Occurrence of Vancomycin Resistant Enterococci (VRE) in two Durban wastewater treatment plants for effluent reuse

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DECLARATION BY STUDENT

I declare that this thesis, submitted for the award of Masters in Biotechnology at the Durban University of Technology, is the original work of the author and has not been submitted for a degree at any other University. Where use is made of any author's work, it has been duly acknowledged.

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ABSTRACT

The presence of enterococci in improperly treated wastewater leads to pollution of the recipient water bodies which directly or indirectly affects the humans especially when antibiotic resistant strains are involved. In 2017 the World Health Organization listed vancomycin resistant enterococci (VRE) among those with highest priority for further surveillance and research, both among humans and in the receiving aquatic environment. The purpose of this study is to determine how efficient the WWTPs are in removing both vancomycin-resistant enterococci (VRE) and vancomycin-sensitive enterococci (VSE) from wastewater. One hundred (60 wastewater and 40 river) samples were collected from July 2016 to June 2017 which covered the warm and cold seasons of South Africa. Primary isolation and enumeration were carried out on Slanetz and Bartley agar supplemented with and without vancomycin (6 µg/mL) for vancomycin resistant enterococci (VRE) and total enterococci (TE) respectively. Presumptive enterococci were selected using Gram staining, growth on bile aesculin agar, catalase and pyrase tests. The presumptive enterococci isolates (202 VRE and 67 VSE) were confirmed and speciated using polymerase chain reaction (PCR). The identified *Enterococcus* isolates were subjected to antibiotic susceptibility testing (AST) to examine their resistance profile against fifteen antibiotics including vancomycin. Antibiotic resistance genes (*van*, *tet* and *emeA*) were detected by PCR. The TE and VRE counts of the two WWTPs influents ranged from 6.1 to 7.2 $\log_{10}$ CFU/100 mL (for TE) and 4.3 to 6.7 $\log_{10}$ CFU/100 mL (for VRE) while the effluent concentration of Plant II contained 1.5 to 4.4 $\log_{10}$ CFU/100 mL and 0.9 to 3.4 $\log_{10}$ CFU/100 mL for TE and VRE respectively. Neither TE nor VRE was detected in Plant I effluent. The TE and VRE counts of the recipient river samples were higher than the effluents. There were no visible seasonal effects based on the counts. The removal efficiencies in the two plants

ranged from 95 to 100%, where chlorination played a major role. Two hundred and sixty-nine (202 VRE and 67 VSE) isolates were identified by PCR as *Enterococcus*. The most abundant species was *E. faecium* followed by *E. faecalis* while other species include *E. hirae, E. gallinarum, E. durans, E. casseliflavus* and *E. cecorium*. MALDI-TOF and PCR were used in parallel for the identification of the isolates, which resulted in 80.1% agreement for genus identification. The AST results showed that a large percentage (39 to 98%) were resistantto all other antibiotics except amoxicillin/clavulanic acid and imipenem to which the isolates showed high sensitivity. Four *van* genes (*vanA, vanB, vanC1, vanC2/3*) and 4 *tet* genes (*tetK, tetL, tetM, tetO*), and also the multidrug efflux pump gene, *emeA* were detected among the 269 enterococci isolates with *vanA* and *tetL* being the most prevalent. At least one virulence gene (*ace, asa1, cylA, efaA, esp, gelE and hyl*) occurred in 74% (67/88) of the isolates. The result showed that the two WWTPs are efficient in removing both enterococci and VRE from their influents. Thus these effluents had little or no effect to enterococci count of their interlinked recipients. Also, a majority of the isolates are not only antibiotic resistant strains but are also virulent. They therefore pose risk to public health.

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DEDICATION

This work is dedicated to my husband, Dr Jonathan Chukwuemeka Madu in appreciation for his love for God and for his sacrifices especially during the course of this study; and also to all who believe in hard work.

CONFERENCES/PAPERS

Oral Presentations

- 1st Interdisciplinary Research and Postgraduate Conference at Durban University of Technology, South Africa, **3-5 October** 2016. "Occurrence of vancomycin resistance enterococci (VRE) in wastewater and receiving water bodies in KwaZulu Natal" **Madu, C E**; Adegoke A A, Reddy P, Stenström T A.

- The 18th WaterNet/WARFSA/GWPSA Symposium held in Swakopmund, Namibia, **25-28 October, 2017**. "Prevalence of vancomycin resistant *Enterococcus* species in wastewater treatment plants and their receiving water bodies". **Madu, CE**; Adegoke, AA; Reddy P and Stenström TA

- SASM Conference, Muldersdrift, Johannesburg, South Africa**, 4-7 April, 2018**. "Pathogenic attributes, antibiogram and antibiotic resistant genes in *Enterococcus* from Wastewater treatment plants and their recipients". **Madu, C E**; Adegoke A A, Reddy P, Stenström T A.

Papers in manuscript

- **Madu, C E**; Adegoke, A A; Reddy P and Stenström T A "Prevalence of vancomycin resistant *Enterococcus* in wastewater treatment plants and their recipients". ***Submitted to the Journal of Microbiology: Submission No; TJOM-D-18-00626***.

This manuscript covers the results presented in chapter 4 and some of the chapter 5

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ABBREVIATIONS

ABA	Aesculin-bile-azide
ANOVA	Analysis of Variance
APHA	American Public Health Association
ARDs	Antimicrobial Resistance Determinant
ARGs	Antibiotic Resistance Gene
AS	Aggregation Substance
AST	Antibiotic Susceptibility Testing
ATTC	American Type Culture Collection
BAA	Bile Aesculin Agar
BHI(A)	Brain Heart Infusion (Agar)
CARD	Catalysed Reporter Deposition
CATC	Citrate Azide Tween Carbonate
CFU	Colony Forming Unit
DAPI	4′,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
ECSA	Enterococcosel agar
EDTA	Ethylenediaminetetraacetic acid
EI	*Enterococcus* Indoxyl –β-D-glucoside
ELISA	Enzyme-Linked Immunosorbent Assay
ESP	Enterococcal surface protein
EU	European Union
FIB	Faecal Indicator Bacteria

FISH	Fluorescent *in situ* Hybridization
GRE	Glycopeptide Resistant Enterococci
GTI	Genital Tract Infection
HRP	Horseradish peroxidise
IBD	Inflammatory Bowel Disease
IS	Insertion Sequence
KAA	Kanamycin Aesculin Azide
LAB	Lactic Acid Bacteria
MALDI-TOF	Matrix-Assisted Laser Desorption Ionisation-Time of Flight Mass
MAR	Multiple Antibiotic Resistance
MGE	Mobile Genetic Elements
MHA	Muller-Hinton Agar
ND	Not Detected
OD	Optical Density
ORFs	Open Reading Frames
PAI	Pathogenicity Island
PBP	Penicillin Binding Proteins
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulse-Field Gel Electrophoresis
RAPD-PCR	Random Amplified Polymorphic DNA -PCR
RE	Removal Efficiency
REA	Restriction Endonuclease Analysis

RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SA	South Africa
SARChI	South African Research Chairs Initiative
SD	Standard Deviation
SDS	Sodium Dodecylsulfate
SDS-PAGE	Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
S&B	Slanetz and Bartley
TAE	Tris-acetate- EDTA
TCP	Tissue Culture Plate
TE	Total Enterococci
TlTG	Thallous Acetate Tetrazolium Glucose
UK	United Kingdom
UTI	Urinary Tract Infection
UWTPs	Urban Wastewater Treatment Plants
US	United States
UV	Ultraviolet
USEPA	United States Environmental Protection Angency
VBNC	Viable But Non Culturable
VRE	Vancomycin Resistant Enterococci
VSE	Vancomycin Sensitive Enterococci
WHO	World Health Organization
WSP	Waste Stabilization Pond
WWTPs	Wastewater Treatment Plants

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1 CHAPTER 1: INTRODUCTION

1.1 Background Study

Access to potable water is very important and an indispensable factor in preventing risk of infections with waterborne pathogens. Due to the dwindling sources of fresh water, reclaimed water from wastewater treatment plants (WWTPs) is important to alleviate the pressures on fresh water sources in many countries including South Africa. Effluents from WWTPs are discharged into recipient waters and may subsequently affect recreational waters, community water supply or irrigation water for agricultural purposes. If wastewater effluents are not properly treated due to inefficiency of the WWTPs, harmful pathogens will be transferred and affect secondary use (Okoh et al., 2007). When the water is used for irrigation, these harmful bacteria are transferred to the food crops and subsequently potentially to humans through consumption of the contaminated food product. In addition, improperly treated effluents affect recreational water areas or community water supplies and may impact the overall health of the community that come in contact with the water (Naidoo and Olaniran, 2014). Regular assessment of the operational standards of the wastewater treatment plants will help to reduce the risks associated with the use of their effluents and thus enhance the efficient use of this valuable resource. Some individual species or microbial groups including enterococci are used as faecal indicators of water quality (WHO, 2006). These faecal indicators are also used to determine the microbial quality of the WWTP effluents and subsequently the efficiency of the treatment processes.

Enterococci are Gram-positive coccoidal bacteria that mainly occur in pairs or short chains, and share some phenotypic attributes with streptococci (Vu and Carvalho, 2011). The group contain several species. Among these, *E. faecalis* and *E. faecium* are normal commensals of

human gastrointestinal tract and those of some other animals with typical concentration in human stool of 10^4 to 10^7 CFU/ g (Boehm and Sassoubre, 2014; Fisher and Phillips, 2009). Enterococci may be present on vegetation and surfaces in contact with water, partly due to contamination by animal excreta or raw sewage (Jett et al., 1994). However, species like *E. hirae, E. faecalis, E. casseliflavus, E. faecium, E. mundtii* and *E. sulfureus* have also been shown to be associated with vegetation such as forage crops (Cai, 1999; Muller et al., 2001).

Enterococci do not multiply easily in water environment and thus their quantitative detection may reflect possible faecal pollution. Enterococci are resilient organisms and can tolerate some environmental stressors such as temperature extremes, variable pH, desiccation, and oxidative stress (Arias and Murray, 2012; Fisher and Phillips, 2009) and so can survive longer in different water environments affected by faecal pollution. Considering the above attributes and also the high concentration of enterococci in faeces, it is suitable to use enterococci as indicator of faecal pollution of water (Boehm and Sassoubre, 2014; EU, 2006; USEPA, 2006; and WHO, 2006). Their resilient ability may also help them to withstand some less stringent WWTP processes. This can be exemplified by the detection of enterococci from all chlorinated effluent samples tested by Rosenberg- Goldstein et al. (2014). Therefore, the concentration of enterococci in the final wastewater effluents and their receiving water bodies will reflect the effectiveness of the WWTP processes and their subsequent contamination of the surface water (Momba et al., 2006; Naidoo and Olaniran, 2014).

In addition to the importance of enterococci as a faecal indicator of water quality and the efficiency of treatment processes, they are also human pathogens. Many species of enterococci are opportunistic pathogens and can cause some life threatening infections like

nosocomial endocarditis, bacteraemia, urinary tract infection and wound infection. The ability to cause infection is linked to their possession of some virulence genes. Several virulence genes have been detected in enterococci. These include *gelE* encoding gelatinase production, *esp* for enterococcal surface protein, *asa1* for aggregation substance, *cyl* and *hyl* genes which code for cytolysin and hyaluronidase respectively (Chuang et al., 2009; Vankerckhoven et al., 2004). While enterococcal surface protein (*Esp*) and hyaluronidase are common in *E. faecium*, aggregation substance, gelatinase, cytolysin and also *Esp* are found in *E. faecalis* (Vankerckhoven et al., 2004). The severity of enterococcal infections also lies in their resistance to antibiotic therapy. Mortality associated with antibiotic resistant enterococcal infections is higher than when associated with susceptible enterococcal infections (Billington et al., 2014). Enterococci can easily develop resistance to antibiotics due to possession of some intrinsic genes, which confer resistance. This is reflected in the occurrence in environmental isolates as well as in clinical ones.

As an important glycopeptide antibiotic, vancomycin is a valuable and the drug of choice against enterococcal infection due to resistance to other antibiotics (Varela et al., 2013). Vancomycin affects the synthesis of cell walls by targeting the D-Alanyl-D-Alanine ends. Thus, resistance by enterococci involves altering the bacterial cell wall precursor D-Ala-D-Ala (normal cell wall component) to D-Ala-D-lactate or D-Ala-D-Serine (resulting to resistance to vancomycin) (Fisher and Philip, 2009). There are different types of vancomycin resistance controlled by *van* genes. The differences between these phenotypes are based on the composition of their peptidoglycan precursors in response to vancomycin, the level of resistance conferred, and their inducibility. The *van* genes prevalent in VRE include *vanA, vanB, vanC1* and *vanC2/3* (Nam et al., 2013) while others like *vanD, vanE,*

vanG, vanL, vanM and vanN have also been detected in *Enterococcus* but are less prevalent (Eshaghi et al., 2015; Xu et al., 2010).

Development and spread of vancomycin resistant enterococci (VRE) is of public health importance and a general threat to antibiotic therapy. The first report of VRE was from Great Britain in 1988 and was later detected in many other countries including United States (Nam et al., 2013). The occurrence of VRE has continually been increasing (Varela et al., 2013). VRE in poorly treated wastewater recycle back directly or indirectly to humans (Rizzo el al., 2013; Okoh et al., 2007) and so constitutes a serious danger to public health.

The occurrence of antibiotic resistant bacteria in WWTPs and effluents has been the focus of several investigations and has detected VRE at different treatment steps in the wastewater treatment processes including the final effluents (Araujo et al., 2010; Kotzamanidis et al., 2009; Luczkiewicz et al., 2010; Morris et al., 2012; and Nagulapally et al., 2009). Also, VRE has been detected from both hospital and domestic WWTP effluent from Alice, South Africa (Iweriebor et al., 2015). Sadowy and Luczkiewicz (2014) showed that WWTPs is an important source of enterococcal strains carrying antimicrobial resistance determinants (ARDs). The ARDs are usually connected with the presence of mobile genetic elements (MGE), thus increasing the chances of such genes for potential transfer to other organisms in the environment.

Managing and minimizing the level of VRE in the effluents from wastewater treatment is an important aspect in reducing infection risks among the exposed individuals. It will also reduce the risk of acquiring antimicrobial resistance by other bacterial species through genetic recombination in the environment. It is therefore important to assess the efficiency

of WWTPs in removing VRE and subsequent measures to be taken to curb dissemination of these high-risk bacteria in South Africa.

The first outbreak of VRE in a haematology unit in Durban, South Africa was reported in 2016 (Mahabeer et al., 2016). This has raised the need for increased surveillance in clinical settings and the environment. Determination of the prevalence of VRE from the WWTPs and their receiving water bodies will help to reinforce control measures to avoid subsequent outbreaks. The detection of virulence genes in enterococci especially the VRE will indicate higher risk associated with their presence in the environment. The expression of virulence factor in an organism is an indication of its pathogenic ability, and also being highly resistant to antibiotics makes it more dangerous. Also, virulence factor such as biofilm formation has been shown to contribute to antimicrobial resistance (Ballering et al., 2009). Therefore, the work on determination of the antibiotic resistance profile and their resistance genes, putative virulence genes and their possible expression on the enterococci isolates is a worthwhile study. This will help to assess the public health implication associated with wastewater effluents reuse.

1.2 Research aims and objectives

This study is designed to assess the efficiency of two Durban waste treatment systems in removing VRE from wastewater for safe effluent reuse. To achieve this aim, the following objectives were used.

1. To evaluate wastewater influents and effluents from two Durban wastewater treatment plants for the presence and quantities of total vancomycin resistant and vancomycin susceptible enterococci.
2. To assess the effectiveness of treatment process by cultural methods.

3. To determine the occurrence of VRE in the interlinked recipients.
4. To determine the presence of putative antibiotic resistance genes and virulence genes in the VRE isolates.

This thesis is composed of seven chapters including the introduction. Critical review of the relevant literature and the general methodology are in the second and third chapters respectively. Chapter four contains the assessment of the prevalence of vancomycin-resistant enterococci in the wastewater treatment plant and their recipients in relation to the concentrations of VRE as well as the total enterococci (TE) in these sites and the species of *Enterococcus* involved. The antibiotic resistance profile and resistance genes of the purified isolates are documented and discussed in chapter five, while their virulence attributes are treated in chapter six. The last, chapter seven is the general summary and conclusion of the entire work.

2 CHAPTER 2: LITERATURE REVIEW

2.1 The genus *Enterococcus*

Enterococcus is a genus of Gram-positive spherical bacterium that may occur in singles, in pairs or in chains. It comprises of many species, which among others include *E. faecalis, E. faecium, E. casseliflavus, E. avium, E. raffinosus, E. gallinarum,* and *E. durans*. Among these, the first two species are the better-known representatives and are more abundant. Enterococci are lactic acid, homo-fermentative and facultative anaerobic bacteria. They are non-spore forming and non-motile except *E. casseliflavus* and *E. gallinarum* that are motile. Pigmentation varies among species where for example *E. sulfureus*, *E. casseliflavus,* and *E. mundtii* have yellow pigments. Most enterococci are catalase-negative but a few pseudo-catalase species exist (Byappanahalli et al., 2012a), that lack oxidative cytochrome enzyme and are positive for pyrrolidonyl peptidase activity. They produce bacteriocins and possess low G+C content of less than 50mol% (Fisher and Phillips, 2009). Apart from *E. faecalis*, the peptidoglycan of other species of enterococci contains lysine-D-asparagine linkages (with D-asparagine as cross-bridge). *E. faecalis* possesses a peptidoglycan of the lysine-alanine 2-3type (Byappanahalli et al., 2012a).

Enterococci were previously classified as *Streptococcus*, but have been differentiated from *Streptococcus* due to physiological attributes. The term enterococci initially owing to their intestinal habitat include most group D streptococci. The *Enterococcus* was established as a genus when Schleifer and Kilpper-Balz suggested that enterococci should be a separate genus from the *Streptococcus* (Domig et al., 2003a). Presently, *Enterococcus* has about 36 known species, placed into five groups (Byappanahalli et al., 2012a). The members of each of the five groups; *E. faecalis, E. faecium. E. avium, E. gallinarum,* and *E cecorum* are as

shown in Table 2.1. Also the table contains some species such as *E. saccharolyticus* which do not belong to any of the five groups and thus categorized as ungrouped.

Enterococcus grows at a wide temperature range (5- 50ºC) with the temperature of 42.7ºC as the optimum for growth on brain heart infusion agar under aerobic conditions (Fisher and Phillips, 2009). Enterococci also grow within a wide range of pH. *E. faecalis* grows at pH between 4.6-9.9 with 7.5 as the optimum. They tolerate and also grow on 40% (w/v) bile salts and on 6.5% NaCl (Fisher and Phillips, 2009). *Enterococcus* can withstand extremes of temperature and pH due to its structural components of their cell membrane as shown by Park et al. (2016). This is related to the lipid and fatty acid content of the cell membrane.

2.2 Environmental stressors affecting the distribution of enterococci

Sunlight has been shown to affect the viability of enterococci causing a reduction in the concentration in water bodies (Boehm et al., 2009; Schultz-Fademrecht et al., 2008; Sinton et al., 2002). The effect is more pronounced at higher temperatures such as in summer and warm climates. The effects of sunlight diminish by higher turbidity and are affected by dissolved oxygen. Increased salinity will reduce their survival although they are more resistant than faecal coliforms, also used as indicators in water quality assessments (also see 2.4). Increase in salinity leads to corresponding decrease in time taken to achieve reduction (Kay et al., 2005). Sinton et al. (2002) in New Zealand compared enterococci in fresh and marine water from raw sewage and waste stabilization pond (WSP) and showed that enterococci survive longer in fresh water than in marine water, with enterococci from raw sewage being more sensitive to salinity than those from waste stabilization pond. This shows that the ability of enterococci to withstand environmental stressors is influenced by the nature of the environment from which it initially existed

2.3 Extra-intestinal habitats of enterococci

Enterococci, like the *E. faecium* and *E. faecalis* are mainly present in the gastrointestinal tract as part of the normal microbiological flora but these and especially other species of enterococci have other environmental habitats where they can multiply and persist (Byappanahalli et al., 2012a). These include aquatic vegetation for example the macrophytic aquatic green alga *Cladophora,* (Verhougstraete et al., 2010; Whitman et al., 2003). *Enterococcus* derives nourishment from this alga and thus can grow and multiply on it (Byappanahalli et al., 2003). Other habitats of enterococci are decaying seaweed (Anderson et al., 1997; Grant et al., 2001), submerged vegetation (Badgley et al., 2011 & 2010), and in association with planktons (Maugen et al., 2004). Different species of enterococci have also been detected in beach sand (Halliday and Gast, 2011) where their high concentration may be attributed to their ability to persist and multiply in this habitat (Yamahara et al., 2009) or by contamination from, humans, birds and animal faecal matter (Fogarty et al., 2003; Muniesa et al., 1999). Both fresh water and estuarine sediments can also be rich in enterococci (Anderson et al., 2005; Jeng et al., 2005) where their concentration can be far higher than that of the corresponding surface water.

Enterococci are also found in terrestrial habitats. They are found on flowers and buds of different plant species as well as on forage and different crops (Byappanahalli et al., 2012a; Müller et al., 2001). Their abundance is higher in the soils as compared to other faecal indicator bacteria (FIB) (Byappanahali et al., 2012b) probably due to higher resistance to environmental stressors. Similar to the association with aquatic plants, their association in the terrestrial habitat may be higher in the plant rhizosphere region where various compounds such as amino acids and other growth promoting substances are released as exudates (Sorensen, 1997; Walker et al., 2003).

Table 2:1: Groups and species of *Enterococcus* with their known habitats

Group	Species	Habitat
E. faecalis	*E. faecalis*	Human, animal (multiple), plant, insect
	E. haemoperoxidus	Surface water
	E. moraviensis	Surface water
	E. silesiacus	Drinking water
	E. termitis	Animal (termite)
	E. caccae	Human
E. faecium	*E. faecium*	Human, animal (multiple), plant, insect
	E. durans	Human, animal (multiple), insect
	E. hirae	Animal (multiple), plant
	E. mundtii	Soil, plant
	E. villorum	Animal (hog)
	E. canis	Animal (dog)
	E. ratti	Animal (rat)
	E. asini	Animal (donkey)
	E. phoeniculicola	Animal (bird)
	E. canintestini	Animal (dog)
	E. thailandicus	Human, animal (cattle)
E. avium	*E. avium*	Human, animal (multiple)
	E. pseudoavium	Human
	E. malodoratus	Animal (cattle)
	E. raffinosus	Human
	E. gilvus	Human
	E. pallens	Human
	E. hermanniensis	Animal (dog)
	E. devriesei	Animal (cattle)
	E. viikkiensis	Animal (broiler plant)
E. gallinarum	*E. gallinarum*	Human, animal (multiple), insect
	E. casseliflavus	Plant, soil, human, animal (multiple)
E. cecorum	*E. cecorum*	Animal (chickens)
	E. columbae	Animal (pigeon)
Ungrouped	*E. saccharolyticus*	Animal (cattle), sewage
	E. aquimarinus	Seawater
	E. sulfureus	Plant
	E. dispar	Human
	E. italicus	Animal (cattle)
	E. camelliae	Plant

(Byappanahalli et al., 2012a)

In summary, the enterococci as normal microbial flora in the alimentary tract of man (*E. faecalis* and *E. faecium*) and other animals (*E. ratti, E. canis*) can survive in non-enteric aquatic environments (including vegetation and sediments), soil and terrestrial vegetation as shown in Table 2.1. The enterococci may have originated from the gastrointestinal tract through faecal wastes or are autochthonous to the environment.

2.4 Enterococci as faecal indicator of water quality

Enterococcus species especially *E. faecalis* and *E. faecium* are normally used to assess the faecal contamination or pollution of water and other aquatic environments, including sewage, rivers and coastal areas (USEPA, 2013). This is due to their abundance in the faeces of warm-blooded animals and for their long-term survival in the environment. Thus they can conviently serve as faecal indicators of water quality. Their detection indicates possible presence of other enteric pathogens. Enterococci are excreted in faeces and are abundant in large numbers in water polluted by sewage or human and animal waste. They can survive for extended period following pollution and be subsequently detected. *E. faecalis* and *E. faecium* do not multiply in water environments and so their concentration at any point may show the exact and extent of faecal contamination. The above factors make enterococci suitable indicators of faecal contamination (Cabral, 2010). Furthermore, enterococci when compared with *E. coli* survive for longer periods and are more resistant to chlorination, which enhance their suitability as indicators of inefficient disinfection processes (WHO, 2011).

Enterococci as indicators of faecal contamination are employed in recreational water assessments throughout the world both in marine waters and fresh waters (WHO, 2003 and 2009). There is a strong direct relationship between the concentration of enterococci

measured in polluted recreational marine waters and the risk of gastrointestinal illness of swimmers (Boehm and Soller, 2011; Wiedenmann et al, 2006; Wade, et al., 2006). Therefore, both US, EU, and the World Health Organization (WHO) recommend that enterococci be used as an indicator of recreational water quality and risk of swimmer illness (Boehm and Sassoubre, 2014; USEPA, 2012; WHO, 2009). Additionally, in the European Union (EU), enterococci are also used as an indicator of drinking water quality (The Council of the European Union, 1998) with permitted levels of less than 1 in a 100 mL sample of tested drinking water from a tap, and less than 1 in 250 mL samples of bottled water (Boehm and Sassoubre, 2014).

