EVALUATION OF THE FINAL EFFLUENTS OF SOME WASTEWATER TREATMENT PLANTS IN AMATHOLE AND CHRIS HANI DISTRICT MUNICIPALITY OF THE EASTERN CAPE PROVINCE AS SOURCES OF VIBRIO PATHOGENS IN THE AQUATIC ENVIRONMENT

BY

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DECLARATION

I, the undersigned, declare that this dissertation submitted to the University of Fort Hare for the degree of Masters in Microbiology in the Faculty of Science and Agriculture, School of Biological and Environmental Sciences, and the work contained herein is my original work with exemption to the citations and that this work has not been submitted at any other University in partial or entirely for the award of any degree. I certify that this dissertation is devoid of any element of plagiarism and in the event that element(s) of plagiarism is/are detected in this dissertation I and I alone will be held responsible for the offence

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Certain areas in the world still depend on the receiving water bodies as sources of domestic water and for recreational purposes. The discharge of poor quality effluents from wastewater treatment plants can impact negatively on these water bodies, as they can act as vehicles for pathogens to the environment, posing a threat to humans if such water is used without precaution. Vibrio species are amongst those pathogens that can survive wastewater treatment processes, ending up in the environment, hence the aim of this study was to evaluate the final effluents of some wastewater treatment plants as sources of vibrio pathogens. Five wastewater treatment plants (WWTP) located in Amathole and Chris Hani District Municipalities in the Eastern Cape were used in this study. Samples were collected monthly from September 2012 – August 2013 and analysed using the standard membrane filtration technique. Yellow and green colonies on TCBS agar were enumerated as presumptive *Vibrio* species and expressed as CFU/100ml for each plant. Colonies were later picked based on their phenotypic characteristics, sub-cultured on fresh TCBS agar to ascertain purity. These presumptive isolates were then subjected to Gram staining and Oxidase test. Gram negative and Oxidase positive isolates were selected for further confirmation using Polymerised Chain Reaction (PCR). PCR was also employed for characterisation of *Vibrio* into three species viz *V. parahaemolyticus, V. fluvialis* and *V. vulnificus.* Antibiogram profile of the characterised species was then determined together with the presence of relevant antibiotic resistance genes Vibrio densities for the twelve month period ranged between 0 - 1.48×10^4 CFU/100ml with two of the plants located in East bank and Queenstown characterized by extremely high counts and one plant(Reeston) with very low counts.

Three hundred presumptive Vibrio isolates were screened for identity confirmation. Of these, the dominating species found was *V. fluvialis* (28.6%) followed by *V. vulnificus* (28%) and the least was found to be *V. parahaemolyticus* (11.6%). The remaining unidentified 31.6% were suspected to belong to other Vibrio species not covered within the scope of this study. All the confirmed isolates i.e., *V. parahaemolyticus, V. vulnificus* and *V. fluvialis* were susceptible to imipenem, gentamicin and meropenem and resistant to only tetracycline. Between 60-100% of the *V. parahaemolyticus* isolates, 7.1% to 100 % *V. vulnificus* isolates and 2.5 to 100 % *V. fluvialis* showed resistances to polymixin B, sulfamethazole, erythromycin, penicillin G, chloramphenicol, trimethroprim and trimethroprim & sulfamethazole. Antibiotic Resistance Genes that were assessed included *dfR*A, SXT, *flo*R and *Sul*2 varying in proportion with each species showing diversity in the Vibrio community. The *dfR* A gene was detected in all the *V. parahaemolyticus* isolates while *flo*R gene was not detected in any of the isolates belonging to the three species. The distribution of *sul2* gene cut across the species being 1% (1) in *V. fluvialis,* 3% (1) *in V. parahaemolyticus* and 4% (3) in *V. vulnificus*. The *SXT* gene was only determined in *V. parahaemolyticus.* It is clear that the final effluents of the selected plants are reservoirs for Vibrio pathogens as well as antibiotic resistance genes in the environment. The isolation of *Vibrio* from WWTP shows that this pathogen is in circulation in some pockets of the population**.** Therefore, wastewater treatment plants need to be properly monitored to ensure that they comply with set guidelines.

CHAPTER 1: INTRODUCTION

1.1 General introduction

Despite advances in water and wastewater treatments, waterborne diseases still pose a major threat to public health worldwide (Zhou and Smith, 2002). Water contaminated by effluents from various sources has been associated with heavy disease burden (Okoh *et al*., 2007) and in the USA, because of the Federal Clean Water Act, the requirements for wastewater treatment are set on a plant-by-plant basis determined by the National Pollutant Discharge Elimination System (NPDES) (EPA, 2008). If the final effluent does not meet the standards, drinking water may be difficult to treat with normal water treatment methods (Osode and Okoh, 2009), thus posing a health risk to several communities which rely on the receiving water bodies primarily as their source of domestic water (Igbinosa *et al*., 2009).

The genus Vibrio is a member of the family *Vibrionaeceae* which includes opportunistic pathogens of humans and animals (Daniels *et al*., 2000; Thompson *et al*., 2004). The species is amongst those enteric pathogens which are a threat to human health and have been mostly known for causing cholera. *Vibrio* includes more than 60 species, mostly marine in origin (Sawabe *et al*., 2013; Igbinosa and Okoh, 2010), and its taxonomy is continuously being updated due to the addition of new species. The role of Vibrios in the marine environment has been shown to include biodegradation, nutrient regeneration and biogeochemical cycling (Colwell, 1994; Ducklow, 1983). Vibrio species can be widely distributed in effluent environments associated with domestic sewage (Mezrioui and Oufdou, 1996). They are commonly associated with aquatic living species and include many important pathogens for aquatic animals and humans who consume contaminated seafood or polluted drinking water (Thompson *et al*., 2004). They are present in the environment either as free-living, or are

ssociated with different biofilms (Tamplin *et al*., 1990) which enables them to survive in the natural environment longer than free-living forms, by means of adhesive strategies, thus improving their adaptability to adverse conditions (Carman and Dobbs, 1997). Enormous numbers of vibrios are associated with zooplankton (Huq *et al*., 1983), thus suggesting that Vibrio species have a competitive advantage when chitinous zooplankton is present (Heidelberg *et al*., 2002). Previous research has focused on *Vibrio cholerea* because of the severity of the disease it causes (Kaper *et al*., 1982; Nair *et al*., 1994; Mishra *et al*., 2004); but over the past years, several studies have involved relatively minor Vibrio species of medical interest (Daniels *et al*., 2000), some of which are described as emerging pathogens able to cause mild to severe human diseases (Igbinosa and Okoh, 2008). *Vibrio* spp. are Gram-negative, facultative anaerobes that test positive for oxidase and are typically found in seawater. All members of the genus are motile and have polar flagella with sheaths (Colwell, 1989).

Several species of *Vibrio* including *V. cholerae, V. parahaemolyticus* and *V. vulnificus* are human pathogens (Hogan, 2010). *V. parahaemolyticus* and *V. cholerea* both produce diarrhea, but in ways that are entirely different. *V. parahaemolyticus* is an invasive organism affecting primarily the colon while *V. cholerae* is noninvasive, affecting the small intestine through secretion of an enterotoxin (Todar, 2005). *Vibrio cholerae* serogroups O1 (classical and El Tor biotypes) and O139 are associated with epidemic and pandemic cholera while other serogroups are known to cause non-epidemic cholera (Banwell et al., 1970; Holmgren, 1981). *V cholerae* O1 is the primary causative agent of cholera and its strains have been further differentiated into 3 serotypes designated Ogawa, lnaba and Hikojima, which have antigenic formulae of AB, AC and ABC types, respectively (Greenough, 1995). Infection with *V.cholerae* can cause profuse watery diarrhea, vomiting, and muscle cramps which results in substantial loss of fluid, electrolytes and stool volumes may approach 1 L/h. Severe illness has been associated with high-dose exposure, low gastric acidity, and blood group O (Blake, 1993).

V. vulnificus is common in warm seawater and thrives in water temperatures greater than 20°C (Bross *et al*., 2007) and is directly associated with pollution or faecal waste. The taste, appearance, and odour of seafood are not affected by *V. vulnificus* contamination, and proper cooking methods readily kill the organism (Bross *et al*., 2007). The species is known to cause wound infections, gastroenteritis, or a syndrome known as primary septicaemia (Morris, 1988). *V. vulnificus* causes disease in individuals who eat contaminated seafood (usually raw or undercooked oysters) or have an open wound that is exposed to seawater (Todar, 2009). Among healthy people, ingestion of *V. vulnificus* can cause vomiting, diarrhea, and abdominal pain. Most *V. vulnificus* infections are acute and have no long-term consequences.

