Characterization of enterococci isolates from water sources in the North West Province

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Blessed is she who has believed that the Lord would fulfil his promises to her!

Luke 1: 45

I praise you, for I am fearfully and wonderfully made. Wonderful are your works; my soul knows it very well.

Psalm 139:14

He will certainly accomplish what he has decreed for me, and he has many more things like these in mind.

Job 23: 14

Bless the Lord oh my soul, and forget not all his benefits!

Psalm 103: 2

This work is dedicated to the reason for my existence, my parents Sarah and Lekopane Molale. Thank you for a lifetime of unconditional love and allowing me to realize my potential. The love, devotion, support and guidance you have provided me over the years is by far the greatest gift anyone has ever given me. I am humbled to be the fruit of your womb and an imprint of your seed.

Enkosi ma'Mpinga

Kealeboga Morolong wa ga Sehuba, mmina Tshipi encho

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Abstract

Enterococci are a diverse and complex group of ubiquitous bacteria that can survive extreme conditions. Despite constituting a large portion of the gastrointestinal tract in warm blooded animals and humans, they are responsible for numerous infections and diseases globally. Enterococcal pathogens from point and nonpoint sources such as agricultural runoff and wastewater treatment plants (WWTPs) can be mobilized into receiving water bodies via storm water. This is a cause for concern since epidemiological studies have demonstrated a positive correlation between *Enterococcus* concentrations and swimmer associated gastroenteritis. Few studies have characterised *Enterococcus* spp. in South Africa, particularly the North West Province. Thus, there is a paucity of data reporting the prevalence and susceptibility patterns of *Enterococcus* spp. isolated from surface water systems in the province. In addition, the presence of potentially pathogenic *Enterococcus* spp. harbouring efflux pumps, plasmids and virulence genes, in surface water systems of the North West Province is unknown. To increase our understanding of *Enterococcus* spp. in surface water systems of the North West Province, and to highlight their importance in water quality assessments, an overview study was performed in order to determine whether *Enterococcus* spp. present in environmental water systems of the North West Province were potential pathogens. In the first part of the study, the presence of five clinically relevant virulence genes in six *Enterococcus* species isolated from environmental water systems was determined. In addition, the presence of virulence genes was correlated with their extracellular enzyme production. Sixty five percent of the isolates were positive for one virulence gene and 13% for two or more. Most frequently detected genes were *gelE* (32%) and *cylA* (28%). Enterococcal surface protein was absent in all isolates screened. The results show that a large percentage of these environmental *Enterococcus* spp. possess virulence factors that could be expressed *in vitro*. This is a cause for concern and could have implications for individuals using this water for recreational and cultural purposes. It requires further investigation into the sources of these potential pathogenic Enterococcus isolates and measures to minimize their presence in water sources. In the second part of this study the potential source of *Enterococcus* spp. found in these environmental water systems was determined. The final effluent of three WWTPs and points downstream were analysed for the presence of

Enterococcus spp. while their antibiotic susceptibilities and presence of virulence genes were also determined. Furthermore, the multiple antibiotic resistance (MAR) phenotypes were identified for all profiles for pre- and post plasmid curing. The antibiotic inhibition zone diameter data was subject to cluster analysis. Sixty eight percent of the screened *Enterococcus* spp. were resistant to three or more antibiotics and harboured plasmids. All five virulence genes were detected and six multi-virulence profiles observed. Cluster analysis indicated grouping of isolates from all three WWTPs final effluent together, points downstream together, and between plants one and two together. The findings of this study have demonstrated that *Enterococcus* spp. harbouring virulence factors and plasmids that mediate multiple antibiotic resistance are present in WWTPs final effluent and receiving water systems that support various social needs. This is a cause for concern and it is recommended that *Enterococcus* be used as an additional faecal indicator when microbiological quality of water is assessed. The third part of the study analysed the antimicrobial susceptibility of *Enterococcus* species isolated from five surface water systems in the North West Province. In addition, the presence of four efflux pump genes was determined. Thereafter, the presence of virulence genes was determined. Efflux genes *mefA* and *tetK* were not detected in any of *Enterococcus* spp. screened. However, *tetL* and *msrC* were detected in 17% of the *Enterococcus* spp. Virulence determinants were detected in 86% of the *Enterococcus* spp. harbouring efflux pump genes. Of the screened five virulence determinants (*asa1, cylA, esp, gelE* and *hyl*), four were detected. The findings of this study have demonstrated that *Enterococcus* spp. from South African surface water systems are resistant to multiple antibiotics, some of these are frequently used for therapy. Furthermore, these isolates not only harbour efflux pump genes coding for resistance to antibiotics but also harbour virulence factors which enhance their pathogenicity potential. These genetic determinants may be useful for the survival of these isolates in surface water environments allowing for their dissemination to humans and animals. Data generated in this study is valuable as it indicates the presence of potentially pathogenic *Enterococcus* spp. harbouring genetic determinants in surface water systems used for a wide variety of activities. In addition, this study has generated readily available information on the local antimicrobial resistance patterns of potential bacterial pathogens which could assist in improved assessments of human health risks while guiding empirical and pathogen specific therapy. Lastly, data produced in

this study could be used for making optimal decisions regarding future water quality monitoring practices.

Keywords: *Enterococcus* spp.; environmental water systems; virulence factors; efflux pump genes.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

***asa1*:** Aggregation substance

***cylA*:** Cytolysin

DNA: Deoxyribonucleic acid

DS: Downstream the final effluent

***esp*:** Enterococcal surface protein

FE: Final effluent

gelE: Gelatinase

hyl: Hyaluronidase

PCR: Polymerase Chain Reaction

WWTP: Wastewater treatment plant

CHAPTER 1

Introduction and Problem Statement

Environmental water systems harbouring multiple antibiotic resistant bacterial species possessing virulence genes are a human and animal health concern (Santiago-Rodriguez *et al.,* 2013). The latter is in view of the ability of these bacterial species to disseminate antibiotic resistance and virulence genes to pathogenic or opportunistic pathogenic bacteria found in the environment (Pal *et al.,* 2015). Furthermore, the treatment of diseases caused by such bacterial strains has over the years proven difficult (Pal *et al.,* 2015). Thus, the microbial quality and safety of these environmental water systems cannot be overemphasised. The latter is particularly important seeing that these environmental water systems support a variety of economic, social and in some instances domestic activities (DWAF, 2004).

Various microbial indicator species are used to monitor faecal pollution, amongst these are enterococci (Franz *et al.,* 1999). This species is ubiquitous in nature and has been identified in various aquatic ecosystems (Micallef *et al*., 2013). In addition, the study of this bacterial groups presence in environmental waters as well as the determination of isolates harbouring antibiotic resistant and virulence genes has also received much attention lately (Lata *et al.,* 2009; Sidhu *et al.,* 2014). However, the study of, and information on, *Enterococcus* spp. harbouring antibiotic resistance and virulence genes in South African environmental water systems, particularly the North West Province is limited and warrants investigation. Thus, an overview of the environmental water situation in South Africa and the North West Province is provided in this chapter. In addition, an introduction to enterococci, an overview of the antibiotic and virulence features as well as genes relevant to the thesis is provided. Lastly, a problem statement followed by the structure of the thesis is presented.

1.1 Current state of surface water systems in South Africa and the North West Province

Water is fundamental for the sustenance of all living matter and is deemed one of South Africa's precious resources (Stats SA, 2010). However, numerous challenges in terms of water quantity and quality are faced in South Africa (DWA, 2013). Consequently, water is not always accessible to meet South Africa's developmental demands (DWA, 2013). According to DWA (2013), South Africa is the 30th driest country in the world. Relative to the world's average rainfall, South Africa has low rainfall levels (DWAF, 2004). In addition, high levels of rainfall variability are experienced in South Africa while high evaporation levels result from the hot climate (DWAF, 2004).

Currently, South Africa is experiencing one of its driest rainy seasons resulting in a severe drought (http://www.news24.com/SouthAfrica). This has affected 2.7 million households having the greatest impact on inhabitants of rural areas who relying on water from nearby surface systems for domestic purposes (http://www.aljazeera.com/news/, 2015; NWDAC-SoER, 2008). On the 7th of November 2015, Minister Pravin Gordhan announced that the current water crisis experienced in the midst of an El Nino has had the greatest effect on the North West, KwaZulu-Natal, Mpumalanga, Limpopo and Free State Provinces (http://www.news24.com/SouthAfrica, 2015). The minister of Cooperative Governance and Traditional Affairs further declared these provinces drought disaster stricken areas (http://www.news24.com/SouthAfrica, 2015). According to eNCA (https://www.enca.com/south-africa, 2015), the drought is only one of the contributing factors to the current water crisis experienced in the North West Province. Despite water sources in the North West Province comprising groundwater, surface water, imported water and re-useable effluent, significant water quantity and quality challenges are experienced in this province (Kalule-Sabiti and Heath, 2008; NWDETEA, 2014). Moreover, most surface water systems in the province are non-perennial and comprise rivers, dams, pans as well as wetlands which are all regarded a socio-economic entity.

Water in the North West Province has been attributed a key constraint for the development of this province (NWDETEA, 2014). A large portion of these surface

water resources are attributed to mining and agriculture however, they also play significant roles in numerous recreational, cultural and religious activities (Molale, 2012; NWDETEA, 2014). However, when the inadequate treatment of water, its misuse as well as the inadequate recharge rate of surface water systems are considered, the consequence of water quantity and quality come at a great cost (DWA, 2012; NWDETEA, 2014).

In spite of advances and improvements in wastewater treatments, numerous WWTPs in South Africa have been reported to be inefficiently functioning (DWA, 2012). Furthermore, despite the introduction of the Green Drop in 2008, the status of some WWTPs in the North West Province was still unsatisfactory in 2012 (DWA, 2013). This is a cause for corncern because ineffectively functioning WWTPs in the province discharge inadequately treated effluents into surface water systems decreasing the quality of water consequently posing a public health threat (NWDETEA, 2014). Consequently, faecal pollution from WWTPs has emerged as one of the major water quality problems experienced in the North West Province (NWDETEA, 2014).

In addition to the the degradation of surface water systems in the North West Province, water supply is a key challenge in the province (NWDETEA,2014). The North West Province has in recent times experienced protests by communities expressing their unhappiness with regard to the water supply shortages (NWDETEA, 2014). Furthermore, Kalule-Sabiti and Heath (2008) stated that the microbial variables of the North West Province water resources were not being monitored on a routine basis despite their importance in providing information for the compilation of the North West Department of Agriculture, Conservations and Environment-State of the Environment Report (NWDACE-SoER, 2013). This is evident as the latest NWPSoER (2013) has limited information on the current state of surface water systems in the North West Province.

Therefore, availability and quality in the North West Province water will depreciate if improvements regarding the manner in which its water systems are managed are not improved. Of the possible management regimes, scientific principles could be used in order to elucidate the current state of surface water systems (WRC, 1998).

Thereafter, health risk mitigation strategies as well as effective WWTP strategies can be devised and implemented.

1.2 Enterococci

Enterococci are a diverse and complex group of bacteria first described in the late 1800's and early 1900's (Murray, 1990). Theiercelin (1899) originally named a Gram-positive diplococcus organism of intestinal origin 'enterocoque' (reviewed in Murray, 1990 Thereafter, the group *Enterococcus* was proposed by Theircelin and Jouhaud in 1903 and the group derived (Manero and Blanch, 1999). In 1906, Andrewes and Horder identified a potentially pathogenic bacterium isolated from a patient with endocarditis and named it *Streptococcus faecalis.* In 1919, a faecal organism *Streptococcus faecium* was described and included in the *Enterococcus* group (Orla-Jensen, 1919). In 1933 a serological typing system for streptococci was developed by Lancefield (1933), and isolates of faecal origin (enterococci) were classified as group D streptococci. This was decided on the basis of this bacterial group possessing a group D cell wall antigen (Franz *et al.,* 1999). Thereafter, Sherman (1937) developed a serological typing system which divided streptococci into four groups: pyogenic, viridans, lactic and *Enterococcus*.

In 1970, Kalina recommended *Streptococcus faecalis, Streptococcus faecium* as well as their subspecies be named *Enterococcus* and classified in their own genus. Scheleifer and Kilpper-Balz (1984) supported this suggestion when the findings of their study proved that the DNA-DNA and DNA-RNA hybridization of *Streptococcus faecalis and Streptococcus faecium* were significantly different from the other members of the genus. Thereafter enterococci were reclassified into their own genus and since then the genus has been valid.

There are, to date, more than 48 species within the *Enterococcus* genus and these are classified into four species groups based on 16S rRNA sequences (Franz *et al.,* 1999). The first group is the *faecium* group and it includes species such as *E. faecium, E. durans, E. hirae* and *E. mundtii.* The *avium* group comprises, amongst others, *E. avium, E. raffinosus, E. malodoratus* and *E. pseudoavium.* Thirdly is the *gallinarum* group which is made up of species such as *E. casseliflavus* and *E.*

gallinarum. The last group comprises *E. columbae* and *E. cecorum* (Williams *et al.,* 1991).

1.3 Taxonomy of *Enterococcus*

The genus *Enterococcus* is composed of Gram-positive non-spore forming, catalase negative cocci that occur singly, in pairs or chains (Teixeria and Merquior, 2013). Species in this group have intrinsic characteristics that enable them to grow, proliferate and survive a variety of hostile conditions (Teixeria and Merquior, 2013). Enterococci are anaerobic organisms but have the ability of growing under both oxygen and reduced oxygen environments by means of readily converting respiration to fermentation (Huycke *et al.,* 1998). Furthermore, enterococci constitute a large portion of the gastrointestinal tract in warm blooded animals and humans (Lanthier *et al.,* 2010).

Species in this genus can grow at extreme temperatures ranging from 5 °C to 50 °C while also being able to survive heating of 30 minutes at 60°C (Fisher and Phillips, 2009). Additionally, enterococci are able to withstand broad pH ranges, growing between 4.0 and 9.6 while growing optimally at pH 7.5 (Schleifer and Klipper-Balz, 1984). This bacterial group can survive conditions, which inhibit growth of other Gram positive organisms, such as growth at high salt concentrations of 6.5% NaCl and 40% (w/v) bile salts (Schleifer and Klipper-Balz, 1984). The latter described extreme conditions within which enterococci can grow, proliferate and survive enable them to colonize various niches which could have an influence in their clinical relevance (Vu and Carvalho, 2011). Furthermore, these intrinsic characteristics enable enterococci to fulfil diverse roles as commensals and opportunistic pathogens (Teixeria and Merquior, 2013).

1.4 Clinical significance

Although enterococci are normal inhabitants of the gastrointestinal tract of humans and animals, they are responsible for numerous infections and diseases (Jett *et al.,* 1994). Enterococci are opportunistic pathogens and target patients with underlying diseases as well as those treated with a broad spectrum of antibiotics, the elderly and immune-compromised persons (Ogier and Serror, 2008; Vu and Carvalho, 2011).

Infections caused by enterococci include endocarditis, bacteremia, biliary and urinary tract, intra-abdominal, pelvic as well as burn and surgical wound infections (Jett *et al.,* 1994; Vu and Carvalho, 2011). According to Teixeria and Merquior (2013), enterococci can cause sinusitis, septic arthritis, otitis and endophthalamitis as well as infections in the respiratory tract and central nervous system, however these occur very rarely. *Enterococcus faecalis* is the most prevalent *Enterococcus* species isolated from human infections and accounts for 80-90% of *Enterococcus* clinical infections. Furthermore, *E. faecium* strains account for the remaining 5-20% (Franz *et al.,* 1999). The remaining *Enterococcus* species infrequently cause infections (Jett *et al.,* 1994; Ogier and Serror, 2008).

However, according to Teixeria and Merquior (2013), enterococcal infections are amongst the leading therapeutic challenges. Furthermore, infections caused by *Enterococcus* species are difficult to treat (Murray *et al.,* 1990). This is a result of *Enterococcus* spp. acquiring specific mechanisms of resistance to different antibiotics (Murray *et al.,* 1990). According to Ogier and Serror (2008), the most important contributor to the pathogenesis of enterococci is their resistance to multiple antibiotics. Furthermore, much attention has been given to them because of their importance in community-acquired and hospital-acquired infections (Aznar *et al.,* 2004; Olawale *et al.,* 2011).

Enterococci are equipped with a variety of antibiotic resistance genes, some inherent and some acquired (Jonas *et al.,* 2001). The resistance of enterococci to different antibiotics has over the years led to their emergence as important bacterial pathogens (Teixeira and Merquior, 2013). Furthermore, enterococci have, in the past two decades, emerged as important nosocomial pathogens (Rice, 2001). Therefore, the intrinsically resistance of enterococci to a variety of antibiotics allows for the assumption that multidrug resistance efflux pumps could be contributing to the drug resistance.

1.5 Antibiotic resistance

Since the introduction of antibiotic therapy, scientists have studied the resistance of bacteria against antimicrobial agents as well as their remarkable ability to adapt to new agents (Köhler *et al.,* 1999). Although the targets of antibiotics are limited to few

cellular structures and enzymes, the mechanisms by which bacteria can become resistant are versatile (Köhler *et al.,* 1999). These mechanisms could be a result of the acquisition of resistance genes located on mobile genetic elements such as transposons, plasmids or integrons (Verraes *et al.,* 2013). Mechanisms accounting for intrinsic resistance could result from spontaneous mutations, occurring in the target gene, which activate or modify chromosomal determinants. Whereas, mechanisms of acquired resistance include chemical modification of the antibiotic, inactivation of the drug as well as a decrease in the internal concentration of the antibiotic through a process known as active efflux (Portillo *et al.,* 2000).

According to Marquez (2005), active efflux is an energy dependent process mediated by efflux pumps: transporter proteins responsible for decreasing the intracellular concentration of an antibiotic by extruding it into the external environment. Although the primary functions and mechanisms of action for most efflux systems remain unclear, as antibiotic susceptible and resistant bacteria carry and express these genes, these proteins are known to actively transport toxic compounds out of the cell (Jonas *et al.,* 2001). Secondly transporters that efflux multiple substrates, including antibiotics, have not evolved in response to the stresses of the antibiotic era but rather all bacterial genomes studied contain several different efflux pumps indicating their ancestral origins (Saier and Paulsen, 2001).

Efflux systems are found in both Gram positive and negative bacteria as well as eukaryotic organisms (Bambeke *et al.,* 2000). In the prokaryotic kingdom, five major families of efflux transporter systems are found, these include the MF (major facilitator), MATE (multidrug and toxic efflux), RND (resistance-nodulation-division), SMR (small multidrug resistance) and ABC (ATP binding cassette) (Köhler *et al.,* 1999). These systems derive their energy source from proton motive force while the ABC family, utilizes ATP hydrolysis.

According to Lee et al. (2000) genes encoding specific efflux pumps are usually found on transmissible plasmids and transposons. Whereas, most efflux pumps genes are encoded on the bacterial chromosome providing the bacterium an intrinsic mechanism that allows survival in a hostile environment (Webber and Piddock, 2003). Furthermore, according to Webber and Piddock (2003) efflux pumps have a ubiquitous nature while they possess a broad substrate range. Thus, these

characteristics make the presence of efflux systems a concern because when over-expression of a pump occurs, resistance to antibiotics of more than one class results (Webber and Piddock, 2003).

1.5.1 *mefA/E*

The mef pump made up of two variants *mefA* and *mefE* confers low and moderate resistance to 14- and 15-membered macrolides (del Grosso *et al.,* 2002). The *mefA* and *mefE* efflux pump genes are part of the major facilitator (MF) superfamily and are amongst the most prevalent transporter proteins in Gram positive bacteria (Poole, 2012). According to Webber and Piddock (2003), the *mefA* gene, encodes a hydrophobic 44.2-kDa protein made up of 12 putative transmembrane stretches. Furthermore, this gene is carried on a 7.2-kb defective transposon containing eight open reading frames (ORFs), with one being a putative site-specific recombinase (Santagati *et al.,* 2000). This gene was first cloned from *Streptococcus pyogenes* while the *mefE* was first identified in *Streptococcus pneumoniae* (Clancy *et al.,* 1997; Tait-Kamradt *et al.,* 1997). The *mefE* efflux gene has a 90% amino acid identity similarity to *mefA* (Roberts *et al.,* 1999). However, the *mefE* gene is carried on a macrolide efflux genetic 5.5 kb mega element containing five ORFs (Santagati *et al.,* 2000). These two mef variants were once considered species specific however; several studies have indicated their presence in group C *Streptococcus* spp. and *Enterococcus faecium* spp. (Kataja *et al.,* 1998; Köhler *et al.,* 1999).