2.5 Concentration and distribution of enterococci species in WWTP

The concentration of enterococci in human faeces is in the range of 10^5 to 10^7 CFU/g and the expected corresponding concentration range in raw untreated wastewater will be approximately 10^5 to 10^7 CFU/100 mL. This concentration was similar to that shown by Rosenberg-Goldstein et al. (2014). Sadowy and Luczkiewicz (2014) showed a more than 99% removal efficiency of enterococci in non-disinfected wastewater with remaining high counts (up to $6.1x10^5$ CFU/100 mL) in the effluent. Also, Łuczkiewicz et al. (2010) found enterococci count of more than 10^4 CFU/100 mL in the effluent of municipal wastewater treatment plant using activated sludge system.

Species identified among wastewater isolates were *E. faecium* (57%), *E. hirae* (26.4%) and *E. faecalis* (14.7%) and less frequently *E. gallinarum/casseliflavus, E. durans* and *E. avium* (1.9%) (Sadowy and Luczkiewicz, 2014). For Łuczkiewicz et al. (2010), the species abundance among the isolates were; *E. faecium* (60.8%) and *E. faecalis* (22.1%), *E. hirae* (12.1%), *E. casseliflavus/gallinarum* (4.5%), and *E. durans* (0.5%). Said et al. (2015) did

not quantify enterococci from WWTP but showed *E. faecium* as the most abundant species followed by *E. faecalis*. The concentration of *Enterococcus faecium* and *Enterococcus faecalis* are higher in human faeces than other enterococcal species, while others such as *Enterococcus casseliflavus* and *Enterococcus mundtii* are isolated more frequently from environmental surfaces (such as on plants) than other species (Boehm and Sassoubre, 2014; Ferguson et al., 2005)

In as much as the concentration of enterococci in the WWTPs is high, the proportion of VRE remained low in several studies. The VRE count in the influent ranged from 2.5×10^3 to 8.6×10^4 CFU/100 mL and 0 to 3.3×10^0 CFU/100 mL in the effluent (Rosenberg-Goldstein et al, 2014). Several studies (Nagulapally et al., 2009; Kotzamanidis et al., 2009; Luczkiewic et al., 2010; Araujo et al., 2010; and Morris et al., 2012), have detected VRE from various stages of the wastewater treatment process and in the treated effluents. Carey et al. (2016), in their study on occurrence of vancomycin-resistant and susceptible *Enterococcus* spp. in reclaimed water used for spray irrigation detected enterococci from most of the reclaimed water samples. The prevalence of VRE was low while *E. faecalis* still remained most abundant species isolated.

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Microbial source tracking is defined as the use of indicator organisms or pathogens to trace the origin of pollution. Proper pollution assessment and control require identification and management of point- and diffuse microbial contamination. Different species of Enterococci are linked to different animal origins. Enterococci and their phages are valuable tools in microbial source tracking. It was shown that some strains of enterococci were 100% specific to either cattle, human, or pig samples. *Enterococcus casseliflavus*, *E. mundtii*, and *E.*

gallinarum were found to be related to cattle while those specific to human and pig belonged to either *Enterococcus faecium* or *Enterococcus faecalis* (Purnell et al., 2011).

2.6 Isolation and enumeration of enterococci

Isolation of enterococci from samples is usually by culturing on selective media (APHA, 2005) and subsequent confirmation using biochemical tests (Facklam and Elliot, 1995; Manero et al., 2002) and molecular techniques (Patel et al., 1998). The choice of media or method depends on the nature or type of sample, the degree of the contamination and the purpose of the study. The choice of a particular medium may be as a result of personal experience or convenience. Enumeration of bacteria from less contaminated sample such as drinking water requires concentration of the water sample for example by membrane filtration and subsequent cultivation on membrane selective medium (USEPA 2006). Highly contaminated samples will require serial dilution followed by spread plate or filtration of diluted sample as employed by Rosenburg-Goldstein et al. (2014). *Enterococcus* grows very well in rich complex liquid media such as Brain Heart Infusion (BHI) broth or Trypticase Soy broth (TSB) and on their corresponding agars that also support the growth of a general heterotrophic flora of bacteria. Selective media are mainly used for quantification of enterococci. A wide range of these exist including Aesculin-bile-azide medium (ABA), Kanamycin aesculin azide agar (KAA), Citrate azide tween carbonate agar (CATC), Slanetz and Bartley medium (S&B), Thallous acetate tetrazolium glucose agar (TlTG), Crystal violet azide agar (KA) (Reuter, 1995) and *Enterococcus* Indoxyl –β-D-Glucoside agar (EI) (USEPA, 2006). Non-group D streptococci do not grow on media containing high concentration of bile salts for example Aesculin-bile-azide agar. On this medium the colonies turn dark brown or black after 24 hr incubation at 37^{o}C and are counted as presumptive for enterococci. Selective appearance can also be shown by addition of the

chromogen, indoxyl-β-D-glucoside in *Enterococcus* medium where the formation of colonies with a blue halo after 24 hr incubation at 37^{o}C is characterised as presumptive for enterococci. Also the formation of red to marron colonies on Slanetz and Bartley agar following incubation at 44^{o}C for 48 hrs is selective for *Enterococcus*. It has been shown by Domig et al. (2003a) that incubation at 44.5^{o}C were more selective for enterococci using some selected media while lower results were obtained at 37^{o}C.

Membrane-enterococcus agar (Slanetz-Bartley, mSB) were originally devised by Slanetz and Bartley and has since 1957 been used as a selective medium for the enumeration, isolation, and cultivation of enterococci from water, sewage and faeces. It is suitable both for the membrane filter method and direct plating of sample onto the medium in order to detect and enumerate enterococci (Domig et al., 2003a; Atlas, 1995). The medium contains 2, 3, 5, -Triphenyl tetrazolium chloride (TTC) which is the marker substance and sodium azide which makes the medium selective for enterococci. TTC forms formazan, an insoluble red compound as a result of its reduction by microorganisms. The coloured colonies are easily visible especially on the membrane filters and also directly on plates. At pH of 6.2, *Enterococcus faecalis* produces a deep red colouration while others including *E faecium* form lighter red coloured colonies and thus the colour are used to presumptively differentiate between these species (Domig et al., 2003a).

Enterococci form pink, dark red or brownish colonies on Slanetz-Bartley (SB) agar with the membrane filters after 48–72 hrs incubation at 37^{o}C. The method proved to be precise and accurate with good recovery and specificity result in comparison to other tested media (Dionisio and Borrego, 1995). For optimum results with SB medium, plates are pre-incubated for 4 hrs at 37^{o}C before continued incubation for another 44 hrs at 44^{o}C. This has

been used for the enumeration and isolation of enterococci from water samples (Domig et al., 2003a; Fricker and Fricker, 1996).

Addition of extra substances such as the antibiotic vancomycin to the selective medium will enhance the enumeration and isolation of vancomycin resistant enterococci (VRE). Enterococcosel agar (ECSA) agar plates containing 4 or 16 mg vancomycin/L were compared for screening glycopeptide resistant enterococci (GRE) in clinical specimen by Wendt et al. (1999). It was shown that the 16 mg vancomycin/L medium produced a better result. The 4 mg/L vancomycin media showed lower specificity due to growth of some vancomycin susceptible isolates as well from heavy non-standardized inoculum. The 16 mg/L vancomycin media had improved specificity but did not support the growth or isolation of *vanC* resistant *Enterococcus gallinarum* and *E. casseliflavis*. In another study, Reisner et al. (2000) found that pre-inoculation of samples in a liquid enrichment is important for isolating VRE from environmental samples. This may enhance recovery of the desired organism. Though, considering the purpose of the research, inoculating the sample directly on plate without pre-enrichment will give a good and proper count of VRE present in the sample (proper enumeration procedure), but enrichment procedures are usually used to isolate or to detect occurrence in samples with expected low levels of VRE.

On selective media, total enterococci count can be obtained, but the different species involved cannot be ascertained and thus the need for further analysis of the individual isolates. Enumeration of enterococci on selective media may yield non-enterococci isolates and thus still needs some confirmatory tests to avoid false results. Maraccini et al. (2012) showed that there are non-enterococci isolates on membrane *Enterococcus* Indoxyl –β-D-Glucoside (mEI) which were identified as *Aerococcus viridans*, *Streptococcus mutans*, *S.*

galdyticus, *Leuconostoc* sp, and *Pediococcus acidilactici*. Also, Viau and Peccia (2009) reported the growth of *Bacillus* sp., *Vagococcus* sp., and *Desemzia incerta* on mEI medium. So, enumeration of enterococci on selective media remains an initial step in the identification process and thus the result obtained is reported as presumptive enterococci.

2.7 Confirmation and speciation of enterococci

Speciation is very important for microbial source tracking and health risk assessment (Schoen and Ashbolt, 2010). Using phenotypic characteristics only to distinguish *Enterococcus* species from other Gram-positive, catalase-negative coccoid bacteria is difficult and involves a rigorous process. So identification by phenotypic methods is usually established by reverse methodology (elimination of other species traits first) (Fisher and Phillips, 2009). *Enterococcus* belonging to the Lancefield serogroup D is differentiated from other members of this group by growth in 6.5% (w/v) sodium chloride. Other phenotypic characteristics that can be used to differentiate *Enterococcus* from other Gram-positive cocci that are catalase negative include sugar fermentation patterns, pyroglutamyl aminopeptidase enzyme activity (PYRase) (Domig et al., 2003b), growth at defined temperatures and physiological characteristics (Shanks et al., 2006) such as bile aesculin test.

Among the miscellaneous catalase-negative, Gram-positive cocci, *Enterococcus, Streptococcus pyrogenes, Lactococcus lactis, Aerococcus, Gemella,* can be differentiated from other species such as *Streptococcus* and *Lactococcus, Leuconostoc*, *Pediococcus*, and *Lactobacillus* by being PYRase positive. Furthermore, *Aerococcus* and *Gemella* can be eliminated from this group of PYRase positive related *Enterococcus* by forming clusters

instead of chains (Rouff, 2002). Thus the use of a selective medium such as Slanetz and Bartley agar and incubation at 44^{o}C followed by Gram staining, catalase, pyrase and bile asculin tests is a valid test chain in the identification of *Enterococcus*.

Some biochemical tests such as catalase test and pyrrolidonylnase production helps to further differentiate enterococci from related group of Gram- positive cocci. Further identification of different species of the isolates can be reached by comparing their enzyme patterns and ability to grow on and ferment different carbohydrates. These are unique to each enterococcal species, and the production of certain metabolites in specified media can also be applied to differentiate and identify *Enterococcus* species. Traditional methods for *Enterococcus* species identification with conventional biochemical tube testing are time and labour intensive, and thus the use of commercial identification kits such as API 20 Strep and Vitek have gained ground as much easier and quicker. Buschelman et al. (1993) compared these commercial kits and showed that they provided accurate identification of the species in their database but that further modification of these systems would be needed to account for new species. Winston et al. (2004) later had a different view and recommended that API 20 Strep should not be used alone as it might incorrectly speciate enterococci. Morphological and biochemical attributes of enterococci regarded as phenotypic characters may be useful but may also misrepresent the phylogenetic relationship and thus complicate the speciation of enterococci. Therefore, these phenotypic profiles still remain a preliminary step in the identification procedure. Final confirmation relies on the genetic identification which involves DNA-based techniques.

Protein fingerprinting by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a more rapid and precise method than other phenotypic profiles. It compares the

whole-cell protein patterns of an unknown isolates by zone electrophoresis of their proteins under well-defined condition. The protein profiles are fingerprints of the bacterial strain identity. The technique is relatively simple, cheap and has earlier been used by several authors (Müller et al., 2001; Andrighetto et al., 2001) to identify and classify enterococci species and to identify novel species (Vancanneyt et al., 2001; Tyrrell et al., 2002). The result obtained by SDS-PAGE has been shown to agree with that of Random Amplified Polymorphic DNA-PCR (RAPD-PCR) (Andrighetto et al., 2001). The antimicrobial resistance pattern has been considered by some authors, for example Willey et al. (1999) and Da Gloria et al. (1998) as a method of identification. This may not be reliable especially with resistance genes which are carried on a mobile genetic element as illustrated by Young (2016). So resistance to antibiotics cannot be used alone as a method of identification. Therefore, the identification procedure involving the core genetic makeup remains an excellent and confirmation method.

2.7.1 DNA-based methods for confirmation of enterococci

DNA based methods for identification of enterococci are among others, restriction endonuclease analysis (REA), pulse-field gel electrophoresis (PFGE), ribosomal RNA gene restriction analysis (ribotyping), polymerase chain reaction (PCR) and gene sequencing. Various versions or modified protocols have been developed and used both to identify and differentiate enterococci species and to assess the presence of antimicrobial resistance genes as well as virulence genes. Ke et al. (1999), developed a protocol targeting the *tuf* gene for identification of the genus *Enterococcus* which Deasy et al. (2000) improved by designing a specific primer for *Enterococcus* which did not react with other related genera. Dutka-Malen et al. (1995) developed a species-specific PCR assay targeting the D-alanine/D-alanine ligase (*ddl*) gene and Miele et al. (1995) developed a set of primers for the *van* genes

2.7.2 Primers for molecular confirmation of *Enterococcus*

Different primer sequences have been developed to identify *Enterococcus* by targeting either 23S rRNA, 16S rRNA, or functional genes. Among these three, the information for 16S rRNA sequencing is more available than that for 23S rRNA, and only a few non-ribosomal genes have been used in environmental studies to identify enterococci (Ryu et al., 2013). Therefore, the sequence database for functional genes of environmental enterococci and other phylogenetically related genera is much more limiting than that of 16S and 23S rRNA genes. Furthermore, sequence conservancy in functional genes is considerably lower than that in rRNA and thus making ribosomal genes the more reliable target for primers (Ryu et al., 2013). However, non-ribosomal genes have been used to differentiate between enterococcal species (Jackson et al., 2004; Vermette et al., 2010), and for the direct identification of the genus *Enterococcus* (Ke et al., 1999).

Four genus specific primers (*Ent1, Ent2, Ent3,* and *Ent4*) targeting the 16S rRNA developed by Ryu et al. (2013) produced positive results with more than 97% of the environmental strains tested and with few false positive results (0 - 24%). Two of these primers, *Ent2* and *Ent3* did not amplify with non-enterococcal strains during the validation assay but*Ent2* had a limited sensitivity and only detected 59% of the enterococcal strain. Therefore, *Ent3* primer was recommended as useful in confirmatory tests while *Ent2* was limited and gave false negative results (Ryu et al., 2013).

Entero1, a probe based primer targeting 23S rRNA developed by Ludwig and Schleifer (2000), was proposed as an alternative method for the rapid detection of *Enterococcus* in recreational water (USEPA, 2010). However, *Entero1* cross amplified with lactobacilli

resulting in an overestimation of enterococci in recreational waters due to false positive results (Frahm and Obst, 2003).

Deasy et al. (2000) developed a primer based on 16S rRNA gene sequences specially designed to differentiate the members of the genera of *Lactococcus* from *Enterococcus.* This primer as validated by the authors showed no amplification with strains of other lactic acid bacteria (LAB) including *Streptococcus thermophilus,* S. *salivarius, Lactobacillus casei, Lb. plantarum, Lb. acidophilus, Lb. delbrueckii* ssp. *lactis, Lb. helveticus, Pediococcus acidilactici, P. pentosaceus* and *Leuconostoc oenos.*

Enterococcus primer, Ent1 5′-TACTGACAAACCATTCATGAT G-3′ and Ent2 5′-AACTTCGTCACCAACGCGAAC-3′ of a 112-bp DNA sequence was developed Ke et al. (1999). This primer is targeting the *tuf* gene, which encodes the EF-*Tu* known as elongation factor, which is an essential constituent of the bacterial genome. Using the PCR conditions of 30/40 cycles after the initial denaturation step yielded slightly different specificity results. The primer amplified with most species of *Enterococcus* but not *E. solitaries* and had a positive reaction with two *Abiotrophia* species, *A. adiacens* and *A. defective* for 30-cycle PCR. In the 40-cycle regimen, four *Listeria* species, *Listeria innocua*, *L. ivanovii*, *L. monocytogenes*, and *L. seeligeri*, were also positive in addition to the enterococci and *Abiotrophia* species. Since *E. solitaries* is not a common human pathogen, this primer could conveniently be used for clinical enterococci isolates. Also, keeping to a 30 PCR cycle regimen will reduce false positive. This primer has been used by several authors (Iweriebor et al., 2015; Fouad et al., 2005; Getachew et al., 2012) to confirm their presumptive enterococci isolate.

Species-specific primers have been developed to speciate enterococci (Ryu et al., 2013; Vermette et al., 2010; Jackson et al., 2004). The primers and protocol developed by Jackson et al. (2004) seems to be reliable and convenient. It can be used to speciate 23 species of enterococci in a multiplex group of seven. The interesting feature of this assay is that it also incorporates the genus primer and so allows for simultaneous confirmation of both genus and species identification.

2.7.3 Fluorescent *in situ* hybridisation (FISH) for detection and quantification of enterococci in wastewater and river samples.

Fluorescent *in situ* hybridization is a molecular cytogenic method that uses fluorescent probes that specifically bind to only those parts of the chromosome that have high degree of sequence complementarity. The application of this method to identify microorganisms is based on the use of specific fluorescent-labelled DNA probes, which target and hybridize the rRNA of the organism to be identified. FISH is more rapid than cultural identification methods of bacteria. Many microorganisms lose their tendency to grow in cultivation medium but at the same time have the potential to be active (Pommepuy et al., 1996). FISH methods gives a supplementary assessment of presumptive viability in this context. In the past few decades, the FISH method has been used to detect and enumerate different phylogenic groups of bacteria from both clinical (Gharibi et al., 2010) and environmental samples (Amann et al., 1990; DeLong, 1992; Pernthaler et al., 2002). The limitations of FISH in environmental samples is as a result of low rRNA content of the target organism due to low metabolic activities imposed by environmental stress and thus organisms may not be detected (Amann et al., 1995).

According to Bouvier and Giorgio (2003), many factors affect the efficiency of the fluorescent *in situ* hybridization technique, which are due to methodological procedures and environmental factors or conditions. The rate of growth of the bacteria, phylogenetic groups and the type of ecosystem are the environmental factors while the methodological factors include temperature, concentration and nature of the reagents used. However, there have been some improved methodology protocol to increase the efficiency of FISH (Pernthaler et al., 2002; Glöckner et al., 1996; Fuchs et al., 2000; DeLong et al., 1999).

2.7.4 Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry

Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry has become an efficient tool for identification of microbes. The isolate to be identified is mixed with solution of an energy-absorbent, organic compound known as matrix. The principle of MALDI-TOF is based on ionization and desorption of a matrix in an automated mode with a laser beam which generates protonated ions from the analyte in the sample embedded in the matrix. The protonated ions when accelerated at a fixed potential separate from each other based on their mass-to-charge ratio. The mass-to-charge ratio is measured by determining the time required for it to travel the length of the flight tube (TOF). The measured TOF is used to generate peptide mass fingerprint (PMF) for analytes in the sample. The generated PMF of the unknown isolate is compared to PMF in the database to identify the isolate (Singhal et al., 2015).

It has been shown that culture condition and time of the isolate have no effect on its identification using MALDI-TOF (Carbonnelle et al., 2007). MALDI-TOF is a fast and

accurate method that can be used to identify enterococci. Apart from the initial cost of the MALDITOF equipment, it is less expensive than the molecular based methods. However, its accuracy depends on the availability of the database such that new organism or strains that are not in the database may not be correctly identified.

2.8 Estimation of prevalence of antibiotic resistance bacteria in environmental samples

Culture based methods are most often used for determining the antibiotic resistance and susceptibility of most bacteria from environmental samples by using disc-diffusion or micro-dilution methods compared to standardized available references (CLSI, EUCAST). This procedure is tedious and takes a long time (Łuczkiewicz et al., 2010; Rizzo et al., 2013) due to isolation, purification and subsequent susceptibility testing procedures involved. However, with certain modification such as using selective media supplemented with antibiotics at concentrations inhibitory for the target organism, the percentage resistance can be calculated as the ratio of bacterial counts on the antibiotic medium with the counts on the medium without the antibiotic (Figueira et al., 2011; Novo and Manaia, 2010; Watkinson et al., 2007). This has proved to be reliable and convenient for quick characterization of a large number of samples. Watkinson et al. (2007) used it to estimate the frequency of resistant strains of *E. coli* against ampicillin, tetracycline, ciprofloxacin and sulfamethoxazole. Novo and Manaia (2010) also employed this method to estimate the prevalence to amoxicillin, tetracycline and ciprofloxacin resistance in heterotrophs, enterobacteria and enterococci and compared the antibiotic resistance between the influent and efflulent of three urban wastewater treatment plants (UWTPs). The methodological approach also allows for the

selection and concentration of the target organism thereby making further analysis easier. As shown in the work by Figueira et al. (2011), on characterization of quinolone resistance in *Aeromonas* isolated from water habitats, the gene *aacA6-ib-cr* was detected mainly in bacteria isolated in the presence of ciprofloxacin. This was due to concentration of the quinolone resistant *Aeromonas* by selection using ciprofloxacin supplemented medium where after colonies were picked from a large pool of the both resistant and non-resistant isolates. The use of media supplemented with antibiotic is easy and reliable for estimation of the prevalence of antibiotic resistant bacteria, but Kariyama et al. (2000), experienced a slower growth by enterococci isolated using vancomycin supplemented medium. Thus, the antibiotic may have caused some adverse effect on the physiological and morphological characteristics of the bacteria.

Multiple antibiotic resistance (MAR) index is a useful tool in determining the extent to which an isolate is resistant to various antibiotics. It is calculated as the ratio of number of antibiotics to which organism is resistant to total number of antibiotics to which organism is tested. It may also serve as a valid method of bacteria source tracking. A MAR index of above 0.2 shows that the organism was isolated from antibiotic laden environment and so of a 'high-risk' source (Davis and Brown 2016).

2.9 Enterococci as pathogens

The pathogenic importance of enterococci greatly outweighs that they are ordinary commensal of the gastrointestinal tract of humans (Tendulkar et al., 2003). The mobile genetic elements (MGEs) are among the factors that contribute to the transition of enterococci from commensals to pathogens (La Rosa et al., 2015). Enterococci can become

pathogenic due to their ability to easily acquire genes. Upon acquisition of virulence gene, a non-virulent *Enterococcus* becomes pathogenic. This is the underlying reason for many authors to describe them as emerging pathogens (Giridhara-Upadhyaya et al., 2009) and/or opportunistic pathogens (Al-Ahdal et al., 2012; Comerlato et al., 2013). Over 25% of the enterococci genome is composed of mobile and/or exogenously acquired DNA which consists of conjugative and composite transposons, a pathogenicity island (PAI), integrated plasmid genes phage regions, and high number of insertion sequence (IS) (Giridhara-Upadhyayaet al., 2009). About one-third of the PAI seem to have evolved from integration into chromosome of a conjugative plasmid sequence (Giridhara-Upadhyaya et al., 2009). The PAI also encode 18 open reading frames (ORFs) without any well-defined functions. The ORFs is not present in commensal-derived enterococci which may suggest that they contribute to the survival of enterococci in the hospital environment or in the process of disease transmission or pathogenesis (Giridhara-Upadhyayaet al., 2009).

As pathogens enterococci have been shown to cause urinary tract infections, wound infections, bacteraemia and endocarditis (Carey et al., 2016), hepatobiliary sepsis, neonatal sepsis (Fisher and Phillips, 2009), tooth infection (Vidana et al., 2011; Anderson et al., 2016), and has been shown to be associated with inflammatory bowel disease (IBD) in human (Golinska et al., 2013). Between 2006 and 2007, *Enterococcus* spp. was the third most commonly reported pathogen causing healthcare-acquired infections in the United States (Carey et al., 2016). They are presently recognised as important nosocomial pathogens and the second-most common cause of urinary tract infections and the third-most common cause of nosocomial bacteraemia (Comerlato et al., 2013).

The severity of enterococcal infections lies on their resistance to available antibiotics and therefore is difficult to treat. Reports show that mortality due to enterococcal infection is higher when resistance strains are involved. For vancomycin, mortality increased from 27% in vancomycin-susceptible to 57% in vancomycin-resistant infections. The species commonly associated with clinical infections are *E. faecalis* and *E. faecium* with *E. faecalis* being more prevalent while *E. faecium* is known for increased antibiotic resistance (Fisher and Phillips, 2009).

2.9.1 Virulence attributes in enterococci

Virulence factors contribute to the pathogenic ability of an organism. Virulence factors associated with enterococci include gelatinase production, *Enterococcus* surface protein (*Esp*), aggregation substance (AS), cytolysin, hyaluronidase, and biofilm formation (Chuang et al., 2009; Vankerckhoven et al., 2004; Iweriebor et al., 2015). While *Esp* and hyaluronidase are specific to *E. faecium*, aggregation substance, gelatinase, cytolysin and *Esp* are found in *E. faecalis* (Vankerckhoven et al, 2004). Other factors that are associated with virulence in *Enterococcus* include; *E. faecium* endocarditis antigen *efaA*, *Ace* (Microbial surface component recognizing adhesive matrix molecule adhesin of collagen from enterococci), serine protease, capsule, cell wall polysaccharide and superoxide (Iweriebor et al., 2015; Giridhara Upadhyayaet al., 2009).

Gelatinase, a zinc metalloprotease, is linked with the gene *gelE* (Lindenstrau et al. 2011). Gelatinase liquefies gelatine and as a virulence factor, it hydrolyses the collagen, casein, haemoglobin and other proteineous substances of the host cell enabling bacterial invasion (Giridhara Upadhyaya et al., 2009). If gelatinase could hydrolyse haemoglobin, its activity may be related or could enhance that of cytolysin when both are present in an organism.