Occupying a variety of niches, *Vibrio parahaemolyticus* is a common bacterium in marine and estuarine environments (McCarter, 1999). It can exist planktonically or attached to submerged, inert and animate surfaces, including suspended particulate matter, zooplankton, fish and shellfish (Kaneko and Colwell, 1975). This organism is recognized as a major worldwide cause of gastroenteritis, particularly in areas of the world where seafood consumption is high such as Southeast Asia (Joseph *et al*., 1982).

Vibrio fluvialis, a halophilic *Vibrio* species, has been associated with sporadic outbreaks of diarrhea worldwide (Huq *et al*., 1980; Hlady and Klontz, 1996; Lesmana *et al*., 2002), which is clinically very similar to cholera. *V. fluvialis* can also pose a significant economic threat for aquaculture for being pathogenic to cultured fish and lobsters (Tall *et al*., 2003). The infection of *V. fluvialis* is generally common in infants, children, and young adults (Bellet *et al*., 1989). Therefore, *V. fluvialis* is becoming a high human public health hazard all over the world, especially in coastal areas of developing countries and regions with poor sanitation.

Among halophilic Vibrios, *Vibrio alginolyticus* and *V. metschnikovii* are also pathogenic to humans, while *Vibrio anguillarum* represents a pathogen for fish and other marine animals (Farmer and Hickman-Brenner, 1992). Other Vibrio species, e.g. *V. cincinnatiensis, V. fluvialis, V. furnisii, V. harveyi*, and *V. mimicus*, have been sporadically found in human infections (Farmer and Hickman-Brenner, 1992; Abbott and Janda, 1994).

1.2 Justification for the study

According to the Department of Water Affairs and Forestry (DWAF, 2013) many of the poorly performing wastewater treatment plants are located in the country's poorer provinces, including the Eastern Cape, Free State, Northern Cape and Limpopo. Effluents released from these wastewater systems sometimes do contain pollutants of concern since even advanced treatment systems are unable to remove all pollutants and chemicals. Several environmental and health impacts resulting from insufficient wastewater treatment have been identified in the scientific literature (Bolong *et al*., 2009; Emmanuel *et al*., 2009), especially with regards to *V. cholerae* (Osode, 2007). Vibrio outbreaks have been common worldwide; in 2000, a report of 272 cases from Somalia resulted in 14 deaths. Samples tested positive for *Vibrio cholerae* 01 Ogawa and over 50% of the cases were children, where the case-fatality rate was the highest (WHO, 2012). In Tanzania, WHO reported 109 cases with 3 deaths between 18 May and 20 July 2001 in Temeke and Ilala districts of Dar es Salaam. As of 30 May 2009, 98424 suspected cases including 4276 deaths (Case Fatality Rate of 4.3%) have been reported in Zimbabwe since August 2008. Fifty-five out of 62 districts in all 10 provinces have been affected (WHO, 2012).

In South Africa a cholera epidemic started in KwaZulu-Natal Province and spread to other provinces in 2001 (Mugero and Hoque, 2001). In 2003, an outbreak in the Eastern Cape

affected O.R. Tambo, Chris Hani and Amathole District Municipality (Department of Health, 2003). Even recently February 2014, SABC News has reported suspected cases of *V. cholerae* in Fort Beaufort under the Amathole District Municipality. Although, Igbinosa *et al*. (2009) previously reported the occurrence of several *Vibrio* pathogens in final effluents of wastewater treatment plants in the Eastern Cape Province, this study was restricted to only three treatment plants located in one district municipality in the province which is grossly inadequate to make an informed statement on the extent of the problem in the province hence other provinces and municipality are still at risk for an outbreak.

1.2 Aim and objectives

This study was aimed at evaluating the incidence of Vibrio species from the final effluents of five wastewater treatment plants located in Amathole and Chris Hani District Municipalities in the Eastern Cape Province. To achieve this aim, the following specific objectives were set:

1.2.1. To assess the occurrence of Vibrio species in the final effluents of the selected wastewater treatment plants.

1.2.2. To carry out molecular confirmation of the isolated presumptive Vibrio species and assess the prevalence of *V. parahaemolyticus*, *V. fluvialis* and *V. vulnificus* amongst the Vibrio bacteria community.

1.2.3. To assess the antibiogram characteristics of the identified Vibrio species and determine the prevalence of antibiotic resistance genes in the isolates

CHAPTER 2: LITERATURE REVIEW

2.1 Wastewater systems in South Africa

Communities across the world have one thing in common – they produce wastewater (WHO, 1996). From a resources viewpoint, DWAF (1996) defines wastewater as the liquid or water carrying wastes removed from formal and informal residences, institutions, commercial and industrial establishments, together with such groundwater, surface water, storm water and potable water as may be present. In South Africa, the majority of wastewater treatment plants are either online or under construction. The dominant problems of wastewater services have thus shifted from those of design and construction to those of infrastructure operation, maintenance and management, particularly in the field of wastewater treatment (W2RAP, 2011). Hence, in South Africa, such programs as the Green Drop and Blue Drop certification have been introduced by the Department of Water Affair and Forestry.

Green Drop Certification is awarded to wastewater systems that obtain scores of 90% when compared against the criteria set for wastewater management. According to a report by Mail & guardian, (2011) this has assisted Water Services Authorities to strive for improvement in their management of wastewater as part of the incentive-based regulation approach. The Green Drop regulation programme seeks to identify and develop the core competencies required for the sector that if strengthened, will gradually and sustainably improve the level of wastewater management in South Africa (Green Drop, 2011).

Although other aspects of the wastewater treatment plants are assessed for the award of Green Drop status, the end product i.e., municipal wastewater effluents, are of major concern because of the many pollutants that they normally contain (DWAF, 1996). Municipal

wastewater effluents can contain grit, debris, and suspended solids, which can discolour the water, make it unfit for recreational, domestic, and industrial use and eventually smoother and contaminate plant and animal life on the bottom of the receiving water body (U.S EPA, 2004). Pathogens (e.g. bacteria and viruses), which can make the water unfit for drinking, swimming, and other recreational uses, can also contaminate aquatic life which is food in other parts of the world (Simpson and Charles, 2000). Constituents of wastewater effluents include; decaying organic wastes (which use up the water's dissolved oxygen and threaten the survival of fish and other aquatic life), nutrients (which over stimulate the growth of algae and other aquatic plants, giving rise to odours and other aesthetic problems, diminished biodiversity, and, in some cases, toxic contamination of shellfish), and about 200 different identified chemicals (many of which may be either acutely or chronically toxic to aquatic organisms) (FAO, 1996). Many of these chemicals may have long-term environmental effects, as they are not easily broken down and tend to accumulate in aquatic or terrestrial organisms through the food chain. Common bacteria, such as *Salmonella*, *E. coli* and *Vibrio cholera* are common wastewater pathogens which can lead to serious gastrointestinal illness and eventually death if not monitored (Tantillo *et al*., 2004). Since faecal coliform bacteria originate mostly in faeces, they have been used as an indicator of possible pathogens which could be found in the receiving water bodies. Standards have been set for faecal coliform to be ≤1000 CFU/100ml whereas the Department of Water Affairs entails pathogenic bacteria to be nil (DWAF, 1996). Same as Feacal coliforms, most waterborne disease-causing organisms originate in humans or animal bodies and are discharged as part of body wastes (FAO, 1992). Due to relatively small numbers of disease causing organisms, it can be difficult to isolate and identify specific disease causing bacteria (Baron, 1996) Moreover, there are many types of pathogens and each requires a unique microbiological isolation technique (Bartram and Pedley, 1996) hence the introduction of faecal coliforms as one of the techniques used to monitor the quality of water.

2.2 Prevalence of Vibrio species in wastewater final effluents

Vibrio spp. have been recognized as the leading cause of foodborne outbreaks in many countries including Japan (Hara- Kudo *et al*., 2003; Alam *et al*., 2003; Yang *et al*., 2008), India (Chakraborty *et al*., 2008; Gopal *et al*., 2005), China (Luan *et al*., 2008; Chen *et al*., 2010), Taiwan (Hara-Kudo *et al*., 2003), Korea (Lee *et al*., 2008) and Malaysia (Paydar et al., 2013). However, Vibrios are also widely distributed in effluent environments associated with domestic sewage (Igbinosa et al., 2009; Naidoo and Olaniran, 2013) and they still pose a major threat to public health worldwide (Zhou and Smith, 2002). Many wastewater treatment plants still discharge significant amounts of faecal coliforms and pathogenic micro-organisms containing effluents which impair the quality of water in the receiving water sheds (Bahlaoui *et al*., 1997; Simpson and Charles, 2000). The poor operational state and inadequate maintenance of most of these municipalities' sewage treatment works, i.e., design weaknesses, overloaded capacity, and faulty equipment and machinery has resulted in major pollution problems, with water quality standards of the receiving bodies consequently not meeting regulatory standards (Momba *et al*., 2006). Wastewater final effluents therefore serve as reservoirs for many pathogens (FAO, 1992). Enteric pathogens are increasingly finding strategic ways to survive wastewater treatment stages through the development of biofilms, making them less susceptible to disinfectants (Donlan, 2002). Several studies have specifically focused on reporting the presence of Vibrio species in treated effluents (Gugliandolo *et al*., 2005; Maugeri *et al*., 2004; Okoh and Igbinosa, 2008) and their resistance to antibiotics. The Widespread use and mis-use of antibiotics in highly medicated societies is leading to the proliferation of antibiotic resistant pathogens (super bugs), increasing the risk of morbidity to those who get infected while using the water either for drinking or recreational purposes (Salyers and McManus, 2001)