1.5.2 *msrA/msrC*

The *msrA* efflux gene encoded on a plasmid confers resistance to macrolides, and streptogramins (Ross *et al.,* 1990). It is part of the ATP-binding cassette (ABC) family which derive their energy from the hydrolysis of ATP (Köhler *et al.,* 1999). According to Ross et al. (1990), erythromycin resistant isolates harbouring *msrA* genes accumulate less erythromycin than susceptible isolates. Furthermore, the *msrA* and *mefA* efflux genes have shown a strong association with TEM-1 β-lactamase among erythromycin- and ampicillin-resistant clinical isolates of *E. faecalis* (Chouchani *et al.,* 2012).

The *msrC* gene encodes an ABC porter for macrolide and streptogramin B antibiotics (Portillo *et al.,* 2000; Singh *et al*., 2001). Studies have illustrated that the

msrC gene is encoded on a chromosome on the ATP- binding cassette (ABC) transporter (Portillo *et al.,* 2000). This gene is believed to be an intrinsic property of *E. faecium* (Portillo *et al.,* 2000; Singh *et al*., 2001).

In recent times, a gene with a 62% DNA and 72% amino acid similarity with *msrA* gene has been identified (Portillo *et al.,* 2000). This gene has been ascribed *msrC* and encodes an ABC porter for macrolide and streptogramin B antibiotics (Portillo *et al.,* 2000; Singh *et al*., 2001). Studies have illustrated that the *msrC* gene shares a sequence similarity with the *msrA* gene and is also encoded on a chromosome on the ATP- binding cassette (ABC) transporter (Portillo *et al.,* 2000). This gene is believed to be an intrinsic property of *E. faecium* (Portillo *et al.,* 2000; Singh *et al*., 2001).

1.5.3 *tetK and tetL*

The ribosomal protection against tetrcyclines is mediated by *tetM* and *tetO* determinants; however the active efflux mechanism is conferred by *tetK* and *tetL* efflux pump genes (Guay *et al.,* 1993). The *tetK* and *tetL* genes form part of the major facilitator (MF) superfamily and are the major efflux determinants of tetracycline resistance in bacteria (Pankey, 2005; Poole, 2012). The *tetK* gene is located on a plasmid coding for a cytoplasmic membrane protein containing 14 transmembrane helices (Guay *et al.,* 1993). Furthermore, it has largely been identified in clinical isolates (Warsa *et al.,* 1996). A study by Ammor et al. (2008) identified the *tetL* gene located on a 5kb plasmid. The expression of this tet variant is dependent on the synthesis of a 20-amino-acid leader peptide that is encoded 22bp upstream the ribosome binding site (Hoshino *et al.,* 1985). The *tetK* and *tetL* efflux genes have demonstrated their role in tetracycline resistance of *Enterococcus* spp. (Zilhao *et al.,* 1988; Rao *et al.,* 2012).

1.6 Virulence factors

Antibiotic resistance cannot solely explain the virulence of enterococci (Oladipo *et al.,* 2014). Thus, the study of these organisms' virulence mechanisms has also become an important aspect (Semedo *et al.,* 2003). The emergence of infections in a susceptible host is determined by the presence of multiple virulence factors, in pathogenic bacteria, functioning collectively or singularly at various stages of

infection (Wu *et al*., 2008). Virulence factors are gene products, traits or effector molecules produced by pathogens in order to enhance the ability of a microorganism to cause tissue damage, disease and systematic inflammation (Mundy *et al.,* 2000; Vu and Carvhalo, 2011). Virulence factors achieve the latter by assisting pathogens with colonization, immunoevasion as well as immunosuppression (Vu and Carvhalo, 2011). In addition, they interact directly with host tissues and have an ability to mask a bacteria's surface from its host's defence mechanisms (Wu *et al.,* 2008).

Enterococcal virulence was first examined and reported in 1899 (Jett *et al.,* 1994). It is now known that virulence genes are either encoded on transposons or located on plasmids. Over 25 virulence factors have been suggested to be present in enterococci (Franz *et al.,* 2011). Furthermore, virulence in enterococci has largely been associated with *E. faecalis* and *E. faecium* species as a result of their clinical relevance (Ogier and Serror, 2008; Hällgren *et al.,* 2009). A number of virulence factors have been described and studied in enterococci. These include: aggregation substance (*asa1*), cytolysin (*cylA*), enterococcal surface protein (*esp*), gelatinase (*gelE*) and hyaluronidase (*hyl*) (Vanckerhoven *et al.,* 2004)

According to Wu *et al.* (2008), virulence factors are classified based on their mechanism of virulence as well as their function. They either interact directly with host tissues or conceal the bacterial host surface (Fischer and Phillips, 2009). Furthermore, studies have demonstrated that enterococcal virulence factors can contribute to diseases in various ways, these include:

- increasing colonization;
- host tissue adherence and invasion;
- modulation of the host immunity;
- increasing the severity of infection by inducing pathological changes in the host (Fischer and Phillips, 2009).

1.6.1 Aggregation substance

Adherence to host tissues is a vital principle step in the infection process such that colonization, internalization and survival within the host are made possible (Rozdziniski *et al*., 2001). This type of adhesion is facilitated by surface adhesions such as aggregation substance a pheromone-inducible plasmid- encoded surface

protein (Semedo *et al.,* 2003). Aggregation substance facilitates the conjugative transfer of sex pheromone gene-containing plasmids of one *Enterococcus* to another through clumping (Jett *et al.,* 1994). It is the most widely studied adhesion of enterococci as a result of the different functions attributed to it (Semedo *et al.,* 2003).

The augmentation of enterococcal virulence by aggregation substance appears to occur at multiple levels. Aggregation substance plays an important role in the adherence ability of this bacterial group to a variety of eukaryotic (heart endocardial, intestinal and renal) epithelial cells (Kreft *et al.,* 1992; Olmsted *et al.,* 1994).The enhanced attachment ability is important because without these specific attachment means *Enterococcus* isolates would be easily eliminated through the bulk flow of intestinal contents (Saarela *et al.,* 2000). Furthermore, animal model studies have illustrated that this virulence factor has ability to increase the abnormal outgrowth on the heart valves in experimental endocarditis (Jett *et al.,* 1999).

Other reports have suggested that aggregation substance is an important multifunctional virulence factor (Muscholl-Silberhorn *et al.,* 2002). This is supported by reports which show that aggregation substance promotes the survival of enterococci in host immune cells (Kreft *et al.,* 1992; Salminen and von Wright, 2004). Added to its adhesion functions, aggregation substance plays an important role in disseminating other plasmid encoded virulence factors such as the enterococcal cytolysin as well as antibiotic resistant determinants within the species group (Gilmore *et al.,* 2002). Furthermore, cytolysin and aggregation factor have the ability to act synergistically in order to increase enterococcal virulence by facilitating the development of a quorum (Franz *et al.,* 1999; Foulquié Moreno *et al.,* 2006). This is achieved when these two virulence factors activate the quorum sensing mode of cytolysin regulation (Foulquié Moreno *et al.,* 2006). The latter permits deep tissue damage and enhanced tissue invasion (Rakita *et al.,* 1999; Foulquié Moreno *et al.,* 2006).

1.6.2 Cytolysin

The virulence factor cytolysin is a post-translationally modified haemolytic cellular toxin located on pheromone-responsive plasmids (Franz *et al.,* 2001). Nonetheless, this virulence factor is occasionally integrated in bacterial chromosomes and pathogenicity islands (Haas *et al.,* 2002; Shankar *et al.,* 2002). Several studies have

reported that the virulence of enterococci is enhanced by cytolysin as it causes a β-hemolytic reaction on certain blood erythrocytes (Haas and Gilmore, 1999). Some authors have also described it as one of the lethal virulence factors as it can destroy the immune system of its hosts (Coburn and Gilmore, 2003; Franz *et al.,* 2001). This virulence factor is able to rupture the target membranes of various bacterial and eukaryotic cells (Haas and Gilmore, 1999).

The production and secretion of cytolysin is dependent on the expression of the cytolysin operon (Coburn and Gilmore, 2003): This operon is made up of five genes *cylL, cylS, cylM, cylB* and *cylA* (Jett *et al*., 1994). In addition, cytolysin is made up of two components: the lysin (L) and activator (A) (Jett *et al*., 1994). According to Vanckerckhoven *et al.* (2004), cytolysin contributes largely to the severity of enterococcal disease in humans.

1.6.3 Enterococcal surface protein

The virulence factor enterococcal surface protein, encoded by the *esp* gene is one of the most significant genes in *Enterococcus* spp. (Eaton and Gasson, 2001). This gene was first discovered in *E. facealis*, however it has also been identified in *E. faecium* isolates (Di rosa *et al.,* 2006). Furthermore, *esp* has been detected in mammal faeces however, it is more prominent in human faeces (Shankar *et al.,* 2001). *Enterococcu*s spp. harbouring this virulence factor are able to cause nosocomial infections such as urinary tract infections, endocarditis and bacteremia (Kafil and Mobarez, 2015). The *esp* gene enhances the survival ability of enterococci in a host by permitting biofilm production, thus improving its colonization abilities. Hällgren *et al*. (2009) further explains that the hydrophobicity of bacterial cells is increased when the *esp* gene is present. Several studies have hypothesised that *esp* and cytolysin may also have an ability to function synergistically (Gilmore *et al.,* 2002; Ogier and Serror, 2008). This has been deduced on the basis that the *esp* gene and cytolysin operon are located in close proximity and their synergy is similar to that of aggregation substance and cytolysin (Gilmore *et al.,* 2002). This hypothesis is also supported by studies illustraing aggregation substance, cytolysin and enterococcal surface protein to be a part of the pathogenicity island of *E. faecalis* and *E. faecium* spp. (Eaton and Gasson, 2002; Ogier and Serror, 2008).

1.6.4 Hyaluronidase

Hyaluronidase is a virulence factor encoded by chromosomal *hyl* gene (Kayaoglu and Ørstavik, 2004). It is known as the “spreading factor” due to its functionality of spreading bacteria and their toxins in host tissues (Hynes and Walton, 2000). Hyaluronidase is a degradative enzyme that acts on hyaluronic acid (hyaluronate, hyaluronan) associated with causing tissue damage (Kayaoglu and Ørstavik, 2004). This is achieved by the action of hyaluronidase when it depolymerizes the mucopolysaccharide functional region of connective tissues, thus increasing bacterial invasiveness (Kostyukova *et al.,* 1995).

A study by Fitzgerald and Repesh (1987), reported hyaluronidase as a critical causative micro-organism of syphilis due to its role in assisting the dissemination of *Treponema pallidum*. Studies have also suggested that hyaluronidase may also be produced by bacteria which are believed to play a critical role of invasion in the nasopharynx and pneumococcal pneumonia (Berry and Paton, 2000). Hynes and Walton (2000) also suggested that hyaluronidase may play a role in providing bacteria with nutrients. This is because the degradation products of its target substrates are disaccharides which have the ability to be transported and metabolized intracellularly by bacteria (Hynes and Walton, 2000).

1.6.5 Gelatinase

The virulence factor gelatinase is encoded by chromosomal *gelE* an extracellular zinc-endopeptidase. It is located on an operon and acts on various substrates such as the insulin-β chain and haemoglobin (Su *et al.,* 1999; Pillai *et al.*, 2002). Additionally, this virulence factor has the ability to break down host tissues by hydrolysing gelatin, collagen, casein and other peptides (Su *et al.,* 1999). Furthermore, it has shown to be involved in enterococcal invasion of the immune systems of the host (Park *et al.,* 2008). When this virulence factor is expressed, it is able to cleave the fibrin layer surrounding bacteria thus permitting an enhanced dissemination of the organism (Waters *et al.,* 2003). Gelatinase has been reported mainly in *E. faecalis* and *E. faecium* isolates (Macedo *et al.,* 2011). Furthermore, it has also been identified in both clinical and environmental enterococcal isolates

(Franz *et al.,* 2001). The phenotypic expression of this gene is, however, more active in clinical than environmental isolates (Semedo *et al.,* 2003).

1.7 Genotypic methods for characterization of enterococci

According to Moore *et al*. (2006) culture based methods are often time consuming and difficult. Furthermore, the inability to detect non-culturable as well as specific environmental *Enterococcus* spp. has rendered this technique limiting (Ryu *et al.,* 2013). Thus, full and partial 16S rRNA gene sequencing methods have emerged as useful tools for identifying microorganisms (Petti *et al.,* 2005).

Ryu *et al.* (2013) explains that multiple copies of 16S and 23S rRNA genes are found in the genome of most bacterial species. Thus, using these genes can prove advantageous by enhancing the detection of environmental *Enterococcus* spp. (Maheux *et al.,* 2011). However there is a larger 16S rRNA gene database as compared to the 23S rRNA component (Ryu *et al.,* 2013). Thus most studies have focused on 16S rRNA gene sequencing which allows for robust sequence validation (Ryu *et al.,* 2013). Furthermore, 16S rRNA sequencing has been widely used as a result of its accuracy and objective manner of identifying microorganisms (Facklam and Elliott, 1995; Clarridge, 2004; Manero *et al.,* 2006).

PCR has been used to confirm the presence of *Enterococcus* spp. in environmental and food studies (Domingo *et al.,* 2003; Macovei and Zurek, 2006). Furthermore, this technique has been used to detect enterococcal diversity in environmental water systems (Badgley *et al.,* 2010; Moore *et al.,* 2008). The detection of *Enterococcus* spp. prevalence is particularly important for identifying sources of faecal pollution (Wheeler *et al.,* 2002).

1.8 Problem statement

Water sources in the North West Province comprise groundwater, surface water, imported water as well as re-useable effluent (Kalule-Sabiti and Heath, 2008). However, significant water quantity and quality challenges are experienced in this province. Despite improved infrastructure, water in the North West Province is a limited resource and the consequences of demand exceeding supply come at a great cost (NWP-SoER, 2002; DWAF, 2004; DWA, 2013). Eighty percent of the poor and marginalized in the rural areas of the province experience water scarcity most

intensely because groundwater sources account as their sole reliable source of domestic water supply (Kalule-Sabiti and Heath, 2008). Furthermore, surface water systems in the province are non-perennial and a large portion of the available surface water contributes to the mining, agricultural, industrial and property sectors (Kalule-Sabiti and Heath, 2008; NWDACE-SoER, 2008). While the provinces surface water resources are used for a wide variety of activities such as full contact water sports and recreational activities; these surface waters also play a central role in many cultural ceremonies (Zenani and Mistri, 2005).

Although mining and agriculture contribute largely to the country's as well as the province's economies, they are amongst the main contributors of water quality problems experienced in the North West Province (DWA, 2012; NWDAC-SoER, 2008). Large amounts of faecal matter and chemical compounds from the mining and agricultural sectors pollute the provinces water systems (Kalule-Sabiti and Heath, 2008).

Several studies have indicated the presence of opportunistic pathogenic enterococci such as *E. faecalis* and *E. faecium* in surface and groundwater sources of the North West Province (Ateba and Maribeng, 2011; Ferreira, 2011; Molale, 2012). Enterococci are facultative anaerobic Gram-positive cocci that naturally inhabit the gastrointestinal tract, oral as well as vaginal cavity of humans (Kayaoglu and Ørstavik, 2004).These organisms cause a variety of infections in humans including: urinary tract, bloodstream, endocardium, abdomen, biliary tract, burn wounds, indwelling foreign device, soft tissue and pelvic infections (Jett *et al.,* 1994; Semedo *et al.,* 2003). Therefore, the presence of enterococci in water systems used for a wide range of activities is a cause for concern when considering their medical relevance. This is because enterococci have an increased incidence of nosocomial infections and their ability to cause diseases is heightened when their resistance to multiple antibiotics is considered (Jett *et al.,* 1994).

Enterococcal antibiotic resistance has spread rapidly over the years making it an important public health concern (Iweriebor *et al.,* 2015b). Contrary to the limited enzymatic and cellular targets of antibiotics, bacteria possess versatile mechanisms with which their resistance to antibiotics is associated (Köhler *et al.,* 1999).The biochemical mechanisms for antimicrobial resistance are divided into three main

categories. These include: alteration of the antibiotic's target, inactivation of the antibiotic as well as the active efflux of the antibiotic from the target (Portillo *et al.,* 2000). Active efflux is the mechanism of interest in this study.

Active efflux is an energy dependant process where bacteria limit the internal concentration of antibiotics by extruding the antibiotics outside the cell by means of efflux pumps (Li and Nikaido, 2004).The main problem with enterococci that have efflux pumps is that, over-expression of these ubiquitous transport proteins results in the resistance of enterococci to a wide variety of antibiotics (Webber and Piddock, 2003). For this reason, the study of these organisms' virulence mechanisms has also become an important aspect (Semedo *et al.,* 2003). The ability for a pathogenic bacterium to establish infection in a susceptible host is determined by multiple virulence factors (Wu *et al.,* 2008).

The potential presence of *Enterococcus spp.* that have virulence factors and efflux pump systems coding for antibiotic resistance, in water sources used for various recreational activities, religious and cultural practices as well as domestic purposes in the North West Province is worrisome. Particularly because the increased resistance of enterococci to multiple antibiotics has made treatment of these microorganisms difficult (Dennesen *et al.,* 1998; Murray, 1990). Therefore, the microbial safety of these water systems cannot be overemphasised, more so in this era where a large proportion of communities are HIV positive (Momba *et al.,* 2010).

The aim of this study was thus to characterize *Enterococcus spp.* isolated from various surface systems as well as effluent from waste water treatment plants in the North West Province, that decant into these water sources. The objectives were to:

(i) Determine presence of virulence genes and production of extracellular enzymes in *Enterococcus* spp. from surface water sources

(ii) Investigate the prevalence, antibiotic resistance and virulence of *Enterococcus* spp. from WWTP effluent and receiving waters in the North West Province, South Africa

(iii) Study antibiotic resistance, efflux pump genes and virulence determinants in *Enterococcus* spp. from surface water systems

1.9 Outline of the thesis

Chapter 1 provides an overview on the current state of surface water systems in the North West Province and South Africa. In addition it provides information on enterococci, their taxonomy, clinical significance, antibiotic resistance efflux as well as virulence mechanisms. It concludes with a problem statement and provides the current study's research hypothesis. Thereafter, the general aim and specific objectives of the current study are presented.

Chapter 2 reports the presence of *Enterococcus* spp. harbouring selected virulence genes in environmental waters in the North West Province of South Africa. In addition, the relationship between the virulence genetic determinants and their expression was reported. The presence of virulence genes was acheived by PCR whereas the expression potential of three virulence genes was determined using biochemical methods.

Title: Virulence determinants and production of extracellular enzymes in *Enterococcus* spp. from surface water sources

Authors: Molale, L., Bezuidenhout, C.C.

Journal: Water Science and Technology

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Chapter 3 describes the antimicrobial resistance patterns and presence of virulence genes in *Enterococcus* spp. isolated from three municipal WWTPs final effluents and receiving waters in the North West Province, South Africa. The disk diffusion method was used to determine susceptibility patterns while the antibiotic inhibition zone diameter data was subject to cluster analysis. Furthermore, the *Enterococcus* spp. were screened for the presence of plasmids. Plasmid curing was used to determine whether these were responsible for multiple antibiotic resistance. Additionally, polymerase chain reaction was used to screen WWTP isolated *Enterococcus* spp. for the presence of five clinically relevant virulence determinants.

Title: Prevalence, antibiotic resistance and virulence of *Enterococcus* spp. from WWTP effluent and receiving waters in the North West Province, South Africa

Authors: Molale, L., Bezuidenhout, C.C.