Gelatinase is an invasive factor. It causes damage to host tissues and help the advancement and survival of enterococci in newly infected sites (Strateva et al., 2016). It has been shown to be the principal mediator of pathogenesis in endocarditis (Thurlow et al., 2010). Detection of gelatinase production in the laboratory can be done by inoculating the enterococci isolate on tubes of freshly-prepared peptone-yeast extract or brain heart infusion agar containing about 4% gelatine. Liquefaction after 24hr incubation at 37^{o}C upon cooling at ambient temperature is reported as positive (Comerlato et al., 2013; Marra et al., 2007). The presence of virulence genes in enterococci may not be expressed phenotypically. Only 60 % of the *E. faecalis* isolates that have *gelE* genes produced gelatine (Qin et al., 2000), and 77 out of 88 strains that possess *gelE* gene expressed it phenotypically (Revathy et al., 2010). Also, the numbers of VRE strains genotypically positive for *gelE* were higher than those showing positive reaction by phenotypic tests (Biswas et al., 2016). It is therefore important to use both phenotypic and genotypic assays for proper determination of virulence of a strain.

Aggregation substance (AS), encoded by a plasmid gene, ordinarily involved in bacterial aggregation during conjugation which facilitates plasmid exchange is shown to mediate binding to the host epithelium during the infection process (Schlievert et al., 2010). AS significantly helps enterococci to resist phagocytosis by inhibiting the respiratory bursts of macrophages (Golińska et al., 2013; Süssmuth et al., 2000).

The *esp* gene codes for the *Esp* which are implicated in the colonisation and persistence of *E. faecalis* strains in the urinary tract. Enterococcal surface protein is also shown to participate in or enhance biofilm formation (Chuang-Smith et al., 2010), which strongly helps the survival of bacteria in biopolymers and seem to also increase in antimicrobial resistance (Ballering et al., 2009). *Esp* is linked to increased virulence and its structure might

help the organism for immune evasion in case of immune deficiency (Vankerckhoven et al., 2004). The *esp* gene has been shown to be more prevalent among clinical isolates than commensal isolates (Ira et al., 2013; Upadhyaya et al., 2011).

Enterococcal cytolysin also known as haemolysin is lethal to prokaryotic and eukaryotic cells (Cox et al., 2005; Giridhara Upadhyaya et al., 2009) and has been shown to exacerbate the severity of human infections (Vankerckhoven et al., 2004). Expression of cytolysin is regulated by a novel, two-component regulatory system via the quorum sensing mechanism, components lysin (L) and activator (A). Five genes are required for the expression of cytolysin in which *cylL1, cylL2*, *cylM,* and *cylB* code for component L while *cylA* codes for component A. Detection of haemolysin relies on formation of a clear zone of β haemolysis around colonies on 5-10% human or horse blood agar after 24hrs incubation at 37ºC, which positively relates to cytolysin. Hyaluronidase is a degradative enzyme, which causes tissue damage and thus contributes to invasion (Kayaoglu and Ørstavik, 2004). The presence of these virulence genes has been shown in *Enterococcus* especially in clinical strains. They have also been detected in food and environmental enterococci isolates (Said et al., 2015; Iweriebor et al., 2015).

The relationship between the ability to form biofilm and the presence of *esp*, *gelE* and *asal* genes have been investigated and is looked upon differently by various authors (Chuang-Smith et al., 2010; Dworniczek et al., 2012). The *E. faecalis fsr* quorum-sensing system was shown to controls biofilm development through the production of gelatinase. Inactivation of the *fsr*-controlled gene *gelE* encoding the zinc-metalloprotease gelatinase (La Rosa et al., 2015) was found to prevent biofilm formation **(**Hancock and Perego, 2004**).** *Esp* was shown to participate in or enhance biofilm formation by some authors (Chuang-Smith et al., 2010;

Heikens et al., 2007). However, in a study on six clinical isolates of *E. faecalis* for the expression of *esp*, gelatinase activity, and biofilm formation, it was shown that the isolates with and without *esp* gene and/or gelatinase activity were able to form biofilms *in vitro*. Thus, there is no correlation between the expression of these virulence factors and biofilm formation (Marra et al., 2007). Also according to Sillanpää et al. (2010), *esp* negative *E. faecium* isolates efficiently formed biofilm. Furthermore, Dworniczek et al. (2012) showed that no clear relationship exists between the expression of *esp* or *gelE* and biofilm formation. Also, Comerlato et al. (2013), in their study on clinical isolates could not show an association between the presence of *esp* and biofilm production, and thus assumed that other factors are associated with biofilm formation. Biswas et al. (2016) reported that 61.1% of their clinical VRE isolates formed biofilm while *asa1* and *esp* genes were detected in 30.5% and 27.5% of the VRE isolates respectively. Since the numbers of VRE isolates that contained the *asa1* and *esp* genes presumed to be responsible for biofilm formation were less than the number that actually formed it, they concluded that these genes are not really responsible for biofilm formation.

The virulence of an organism is controlled by virulence genes, which occupy specific regions on the genome known as pathogenicity islands. It has been proven that at least one-third of the PAI in certain strains of *Enterococcus* may have resulted from integration into chromosome of a conjugative plasmid sequence (Giridhara Upadhyaya et al., 2009). Therefore, it may be concluded that some of the above virulence attributes of *Enterococcus* are not intrinsic but acquired from the environment. Consequently, these virulence factors may not be species specific but rather source specific. Thus, certain virulence factors are prevalent in clinical isolates or associated with a particular disease.

Although some virulence genes may be more prevalent in clinical strains which comprises of *E. faecalis* and *E. faecium*, or are peculiar to some species, their detection across other species of *Enterococcus* such as *E. gallinarum*, *E. mundtii*, *E. raffinosus*, *E. solitaries*, *E. malodoratus*. *E. dispar* and *E. hirae* have been shown (Biswas et al., 2016). This is due to the ability of *Enterococcus* to easily acquire and transfer genes. The prevalence of *gelE*, *esp*, and *hyl* genes were significantly higher in clinical VRE than VSE isolates (Biswas et al., 2016).

The presence of virulence genes in *Enterococcus* was detected by a molecular method using relevant primers. Multiplex PCR for detection of five virulence genes (*asa1, esp, cylA, gelE* and *hyl*) in *Enterococcus* was developed by Vanckerhoven et al. (2004) and used by several authors (Biswas et al., 2016). Thus the method has proven reliable and convenient.

2.9.2 Mechanism of antibiotic resistance and antibiotic resistance genes in enterococci

The mechanism of drug resistance in enterococci is related to genes, which may be intrinsic or acquired such as in glycopeptide antibiotics. Some strains of enterococci possess a natural, low-level intrinsic resistance to beta-lactam antibiotics as a result of low affinity to penicillin binding proteins (PBPs) for the beta-lactam agents (Taucer-Kapteijn et al., 2016) or that they produce beta-lactamase that confer resistance to beta lactam antibiotics such as penicillins (Murray, 1990). Resistance by enterococci can also be by active efflux. Multi-drug efflux pump gene and 34 efflux pump genes have been detected in *Enterococcus* (Jia et al., 2014; Davis et al., 2001; Jonas et al., 2001). Enterococci lack cytochrome enzymes and do not have enough energy for active uptake of substances like antibiotics into the cell.

This naturally confers on them some resistance to aminoglycosides at low levels (Klare et al., 2003). Genes conferring resistance to ciprofloxacin are *gyrA* and *parC* (Nowroozi et al., 2014) and resistance to fluoroquinones by enterococci has been shown to be by active efflux (Lynch et al., 1997).

Intrinsic resistance (inherent) traits in enterococci include resistance to semisynthetic penicillins, cephalosporins, low levels of aminoglycosides, and low levels of clindamycin, while acquired resistance include resistance to chloramphenicol, erythromycin, high levels of clindamycin, tetracycline, high levels of aminoglycosides, penicillin by means of penicillinase, fluoroquinolones, and vancomycin (Murray, 1990). Acquired resistance in enterococci occur through plasmids and transposons and different antibiotic resistance genes have been shown to be carried by plasmids and transposons in *Enterococcus* (Fisher and Phillips, 2009).

Up to 60 to 80% of enterococci can be resistant to tetracycline and tetracycline resistance genes detected are *tetL, tetM, tetN*, and *tetO*. They confer resistance by two different mechanisms; *tetL* mediates active efflux of tetracycline from cells (Molale and Bezuidenhout, 2016) while *tetM* and *tetN* mediate resistance by a mechanism that protects the ribosomes from inhibition by tetracycline. *tetL* is contained in the well- studied plasmid *pAMa*.1 and *tetM* is on the conjugative transposon Tn916 (Murray, 1990).

Antibiotic resistance genes (ARGs) have been detected using different molecular methods, which include but are not limited to DNA hybridization, DNA sequencing, and polymerase chain reaction (PCR). Both simple and multiplex PCR assays have been extensively used in pure cultures and mixed environmental samples for detection of specific ARGs. However,

a false-positive result is often obtained in the PCR assay (Zhang et al., 2009). Southern hybridization of PCR products labelled and used as DNA probes to plasmid or chromosome DNA samples from strains harbouring target genes, can avoid the false-positive PCR results (Zhang et al., 2009; Ahmed et al., 2006; Akinbowale et al.,2007). Well-developed multiplex PCR is more convenient than simple PCR because it allows for simultaneous detection of more than one ARG. With multiplex PCR, some DNA amplifications may be inhibited resulting in false-negative results and the dimer formation between primer pairs disturbs experimental results leading to poor sensitivity (Markoulatos et al., 2002). Despite these limitations, multiplex PCR is still a convenient and rapid method for detection of multiple genes in an isolate (Agersø et al., 2007). Various multiplex PCR protocols have been used by different authors for the detection of vancomycin resistance genes (Nam et al., 2013; Bell et al., 1998), macrolide resistance genes (Jensen et al.,2002), and tetracycline resistance genes (Agersø et al.,2007; Ng et al**.,** 2001).

2.9.3 Vancomycin antibiotic and its resistance in enterococci

The glycopeptides are structurally complex antibiotics, which are effective against Gram positive bacteria. The basic chemical structure of natural glycopeptides is a cyclic peptide consisting of seven amino acids, to which two sugars are bound, hence the name glycopeptides. Vancomycin and teicoplanin are natural glycopeptide isolated from the actinomycetes, *Nocardia orientalis* and *Actinoplanes teichomyceticus* respectively that are used in human medicine (Binda et al., 2014).

Glycopeptides affect the cell wall synthesis in dividing organisms by interfering with the formation of peptidoglycan, which is the main structural component of bacterial cell walls. They inhibit the transpeptidation reaction which is important for the elongation of the peptidoglycan backbone. Vancomycin target the D-Alanyl-D-Alanine terminus of

pentapeptidic forming a stoichiometric complex with the D-Ala D-Ala dipeptide via the formation of five hydrogen bounds with the peptidic backbone of the glycopeptide. The formation of this complex prevents the transpeptidation reactions by steric hindrance. Teicoplanin inhibits polymerisation of peptidoglycan in bacterial cell walls by binding non-specifically to saturate the outer layers of the bacterial peptidoglycan. It then binds to the terminal amino acyl- D-alanyl-D-alanine precursor, which fits into a cleft in the teicoplanin molecule.

The susceptibility breakpoints of vancomycin is S≤2 mg/L; I : 4-8 mg/L; R≥16 mg/L in North America CLSI (www.clsi.org) and S≤2 mg/L; R≥4 mg/L in Europe (www.eucast.org). Glycopeptides antimicrobial activity is limited to Gram-positive organisms because the molecule cannot cross the external membrane of Gram-negative bacteria (Johnson et al, 1990) and some anaerobic species due to their voluminous structure. Clinically achievable concentrations of vancomycin are bacteriostatic rather than bactericidal to enterococci. This may have contributed to rapid development of resistance by enterococci to vancomycin. Vancomycin has also been shown to increase the bactericidal activity of linezolid in *in vitro* pharmacokinetic models (Allen et al., 2002) and of quinupristin/dalfopristin in *in vitro* models and animal infective endocarditis (Pavie et al., 2002). The combination of vancomycin and an aminoglycoside is bactericidal against enterococci unless high level aminoglycoside resistance is present.

Resistance to glycopeptide by enterococci involves a two component system where the cell wall composition is altered from the peptidoglycan precursor D-Ala-D-Ala (vancomycin-susceptible) to D-Ala-D-lactate (D-Lac). The genes involved in this two-component system are *vanS/vanR*. The presence of vancomycin induces a response on the *vanS* sensor kinase, which leads to the activation of D-Lac or D-Ser peptidoglycan precursor and the repression

of D-Ala-D-Ala (Stephenson & Hoch, 2002). Thus the resultant peptidoglycan precursor is composed of D-Ala-D-lactate (D-Lac) or D-Ala-D-Serine (D-Ser) having 1000 times less affinity for vancomycin, while D-Ala- D-Ser has a sevenfold decrease in affinity for vancomycin, thus removing the susceptible target (Fisher and Phillips, 2009: Gilmore, 2002).

There are several types of genes responsible for resistance to vancomycin that have been described in enterococci. They are designated according to the ligase gene involved in the gene operon responsible for resistance. They are *vanA, vanB, vanC, vanD, vanE, vanG, vanL* and recently *vanM* and *vanN*. The differences between these phenotypes are based on the composition of their peptidoglycan precursors in response to vancomycin, their inducibility, and in the level of resistance conferred. *VanA vanB, vanD,* and *vanM* types of resistance result from the preferential incorporation of D-alanyl – D-lactate precursors in peptidoglycan, while *vanC, vanE, vanG*, and *vanL* the ligase produces D-alanyl – D-serine rather than D-alanyl – D-lactate (Xu et al, 2010). Although *vanA*, *vanB*, and *vanD* gene clusters are similar by having D-alanyl – D-lactate precursors and also inducible. Thus, their gene operon contains *vanR* and *vanS* which are the two-component regulatory system. They are different based on the location of the gene, the level and inducibility of resistance to glycopeptide. *VanA* and *vanB* are acquired and carried on transposons, which may be on plasmids or on chromosomes while *vanD* is present on chromosome and not transferrable. Thus *vanD* is less widespread and less prevalent than *vanA* and *vanB*. *VanA* is characterized by resistance to high level vancomycin and teicoplanin, and can be induced by either vancomycin or teicoplanin. *VanB* resist various concentrations of vancomycin but not to teicoplanin and so can be induced only by vancomycin. *VanD* is known by resistance to intermediate levels of vancomycin and to low levels of teicoplanin. *VanM* is inducible,

related to *vanA, vanB,* and *vanD* and could be transferred by conjugation. *VanC* and *vanD* seems to be similar by showing resistance to low level glycopeptide and by being constitutive, located on the chromosome and are not transferable to other enterococci. However, they differ in the peptidoglycan precursor.

The *vanA, vanB* and *vanC* clusters are the main types of resistance. There are three types of *vanC: vanC1, vanC2,* and *vanC3*. The *vanC2* and *vanC3* are closely related and are most often referred to as *vanC2/3*. The *vanA* and *vanB* are more common due to their transferability. The *vanA* resistance operon comprises seven genes (*vanH, vanA, vanX, vanR, vanS, vanY* and *vanZ*) and is acquired through the Tn1546 transposon (while *vanB* operon which is acquired through Tn1547 and/or Tn5382, (Gilmore, 2002) has *vanW* instead of *vanZ* (Courvalin, 2006).

Most vancomycin-resistant enterococcal isolates are *E. faecium*, (Bertics and Wiepz, 2009) but glycopeptide resistance has also been seen in *E. faecalis, E. gallinarum, E. casseliflavus, E. avium, E. durans, E hirae,* and *E. raffinosus* (Nam et al., 2013). Intrinsic *vanC* resistance is specific to *E. gallinarum, E. casseliflavus* and *E. flavescens* (Fisher end Phillip, 2009). Several authors (Iweriebor et al., 2015: Nam et al., 2013; Getachew et al., 2012; Kühn et al., 2005) have detected *van* genes among these species. In Iweriebor et al. (2015), *vanB, vanC1* and *vanC2/3*genes were detected in all isolates phenotypically resistant to vancomycin and no *vanA* was detected. Getachew et al. (2012) showed higher prevalence of *vanA* among their isolates.

The presence of vancomycin induces an organism to change its cell wall composition by replacing the D-Ala-D-Ala with D-Ala-D-lactate (D-Lac) or D-Ala-D-Serine (D-Ser) thus

acquiring appreciable degree of resistance to vancomycin. Multiplication of this resultant strain further adds to the pool of VRE in the environment. This invariably supports the view that emergence of vancomycin resistance is a result of the use of vancomycin and its derivatives in animal husbandry and treatment of infections in the hospital environment. These genes, carried as mobile genetic elements, can easily to be acquired and transferred horizontally within and between organisms. *Enterococcus* easily acquires and transfers genes thus facilitating transfer to the entire environment. Thus, VRE is like a nuclear weapon fighting against antibiotic therapy.

2.10 Emergence and spread of vancomycin resistant enterococci

The use of antibiotic therapy may have contributed to the emergence of antibiotic resistant organisms such as enterococci. It has been discovered that the microbiota of the gut changed as a result of administration of antibiotics. The extent of the alteration varies depending on the nature, dosage, route of administration and other properties of the antimicrobial agent. This may lead to decreased colonization resistance of the microbiota, proliferation of the resistant bacteria with due advantage of selective pressure by the antibiotics, and emergence of antibiotic-resistant strains (Sjölund et al., 2003; Sullivan et al., 2001). Invariably, those organisms, which used to be commensal suddenly become harmful to the host because of the number and its resistance to the antibiotics. The resistant strain can serve as potential source of resistant genes to other organisms.

Also, the use of antibiotics in agriculture has contributed to the emergence of antibiotic resistant strains. Many antibiotics are used as growth promoters in animal husbandry. Some of these include tylosin, advocin (danofloxacin), avoparcin, ampicillin, and penicillin G.

The use of avoparcin is assumed to be related to the high level of vancomycin-resistance in farm animals (Boerlin et al., 2001). A study by Iweriebor et al. (2015) on antimicrobial resistance factors of *Enterococcus* species isolated form faecal samples from pigs from piggery farms shows very high antimicrobial resistance. These pigs were exposed to advocin, tylosin, ampicillin and penicillin G. The *Enterococcus* isolates showed 100% resistance to vancomycin, streptomycin and cloxacillin, 91% to penicillin G, 77.5% to ciprofloxacin and 16.3% to imipenem.

Enterococci being able to easily acquire resistance to antibiotics will build up resistance to these antibiotics used as growth promoters. Also being thermo-tolerant, and some can survive pasteurization temperatures combined with its tolerance to environmental stressors may contribute its survival in processed meat products (Fisher and Phillips, 2009). Several studies have detected enterococci in processed food products. Fermented meat are ready to eat food and thus may be an assured route of transmission of these antibiotic resistant bacteria from animal origin to human gastrointestinal microflora and subsequent transfer of the acquired resistance to those human microflorae. The transfer of antibiotic resistance from *Enterococcus* of animal origin to human microflora was shown by Jahan et al. (2015). In their study, the relationship between enterococci from fermented meat and clinical samples using PFGE showed high levels of heterogeneity among the isolates from each source belonging to the same species. However, there were still some similarity and mating resulted in genetic transfer. Phenotypic and genotypic tests established that tetracycline resistant gene *tet(M)* had been transferred from *E. faecium* of meat origin to clinical strains. The mechanism of transfer was also established to be by natural conjugation through integron as experimental evidence by PCR confirmed that the transconjugants were plasmid

negative and the donor cell lacked integrase genes which determine the possibility of transposon.

3 CHAPTER 3: METHODOLOGICAL OVERVIEW

3.1 Sample collection

The sampling sites selected for this study were two wastewater treatment plants receiving municipal wastewater from Durban metropolis and their effluent recipients. The wastewater treatment plants (WWTP) were chosen based on their size, plant configuration and the sources of their wastewater. They are among the larger plants in Durban and mainly receive and treat municipal wastewater. The recipient rivers flow past various settlements where the water is used for agricultural and other purposes. The WWTP I (referred to as Plant I) has a design capacity of 15 ML/d and a working capacity of 10.9 ML/d. The biological treatment is based on trickling filter with four primary settling tanks, six trickling filters, six settling tanks (humus tanks) and three (2000 m^3) anaerobic digesters (unheated and unmixed). The WWTP II (Plant II) is larger with the biological treatment based on activated sludge and with a design and working capacity of 25 ML/d and 22.5 ML/d respectively. It has two primary settling tanks, the activated sludge reactors, three clarifiers, and three (2600 m^3) anaerobic digesters (heated and mixed).

Samples collected include wastewater samples from the two treatment plants and water samples from their receiving water bodies. Wastewater samples were collected from the influent, from biofilter/clarifiers respectively and the final effluent at the point where it is discharged to the recipient. These samples from different treatment steps were used to determine the effectiveness of the different steps in removing VRE from the wastewater. Recipient samples; upstream (1 km) before and downstream (at least 1 km) after the effluent discharge point were collected. Composite samples were taken at each sampling point at 1 min interval for 10 min. All the required samples (wastewater and river) for each month

were collected on the same day between 9 AM to 12 noon. They were collected in 1L sterile plastic sample bottles, transported and stored at 4°C and analyzed within one day. Samples were taken monthly from July 2016 to June 2017 with the aim to cover the warm and cold seasons. The illustration of the wastewater treatment steps of the two plants sampled showing the different sampling points are shown in Figure 3.1 and 3.2

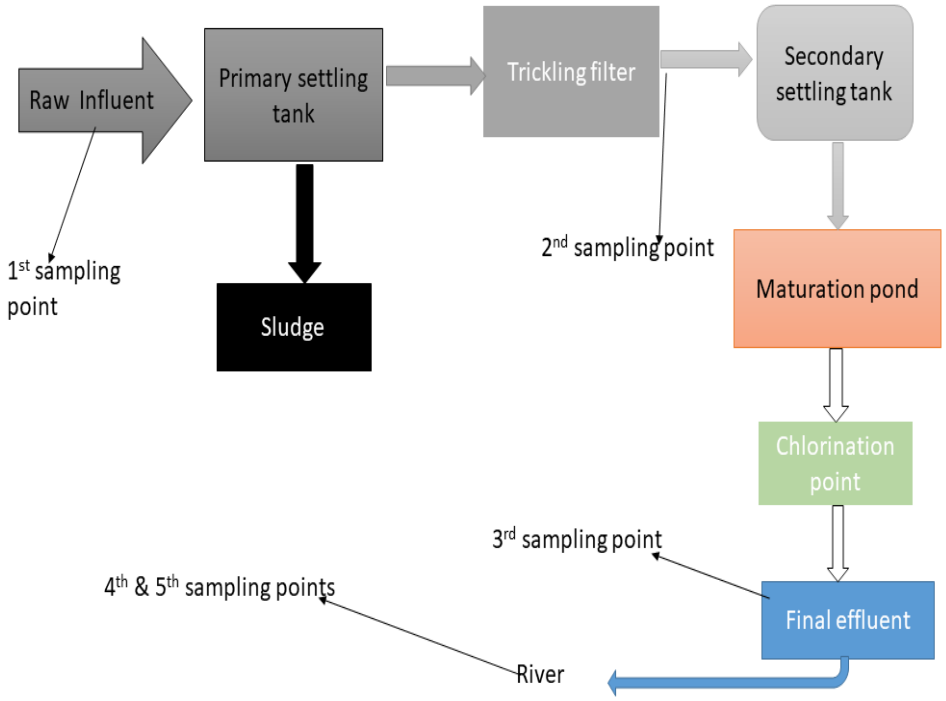


Figure 3.1: Illustration of Plant I (Trickling filter) showing the sampling points

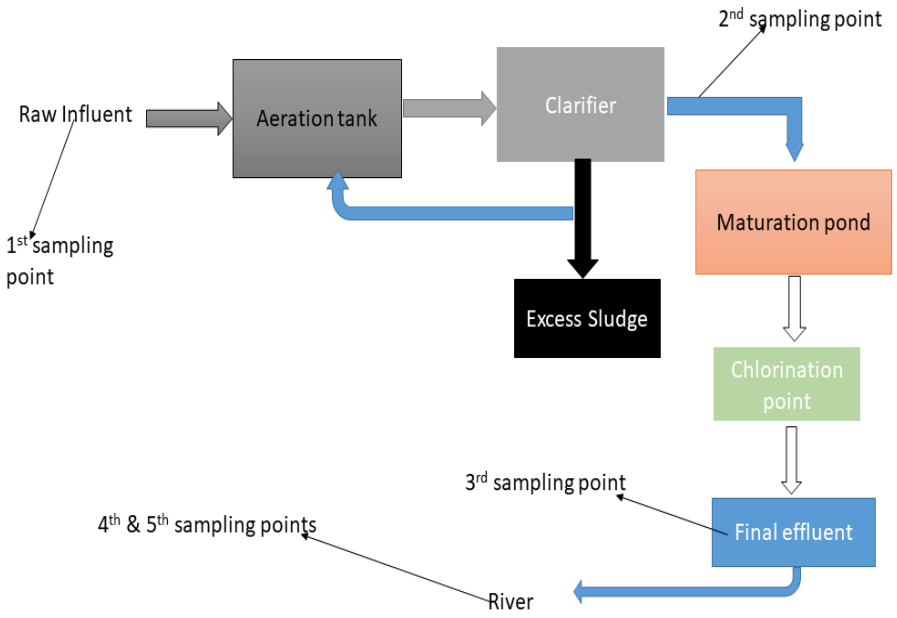


Figure 3.2: Illustration of Plant II (Activated sludge) showing the sampling points

3.2 Isolation of enterococci from samples

Standard membrane filtration (USEPA, 2006) and the spread plate methods were used to isolate total enterococci (TE) and vancomycin-resistant enterococci (VRE) from the samples collected based on modified procedure according to Rosenberg-Goldstein et al. (2014). Briefly, for influent and biofilter samples, ten-fold serial dilutions in the range of 1 to 0.001mL were prepared using sterilised phosphate buffered saline (PBS). Then, 100 µL portions of each dilution was used to inoculate the medium by the spread plate method in duplicate on membrane-Enterococcus Slanetz and Bartley (mS&B) agar to isolate total enterococci, and mS&B agar modified with 6 µg/mL of vancomycin to isolate VRE. Hundred millilitres (100 mL) of each non-diluted effluent samples and 100 mL of diluted

river samples (10 in 100 mL) from the receiving water bodies were also filtered through 0.45 µm, 47mm Ø membrane filters (Millipore, Billerica, MA). These were aseptically placed in duplicate on membrane-*Enterococcus* Slanetz and Bartley (mS&B) agar plates to isolate total enterococci, and mS&B agar modified with 6 µg/mL of vancomycin to isolate VRE. Plates were incubated at 35°C for 4 hrs and then at 44°C for 44 hrs. Colonies with a pink to dark red colouration were considered presumptive total enterococci and VRE respectively.