Currently there are twelve pathogenic Vibrio species implicated in human infections (Summer *et al*., 2001), eight of which are associated with foodborne infections of the gastrointestinal tract (Oliver and Japer, 1997). Several species have been reported to be pathogenic to marine vertebrates and invertebrates (Maugeri *et al*., 2000). *Vibrio* spp. that are pathogenic to humans include *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Hogan, 2010). According to a report by the Health Protection Agency (2007) and Farmer and Hickman (1992), Vibrio species reported to have previously caused human disease include *Vibrio alginolyticus, Vibrio furnissii, Vibrio carchariae, Vibrio hollisae, Vibrio cholerae, Vibrio metschnikovii, Vibrio cincinnatiensis, Vibrio mimicus, Vibrio damsel, Vibrio parahaemolyticus, Vibrio fluvialis,* and *Vibrio vulnificus*. The most common clinical presentation of *Vibrio* infection is self-limiting gastroenteritis, though wound infections and primary septicemia may also occur (Levine *et al*., 1993). Those most commonly isolated from patients are *V. parahaemolyticus, V. cholera* and *V. vulnificus,* while numerous case reports and reviews of these and other human pathogenic vibrios have been published (Rubin and Tilton, 1975, Schmidt *et al*., 1979, Shandera *et al*., 1983, Colwell, 1996, Shinoda *et al*., 2004). Pathogenicity is usually brought by production of toxins and virulence genes.

2.3 Some human pathogenic Vibrio species and their pathogenesis

Vibrio infections are becoming increasingly common in most of the countries, including the United States (Daniels et al., 2000), Taiwan (Ko *et al*., 1998), Germany (Frank *et al*., 2006) and South Africa (Igbinosa *et al*., 2009). Pathogenic members of this genus have been found out to cause three major syndromes of clinical illness namely wound infections, gastroenteritis and septicaemia (Daniels and Shafaie, 2000). Many cases of Vibrio associated gastroenteritis can be hard to identify in routine stool cultures as laboratories use TCBS agar when an outbreak has occurred already (Marano *et al*., 2000).

2.3.1. *V. parahaemolyticus*

This organism is recognized as a major cause of gastroenteritis particularly in regions where seafood consumption is high such, as Southeast Asia (Joseph *et al*., 1982). It is generally undetectable in marine water below 19 ºC but may grow in culture at temperatures as low as 5 ºC and on food at 10 ºC (Kaneko and Colwell, 1978). There is no guideline that describes the minimal level of *V. parahaemolyticus* in seawater, fish and shellfish that could potentially be hazardous to humans and not all strains of this species are considered to be truly pathogenic (Aberoumand, 2010). *V. parahaemolyticus* is halophilic and can be subtyped based on its somatic (O) and capsular (K) antigen patterns. The O3:K6 serovar is a predominant strain that is distributed globally (Osawa *et al*., 2002). Although the gastroenteritis caused by *V. parahaemolyticus* is self-limiting, the infection is capable of causing life-threatening septicaemia in people with underlying conditions, such as liver disease or immune disorders (Su and Liu, 2007).

2.3.1.1 Virulence Properties of *V. parahaemolyticus*

a) Kanagawa Phenomenon

Clinical strains of *V. parahaemolyticus* have been observed to produce haemolysis on special Blood Agar Medium (Park *et al*., 2004). In 1968, Wagatsuma developed a special medium for measuring the haemolytic character of *V. parahaemolyticus* called the Wagatsuma agar, a high-salt (7%) blood agar (defibrinated human or rabbit blood) medium containing d-mannitol as the carbohydrate source (Wagatsuma,1968) . The haemolysis observed on Wagatsuma agar medium, referred to as the Kanagawa phenomenon (KP), has diagnostic as well as pathogenic significance for *V. parahaemolyticus*. KP is known to occur due to the expression of thermostable direct haemolysin (TDH) that is more frequently detected in

clinical strains of *V. parahaemolyticus*. Only 1–2% of the environmental strains of *V. parahaemolyticus* express the haemolytic protein and therefore most non-clinical isolates of *V. parahaemolyticus are* KP-negative (Miyamoto *et al*., 1969). Studies have shown that ingestion of $2 \times 10^5 – 3 \times 10^7$ CFU of KP-positive *V. parahaemolyticus* can lead to the rapid development of gastrointestinal illness, whereas 1.6×10^{10} CFU of KP-negative *V. parahaemolyticus* ingested has exhibited no signs of diarrhea (Sanyal and Sen, 1974; Oliver and Kaper, 1997).

b) Thermostable Direct Haemolysin *(TDH)*

The pathogenicity of *V. parahaemolyticus* is well correlated, since a long time, to the presence of TDH that produces beta-type haemolysis on Wagatsuma agar (Sakazaki, 1968; Miyamoto *et al*., 1969). The haemolysin is a homodimer protein with a molecular mass of 46 kDa, each peptide being composed of 165 amino acids (Tsunasawa *et al*., 1987; Honda and Iida, 1993). The biologically active haemolysin is formed by non-covalent association of subunits that are not linked together by disulfide bonds (Tsunasawa *et al*., 1987). Haemolysis of human erythrocytes by TDH is a two-step process consisting of adsorption of the haemolysin to human erythrocytes and the step(s) following adsorption (Sakurai *et al*., 1975). The two amino acids, Trp65 and Leu66 are essential for the haemolytic property of TDH (Toda *et al*., 1991; Baba *et al*., 1991) that acts as a "pore-forming toxin" in temperature-dependent and independent steps (Honda *et al*., 1992). In addition to its haemolytic nature, TDH has been found to be cytotoxic to a variety of cell types (Takeda, 1983). A study investigating the enterotoxicity of TDH for human colonic epithelial cells showed that TDH increases Cl^- secretion through mechanisms involving cell binding and Ca^{2+} influx, followed by the elevation of Ca^{2+} concentration in association with protein kinase C phosphorylation (Takahashi *et al*., 2000). A dose-dependent increase in the intracellular free calcium has been reported in Caco-2 and IEC-6 cells (human and rat cell monolayers) (Raimondi *et al*., 2000).

TDH has also been shown to induce cytotoxicity on cultured rat embryonic fibroblast cells both from outside and inside the cells and could kill the cells through apoptosis (Naim *et al*., 2001). TDH is encoded by the *tdh* gene located in the chromosome, and all KP-positive *V. parahaemolyticus* strains contain two *tdh* gene copies, *tdh1* and *tdh2*, that are 97% homologous (Nishibuchi and Kaper, 1990). Construction of isogenic mutants defective in either *tdh1* or *tdh2* revealed that the KP and >90% of the total TDH protein production were attributable to expression of the *tdh2* gene (Nishibuchi and Kaper, 1990; Nishibuchi *et al*., 1991). This was possibly due to the increased transcriptional activation of *tdh2* gene copy by the activator protein Vp-ToxRS encoded by the *toxRS* gene (Lin *et al*., 1993; Nishibuchi and Kaper, 1995). Although the protein products of the two *tdh* loci are immunologically indistinguishable, the predicted amino acid sequences of the gene products (mature proteins) vary by seven amino acid residues (Nishibuchi and Kaper, 1990). Most of the *V. parahaemolyticus* strains that showed weak haemolysis on Wagatsuma agar have been shown to possess a single copy of the *tdh* gene in contrast to the gene duplication observed in the KP-positive strains (Nishibuchi and Kaper, 1990). On the other hand, only a few KP-negative strains were found to possess a single copy of the *tdh* gene (*tdh5*) and only one strain was found to carry an additional *tdh* copy on a 35 kb plasmid (*tdh4*) apart from the chromosomal copy (*tdh3*) (Nishibuchi and Kaper, 1995). Despite variations observed in the nucleotide sequences, the genes of the KP-negative strains encoded TDH proteins were found to be very similar to the ones encoded by the *tdh1*and *tdh2* genes of KP-positive strains, i.e., they had haemolytic and other biological activities and were immunologically indistinguishable (Baba *et al*., 1991; Honda *et al*., 1991; Yoh *et al*., 1991). These results suggested that low-level expression of the *tdh* genes in the *tdh*-bearing KP-negative strains could be the reason for the manifestation of such a phenotype.

c) TDH-related Haemolysin (TRH)