Journal: International Journal of Environmental Research and Public Health

Manuscript number ijerph-105969

Chapter 4 reports the antibiotic susceptibility patterns and highlights the presence of efflux pump genes and virulence genetic determinants in *Enterococcus* spp. isolated form a five of socio-economically important surface water systems in the North West Province. The presence of efflux pump genes and virulence factors was determined using PCR. Where as antibiotic susceptibility patterns were determined using the disk diffusion method.

Title: Antibiotic resistance, efflux pump genes and virulence determinants in *Enterococcus* spp. from surface water systems

Authors: Molale, L., Bezuidenhout, C.C.

Target Journal: Journal of Water and Health

Overlaps in the study were unavoidable, particularly in Chapter 2 and 4.

Chapter 5 provides a summary of the relevant conclusions of the current study. Furthermore, substantial recommendations for future research in this field are provided.

CHAPTER 2

Virulence determinants and production of extracellular enzymes in *Enterococcus* spp. from surface water sources

2.1 Introduction

Enterococcus spp. are natural inhabitants of the intestinal flora of warm blooded animals and humans (Lanthier *et al.,* 2010). However, their presence in soil, surface waters, plants and vegetables has also been reported (Micallef *et al*., 2013). This ubiquitous nature of *Enterococcus* is a result of several intrinsic traits that enable their survival and adaption under harsh environments (Teixeira and Merquior, 2013). These traits are attributed to their possession of easily transferable genes and highly effective gene transfer mechanisms such as conjugation and conjugative transposition (Eaton and Gasson, 2001). According to Johnson (1994), the ability of *Enterococcus* to acquire and share elements encoding virulence traits has resulted in their increased importance as pathogens.

Virulence factors have been described as gene products that increase the ability of microorganisms to cause disease beyond the microorganism's intrinsic properties (Mundy *et al.,* 2000). Furthermore, they enhance the pathogenicity of a microorganism by permitting adhesion and invasion to a hosts' tissue, abscess formation, translocation through epithelial cells, evading host immune responses and secretion of toxic products (Eaton and Gasson, 2001). Studies of *Enterococcus* virulence mechanisms have become increasingly important because infections caused by this group of bacteria have, over the years, proven difficult to treat (Semedo *et al.,* 2003).

Various studies have reported the presence of virulence factors such as aggregation substance, gelatinase, cytolysin, enterococccal surface protein and hyaluronidase in *Enterococcus* spp. (Eaton and Gason 2001; Mundy *et al.,* 2000; Mannu *et al.,* 2003). However, majority of these studies have linked the subject of virulence to *E. faecalis*

and *E. faecium* because of their significance in the clinical setting (Eaton and Gasson *et al.,* 2001; Ogier and Serror, 2008). Nonetheless, reports of infection caused by other *Enterococcus* spp. such as *E. gallinarum*, *E. caseliflavus*, *E. mundtii*, and *E. hirae* have occasionally been documented (Ahmed *et al.,* 2012; Mundy *et al.,* 2000; Sidhu *et al.,* 2014). The use of *Enterococcus* as indicators of faecal pollution is well documented and their presence in recreational and marine waters correlates with health risks (Cabelli *et al.,* 1983). However, the relationship between the presence of virulent genes in *Enterococcus* spp. isolated from fresh water sources and whether these genes are expressed remains largely unknown (Sidhu *et al.,* 2014).

The aim of this study was to determine the presence of five clinically relevant virulence genes (*asa1, cylA, esp, hyl* and *gelE*) in *Enterococcus* spp. isolated from environmental water sources in the North West Province, South Africa. In addition, the expression of three genes (*hyl, cylA* and *gelE*) was determined. This will aid in determining the potential pathogenicity of *Enterococcus* spp. isolated from environmental water sources and whether these may pose health risks to users.

2.2 Materials and methods

2.2.1 Bacterial strains

A total of 124 *Enterococcus* isolates were investigated in this study. Eighty *Enterococcus* isolates were obtained from five surface water systems located in the North West Province, South Africa during 2010 and 2011. Furthermore, and 44 *Enterococcus* isolates were obtained from the Upper Harts River in 2014. These included four rivers (Harts, Vaal, Mooi, Schoonspruit rivers) and an inland lake (Baberspan). In addition, all isolates were identified to species level by phenotypic and molecular methods (Molale, 2012). *Enterococcus* spp. analysed during this study included *E. faecium* 30 (24%)*, E. faecalis* 37 (30%)*, E. mundtii* 36 (29%), *E. casseliflavus* 14 (11%)*, E. gallinarum* 5 (4%), *E. hirae* 1 (0.8%) and *E. sulfureus* 1 (0.8%).

2.2.2 Detection of virulence genes using PCR

Sequences of the virulence genes specific primers used in this study were from Vankerckhoven *et al*. (2004). The expected amplicon sizes of interest as well as all five oligonucleotide primer pairs (Applied Biosystems, UK) used to amplify the genes *asa1*, *cylA, esp*, *gelE*, and *hyl* are listed in Table 1.

Table 1: PCR primer sequences and products used in this study for verification of virulence determinants.

Virulence factor	Gene	Oligonucleotide Sequence (5'-3')	Product size (bp)
Aggregation substance	*asa1*	**ASA 11F-** GCACGCTATTACGAACTATGA **ASA 12R-** TAAGAAAGAACATCACCACGA	375
Cytolysin	*cylA*	**CYT IF -** ACTCGGGGATTGATAGGC **CYT IIbR -** GCTGCTAAAGCTGCGCTT	688
Enterococcal surface protein	*esp*	**ESP 14F -** AGATTTCATCTTTGATTCTTGG **ESP 12R -** AATTGATTCTTTAGCATCTGG	510
Gelatinase	*gelE*	**GEL 11F -** TATGACAATGCTTTTTGGGAT **GEL 12R -** AGATGCACCCGAAATAATATA	213
Hyaluronidase	*hyl*	**HYL n1F -** ACAGAAGAGCTGCAGGAAATG **Hyl n2R -** GACTGACGTCCAAGTTTCCAA	276

Identification of virulence genes for each isolate was performed by separate polymerase chain reaction (PCR). PCR amplifications were performed in a Techne Prime Elite thermocycler (Cambridge, UK), in 0.2 ml reaction tubes. Each PCR assay was performed in a total volume of 25 µl containing 1µl bacterial DNA template (50-100 ng/µl), RNase/DNase free water (Fermentas Life Sciences, US), 2x DreamTaq PCR Master Mix (0.05 U/µL *Taq* DNA polymerase in reaction buffer, 0.4 mM of each dNTP and 4 mM MgCl2), 0.2 µM of primers *asa1* and *gelE* as well as 0.4 µM of primers *cylA*, *esp*, and *hyl*. Samples were amplified by denaturing at 95°C for 180 seconds, followed by 30 cycles of 95°C for 30 seconds, annealing at 56˚C for 30 seconds and at 72°C for 60 seconds. This was followed by a final step of 72°C for 600 seconds. PCR products were confirmed by gel electrophoresis using a 1% (w/v) agarose gel in 1 x TAE buffer (40 mM Tris, 20 mM Acetic acid, 1 mM EDTA, pH 8.0)

at 60 V for 90 minutes. A 100bp molecular weight marker was used (O'GeneRulerTM 100bp DNA ladder, Fermentas Life Sciences, US). Agarose gel images were captured under UV light using a Bio-Rad ChemiDoc imaging system (Hercules, CA).

2.2.3 Sequence validation

The PCR products of representative isolates that were positive for the virulence genes *asa1, cylA, gelE* and *hyl* were purified as described by Li *et al* (2010) followed by using the ZR DNA sequencing clean-up kit (Zymo Research, USA) according to the instructions of the manufacturer. Sequencing was performed and subsequent analysis was performed as described in Jordaan and Bezuidenhout (2013). Sequences were submitted to the GenBank database under accession numbers: KT598460 – KT98465 and KT724720 - KT724721.

2.2.4 Phenotypic assays

2.2.4.1 Cytolysin

Cytolysin production was evaluated by observing β-haemolysis on blood agar plates. The haemolysis test was performed as described by Frobisher *et al*. (1928). Briefly, purified overnight cultures were spot inoculated on 5% (v/v) sheep blood agar plates (National Health Laboratories, SA) and incubated at 37°C for 24 hours. The β-haemolysis isolates caused complete cell lysis and were identified by a clear zone of hydrolysis around the colonies where inoculation occurred (Health Protection Agency, 2008).

2.2.4.2 Gelatinase

The production of gelatinase was determined by the gelatin liquefaction protocol. Briefly, purified 18 hour streak plate cultures were stab inoculated into nutrient agar slants (Merck, Germany) supplemented with 12% gelatin (Oxoid, UK). After incubation at 28°C for seven days, the tubes were chilled for 30 min in cold conditions (5-10°C). Cultures that remained liquefied were considered positive for gelatin hydrolysis (dela Cruz and Torres, 2012).

2.2.4.3 Hyaluronidase

Hyaluronidase production was determined by spot inoculations on Brain Heart Infusion Broth (Merck, Germany) supplemented with 1.0 g of Noble agar (Difco Laboratories, US) per 100 ml. Furthermore, an aqueous solution containing 5% (w/v) bovine albumin fraction V (Sigma Aldrich, US) and 2 mg/ml of hyaluronic acid (Sigma Aldrich, US) was filtered using minisart filters (0.20 µm; Separations, US) and added to the media. A zone of clearing around the bacterial colonies was regarded a positive result (Hynes and Walton, 2000).

2.2.5 Statistical analysis

Comparison of all *Enterococcus* spp. and the presence of virulent genes was achieved by converting data per enterococcus isolate into binary variables according to presence (1) and absence (0). These were ordered in Microsoft Office Excel 2007. All statistical analyses and graphs were performed using Statistica 12.0 (StatSoft, US). The statistical association of *Enterococcus* spp. and virulence genes was determined using basic statistics correlation matrices and marked correlations were significant at $P < 0.05$ and $P < 0.001$.

2.3 Results

PCR amplification and agarose gels were used to analyse the DNA of 124 *Enterococcus* isolates for the presence of the following virulence genes: *asa1, cylA, esp, gelE* and *hyl*. Figure 1 is a negative image of an agarose gel illustrating the PCR fragments of the 4 virulence genes identified in this study. The image also indicated the expected fragment lengths. No primer dimers or non-specific products were observed in any of the products. Furthermore, representative *Enterococcus* isolates positive for virulence the genes *asa1, cylA, gelE* and *hyl* were sequenced and submitted to the GenBank database under accession numbers: KT598460 – KT98465.

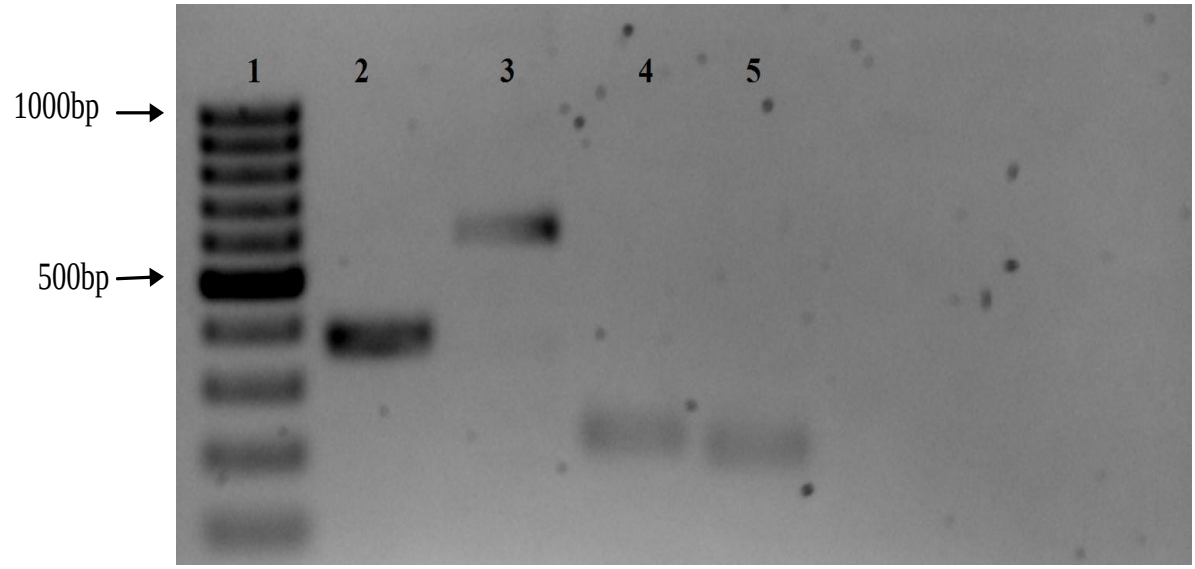

Figure 1: A negative image of a 1% agarose gel indicating the amplified virulence gene fragments. A 100bp molecular weight marker was used in lane 1. Lane 2 *asa1*(375bp); lane 3 *cylA* (688bp), lane 4 *hyl* (276bp) and lane 5 *gelE* (213bp).

2.3.1 Occurrence of 5 virulence determinants in *Enterococcus* spp.

The *Enterococcus* spp. of interest comprised 30 *Enterococcus faecium*, 37 *E. faecalis*, 5 *E. gallinarum*, 14 *E. casseliflavus*, 36 *E. mundtii,* 1 *E. hirae* and 1 *E. sulfureus* isolate. Of the 124 *Enterococcus* spp. screened, 81 (65%) harboured at least one virulence gene. While 16 (13%) carried two or more virulence genes.

Prevalence levels of virulence genes per species are listed in Table 2. In addition, this table also illustrates the statistical association of the screened *Enterococcus* spp. with virulence genes. As depicted in Table 2, the most frequently detected virulence determinants were *gelE* and *cylA*. These genes accounted for 60% of the detected virulence determinants. Furthermore, virulence genes *asa1* and *hyl* were also detected, though at lower levels. However, enterococcal surface protein (*esp*) was not detected in any of the *Enterococcus* spp. screened.

A high prevalence of virulence genes was observed in *E. faecalis* isolates proceeded by *E. faecium.* The virulence gene *cylA* was present in 6 (86%) *Enterococcus* spp. and predominantly carried by *E. gallinarum* and *E. casseliflavus* spp. (Table 2). More so, the presence of *cylA* was statistically significant in four of the six species it was detected in (Table 2). The virulence genes *asa1*, *gelE* and *hyl* were each detected in 4 (57%) *Enterococcus* spp (Table 2). Furthermore, the presence of *asa1* was

significantly higher in *E. mundtii* isolates compared to the others (Table 2). Whereas, the presence of *gelE* was frequently and significantly detected in *E. faecalis* spp. Lastly, a statistically significant high frequency of the *hyl* gene was observed in *E. gallinarum* spp.

2.3.2 Production of extracellular enzymes

The production of three extracellular enzymes (haemolysin, gelatinase and hyaluronidase) was determined in the *Enterococcus* isolates. As depicted in Table 2, the most frequently detected extracellular enzymes were β-haemolysis and gelatinase. Haemolysin activity was observed in four *Enterococcus* spp. representing 57% of the isolates. However, only two of the *Enterococcus* spp., representing 29% of the isolates produced gelatinase and hyalunronidase (Table 2). Furthermore, haemolysin activity was predominantly observed in *E. gallinarum* spp, however, this characteristic also showed a statistically significant association with *E. casseliflavus* and *E. mundtii* spp. (Table 2). On the other hand, gelatinase production was statistically significant amongst *E. faecalis* as compared to the other *Enterococcus* spp.

Table 2: Observed presence of virulence determinants and the production of extracellular enzymes in seven *Enterococcus* spp.

		Virulence genes					**Extracellular enzyme activity**		
Species	No. of isolates (N)	*asa1*	*cylA*	*esp*	*gelE*	*hyl*	*β*-haemolysis	*Gelatinase*	*Hyaluronidase*
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
E. faecium	30	3 (10.0)	12 (40.0) *	0	11 (37.0)	3 (10.0)	2 (6.0)	4 (13.3)	1 (3.3)
E. faecalis	37	4 (11.0)	3 (8.1)*	0	25 (68.0)**	1 (3.0)	2 (5.0)	5 (14.0)**	1 (3.0)
E. gallinarum	5	0	4 (80.0)**	0	0	4 (80.0)**	4 (80.0)**	0	0
E. casseliflavus	14	1 (7.1)	11 (79.0)**	0	1 (7.1)	1 (7.1)	6 (43.0)**	0	0
E. mundtii	36	8 (22.0)*	4 (11.0)	0	3 (8.3)	0	0	0	0
E. hirae	1	0	1 (100.0)	0	0	0	0	0	0
E. sulfureus	1	0	0	0	0	0	0	0	0
Total	**124**	**16**	**35**	**0**	**40**	**9**	**14**	**9**	**2**

Percentage (%) was determined as a function of the number of isolates for the specific *Enterococcus* spp. thus ($\frac{n}{N}$ x 100)**;** Correlation significance: ** p <.001, *p <0:05.

2.3.3 Presence of virulence genes and production of extracellular enzymes

The presence of virulence genes and production of extracellular enzymes was analysed. Figure 2, is a comparative analysis of the presence of virulence genes and their expression and non-expression.

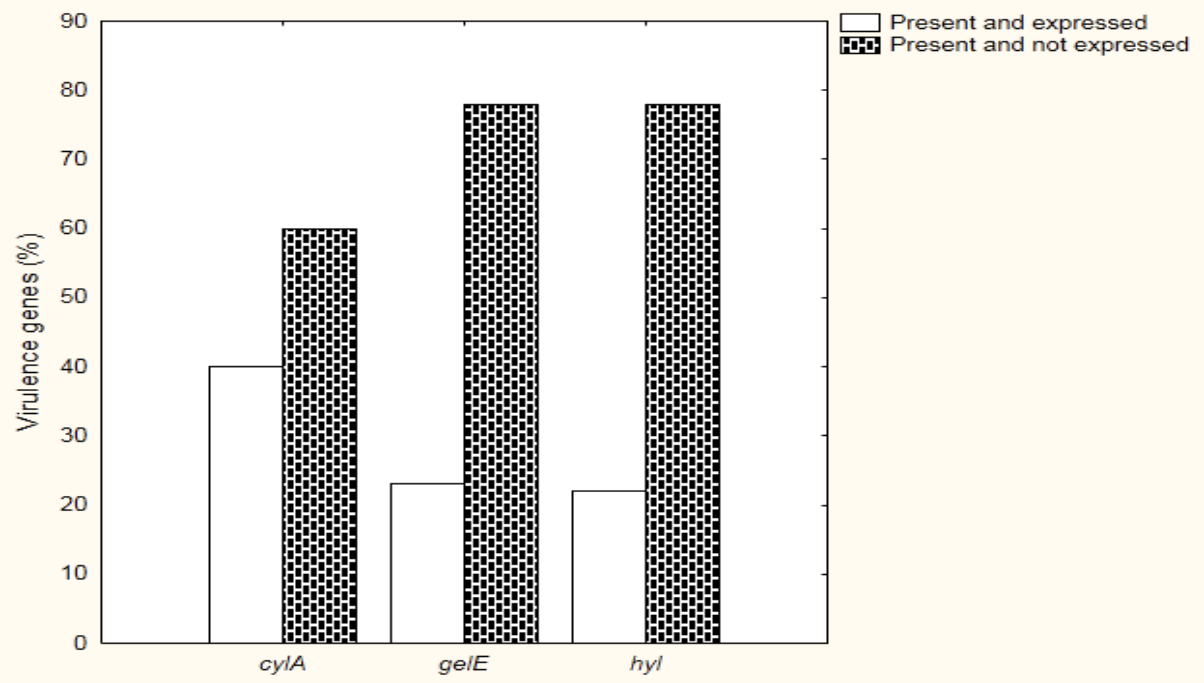

Figure 2: Comparative illustration of the presence of virulence genes and their expression as well as non-expression.

Twenty five (29%) of the *Enterococcus* spp. carrying virulence genes also produced the respective extracellular enzymes. Of the three genes screened for the production of extracellular enzymes, the *cylA* gene was expressed by 40% of the *Enterococcus* spp. that were positive for this gene. Whereas, only 20% of the isolates that were positive for the *gelE* and *hyl* genes also produced gelatinase and hyaluronidase.

