8016.

USEPA 2009. U.S. Environmental Protection Agency. *Analytical methods approved for drinking water compliance monitoring under the total coliform rule. National Primary Drinking Water Regulations*, 1-18.

Vinjé, J., Oudejans, S.J., Stewart, J.R., Sobsey, M.D., Long, S.C. 2004. Molecular detection and genotyping of male-specific coliphages by reverse transcription-PCR and reverse line blot hybridization. *Applied and Environmental Microbiology,* 70, 5996-6004.

Weigel, L.M., Clewell, D.B., Gill, S.R., Clark, N.C., McDougal, L.K., Flannagan, S.E., Kolonay, J.F., Shetty, J., Killgore, G.E., Tenover, F.C. 2003. Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. *Science,* 302, 1569-1571.

Weinbauer, M.G. 2004. Ecology of prokaryotic viruses. *FEMS Microbiology Reviews,* 28, 127-181.

Wheeler, A.L., Hartel, P.G., Godfrey, D.G., Hill, J.L., Segars, W.I. 2002. Potential of *Enterococcus faecalis* as a human fecal indicator for microbial source tracking. *Journal of Environmental Quality,* 31, 1286-1293.

WHO 2003. Emerging Issues in Water and Infectious Disease, World Health Organization, Geneva, Switzerland.

WHO 2004. Guidelines for drinking-water quality, 3rd Edition, Volume 1. Recommendations. World Health Organization, Geneva, Switzerland.

WHO 2008. Guidelines for Drinking-water Quality, Incorporating 1st and 2nd Addenda, Volume 1, Recommendations. 3rd Edition. World Health Organization, Geneva, Switzerland.

Willems, R.J., Van Schaik, W. 2009. Transition of *Enterococcus faecium* from commensal organism to nosocomial pathogen. *Future Microbiology,* 4, 1125-1135.

Willey, B.M., Jones, R.N., McGeer, A., Witte, W., French, G., Roberts, R.B., Jenkins, S.G., Nadler, H., Low, D.E. 1999. Practical approach to the identification of clinically relevant *Enterococcus* species. *Diagnostic Microbiology and Infectious Disease,* 34, 165-171.

Willey, J.M., Sherwood, L.M., Woolverton, C.J. 2008. Prescott, Harley, and Klein's Microbiology. 7th Edition. Bacteriophages.

Yang, Y., Griffiths, M.W. 2013. Comparative persistence of subgroups of F-specific RNA phages in river water. *Applied and Environmental Microbiology,* 79, 4564-4567.

APPENDIX

Appendix Table 1: Antibiogram data of all isolates derived from the inhibition zone data

Sample ID	AP	A	E	T	VA	C	GM
SWEFAE3A	Resistant	Sensitive	Intermediate	Intermediate	Sensitive	Intermediate	Resistant
SWEFAE3B	Resistant	Sensitive	Intermediate	Intermediate	Sensitive	Intermediate	Resistant
SWEFAE4A	Resistant	Sensitive	Intermediate	Intermediate	Sensitive	Intermediate	Intermediate
SWEFAE4B	Resistant	Sensitive	Intermediate	Intermediate	Sensitive	Intermediate	Intermediate
SWEFAE5A	Resistant	Sensitive	Intermediate	Intermediate	Intermediate	Intermediate	Resistant
SWEFAE5B	Resistant	Sensitive	Intermediate	Intermediate	Sensitive	Intermediate	Intermediate
MTEFAE8A	Resistant	Sensitive	Intermediate	Intermediate	Sensitive	Intermediate	Intermediate
DBEFAE1A	Resistant	Sensitive	Intermediate	Intermediate	Sensitive	Intermediate	Intermediate
DBEFAE1B	Resistant	Sensitive	Intermediate	Intermediate	Intermediate	Intermediate	Sensitive
DBEFAE2A	Resistant	Sensitive	Intermediate	Intermediate	Sensitive	Intermediate	Sensitive
DBEFAE6A	Resistant	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Sensitive
DBEFAE6B	Resistant	Sensitive	Intermediate	Intermediate	Sensitive	Intermediate	Intermediate
STEFEC1A	Sensitive	Sensitive	Intermediate	Sensitive	Intermediate	Resistant	Sensitive
STEFEC1B	Sensitive	Sensitive	Intermediate	Sensitive	Sensitive	Resistant	Sensitive

STEFEC2A	Sensitive	Sensitive	Intermediate	Intermediate	Sensitive	Resistant	Sensitive
STEFEC2B	Sensitive	Sensitive	Intermediate	Sensitive	Sensitive	Resistant	Sensitive
STEFEC3A	Sensitive	Sensitive	Intermediate	Intermediate	Sensitive	Resistant	Sensitive
STEFEC3B	Sensitive	Sensitive	Intermediate	Sensitive	Intermediate	Resistant	Sensitive
STEFEC4A	Sensitive	Sensitive	Intermediate	Intermediate	Intermediate	Resistant	Sensitive
DDEFAEB	Sensitive	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
DDEFAEC	Sensitive	Sensitive	Resistant	Resistant	Intermediate	Intermediate	Resistant
DDEFAED	Sensitive	Sensitive	Resistant	Resistant	Intermediate	Intermediate	Resistant
DDEFAEE	Sensitive	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
DDEFAEF	Sensitive	Sensitive	Intermediate	Resistant	Intermediate	Intermediate	Resistant
SDEFECA	Sensitive	Sensitive	Intermediate	Sensitive	Sensitive	Resistant	Sensitive
SDEFECB	Sensitive	Sensitive	Intermediate	Sensitive	Sensitive	Resistant	Sensitive
LDEFAEA	Resistant	Resistant	Resistant	Resistant	Sensitive	Intermediate	Resistant
LDEFAEB	Resistant	Sensitive	Resistant	Resistant	Intermediate	Intermediate	Resistant
LDEFAEC	Resistant	Sensitive	Resistant	Resistant	Intermediate	Intermediate	Intermediate
LDEFAED	Sensitive	Sensitive	Resistant	Resistant	Intermediate	Intermediate	Intermediate
LDEFAEF	Sensitive	Sensitive	Intermediate	Resistant	Intermediate	Intermediate	Intermediate