The role of TRH in *V. parahaemolyticus* pathogenesis was first identified during an outbreak of gastroenteritis in the Maldive Islands; KP-negative isolates of *V. parahaemolyticus* associated with the outbreak were found to produce TRH but not TDH (Honda *et al*., 1988). Biological, immunological, and physicochemical characteristics of TRH have been found to be similar but not identical to those of TDH (Honda *et al*., 1988). TRH is encoded by the *trh* gene, two copies (*trh1* and *trh2*) of which are found to be chromosomally located in the *V. parahaemolyticus* genome. The two *trh* loci share 84% sequence identity (Kishishita *et al*., 1992).

d) Other Putative Virulence Factors

Isolation of strains of *V. parahaemolyticus* that express neither TDH nor TRH has indicated the possibility of existence of other virulence factors (Belkin and Colwell, 2006). Studies on the invasive ability of *V. parahaemolyticus* indicated that active processes in cells, such as signal transduction by tyrosine protein kinase, may be involved in the internalization of this bacteria in Caco-2 cells and that actin filaments and cytoskeletal structure may have important roles in this process (Akeda *et al*., 1997). These results indicate that the disease caused by some isolates of *V. parahaemolyticus* could be attributable not only to toxin production but also to invasion into intestinal epithelium. A serine protease (protease A) has been purified directly from the supernatant of *V. parahaemolyticus* and identified as a potential virulence factor (Lee *et al*., 2002). The protease A was a monomeric protein having a molecular mass of 43 kDa and an isoelectric point of 5.0. The protease could be inhibited by the serine protease inhibitors, and was found to have significant effects on the growth of

Chinese hamster ovary, HeLa, Vero, and Caco-2 cells. The purified protease-induced tissue haemorrhage and caused death of experimental mice when injected intravenously and intraperitoneally (Lee *et al*., 2002).

2.3.2 *V. vulnificus*

Vibrio vulnificus is an emerging pathogen of humans (Todar, 2009). It was first recognized as an agent of disease in 1976 (Reichelt *et al*., 1976). The bacterium thrives in warm seawater and is part of a group of vibrios that are "moderate halophiles", meaning they require salt for growth (Todar, 2009). Unlike other members of this family, *V. vulnificus* infection is extremely invasive (Bisharat *et al*., 1999). This species is heterogeneous and has been subdivided into three biotypes and more than eight serovars. In the event of an infection, even with prompt diagnosis and aggressive therapy, case-fatality rates are usually around 30 to 40 percent (Centers for Disease Control and Prevention, 2004; Hsueh *et al*., 2004). According to a review by Bross *et al*. (2007), the organism is not associated with pollution or faecal waste, but infections are attributed to consuming raw seafood especially oysters and exposure of an open wound to seawater contaminated with the pathogen which is usually fatal owing to development of septicemia (Iwamoto *et al*., 2010; Oliver, 2005). Also, persons who are immunocompromised, especially those with chronic liver disease, or hepatitis B or C are a high risk (Hsueh *et al*., 2004; Todar, 2009). However, proper cooking methods readily kill the organism and eliminate food-related infections (Hlady *et al*., 1993; Mead *et al*., 1999).

2.3.2.1 Virulence properties

At least two distinct biotypes of this organism have been identified based on lipopolysaccharide composition (Biosca *et al*., 1996). Biotype 1 strains are most often found in association with shellfish and in the intestinal contents of fish and are a common cause of

human infection, either through ingestion of raw or undercooked shellfish or by wound exposure to the organism (Strom and Paranjpye, 2000). *V. vulnificus* biotype 2 commonly infects marine vertebrates, although infections in humans have been reported (Veenstra *et al*., 1992).The existence of a third biotype causing wound infections and bacteremia in people handling cultured tilapia in Israel has been proposed (Bisharat *et al*., 1999).

Among *V. vulnificus* biotype 1 strains, it has long been recognized that there is a wide range of virulence as measured in various animal models (Stelma *et al*., 1992). Most strains isolated from environmental reservoirs appear to be as virulent as clinical strains in animal models (DePaola *et al*., 2003; Starks *et al*., 2000). Virulent strains can be distinguished by opaque colony morphology (Simpson *et al*., 1987), which reflects expression of a protective capsular polysaccharide (CPS); however, both clinical and environmental strains are generally encapsulated (Wright *et al*., 1996)

Several biomarkers, e.g., the virulence-correlated gene (*vcg*), 16S rRNA, and the capsular polysaccharide operon (CPS) have been used to differentiate virulent- from non-virulent-type *V. vulnificus* strains (Han and Ge, 2010). A number of molecular-based detection methods, primarily PCR and real-time PCR targeting the *V. vulnificus* cytolysin/haemolysin gene (*vvhA*) have been described (Hill *et al.,* 1991; Coleman *et al.*, 1996; Panicker and Bej 2005; Wright *et al.,* 2007). Although widely used and highly specific to *V. vulnificus*, this species-specific gene is not capable of predicating the virulence potential of *V. vulnificus* strains.

The first biomarker is the virulence-correlated gene (*vcg*), which has been identified using randomly amplified polymorphic DNA (Warner and Oliver, 1999). A follow-up study showed that 90% of clinical strains had the *vcgC* sequence variant, whereas 93% of environmental isolates possessed the *vcgE* sequence variant (Rosche *et al*., 2005). Second, polymorphism in

17 nucleotides of the *V. vulnificus* 16S rRNA gene was used to differentiate clinical- from environmental-type strains using restriction fragment length polymorphism (Nilsson *et al*., 2003). By real-time PCR, the majority of clinical isolates have been determined to be 16S rRNA type B, while most environmental strains belonged to 16S rRNA type A (Vickery *et al*., 2007; Gordon *et al*., 2008). Thirdly, the capsular polysaccharide (CPS) operon has been examined in a study by Chatzidaki-Livanis *et al*. (2006), and significant associations were identified between clinical isolates and CPS allele 1, as well as between environmental isolates and CPS allele 2. In most studies using biomarkers to differentiate *V. vulnificus* strains, single PCR or real-time PCR assays have been used (Gordon *et al*., 2008). Recently, multiplex PCR has been used so as to target multiple biomarkers at one time. Han and Ge (2010) have performed multiplex PCR assays that targeted *vvhA* and a combination of several potential virulence biomarkers (*vcg*, 16S rRNA, and CPS) to simultaneously detect and characterize *V. vulnificus* strains, either virulent type or non-virulent type.

2.3.3 *V. fluvialis*

V. fluvialis has emerged as a potential enteropathogen and a notable outbreak of diarrhoeal disease involving *V. fluvialis* in Bangladesh was described by Huq *et al*. (1980). On numerous occasions, *V. fluvialis* has also been isolated from marine and estuarine environments (Seidler *et al*., 1980; Lee *et al*., 1981; Lockwood *et al*., 1982). However, the public health significance of this pathogen has not been studied in detail due to the lack of simple and reliable diagnostic tests. Although the bacteria is known to produce several potent toxins, their role in pathogenesis is not well established (Lockwood *et al*., 1982; Huq *et al*.,1985; Kothary *et al*., 2003; Chakraborty *et al*., 2005). Information regarding virulence genes and standard genetic markers for the identification of this organism has not been fully exploited to date.

2.3.3.1 Virulence properties

In a recent study by Liang *et al*. (2013), virulence phenotypes of *V. fluvialis* with regards to the ability to produce haemolysin, cytotoxin, protease and biofilm formation have been reported. In spite of many pathogenic factors being characterized, their precise role in producing the clinical manifestations remains to be unknown and little definitive information about the pathogenic mechanism of *V. fluvialis* has been achieved. Several toxins that may be important in pathogenesis have been reported in *V. fluvialis* include a Chinese hamster ovary (CHO) cell elongation factor, CHO cell-killing factor, enterotoxin-like substance, lipase, protease, cytotoxin, and hemolysin (Chikahira and Hamada, 1988; Liang *et al*., 2013) The cell-free culture filtrate of *V. fluvialis* strains has been demonstrated to evoke distinct cytotoxicand vacuolation effects on HeLa cells (Chakraborty *et al*., 2005) illustrating its toxicity.

2.4 Aquatic pathogenic Vibrio species

Members of the genus *Vibrio* are widespread in many natural aquatic environments, often forming a major component of microbial populations associated with recycling of organic compounds such as chitin (Baumann and Baumann, 1981). Because municipal wastewater can be discharged into the sea, seawater is thus highly susceptible to contamination (Kim and Bang, 2008). A few species are economically important pathogens of fish and shellfish (Colwell and Grimes, 1984). Interactions among *Vibrio* species and aquatic animals, both vertebrate and invertebrate, have been of interest to marine biologists for many years. Studies of interactions include crustaceans (Wang ,2011), shellfish (Colwell and Liston, 1961, Grimes *et al*., 1984; Tubiash *et al*., 1970, Hada *et al*., 1984), copepods (Huq *et al*., 1983; Kaneko and Colwell, 1978), sea urchins (Guerinot *et al*., 1982) and fin fish (Toranyo *et al*.,

1983). While mutualism has been hypothesized e.g., between *Vibrio* spp and shellfish (Colwell and Liston, 1962), pathogenicity involving other aquatic animals has been established.