2.4 Discussion

In spite of major improvements in water and wastewater treatments as well as recommendations made by regulatory bodies, several studies in South Africa have reported the poor operational state of waste water treatment plants resulting in pollution of receiving water bodies (Momba 2009; Hendriks and Pool, 2012). Additionally, studies reporting the discharge of wastewaters containing significant

pathogenic micro-organisms into receiving water bodies have indicated that this is a worldwide problem (Okoh *et al.,* 2007).

Water systems receiving wastewater effluents may be used for agricultural, recreational as well as industrial purposes, land application or recycled and used as drinking water (Weinberg *et al.,* 2004; Sidhu *et al.,* 2014). Exposure to environmental surface water systems contaminated by various sources of pollution could lead to transmission of potentially pathogenic multiple antibiotic resistant *Enterococcus* spp. which have previously been isolated from surface water systems of the North West Province (Molale, 2012).

In the present study, 124 *Enterococcus* spp. isolated from environmental surface water systems in the North West Province, South Africa, were screened for the presence of five clinically relevant virulence factors (*asa1, cylA, esp, gelE* and *hyl*). The high incidence of *gelE* genes in *Enterococcus* isolates screened in this study is in accordance with several other studies (Eaton and Gasson, 2001; Doğru *et al.,* 2010; Di Cesare *et al.,* 2014). Furthermore, the observation of *gelE* positive isolates being significantly more common in *E. faecalis* isolates agrees with the findings of similar other studies (Ahmed *et al.,* 2012; Hammad *et al.,* 2014; Sidhu *et al.,* 2014). In the case of the present study *gelE* detection was not confined to *E. faecalis*. It was also associated with several *Enterococcus* spp. This is supported by several studies that reported the presence of *gelE* genes also in *E. faecium, E. mundtii, E. durans* and *E. casseliflavus* (Macedo *et al.,* 2011; Sidhu *et al.,* 2014).

The high prevalence of *cylA* positive *Enterococcus* spp. identified in this study is in contrast to numerous studies where no or very few *cylA* genes were detected in *Enterococcus* isolates screened (Lanthier *et al.,* 2010; Ahmed *et al.,* 2012; Iweriebor *et al.,* 2015a). However, the presence of the *cylA* gene amongst six of the seven *Enterococcus* spp. that were screened in the present study confirms an observation by Semedo et al*.* (2003) that *cylA* genes are widely distributed throughout the *Enterococcus* genus. A study by Ahmad *et al*. (2014) predicted that *cylA* positive isolates from river water systems are a result of urban flow. They advocated the use of this gene as a marker for human faecal pollution. Taking this into account suggests that isolates in the present study could have originated from human faecal matter. This is an aspect that should be further investigated.

Similar to the findings of Sidhu *et al*. (2014) the plasmid bound *asa1* gene was more frequently prevalent in *E. faecalis* and *E. mundtii* isolates. In the present study this gene was also detected in *E. faecium* and *E. casseliflavus.* These findings support suggestions of previous studies in that the *asa1* gene has a significant distribution amongst *Enterococcus* spp. isolated from fresh water sources (Macedo *et al.,* 2011; Sidhu *et al.,* 2014). Since this gene is located on a plasmid (Sidhu *et al.,* 2014) it may explain the wide distribution thereof among *Enterococcu*s spp. from water systems as plasmids provide a means for the dissemination of such genes among aquatic bacteria.

The *hyl* gene was detected among various *Enterococcus* species (Table 2). This is in accordance with the findings of Gonçalves *et al.* (2011) and Trivedi *et al.* (2011). The latter authors showed that *E. faecalis, E. casseliflavus, E. mundtii, E. durans* and *E. gallinarum* could all be associated with carrying this gene. However, the low prevalence of the *hyl* gene among environmental isolates should not be regarded as uncommon. Even in the case of clinically relevant isolates, Rice *et al*. (2003) reported lower prevalence levels of *hyl* positive *Enterococcus* spp. from community acquired isolates when compared to isolates from a hospital environment. In the present study, none of the 36 *E. mundtii* isolates tested were positive for the *hyl* gene. This may not be surprising as recent studies of Iweriebor *et al.* (2015ab) demonstrated that various *Enterococcus* spp. isolated from two different environments in South Africa were also negative for the *hyl* gene.

The *esp* gene was not detected amongst any of the various environmental *Enterococcus* isolates. This gene is associated with a 150-kb putative pathogenicity island (PAI) usually found in clinical isolates (Leavis *et al.,* 2004) contributing to urinary tract infections (Shankar *et al.,* 2001). In various previous studies, *esp* positive *Enterococcus* sp. were isolated from human sewage, faecal samples, fresh water, wastewater and septic samples (Scott *et al.,* 2005; Sidhu *et al.,* 2014; Iweriebor *et al.,* 2015b). These were all associated with severe human faecal pollution. Not finding the *esp* gene amongst any of the 124 *Enterococcus* spp. suggests that (i) the isolates were not from fresh human faecal pollution events or (ii) that the gene PAI was absent or (iii) that it was incomplete. This is in agreement with the findings of Shankar *et al*. (2006) who illustrated that esp-negative isolates can contain fragments of the PAI. Furthermore, Shankar *et al*. (2006) demonstrated

variation in the genetic content of the PAI. Thus, the absence of the esp gene could imply that the PAI, if present, was incomplete.

2.4.1 Presence of virulence genes and production of extracellular enzymes

Forty percent of the *cylA* gene carrying isolates produced β-haemolysis on blood agar. The low β-haemolysis positive isolates from this study are similar to results of Sidhu *et al*. (2014). The latter authors suggested the low spread of β-haemolysis activity in *Enterococcus* spp. isolated from water sources. Furthermore, it has been demonstrated that cytolysin expression requires the products of eight genes on the *cyl*$L_L L_S$ABM operon (Semedo *et al.,* 2003; Poeta *et al.,* 2008). It is thus possible that isolates incapable of producing β-haemolysis in this study did not harbour all genes of the *cyl*$L_L L_S$ABM operon. However, according to Franz *et al*. (1999), the absence of haemolytic activity in *Enterococcus* isolates does not mean they are not virulent. This is in agreement with a study by Macovei and Zurek (2006) which showed a 100% correlation between *cylA* and β-haemolysis on human blood as opposed to cattle blood. In the present study, sheep blood agar plates were used and this could have played a role in the low levels of β-haemolysis production.

Similar to the findings of Creti *et al*. (2004), the presence of *gelE* positive *Enterococcus* spp. in this study was not correlated with the production of gelatinase. Eaton and Gasson (2001) suggest that this minimal expression of the *gelE* gene is due to existence of silent *gelE* genes. Furthermore, it has been illustrated that the presence of the *gelE* gene is not enough for gelatinase production if the 23.9-kb region including most of the fsr locus, a positive gelE expression regulator, is absent (Bourgogne *et al.,* 2006). Thus, in the present study, the expression regulator might have been absent or repressed among the gelatinase negative isolates.

Hyaluronidase activity was observed among 22% of the *hyl* gene carrying isolates. According to Eaton and Gasson (2001) the absence of phenotypic activity in virulence gene carrying isolates may be a result of down regulation of gene expression or may be indicative of an inactive gene product. These two processes could have been responsible for the low hyaluronidase activity among the *hyl* gene carriying isolates from the present study.

2.5 Conclusions

Water pollution is amongst the key environmental problems experienced in South Africa. Municipal WWTPs not working efficiently or fully operational are amongst the many sources responsible for polluting environmental water systems by spilling poor quality effluents into receiving surface water bodies. This results in the degradation of the county's surface water systems, which support various social needs. In this study, PCR was used to screen for the presence of five clinically relevant virulence factors among seven species from the genes of *Enterococcus* isolated from surface water systems of the North West Province, South Africa. Four of these factors were detected among the various species and showed statistical significant associations with the species. The isolates were also tested to determine if three of the genes (*cylA, gelE* and *hyl*) are expressed into functional units that could be associated with virulence. The number of isolates that were positive for these three virulence factors were much lower than those that produced the functional gene products causing the phenotypic characteristics. There could be various reasons for the non-expression of the phenotypic trait, among those, repression of the gene or absence of critical factors that may be present/provided under *in vivo* conditions. Finding these genes amongst a large number of *Enterococcus* spp. isolated from surface water may thus constitute a human infection risk. On the other hand, if these genes are not directly involved in causing infections, their presence in surface water should be considered a cause for concern. If these genes are associated with mobile elements, such as plasmids, in environmental populations; environmental *Enterococcus* spp. could act as reservoirs and sources of such virulence genes for dissemination. Dissemination of such carriers of virulence genes in environmental surface water sources that are used for various agricultural, religious, cultural and recreational activities expose those individuals and animals to serious health risks. It is thus recommended that recreational and agricultural water be regularly tested for the presence of *Enterococcus* and that appropriate intervention methods be put in place if the water does not comply with specified standards.

CHAPTER 3

Prevalence, antibiotic resistance and virulence of *Enterococcus* spp. from WWTP effluent and receiving waters in the North West Province, South Africa

3.1 Introduction

According to the River Health Programme (DWA, 2011), South African rivers provide services important for human survival. Also, these rivers support social needs such as recreational and religious activities as well as subsistence fishing while also playing an important economic role in agricultural and industrial production. To date, *E. coli* is used as faecal indicator organism in South Africa despite current doubts regarding its applicability as an indicator of faecal pollution (Murray *et al.,* 2004 Boehm *et al*., 2009). The WHO (2008) has suggested enterococci to be used as a supplementary indicator as they have proven to be more reliable than *E. coli*.

Enterococci are a diverse, complex and important group of bacteria with regard to their interaction with humans. They are commensal organisms in the mammalian gastrointestinal tract but are also found in soil, water and on plants (Byappanahalli *et al.,* 2011). Despite being harmless in vivo, their spread from the intestinal tract to other mucosal and skin surfaces can result in them causing disease (Vu and Carvalho *et al.,* 2011). It has been reported that *Enterococcus* spp. are the most frequent causative agents of a variety of infections in humans including: urinary tract, bloodstream, endocardium, abdomen, biliary tract, burn wounds, indwelling foreign device, soft tissue and pelvic infections (Jett *et al.,* 1994). According to Teixeria and Merquior (2013), enterococci are amongst the leading therapeutic challenges with regard to life-threatening infections and are becoming significant pathogens worldwide. For this reason, the study of these organisms' virulence mechanisms has also become an important aspect (Semedo *et al.,* 2003). Studying these organisms' virulence mechanisms allows for determining their ability to establish infection in a susceptible host (Wu *et al.,* 2008).

Enterococci have been exposed to multiple antibiotics in the hospital setting that have provided them an evolutionary pressure for selection. Their resistance to various antibiotics allows them to survive in the hospital environment which provides

an opportunity for their dissemination (Murray, 1990). This dissemination can be via storm events which are capable of mobilizing enteric pathogens from various point and non-point sources such as WWTPs and animal feeds (Sidhu *et al.,* 2014). Furthermore, it has been reported that many WWTPs in South Africa are not functioning efficiently (DWA: 2012). Consequently, the WWTPs don't remove pollutants adequately allowing for bacteria as well as antimicrobial residues to enter receiving environmental water bodies (Michael *et al.,* 2013).

Several studies have reported the presence, emergence as well as outbreak of antibiotic resistant clinical enterococci in South Africa (McCarthy *et al.,* 2000; von Gottberg *et al.,* 2000); while only one reported the presence of antibiotic resistant *Enterococcus* spp. in hospital and domestic wastewater effluents (Iweriebor *et al.,* 2015b). Therefore, there is paucity of data on susceptibility patterns and epidemiology of antibiotic resistant enterococci isolated from environmental sources in South Africa. The presence of enterococci exhibiting antibiotic resistance and harbouring clinically relevant virulence genes is of particular interest due to the possible link of community acquired enterococcal infections and recreational activities. The aim of this study was to determine the antimicrobial resistance patterns and presence of virulence genes in *Enterococcus* spp. isolated from three municipal WWTPs final effluent as well as receiving environmental water systems in the North West Province, South Africa.

3.2 Materials and methods

3.2.1 Sample collection and bacterial isolation

Three WWTPs in the North West Province which receive wastewater from urban households, industries, farms and hospitals were sampled. All samples were collected at the final effluent, points downstream and a point between two of the WWTPs. The GPS co-ordinates illustrating the position of the three WWTPs are displayed in Table 3. The dip sampling technique was employed at each sampling site (US EPA, 1994). Membrane filtration was employed for *Enterococcus* isolation and enumeration. KF-Streptococcus agar plates supplemented with Triphenyltetrazolium chloride (TTC) were incubated at 37°C for 48 hours. Single well isolated pink colonies were aseptically sub-cultured three times on nutrient agar using the streak plate technique and incubated for 24 hours at 37°C.

3.2.2 Genomic *Enterococcus* DNA isolation and identification

For each *Enterococcus* isolate screened, genomic DNA was extracted using an isolation kit according to the manufacturer's instructions (Macherey-Nagel, Germany). The quantity and quality of the isolated total genomic DNA was determined using a NanoDrop TM 1000 Spectrophotometer (Thermo Fischer Scientific, US) and agarose electrophoresis. DNA was then amplified using an ICycler thermal cycler (Bio-Rad, UK). A PCR reaction mixture with a final volume of 25 µl was used and it contained RNase/DNase free water (Fermentas Life Sciences, US), 12.5 µl 2x PCR Master Mix (0.05U/µl Taq DNA Polymerase in reaction buffer, 0.4mM of each dNTP, 4mM $MgCl_2$) (Fermentas Life Sciences, US), 50µM primer and 50-100 ng/µl bacterial DNA template. The primer set used for 16S rRNA amplification in this study is listed in Table 4 (Muyzer *et al.,* 1993). Furthermore, the PCR reaction was set such that initial denaturation occurred at 95°C for 30 seconds. Thereafter, the PCR mixture was subjected to 35 cycles of 30 seconds at 95°C, 30 seconds at 52°C and 60 seconds at 72°C, followed by a final extension step of 180 seconds at 72°C. All PCR amplifications were confirmed using electrophoresis.

Table 3: GPS co-ordinates of all sampling sites.

WWTPs	Sampling sites	GPS Co-ordinates	
		Longitude (S)	Latitude (E)
.	P1 Final effluent	26°19’14.4”	026°48’13.7”
	P1 Point downstream	26° 19’ 24.7”	026°48’10.5”
	Point between Plants 1 and 2	26°19’32.7”	026°48’083”
Plant 2	P2 Final effluent	26°53’50.0”	026°37’24.3”
	P2 Point downstream	26°53’53.5”	026°38’30.4”
Plant 3	P3 Final effluent	26°45’05.5”	27°05’40.7”
	P3 Point downstream	26°45’48.5”	27°05’25.4”

Table 4: PCR primers and products used in this study.

Gene	Forward primer sequence 5’-3’	Reverse primer sequence 5’-3’	Amplicon length (bp)
16S rRNA	CCTACGGGAGGCAGCAG	CCGTCAATTCCTTTGAGTTT	550
asa1	GCACGCTATTACGAACTATGA	TAAGAAAGAACATCACCACGA	375
cylA	ACTCGGGGATTGATAGGC	GCTGCTAAAGCTGCGCTT	688
esp	AGATTTCATCTTTGATTCTTGG	AATTGATTCTTTAGCATCTGG	510
gelE	TATGACAATGCTTTTTGGGAT	AGATGCACCCGAAATAATATA	213
hyl	ACAGAAGAGCTGCAGGAAATG	GACTGACGTCCAAGTTTCCAA	276

3.2.3 Sequencing

PCR products were purified using ZR DNA sequencing clean-up Kit (Zymo Research, USA) and as described by Li *et al*. (2010). Genomic DNA sequencing was performed and sequences analysed as described in Jordaan and Bezuidenhout (2013). A total of 63 *Enterococcus* spp. nucleotide sequences were submitted to the GenBank database under accession numbers: KT598397-KT598459.

3.2.4 Detection of virulence factors using PCR

Five oligonucleotide primer pairs (Applied Biosystems, UK) used for the detection of virulence genes are presented in Table 4 (Vankerckhoven *et al.,* 2004). Identification of virulence genes for each isolate was performed by separate polymerase chain reaction (PCR) for *asa1*, *hyl* and *gelE* while a duplex reaction was used for *cylA* and *esp*. PCR amplifications were performed in a Techne Prime Elite thermocycler (Cambridge, UK), in 0.2 ml reaction tubes. PCR assays were performed in a total volume of 25 µl containing 1µl bacterial DNA template (50-100 ng/µl) for the single assays and 2ul bacterial DNA template (100-150 ng/µl) for the duplex reactions. Additionally, the reaction contained RNase/DNase free water (Fermentas Life Sciences, US), 2x DreamTaq PCR Master Mix (0.05 U/µL Taq DNA polymerase in reaction buffer, 0.4 mM of each dNTP and 4 mM $MgCl_2$), 0.2 µM of primers *asa1* and *gelE* and 0.4 µM of primers *cylA*, *esp*, and *hyl*. The PCR cycling conditions consisted of denaturing at 95°C for 180 seconds, followed by 30 cycles of 95°C for 30 seconds, annealing at 56°C for 30 seconds and at 72°C for 60 seconds. This was followed by a final step of 72°C for 600 seconds. PCR products were confirmed by gel electrophoresis.

3.2.5 Antimicrobial susceptibility testing

Antimicrobial susceptibility patterns of *Enterococcus* isolates were determined using the disk diffusion method (Bauer *et al*., 1966). Assays were performed on Mueller Hinton agar (Merck, Germany) using Ampicillin (10 µg), Amoxicillin (10 µg), Penicillin G (10 µg), Neomycin (30 µg), Streptomycin (300 µg), Vancomycin (30 µg), Chloramphenicol (30 µg), Ciprofloxacin (5 µg), Tetracycline (30 µg), Trimethoprim (2.5 µg) and Erythromycin (15 µg). All antibiotics were obtained from Mast Diagnostics (UK). *Enterococcus* isolates were classified resistant, susceptible or

intermediate according to the criteria from Clinical and Laboratory Standards Institute (CLSI, 2012).

3.2.6 Plasmid isolation and curing

Pure *Enterococcus* isolates showing resistance to two or more antibiotics were grown on nutrient agar, cultured overnight at 37°C in 20 ml Brain Heart Infusion broth (BHI, Merck, Germany), and harvested by centrifugation. A plasmid isolation kit was used to extract total plasmids according to the manufacturer's instructions (Macherey-Nagel, Germany). All plasmid products were electrophoresed for 1 hour at 60V and agarose gel images were captured under a UV light using a Bio-Rad ChemiDoc imaging system (Hercules, CA). *Enterococcus* isolates resistant to two or more antibiotics were subjected to plasmid curing according to Molina-Aja *et al.* (2002) and Carvalho *et al.* (2014). The isolates were once again subjected to antibiotic susceptibility testing and resistance classified as plasmid dependent when affected by curing.

3.2.7 Statistical analysis

The multiple antibiotic resistance data of *Enterococcus* spp. was subject to cluster analysis using Ward's method and Euclidean distances in Statistica 12.0 (StatSoft, US).

3.3 Results

3.3.1 Identification of *Enterococcus* spp.

The most frequently detected *Enterococcus* spp. were *E. hirae* (21%), *E. faecalis* (21%) and *E. faecium* (19%). The remaining detected species were *E. gallinarum* (13%), *E. casseliflavus* (16%), *E. mundtii* (8%) and *E. durans* (3%).

The antibiotic inhibition zone diameter data from the antimicrobial susceptibility test performed on all 63 *Enterococcus* spp. was subject to cluster analysis which is presented in Figure 3. Two main clusters, A and B, were generated and cluster A subdivided into minor clusters A1 and A2.