3.3 Purification of the isolates

For further analysis, representative colonies were picked from the primary culture plates based on the colonial appearance and size. Distinct colonies with various colours (pink to dark red) and of various sizes were isolated. Depending on the count, one out of every five colonies was picked with at least two isolates from each plate. These colonies were purified on Brain Heart Infusion agar (BHIA) and stored in BHI broth with 25% glycerol at -80^{o}C for further studies.

3.4 Vancomycin sensitive enterococci (VSE) from total enterococci

In order to obtain vancomycin sensitive enterococci (VSE), 100 colonies were picked at random from TE culture plates (S&BA without vancomycin) from sampling points that produced VRE colonies on the primary isolation plates. The selected colonies were numbered 1 to 100 for convenience. These were first purified once on S&BA. After 24 hrs incubation at 37^{o}C, they were streaked on S&BA supplemented with vancomycin and incubated for another 24 hrs at 37^{o}C. The number of isolates that showed growth on

S&BA+V was noted. Following the numbering, one out of every five isolates, which did not grow on S&BA+V were picked from the first S&BA plate and further purified on BHIA. It was assumed that these colonies represent VSE. This was done only for two sampling rounds and done to ascertain the likely proportion of VSE among the TE. The VSE were further compared with the VRE in relation to antibiotic susceptibility testing, vancomycin resistance genes and virulence genes.

3.5 Preliminary identification of *Enterococcus* isolates

Isolates were further subjected to a set of further identifications involving growth on Bile Aesculin Agar (BAA), Gram stain, catalase test, detection of pyrrolidonyl peptidase activity, and growth on 6.5% NaCl medium.

Pure colonies from BHIA were inoculated on BAA and incubated at 37^{o}C for 24 hrs. The ability to grow and hydrolyse aesculin to produce dark colouration was noted. Basic Gram staining procedure was employed. Catalase test was done using a drop of 3% H_2O_2 on the slide smear of pure colonies of 24 hrs culture from BHIA while Pyrase strips (Sigma-Aldrich, Singapore) was used to detect pyrrolidonyl peptidase activity. Pure colonies of the isolate were streaked on BHIA containing 6.5% NaCl to check for growth. The procedures used for the isolation and preliminary identification of presumptive enterococci (VRE and VSE) are illustrated in Figure 3.3.

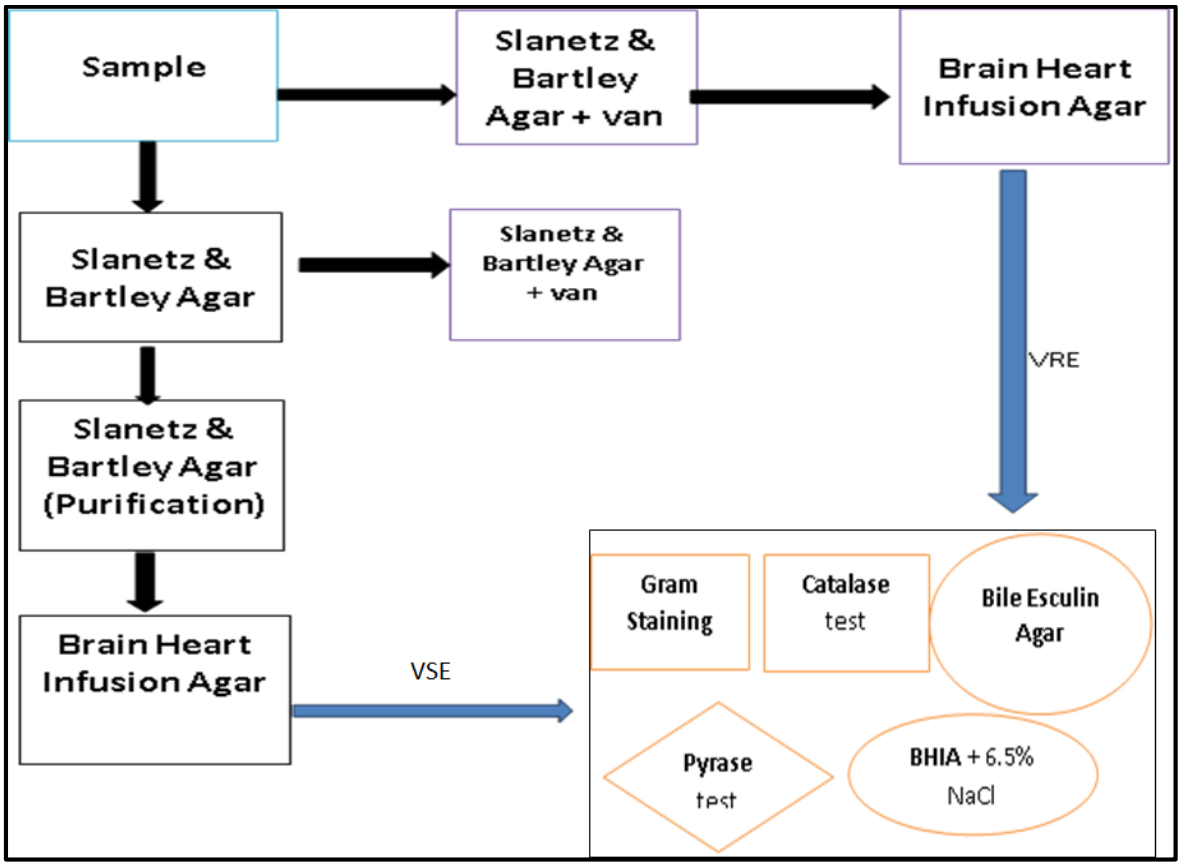


Figure 3.3: Systematic work flow chart for isolation, purification and preliminary identification of presumptive enterococci

3.6 Confirmation of *Enterococcus*

3.6.1 DNA extraction

DNA extraction was carried out using the boiling method described by Dashti et al. (2009) but with modification in the centrifugation procedure. The glycerol stock isolates were streaked on Brain Heart Infusion Agar. After 24 hrs a loopful of this BHIA culture was

transferred into a 2 mL Eppendoff tube with 500 µL of sterile distilled water. This was heated in a heat block at 100^{o}C for 10 min and then centrifuged at 14,000 rpm for 15 min. Then 300 µL of the supernatant containing the DNA was carefully taken and stored at -20^{o}C. The quality of the DNA obtained was measured or determined using Nanodrop technology. This served as DNA template for all PCR based reactions.

3.6.2 Genus identification

For genus confirmation, two sets of genus primers were used independently to verify the results. These were *Enterococcus* primer A and PCR condition developed by Deasy et al. (2000), and primer B by Ke et al. (1999). Primer A targets the 16S rRNA and can be used in a multiplex PCR for speciation of *Enterococcus* (Jackson et al., 2004). Primer B is based on a functional gene targeting the *tuf* gene, which encodes the EF-Tu, elongation factor. (Iweriebor et al., 2015; Getachew et al., 2012).

3.6.2.1 PCR conditions for genus identification

For primer A, the DNA amplification was carried out in a final volume of 20 µL containing 10 µL of Hot-start green master mix (Thermo Fisher), 20 µM each of the forward and reverse primers El (5' TCA ACC GGG GAG GGT 3') and E2 (5' ATT ACT AGC GAT TCC GG 3') (bp 733), water as well as 5µL of DNA template. The cycling condition in a thermocycler (BIORAD) consisted of an initial denaturation step of 95°C for 5 min, 25 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, followed by polymerization at 72°C for 1 min. For primer B, Ent1 5'-TACTGACAAACCATTCATGATG-3' and Ent2 5'-AACTTCGTCACCAACGCGAAC-3' (bp 112), the reaction volume, concentration of primers, master mix and DNA was the same as for primer A. The PCR cycling condition comprised of the initial denaturation 94 °C/3

min, amplification—30 cycles (94 °C/30 s, 53 °C/45 s, 72 °C/60 s), final extension 72 °C/7 min. *Enterococcus faecalis* ATCC 19433 and nuclease free water were used as positive and negative control respectively. Five microlitres of the PCR product was electrophoresed on a 2% (w/v) agarose gel using Ix TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8) stained with gel red and a 100-bp ladder was used as a marker. The gel was run at 100 V for 60 min and visualized with UV transilluminator (BIORAD).

3.6.3 Speciation of the *Enterococcus* isolates

A multiplex conventional PCR as adapted from Jackson et al. (2004) was used to identify the species of the confirmed enterococcal isolates. This was modified by using a DNA template instead of the whole cell suspension. The twenty-three species of enterococci and the multiplex groups (see Table 3.1) were as used by the authors (Jackson et al., 2004). Both the genus and species primers were synthesized by Inqaba Biotech SA. The PCR was done in a 25 µL reaction volume consisting of 14 µL PCR master mix (DreamTag MM Thermo fisher), 0.2 µL (20 µM) each of the forward and reverse primers for genus and species; 4 µL Nuclease free water; and 5 µL of DNA template. The thermocycling conditions included initial denaturation at 95°C for 4 min, products were amplified by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C (groups 1, 2, 5, and 6) or 60°C (groups 3, 4, and 7) for 1 min, and elongation at 72°C for 1 min. Amplification was followed by a final extension at 72°C for 7 min. Five microliters of product was electrophoresed on a 2% 1× Tris-acetate-EDTA agarose gel containing gel red stain. DNA molecular weight marker (100 bp; Roche) was used as the standard. The gel was run for 1 hr at 100 V.

Table 3:1: Primers for species-specific identification of *Enterococcus*

Species	**Primer**	**Sequence (5'-3')**	**Product size (bp)**	**Multiplex grp**
E. asini	AS1	GCATCATGACAAGCATCACGC	365	7
	AS2	GGCTTTTTGCCTTCAGATAAA		
E. avium	AV1	GCTGCGATTGAAAAATATCCG	368	5
	AV2	AAGCCAATGATCGGTGTTTTT		
E. casseliflavus	CA1	TCCTGAATTAGGTGAAAAAAC	288	2
	CA2	GCTAGTTTACCGTCTTTAACG		
E. cecorum	CE1	AAACATCATAAAACCTATTTA	371	6
	CE2	AATGGTGAATCTTGGTTCGCA		
E. columbae	CO1	GAATTTGGTACCAAGACAGTT	284	5
	CO2	GCTAATTTACCGTTATCGACT		
E. dispar	DI1	GAACTAGCAGAAAAAAGTGTG	284	3
	DI2	GATAATTTACCGTTATTTACC		
E. durans	DU1	CCTACTGATATTAAGACAGCG	295	1
	DU2	TAATCCTAAGATAGGTGTTTG		
E. faecalis	FL1	ACTTATGTGACTAACTTAACC	360	1
	FL2	TAATGGTGAATCTTGGTTTGG		
E. faecium	FM1	GAAAAAACAATAGAAGAATTAT	215	1
	FM2	TGCTTTTTTGAATTCTTCTTTA		
E. flavescens	FV1	GAATTAGGTGAAAAAAAAGTT	284	4
	FV2	GCTAGTTTACCGTCTTTAACG		
E. gallinarum	GA1	TTACTTGCTGATTTTGATTCG	173	2
	GA2	TGAATTCTTCTTTGAAATCAG		
E. gilvus	GI1	CTGGCTGGGCTTGGCTAGTGA	98	7
	GI2	ATAATCGGTGTTTTACCGTCT		
E. hirae	HI1	CTTTCTGATATGGATGCTGTC	187	6
	HI2	TAAATTCTTCCTTAAATGTTG		
E. malodoratus	MA1	GTAACGAACTTGAATGAAGTG	134	1
	MA2	TTGATCGCACCTGTTGGTTTT		
E. mundtii	MU1	CAGACATGGATGCTATTCCATCT	98	4
	MU2	GCCATGATTTTCCAGAAGAAT		
E. pallens	PA1	TGGCACCAAATGCTGGCGGAA	160	7
	PA2	TGGTGTAGAAGTAATTTCAAG		
E. porcinus/villorum	PO1	TGGTTTCTGATATGGATGCGA	280	7
	PO2	GTAATCGCTAATTTCTCTCCA		
E. pseudoavium	PV1	TCTGTTGAGGATTTAGTTGCA	173	3
	PV2	CCGAAAGCTTCGTCAATGGCG		
E. raffinosus	RF1	GTCACGAACTTGAATGAAGTT	287	6
	RF2	AATGGGCTATCTTGATTCGCG		

E. saccharolyticus	SA1	AAACACCATAACACTTATGTG	371	3
	SA2	GTAGAAGTCACTTCTAATAAC		
E. seriolicida	SE2	ACACAATGTTCTGGGAATGGC	100	5
	SE2	AAGTCGTCAAATGAACCAAAA		
E. solitarius	SO1	AAACACCATAACACTTATGTGACG	371	2
	SO2	AATGGAGAATCTTGGTTTGGCGTC		
E. sulfureus	SU1	TCAGTGGAAGACTTAATCGCA	173	4
	SU2	CCAAATGTATCTTCGATCGCT		

(Jackson et al., 2004)

3.6.4 Genus and species identification with MALDI-TOF

Two hundred and eighty-eight (288) isolates were identified to species level with MALDI-TOF in parallel to the PCR identifications. The 288 isolates were made up of 230 PCR confirmed enterococci and 58 isolates, which had strong basic features of enterococci but could not amplify with the *Enterococcus* primers. The identification with MALDI-TOF was done by the Centre for Antibiotic Resistance Research (CARe) at University of Gothenburg, Sweden.

3.7 Antibiotic resistance profiling, detection of resistance genes, virulence factor and virulence genes

3.7.1 Antibiotic susceptibility testing

Antibiotic susceptibility testing (AST) was done using the disk diffusion method (CLSI, 2016). Two hundred and forty-five (245) genus confirmed isolates consisting of 202 VRE and 43 VSE were tested for susceptibility to 15 selected antibiotics. Inoculum for AST was standardized to 0.5 McFarland standards by the methods of Komolafe and Adegoke (2008). Four pure colonies of each isolate from a 24 hrs Brain Heart Infusion plate culture were

inoculated into 2 mL sterile peptone water broth. This was incubated at 37^{o}C for 6 hrs and the turbidity adjusted by diluting with PBS (pH 7.2) to 0.5 McFarland (10^5 CFU/mL). Hundred microliters (100 µL) of this culture dilution was inoculated on Muller-Hinton agar (MHA) using sterile swab sticks to obtain a uniform spread. Antibiotic discs were aseptically placed on the inoculated MHA plate and cultures read after 24 hrs incubation at 37^{o}C. The following antibiotics were used; azithromycin (15 µg), vancomycin (30 µg), imipenem (10 µg), teicoplanin (30 µg), tetracycline (30 µg), doxycline (30 µg), amoxyl-clav (30 µg), gentamicin (10 µg), streptomycin (10 µg), ciprofloxacin (5 µg), cefixime (5µg), quinupristin-dalfopristin (15 µg), erythromycin (15 µg), penicillin G (10 units) and ampicillin (10 µg). The choices of these antibiotics were based on their reported activity against Gram-positive bacteria (Luczkiewicz et al. 2010). They also appear on the WHO list of first line antibiotic therapy (WHO, 2015). Since vancomycin used in the primary isolation medium was 6 µg/mL, a higher dose was used during susceptibility testing to evaluate if a growth inhibition was dose dependant. The zone of inhibition was measured and then interpreted using the CLSI (2016) standard.

3.7.2 Multiple antibiotic resistance index (MAR Index)

The multiple antibiotic resistance index (MAR Index) was determined following the methods of Adegoke and Okoh (2014). This was calculated as the ratio of the number of the antibiotics to which resistance occurred by the isolates (A) to the total number of antibiotics to which the isolates were tested (B).

$$MAR\ Index = A/B$$

All the antibiotics except vancomycin and teicoplanin used in the AST were also considered for MAR Index determinations as organisms exhibit unequal profile to antibiotics within the same class. Teicoplanin belongs to the same group of antibiotics (the glycopeptides) as

vancomycin. Vancomycin was excluded in the MAR Index determination, because it was the original supplement in the isolation medium.

3.7.3 Detection of *van A, B, C1* and *C2/3* genes

Multiplex PCR as adapted from Nam et al. (2013) was used for the detection of *Van A, B, C1* and *C2/3* genes. The reaction mix was composed of 12.5 µL PCR master mix (DreamTag MM Thermo Fisher), 20 µM of each forward and reverse primers, water, 5µL of the DNA template in a final reaction volume of 25 µL. The cycling condition involves the initial denaturation at 94^{o}C for 5 min, 35 cycles consisting of 45 s at 94^{o}C, 60s at 54^{o}C, and 90 s at 72^{o}C, with final extension for 15 min at 72^{o}C. Products were resolved by electrophoresis in 2% agarose gel using 110 V for 45 min. Results were determined using the expected band size on 100 bp ladder. The list of the primers as adapted by Nam et al. (2013) are summarised in Table 3.2.

3.7.4 Detection of other resistance genes *tet, gyrA, parC* and *emeA* genes

Multiplex PCR was used for the detection of 14 tetracycline resistance (*tet)* genes. The multiplex groups, primer concentrations, and amplification conditions are as adapted by Ng et al. (2001). Group I contained primers for *tet*(B) (0.25 µM), *tet*(C) (0.25 µM) and *tet*(D) (2.0 µM) each. Group II contained primers for *tet*(A) (1.0 µM), *tet*(E) (1.0 µM) and *tet*(G) (1.0 µM) each. Group III contained primers for *tet*(K) (1.25 µM), *tet*(L) (1.0 µM), *tet*(M) (0.5 µM), *tet*(O) (1.25 µM) and *tet*(S) (0.5 µM) each. Group IV contained primers for *tetA*(P) (1.25 µM), *tet*(Q) (1.25 µM) and *tet*(X) (1.25 µM) each). This was done in a reaction volume of 25 µL consisting of 12.5 µL master mix (OneTag Quick Load Master mix with standard buffer, New England BioLabs), and 5 µL of DNA template. Cycling conditions was as

follows; a 5 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min.

For *gyrA* and *parC* genes, the primers and conditions adopted by Nowroozi et al. (2014) were used to detect the ciprofloxacin resistance genes. The PCR volume of 20 µL made up of 10 µL master mix (OneTag Quick Load Master mix with standard buffer, New England Bio Labs), 1 µL (10 µM) of forward and reverse primers, and 5 µL of DNA template. For *gyrA*, the following parameters were used: an initial template denaturation at 95°C for 1 min; 36 cycles consisting of 30s of denaturation at 95°C, 30s of annealing at 53°C and 2 min of extension at 72°C; and a final extension at 72°C for 10 min. For *parC*, an initial template denaturation at 95°C for 2 min; 36 cycles consisting of 1 min of denaturation at 95°C, 1 min of annealing at 60°C and 2 min of extension at 72°C; and a final extension at 72°C for 10 min.

The detection of multidrug efflux pump, *emeA* gene, was done following the method used by Jia et al. (2014). The reaction mixtures of 20 µL volume consisted of 10 µL Quick load master mix (OneTag Quick Load Master mix with standard buffer, New England Bio Labs), 0.5 µL of the forward and reverse primers, 4 µL of DNA free water and 5 µL of DNA template. Initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 60 s, extension at 72°C for 90 s and final extension at 72°C for 10 min (Jia et al., 2014). Five microlitres of the PCR product was electrophoresed on a 2% (w/v) agarose gel using Ix TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8) stained with gel red and a 100-bp ladder was used as a marker. This was run at 100 V for 60 min and visualized with UV transilluminator (BIORAD). Table 3.2 shows the primers used to detect resistance genes.

Table 3:2: List of primers for detection of resistance genes

Gene	Primer sequence (5'-3')	Product size (bp)	Reference
vanA	F-GCGCGGTCCACTTGTAGATA	314	Nam et al, (2013)
	R-TGAGCAACCCCCAAACAGTA		
vanB	F-AGACATTCCGGTCGAGGAAC	220	Nam et al, (2013)
	R-GCTGTCAATTAGTGCGGGAA		
vanC1	F-ATCCAAGCTATTGACCCGCT	402	Nam et al, (2013)
	R-TGTGGCAGGATCGTTTTCAT		
vanC2/3	F-CTAGCGCAATCGAAGCACTC	582	Nam et al, (2013)
	R-GTAGGAGCACTGCGGAACAA		
gyrA	F-AAATCTGCCCGTGTCGTTGGT	343	Nowroozi et al, (2014)
	R-GCCATACCTACGGCGATACC		
parC	F-AAAAATCAGCGCGTACAGTG	327	Nowroozi et al, (2014)
	R-CGAGAGTTTGGCTTCGGTAT		
tetA	F-GCTACATCCTGCTTGCCTTC	210	Ng et al, (2001)
	R-CATAGATCGCCGTGAAGAGG		
tetB	F-TTGGTTAGGGGCAAGTTTTG	659	Ng et al, (2001)
	R-GTAATGGGCCAATAACACCG		
tetC	F-CTTGAGAGCCTTCAACCCAG	418	Ng et al, (2001)
	R-ATGGTCGTCATCTACCTGCC		
tetD	F-AAACCATTACGGCATTCTGC	787	Ng et al, (2001)
	R-GACCGGATACACCATCCATC		
tetE	F-AAACCACATCCTCCATACGC	278	Ng et al, (2001)
	R-AAATAGGCCACAACCGTCAG		
tetG	F-GCTCGGTGGTATCTCTGCTC	468	Ng et al, (2001)
	R-AGCAACAGAATCGGGAACAC		
tetK	F-TCGATAGGAACAGCAGTA	169	Ng et al, (2001)
	R-CAGCAGATCCTACTCCTT		
tetL	F-TCGTTAGCGTGCTGTCATTC	267	Ng et al, (2001)
	R-GTATCCCACCAATGTAGCCG		
tetM	F-GTATCCCACCAATGTAGCCG	406	Ng et al, (2001)
	R-CGGTAAAGTTCGTCACACAC		
tetO	F-AACTTAGGCATTCTGGCTCAC	515	Ng et al, (2001)
	R-TCCCACTGTTCCATATCGTCA		
tetS	F-CATAGACAAGCCGTTGACC	667	Ng et al, (2001)
	R-ATGTTTTTGGAACGCCAGAG		
tetA(P)	F-CTTGGATTGCGGAAGAAGAG	676	Ng et al, (2001)
	R-ATATGCCCATTTAACCACGC		
tetQ	F-TTATACTTCCTCCGGCATCG	904	Ng et al, (2001)
	R-ATCGGTTCGAGAATGTCCAC		

tetX	F-CAATAATTGGTGGTGGACCC R-TTCTTACCTTGGACATCCCG	468	Ng et al, (2001)
emeA	F-GTGACAGCCTTTGTGGCAGAT R-TAGTCCGTTGATGGTTCCTTG	687	Jia t al, (2014)

3.7.5 Detection of virulence genes

DNA samples from the isolates were assessed to detect the presence of *asa1 (*Aggregation substance), *gelE (*Gelatinase), *cylA* (Cytolysin), *esp* (Enterococcal surface protein), and *hyl* (Hyaluronidase) using a multiplex PCR according to the methods used by Vankerckhoven et al. (2004) with modifications. This method was modified by using extracted DNA from the isolates instead of a whole cell suspension as DNA template and by subsequent modifications of the initial denaturation temperature, the reaction volume and gel condition. Each 25 µL PCR mixture consisted of 5 µL of DNA template; 0.1 µM concentration (each) of primers specific for *asa1*, *gelE*, and *hyl*; 0.2 µM concentration (each) of primers specific for *cylA* and *esp*; 12.5 µL of Hot-Star Taq master mixture (Thermo Fisher), which consisted of 2.5U of Hot-StarTaq DNA polymerase, 1.5 mM $MgCl_2$, and 200 µM deoxynucleoside triphosphates; and an additional 1.0 mM $MgCl_2$. Cycling conditions were an initial activation step at 95°C for 5min, followed by 30 cycles of denaturation (94°C for 1 min), annealing (56°C for 1 min), and extension (72°C for 1 min), followed by one cycle consisting of 10 min at 72°C. Monoplex PCR was used to detect *ace* and *efa*A gene with primer and cycling condition as used by Iweriebor et al. (2015). Reaction volume for each was 20 µL containing 10 µL master mix, 1 µL each of the forward and reverse primers, and 5 µL of DNA template. Cycling conditions for *ace* were as follows: initial denaturation at 94 °C for 3 min; followed by 35 cycles of amplification (93°C/1 min, 50 °C/1 min, 73°C/1 min) and final extension at 72°C/10 min. The same conditions were used for *efa*A but with

the annealing temperature of 56.5 °C/1 min. Products were resolved as above. The primers used for the detection of the virulence genes are shown in Table 3.3.