2.4.1 *Vibrio penaeicida*

Vibriosis is a major disease problem in shrimp aqua-culture (Lightner, 1988; Brock and LeaMaster, 1992; Mohney *et al.*, 1994) especially Syndrome 93. According to a study by Goarant and Merien (2006), "Syndrome 93" has been affecting New Caledonian shrimp farming industry every cold season, causing significant losses and severe epizootic mortalities in grow-out ponds. Highly pathogenic strains of *Vibrio penaeicida* are considered the etiological agent of the disease in *Litopenaeus stylirostris*. Another study by Goarantl *et al.* (2000) assessed the toxic activities of extracellular products (ECPs) from *V. penaeicida*, *V. alginolyticus* and *V. nigripulchritudo* using *in vivo* injections in healthy juvenile *L. stylirostris* (*Penaeus stylirostns*) and *in vitro* assays on shrimp primary cell cultures and the fish cell line epithelioma papulosum cyprini (EPC).Toxic effects of ECPs were demonstrated for all pathogenic *Vibrio* strains tested both *invivo* and *invitro*.

2.4.2 *Vibrio harveyi*

V. harveyi is a ubiquitous, Gram-negative luminous organism which grows in warm marine waters (Lavilla-Pitogo *et al.,* 1992) and a part of the intestinal floras of marine animals (O'Brien and Sizemere, 1979). With the rapid developments in aquaculture, particularly in Asia and South America, the organism has become recognized as a serious cause of disease, particularly of marine invertebrates, and especially the economically important penaeid shrimp (Austin and Zhang, 2006). A similar organism was recovered from lemon sharks (*Negraprion brevirostris*) (Colwell and Grimes, 1984). Then as a result of phenotypic

and genotypic studies, including 16S rDNA sequencing (Gauger and Gomez-Chiarri, 2002), it was recognized that *V. harveyi* and *V. carchariae* were synonymous, with *V. harveyi* having precedence as the senior synonym (Farmer and Hickman-Brenner, 1992; Pedersen *et al*., 1998; Gauger and Gomez-Chiarri, 2002). Also, isolates identified as *V. carchariae* have been recovered from humans with wounds caused by shark bites (Pavia *et al*., 1989). Thus, *V. harveyi* is a pathogen of both invertebrates and vertebrates

2.4.3 *Vibrio alginolyticus*

Vibrio alginolyticus is considered to be a part of normal marine flora (Austin *et al*., 1995; Vandenberghe *et al*., 1998). Wound infections account for 71% of *Vibrio alginolyticus* infections (Hlady and Klontz, 1996) with Gastroenteritis thought to be a rare presentation. However, some studies have shown its virulence to aquatic animals (Lightner, 1993). According to Xie *et al*. (2005) the whole industry in Guangdong China, was badly hampered by the fish mortality because of vibrosis, where *V. alginolyticus* is the dominant causative species.

2.4.4 *Vibrio anguillarum*

Vibrio anguillarum is a marine pathogen that causes vibriosis in close to 50 species of fish, including cultured and wild fish, mollusks, and crustaceans, in marine, brackish, and fresh water (Actis *et al*., 2011). It is a polarly flagellated, non-sporeforming, halophilic and facultative anaerobe (Buller, 2004) that grows rapidly at temperatures between 25 and 30ºC on rich media containing 1.5–2% sodium chloride (NaCl). In total, 23 serotypes of *V. anguillarum* have been reported but the O1 and O2 are the major causes of fish vibriosis (Larsen *et al*., 2002; Toranzo and Barja, 1990; Naka *et al*., 2011). The O1 serotype strains have been

reported to be the most important in causing disease in salmonid fish (Larsen *et al*., 1994). Serotype O2 can be divided into O2 and O2β. These O2 strains have been isolated from both salmonid and marine fish, while O2β strains are usually isolated from cod and other nonsalmonids (Mikkelsen *et al*., 2007). In early stages of infection, *V. anguillarum* strains cause histopathological changes in blood, loose connective tissue, kidney, spleen, gills, and posterior gastrointestinal tract, and these bacteria are most abundant in the blood, although they appear uniformly dispersed throughout the affected tissues (Naka *et al*., 2011). The infection spreads so rapidly that most of the infected fish die without showing any clinical signs (Actis *et al*., 1999; Toranzo *et al*., 2005; Austin and Austin, 2007). Different selective media, such as thiosulphate citrate bile salts sucrose agar medium (TCBS) and *V. anguillarum* medium (VAM), have been developed for the detection of *V. anguillarum* in water samples. However, these media are not conclusive as other *Vibrio* spp. are also able to grow on TCBS and VAM (Bolinches *et al*., 1988; Alsina and Blanch, 1994). Furthermore, *V. anguillarum* can also be present in a viable but non-culturable state during the winter months, resulting in false-negative results (Eguchi *et al*., 2003; Frans *et al*., 2011).

2.5) Emergence of antibiotic resistance in Vibrio species

Antimicrobial resistance has become a major medical and public health problem as it has direct links with disease management (Faruque and Nair, 2008), which has led to an intensification of discussion about the prudent use of antimicrobial agents, especially in veterinary medicine, nutrition and agriculture (Hossain *et al*., 2012). The main source of *Vibrio* is seafood and there are many reports from all over the world on seafood associated vibriosis outbreaks (Hoi *et al*., 1998; Daniels and Shafaie, 2000; Nascimento *et al*., 2001; Rahimi *et al*., 2010) hence the use of antibiotics to prevent infections to consumable aquatic life such as shrimps. The

inappropriate use of antibiotics in aquaculture becomes one of the causes for the high incidence of antimicrobial resistant bacteria isolated from aquatic environments that represent a danger for aquatic organisms and human health (Reboucas *et al*., 2011) as seafood is popular to most parts of the world. Over time vibrios exposed to antibiotics inside or outside the aquatic farming environment can acquire antimicrobial resistance transferable by mobile genetic elements and horizontal gene transfer (Serrano, 2005). Thus, due to the presence of R-factors in the population, resistance developed through gene regulation of plasmids and chromosomes may be transferred vertically (by heredity) or horizontally (Madigan *et al.*, 2003). According to a report by Urbanczyk *et al*. (2008) bacterial luminescence is a distinctive, easily observable phenotype of members of *Vibrionaceae* and certain other bacteria and it can provide a readily tractable subject for evaluating the frequency of HGT events in nature.

2.5.1 Multidrug resistance in *Vibrio*

In recent times, higher frequency of multidrug-resistant *Vibrio* has been reported (Raissy *et al.*, 2012, Okoh and Igbinosa, 2010). Emergence of microbial resistance to multiple drugs is a serious clinical problem in the treatment and containment of the cholera-like diarrhoea, as reflected by the increase in the fatality rate from 1% to 5.3% after the emergence of drug-resistance strains in Guinea-Bissau during the cholera epidemic of 1996-1997 (Dalsgaard *et al*., 2000). The genetic element, termed SXT element, which has properties similar to those of the conjugative transposons, has been found to carry genes encoding resistance to sulfamethoxazole, trimethoprim and streptomycin in *V. cholerae* O139 and O1 strains isolated in India and was responsible for the multi-drug resistance (Waldor and Mekalanos, 1996) Currently, SXT belongs to a large and diverse class of mobile genetic elements known as integrative and conjugative elements (ICEs) (Burrus and Waldor, 2003). The genetic elements in this family are excised from the chromosomes of their hosts, transferred to a new

host through conjugation, and integrated into the chromosome again. SXT integrates site is specifically into the 5' end of *prfC*, the gene encoding peptide chain release factor 3 (RF3), which is involved in translation regulation (Hochhut and Waldor, 1999). It forms a circular extrachromosomal intermediate through specific recombination of the left and right ends of the integrated element. Chromosomal integration of the element occurs via site-specific recombination of a 17-bp sequence found in the circular form of SXT and recombination of a similar 17-bp sequence in *prfC* (Hochhut and Waldor, 1999). Formation of this circular extrachromosomal form of SXT requires the SXT-encoded site-specific recombinase (Int), which is closely related to the integrases found in lambdoid bacteriophages (Hochhut and Waldor, 1999). Presence and transfer of SXT element and resistance gene in class 1 integrons has been studied in South Africa reporting for the first time the presence of SXT element in *V. cholerae* O1 clinical isolates in Africa (Dalsgaard *et al*., 2001).