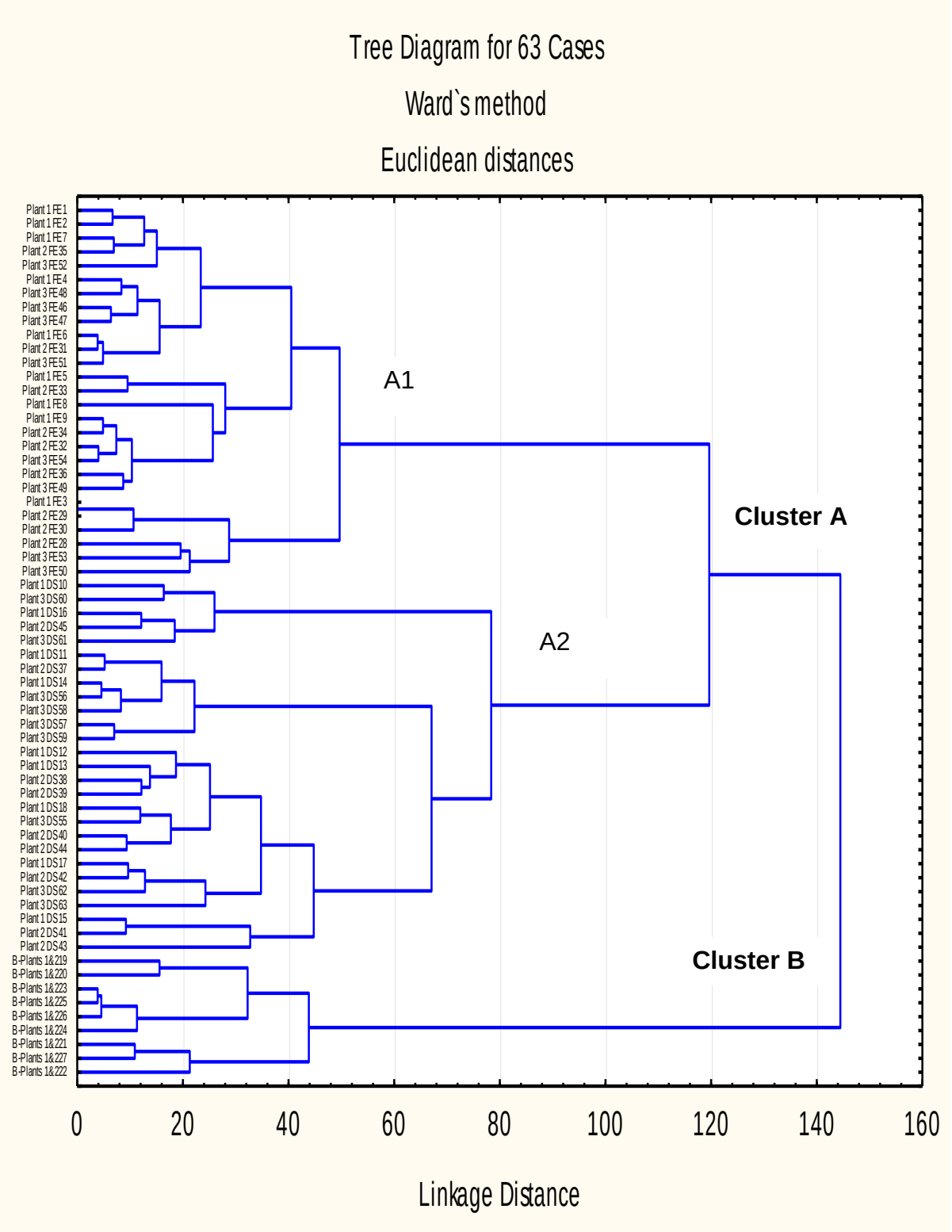

Figure 3: Dendogram showing the relationship of 63 *Enterococcus* spp. obtained from three WWTPs final effluent and points downstream, based on inhibition zone diameter data. FE: final effluent, DS: downstream the final effluent, B-Plants 1 & 2: between Plants 1 and 2.

Furthermore, the species composition of both clusters was determined and is presented in Table 5. Differences in species diversity and prevalence levels at the various sites were observed. Furthermore, as depicted in Figure 3, the cluster analysis indicated grouping of the antibiotic inhibition zone results of *Enterococcus* spp. isolated at the final effluent and points downstream, respectively. Cluster A1 comprised *Enterococcus* spp. from all sampled WWTPs final effluent. This cluster was predominated by *E. faecalis* isolates followed by *E. faecium* and *E. hirae* isolates (Table 5). Furthermore, as depicted in Figure 3, cluster A2 comprised *Enterococcus* spp. isolated from the points downstream the WWTPs effluents. The most predominant species in this cluster (A2) were *Enterococcus gallinarum*, *E. casseliflavus* and *E. mundtii* (Table 5). Lastly cluster B represented *Enterococcus* isolates from the point located between Plants 1 and 2. In this cluster the most predominant species, as presented in Table 5, were *E. faecium* and *E. hirae*.

Table 5: *Enterococcus* spp. composition of clusters A and B.

Species	**Cluster A**		**Cluster B**
	Minor cluster A1	**Minor cluster A2**	
	N = 27	N = 27	N = 9
E. faecalis	9 (33%)	4(15%)	0
E. faecium	6 (22%)	2(7%)	4(44%)
E. hirae	6 (22%)	4(15%)	3(33%)
E. gallinarum	1 (4%)	7(26%)	0
E. casseliflavus	4 (14%)	5(18.5%)	1(11%)
E. mundtii	0	5(18.5%)	0
E. durans	1 (4%)	0	1(11%)

3.3.2 Identification of virulence genes and report of MAR phenotypes prior and post plasmid curing

Presented in Table 6 are the characteristics of *Enterococcus* spp. isolated from three WWTPs final effluent and points downstream with reference to the presence of virulence genes as well as the MAR phenotypes prior and post plasmid curing. Figure 4 is an agarose gel with representative isolates confirming the presence of virulence genes in some *Enterococcus* spp. screened in this study. All five virulence genes screened for were detected and *Enterococcus* isolates harbouring virulence genes are presented in Table 6.

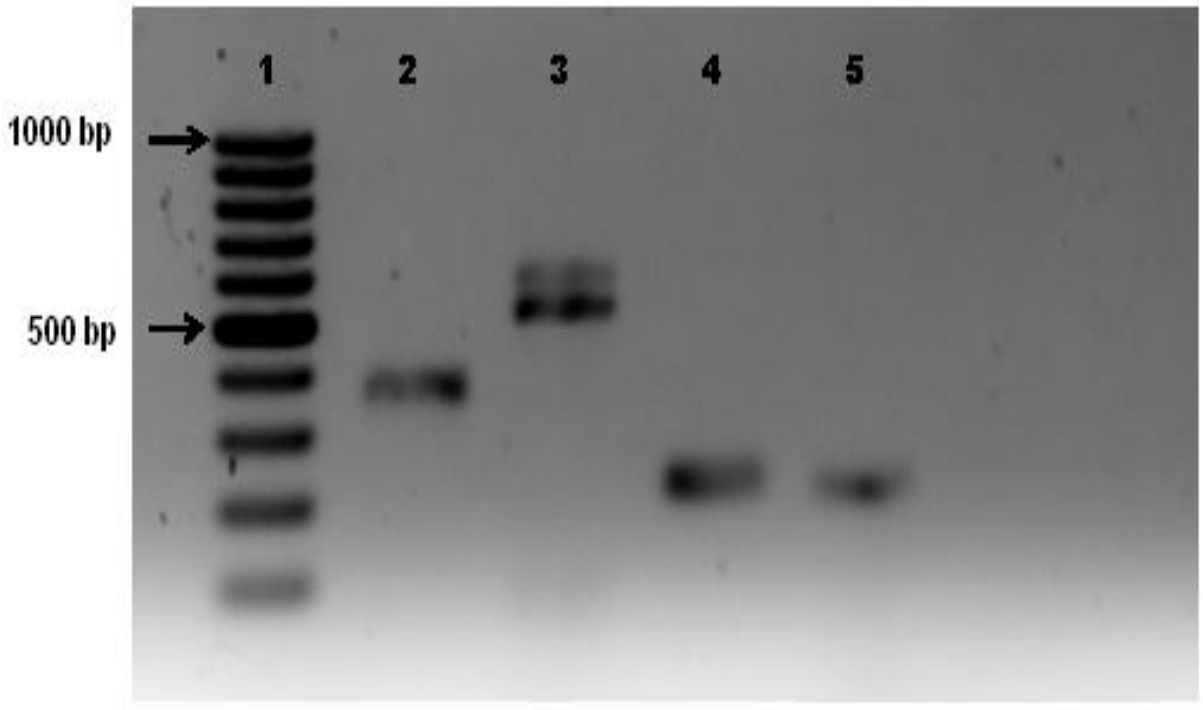

Figure 4: A negative 1% (w/v) agarose gel image of four representative *Enterococcus* isolates harboring the five virulence genes of interest. Lane 1: 100bp molecular weight marker; 2: *asa1* (375bp); 3: *esp* (510bp) & *cylA* (688bp); 4: *hyl* (276bp); 5: *gelE* (213bp).

Table 6: Characteristics of *Enterococcus* spp. isolated from three WWTPs final effluent and points downstream.

Isolate	Species	Virulence gene	Antimicrobial resistance profiles	
			Before plasmid curing	After Plasmid curing
VE1 VE5	*E. casseliflavus* *E. faecium*	*esp* *gelE*	ERY-VA	- VA
VE2	*E. casseliflavus*	-	CHL-PEN-VA	-
VE4 KE31	*E. faecium* *E. faecalis*	*cylA, esp, gelE* *cylA, esp*	AMP-ERY-PEN-TET-VAN	VA -
VE7 H VE8 F	*E. hirae* *E. faecalis*	- *cylA, esp*	AMP-ERY-PEN-CIP-VA	AMP-PEN -
VDS10	*E. casseliflavus*	-	AMP-ERY-PEN-GEN-TET-VA	AMP-PEN
VDS11 VDS14 OV19 OV20 KDS37 PDS56 PDS57 PDS58 PDS59	*E. mundtii* *E. faecalis* *E. faecium* *E. faecium* *E. casseliflavus* *E. gallinarum* *E. gallinarum* *E. gallinarum* *E. gallinarum*	*asa1* *gelE* - - *esp* *hyl* *cylA, esp* - -	ERY-STREP-PEN-TET	- - - - - - - - -
VDS12 OV21 OV24	*E. hirae* *E. casseliflavus* *E. gallinarum*	- *hyl* *cylA*	AMP-PEN-TET	- - -
VDS13	*E. faecalis*	-	AMP-TET-VA	-
VDS15 PE46	*E. faecium* *E. faecium*	*hyl* -	AMP-STREP-TET-VA	- -
VDS16	*E. faecium*	*gelE*	AMP-CHL-ERY-PEN-	PEN

			GEN-TET-VA	
OV22	*E. hirae*	*esp, hyl*	AMP-PEN-TET-VA	-
OV23	*E. hirae*	*asa1, gelE*		-
OV25	*E. faecium*	-		-
OV26	*E. faecium*	*hyl, gelE*		-
KE33	*E. casseliflavus*	*hyl*	AMP-ERY-TET-VA	-
KE36	*E. faecalis*	-		-
KDS41	*E. gallinarum*	-		-
KDS45	*E. gallinarum*	-		VA
PE46	*E. faecium*	-		-
PE47	*E. faecalis*	*hyl*		ERY-TET
KE32	*E. hirae*	*hyl*	AMP-ERY-VAN	VA
KE34	*E. faecalis*	*cylA, esp*		-
PE51	*E. faecium*	*asa1*		VA
KE35	*E. faecium*	*gelE*	AMP-PEN-CIP-VA	VA
KDS38	*E. casseliflavus*	*cylA, esp, gelE*	AMP-VA	VA
KDS40	*E. casseliflavus*	-	AMP-ERY-TET	VA
PE48	*E. hirae*	*esp, gelE*	ERY-PEN-TET-VA	TET-VA
PE52	*E. faecalis*	*hyl*	AMP-CHL-CIP-TET-VA	-
PE53	*E. faecalis*	*hyl*	CHL-TET-VA	-
PDS60	*E. mundtii*	-	AMP-CHL-ERY-GEN-	-
PDS61	*E. mundtii*	-	TET	VA
PDS62	*E. mundtii*	-	AMP-CHL-PEN	

As presented in Table 6, the most frequently detected virulence determinants were *esp* (25%, 10/39), *hyl* (25%, 10/39), *gelE* (23%, 9/39) and *cylA* (17%, 7/39). Nonetheless, *asa1* (7%, 3/39) was also detected although at lower prevalence levels. *Enterococcus faecium* spp. predominately harboured the *gelE* (55%, 5/9) and *hyl* (3%, 3/10) genes (Table 6). The *esp* gene was predominately harbored by *E. faecalis*, *E. casseliflavus* and *E. hirae* spp. at prevalence levels of 3% (3/10) each. Furthermore, virulence gene *cylA* was predominately detected in *E. faecalis* and *E.*

gallinarum spp. at prevalence levels of 29% (2/7) each (Table 6). In addition, six multi-virulence profiles were observed with *cylA-esp* being the most prominent.

3.3.3 Antibiotic resistance

Analysis of the antimicrobial susceptibility of the 63 *Enterococcus* isolates revealed that resistance to ampicillin (67%), vancomycin (62%), tetracycline (58%), penicillin (52%) and erythromycin (51%) were most frequent. Furthermore, resistance to chloramphenicol (22%) and streptomycin (17%) was also observed, although at slightly lower levels. Additionally, low frequencies of resistance to ciprofloxacin (8%) and gentamycin (6%) were detected.

Sixty eight percent of the screened *Enterococcus* spp. were resistant to three or more antibiotics (Table 6). While, resistance to multiple antibiotics was more common among the *E. faecium* isolates followed by *E. faecalis* and *E. casseliflavus* spp. As depicted in Table 6, a total of 19 multiple antibiotic resistance (MAR) phenotypes were identified with the most frequently identified MAR phenotype being ERY-STREP-PEN-TET. The latter MAR phenotype was observed in nine *Enterococcus* isolates. In addition, AMP-PEN-TET-VA was the second most prevalent MAR phenotype and was identified in six *Enterococcus* isolates screened. Similarities in species composition of these MAR phenotypes was observed as they were composed of *Enterococcus faecalis*, *E. faecium*, *E. casseliflavus* and *E. gallinarum* spp. (Table 6).

Screening for the presence of plasmids revealed that 76% (35/46) of the screened *Enterococcus* isolates resistant to two or more antibiotics had plasmids. The plasmids were of high molecular weight. Furthermore, In Table 6 MAR phenotypes of the screened *Enterococcus* spp. pre- and post-plasmid curing are presented. Differences in MAR phenotypes were observed for all profiles pre- and post-plasmid curing. Thirty three percent (10/30) of the *Enterococcus* spp. resistant to vancomycin were still resistant to this antibiotic after plasmid curing. While, resistance to penicillin, ampicillin erythromycin and chloramphenicol were observed in few isolates post plasmid curing (Table 6).

3.4 Discussion

Although antibiotic resistance profiles have often been detected in recreational and wastewaters (Soge *et al.,* 2009; Araújo *et al.,* 2010), the current knowledge on prevalence of antibiotic resistant bacteria and types of antibiotic resistance in the environment is insufficient (Rizzo *et al.,* 2013). Urban WWTPs provide environmental waters, used for various agricultural, religious, cultural and recreational activities, effluent for reuse. However, ineffectively functioning plants are amongst the main sources of antibiotics released into the environment. This contamination of environmental surface water systems with antibiotics consequently facilitates the selection of antibiotic resistance genes and antibiotic resistant bacteria (Martinez, 2009) which is a concern when the health and well-being of both humans and animals is considered.

In this study, antimicrobial resistance patterns and presence of clinically relevant virulence genes were screened in 63 *Enterococcus* spp. isolated from the final effluent of three municipal WWTPs and receiving environmental water systems. Differences in species composition and diversity at varying prevalence levels were observed for the various sites. Graves and Weaver (2010) explain that the observed differences are a result of influences exerted by environmental factors as well as the sources of a sewer system. Carvalho *et al*. (2014) reported higher species diversity in raw sewage compared to sites more distant from the raw sewage. The latter findings support the findings of this study where species diversity was higher at the final effluent sites as compared to the site between Plants 1 and 2. Furthermore, similar to the findings of other studies, *E. hirae*, *E. faecalis* and *E. faecium* were the most frequently identified *Enterococcus* spp. (da Silva *et al.,* 2006; Łuczkiewicz *et al.,* 2010).

The high prevalence of *E. hirae* isolates identified in this study, correlates with the findings of Bonilla *et al*. (2006) and Ferreira da Silva *et al*. (2006), who reported high *E. hirae* levels in recreational and urban wastewater. In addition, studies have also reported the dominance of *E. hirae* species in animal faeces (Fisher and Phillips, 2009; Peeters *et al.,* 2015). Thus the high levels of *E. hirae* spp. identified in this study suggest faecal contamination. The presence of *E. hirae* spp. in the WWTPs final effluent and points downstream used for recreational, agricultural and cultural

activities is a cause for concern since several studies have reported the ability of *E. hirae* to cause wound infections, gastritis and bacteraemia (Talarmin *et al.,* 2011; Chan *et al.,* 2012).

Enterococcus faecalis and *E. faecium* were predominantly detected in the WWTPs final effluent. This is in accordance to the findings of several other studies (Cheng *et al.,* 2012 Varela *et al.,* 2013; Iweriebor *et al.,* 2015b). Finding *E. faecalis* and *E. faecium* at high levels in all three sampled WWTPs final effluent suggests poorly disinfected sewage effluent as these two species are the most commonly found *Enterococcus* spp. in the human gastrointestinal tract (Manero *et al.,* 2002; Klein, 2003). Cheng *et al*. (2012) suggested that frequently detecting *E. faecalis* and *E. faecium* in treated effluent may imply these species are naturally present at higher concentrations in the influent or may be more resistant to treatment processes, particularly disinfection. Furthermore, their presence in the WWTPs final effluent and points downstream may suggest the dissemination of these species from the WWTP into the environment. Their presence in the WWTPs final effluent is also a cause for concern seeing that they account for majority of infections caused by *Enterococcus* spp. (Vu and Carvalho, 2011).

Enterococcus gallinarum, *E. casseliflavus* and *E. mundtii* were also isolated in this study and were the most prevalent isolates in surface water bodies receiving the final effluent from the WWTPs. The presence of these species in the downstream sites is not unexpected due to the well documented presence of these *Enterococcus* spp. in plants, soil and water (non-human and animal hosts) hosts (Byappanahalli *et al.,* 2012; Ran *et al.,* 2013). However, their presence in downstream sites of receiving water bodies that are used for various social activities is a cause for concern as *E. gallinarum*, *E. casseliflavus* and *E. mundtii* have been listed amongst *Enterococcus* spp. known to occasionally cause human infection (de Perio *et al.,* 2006). Thus, Implications for the immuno-compromised could have far reaching effects.

3.4.1 Presence of virulence factors

For *Enterococcus* spp. to cause infection, they must possess virulence factors that allow them to colonize and invade the host's tissue, translocate through epithelial cells and evade the host's immune response (Hammad *et al.,* 2014). *Enterococcus* isolates screened in this study displayed the presence of multi-virulence profiles.

This is in accordance to the findings of Poeta *et al*. (2005) and Lata *et al*. (2008). Furthermore, the findings of Lata *et al*. (2008) showed that *Enterococcus* isolates exhibiting two to three virulence-markers per isolate were mostly associated with polluted sites.

The *esp* gene was the most predominant virulence gene identified and was harboured by *E. faecalis*, *E. casseliflavus*, *E. hirae* and *E. faecium* isolates. Enterococcal surface protein (*esp*) is considered a pathogenicity marker in *E. faecium* and *E. faecalis* clinical isolates (Said *et al.,* 2015). Therefore, the predominance of *esp* in *Enterococcus* isolates obtained from the WWTPs final effluent and receiving water bodies is not strange as the WWTPs receive wastewater from the hospitals. Furthermore, the presence of *esp* in *Enterococcus* spp. other than *E. faecalis* and *E. faecium* has not been extensively reported. However, it should be noted that its presence in other *Enterococcus* spp. may possibly be a result of genetic exchange by conjugation (Oancea *et al.,* 2004).

The *cylA*, *gelE* and *hyl* virulence genes were also detected in the various *Enterococcus* spp. screened in this study. This observation is supported by various studies globally that reported the presence of these virulence genes in various *Enterococcus* spp. isolated from different sources (Semedo *et al.,* 2003; Gonçalves *et al.,* 2011; Sidhu *et al*., 2014). However, a recent study in South Africa reported the absence of virulence genes *cylA*, *esp* and *hyl* in *Enterococcus* spp. isolated from WWTPs in the Eastern Cape (Iweriebor *et al.*, 2015b). Thus, in the present study we report on virulence patterns previously observed globally as well as those not previously reported, particular for South African enterococci.