Table 3:3: Primers for the detection of virulence genes

Gene	Primer sequence (5'-3')	Product size (bp)	Reference
Ace	F- AAAGTAGAATTAGATCCACAC R- TCTATCACATTCGGTTGCG	320	Iweriebor et al, (2015)
asa1	F-GCACGCTATTACGAACTATGA R-TAAGAAAGAACATCACCACGA	375	Vankerckhoven et al, (2004)
ClyA	F- ACTCGGGGATTGATAGGC R- GCTGCTAAAGCTGCGCTT	688	Vankerckhoven et al, (2004)
efaA	F- CGTGAGAAAGAAATGGAGGA R- CTACTAACACGTCACGAATG	499	Iweriebor et al, (2015)
esp	F-AGATTTCATCTTTGATTCTTGG R-AATTGATTCTTTAGCATCTGG	510	Vankerckhoven et al, (2004)
Hyl	F- ACAGAAGAGCTGCAGGAAATG R- GACTGACGTCCAAGTTTCCAA	276	Vankerckhoven et al, (2004)
gelE	F-TATGACAATGCTTTTTGGGAT R-AGATGCACCCGAAATAATATA	213	Vankerckhoven et al, (2004)

3.7.6 Detection of virulence factors

Expression of virulence factors such biofilm formation, ability to hydrolyse gelatin and haemolysis was determined.

3.7.6.1 Biofilm formation

Ability to form biofilm was done using tissue culture plate (TCP) method following the procedure as used by Hassan et al. (2011). Ten millilitres (10) mL of tryptose soy broth with 1% glucose was inoculated with a 24 hr agar culture of the isolates. The broth was incubated at 37^{o}C for 24 hrs after which it was diluted 1:50 with fresh broth. Individual wells of sterile flat bottom polystyrene tissue culture treated plates were filled with 200 µL of the diluted

cultures. Sterile broth was used as a negative control. The plates were incubated at 37^{o}C for 24 hr. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) for a total of four times to remove free floating bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionised water and plates were kept for drying. The optical density (OD) of stained adherent biofilm was obtained by using micro ELISA autoreader (model 680, Biorad, UK) at wavelength of 570 nm. The experiment was performed in triplicate. Table 3.4 shows the interpretation of biofilm production.

Table 3:4: Cut-off value for interpreting biofilm formation

Average OD value	**Biofilm formation**
≤ ODc	Non
ODc < ~ ≤ 2x ODc	Weak
2x ODc < ~ ≤ 4x ODc	Moderate
> 4x ODc	Strong

ODc =Optical density cut-off value =average OD of negative control + 3x standard deviation (SD) of negative control.

Haemolysis was determined using blood agar plates. Fresh cultures of the isolates were streaked onto agar supplemented with 5-10% of human blood. The plates were analysed after 24 hrs incubation at 37^{o}C. Un-inoculated blood was also incubated as control, to confirm the sterility of the blood used (lack of bacteraemia). Presence and types of haemolysis were documented.

Gelatinase activity was determined using the method as described Marra et al. (2007) and reported by Comerlato et al. (2013**)**. Briefly, samples were inoculated into tubes containing 4 mL of brain heart infusion broth with 4% gelatin. After incubation at 35-37ºC for 24 hrs, the tubes were cooled at 4ºC for 30 min. The liquefaction of the medium was taken to be positive.

3.8 Statistical analysis

Descriptive statistics was used to analyse the result of the antibiotic susceptibility test, the putative antibiotic resistant genes and virulence genes. T-test was used to compare enterococci count between relevant sampling points and p value of less than or equal to 0,05 was considered to be significant. The putative antibiotic resistance genes and virulence genes were analysed and correlated against the common species involved. Also the virulence factors were statistically compared to the presence of virulence genes.

4 CHAPTER 4: PREVALENCE OF VANCOMYCIN RESISTANT *ENTEROCOCCUS* IN WASTEWATER TREATMENT PLANTS AND THEIR RECIPIENTS

4.1 Introduction

The concentration of enterococci in raw wastewater is expected to be high due to their general presence in human faeces (10^5 - 10^7 CFU/g) (Boehm and Sassoubre, 2014). Due to dilution, the inflow counts in raw wastewater are expected to fall in the range of 10^5 to 10^7 CFU/mL. Being part of the normal flora of the gastrointestinal tract, *Enterococcus* species are faecal indicator used in water quality control (Boehm and Sassoubre, 2014; EU, 2006; USEPA, 2006; and WHO, 2006). They can survive for long periods in the environment after excretion. They do not normally multiply in water environments which aid to their usefulness as faecal environmental indicators (Cabral, 2010).

Vancomycin-resistant enterococci (VRE) have been detected at different stages of the wastewater treatment processes including their effluents (Araujo et al., 2010; Luczkiewicz et al., 2010; Rosenberg-Goldstein et al., 2014). For detection of VRE from samples, some authors (Araujo et al., 2010; Luczkiewicz et al., 2010) employed the method of prior isolation of enterococci followed by vancocomycin resistance testing while other authors (Rosenberg-Goldstein et al., 2014; Taucer-Kapteijn et al., 2016; Varela et al., 2013) incorporated vancomycin in their primary isolation media. This method of using media supplemented with vancomycin seemed reliable and convenient and can be used for rapid analysis of a large number of samples. The use of media supplemented with antibiotic has

been used to estimate the prevalence of antibiotic resistant bacteria in environmental samples (Figueira et al., 2011; Novo and Manaia, 2010; Watkinson et al., 2007). The percentage of resistance can be estimated as the ratio between the number of bacteria growing in the presence and in the absence of the antibiotic (Novo and Manaia, 2010; Watkinson et al., 2007) (section 2.8).

The first reported outbreak of VRE in a haematology unit in Durban, South Africa in 2016 (Mahabeer et al., 2016) has raised the concern and the need for increased surveillance both in clinical settings and the environment. Determination of the prevalence of VRE from the WWTPs and their receiving water bodies will help to reinforce control measures, which in turn may lower the risk for subsequent outbreaks.

Thus this chapter is focused on determining:

1. The removal efficiency in two wastewater treatment plants of vancomycin-resistant and vancomycin sensitive enterococci (VRE and VSE) from the influent.
2. The prevalence of VRE in WWTPs and their receiving water bodies.
3. The extent at which the effluent from the WWTPs contribute to the concentration of VRE count of their receiving water bodies.
4. Species distribution among the isolates.

4.2 Methodology

Wastewater samples were collected from influent, biofilter/clarifier and the chlorinated effluent points of Plant I and Plant II monthly for a period of one year. Upstream (1 km before the effluent discharge point) and downstream (1 km after the effluent discharge point) samples of their receiving water bodies were collected. The sampling points are illustrated

in figure 3.1a and b. A total of 100 (60 wastewater and 40 water) samples were collected. Enumeration and isolation was done using Slanetz and Barley agar with and without vancomycin for vancomycin-resistant enterococci (VRE) and total enterococci (TE) respectively as specified in Section 3.2. Some VSE were picked out from TE culture plate following the procedure described in section 3.4. Pink to maroon red colonies purified on brain heart infusion agar were further identified as presumptive enterococci using preliminary tests (see further at section 3.6). Genus confirmation was based on two different primers (targeting the 16S rRNA and *tuf* gene for clarity) (as described in section 3.7.2), and species identification were done using PCR (see also 3.7.3). Also in parallel with PCR, two hundred and eighty-eight (288) isolates were also identified on the genus and species levels with MALDI-TOF with the assistance of the Centre for Antibiotic Resistance Research (CARe) at University of Gothenburg, Sweden. These 288 isolates consisted of 58 isolates which had strong basic features of enterococci but could not amplify with the *Enterococcus* primers and 230 out of 269 PCR confirmed *Enterococcus.*

Descriptive statistics was used for presentation of data while the T- test was used to compare counts at selected sampling points. A p value of ≤ 0.05 was considered to be statistically significant. The removal efficiency for the treatment steps was calculated as the difference between counts while ANOVA was used to determine the significant reduction in count of the three treatment steps sampled. The prevalence of VRE was estimated according Watkinson et al. (2007) as the percentage resistance.

$$Percentage\ resistance = \frac{enterococci\ count\ on\ plate\ with\ vancomycin}{enterococci\ count\ on\ plate\ without\ vancomycin} \times 100$$

4.3 Results

The following sections contain the result of the samples taken from July 2016 to June 2017 excluding September and December, 2016, due to unreliable variations between parallels. ö

4.3.1 TE and VRE counts of the WWTPs and their receiving water bodies

The total enterococci (TE) and vancomycin-resistant enterococci (VRE) counts in wastewater influent of WWTP I ranged between 6.1 to 6.7 log_{10} CFU/100 mL and 4.3 to 4.9 log_{10}CFU/100 mL respectively excluding the months of July and February when no VRE was detected. The TE and VRE count in the influent of Plant II was higher than that of Plant I with VRE detected in all the month sampled. In plant II, TE count of 6.3 to 7.2 log_{10}CFU/100 mL and VRE count of 4.4 to 6.7 log_{10} CFU/100 mL was detected. The various monthly TE counts of both plants have close range values as shown by their means and standard deviation (Plant I = 6.4±0.2, Plant II = 6.5±0.3). Apart from the wider range of various VRE counts of Plant I than that of Plant II (Plant I = 3.6±1.9, Plant II = 4.8±0.3), the difference between the VRE count of the influents of both plants were significant (P = 0.01). The VRE counts of Plant II influent were higher than that of Plant I influent. Table 4.1 shows the log_{10} counts of the different sampling points of WWTPs. The raw counts are shown in the appendix (Table 0.1)

.

Neither TE nor VRE was detected in effluent samples of Plant I throughout the sampling periods. For Plant II, TE was detected in all except for two months (October and June) with counts ranging from 1.5 to 4.4 log_{10} CFU/100 mL while VRE count of 0.9 -3.4 log_{10} CFU/100 mL was detected only during four months. The upstream TE and VRE counts of both plants were higher than their downstream counts (see table 4.2). The one-year sampling period (July 2016 - June 2017) covered both the hot and cold seasons. The variations in the

counts did not show any similar trend that can be related to the seasonal changes that occurred throughout the sampling period.

Table 4:1: TE and VRE counts of Plant I and Plant II

	Influent				Biofilter				Effluent			
	TE		VRE		TE		VRE		TE		VRE	
Month	Plant I	Plant II	Plant I	Plant II	Plant I	Plant II	Plant I	Plant II	Plant I	Plant II	Plant I	Plant II
Jul	6.7	6.6	0	4.7	3.7	3.8	0	2.8	0	3.8	0	3.4
Aug	6.2	6.5	4.3	4.7	5.2	5.2	3	3.1	0	2	0	0.9
Oct	6.3	6.4	4.3	5	4.7	4.4	0	3.3	0	0	0	0
Nov	6.4	6.4	4.6	4.7	4.1	4.7	0	0	0	2.4	0	1.1
Jan	6.5	6.5	4.8	5	3.9	4.8	0	3.6	0	3.5	0	0
Feb	6.5	7.2	0	6.7	4.3	5.2	0	3.9	0	4.4	0	2.2
Mar	6.3	6.3	4.9	4.9	4.4	5	0	3.8	0	1.6	0	0
Apr	6.3	6.6	4	4.4	4.3	4.6	2.8	3	0	3.9	0	0
May	6.3	6.5	4.4	5.2	4.2	5.3	0	3.8	0	1.5	0	0
June	6.5	6.4	4.7	4.6	4.3	4.5	0	0	0	0	0	0
Mean	6.4	6.5	3.6	4.8	4.3	4.8	0.6	2.7	0	2.3	0	0.8
SD	0.2	0.3	1.9	0.2	0.4	0.5	1.2	1.4	0	1.6	0	1.2
p value	0.1903		0.0123									

The counts expressed as Log_{10} /100 mL

Table 4:2: Comparison of the effluent, upstream and downstream counts of the presumptive enterococci

Month	**Plant I**						**Plant II**					
	TE Counts			VRE Counts			TE Counts			VRE Counts		
	Upstrm	Efflnt	Dwstrm	Upstrm	Efflnt	Dwstrm	Upstrm	Efflnt	Dwstrm	Upstrm	Efflnt	Dwstrm
Aug	2.6	0	1.9	0.9	0	0.8	2.4	2.0	2.3	0.9	0.9	1.0
Oct	3.4	0	3.3	1.8	0	1.6	3.3	0	2.1	1.8	0	0
Nov	3.1	0	2.5	1.0	0	0.4	2.8	2.4	3.3	1.0	1.1	1.7
Jan	3.4	0	2.9	1.4	0	0	3.3	3.5	3.5	1.4	0	2.1
Feb	4.6	0	2.9	2.3	0	0.1	3.0	4.4	3.1	2.3	2.0	2.3
Mar	3.8	0	3.2	1.1	0	1.4	3.1	1.6	0	1.1	0	0
Apr	4.5	0	4.5	3.0	0	2.2	4.4	3.9	4.3	3.0	0	2.3
May	5.5	0	3.6	1.4	0	0	3.4	1.5	3.0	1.4	0	0
Jun	4.4	0	1.8	2.1	0	0	3.5	0	0	2.7	0	0
Mean	3.9	0	2.8	1.4	0	0.7	3.3	2.3	2.4	1.7	0.4	1.0
SD	0.9	0	0.8	0.9	0	0.8	0.6	1.6	1.5	08	0.8	1.1

The counts expressed as Log_{10} /100 mL of water sample

Upstrm = Upsteream, Dwstrm = Downstream, Efflnt = Effluent

*Here the effluent count for July was not included as there were no river counts for that month

P values: Plant I TE Upstrm/Dwstrm = 0.03; VRE Upstrm/Dwstrm = 0.04

Plant II TE Upstrm/Dwstrm = 0.19; VRE Upstrm/Dwstrm = 0.15

4.3.2 Log reduction and removal efficiency of plants I and II

Although the wastewater samples from the three treatment points (influent, biofilter/clarifier, and the final effluent) were collected at the same time, the enterococci count of the biofilter/clarifier and the effluent were the estimation rather than the actual reduction of the enterococci count of that particular influent. However, they could give an indication of the log reduction potential or removal efficiency of the treatment process.

Varying TE- and VRE $\log_{10}$ removal occurred over the various steps of the treatment processes. For Plant I, $\log_{10}$ removal of TE and VRE in influent by the biofilters ranged from $\log_{10}$ 0.99 to 2.47 (89.9-99.9%) and 1.17 to 4.88 (95-100%) respectively. From the biofilter to the final effluent stage (chlorination), there was consistently complete reduction/hundred percent removal of both TE and VRE. For Plant II, reduction of TE and VRE from the influent by clarifier ranged from $\log_{10}$1.22 to 2.55 (94-98%) and1.06 to 4.85 (96-100%) respectively. Between the clarifier to the final effluent, a complete reduction of both TE and VRE were not consistently found over the sampling period when compared to Plant I. Complete TE and VRE reduction was achieved in Plant II only on two (October and June) out of the ten months sampled while complete removal of VRE was achieved on six out of the ten months.

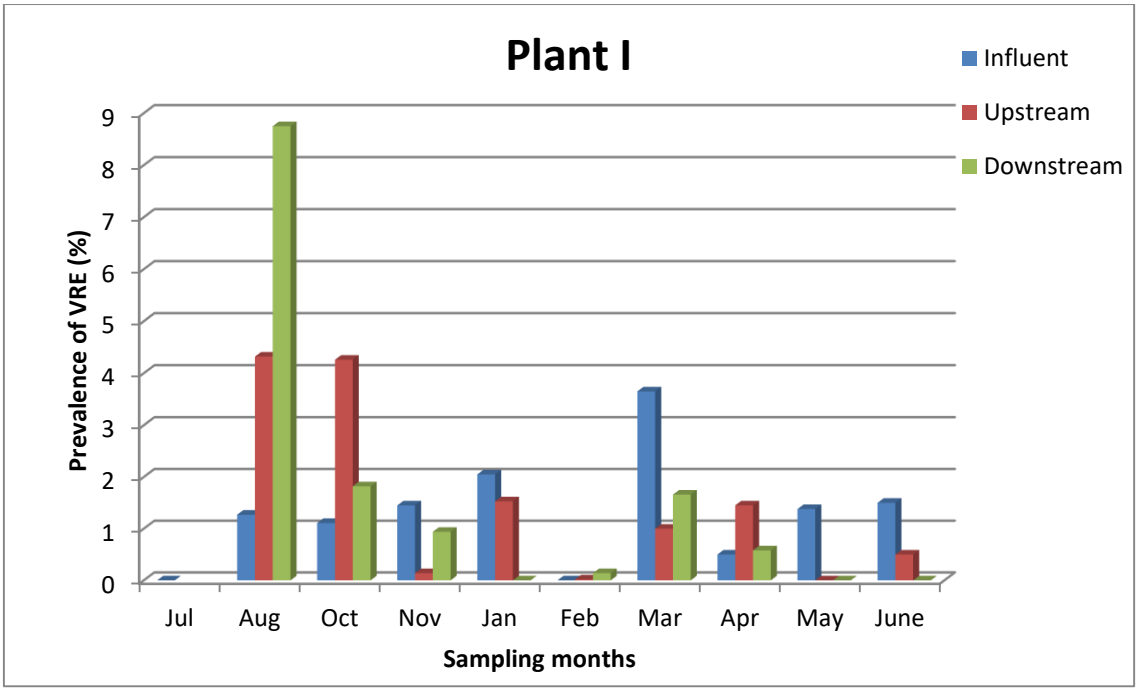
Based on the above facts, the biofilter and clarifier greatly reduced the enterococci from the influent. Further reduction was achieved by subsequent steps including chlorination. The biofilter (Plant I) and the clarifier (Plant II) when compared showed similar removal efficiency. The removal efficiency of biofilter of Plant I ranged between 89.9 -99.7% for TE; 93.3 – 100% for VRE and that of the clarifier of Plant II were 94 -99.9% for TE; 91.4 – 100% for VRE. However, the final treatment stages of the two plants did not show the

same reduction potential. The Plant I showed greater log reduction. Generally, the two WWTPs in this study are efficient in removing both TE and VRE from the influent. The overall removal efficiency (RE 2) of plant I was 100% for both TE and VRE throughout the sampling period, but Plant II had between 94.0-100% removal efficiency. Details are in the Table 0.1 in the appendix section, showing the TE and VRE counts of the three points sampled in the two WWTPs, the log reduction and removal efficiency at each stage of the treatment.

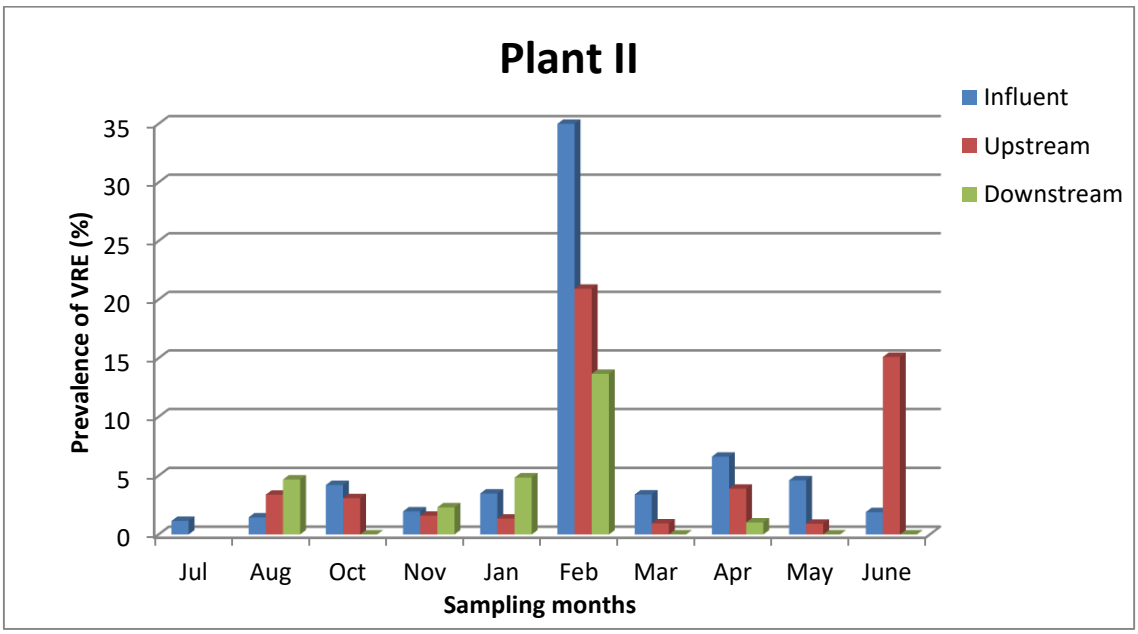
4.3.3 Prevalence of vancomycin resistant enterococci in the samples

The prevalence of VRE was estimated as stated in the methodology (see 4.2) and were as follows: For Plant I, it was zero in the influents for the months of July and February as no VRE was detected in those months. For the remaining months, it ranged from 0.5 -3.7% in the influent. Higher prevalence was shown in Plant II influent, which ranged from 1.2 – 6.61% and a skewed 35% shown in the month of February. VRE was less in the biofilter samples of Plant I than that of the clarifier of Plant II. The percentage proportion of VRE in biofilter of Plant I for 2/10 samples were 0.6% and 3.0% and that of the clarifier of Plant II, in 8/10 samples were in the range of 0.9% - 10.8%. Also, theVRE in the effluents of the two plants was of little or no significant value because they were not detected in the effluent of Plant I and was only detected in 4/10 effluent samples of Plant II. The percentage VRE for these months was 3.8%, 7.3%, 5.4% and 3.9% respectively. Apart from some of the months recorded as 0%, the percentage proportion of VRE in the upstream and downstream of Plant I ranged from 0.5 – 4.3% and 0.6 - 8.8% while those of Plant II were 0.9 – 20.9 and 1.0 -13.7% respectively.

Figure 4.1 shows the percentage VRE for three sampling points; influent, upstream and downstream of Plants I and II which had more consistent VRE counts than the biofilter/clarifier and effluent points. The prevalence of VRE in the influents was presented to show their composition in the wastewater from the environment and the possible effect if this wastewater has been discharged into the rivers without proper treatment.



a) Plant II



b) Plant II

Figure 4.1: Prevalence of vancomycin-resistant enterococci (VRE) present in the samples

The Figure 4.2 shows the mean prevalence of VRE in the various sampling points with Plant II having consistent higher values than Plant I.

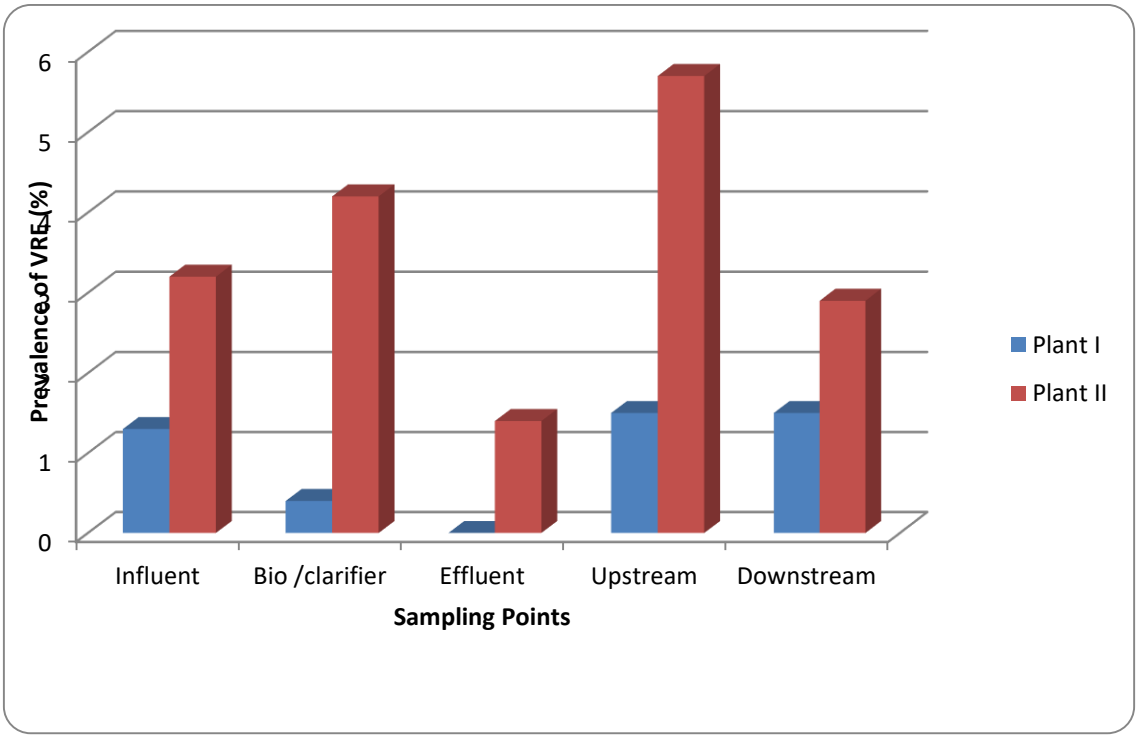


Figure 4.2: Comparison of the prevalence of VRE between Plants I and II

4.3.4 Effect of the WWTP effluents on the recipient

The upstream and downstream samples were taken at the most accessible points before and after the effluent outlet respectively. The upstream point of Plant I is about 1 km away from the WWTP. There is possibility of run-offs from the treatment plant getting into the river following a rainfall event. In Plant II, the upstream is right behind an animal farm and otherwise serve sometimes as a grazing place for cattle. The downstream point of Plant II is less than a kilometre from the effluent point while that of Plant I is 5 km after its effluent outlet.

The upstream count (TE and VRE) of recipients of both plants were consistently higher than their respective downstream location (see Table 4.2). While the difference in the upstream and downstream counts of both TE and VRE of Plant I is statistically significant ($p \leq 0.05$), those of Plant II are not significant ($p \geq 0.5$). Also, the downstream counts of TE and VRE were higher than that of the effluents. Therefore, the effluents of both WWTPs did not lead to increase the TE and VRE counts downstream of the receiving water bodies.