2.6 Environmental conditions influencing the survival of pathogenic Vibrio species

2.6.1 Climate

The quality and quantity of drinking water, irrigation water, environmental or recreational waters and wastewater final effluents can be associated with changes in environmental conditions including weather- or climate-related variables (Lipp *et al*., 2002). Floods may cause the overflow of wastewater treatment plants, failure of septic systems, or combined sewer overflows, which could contaminate nearby surface waters or wells. Furthermore, there is increasing concern about pathogens in storm water runoff (O'Shea and Field, 1992). Severe weather events appear to be correlated with enteric diseases related to excessive demand placed on sewage treatment plants from heavy rains and flooding (Tavana *et al*., 2008). In addition to

enteric pathogens, climate also influences the abundance and ecology of non- enteric and other pathogens which are naturally present in the environment (Lipp *et al*., 2002). The best examples of this include the pathogenic *Vibrio* spp. which are autochthonous in estuarine ecosystems. Therefore, with a changing climate, the geographic range of these pathogens may also change, potentially resulting in increased exposure and risk of infection for humans (Sterk *et al*., 2013). Furthermore, changes in plankton populations, and other hosts for which vibrios are commensals or symbionts, would similarly alter the ecology of these pathogens that are autochthonous to the aquatic environment (Lipp *et al*., 2002).

2.6.2 Salinity and temperature

Over the past years, studies have been done to demonstrate the effect of salinity and temperature to different *Vibrio* species such as *V. vulnificus* and *V. cholerea* (Randa *et al.*, 2004). Kelly (1982) demonstrated seasonal variation in the occurrence of *V. vulnificus* suggesting that growth of the organism is favoured by relatively high temperatures and low salinity. Pathogenic vibrios are found more frequently in environments whose water temperature exceed 10°C (Rhodes *et al*., 1986; Chan *et al*., 1989) and in some regions temperature may be higher (De Paola et al., 1983). However, pathogenic vibrios are less frequently isolated from natural aquatic environments when water temperatures exceed 30°C (Tantillo *et al*., 2004). Yet another study has disagreed with the above facts, when it came to *V. splendidus*, temperature did not affect the survival of this species, showing the efficiency of the species to maintain itself as culturable at low temperatures (Armada *et al*., 2003). Pathogenic *Vibrio* species have halophilic characteristics and occur most frequently in water ranging in salinity from 5% to 30%, significantly limiting their presence to estuarine and inshore coastal areas (Tison and Kelly, 1986; Kelly and Dan Stroh, 1988).

2.6.3 Turbidity

Turbidity in water is often associated with the possibility of microbiological contamination, as high turbidity makes it difficult to disinfect water properly (Obi *et al*., 2007)) and it also increases the possibility for waterborne diseases, since particulate matter may harbour microorganisms and may stimulate the growth of bacteria (Hoko, 2005; Osode and Okoh, 2009), thereby posing some health risk to the effluent users. This could happen also with *Vibrio* species, where they could get shielded from the disinfection process allowing them to end up in the environment.

2.7 Vibrio pathogen control strategy

Disinfection of treated sewage before discharge can substantially reduce the pathogen levels in the receiving waters, thus minimizing the health risks to humans and marine mammals from sewage-borne infection (DWAF, 2009). Chlorine has been the popular disinfectant of choice worldwide and according to WHO (2004), the principal factors that influence disinfection efficiency are disinfectant concentration, contact time, temperature and pH. Other factors that influence microbial sensitivity to disinfection include attachment to surfaces, encapsulation, aggregation and low-nutrient growth (WHO, 2003). Increased resistance to disinfection may result from attachment or association of microorganisms to various particulate surfaces, including: macroinvertebrates (*Crustacea*, *Nematoda*, *Platyhelminthes* and *Insecta*) (Levy *et al*., 1984); particles that cause turbidity (LeChevallier *et al*., 1987; Ridgway and Olson, 1982); algae (Silverman *et al*., 1983) and carbon fines (LeChevallier *et al*., 1990; Camper et al., 1986). The challenge with chlorine is that it can be consumed before it can react with the bacterial components of the biofilm (Chen and Stewart, 1996). Some reactions are beneficial in that they result in unwanted organisms being killed; others merely

consume chlorine and remove it from solution without killing any unwanted organisms (Kobylinski *et al*., 2006). Studies have shown rugose forms of *V. cholerae* within rugose cultures to remain viable in the presence of chlorine (Morris *et al*., 1996). Therefore other methods of disinfection need to be explored or a proper review of the chlorine disinfection since *Vibrio* escape this process.

CHAPTER 3: MATERIALS AND METHODS

3.1 Description of study site

The Eastern Cape Province is one of the poorest and second largest provinces in South Africa and mainly comprised of rural settlements with little or no adequate sanitary facilities (The local government handbook, 2012). It is divided into six district municipalities, namely, Alfred Nzo, Amathole, Chris Hani, Ukhahlamba, O.R. Tambo and Cacadu and the Nelson Mandela Metropolitan Municipality. Two municipalities namely; Amathole D.M (Mdantsane, Reeston and Eastbank) and Chris Hani D.M (Whittleasea and Queenstown) were selected for this study under which a total of five wastewater treatment plants were assessed.

3.1.1 Amathole District Municipality

Amathole District Municipality is situated in the central part of the Eastern Cape stretching along the Sunshine Coast from the Fish River Mouth, along the Eastern Seaboard to just south of Hole in the Wall along the Wild Coast. It is comprised of eight local municipalities: Mbhashe, Mnquma, Great Kei, Amahlathi, Buffalo City, Ngqushwa, Nkonkobe and Nxuba. According to the DWAF (2012), Buffalo City being the largest, is also the best performing district when it comes to Green Drop assessment criteria with 100% (15 out of 15) plants being in low and medium risk positions.

3.1.2 Chris Hani District Municipality

According to the DWAF (2012), Chris Hani represents one of the highest risk municipalities in the Eastern Cape with 93% (15 of 16 plants) in critical and high risk positions

It comprises eight local municipalities: Inxuba Yethemba, Tsolwana, Inkwanca, Lukanji, Intsika Yethu, Emalahleni, Engcobo and Sakhisizwe.

3.1.3 Demographic information

Table 3.1 represents the demographic information of both municipalities with regards to the population in each municipality. Percentages of dwellings with flushing toilets connected to the sewerage and piped houses are shown. With that information, one could estimate the proportion of the population which still rely on the receiving water bodies for water supply. This on its own is reason why the two District Municipalities have been chosen as case studies.

Table 3.1: Demographic Information of Amathole and Chris Hani District Municipality

	Population	*Households*	*Formal Dwellings*	*Flush Toilet Connected To Sewerage*	*Piped Water Inside Dwelling*
AMATHOLE	892637	237776	52.60%	14.80%	12.10%
CHRIS HANI	795 461	210 852	61.90%	31.20%	23.40%

Source: Census 2011 Municipal Fact Sheet, published by Statistics South Africa

Table 3.2 describes each plant with respect to the technology being used, the design capacity and the river into which the effluent is discharged.

Table 3.2: Description of the five wastewater treatment plants selected for this study.

Site	Technology Used	Design Capacity(Ml/d)	River Into Which Effluent Is Discharged
Mdantsane	Biofilters, anaerobic digestion and sludge drying beds	24	Buffalo River
Reeston	Activated sludge and sludge lagoons	2.5	Buffalo River
Eastbank	Activated Sludge	40	Marine
Whittlesea	Biofilters	4.99	Klipplaat river
Queenstown	NI*	NI*	NI*

NI* = denotes no information provided on this plant in the Green drop report 2012

Table 3.3 report the microbiological, chemical and physical compliance according to the Green drop report 2012. The risk rating of each plant is also stipulated including its Green drop status for the 2012 giving background information on the performance of each plant.

Table 3.3: Reports the Green drop status with regards to Microbiological, Chemical and Physical compliance with the Risk Rating of the selected treatment plants

Site	Microbiological compliance (%)	Chemical compliance (%)	Physical compliance (%)	Wastewater Risk Rating (%)	Green Drop status (2012)
MDANTSANE	0	84.5	79.3	37.0	LOW RISK
REESTON	68	92	84.5	35.3	LOW RISK
EASTBANK	74	88.5	84.5	51.9	MEDIUM RISK
WHITTLESEA	84	75	55.7	52.9	MEDUIM RISK
QUEENSTOWN	NI*	NI*	NI*	100	CRITICAL RISK

NI* = denotes no information provided in the Green drop report 2012

3.2 Sample collection

All samples were collected aseptically from the final effluents of each wastewater treatment plant between the months of September 2012 to August 2013, using sterile 1000 ml Nalgene bottles. The samples were transported on ice from the sampling site to the laboratory at the University of Fort Hare for analysis within 6 h of collection. Water samples from the final effluents were dechlorinated by adding 1.7 ml of 1% sodium thiosulfate. Samples were stored at 4 ^{o}C until analysis was complete.