3.4.2 Antibiotic resistance screening

High levels of *Enterococcus* spp. resistant to multiple antibiotics were isolated in this study. This is accordance to the findings of several other studies that also isolated large numbers of enterococci from water sources that were resistant to multiple antibiotics (Da Costa *et al.,* 2006; Carvalho *et al.,* 2014). Furthermore, the observed resistance to vancomycin, tetracycline, penicillin and erythromycin is in agreement to the findings of other studies (Moore *et al.,* 2010; Carvalho *et al.,* 2014; Iweriebor *et al.,* 2015b). On the other hand, resistances to ampicillin, chloramphenicol and streptomycin were also observed in the present study. Resistances to the latter

antibiotics may not be surprising as these antibiotics are frequently prescribed at South African Hospitals (NDoH, 2012).

Similar to the findings of Xu *et al*. (2007) and Carvalho *et al*. (2014) twenty one distinct multiple antibiotic resistance profiles were observed. The presence of *Enterococcus* spp. in wastewater, and receiving water systems that are resistant to multiple antibiotics is a cause for concern as these species are associated with both community- and hospital-acquired infections. If these antibiotics are commonly used for treatment of infections (Xu *et al.,* 2007), then it could be a further health burden as treatment failure could arise.

However, sixty seven percent of the *Enterococcus* spp. with multiple antibiotic resistance were susceptible to the same antibiotics after plasmid curing, implying the resistance is plasmid mediated. Similar results were observed by Amaechi (2015) and Carvalho *et al*. (2014). According to McBride *et al*. (2007) the presence of plasmids is common in the *Enterococcus* genus, as these mobile genetic elements constitute a substantial fraction of their genome and are responsible for horizontal gene transfer. Therefore, *Enterococcus* spp. may acquire antimicrobial resistant genes through mutation or horizontal transfer of mobile genetic elements such as plasmids and transposons (Hasmann *et al.,* 2005). Furthermore, environmental water systems polluted by sewage, may act as vehicles of bacteria carrying and disseminating antibiotic resistant plasmids (Carvalho *et al.,* 2014).

Although only one plasmid with a high molecular weight was observed in all *Enterococcus* spp. screened, studies of Szczepanowski *et al*. (2004) and Schlüter *et al*. (2003) demonstrated that antibiotic resistance genes were commonly contained in high molecular weight plasmids. Therefore, the presence of *Enterococcus* spp. harbouring plasmids in WWTPs final effluent flowing into receiving water systems implies that these aquatic systems could potentially be acting as reservoirs of antibiotic resistant enterococci harbouring high molecular weight plasmids.

3.5 Conclusions

In this study, the antimicrobial resistance patterns and presence of virulence genes in *Enterococcus* spp. isolated from three municipal WWTPs final effluent as well as receiving environmental water systems in the North West Province, South Africa

were determined. The findings of this study have demonstrated that *Enterococcus* spp. harbouring plasmids that mediate multiple antibiotic resistance are not effectively removed during treatment consequently entering environmental water systems that support a variety of social needs. Furthermore, these *Enterococcus* spp. harbour virulence factors, which play a significant role in the rupture of a variety of target membranes, colonization and biofilm production, as well as the catabolism of various target molecules such as gelatin, collagen, fibrinogen, casein, and insulin. Therefore, the data presented here serves as an alert for public health authorities. It is recommended that interventions such as advanced treatment technologies and disinfection processes be implemented in WWTPs while environmental water systems used by humans and animals be tested regularly for *Enterococcus* levels.

CHAPTER 4

Antibiotic resistance, efflux pump genes and virulence determinants in *Enterococcus* spp. from surface water systems

4.1 Introduction

There are concerns regarding the occurrence of bacteria harbouring antibiotic resistance and virulence genes in recreational waters as well as the risk that these may pose to users (Santiago-Rodriguez *et al.,* 2013). Among the environmental bacteria are enterococci, a bacterial group ubiquitously found in the gastrointestinal tract of humans and animals as well as soil, water and plants (Teixeira and Merquior, 2013). Some strains in this bacterial group have been identified as opportunistic pathogens and important etiological agents of nosocomial infections (Top *et al.,* 2008). Consequently, the presence of enterococci in environmental water sources are of particular interest due to the possible link of community acquired infections and recreational activities (APHA, 1995).

Enterococci are known to have intrinsic resistance traits while also possessing acquired specific mechanisms of resistance to different antibiotics (Aslangul *et al.,* 2006). Additionally, coupled to their intrinsic resistance to a variety of antibiotics, enterococci are inherently more resistant to various antimicrobials in comparison to most Gram positive bacteria (Li and Nikaido, 2004). Therefore, this allows for the reasonable assumption that the presence of genes encoding multidrug resistance efflux pumps could be contributing to antimicrobial resistance (Jonas *et al.,* 2001)

Efflux pumps are transporter proteins harboured in both Gram positive and Gram negative bacteria (Bambeke *et al.,* 2000). These transporter proteins extrude various toxic substances including antibiotics from within a cell to its external environment (Webber and Piddock, 2003). The mechanism of efflux has previously been studied in enterococci particularly for the efflux of fluoroquinolones as well as chloramphenicol (Lynch *et al.,* 1997). Furthermore, 34 efflux pump genes have been identified in the genome of *E. faecalis* spp. (Davis *et al.,* 2001; Jonas *et al.,* 2001).

According to Teixeria and Merquior (2013), *Enterococcus* spp. are amongst the leading therapeutic challenges with regard to life-threatening infections and are

becoming significant pathogens worldwide. Infections caused by *Enterococcus* spp. are frequently treated with tetracyclines (Santiago-Rodriguez *et al.,* 2013). However, erythromycin and other macrolides are also generally used for enterococcal infections, particularly where allergy to β-lactams is suspected (Arvanitidou *et al.,* 2001; Duarte *et al.,* 2005).

Nevertheless, antibiotic resistance cannot alone explain the pathogenicity of this bacterial group. *Enterococcus* spp. may possess the genes coding for virulence factors and their protein products which contribute to their infection potential (Hill, 2012). Various virulence factors such as aggregation substance, cytolysin, enterococcal surface protein, gelatinase and hyaluronidase have been considered and could play a role in rendering *Enterococcus* spp. pathogenic (Lata *et al.,* 2009). Moreover, previous studies have demonstrated that *Enterococcus* spp. carrying antibiotic resistance genes can harbour genes encoding virulence factors (Shankar *et al.,* 2001).

Recent studies have contributed to the understanding of the prevalence of antibiotic resistance and virulence genes in *Enterococcus* spp, isolated from human and animal sources (Sidhu *et al.,* 2014). Several studies have reported the presence, emergence as well as outbreak of antibiotic resistant enterococci in South Africa (Budavari *et al.,* 1997; Struwig *et al.,* 1998; McCarthy *et al.,* 2000; von Gottberg *et al.,* 2000). However, similar studies on enterococci isolated from surface water and other environmental sources are limited.

The goal of the present study was thus to determine the prevalence of *Enterococcus* spp. in South African surface water systems, their antibiotic susceptibility patterns as well as the presence of efflux pump genes coding resistance to antibiotics. Furthermore, virulence factors of isolates harbouring antibiotic resistant genes are reported. In this way the importance of enterococci in water quality assessments, was demonstrated.

4.2 Materials and Methods

4.2.1 Study area and sample collection

The study area comprised a total of four rivers (Vaal, Harts, Schoonspruit and Mooi) and an inland lake (Barberspan) flowing in and through the North West Province of South Africa. These surface water resources are important to the province as they largely support the gold and platinum mining, manufacturing industries, agricultural sector well as urban populations. A total of 80 *Enterococcus* isolates were collected between March 2010 and August 2011 were used. However, limited Upper Harts River isolates were available in the 2010/2011 samples. Thus, 44 samples were collected along the Upper Harts River in 2014. The direct and dip sampling techniques were employed depending on the physical setting of each sampling site (US EPA, 1994).

4.2.2 Enumeration and isolation of *Enterococcus*

Membrane filtration was used for *Enterococcus* isolation and enumeration (US EPA, 1997). Triplicates of 100ml water samples were filtered through on 0.45 µm (47 mm grid) PALL Corporation sterilised filter membranes (PALL Life Sciences, Mexico).and placed on KF-Streptococcus agar containing 1ml of 2,3,5-Triphenyltetrazolium chloride (TTC) per 100ml (Sigma Aldrich, South Africa). The KF-Streptoccus agar plates were incubated at 37°C for 48 hours. Single well isolated pink colonies were aseptically sub-cultured 3 times on nutrient agar using the streak plate technique and incubated for 24 hours at 37ºC.

4.2.3 Genomic *Enterococcus* DNA isolation and identification

Pure *Enterococcus* isolates were grown on nutrient agar ,cultured overnight at 37°C in 20 ml Brain Heart Infusion broth (BHI, Merck, Germany), and harvested by centrifugation. A commercial genomic DNA isolation kit (Macherey-Nagel, Germany) was used to isolate total DNA, using the instruction of the manufacturer. The quantity and quality of the isolated total genomic DNA was determined using a NanoDrop TM 1000 Spectrophotometer (Thermo Fischer Scientific, US) and agarose electrophoresis. The 16S rDNA was then amplified using an ICycler thermal cycler (Bio-Rad, UK) and reagents and procedures for the PCR and evaluation of amplification success is described in Jordaan and Bezuidenhout (2013). The

annealing temperature in this case was 52°C. Amplicons were sequenced by Inqaba Biotech (South Africa, Pretoria). Raw sequence data was transferred to Geospiza Finch TV (version 1.4) software which was used to view all chromatograms. All amplified DNA sequences were identified using BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST).

4.2.4 Antimicrobial susceptibility testing

Antimicrobial susceptibility patterns of *Enterococcus* isolates were determined using the disk diffusion method (Bauer *et al*., 1966; CLSI, 2012). Assays were performed on Mueller Hinton agar (Merck, Germany) using Ampicillin (10 µg), Amoxicillin (10 µg), Penicillin G (10 µg), Neomycin (30 µg), Streptomycin (300 µg), Vancomycin (30 µg), Chloramphenicol (30 µg), Ciprofloxacin (5 µg), Tetracycline (30 µg), Trimethoprim (2.5 µg) and Erythromycin (15 µg). All antibiotics were obtained from Mast Diagnostics (UK). *Enterococcus* isolates were classified resistant, susceptible or intermediate according to the criteria from Clinical and Laboratory Standards Institute (CLSI, 2012).

4.2.5 PCR detection of efflux pump genes

Four efflux pump genes were targeted (Table 7) using the primers (Applied Biosystems, UK) in Table 7. The final reaction volumes were 20 µl (*msrA/B, tetK, tetL*) and 25 µl (*mefA*). The PCR reaction for *msrA/B* contained 1 µl DNA template (30-50 ng/µl), RNase/DNase free H_2O (Fermentas Life Sciences, US), 2x DreamTaq PCR Master Mix (0.05 U/µL *Taq* DNA polymerase in reaction buffer, 0.4 mM of each dNTP and 4 mM $MgCl_2$), 0.25 µM primer.

For detection of *tetK* and *tetL*, 1µl bacterial DNA template (50-100 ng/µl), RNase/DNase free H_2O (Fermentas Life Sciences, US), 2x DreamTaq PCR Master Mix (0.05 U/µL Taq DNA polymerase in reaction buffer, 0.4 mM of each dNTP and 4 mM $MgCl_2$), 0.2 µM primer were used. Reaction mixtures for the detection of the *mefA* gene, contained 1 µl DNA template (30-50 ng/µl), RNase/DNase free water (Fermentas Life Sciences, US), 4 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.5 U/µL *Taq* DNA polymerase, 10 mM reaction buffer (200 mM Tris-HCL and 500 mM KCL) and 0.5 µM primer. The PCR cycling conditions, of all primers are described in Table 7.

Table 7: Oligonucleotide primers used in this study.

	Primer	Sequence (5'-3')	Size (bp)	PCR conditions	Reference
16S rRNA gene	*16S RNA*-F	CCTACGGGAGGCAGCAG	550	Denaturation 95°C 300sec, 35 cycles at 95°C 30sec, 52°C 30sec, 72°C 60sec, 72°C 180sec	Muyzer *et al.* (1993)
	16S RNA-R	CCGTCAATTCCTTTGAGTTT			
Efflux pump genes	*mefA-F*	AGTATCATTAATCACTAGTGC	348	Denaturation 94°C 180sec, 35 cycles at 93°C for 60sec, 52°C 30 sec, 72°C 60 sec, 72°C 300sec	Duarte *et al*. (2005)
	mefA-R	TTCTTCTGGTACTAAAAGTGG			
	tetK-F	TATTTTGGCTTTGTATTCTTTCAT	1,159	Denaturation 95°C 60sec, 35 cycles at 50˚C 60sec, 72°C 30sec, 72°C at 300sec	
	tetK-R	GCTATACCTGTTCCCTCTGATAA			
	tetL-F	ATAAATTGTTTCGGGTCGGTAT	1,1077	Denaturation 95°C 60sec, 35 cycles at 50˚C 60sec, 72°C 30sec, 72°C at 300sec	
	tetL-R	AACCAGCCAACTAATGACAATGAT			
	msrA/B-F	GCAAATGGTGTAGGTAAGACAACT	400	Denaturation 95°C 180sec, 35 cycles at 93°C 30sec, 55°C 120 sec, 72°C 90sec	Wondrack *et al.* (1996)
	msrA/B-R	ATCATGTGATGTAAACAAAAT			
Virulence genes	*asa1-F*	GCACGCTATTACGAACTATGA	375	Denaturation 95°C 180sec, 30 cycles at 95°C 30sec, 56˚C 30sec, 72°C 60 sec, 72°C 600sec	Vankerckhoven *et al.* (2004)
	asa1-R	TAAGAAAGAACATCACCACGA			
	cylA-F	ACTCGGGGATTGATAGGC	688		
	cylA-R	GCTGCTAAAGCTGCGCTT			
	esp-F	AGATTTCATCTTTGATTCTTGG	510		
	esp-R	AATTGATTCTTTAGCATCTGG			
	gelE-F	TATGACAATGCTTTTTGGGAT	213		
	gelE-R	AGATGCACCCGAAATAATATA			
	hyl-F	ACAGAAGAGCTGCAGGAAATG	276		
	hyl-R	GACTGACGTCCAAGTTTCCAA			

4.2.6 PCR detection of virulence genes

Five oligonucleotide primer pairs (Applied Biosystems, UK) were used for the detection of virulence genes, and were obtained from Vankerckhoven *et al.* (2004) (Table 7). Separate polymerase chain reaction (PCR) was used for the identification of all virulence genes. The reaction mixtures, in final volumes of 25 µl, for detection of the various virulence genes contained 50-100 ng/µl bacterial DNA and reaction mix was the same as for Vankerckhoven *et al*. (2004). In this case 0.2 µM of primers *asa1* and *gelE*, and 0.4 µM of primers *cylA*, *esp,* and *hyl* were used with PCR cycling conditions described in Table 7.

4.2.7 Electrophoresis and sequencing

The success of all PCR amplifications was determined by electrophoresis (Jordaan and Bezuidenhout, 2014). PCR products of efflux pump genes were purified as described by Li *et al* (2010) as well as using the ZR DNA Sequencing Clean-up Kit (Zymo Research, USA). DNA sequencing was done using the BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, UK). Sequencing was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems-Hitachi), according to manufacturer's instructions.

All sequences obtained were compared to published gene sequences in the National Center of Biotechnology Information Database (NCBI), GenBank, using BLASTN to determine their identity. Representative bacterial nucleotide efflux sequences were submitted to the Genbank database under accession numbers: KU182369-KU182389.

4.3 Results

Five surface water systems in the North West Province, South Africa were screened for the presence of *Enterococcus* spp. Presented in Table 8, are the virulence and efflux pump gene trends as well as antibiotic resistance patterns of the *Enterococcus* spp. *Enterococcus* isolates from the Mooi and Vaal River harboured four of the five virulence genes screened. Whereas, *Enterococcus* spp. from the Upper Harts, Lower Harts and Vaal River harboured two of the five virulence factors of interest.

Table 8: Virulence and efflux pump gene trends as well as antibiotic resistance patterns of *Enterococcus* spp. isolated from five surface water systems, in the North West Province, South Africa.

Water system	**No. of sites**	**No. of *Enterococcus* spp. (n)**	***asa1* n (%)**	***cylA* n (%)**	***gelE* n (%)**	***Hyl* n (%)**	***msrA* n (%)**	***tetL* n (%)**	**MAR**
Upper Harts River	7	34	5(15)	0	20(59)	0	1(3)	0	β-lactams,CIP,ERY,TET,VA β-lactams,CIP,NE,VA β-lactams,CIP,ERY,NE,TET β-lactams,CIP,NE PEN,NE,CIP AMP,PG,ERY,TET
Barberspan	4	18	8(44)	5(28)	6(33)	0	1(6)	3(17)	β-lactams,NE,VA β-lactams,CIP,NE,VA,CHL β-lactams,CIP,NE,STREP,VA PEN,NE,VA β-lactams,CIP,NE β-lactams,CIP,NE,VA,CHL
Lower Harts River	6	20	0	3(15)	0	2(10)	1(15)	0	β-lactams,NE,VA,CHL,TET PEN,VA,CHL,CIP β-lactams,CIP,NE,VA,CHL,TET AMOX,NE,VA,CIP AMP,AMOX,VA,CIP β-lactams,CIP,NE,STREP,VA,CHL,ERY PEN,NE,CHL,CIP AMP,PEN,NE,CIP,TET β-lactams,CIP,VA,CHL, β-lactams,CIP,NE,VA,CHL,ERY,TET AMOX,PEN,NE,VA,CIP,ERY,TET PEN,NE,CIP,ERY,TET
Mooi River	6	13	1(8)	6(46)	4(31)	2(15)	4(31)	4(31)	AMOX,PG,VA,CIP,ERY PEN,NE,ERY PEN,ERY,TET AMOX,NE,ERY,TET

Schoonspruit River	4	17	1(6)	9(53)	6(35)	3(18)	1(6)	6(35)	β-lactams,CIP,NE,VA,ERY,TET β-lactams,NE,VA,ER,TET PEN,NE,CIP β-lactams,CIP,NE,VA AMOX,PEN,NE,VA,CIP,TET β-lactams,CIP,VA,TET PEN,NE,VA,CIP
Vaal River	5	22	0	10(46)	2(9)	0	1(5)	2(9)	AMP,PG,VA,CHL,CIP,ERY,TET NE,VA,CIP,TET PEN,VA,TR,TET β-lactams,CIP,NE,VA,CHL,NE,VA β-lactams,VA β-lactams,CIPNE,ERY β-lactams,CIP,NE,

*Percentage (%) was determined as a function of the number of isolates of the specific *Enterococcus* spp. thus (n/(N) x 100). β-lactam = AMP :Ampicillin (10 µg), AMOX: Amoxicillin (10 µg), PEN: Penicillin G (10 µg); NE: Neomycin (30 µg); STR: Streptomycin (300 µg); VA: Vancomycin (30 µg); CHL: Chloramphenicol (30 µg); CIP: Ciprofloxacin (5 µg): TET: Tetracycline (30 µg), ERY: Erythromycin (15 µg).

Aggregation substance (*asa1)* was most frequently detected among the enterococci from Barberspan. However, *asa1* was also present in isolates from the Upper Harts and individual isolates from the Mooi and Schoonspruit Rivers (Table 8). Furthermore, cytolysin (*cylA*) was frequently observed in *Enterococcus* spp. isolated from the Mooi, Schoonspruit and Vaal Rivers. Gelatinase (*gelE*) was harboured predominantly by isolates from the Upper Harts River. However, this virulence gene was also prevalent in enterococci isolates from Barberspan, Mooi and Schoonspruit Rivers. In addition, hyaluronidase (*hyl*) was not detected in high levels, however, it was observed in *Enterococcus* spp. isolated from the Lower Harts, Mooi River and Schoonspruit Rivers. The presence of enterococcal surface protein (*esp*) was investigated and this virulence gene was not detected in any of the isolates (Table 8).