4.3.5 Confirmation of the isolates

All the isolates verified as Gram-positive cocci in chains, catalase negative, pyrase positive, forming dark colonies on bile esculine agar, and with significant growth on 6.5% NaCl medium were further confirmed as *Enterococcus* species by PCR and MALDI-TOF. The two primers (primer A targeting the 16S rRNA and primer B targeting the *tuf* gene) confirmed the isolates to be *Enterococcus*. However, the result of the two primers did not agree on 18 out of 269 isolates tested. Among the 18 isolates, five were positive with primer A but negative with primer B while the remaining 13 amplified with primer B only. Comparing the results from the two primers with that of MALDI-TOF, the five isolates (primers A+ B-) were identified as *Staphylococcus sciuri.* Then eight out of the 13 isolates (primers B+ A-) were *Enterococcus* while the remaining (5/13) were identified as *Pediococcus acidilactici* by MALDI-TOF. Also, all isolates that amplified with both primers which were tested by MALDI-TOF were confirmed positive for enterococci (not all Primers A and B positives were tested by MALDI-TOF). Thus primer A had five false positives and eight false negatives resulting to specificity (98%) and sensitivity (97%) while primer B had five false positives only with specificity of 98% and sensitivity of 100%.

4.3.6 Comparison of PCR and MALDI-TOF results

4.3.6.1 Genus identification

Two hundred and eighty-eight isolates (made up of 58 non-enterococci and 230 *Enterococcus* by PCR) were sent for confirmation by MALDI-TOF. Apart from 22 /230 isolates which could not grow after sub-culturing, 186 (80.1%) isolates tallied with PCR as *Enterococcus* while 22 (9.5%) were positive by PCR but negative with MALDI-TOF. Eight out of fifty-eight isolates (13.8%) which did not amplify with any of the primers were confirmed to be *Enterococcus* by MALDI-TOF while the remaining 50 tallied with PCR as non-enterococci. These results are further illustrated in Figure 4.3.

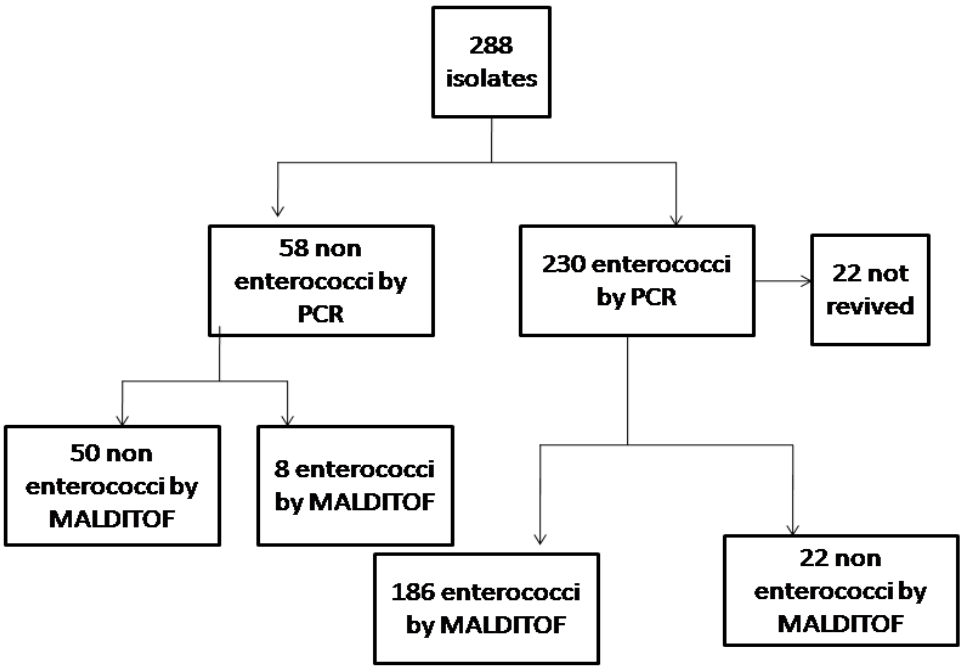


Figure 4.3: Genus confirmation of the enterococci isolates by PCR and MALDI-TOF

4.3.6.2 Speciation

According to MALDI-TOF, the isolates were identified either as *E. faecium, E. faecalis*, *E hirae, E. gallinarum,* or *E. casseliflavus* while PCR also resulted in the identified *E. durans* and *E. cecorum* among the isolates. Among the 186 isolates that were confirmed to be *Enterococcus* by the two methods, 158 agreed on speciation while 28 isolates identified as a member of a species by PCR were placed on different species by MALDI-TOF. Table 4.3 shows the speciation of these 28 isolates according to PCR and MALDI-TOF.

Table 4:3: Differences in species of the isolates between PCR and MALDI-TOF

	PCR	MALDI-TOF
1	*E. durans* (5 isolates)	*E. casseliflavus* (1), *E. hirae* (1), *E. faecalis* (3).
2	*E. faecalis* (4 isolates)	*E. faecium* (1), *E. casseliflavus* (3)
3	*E. cecorum* (7 isolates)	*E. gallinarum* (7)
4	*E. gallinarum* (9 isolates)	*E. faecalis* (2), *E. faecium* (1), *E. casseliflavus* (6)
5	*E. hirae* (2 isolates)	*E. faecalis* (2)
6	*E. faecium* (1 isolate)	*E. casseliflavus* (1)

The two hundred and sixty-nine isolates (202 VRE and 67 VSE) which were confirmed based on the PCR result of either of the two primers, and some of which were confirmed with MALDI-TOF were adopted for further referencing in this thesis. The 202 VRE consisted of 91 wastewater and 111 river isolates while the 67 VSE are made up of 36

wastewater and 31 river isolates. The isolates based on the PCR confirmation were identified as the species *E. faecium* and *E. faecalis* with *E. casseliflavus, E. durans, E. hirae, E. cecorium,* and *E. gallinarum*. The distribution of the species differs in wastewater and river samples. While they differ in the relative abundance in these samples, *E. gallinnarum* was only detected in river samples as shown in Figure 4.4. Also it could be seen that *E. faecium* was detected in almost all the sampling points as evidenced in Table 4.4. The species distribution in relation to the sampled WWTPs and points are shown in Table 4.4 and Figure 4.4 shows the species distribution among wastewater and river isolates. The speciation shown in Figure 4.4 and Table 4.4 were based on PCR.

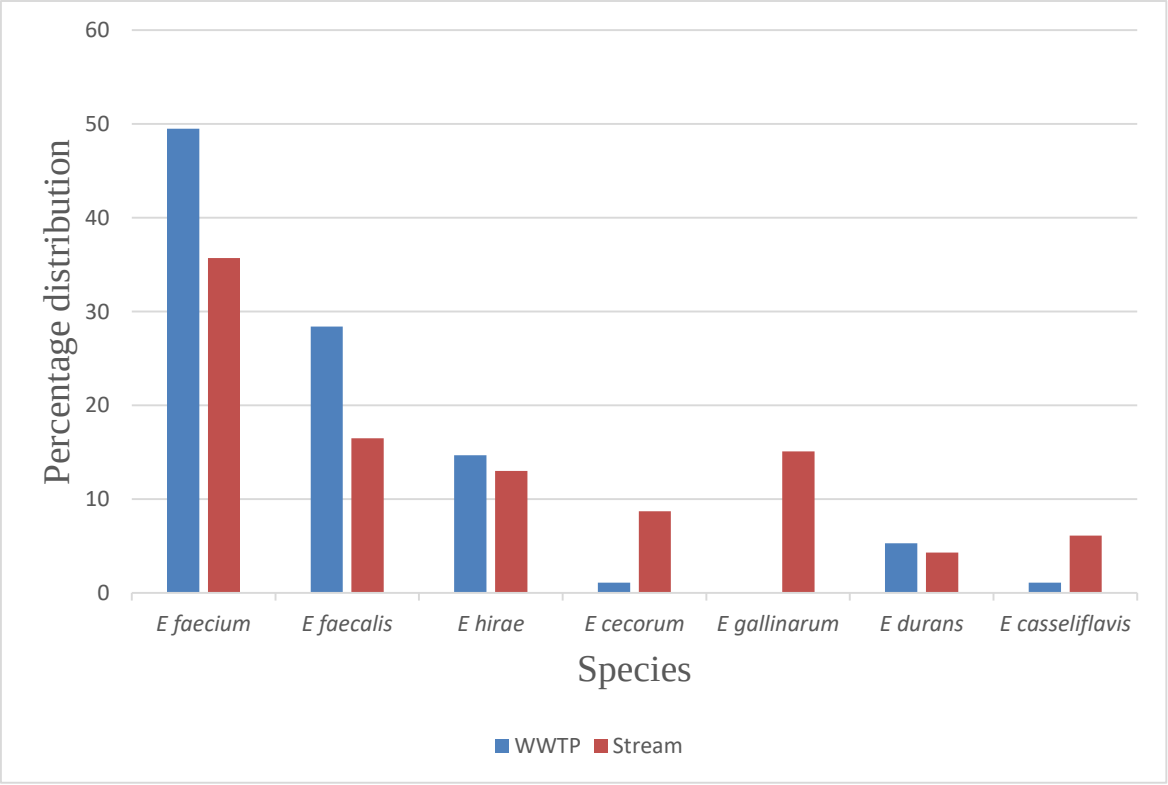


Figure 4.4: Percentage distribution of the *Enterococcus* species among the isolates

Table 4:4: Species distribution of VRE according to sampling points

Sampling Point	Plant I	Plant II
Influent	*E. faecium* 10	*E. faecium* 20
	E. faecalis 7	*E. faecalis* 9
	E. hirae 6	*E. hirae* 6
	E. durans 2	*E. durans* 3
		E. casselilavus 1
Biofilter/Clarifier	*E. faecium* 2	*E. faecium* 7
	E. faecalis 2	*E. faecalis* 7
Effluent		*E. faecium* 6
		E. hirae 1
		E. cecorum 1
Upstream	*E. faecium* 12	*E. faecium* 10
	E. faecalis 6	*E. faecalis* 8
	E. hirae 5	*E. hirae* 5
	E. gallinarum 2	*E. gallinarum* 12
	E.cecorum 1	*E.cecorum* 2
	E. casseliflavus 1	*E. casseliflavus* 2
		E. durans 3
Downstream	*E. faecium* 10	*E. faecium* 8
	E. hirae 1	*E. faecalis* 4
	E. gallinarum 1	*E. hirae* 2
	E.cecorum 7	*E. gallinarum* 3
	E. casseliflavus 2	*E. durans* 2
		E. casseliflavus 2

4.4 Discussion

The raw influent of municipal wastewater contains what comes from the connected population or environment (storm water). Thus, it may represent the different strains and concentrations of pathogens as excreted from the population. Enterococci as normal gastrointestinal flora with the concentration of between $10^5$and 10^7 CFU/g in human faeces (Boehm and Sassoubre, 2014) will be present in large number in influent samples. Hence the total enterococci (TE) count of 6.2 – 6.7$\log_{10}$ CFU/100 mL for Plant I and 6.3– 7.2$\log_{10}$

CFU/100 mL for Plant II falls within the assumed concentration range. The TE counts furthermore are within the same range (5.3×10^5 to 7.2×10^6 CFU per 100 mL) as presented by Rosenberg-Goldstein et al. (2014) on WWTP in United States and Sadowy and Luczkiewicz (2014) on WWTP in Gdansk-Wschod in Poland. The influent VRE count of the two plants ranged between 4.0 and 5.9$\log_{10}$ CFU/100 mL, which also is in the same range as found by Rosenberg-Goldstein et al. (2014) but higher than that of Varela et al. (2013). The difference in VRE count of the influents between the two Plants was significant ($p< 0.05$). This difference could be due to geographic differences in human VRE infection rates (Bouchillon et al., 2005; Rosenberg-Goldstein et al., 2014) or due to their different sources of wastewater. Plant I serves a local township population of about 30,000 while Plant II is in a suburban setting with larger population (about 70,000 people) and thus may account for the higher concentration of VRE Plant II. In addition, the design and working capacity of Plant II is larger than Plant I.

VRE was detected in wastewater samples including effluents with a mean count in the effluent as 0.4±0.8$\log_{10}$ CFU/100 mL in this study leading to the recovery and confirmation of 91 VRE isolates from the wastewater samples. VRE have been similarly detected from wastewater treatment plants and effluents by other authors (Araujo et al., 2010; Kotzamanidis et al., 2009; Luczkiewic et al., 2010; Morris et al., 2012; and Nagulapally et al., 2009) but these authors had fewer VRE isolates than this study. The recovery of higher number of VRE isolates was facilitated by the use of selective media supplemented with antibiotics (vancomycin) at concentrations inhibitory for enterococci. This made it easier to pick VRE isolates from a pool of enterococci colonies on vancomycin supplemented culture plate than going through prior and rigorous antibiotic susceptibility testing procedure. This method, originally developed by Watkinson et al. (2007) was also used to quickly estimate

the percentage of vancomycin resistant enterococci from the samples as have been employed in other studies (Figueira et al., 2011; Novo and Manaia, 2010; Watkinson et al., 2007). This method was found to be reliable, convenient, and thus allowed for rapid characterization of a large number of samples. Furthermore, using the media supplemented with vancomycin during the primary isolation in this study enhanced the presumptive detection and quantification of VRE in the samples. The advantages of the procedure were also addressed by other authors, like Said et al. (2015) and Varela et al. (2013).

On the negative side, the colony morphology (size and colour) were not as distinct compared to those from non-vancomycin medium. The antibiotics also affected the growth-rate, which was experienced during subsequent sub-culturing which impacted on DNA extraction. This was also earlier reported by Kariyama et al. (2000).

Efficient treatment processes are expected to bring about the reduction of pathogen concentration and poor or improperly treated wastewater could lead to pollution of their receiving water bodies with harmful pathogens such as VRE. The log reduction deduced from the TE and VRE counts of the treatment points sampled showed a sequential reduction through to the final effluent. The result showed at least from two log reductions to complete removal of TE and VRE from the influent. Also, the comparison of each plant's counts (TE and VRE) of the three treatment steps sampled showed that the difference between them were very significant with p value of 0.0001 in each case. Thus the two wastewater treatment plants (Plant I and Plant II) are efficient in removing TE and VRE from wastewater with removal efficiency of 100% for Plant I and 94 – 100% for Plant II. Result showed that Plant I was more efficient than Plant II. Since the biological treatment stage (biofilter/clarifier) of both plants had similar removal efficiency values, it could be taken

that Plant I has more steady and effective chlorination based on the fact that neither TE nor VRE was detected in its final chlorinated effluent throughout the sampled period.

Considering the facts that the upstream samples had higher counts than those of the downstream, and the lower or no count of the effluents, there was no visible or quantifiable impact of the receiving water with viable enterococci by the effluent. However, chlorination depending on the concentration or dose may not necessarily kill all bacterial pathogens, but it renders some non-culturable, even when they are still viable (Ding et al., 2017; Oliver et al., 2005; Rizzo et al., 2004). The non-culturable bacteria may still be a threat in conferring both virulence and antibiotic resistance genes on other bacteria in the receiving water environment (Rizzo et al., 2004). Therefore, the effluents from the two WWTPs might have impacted this non-culturable but viable TE and VRE to their receiving water bodies. Enterococci concentration of the receiving surface water might most probably have emanated from other sources other than effluents from wastewater treatment plants. Sources include aquatic algae (Verhougstraete et al., 2010), submerged vegetation (Badgley et al., 2011), from birds and other animal faecal matter (Fogarty et al., 2003), farm yard runoff and other human activities (Pignata et al., 2012).

Although *E. faecium* and *E. faecalis* were the dominant species in both WWTPs and river isolates, the difference in the distribution of *Enterococcus* species in these points was a true reflection of the two environments. The higher percentage of *E. faecium* and *E. faecalis* in WWTPs shows their relative abundance in human faeces (Agudelo and Huycke, 2014; Lebreton et al., 2014) which is reflected in the wastewater. Several other studies have also identified *E. faecium* and *E. faecalis* as the most common species of VRE isolated from wastewater (Morris et al., 2012; Rosenberg Goldstein et al., 2014; Talebi et al., 2008). Other

species such as *E. durans, E. hirae, E. cecorium* and *E. gallinarum* are normally less abundant than *E. faecium* and *E. faecalis* in wastewater as evidenced in this study and in line with the report of Leclercq et al. (2013). Also the relative abundance of other species particularly *E. gallinarum* and *E. casseliflavus* is an evidence of contamination of water by other sources such as animals other than effluents from WWTPs.

The identification of few isolates isolated from this work as *Pediococcus* by MALD-ITOF shows that Slanetz and Barley agar is not 100% selective for *Enterococcus* since *Pediococcus acidilactici* also formed red colonies on it. Similar findings on using other selective medium for *Enterococcus* have been shown (Maraccini et al, 2012; Viau and Peccia, 2009). The two genus primers used in the confirmation of the enterococci isolates in this study were not 100% specific and accurate for *Enterococcus*. Based on the amplification of the isolates using the two primers and their confirmation of the result of the MALDI-TOF, the specificity and sensitivity for primer A (El, 5' TCA ACC GGG GAG GGT 3'; E2, 5' ATT ACT AGC GAT TCC GG 3') are 98% and 97% while primer B (Ent1 5'-TACTGACAAACCATTCATGATG-3' and Ent2 5'-AACTTCGTCACCAACGCGAAC-3') are 98% and 100% respectively. The limitations of PCR resulting in false positives have been documented earlier (Zhang et al., 2009).

4.5 Conclusion

This study was designed to determine the efficiency of two WWTPs in removing TE and VRE from wastewater and thus their impact in their receiving water bodies. The two WWTPs were efficient in removing both TE and VRE from wastewater and the effluents

did not contribute to the viable counts of the enterococci in the recipient water bodies. The VRE present in the river samples were as a result of contamination from other sources. The prevalence of VRE particularly in the river samples serves as potential source of vancomycin resistance gene to other pathogens and thus a threat to public health. The species prevalent among the isolate were *E. faecium*, *E. faecalis*, *E. casseliflavus*, *E. durans*, *E. hirae*, *E. cecorium* and *E. gallinarum*.

5 CHAPTER 5: ANTIBIOTIC RESISTANCE AND RESISTANCE GENES FROM ENTEROCOCCI ISOLATES FOUND IN WASTEWATER TREATMENT PLANTS AND THEIR RECIPIENTS

5.1 Introduction

Multidrug resistant enterococci have been detected in wastewater treatment plants (Iweriebor et al., 2015) and from surface waters (Molale and Bezuidenhout, 2016). Antibiotic resistance in enterococci occurs as a result of specific genes conferring resistance. These resistance genes may be intrinsic or acquired as in glycopeptide antibiotics such as vancomycin. Some strains of enterococci possess a natural, low-level intrinsic resistance to beta-lactam antibiotics which includes the penicillins due to a low affinity to penicillin binding proteins (PBPs) (Klare et al., 2003). Alternatively, they may produce the enzyme beta-lactamase and are thus resistant to beta-lactam antibiotics such as penicillin (Miller et al., 2014). Enterococci can also resist antibiotics by active efflux. Multi-drug efflux pump gene and thirty-four efflux pump genes have been detected in *Enterococcus* (Jia et al., 2014; Davis et al., 2001; Jonas et al., 2001). Enterococci lack cytochrome enzymes which are necessary for high energy production needed for active uptake of substances such as antibiotics into the cell. This confers some resistance to aminoglycosides at low levels (Klare et al., 2003). Even though the genes conferring resistance to ciprofloxacin, *gyrA* and *parC* (Nowroozi et al., 2014) do exist in enterococci, their resistance to fluoroquinolones has been shown to be by active efflux (Lynch et al., 1997).

Intrinsic resistance (inherent) traits in enterococci include resistance to semisynthetic penicillinase-resistant penicillins, cephalosporins, low levels of aminoglycosides, and low levels of clindamycin. The acquired resistance includes resistance to chloramphenicol, erythromycin, high levels of clindamycin, tetracycline, high levels of aminoglycosides, penicillin by means of penicillinase, fluoroquinolones, and vancomycin (Murray, 1990).

Between 60 to 80% of enterococci are resistant to tetracycline (Molale and Bezuidenhout, (2016). The resistance is mediated through two different mechanisms related to different genes. The tetracycline resistance genes are *tetL, tetM, tetN*, and *tetO* where *tetL* mediates active efflux of tetracycline from cells while *tetM* and *tetN* protects the ribosomes from inhibition by tetracycline (Molale and Bezuidenhout, 2016). Vancomycin, a glycopeptide brand of antibiotics, has been the drug of choice in the treatment of enterococci infection due to is resistance to other antibiotics. Vancomycin kills bacteria by inhibiting the cell wall synthesis. The mechanism of resistance to vancomycin and various *van* genes detected in enterococci have been discussed (see section 2.9.3).

The aim of this chapter was to

- determine the antibiotic resistance profile of enterococci isolated from the two wastewater treatment plants and their recipients
- identify the vancomycin resistance genes and other resistance genes present in the isolates
- and to compare the antibiotic resistance between VRE and VSE with focus on their resistance profile and putative van and tet genes

5.2 Methodology

Two hundred and sixty-nine enterococci isolates made up of 202 vancomycin resistant enterococci (VRE) and 67 vancomycin sensitive enterococci (VSE) were isolated from wastewater and recipient river samples from the two wastewater treatment plants following the earlier described isolation procedure (Chapter 3, section 3.2 and 3.4) and confirmed using molecular method (see further section 3.7). The 202 VRE consisted of 91 wastewater and 111 river isolates while the 67 VSE are made up of 36 wastewater and 31 river isolates. Antibiotic susceptibility testing (AST) was done using the disk diffusion method as described in section 3.8.1 and interpreted according to Clinical and Laboratory Standards Institute (CLSI, 2016) procedure. All the 202 VRE and 43 VSE were tested for susceptibility to twelve antibiotics which were azithromycin (15µg), vancomycin (30 µg), imipenem (10 µg), teicoplanin (30 µg), tetracycline (30 µg), doxycline (30 µg), amoxicillin/clavulanic acid (co-amoxiclav) (30 µg), gentamicin (10 µg), streptomycin (10 µg), ciprofloxacin (5 µg), cefixime (5 µg), quinupristin-dalfopristin (15 µg). A subset of 60 VRE and 20 VSE isolates were tested for susceptibility to erythromycin (15 µg), penicillin G (10 units) and ampicillin (10 µg). The 43 VSE strain selected for AST was made to represent the different species and included the VSE isolates that possessed a *van* gene (22 VSE) and some without the *van* gene (21 VSE). The detection of vancomycin resistance genes was done using the multiplex PCR by Nam et al. (2013) on all PCR confirmed *Enterococcus* isolates (202 VRE and 67 VSE). The tetracycline resistance *(tet)* genes were detected by multiplex PCR according to Ng et al. (2001). For the ciprofloxacin resistance (*gyrA* and *parC)* genes, the primers and conditions as adopted by Nowroozi et al. (2014) were used, and multidrug efflux pump gene (*emeA*) was detected as adopted by Jia et al. (2014). The details are included in chapter three (3.8.4).

The multiple antibiotic resistance index (MAR Index) was calculated as the ratio of the number of the antibiotics to which an isolate was resistant to (A) to the total number of antibiotics to which the isolates were tested (B).

$$MAR\ Index = A/B$$

All the antibiotics except vancomycin and teicoplanin used in the AST were also considered for the MAR Index. Teicoplanin and vancomycin are both glycopeptides and vancomycin was excluded in the MAR Index determination, because it was the original supplement in the isolation medium.

5.3 Results

5.3.1 Antibiotic susceptibility testing

The antibiotic susceptibility test showed that both VRE and VSE were susceptible to co-amoxiclav and imipenem with the VRE being more susceptible to imipenem than the VSE. The eight isolates that showed resistance to co-amoxiclav were resistant to all the antibiotics tested. Both VRE and VSE were 100% resistant to cefixime (see Table 5.1). Higher resistance to tetracycline doxycycline, erythromycin, streptomycin and ciprofloxacin than the other antibiotics were also common within both groups although VSE had consistent lower percentage resistance than the VRE (see Figure 5.1). In addition to high percentage resistance to erythromycin (45%) and ciprofloxacin (58.1%) by the VSE group, the percentage that showed intermediate resistance within this group were still high. There were significant differences in the resistance pattern to some antibiotics between the isolates subjected to vancomycin during primary isolation (VRE) and those from non-vancomycin plates (VSE). The VREs were more sensitive to gentamicin, azithromycin, and

quinopristin/dalfopristin than the VSEs. The percentage resistance of VRE were; gentamicin (25.7%), azithromycin (24.3%), and quinopristin/dalfopristin (45%), while that of VSE were 97.7%, 97.7% and 86% respectively. Comparatively, VRE were more resistant to tetracycline (90.1%), streptomycin (91.1%), than VSE (tetracycline 65.1%, streptomycin 74.4%).

All except four VRE isolates were still resistant to 30 µg of vancomycin now exposed through the discs. It is expected that all the VSE isolates will be susceptible to 30 µg of vancomycin. However, a majority of the VSE had clear zone of inhibition but these zones were not large enough to be interpreted as sensitive. Consequently, 39.5% of VSE were resistant to vancomycin (30 µg). The antibiotic resistance profile of the VRE and VSE isolates are shown in Tables 5.1. Figure 5.1 is the graphical presentation of percentage resistance of VRE and VSE to fifteen antibiotics tested.