3.3 Isolation of presumptive Vibrio species

Appropriate dilutions of effluent samples were prepared, with dilutions ranging from undiluted sample (10^{0}) to 10^{-3}. The Membrane filtration method was employed whereby 100 ml of appropriate dilution was filtered through a 0.45 μm filter membrane with the aid of a vacuum pump. The filter membrane was transferred onto thiosulphate citrate bile salts sucrose (TCBS) agar and incubated at 37 ^{o}C for 24 h - 48 h. At the end of the incubation period, typical yellow and green colonies were counted as presumptive Vibrio species and expressed as colony forming units per 100 ml (CFU/100 ml). Five to 10 isolated colonies per plate were then randomly picked and subsequently subcultured on sterile TCBS agar plates. Glycerol stocks (50%) where prepared and stored at -80 ^{o}C.

3.4 Presumptive identification of Vibrio species

The pure isolates were subjected to Gram staining and oxidase test. Only Gram-negative, oxidase positive isolates were selected for confirmation using polymerase chain reaction (PCR).

3.5 Molecular identification of Vibrio species

Polymerase chain reaction (PCR) was used to confirm the identities of the Vibrio species using the species-specific primers targeting a specific sequence within the 16S rRNA (Igbinosa *et al*., 2009

3.5.1 Isolation of genomic DNA

Genomic DNA was extracted following the method of Queipo-Ortuño *et al*. (2008). Single colonies of presumptive *Vibrio* grown overnight at 37 °C on nutrient agar plates were picked, suspended in 200 µl of sterile distilled water and the cells lysed using AccuBlock (Digital dry bath, Labnet) for 15 min at 100 °C. The cell debris was removed by centrifugation at 11 000 ×*g* for 2 min using a MiniSpin micro centrifuge. The cell lysates (5 µl) was used as template in the PCR assays immediately after extraction or following storage at -80 °C.

3.5.2 PCR Assay

The thermal cycling profile was as follows: a single round of enzyme activation for 15 min at 93 ºC followed by 35 cycles at 92 ºC for 40 s, 57 ºC for 1 min and 72 ºC for 1.5 min and final extension at 72 ºC for 7 min. Electrophoresis of the PCR products was done in 1.5% agarose gel containing 0.5 mg/l ethidium bromide for 1 h at 100 V and then visualized using a UV trans illuminator.

3.5.3. Species Differentiation

Polymerase chain reaction (PCR) also was used to differentiate the identities of the Vibrio species using the species-specific primers in Table 3.4. Isolation of DNA and PCR conditions are the same as the ones described in 3.5.1 and 3.5.2

Table 3.4: All primers used for confirmation and characterization of Vibrio species

Target species	Primers	Sequences (5 → 3')	Target gene	Amplicon size (bp)	Reference
All *Vibrio* spp.	V. 16S-700F V. 16s-1325R	CGG TGA AAT GCG TAG AGA T TTA CTA GCG ATT CCG AGT TC	16SrRNA	663	Kwok *et al*.,2002
V. parahaemolyticus	Vp.toxR R Vp.toxR F	GTC TTC TGA CGC AAT CGT TG ATA CGA GTG GTT GCT GTC ATG	toxR	368	Tarr *et al*., 2007
V. vulnificus	Vv. hsp-326F Vv. hsp-697R	GTC TTA AAG CGG TTG CTG C CGC TTC AAG TGC TGG TAG AAG	hsp60	410	Wong *et al*., 2002
V. fluvialis	Vf- toxR F Vf- toxR R	GAC CAG GGC TTT GAG GTG GAC AGG ATA CGG CAC TTG AGT AAG ACT C	toxR	217	Osori and Klose., 2000

3.5 Antibiogram of the identified Vibrio species

3.6.1 Antibiotic Susceptibility Test

Bacterial susceptibilities to the test antibiotics were performed by the disk diffusion method using guidelines recommended by Clinical and Laboratory Standards Institute (2002) using commercial antibiotic discs. A total of 13 antibiotic discs (Mast Diagnostics, Merseyside, United Kingdom) which include ampicillin (25 μg), cotrimoxazole (25 μg), amikacin (30 μg), imipenem (10 μg), erythromycin (15 μg), meropenem (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), cephalothin (30 μg), nalidixic acid (30 μg), tetracycline (30 μg), trimethoprim (30 μg), norfloxacin (10 μg), sulfamethoxazole (25 μg), gentamicin (10 μg), neomycin (30 μg), penicillin G (10 unit), nitrofurantoin (200 μg), polymyxin B (300 units) and cefuroxime (30 μg) were used. Results were read by measuring the zones of inhibition and then compared with the interpretative chart to determine the sensitivity of the isolates to the antibiotics.

3.7 Evaluation of Antibiotic Resistance Genes

3.7.1 Isolation of genomic DNA

Genomic DNA was extracted as outlined section 3.6.1 above.

3.7.2 PCR Assay

Polymerase chain reaction (PCR) was used to detect antibiotic resistance genes. Table 3.5 represents specific primer pairs and their sequences used for the detection of the SXT integrase, *floR, sul2, and dfrA1*. The possible antimicrobials which are responsible for the organism's resistance are also indicated.

Table 3.5: Primers used for detection of antibiotic resistance genes and the SXTelement.

Primer	Sequence (5 '→ 3')	Target Gene	Antibiotic Resistant	Reference
SXT-F	ATGGCGTTATCAGTTAGCTGGC	*SXT* INTEGRASE	sulfamethozole, trimethoprim, strptomycin,erythromycin,tetracycline	Bhanumathi *et al*., 2003
SXT-R	GCGAAGATCATGCATAGACC			
sul2-F	AGGGGGCAGATGTGATCGC	*sul2*	tetracycline,streptomycin, chloramphenicol, sulfamethozole	Falbo *et al*., 1994
sul2-R	TGTGCGGATGAAGTCAGCTCC			
floR-F	TTATCTCCCTGTCGTTCCAGCG	*floR*	tetracycline,streptomycin, chloramphenicol, sulfamethozole	Iwanaga *et al*., 2004
flor-R	CCTATGAGCACACGGGGAGC			
dfrA-F	CGAAGAATGGAGTTATCGGGTG	*dfrA*	trimethoprim	Iwanaga *et al*., 2004
dfrA-R	CTGGGGATTTCAGGAAG			

All reactions were set in 25 µl volumes. Cycling conditions (Bio-Rad My Cycler™ Thermal Cycler) were as follows; *Taq* polymerase activation at 94 °C for 2 min was followed by 35 cycles of 94 °C for 1 min, 60.5 °C for 1 min and 72 °C for 1 min with a final extension at 72 °C for 10 min and cooling to 4 °C. Amplicons were electrophoresed in 1% agarose gel containing 0.5 mg/l Ethidium Bromide (EtBr) 1 h at 100 V in 0.5 TAE buffer (40 mM Tris-HCl, 20 mM Na-acetate, 1 mM EDTA, pH 8.5) and visualized under an UV trans-illuminator.

3.8 Statistical analysis

To determine seasonal variation in *Vibrio* species, mean values and their significance were determined using SPSS (Statistical Package for the Social Sciences) **IBM SPSS Statistics**, from the monthly collected data in all the wastewater treatment plants.

CHAPTER 4: RESULTS

4.1 Occurrence of Vibrio spp. in the final effluent of selected wastewater treatment plants

The cell densities of *Vibrio* spp. varied over time in all the study sites. Nonetheless, high cell densities of 1.28 x 10^4 CFU/100 ml and 1.48 x 10^4 CFU/100 ml were obtained for the months of November 2012 and May 2013 at the East Bank and Queenstown WWTPs, respectively. Significantly high counts of *Vibrio* spp. were similarly observed in the months of April (7.7 × 10^3CFU/100 ml), July (4.5×10^3 CFU/100 ml) and August (1.29× 10^3 CFU/100 ml) respectively, in the 2013 sampling period from the East Bank WWTP. The Queenstown WWTP likewise show a comparable high *Vibrio* spp. count in November (2.26 × 10^3 CFU/100 ml) and December (1.48 × 10^3 CFU/100 ml) 2012 as well as in February (8.3 x 10^3 CFU/100 ml) and March (1.24 x 10^3 CFU/100 ml) 2013 sampling periods, respectively. Generally, the count of *Vibrio* spp. in final effluent samples ranged from 1.3× 10^1 to 9.2 ×10^2 CFU/100 ml (Mdantsane WWTP), 3.5×10^1 to 1.28×10^4 CFU/100 ml (East Bank WWTP) and 1 - 9 CFU/ 100 ml (Reeston WWTP) respectively in the Amathole District Municipality. While the WWTPs located in Chris Hani District municipality showed a range of 1 – 1.8× 10^2 CFU/ 100 ml and 7 - 1.4× 10^4 CFU/100 ml against Whittlesea and Queenstown WWTPs respectively (Table 4.1). Suffice to note that effluent sample was not collected from Whittlesea WWTP in September 2012 as the plant was under maintenance.