Efflux pump genes for *msrC* and *tetL* were detected in some of the isolates (Table 8). The *msrC* efflux pump gene was identified present among isolates from all the water systems. On the other hand, the *tetL* efflux pump gene was predominant in *Enterococcus* spp. isolated from the Schoonspruit and Mooi Rivers. It was also detected among isolates from the Vaal River and Barberspan.

The antibiotic MAR phenotypes of isolates across the five surface water systems of interest were determined. As depicted in Table 8, resistance of *Enterococcus* spp. to β-lactam, fluoroquinolones and vancomycin was common across all surface water systems. However, some β-lactam resistant isolates were susceptible to one or more of the other β-lactam antibiotics. Furthermore, the highest MAR diversity patterns were observed in the Lower Harts River.

The overall antimicrobial resistance of the 124 isolates was determined and results are expressed as percentages of isolates that were resistant to the various antibiotics (Table 9). Most of these isolates were resistant to penicillin (70%) and vancomycin (69%). Between 40 and 55% were resistant to ampicillin, amoxicillin, erythromycin, neomycin, tetracycline, and ciprofloxacin. Lower *Enterococcus* numbers were also resistant to chloramphenicol (19%) and streptomycin (6%).

When considering the antibiotic resistance patterns of various species results, in Table 9 it is demonstrated that a high percentage of *E. faecalis* spp. were resistant to β-lactam antibiotics: ampicillin (62%), amoxicillin (64%), and penicillin (81%) as well

as the fluoroquinolone, ciprofloxacin (58%). Furthermore, 73% of this species were resistant to vancomycin, 57% to neomycin and 48% to erythromycin and tetracycline. Although the exact percentages are different, similar trends of *E. faecium* and *E. mundtii* isolates were resistant to the antibiotics listed above. Low percentages of the various *Enterococcus* spp. were resistant to streptomycin and chloramphenicol.

All the *Enterococcus* spp. isolated were screened for the presence of selected efflux pump and virulence genes. In Table 10, the genotypic characteristics of virulence genes and phenotypic characteristics of 22 antimicrobial resistant *Enterococcus* spp. that carried efflux pump genes are provided. Four of the predominantly isolated *Enterococcus* spp. tested positive for efflux pump genes. Individual strains of *E. faecium*, *E. faecalis* and *E. mundtii* were resistant to β-lactam antibiotics and at least one antibiotic from another class. Eighty six percent (19/22) of the MAR phenotypes observed comprised tetracycline while 55% (19/22) and 45% (10/22) comprised ciprofloxacin and erythromycin. The highest antibiotic resistance phenotypes were observed in *E. faecium* spp. with three isolates being resistant to seven of the ten screened antibiotics. However, one *E. mundtti* isolate also displayed resistance to seven antibiotics.

Of the 124 *Enterococcus* isolates screened, 22 (18%) isolates harboured at least one efflux pump gene (Table 10). While amongst these, 4 (18%) harboured two efflux pump genes. The antibiotic efflux pump genes were predominantly detected in *Enterococcus faecium* (33%, 10/30) followed by *E. casseliflavus* (29%, 4/14), *E. mundtii* (14%, 5/36) and *E. faecalis* (8%, 3/37) spp. In contrast, no antibiotic efflux pump genes were detected in *E. gallinarum, E. hirae* and *E. sulfureus* spp.

The *tetL* efflux gene was the most frequently determined gene amongst the 59 tetracycline resistant *Enterococcus* spp. and was found in 17 (28%) isolates. However, the *tetK* efflux gene was not detected. Furthermore, the *msrC* efflux gene was detected in 9 (13%) of the erythromycin resistant isolates. However, none of the *Enterococcus* spp. harboured the *mefA* efflux gene, despite 68 of them showing resistance to erythromycin.

Table 9: Antibiotic resistant *Enterococcus* spp. isolated from surface water samples.

Species	No. of isolates	Antibiotics									
		AMP	**AMOX**	**PEN**	**NE**	**STR**	**VA**	**CHL**	**CIP**	**ERY**	**TET**
	(N)	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*
E. faecium	30	11 (36)	15 (50)	20 (66)	13 (43)	3 (10)	19 (63)	6 (20)	14 (47)	22 (73)	14 (46)
E. faecalis	37	23 (62)	24 (64)	30 (81)	21 (57)	2 (5)	27 (73)	6 (6)	9 (24)	18 (48)	18 (48)
E. mundtii	36	17 (47)	8 (22)	26 (72)	21 (58)	1 (3)	23 (64)	6 (17)	21 (58)	16 (44)	20 (55)
E. casseliflavus	14	5 (35)	4 (29)	8 (57)	7 (50)	1 (7)	11 (78)	3 (21)	1 (7)	8 (57)	4 (28)
E. gallinarum	5	2 (40)	1 (20)	2 (40)	2 (40)	0	5 (100)	1 (20)	0	3 (60)	2 (40)
E. hirae	1	0	0	0	1 (100)	0	0	0	1 (100)	0	0
E. sulfureus	1	1 (100)	1 (100)	1 (100)	0	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Total	**124**	**59 (48)**	**53 (43)**	**87 (70)**	**65 (52)**	**8 (6)**	**86 (69)**	**23 (19)**	**47 (38)**	**68 (55)**	**59 (48)**

AMP: Ampicillin (10 µg); AMOX: Amoxicillin (10 µg); PEN: Penicillin G (10 µg); NE: Neomycin (30 µg); STR: Streptomycin (300 µg); VA: Vancomycin (30 µg); CHL: Chloramphenicol (30 µg); CIP: Ciprofloxacin (5 µg): TET: Tetracycline (30 µg), ERY: Erythromycin (15 µg). *Percentage (%) was determined as a function of the number of isolates of the specific *Enterococcus* spp. thus (n/(N) x 100).

Table 10: Genotypic and phenotypic characteristics of *Enterococcu*s spp. that carried efflux pump genes.

Species	Antibiotic resistance phenotype	Efflux pump genes	Virulence factors
E. faecium			
SR12	AMP,AMOX,PG,VA,CIP,ERY,TET	*msrC, tetL*	*cylA*
SR4	AMP,AMOX,PG,VA,CIP,ERY,TET	*tetL*	*cylA*
VR5	AMP,PG,VA,CHL,CIP,ERY,TET	*msrC*	*cylA*
MR2	AMOX,PG,VA,CIP,ERY	*msrC*	*cylA, hyl*
MR6	AMOX,PG,CIP,ERY,TET	*msrC, tetL*	*cylA, hyl*
MR13	AMOX, ERY,TET	*msrC*	None detected
MR18	AMOX,CIP,ERY	*msrC*	*cylA*
MR14	AMOX,CIP	*tetL*	*cylA*
LHR6	AMOX,NE,VA,CIP,TET	*tetL*	*gelE, hyl*
UHR37	PG,CIP,ERY,TET	*msrC*	*asa1*
E. faecalis			
VR8	AMOX,PG,NE,VA,ER,TET	*tetL*	*asa1, gelE*
VR10	AMP,AMOX,PG,NE,TET	*tetL*	*gel*
MR11	AMOX,VA,CIP,ERY,TET	*msrC, tetL*	*cylA*
E. casseliflavus			
SR9	NE,VA,TET	*tetL*	*cylA*
VR2	NE,VA,ER,TET	*tetL*	*cylA*
VR3	PG,VA,TET	*tetL*	*cylA*
MR9	CIP, ERY,TET	*msrC, tetL*	*asa1, gelE*
E. mundtii			
B1	PG,NE,VA,TET	*tetL*	*cylA*
B4	AMP,AMOX,PG,NE,TET	*tetL*	*cylA*
B9	PG,TET	*tetL*	*asa1*
LHR3	AMP,AMOX,PG,NE,VA,CHL,TET	*tetL*	None detected
LHR12	PG,NE,TET	*tetL*	None detected

Additionally, the presence of virulence factors in the *Enterococcus* spp. harbouring resistance genes was also determined. As depicted in Table 10, of the *Enterococcus* spp. harbouring efflux pump genes also harboured one (14; 63%) or two (5; 23%) virulence factors. Cytolysin (*cylA*) was the most predominant virulence gene detected in 13 (59%) of *Enterococcus* spp. harbouring efflux pump genes. The virulence genes *asa1* (18%), *gelE* (14%) and *hyl* (14%) were also detected. *Enterococcus faecium* spp. harboured the most virulence genes. No virulence genes were detected in 3 (14%) of the isolates harbouring efflux pump genes.

4.4 Discussion

To date, research on enterococci has focused mainly on clinical isolates with the assumption that these mediate higher public health threats (Dada *et al.,* 2013). However, recent reports have illustrated that isolates obtained from municipal sewerage polluted environmental sources could directly pose threats to the health of users (Iweriebor *et al.,* 2015b). Such sources may permit the dissemination of antibiotic resistance and virulent bacteria (Carvalho *et al.,* 2014). Numerous studies have reported the presence of antibiotic resistant genes in *Enterococcus* spp. isolated from water systems (Schwartz *et al.,* 2003; Santiago-Rodriguez *et al.,* 2013; However, little is known about the genotype of antibiotic resistant and virulence genes and their distribution among enterococci isolated from South African waters. Furthermore, a number of reports, globally, affirm that enterococci with the highest virulence are of clinical origin, followed by industrial isolates (Fisher and Phillips, 2009). However, notably missing are the environmental strains. In this study, the presence of antibiotic resistance and virulence genes in *Enterococcus* spp. isolated from surface water systems was determined.

4.4.1 Virulence and efflux pump gene trends as well as antibiotic resistance patterns

Virulence genes were mostly prevalent in *Enterococcus* spp. of the Mooi River and Vaal River. These two water systems support a variety of urban centres, agricultural and recreational activities (DWAF, 2004; Hendriks and Rossouw, 2009). The recreational activities supported include swimming, fishing and angling (le Roux, 2005; Pantshwa, 2006). Thus, the presence of these virulence genes in these river systems could allow for the dissemination of virulence genes from the environment

to humans through open wounds and other routes. Furthermore, efflux pump genes were identified in all five surface water systems. The *tetL* gene was, however, present in the surface water systems screened with the exception of the Harts River. To our knowledge, this is the first report illustrating the presence *Enterococcus* spp. harbouring efflux pump genes in the five surface water systems of interest. Furthermore, several of the *Enterococcus* spp. isolated were resistant to β-lactam, fluoroquinolone and vancomycin antibiotics. This is not surprising seeing that the fluoroquinolone antibiotic ciprofloxacin is regularly prescribed in South Africa to females between ages of 12 and 18 years to treat urinary tract infections (Agyakwa, 2014). Furthermore, increased intrinsic resistance to β-lactams have been reported in clinical *Enterococcus* spp. as a result of their penicillin-binding proteins (Kak and Chow 2002; Chen and Zervos, 2009; Hollenbeck and Rice, 2012). Furthermore, the presence of vancomycin resistant bacteria in South African clinical enterococcus isolates has previously been reported (Budavari *et al.,* 1997; McCarthy *et al.,* 2000; Iweriebor *et al.,* 2015b). The presence of vancomycin resistant enterococci is, however, a cause for concern because of their ability to transfer the vancomycin resistance determinant *van* to other bacterial species posing a public health threat (Iweriebor *et al.,* 2015b).

According to Chen and Zervos (2009), cell-wall inhibitors such as penicillin, ampicillin, vancomycin alongside aminoglycosides are used for the treatment of serious enterococcal infections. Thus, the observed multiple antibiotic resistance of *Enterococcus* spp. screened in this study is worrisome as it relates to the potential therapeutic failure when antibiotics from several classes are used to attain synergistic bactericidal activity (Chen and Zervos, 2009).

Previous studies have also reported the resistance of *Enterococcus* spp. to fluoroquinolones in water and wastewater samples (Martins da Costa *et al.,* 2006; Moore *et al.,* 2008). Carvalho et al. (2014), ascribed resistance of vancomycin resistant *Enterococcus* isolates present in marine ecosystems to be the result of faecal pollution. Resistance to erythromycin, predominantly found among *E. faecium* spp, was also observed in *E. faecalis*, *E. gallinarum* and *E. casseliflavus.* This is in accordance with findings of Łuczkiewicz et al. (2010). The observed resistance of *Enterococcus* spp. to erythromycin can be a result of its extensive use in livestock

breeding programmes and treatment of infections where resistance or hypersensitivity to penicillin is suspected (Blanch *et al.,* 2003; Duarte *et al.,* 2005).

According to Łuczkiewicz *et al.* (2010), the presence of fluoroquinolones in MAR phenotypes of Gram-positive organisms is an increasing problem. Furthermore, the presence and association of ERY-TET in the MAR phenotypes supports suggestions of a co-selection mechanism between erythromycin and tetracycline resistant organisms (Martins da Costa et al. 2006). Luczkiewicz *et al.* (2010) suggested that resistance to erythromycin could influence and raise resistance to tetracycline. Considering the promiscuity of *Enterococcus* spp., this latter suggestion is not an abnormal phenomenon as macrolides and tetracyclines are commonly and extensively used in clinical and animal health therapy, allowing for the transferal of bacteria resistant to such antibiotics into the environment (Blanch *et al.,* 2003; da Silva *et al.,* 2006).

Multiple antibiotic resistance phenotypes observed among the *Enterococcus* isolates that also harbour antibiotic resistance efflux pump and virulence genes respectively is a cause for concern. The water systems that were sampled are used for various purposes in which direct exposure is common. Such water may pose a health threat to the individuals especially the immuno-compromised.

4.4.2 Presence of efflux pump genes

Of the four (*mefA, msrC, tetL, tetK*) efflux pump genes screened, *mefA* and *tetK* genes were not detected. The absence of these two efflux pump genes in *Enterococcus* spp. has been reported previously (López *et al.,* 2010; Portillo *et al.,* 2000; Roberts *et al.,* 1999). In the case of erythromycin resistance, it could be that different mechanism or efflux pumps could be conferring the resistance phenotype (Chouchani *et al.,* 2011). However, Portillio *et al*. (2000) has reported the occurrence of *mefA* genes in *Enterococcus* spp. Thus, the justification of including these genes when testing for erythromycin resistance determinant is provided.

In this study *tetL* was the main genetic determinant associated with the resistance to tetracycline. This is similar to the study of Valenzuela *et al*. (2013) that also found that *tetL* genes were the most predominant among tetracycline resistant enterococci. However, in the present study this gene as well as *tetK* could not be detected in

some of the tetracycline resistant isolates. In these cases other tetracycline resistance determinants (e.g., *tetM, tetO, tetQ*, or *tetS*) could have been responsible for the observed phenotype. However, detecting *tetL* efflux pump genes in several *Enterococcus* spp. in this study is significant because few studies have focused on the prevalence of tetracycline resistance genes in recreational waters (Santiago-Rodriguez et al., 2013).

The *msrC* gene was detected in some of the erythromycin resistant *Enterococcus* spp. screened. This gene *msrC* codes for an efflux pump that confers low resistance against macrolides and type B streptogramins (Singh *et al.,* 2001). The *msrC* gene was predominantly harboured by *E. faecium* isolates. Portillo *et al (*2000) also reported that the *msrC* gene is distributed among erythromycin resistant *E. faecium*. These authors suggested that this gene is species-specific. However, in the present study, *msrC* also was detected in one *E. faecalis* and one *E. casselflavus* isolate. This finding is supported by previous studies that reported the presence of *msrC* genes conferring resistance to erythromycin in *E. faecalis*, *E. durans*, *E. lactis* and *E. casseliflavus* spp (Aakra *et al.,* 2005; Thumu and Halami, 2012). Thumu and Halami, (2012) further advocated that the presence of *msrC* in different *Enterococcus* spp. could be a result of horizontal gene transfer. However sequencing regions surrounding this gene would be mandatory in order to reveal the presence of elements that would suggest the potential mobility of this gene.

4.4.3 Presence of virulence determinants

Virulence genes were detected among *E. faecium*, *E. faecalis*, *E. casselfilavus* and *E. mundtii* spp. This finding is similar to that of Dada *et al.* (2013), Iwerebor *et al.* (2015) and Sidhu *et al.* (2014). The cytolysin determinant (*cylA*) was detected most frequently. This virulence factor is of interest due to its ability to enhance enterococcal virulence (Vankerckhoven *et al.,* 2004). Furthermore, few of the MAR enterococci also carried the virulence genes *asa1, gelE*, and *hyl*. The virulence factors, *asa1* and *gelE* are involved in bacterial adhesion and the catabolism of various molecules such as gelatin, collagen, fibrinogen (Vankerckhoven *et al.,* 2004). Furthermore, the low levels of virulence genes in surface water systems is in agreement to the findings of a study recently performed in South Africa (Iweriebor *et al.,* 2015b). Nonetheless, the presence of virulence genes in *Enterococcus* spp.

isolated from surface water sources is a cause for concern. These virulence factors permit colonization and invasion of a host's tissue as well as translocation through epithelial cells in order to evade the host's immune response (Vu and Carvhalo, 2011). Therefore, their presence in *Enterococcus* spp. isolated from surface water sources used for various agricultural and recreational activities poses a health risk, particularly for the immune-compromised sector of the population.

4.5 Conclusions

In the current study, the antimicrobial susceptibility patterns of *Enterococcus* spp. isolated from various surface water systems in the North West Province, South Africa was determined. Many of these enterococci were resistant to multiple antibiotics and resistance to tetracycline and erythromycin may be linked to efflux pump genes. A large proportion of the MAR *Enterococci* spp. also tested positive for virulence genes. The presence of efflux pumps conferring resistance to multiple antibiotics is a cause for concern since efflux pumps are known for their remarkable ubiquitous nature and that they a have broad substrate range. The expression and up regulation of these efflux pump genes is particularly important as they could be contributing to multiple antibiotic resistance of the potential pathogenic bacteria. This is a cause for concern as these isolates could be a public health risk particularly for immune-compromised individuals. The potential source of these isolates needs to be investigated. In addition to this it is recommended that *Enterococcus* spp. should be used as an additional indicator of faecal pollution particularly in water systems in which humans are directly exposed to the water.

CHAPTER 5

Conclusions and Recommendations

5.1 Conclusions

Post 1994, a new water act the National Water Act –NWA (Act 36 of 1998) was adopted and accepted in South Africa (Perret, 2002). The key objectives of this water resource management regulation are social development, economic growth, ecological integrity and equal access to water (Farolfi, 2004). For these objectives to be fulfilled, institutions were established at decentralized levels in order to implement water resource management (Farolfi, 2004). Furthermore, South Africa was divided into 19 Water Management Areas (WMAs) and different government departments are responsible for microbial water quality monitoring (Luyt *et al.,* 2012). In addition to adopting the NWA, increased threats of faecal pollution in water resources and their associated health risks lead to the development of the national microbial water quality monitoring programme (NMMP) (Kühn *et al.,* 2000). The NMMP is a conceptual design for monitoring the microbial quality of national surface water systems and is based on the enumeration of *E. coli* (Murray, 1999). This organism was chosen on the basis that faecal coliforms excreted in faeces of healthy mammals are accounted for by *E. coli.* However, numerous studies have questioned the applicability of using faecal coliforms as indicators of faecal pollution due to the several limitations found in their use (US EPA, 2000; Tyagi *et al.,* 2006; Savichtcheva and Okabi, 2006). Furthermore, Luyt et al. (2012) compared South African and international data and identified several shortcomings in the NMMP. According to Luyt et al. (2012), the Colilert® system currently used in South Africa is prone to false positive and false negative results as compared to the membrane filtration technique. Added to the reliability of the Colilert® system, it is more expensive. This is in agreement with the findings of calculation cost studies that reported the standard membrane filtration method to be 5.63 times more cost effective than the Colilert® system (Sundram *et al.,* 2000).