Table 5:1: Antibiotic resistance profile of the VRE and VSE isolates

	Number of Isolates		Percentage Resistant (%)		Percentage Sensitive		Percentage Intermediate	
Antibiotics	VRE	VSE	VRE	VSE	VRE	VSE	VRE	VSE
Gentamicin	202	43	25.7	97.7	74.3	0	0	2.3
Azithromycin	202	43	24.3	97.7	75.7	2.3	0	0
Ciprofloxacin	202	43	88.6	58.1	3	4.7	7.9	37.2
Teicoplanin	202	43	88.6	14	5	27.9	6.4	58.1
Co-amoxiclav	202	43	4.5	4.7	95.5	95.3	0	0
Vancomycin	202	43	93.6	39.5	2	16.3	4.5	44.2
Tetracycline	202	43	90.1	65.1	6.4	11.6	2.5	23.3
Cefixime	202	43	100	100	0	0	0	0
Streptomycin	202	43	91.1	74.4	5	4.7	4	20.9
Quinupristin-dalfopritin	202	43	45	86	21.3	4.7	33.7	9.3
Imipenem	202	43	4.5	16.3	91.6	46.5	4	37.2
Doxycycline	202	43	88.6	67.4	7.9	16.3	3.5	16.3
Erythromycin	60	20	68.3	45	3.3	0	28.3	55
Ampicillin	60	20	43.3	25	56.7	75	0	0
Penicillin G	60	20	36.7	11	63.3	9	0	0

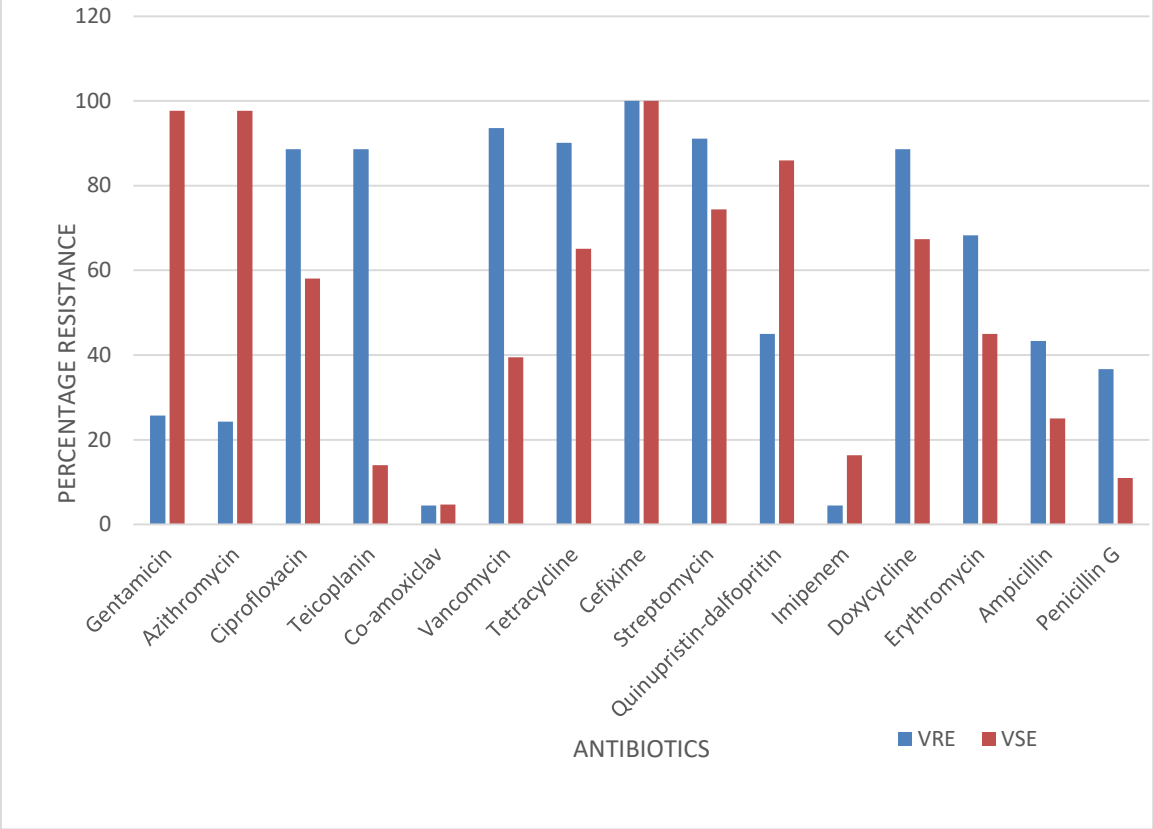


Figure 5.1: Comparison of antibiotic resistance between the VRE and VSE isolates

A MAR Index of ≤ 0.2 shows that an isolate is not a multidrug resistant organism. The MAR Index of ≥ 0.2 shows resistance to many of the antibiotics tested and this was the case among the VRE isolates. About 50.3% of the isolates had a MAR Index of 0.5. They are thus resistant to about half of all the antibiotics tested. About 3.4% of the isolates showed resistance to all the antibiotics used in the study (MAR Index = 1). The details of the MAR Index and the percentage of the isolates are depicted in Fig 5.2.

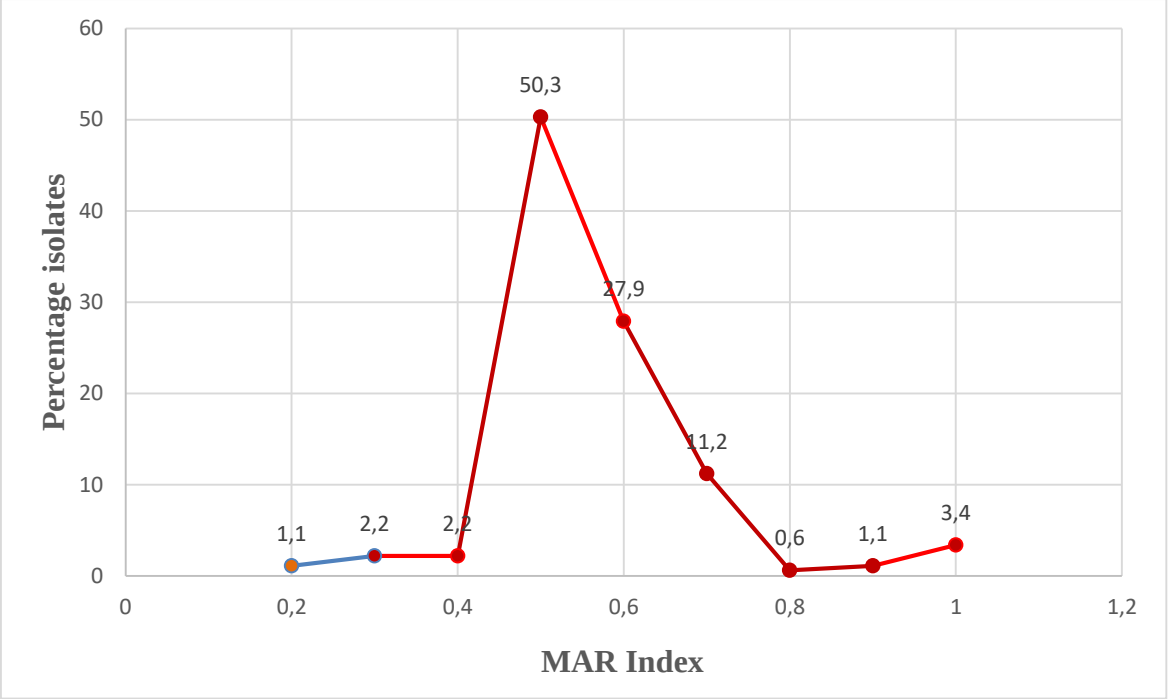


Figure 5.2: Multiple antibiotic resistant index (MAR Index) of the VRE isolates and percentage involved

*Red zone revealed the epidemiological high-risk isolates zone (MAR Index>0.2). The subsequent MAR Index value of the VRE isolates after 0.2 is 0.3 from which the red zone on the figure started.

5.3.2 Resistance across the sampling points and species

There was no remarkable difference in the resistant pattern of the isolates, related neither to the sampling points nor to the species. However, eight isolates were resistant to all the fifteen antibiotics tested. Seven of these isolates were *E. faecium* and one *E. faecalis*. Among the seven *E. faecium* super resistant isolates, five were isolated from the chlorinated effluent and the clarifiers of Plant II on the same sampling date. The *E. faecalis* was isolated from Plant II influent while the remaining two *E. faecium* isolates came from Plant I influent.

5.3.3 Vancomycin resistance genes

Four different vancomycin resistance genes were detected within the group of VREs. All the VRE isolates except one was shown to possess a *van* gene, with a dominance of *vanA* in 149 (73.8%) (Table 5.2). This gene also occurred within the VSE group but in a lower frequency. *Van C2/3* occurred in both groups in similar frequencies. In the sensitive group (VSE) no resistance genes were found in a dominant number of the isolates (67%). It is interesting to note that *vanC* was detected in all *E. gallinarum* of VRE and VSE isolates but not in VRE *E. faecium/faecalis*. In addition, no VSE without a *van* gene belonged to *E. gallinarum*, and *vanA* was not detected in any of the *E. gallinarum isolates. vanC* was detected across all species of VSE. Not more than one *van* gene was detected in any of the isolates.

Table 5:2: Vancomycin resistance genes detected among species of VRE and VSE

Group	*Van* gene	No of Isolates	Percentage	Species involved
VRE	*vanA*	149	73.8	*E. faecium, E. faecalis, E. hirae, E. durans*
	vanB	3	1.5	*E. faecalis*
	vanC1	33	16.3	*E. gallinarum, E. casseliflavus E. cecorum*
	vanC2/3	16	7.9	*E. gallinarum, E. casseliflavus E. faecium*
VSE	*vanA*	14	20.9	*E. faecium, E. hirae, E. faecalis, E. durans*
	vanB	0	0	no species
	vanC1	1	1.5	*E. casseliflavus*
	vanC2/3	7	10.4	*E. gallinarum, E. casseliflavus E. faecium/ faecalis*
	none	45	67.2	*E. faecium, E. hirae, E. faecalis, E. durans*

- Number of isolates tested: VRE=202; VSE=67

5.3.4 Detection of other resistance genes

Although there is remarkable high resistance to ciprofloxacin among the isolates, the detection of the two ciprofloxacin resistance genes, *gyrA* and *parC* was very low. The *gyrA* gene was detected only in 14 VRE isolates while *parC* in five. Contrastingly, tetracycline resistance genes were detected in greater number among the isolates. Four out of the 14 *tet* gene tested were detected. These are; *tetK, tetL, tetM,* and *tetO.* One hundred and twenty-seven (62.9%) of the 202 VRE, possessed at least one of these *tet* genes while 22 (which included the eight isolates that were resistant to all the antibiotics) out of 127 had two *tet* genes (*tetL* and *tetM*). Among the 67 VSE, 21 (31.3%) had at least one *tet* gene while two *tet* genes were detected in 4 out of 21 (three with *tetL* and *tetM* and one with *tetK* and *tetL*). Multidrug efflux pump gene, *emeA* was also detected in appreciable number of the isolate. Table 5.3 summarize the detection of the resistance genes.

Table 5:3: Tetracycline resistance and multidrug efflux pump genes detected in *Enterococcus* isolates

	VRE no (%)	VSE no (%)
tet(K)	16/202 (7.9)	2/67 (3)
tet(L)	61/202 (30.2)	7/67 (10.4)
tet(M)	40/202 (19.8)	12/67 (17.9)
tet(O)	10/202 (5.0)	0 (0)
emeA	27/202(13.4)	8/67 (11.9)

5.3.5 A comparison between antibiotics resistance and the detection of the corresponding genes

Vancomycin resistance genes were detected in all the 202 (100%) VRE isolates. Each isolate possessed only a *van* gene where the *vanA* gene dominated (73.8%) This corresponded to 93.6% and 88.6% of them which expressed resistance to vancomycin and teichoplanin respectively. For tetracycline resistance genes, 62.9% of the VRE isolates possessed at least one *tet* gene. However, there was higher resistance to tetracyclines among the VRE isolates (tetracycline 90.1% and doxycycline 88.6%) than the corresponding *tet* genes detected. Similar findings were seen among the VSE isolates (see Table 5.4).

Table 5:4: Vancomycin and tetracycline resistance and the detection of their corresponding genes among the isolates

Antibiotics	VRE		VSE	
	Resistance (%)	Resistance gene (%)	Resistance (%)	Resistance gene (%)
vancomycin	93.6	100 (73.8 for *vanA*)	39.5	32.8
teichoplanin	88.6		14	
tetracycline	90.1	62.9	65.1	31.3
doxycycline	88.6		67.4	

5.4 Discussion

Wastewater treatments plants (WWTPs) provide an enabling environment for dissemination of resistance genes. This is due to high concentration and variety of bacteria carrying resistance genes from different sources and thus create an enabling environment for horizontal gene transfer. Surface water is exposed to pollution from multiple sources in addition to wastewater effluents like farm yard runoff, animal droppings, and from other human activities (Pignata et al., 2012). The enterococci isolates from the river samples may have originated from these sources. The isolates from the two WWTPs and their connected surface water recipients in this study were multidrug resistant. About 50% of the isolates were resistant to six antibiotics tested apart from vancomycin and teichplanin while 3.4% were resistant to all the antibiotics.

An increasing resistance to antibiotics by enterococci due to acquisition of resistance genes has been reported elsewhere (Osman et al., 2016; Iweriebor et al., 2015; Kotzamanidis et al., 2009). Although the intrinsic resistance to quinupristin-dalfopritin by *E. faecalis* (Hershberger et al., 2004; Johnson et al., 2000), contributed to some extent to the high resistance against this antibiotic, its resistance by *E. faecium* is of concern as this has been an alternative drug in the treatment of vancomycin-resistant *E. faecium* infections (Hershberger et al., 2004). However, the total resistance against the fifteen antibiotics by eight isolates consisting of seven *E. faecium* and one *E. faecalis* is a serious concern. Each of these isolate had *vanA* and two *tet* genes confirming their strong antibiotic resistance ability. *E. faecium* and *E. faecalis* are abundant species among the isolates and their importance and severity in human infection and their antibiotic resistance are well known.

E. faecium is known for its high resistance to antibiotics while *E. faecalis* is more virulent (Fisher and Phillips, 2009).

It is known that some strains of enterococci possess an intrinsic resistance to beta-lactam antibiotics such as penicillins as a result of their low affinity to penicillin binding proteins (PBPs) or by producing the enzyme, beta-lactamase against the beta-lactam agents (Klare et al., 2003; Murray, 1990). While most isolates of *E. faecalis* are inhibited by concentrations of penicillin or ampicillin (1 to 8 µg/mL), isolates of *E. faecium* usually require an average of 16 to 64 µg/mL to inhibit growth, although some isolates are even more resistant (Murray, 1997). As seen in this study also, resistance to the penicillins ranged from 25% to 55% among the two groups of isolates (VRE and VSE) and a majority of these resistant isolates were *E. faecalis* and *E. faecium* with a few *E. gallinarum* in addition. However, the higher sensitivity to co-amoxiclav (with only 4.5/4.7% VRE/VSE isolates being resistant) compared to higher resistance to ampicillin and penicillin G by the same enterococci isolates showed potential activity of a protease inhibitor (clavulanic acid) against beta lactamase activity. This showed that these isolates were resistant against the penicillins due to beta-lactamase activity.

Although the majority of the isolates were resistant to ciprofloxacin, the frequency of detection of *gyrA* and *parC* genes was low. Thus, the resistance to ciprofloxacin by the enterococci isolates could not be linked to the possession or presence of the corresponding resistance gene. The reason could be either that the genes were present but were not detected or that enterococci resist ciprofloxacin by another mechanism as shown by Lynch et al. (1997). According to Lynch et al. (1997), enterococci are resistant to fluoroquinolones by active efflux. This could be partly affirmed by detection of a number of efflux genes, *emeA*

and *tet(L)* among the isolates and have also been shown by other authors (Jia et al., 2014; Molale and Bezuidenhout, 2016). Additional mechanisms may also contribute to the high resistance to ciprofloxacin since the number of isolates that possess either *gyrA, parC*, or *emeA* genes were lower than those that exhibited resistance to the ciprofloxacin.

The selective media supplemented with antibiotics at concentrations inhibitory for the target organism promotes the enrichment of antibiotic resistant bacteria (ARB), and facilitate the detection of resistance genetic determinants. In a study by Figueira et al. (2011), on characterization of quinolone resistance in *Aeromonas* isolated from water habitats, the gene *aacA6-ib-cr* was detected mainly in bacteria isolated in the presence of ciprofloxacin. This was similar to the detection of *vanA* gene in this study. The *van* genes particularly *vanA* were detected mostly among VRE which were isolated on Slanetz and Bartley agar supplemented with vancomycin. However, for bacteria isolated with antibiotic supplemented medium, their true resistance profiles to other antibiotics seem to be affected. The antibiotic in the primary isolation medium seemed to play a pre-synergic effect when exposed to other antibiotics during AST, which explain the difference in the resistance pattern to some antibiotics between the VRE and VSE isolates in this study. The isolates from vancomycin supplemented media were more susceptible to gentamicin, azithromycin, quinupristin-dalfopristin, and imipenem than those from non-vancomycin media. This also could be due to the fact that the organism must have been weakened by the antibiotics in the primary medium and then succumbed more easily to some other antibiotics during AST. Furthermore, synergic effect of vancomycin with an aminoglycoside (Said et al., 2015), and quinopristin-dalfopristin with a cell wall-active agent (Hershberger et al., 2004) against enterococci has been shown.

The detection of *vanA* gene on some VSE isolates may seem unexpected. The presence of these genes in organisms without subsequently being expressed is well documented (Qin et al., 2000). However, resistance marked by these isolates upon AST could be explained by that the putative *vanA* gene was not actively expressed. This slow expression could lead to misinterpretation. Enterococci isolates, which did not grow upon sub-culturing on vancomycin-supplemented medium, were taken to be VSE. Therefore, those isolates, which carry *van* genes that were poorly expressed may not grow on vancomycin, supplement medium and thus be classified as VSE. Hence, upon AST, following the standard reference guideline, the measured zones of inhibition were not large enough to be interpreted as susceptible and thus were regarded as resistant. Therefore, for proper determination of antibiotic resistance/susceptibility of an organism, both AST and detection of resistance genes when possible should be employed.

The presence of v*anC1* or *vanC2/3* detected in all the *E gallinarum* and *E. casseliflavus* isolates portrays the intrinsic *vanC* resistance specific to these species of enterococci. This natural attribute of these two species is also evident in this study as none of the isolates in which no *van* gene was detected belonged to *E gallinarum* or *E. casseliflavus.* Furthermore, *vanA* gene was not detected in these although some authors (Eshaghi et al., 2015; Osman et al., 2016; Corso et al, 2005) have in a few instances detected *vanA* in *E gallinarum.* The *vanA* is linked with high resistance to vancomycin and teichoplanin. The detection in 73.8% of the VRE isolates could explain the resistance to teichoplanin by 88.6% of this group. However, detection of *vanA* in a majority of the isolates in this study is in line with its reported relative abundance (Xu et al., 2010; Getachew et al., 2012) but differs from other authors' findings such as Iweriebor et al., (2015). These authors detected *vanB* instead of *vanA* from *Enterococcus* isolated from wastewater and their effluents in Alice, South Africa.

Nam et al., (2013) also showed a slightly different picture and only detected *vanC1* and *vanC2/3* genes from Korean aquatic environments.

The *vanA* type of resistance is mostly inducible and confers high level of resistance (resistance to high concentration) of both vancomycin and teicoplanin (Fisher and Phillips, 2009). The *vanA* gene has been shown to be on transposons (Arthur et al., 1993) and thus can easily be transferred. This may explain their detection in the majority of the VRE isolates in this study. According to Giraffa (2002), resistant genes can be transferred to both antibiotics susceptible enterococci and other pathogens. In the same way, *vanA* resistant gene can easily be transferred to other pathogens within the environment. Thus, a high prevalence of *vanA* genes among the isolates is a public health risk.

In conclusion, enterococci isolated from wastewater treatment plants and their receiving water bodies are multidrug resistant showing high and remarkable resistance to most of the antibiotics except amoxyl-clav and imipenem. Abundant detection of four types of *van* genes and other resistance genes, *emeA*, four *tet* genes especially those contributing to active efflux of antibiotics explained the high multidrug resistance detected in these isolates. It is therefore a matter for serious concern.

6 CHAPTER 6: PATHOGENIC ATTRIBUTES OF ENTEROCOCCI ISOLATED FROM WASTEWATER TREATMENT PLANTS AND THEIR RECIPIENTS

6.1 Introduction

Pathogenic enterococci have become an emerging public health concern due to increased resistance to anti-microbial therapy (Tendulkar et al., 2003). Mobile genetic elements (MGEs) are among the factors that contribute to the transition of enterococci from commensals to pathogens (La Rosa et al., 2015). Enterococci which were ordinarily commensals became pathogenic due their ability to easily acquire genes. Thus they are referred to as emerging pathogens (Giridhara-Upadhyaya, 2009) or opportunistic pathogens (Al-Ahdal et al., 2012; Comerlato et al., 2013). Enterococci possess a number of pathogenic attributes some of which are intrinsic while some are acquired from other bacterial strains (see section 2.9). They are implicated in a variety of infections (see further 2.9) and the morbidity and mortality caused by these infections are higher when resistant strains are involved (Billington et al., 2014: Fisher and Phillips, 2009; DiazGranados et al., 2005).

Virulence factors that have been described in enterococci include gelatinase production, enterococcal surface protein (*Esp*), aggregation substance (AS), cytolysin, hyaluronidase, and biofilm formation (Chuang et al., 2009; Vankerckhoven et al., 2004). While the genes, *gelE, esp, asa1, cylA,* and *hyl* are linked to gelatinase, enterococcal surface protein, aggregation substance, cytolysin and hyaluronidase production respectively, that of biofilm formation involves several genes which is complex and multifactorial (Biswas et al., 2016).

Other substances and factors that are associated with virulence in *Enterococcus* include; endocarditis-specific antigen A (*efaA*), collagen-binding protein (*ace*), serine protease, capsule, cell wall polysaccharide and superoxide (Iweriebor et al., 2015; Giridhara Upadhyayaet al., 2009) (see 2.9.1). These virulence factors promote infection through mediation of adhesion, colonization and invasion into the host tissues, modulation of the host immunity, and extracellular production of enzymes and toxins, and thus enhance the severity of the infections (Strateva et al., 2016). The adhesins include aggregation substance, enterococcal surface protein, endocarditis-specific antigen A and collagen-binding proteins help enterococci to adhere to their host's tissue while cytolysin, gelatinase, and hyaluronidase affect the host's tissue leading to degradation and invasion (Strateva et al, 2016).

The importance of enterococci as a pathogen includes both their virulence attributes and their resistance to antibiotic therapy. This chapter evaluates some virulence factors and the virulence genes present on the enterococci isolated from the two wastewater treatment plants and their receiving water bodies. These isolates have been shown to be multidrug resistant by antibiotic susceptibility testing and detection of some resistant genes such as *van*, *tet* and multidrug efflux pump, *emeA* genes. Their multidrug resistance and the resistance genes were presented and discussed in chapter five (see sections 5.3 and 5.4).

6.2 Methodology

One third (88) of the total isolates were used for the detection of virulence genes and virulence factors. Systematic random selection of the isolates was done that ensured the proper representation of different species across sampling points including both VRE and

VSE. The isolates represented sampling points (wastewater = 25, receiving river = 63); response to the vancomycin groups (VRE = 62, VSE = 26) and species (*E. faecium* = 50, *E. faecalis* = 12, *E. gallinarum* = 14, *E. casseliflavus* = 7, *E hirae* = 5).

Multiplex PCR was used for the detection of the genes *asa1, hyl, cylA, gelE,* and *esp* while *efaA* and *ace* genes were detected by singleplex PCR and detection of resistance factors was done as previously described in the methodology chapter (section 3.8.5). Ability to form biofilms, gelatinase activity and haemolysis by isolates were also determined (see also 3.8.6). Descriptive statistics was used to assess the prevalence of each virulence gene among the isolates and to compare prevalence of virulence genes among vancomycin resistant enterococci (VRE) and vancomycin sensitive enterococci (VSE), isolates from wastewater and river samples, and between *E. faecalis* and *E. faecium*.

6.3 Results

6.3.1 Detection of *gelE* and gelatinase activity

GelE was detected in 16 (18.1%) isolates while 23 (26.1%) were positive for gelatinase activity. Only eight (50%) of the 16 isolates that possessed *gelE* were positive for gelatinase activitywhile the additional 15 (65%) isolates that hydrolysed gelatine had no detectable *gelE* gene. The eight isolates that were both positive for *gelE* and gelatinase activity comprised of six *E. faecalis* and two *E. faecium*. However, not all *gelE* positive *E. faecalis* were positive for gelatinase activity.

6.3.2 Detection of *cylA* and haemolytic activity

The cyl gene was detected only in one isolate (*E. casseliflavus*) which did not show haemolytic activity. Rather, haemolysis was seen in 24 isolates in which *cyl* gene was not detected. These isolates included *E. faecalis* and *E. faecium* and other species such as *E. gallinarum, E. casseliflavus* and *E. hirae*.

6.3.3 Detection of *ace, efaA, asa1, hyl, esp* genes and biofilm formation

Ace was detected in 13.6% (12) of the isolates while *efaA* was the most prevalent virulence gene among the isolates present in 25% (22) of them. The species that harboured these genes were mostly *E. faecalis* followed by *E. faecium* and *E. hirae*. The *asa1* was detected in 10 (11.4%) of the isolates with a majority of *E faecalis*. *Hyl* and *esp* genes were also detected in 9.1% (8) and 4.4% (4) of the isolates respectively. The *hyl* gene was detected only in *E. faecium* isolates and the *esp* gene only in *E. faecalis*. The isolates were all either weak or non-biofilm formers except for two isolates, one each of *E. faecalis* and *E. faecium* which exhibited strong and moderate biofilm formation respectively. Table 6.1 shows the prevalence of virulence genes/factors detected among the isolates while the virulence genes within different species are presented in Table 6.2.