KDEFAEA	Resistant	Sensitive	Resistant	Resistant	Sensitive	Intermediate	Intermediate
KDEFAEB	Resistant	Sensitive	Resistant	Resistant	Sensitive	Intermediate	Intermediate
KDEFAEC	Sensitive	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Intermediate
MDEFECA	Sensitive	Sensitive	Intermediate	Sensitive	Sensitive	Resistant	Sensitive
MDEFECB	Sensitive	Sensitive	Intermediate	Intermediate	Sensitive	Resistant	Sensitive
MDEFECC	Sensitive	Sensitive	Intermediate	Intermediate	Sensitive	Resistant	Sensitive
MDEFECD	Sensitive	Sensitive	Intermediate	Intermediate	Sensitive	Resistant	Sensitive
MDEFECE	Sensitive	Sensitive	Intermediate	Sensitive	Sensitive	Resistant	Sensitive
MDEFECF	Sensitive	Sensitive	Intermediate	Sensitive	Sensitive	Resistant	Sensitive
PLEFAE5A	Resistant	Sensitive	Intermediate	Intermediate	Sensitive	Intermediate	Resistant
SLEFAE1A	Resistant	Sensitive	Intermediate	Intermediate	Sensitive	Intermediate	Resistant
SLEFAE2A	Resistant	Sensitive	Intermediate	Intermediate	Intermediate	Intermediate	Resistant
PDEFAEB	Resistant	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Intermediate
PDEFAED	Sensitive	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Intermediate
PDEFAEE	Resistant	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Intermediate
PDEFAEF	Resistant	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Intermediate
CDEFAEA	Resistant	Sensitive	Resistant	Resistant	Intermediate	Intermediate	Resistant

CDEFAEB	Resistant	Resistant	Resistant	Resistant	Intermediate	Intermediate	Resistant
CDEFAEC	Resistant	Resistant	Resistant	Resistant	Resistant	Intermediate	Intermediate
CDEFAED	Resistant	Resistant	Intermediate	Resistant	Intermediate	Intermediate	Intermediate
CDEFAEE	Resistant	Resistant	Intermediate	Resistant	Intermediate	Intermediate	Resistant
CDEFAEF	Resistant	Resistant	Resistant	Resistant	Intermediate	Intermediate	Resistant
VTEFAE1A	Resistant	Sensitive	Resistant	Intermediate	Sensitive	Sensitive	Sensitive
VTEFAE3A	Resistant	Resistant	Resistant	Intermediate	Sensitive	Sensitive	Sensitive
VTEFAE3B	Resistant	Resistant	Intermediate	Intermediate	Intermediate	Intermediate	Intermediate
VTEFAE4A	Resistant	Resistant	Resistant	Intermediate	Intermediate	Intermediate	Intermediate
MREFAEA	Resistant	Resistant	Intermediate	Intermediate	Intermediate	Intermediate	Resistant
MREFAEB	Resistant	Sensitive	Intermediate	Intermediate	Intermediate	Intermediate	Resistant
MREFAEC	Resistant	Sensitive	Intermediate	Intermediate	Intermediate	Intermediate	Resistant
POEFECB	Sensitive	Sensitive	Intermediate	Sensitive	Intermediate	Resistant	Sensitive
SEEFAE1A	Resistant	Sensitive	Intermediate	Intermediate	Intermediate	Intermediate	Resistant
SEEFAE1B	Resistant	Sensitive	Intermediate	Intermediate	Sensitive	Intermediate	Resistant
SEEFAE2A	Resistant	Sensitive	Intermediate	Intermediate	Sensitive	Intermediate	Resistant
MOEFAE2A	Resistant	Resistant	Resistant	Sensitive	Sensitive	Resistant	Sensitive

HREFAEB	Resistant	Resistant	Intermediate	Intermediate	Intermediate	Intermediate	Resistant

SWEFAE3A = Seweding; MTEFAE8A = Motlhabeng; DBEFAE1A = Dibate; STEFEC1A = Setlopo; PLEFAE5A = Pella; SEEFAE1A = Setlagole; SLEFAE1A = Silwerkrans; MOEFAE2A = Moretele; VTEFAE1A = Ventersdorp; DDEFAEB = Disaneng Dam; SDEFECA = Setumo Dam; LDEFAEA = Lotlamoreng Dam; KDEFAEA = Klein-Maricopoort Dam; MDEFECA = Marico-Bosveld Dam; PDEFAEB = Pella Dam; CDEFAEA = Coligny Dam; MREFAEA = Mooi River; POEFECB = Poortjies Dam; HREFAEB = Harts River