However, in some parts of the world there have been questionable doubts regarding the applicability of using faecal coliforms as faecal indicator bacteria (FIB). This has been coming on for a long time and suggestion of using enterococci as an additional

indicator has been made (Ostrolenk *et al.,* 1947; WHO, 1996). Furthermore, studies conducted in the 1970 have confirmed the applicability of enterococci as FIB in marine waters (Cabelli et al., 1979). Nonetheless, several studies criticised the use of enterococci as faecal indicator bacteria (Colford *et al.,* 2007). However, limitations of the latter studies were identified and an increase in information elucidating the relationship of enterococci to human health in recreational waters argued these findings (Fujioka and Byappanahalli, 2003; Byappanahalli *et al.,* 2012). The suitability and use of enterococci as FIB is supported by their correlation with human health outcomes over other FIB (Wade *et al.,* 2010). In addition, Byappanahalli et al. (2012) explains that the higher salt tolerance of enterococci justifies their use as better FIB than *E. coli* in recreational waters. Thus, the use of enterococci as FIB is widely accepted and this bacterial group are the only FIB recommended by the U.S. Environmental Protection Agency (EPA) for brackish and marine waters (Wade *et al.,* 2010).

In light of the above information and despite the numerous endorsements and adoption of policies and programmes for water resource management, much still needs to be done for the implementation of water resource protection programmes in South Africa (DWA, 2013). Water resource protection programmes will allow for the appropriate management of South Africa's water systems (DWA, 2013). Furthermore, these will allow for the sustainable use and production of significant water quality and quantity returns (DWA, 2013). An improved understanding of the prevalence and distribution of potentially pathogenic *Enterococcus* spp. in water sources used for various economic and social purposes could be an essential tool in the development of public health risk mitigation strategies. Thus, the hypothesis of this thesis is that high levels of opportunistic, pathogenic and antibiotic resistant enterococci are present in water sources of the North West Province. To investigate this aspect, three studies were conducted in supporting this hypothesis. The studies aimed at characterizing *Enterococcus* spp. isolated from various surface water systems as well as effluent from waste water treatment plants (WWTPs) in the North West Province using molecular and biochemical methods. Furthermore, genes encoding for various efflux transporters were identified and the presence of five virulence factors aiding in pathogenicity determined.

5.1.1 Characteristics of environmental *Enterococcus* spp. with virulence factors

A general study was performed to determine the pathogenic potential of *Enterococcus* spp. isolated from environmental water systems of the North West Province, South African. The aim of this study was to determine the presence of clinically relevant virulence genetic determinants in *Enterococcus* spp. isolated from environmental waters in the North West Province of South Africa. Furthermore, the expression of these virulence factors was also determined.

A total of 93 *Enterococcus* isolates consisting of six species were screened for the presence of five virulence determinants (*asa1, cylA, esp, gelE* and *hyl*) using PCR (polymerase chain reaction). Furthermore, the expression of *cylA, hyl* and *gelE* were determined by phenotypic assessments illustrating β-haemolysin, hyaluronidase and gelatianse activity.

Seventy percent of the screened isolates harboured at least one virulence gene whilst 15% harboured two or more. The most frequently detected virulence genes were *cylA* and *gelE*. However, *asa1* and *hylA* were also detected. Enterococcal surface protein (*esp*) was not detected in all screened *Enterococcus* spp. The presence of virulence genes was associated with their extracellular enzyme production. Less than 50% of the *cylA, gelE* and *hyl* gene carrying isolates produced β-haemolysin, gelatinase and hyaluronidase, respectively.

The results of this study confirmed that a large percentage of environmental enterococci isolated in South African surface water systems possess virulence factors. Furthermore, several isolates harboured virulence genes which could be expressed into functional units associated with pathogenicity. In addition, although the levels of virulence genes reported in this study are lower than those reported in clinical isolates, their presence and prevalence should not be overlooked. The reason is that there is a paucity of information on the presence of virulence genes in *Enterococcus* spp. isolated from surface water systems (Sidhu *et al.,* 2013) both in South Africa and on a global scale. Furthermore, a small percentage of the *Enterococcus* spp. expressed the virulence genes by producing enzymes which cause damage to host tissues and permit microbial invasion. Thus, this study also illustrated that although virulence factors are present in environmental isolates, they

may not always be expressed into functional units under the conditions tested. However, finding these genes in *Enterococcus* spp. isolated from environmental waters provides key information regarding recognising the aquatic environment as a possible harbour and transmission route, of these genes, to humans and animals. The environmental waters within which the *Enterococcus* spp. in this study were isolated support a variety of activities including livestock watering, full contact recreational, cultural and religious activities (Zenani and Mistri, 2005). Thus, the presence of these genes in *Enterococcus* spp. isolated from these environmental water sources is a cause for concern particularly when the transmission of these genes to animals and immune-compromised humans is considered. Therefore, their presence in environmental water sources could have implications for individuals using this water directly since virulence factors trigger the pathogenic potential of an infecting strain by permitting colonization, invasion and translocation of host tissues (Švec *et al.,* 2014).

It is thus important to determine the possible sources of these potential pathogenic enterococci. In this way information will be made available for regulatory bodies to devise measures aimed at improving the current management of the country's environmental waters by minimizing the presence of these potential pathogens in water sources. Additionally, because the presence of virulence factors alone is insufficient to determine pathogenicity, the need to identify the significance of *Enterococcus* spp. in terms of health implications was identified. This would be accomplished by further characterizing *Enterococcus* spp. for multiple antibiotic resistance.

5.1.2 Potential source of *Enterococcus* spp. found in environmental water systems and their antibiotic and virulence characteristics

Following the confirmation that *Enterococcus* spp. harbouring virulence factors were present in South African environmental water systems; the possible source of these potential pathogenic enterococci was determined in this study. Of the possible sources of microbial pollution, this study focused on WWTPs. This follows a major concern that South African WWTPs are overloaded and consequently polluting rivers (DWA, 2012; DWA, 2013). Additionally, WWTPs are also known to be amongst

anthropogenic activities responsible for the release of antibiotics into the environment (Rizzo *et al.,* 2013).

Therefore, the aim of this study was to determine the antimicrobial resistance patterns and presence of virulence genes in *Enterococcus* spp. isolated from three municipal WWTPs final effluent and receiving waters in the North West Province, South Africa.

Sixty three *Enterococcus* isolates consisting of seven spp. *E. hirae*, *E. faecalis, E. faecium, E. gallinarum, E. casseliflavus*, *E. mundtii* and *E. durans* were detected. Antimicrobial susceptibility test was performed on the 63 *Enterococcus* spp. and antibiotic inhibition zone diameter data subject to cluster analysis. Cluster analysis indicated grouping of isolates from all three WWTPs final effluent together, isolates from points downstream from the final effluent clustered together, and isolates obtained from the site between plants one and two formed a cluster. The cluster of *Enterococcus* spp. from all sampled WWTPs final effluent was predominated by *E. faecalis* spp. followed by *E. faecium* and *E. hirae* spp. Whereas, the cluster of *Enterococcus spp.* from downstream sites of receiving water bodies were predominated by *E. gallinarum, E. casseliflavus* and *E. mundtii.* All 63 *Enterococcus* spp. were screened for the presence of five virulence determinants (*asa1, cylA, esp, gelE* and *hyl*). All five virulence genes were detected and six multi-virulence profiles observed. Analysis of the antimicrobial susceptibility of the 63 *Enterococcus* isolates revealed that resistances to ampicillin, vancomycin, tetracycline, penicillin and erythromycin were most frequent. Sixty eight percent of the screened *Enterococcus* spp. were resistant to three or more antibiotics. Isolates were screened for the presence of plasmids and large plasmids were identified. Furthermore, the plasmids of isolates identified were cured in order to determine whether antibiotic resistance was plasmid mediated or not. Differences in MAR phenotypes were observed for all profiles pre- and post-plasmid curing with lower resistance profiles post plasmid curing.

The results of this study illustrated that WWTPs are contributing sources of *Enterococcus* spp. in environmental water systems of the North West Province, South Africa. Furthermore, this study illustrated that the WWTPs effluent contained multiple antibiotic resistant *Enterococcus spp.* harbouring virulence factors. This is a

cause for concern as improperly treated effluent has detrimental effects on the overall health of receiving environmental water systems (Naidoo and Olaniran, 2014). Additionally, contaminated receiving environmental water systems may act as a vehicle for the transmission of pathogenic enteric organisms and serve as a source of infection causing *Enterococcus* as well as other enteric bacteria (Moon *et al.,* 2014).

Furthermore, high levels of multiple antibiotic resistant *Enterococcus* spp. were isolated and these were resistant to antibiotics often prescribed at South African Hospitals (NDoH, 2012). The present study supports previous reports that human activities related to clinical practices influence the transfer and selection of resistant bacteria into the environment via hospital wastewater (Varela *et al.,* 2013; Iweriebor *et al.,* 2015b). Furthermore, differences observed in the presence of enterococcal surface protein (*esp)* in *Enterococcus* spp. screened in this study as opposed to the previous, confirms reports that this gene is a predominant pathogenicity marker in clinical *Enterococcus* isolates (Said *et al.,* 2015). This gene may thus not be widely distributed among environmental isolates. Finding this gene among environmental isolates may thus indicate pollution from a clinical source.

The presence of plasmids in *Enterococcus* spp. screened in this study agrees with reports that these mobile genetic elements constitute a fraction of this bacterial group's genome (McBride *et al.,* 2007). Furthermore, the change of MAR phenotypes in terms of susceptibility to many of the antibiotics after plasmid curing in some *Enterococcus* isolates confirms that their resistance is plasmid mediated (Hasman *et al.,* 2005). The presence of *Enterococcus* spp. harbouring plasmids mediating antibiotic resistance is also a cause for concern when gene transfer mechanisms of this bacterial group are considered (Eaton and Gasson, 2001). Thus, the presence of *Enterococcus* spp. harbouring virulence genes and plasmids mediating antibiotic resistance is also a cause for concern as they may play a role in the spread of these genetic elements in the environment.

Findings of this study serve as an alert for public health authorities because poor operating wastewater treatment plants result in faecal pollution of receiving water bodies posing a health risk not only to humans and animals but also the surrounding environment. Furthermore, this study emphasizes the importance of managing

municipal wastewater sources. A strategic approach aimed at minimising the contamination of receiving surface water systems by improving the treatment processes is thus imperative.

This study determined the overall presence and distribution of *Enterococcus* spp., their antibiotic resistance profiles and presence of virulence genes in significant environmental water systems across the North West Province, South Africa. In addition, because not all resistance to antibiotics in the *Enterococcus* isolates was plasmid mediated; it was considered important to identify another possible resistance mechanism responsible for resistance in this bacterial group.

5.1.3 Overview of *Enterococcus* spp. in significant environmental water sources across the North West Province

The aim of this study was to report on antibiotic susceptibility patterns and highlight the presence of efflux pump and virulence genes in *Enterococcus* spp. isolated form a variety of surface water systems located in the Upper, Middle and Lower Vaal water management areas of the North West Province.

Briefly, water is a precious yet limited resource in the North West Province (as well as South Africa) with its demand exceeding supply (DWA, 2004; DWA, 2013). Furthermore, a large portion of the available surface water is attributed to the sectors which contribute a huge quantity of the country's Gross Domestic Product (Kalule-Sabiti and Heath, 2008). Furthermore, these water systems support various recreational, religious and cultural activities (Molale, 2012). Thus, the role of the provinces rivers in economic and social development cannot be overlooked. Additionally, sustaining healthy water ecosystems is imperative when the major contribution this natural resource makes is considered (DWA, 2013).

One hundred and twenty four *Enterococcus* isolates consisting of seven spp. were identified. Antimicrobial susceptibility testing revealed that large numbers of these isolates were resistant to the β-lactam antibiotic group as well as vancomycin. However, the resistance of these isolates was not limited to these antibiotic groups. A total of 20 antibiotic resistant profiles were observed. These isolates were further screened, using PCR, for the presence of four well-known efflux pump genes (*mefA*, *tetK*, *tetL* and *msrC*). Efflux genes *mefA* and *tetK* were not detected in any of

Enterococcus spp. screened. However, *tetL* and *msrC* were detected in 17% of the *Enterococcus* spp. Additionally, the presence of virulence factors in the *Enterococcus* spp. harbouring efflux pump genes was determined. Virulence determinants were detected in 86% of the *Enterococcus* spp. harbouring efflux pump genes. Of the screened five virulence determinants (*asa1, cylA, esp, gelE* and *hyl*), four were detected.

This study focuses on four rivers and an Inland Lake located in regions that have been labelled “hot spots” recommended for surface water monitoring in the North West Province (Kalule-Sabiti and Heath, 2008). The findings of this study have demonstrated that *Enterococcus* spp. isolated from the latter surface water systems are resistant to multiple antibiotics, some of which are frequently used for therapy in South Africa (NDoH, 2012). Furthermore, these isolates not only harbour efflux pump genes coding for resistance to antibiotics but also harbour virulence factors which enhance their pathogenic potential. Owing the findings of the previous and current study it is evident that the microbial quality of rivers across the province is affected. This is worrisome particularly when the significant water quantity and quality challenges experienced in the North West Province are considered (DWA, 2013). The presence of efflux pump genes coding for antibiotic resistance in *Enterococcus* spp. isolated from surface water systems used for various social needs is also cause for concern. Efflux pumps are ubiquitous transporter proteins with a broad substrate range. Their presence in *Enterococcus* spp. isolated from environmental surface water systems used for various social needs is worrisome as they have the ability to render antibiotic treatment regimes against *Enterococcus* infections ineffective or difficult.

The environmental water systems screened in this study support a variety of full contact recreational, religious and cultural activities (Molale, 2012). The exposure of enterococci is associated with increased risk of gastrointestinal and respiratory illnesses (Haile *et al*., 1999). Furthermore, exposure to faecally contaminated water can result in fever, chills, coughing, ear discharge as well as respiratory disease (Haile *et al*., 1999). However, although the infections may not be life threatening, children and immuno-compromised persons can be significantly affected (Clark *et al.,* 2003).

In addition, these water systems also support farming activities, such as livestock watering and irrigation (DWAF, Anon; le Roux, 2005). Although environmental enterococci are a minor component of the environmental mastitis complex, they have the ability to cause intramammary infection and clinical mastitis in dairy herds (Smith and Hogen, 2008). Furthermore, few interventions aimed at controlling these pathogens are available and one of them is decreasing the exposure of dairy cows to potential pathogens (Smith and Hogen, 2008). A study by Goosen (2012) isolated *Enterococcus* spp. amongst other common mastitis causing pathogens such as *E. coli, Streptococcus* and *Staphylococcus aureus* spp. This study was conducted in the Western Cape, thus, mastitis causing streptococci are not foreign in South African. In addition, a study by Chidamba et al. (2015) reported the presence of *E. coli, Streptococcus*, *Staphylococcus* and *Enterococcus* spp. across the Lower Vaal River catchment. Thus, the possibility of these isolates being distributed amongst the other rivers of interest is high since water from the Upper and Middle Vaal water management areas flows into the Lower Vaal River and management area (Le Roux *et al*., 2010; Goodwin *et al*., 2012).

Furthermore, several studies, including the latter, have reported a positive association between *S. aureus* and *Enterococcus* spp. (Viau *et al.* 2011; Goodwin *et al.,* 2012; Chidamba *et al.* 2015). Thus, even though the current study did not report the presence of other bacterial pathogens, the promiscuous nature of *Enterococcus* spp. allows for the assumption that: when other environmental bacterial pathogens capable of causing mastitis are present and in contact with *Enterococcus* spp., then mastitis causing genetic traits could easily be transferred to this bacterial group. Furthermore, the presence of adhesion enhancing genetic traits such as aggregation substance (*asa1*) support this assumption. This virulence factor plays a major role in adhesion and colonization. Aggregation substance allows for bacterial adherence to host tissues, a crucial first step in the infection process (Jett *et al.,* 1994). Thus, it can also be speculated that *Enterococcus* spp. screened in this study harbour genetic properties allowing for colonization of the teat canal consequently increasing their ability to cause mastitis.

However, irrigation is not the only cause for concern, the exposure of contaminated water sources to crops by means of irrigation is also to be considered. Numerous

studies have indicated the presence of pathogens on the surfaces of vegetables that were irrigated with contaminated surface water (Heaton and Jones, 2007; Steele and Odumeru, 2004). Furthermore, these studies have demonstrated that these pathogenic bacteria can survive days, weeks and months in irrigation water as well as on the fresh fruits and vegetables consumed by humans (Gemmell and Schmidt, 2010). Thus, the exposure of these pathogens to humans may pose a health threat particularly to the immuno-compromised. This study underscores the importance of identifying *Enterococcus* levels in environmental water systems by highlighting the various socio-economic sectors reliant on this natural resource. Furthermore, this study highlights the impact water contaminated with these potential pathogens can have on humans and animals using it for various recreational, religious and agricultural activities.

Data presented in this study is important as it has bridged a knowledge gap on *Enterococcus* spp. found in environmental sources of the North West Province. Additionally, the three mutually supporting studies have provided a better understanding of the potential source, prevalence and distribution of *Enterococcus* spp. in environmental water sources of the North West Province. Furthermore, their significance to health implications has also been highlighted and their resistance to multiple antibiotics was also illustrated. In addition, their possession of genetic elements such as efflux pumps and those enhancing their pathogenicity (virulence factors) was confirmed. Providing information of susceptibility patterns of environmental enterococci in the North West Province could be useful to public health officials seeing that information provided on local antimicrobial resistance patterns of *Enterococcus* spp. could prove helpful in guiding empirical pathogen specific therapy.

5.2 Recommendations

The following recommendations, providing opportunities for future research are proposed:

- Future studies should focus on whole genome sequencing of environmental enterococci in comparison to enteric and clinical enterococci. This will allow for determining genetic distinctions between enteric, clinical and environmental enterococci. Furthermore, whole genome sequencing will elucidate discrete gene signatures of the enteric and environmental enterococci such that biomarkers for discriminating faecal contaminants in environmental sources can be elucidated. This confirmation will address fundamental gaps in microbial monitoring and provide important confirmation and detection tools for using this bacterial group as an indicator of faecal pollution in environmental water systems. In addition, the associated human health risks of environmental enterococci by determining the genetic distinctness of environmental enterococci as opposed to those of enteric, clinical and origin. This would be achieved by also using whole genome sequencing which would allow for a comprehensive analysis of the complete environmental *Enterococcus* genome analysis. Furthermore, whole genome sequencing will determine the variation of variable gene contents between enteric and environmental enterococci. This comparative analysis will also enhance the motivation of using enterococci as a faecal indicator bacterium.
- Conducting an epidemiological investigation in order to determine the likelihood of humans being infected with enterococci isolated from the various water sources used for recreational, religious and cultural activities could suuport using enterococci as an additional FIB. This could be achieved by determining both favourable and unfavourable conditions in recreational waters for infection of humans by enterococci consequently allowing for the dissemination of virulence genes. Furthermore, determining the differential expression of environmental and enteric *Enterococcus* spp. virulence genes could be determined using DNA microarray technology. This array will allow for the determination of the various *Enterococcus* spp. differential virulence gene expression levels in response to various growth temperatures. This is important because one critical observation in this study was that the phenotypic expression of identified virulence genes

was low. Thus, determining whether temperature plays a role in the expression of virulence genes would be beneficial seeing that the average surface water temperature is 25°C while the gastrointestinal tract of mammals is 37°C.

- Determining the *in vivo* expression level of the various efflux pump genes identified in environmental enterococci by means of real time quantitative real time PCR (q-PCR) could be beneficial. This would elucidate whether these genes are functional in environmental isolates, and provide information as to under what conditions are they functional. According to Muller et al. (2002), q-PCR is a highly powerful and sensitive technique that allows for high-throughput analysis of gene expression. This technique has proved to yield accurate quantitation of gene expression and has been implemented in numerous studies (Freeman *et al.,* 1996; Ruzin *et al.,* 2008).
- A limitation in this study was the inability to determine whether genetic elements found in the isolates of interest are possibly disseminated into the environment. Thus, future studies should make use of metagenomics in order to assess the relationship of *Enterococcus* spp. found in environmental water systems to other bacteria. A comprehensive microbial community profile would be determined in order to get more insight on whether genetic elements found on the environmental enterococci are possibly disseminated into the environment.
- Future studies should make use of next generation sequencing of environmental enterococci. This would assist with developing and validating source specific assays in order to enhance environmental water monitoring.

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