Among the 88 isolates, 69 (78.4%) possessed at least one of the virulence determinants tested. Among these 69 isolates, ten (11.4%) and seven (8%) were positive for haemolysis and gelatinase activity respectively but were not positive for any of the virulence genes tested. None of these last two groups included any *E. faecalis* isolates. Furthermore, nine out of the 69 positive isolates were *E. faecalis* and found to be highly virulent possessing between four to seven virulence genes/factors. There was no significant difference in the

prevalence of any of the six virulence genes (*gelE, esp, asa1, hyl, efaA*, and *ace*) between enterococci isolates when wastewater and river samples were compared (p>0.05), and between VRE and VSE (p>0.05) but the prevalence of these virulence genes among *E. faecalis* and *E. faecium* was significantly different (p=0.05). Tables 6.1 and 6.2 show the prevalence of the virulence gene among the isolates and species respectively.

Table 6:1: Virulence determinants detected among the *Enterococcus* isolates

Virulence gene/factor (*)	Total (%)	Wastewater (%)	River (%)	VRE (%)	VSE (%)
efaA (22)	25	28	23.8	16.1	46.2
ace (12)	13.6	20	11.1	12.9	15.4
asa1 (10)	11.4	8	12.7	7.2	19.2
hyl (8)	9.1	20	4.8	12.9	0
esp (4)	4.5	8	3.2	3.2	7.7
gelE (16)	18.1	12	20.6	17.7	19.2
cyl (1)	1.1	0	1.6	1.6	0
gelatinase (23)	26.1	24	27	29	19.2
haemolysis (24)	27.3	16	31.7	21	42.3
biofilm formation (2)	2.3	8	0	0	7.7

(*) the number positive out of 88 isolates tested

Note: number of isolate from wastewater = 25; river = 63; VRE = 62; VSE = 26

Table 6:2: Prevalence of the virulence genes and the *Enterococcus* species involved

Virulence gene	*E. faecalis* (12)	*E. faecium* (50)	*E. hirae* (5)	*E. casseliflavus* (7)	*E. gallinarum* (14)
efaA	10 (83.3%)	5 (10%)	4 (80%)	2 (28.6%)	1 (7.1%)
ace	8(66.7%)	2(4%)	1 (20%)	-	1 (7.1%)
asa1	7 (58.3%)	1 (2%)	-	-	2 (14.3%)
hyl	-	8 (16%)	-	-	-
esp	4 (33.3%)	-	-	-	-
gelE	9 (75%)	5 (10%)	-	1 (14.3%)	1 (7.1%)
cylA	-	-	-	1(14.3%)	-

6.4 Discussion

Seven virulence genes (*ace, asa1, cylA efaA, esp, gelE,* and *hyl*) were detected among the isolates with 74% of the isolates positive for at least one virulence gene/factor. The *efaA* gene was the most prevalent (25%) while *cylA* gene was the least (1.1%) virulence gene detected. In as much as the *cylA* gene was the least prevalent, haemolysis (27.3%) was the highest among the three virulence factor detected. In addition, the number of isolates positive for gelatinase activity was higher than the number of isolates in which the associated gene (*gelE*) was detected. Therefore, there may be possibility of false negative results in the detection of the virulence genes.

The detection of the *gelE* gene in enterococcal strains do not correlate with the ability to produce gelatinase as shown in several investigations (Biswas et al., 2016; Qin et al., 2000; Revathy et al., 2010). This was also the situation in this study where only 50% of the isolates in which *gelE* gene was detected actually expressed gelatinase. This could be as a result of "silent genes". However, the *E. faecalis fsr* quorum-sensing system was shown to control the production of gelatinase (Qin et al., 2000). Inactivation of the *fsr* controlled gene *gelE* encoding the zinc-metalloprotease gelatinase (La Rosa et al., 2015) which led to that *gelE* was not expressed. *GelE* is shown to be associated with *E. faecalis* (Vankerckhoven et al, 2004) but Lopes et al. (2005) reported the detection of *gelE* in species other than *E. faecalis* or *E. faecium,* which was also the case in this study.

Cytolysin is made up of two components lysin (L) and an activator (A). The cytolysin operon contains five genes; *cylL1, cylL2, cylM, cylB* that are responsible for the expression

of the component L, and *cylA* for the expression of the activator protein A (Vankerckhoven et al, 2004). This activator (A) is a serine protease, which is responsible for further cleavage and activation of *cyl1* and *cyl2* subunit extracellularly for subsequent full expression (Kayaoglu and Ørstavik, 2004). In this study, detection of *cylA* gene was chosen (because of the role of the activator protein A in cytolysin expression) rather than the whole cytolysin gene operon. Cytolysin is associated with haemolytic activity (Chajecka-Wierzchowska et al, 2017; Kayaoglu and Ørstavik, 2004), thus β-haemolytic isolates indicates possession of *cylA.* However, β-haemolysis in 24 isolates and the detection of *cylA* gene only in one isolate could not be linked. Either the isolates have this gene but it was not detected in the PCR used or the haemolytic activity by these isolates is as a result of factors other than cytolysin. Multiplex PCR is advantageous as it saves time and cost but some DNA amplifications can be inhibited and so false negative result may be obtained (Zhang et al., 2009). Also, if gelatinase as a virulence factor is said to hydrolyse a range of substance including haemoglobin in the body to enable bacteria invasion (Giridhara Upadhyaya et al., 2009), any bacterial strain capable of expressing gelatinase may cause haemolysis in blood *in vitro*. Therefore, the expression of haemolysis by some enterococci isolates in this study could also be due to gelatinase activity.

The presence of virulence genes in enterococci has been shown to be statistically different between species (Comerlato et al, 2013). As shown in this study (Table 6.2), virulence genes were detected across all species of the enterococci isolates but their prevalence in *E. faecalis wa*s higher compared with the other species. Also, hyaluronidase was detected only in *E. faecium* in this study and has been stated to be a virulence factor unique to *E. faecium* (Vankerckhoven et al., 2004). However, Trivedi et al. (2011) detected *hyl* gene in other species such as *E. casselifavus*, *E. mundtii*, and E. *durans* isolated from food. It has also

been detected in clinical isolates of *E. faecalis* (Strateva et al., 2016). While *E. faecalis* is more virulent, *E. faecium* is more resistant to antibiotic therapy. Isolates possessing four or more virulence factors were only among the *E. faecalis* isolates and result from this study showed that the prevalence of virulence genes among *E. faecalis* was higher than among *E. faecium* (Table 6.2).

Although Comerlato et al. (2013) reported that there was no association between the source of isolation and the presence and activity of any virulence factor; some virulence attributes may be more common in enterococci isolated from a particular kind of infection. Some adhesins (*esp; agg/asa1* and *efaA*) were significantly more prevalent among the non-invasive isolates of *Enterococcus* spp. such as in the genital tract infection (GTI) isolates, wound isolates and urinary tract infection (UTI) isolates compared to the invasive bacteraemia isolates ($p < 0.001–0.05$) (Strateva et al., 2016). The *ace/acm* revealed a significantly higher frequency ($p < 0.02–0.05$) in invasive isolates compared to the non-invasive GTI and wound isolates. However, for environmental isolates, the type and frequency of virulence genes might not differ among isolates from different sites. This study did not show any difference in type and the frequency of the virulence genes between the isolates from wastewater treatment plants and those of the river isolates. Previous reports have shown no difference in presence of virulence genes among VRE and VSE isolates in this study (Comerlato et al., 2013). However, since, Biswas et al. (2016) showed higher prevalence of *hyl* gene among VRE than VSE, the detection of *hyl* genes only among the *E. faecium* isolates of VRE may be considered relevant.

Considering the possession of virulence genes or expression of virulence factors as a confirmation of an isolate being virulent, it can be concluded that 74% of the isolate from

wastewater and river samples are virulent and thus they are considered as potential risk to the health of the community. The isolates exhibited high resistance not only to vancomycin but to a number of antibiotics (see 5.3.1) and possessed relevant genes conferring resistance (section 5.5.5). With the majority of the isolate coming from river samples (section 4.3.4), irrigation and other uses that are associated with these recipient water bodies are invariably contributing to the spread of pathogenic and resistant enterococci to the environment. So frequent screening would for and adequate precautions in the use of the water from these rivers may contribute to infection control measures.

7 CHAPTER 7: SUMMARY AND CONCLUSION

7.1 Summary

This study was motivated by the increasing public health risk associated with antibiotic resistant bacteria with focus on vancomycin resistant enterococci, and the wastewater effluents as possible means of dissemination of antibiotic resistance to the receiving environment. The monitoring of the operational efficiency of the wastewater treatment plants (WWTPs) and receiving water bodies is also very important. Detection of point sources of microbial contamination of surface waters will help in controlling subsequent pollution. Enterococci are a reliable faecal indicator of water quality (WHO, 2006) due to its resilient characteristics and concentration in faeces of human and other animals (Boehm and Sassoubre, 2014). The presence of enterococci in water indicates the prevalence of other harmful pathogens (USEPA, 2006). Enterococci are opportunistic pathogens causing some life threatening infections of which vancomycin has been the drug of choice in the treatment, due to its resistance to other antibiotics. Emergence of vancomycin resistance is a serious public health concern that requires further research and surveillance (WHO, 2017). One of the aims of this research was to determine the efficiency of the WWTPs in removing both enterococci and vancomycin-resistant enterococci (VRE) from wastewater. Two WWTPs in the Durban metropolis were chosen as test sites based on their size and plant configuration. The biological treatment in plant I was based on trickling filters while plant II uses the Activated Sludge System. Four objectives were set up to achieve the aims of the work and the summary of the findings are as follows:

7.1.1 Objective 1, 2 and 3

Both vancomycin resistant and vancomycin susceptible enterococci were enumerated and isolated from samples collected from the two WWTPs and their receiving water bodies. The mean count of total Enterococci (TE) and vancomycin-resistant enterococci (VRE) counts in wastewater influents were: Plant I = 6.4±0.2 and 3.6±1.9 $\log_{10}$ CFU/100 mL respectively; Plant II = 6.5±0.3 and 4.8±0.2 $\log_{10}$ CFU/100 mL. Neither TE nor VRE was detected in final effluent of Plant I but the counts in the final effluents for Plant II ranged from 0-4.4 $\log_{10}$ CFU/100 mL (mean = 2.3±1.6) for TE and 0-3.4 $\log_{10}$ CFU/100 mL (mean = 0.4±0.8) for VRE. Chlorination of the effluents heavily influenced in the reduction of enterococci. The influent VRE count of Plant II was significantly higher than that of Plant I ($p \leq 0.05$) potentially due to human connections and other sources of pollution, like storm water impact. For the receiving water bodies, TE and VRE concentrations were higher upstreams the effluent point than the downstream count. The two sets of *Enterococcus* primers used to confirm the isolates showed little differences in the specificity and sensitivity when compared with the MALDI-TOF confirmation result of the isolates. The primer A had five false positives and eight false negatives resulting to specificity (98%) and sensitivity (97%) while primer B had five false positives only with specificity of 98% and sensitivity of 100%. Based on their counts, there were significant reduction of TE and VRE over the three treatment steps of the two WWTPs and thus showed an effective treatment processes. The effluent counts of the two plants were low such that they had little or no effect on the enterococci concentration of the receiving water bodies.

7.1.2 Objective 4

The isolates both VRE and VSE were generally resistant to multiple antibiotics with 100% resistant to cefixime. There was remarkable resistance to other antibiotics including vancomycin and teichoplanin but showed sensitivity to imipenem and amoxicillin/clavulanic acid. All except one VRE isolates (202) had either of the *vanA, vanB, vanC1* or *vanC2/3* gene with *vanA* most abundant. Tetracycline resistance *(tet)* genes were also detected in more than 62% of the isolates. Multidrug resistance among the isolates were also evident by the detection of multidrug efflux pump gene *(emeA)* in 13.4% of VRE isolates and 11.9% of the VSE. Eight isolates (6 *E. faecium* and 2 *E. faecalis*) were resistant to all the antibiotics tested and each possessed a *van* gene and 2 different *tet* genes.

Seventy-four percent of the isolates possessed at least one of the virulence genes, *asa1, hyl, cylA, gelE, esp, efaA* and *ace*. Among the eight isolates that were resistant to the entire antibiotics tested superbug), the six *E. faecium* had a virulence gene, (*hyl*) while the two *E. faecalis* had at least four virulence determinants.

7.2 Conclusions

Raw wastewater contains high concentration of enterococci harbouring a variety of resistant and virulence genes. The WWTPs are efficient in reducing the viable counts through the various treatment processes such that the effluents contain negligible viable enterococci counts. Thus, the effluents of these WWTPs do not contribute to the pollution of their receiving water bodies because the upstream and downstream counts were higher than that of the effluents. So the rivers were also polluted from other sources, which could be runoffs, wildlife, wash-off from animal farms and from surrounding agricultural farm lands.

Considering the fact that these isolates were multidrug resistant organisms and possessed a number of antibiotic resistance and virulence genes, there is a possibility of transferring these genes to other bacteria in the receiving water bodies. In addition, the concentration of enterococci in the river is more than the 200 CFU/100 mL, which is above the recreational water guideline value and is thus not safe for recreational purposes. However, the water may be used for irrigation purposes if routine monitoring is performed.

7.3 Recommendations

Apart from external monitoring of the operational efficiency of the WWTPs that provide effluent for reuse, I recommend regular assessment of surface water quality to limit the spread of VRE in the environment. Since the surface water has been shown to be also contaminated by sources other than by wastewater effluents, further research on the possible sources leading to high concentration of enterococci in these water bodies would be of great value. Molecular methods should be used in parallel with the cultural methods for determining the quality of the final effluents and river samples. This will also account for the viable but non-culturable bacteria and so ensure proper risk assessment.

7.4 Limitations of this study

The study had the following limitations:

1. Only three points of the WWTPs were sampled. Samples collected at the maturation pond and before the chlorination point would have given more detailed information on the efficiency of the treatment process and the subsequent disinfection effect of chlorination.

2. Samples of the three treatment steps (influent, biofilter/clarifier, and final effluent) were collected almost at the same time. Therefore, the samples from the subsequent steps do not actually represent the reduction of the influent sample collected but rather, they only give an estimated reduction value. However, with sampling over a period, as done, this estimated value would show an actual reduction value.

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APPENDICES

Appendix 1: Further details relating to chapter four

The Table 0.1 contains the raw counts obtained in each of the wastewater treatment point sampled, the log reduction and removal efficiency as were deduced for the counts. The Figure 0.1 illustrates the proportion of VSE and VRE in the influents. The VSE and VRE constituted the total enterococci (TE) counts.

Table 0:1: The raw TE and VRE counts of the three WWTP steps, log reduction and removal efficiency

Month	**Plant**	**Bacteria**	**Influent (CFU/100 mL)**	**Biofilter (CFU/100 mL)**	**Log_{10} red 1**	**RE 1 (%)**	**Effluent (CFU/100 mL)**	**Log_{10} red 2 In - Ef**	**RE 2 (Inf-Eff) (%)**
Jul	Plant I	TE	8.0×10^5	-	-	-	0	5.90	100
		VRE	0	-	-	-	0	0	
	Plant II	TE	3.23×10^6	9.0×10^3	2.55	99.7	7.0×10^3	2.66	99.8
		VRE	5.01×10^4	1.0×10^3	1.67	98.0	3.0×10^3	1.22	94.0
Aug	Plant I	TE	1.6×10^6	1.62×10^5	0.99	89.9	0	6.20	100
		VRE	2.0×10^4	1.0×10^3	1.30	95	0	4.30	100
	Plant II	TE	4.01×10^6	1.05×10^5	1.58	97.4	110	4.56	99.9
		VRE	7.0×10^4	1.0×10^3	1.85	98.6	8	3.94	99.9
Oct	Plant I	TE	2.03×10^6	5.7×10^4	1.55	97.2	0	6.31	100
		VRE	2.0×10^4	0	4.30	100	0	4.30	100
		TE	2.76×10^6	2.4×10^4	2.06	99.1	0	6.4	100

	Plant II	VRE	7.0×10^4	1.0×10^3	1.85	98.6	0	4.85	100
Nov	Plant I	TE	3.1×10^6	1.2×10^4	2.41	99.6	0	6.49	100
		VRE	4.0×10^4	0	4.60	100	0	4.60	100
	Plant II	TE	3.0×10^6	4.95×10^4	1.79	98.4	241	4.10	99.9
		VRE	7.0×10^4	0	4.85	100	13	3.73	99.9
Jan	Plant I	TE	2.95×10^6	1×10^4	2.47	99.7	0	6.47	100
		VRE	6×10^4	0	4.78	100	0	4.78	100
	Plant II	TE	2.7×10^6	5.8×10^4	1.67	97.9	2.76×10^4	2.00	99.0
		VRE	1.6×10^5	4×10^3	1.60	97.5	0	5.20	100
Feb	Plant I	TE	3.91×10^6	2.2×10^4	2.25	99.4	0	6.59	100
		VRE	0	0			0		
	Plant II	TE	1.46×10^7	1.57×10^5	1.97	98.2	2.9×10^4	2.70	99.8
		VRE	6.25×10^6	9.0×10^3	2.84	99.9	1.06×10^2	4.77	99.9
March	Plant I	TE	2.01×10^6	2.47×10^4	1.91	98.8	0	6.30	100
		VRE	7.67×10^4	0	4.88	100	0	4.88	100
	Plant II	TE	2.16×10^6	9.70×10^4	1.35	95.5	3.67×10^1	4.77	99.9
		VRE	7.34×10^4	6.33×10^3	1.06	91.4	0	4.87	100
April	Plant I	TE	2.10×10^6	2.22×10^4	1.98	98.9	0	6.32	100
		VRE	1.00×10^4	6.70×10^2	1.17	93.3	0	4.0	100
	Plant II	TE	4.04×10^6	4.20×10^4	1.98	99	8.75×10^3	2.66	99.8
		VRE	2.67×10^4	1.00×10^3	1.43	96.3	0	4.43	100
May	Plant I	TE	1.93×10^6	1.55×10^4	2.10	99.2	0	6.29	100
		VRE	2.67×10^4	0	4.43	100	0	4.43	100

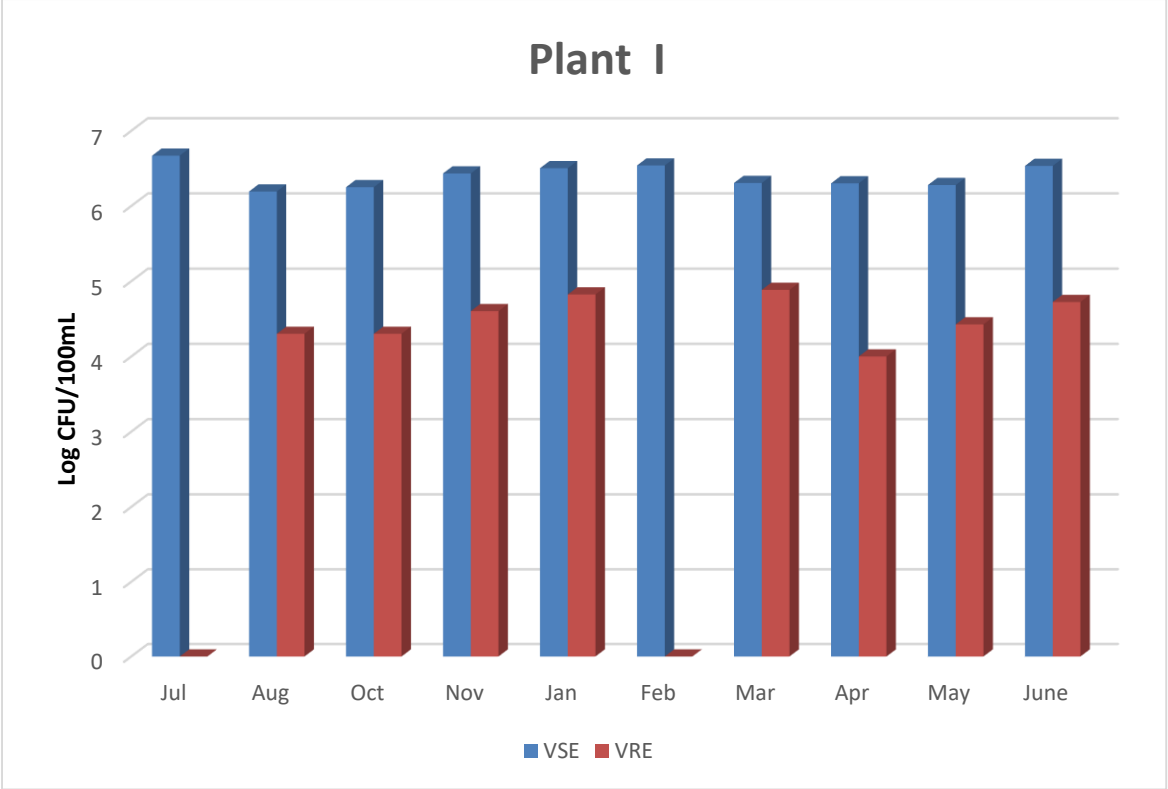
	Plant II	TE	3.32×10^6	1.98×10^5	1.22	94.0	3.0×10^1	5.04	99.9
		VRE	1.53×10^5	6.00×10^3	1.41	96.1	0	5.18	100
June	Plant I	TE	3.47×10^6	2.07×10^4	2.22	99.4	0	6.45	100
		VRE	5.30×10^4	0	4.72	100	0	4.72	100
	Plant II	TE	2.26×10^6	3.10×10^4	1.86	98.6	0	6.35	100
		VRE	4.30×10^4	0	4.63	100	0	4.63	100

Log_{10} red 1and RE 1= Log_{10} / Removal efficiency from Influent to Biofilter (Clarifier)

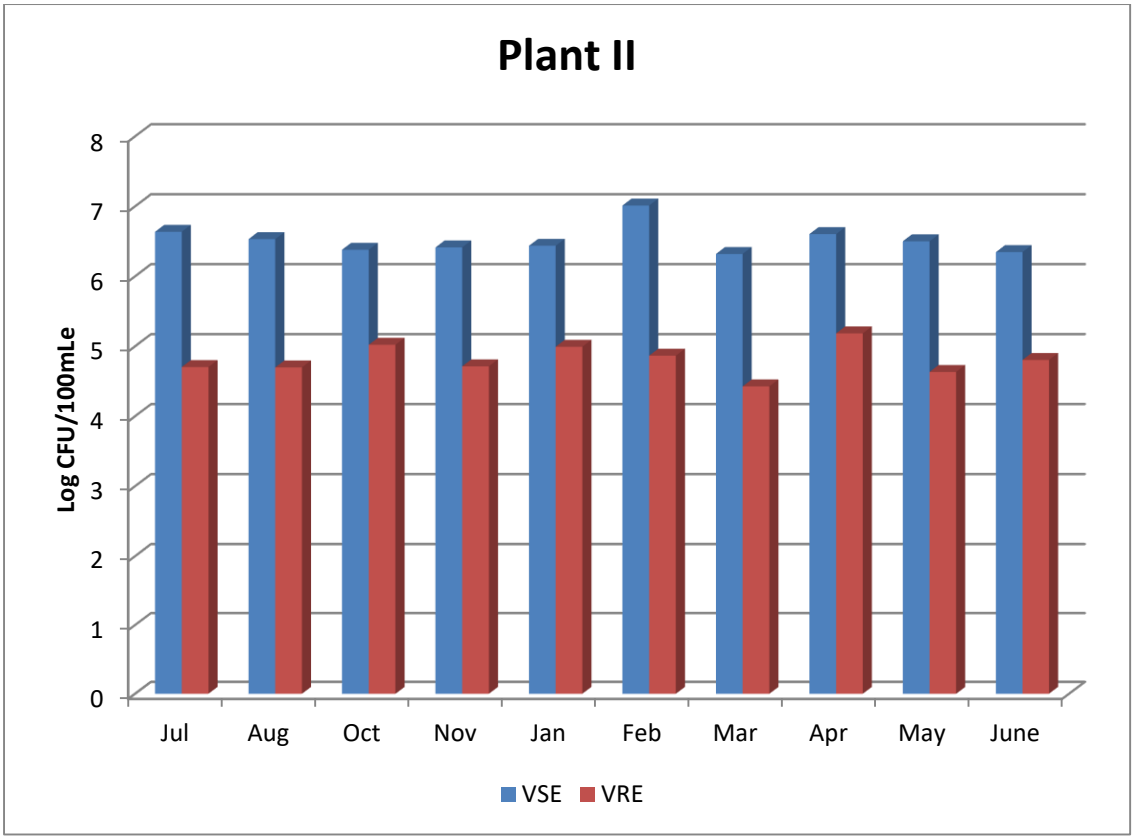
Log_{10} red 2 and RE 2 (overall removal efficiency) = Log_{10}reduction/ Removal efficiency from Influent to Effluent

The removal efficiency (RE) was calculated as shown below.

$$R\,E = \frac{the\ difference\ in\ counts\ between\ two\ treatment\ steps}{the\ count\ of\ the\ initial\ treatment\ step} \times 100$$


Plant I
Log CFU/100mL
0
1
2
3
4
5
6
7
Jul
Aug
Oct
Nov
Jan
Feb
Mar
Apr
May
June
VSE
VRE

a) Plant I



b) Plant II

Figure 0.1: Proportion of VSE and VRE in the influent samples