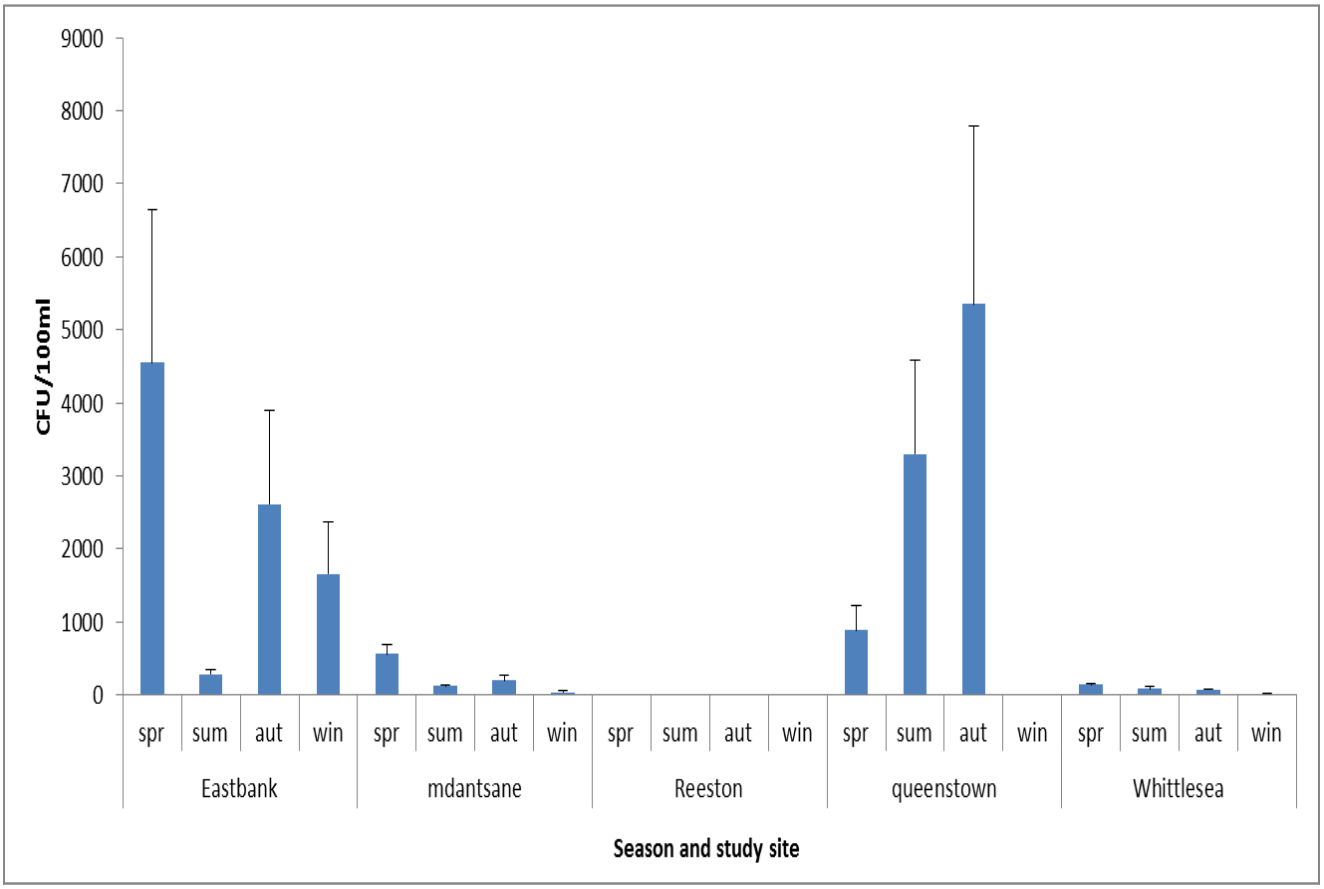
Table 4.1: Monthly distribution of Vibrio spp. in selected WWTPs from the Amathole and Chris District Municipalities

WWTP	*Vibrio* spp. (CFU/100ml)											
	SEPT '12	**OCT '12**	**NOV '12**	**DEC '12**	**JAN'13**	**FEB'13**	**MAR'13**	**APR'13**	**MAY'13**	**JUNE '13**	**JULY'13**	**AUG'13**
MDANTSANE	9.2×10^{2}	4.8×10^{1}	6.2×10^{2}	1.6×10^{2}	1.0×10^{2}	2.6×10^{1}	5.2×10^{2}	6.0×10^{1}	1.3×10^{1}	1.4×10^{1}	9.5×10^{1}	1.3×10^{1}
REESTON	0	0	1.3	2	1	0	0	8.7	9	0	5	0
EASTBANK	3.6×10^{1}	8.5×10^{2}	1.28×10^{4}	6.1×10^{2}	5.2×10^{2}	3.5×10^{1}	6.2×10^{1}	7.7×10^{3}	6.0×10^{1}	3.5×10^{2}	4.5×10^{3}	1.29×10^{3}
WHITTLESEA	N/S	1.14×10^{2}	1.61×10^{2}	1.8×10^{2}	4.0×10^{1}	3.9×10^{1}	7.2×10^{1}	1.6×10^{1}	2.1×10^{1}	0	1.6×10^{1}	7
QUEENSTOWN	6.8×10^{1}	3.4×10^{2}	2.26×10^{3}	1.48×10^{3}	9.6×10^{1}	8.3×10^{3}	1.24×10^{3}	5.8×10^{1}	1.48×10^{4}	2.5×10^{1}	1	0

NS = not sampled; CFU = colony forming units; WWTP = wastewater treatment plant

4.2 Seasonal variation of *Vibrio* spp. in the final effluent of selected wastewater treatment plants (WWTPs)

The variation in the distribution of *Vibrio* spp. in accordance with the sampling season is shown in Figure 4.1. The highest count was obtained in autumn and spring from Queenstown (5.4×10^3 CFU/100 ml) and East Bank (4.6×10^3 CFU/100 ml) respectively at an alpha value of 0.05. All in all, the distribution of *Vibrio* spp. in the five WWTPs which were significantly different ($P \leq 0.05$) across all seasons included the following; Queenstown: 3.3×10^3 CFU/100 ml (summer) and 8×10^2 CFU/100 ml (spring). Similarly, the *Vibrio* spp. counts from the Whittlesea WWTP were; 1.37×10^2 CFU/100ml (spring), 8.8×10^1 CFU/100 ml (summer) and 7.6×10^1 CFU/100 ml (autumn). East Bank likewise showed high counts of *Vibrio* spp., particularly in autumn (2.6×10^3 CFU/100ml) and winter (1.7×10^3 CFU/100ml) as would be seen in Figure 4.1. The *Vibrio* spp. counts obtained from Mdantsane were 5.65×10^2 CFU/100ml (spring), 1.97×10^2 (winter) and 1.26×10^2 CFU/100ml (summer) respectively. *Vibrio* spp. cell count showed large variation which was significantly different (P=0.05) across the seasons without a very distinct sequence. Principally, the Reeston WWTP showed the least *Vibrio* spp. count of all the experimental season.



spr=spring; sum= summer; aut= autumn; win= winter

Figure 4.1: Seasonal distribution of *Vibrio* spp. in selected wastewater treatment plants

4.3 Molecular confirmation of Vibrio species

Molecular confirmation of the presumptive Vibrio isolates resulted in the confirmation of 300 isolates as belonging to the Vibrio genus. The PCR products of the expected amplicon size (663bp) for some of the positive isolates are as shown in Figure 4.2.

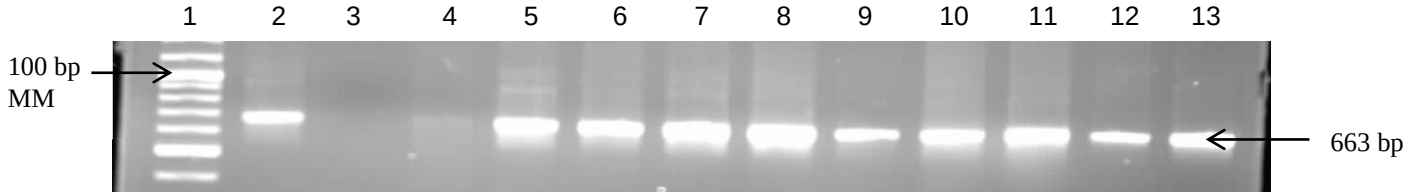


Figure 4. 2: PCR products of some of the confirmed *Vibrio* spp

Lane 1: Molecular Marker Thermo scientific (100 bp); Lane 2: Positive control (*V. fluvialis DSM* ; Lane 3: Negative control (-ve); Lane 4-13: Samples

4.4 Species differentiation

The 300 isolates confirmed to belong to the Vibrio genus were further delineated into different species. About 29% of the isolates were found to be *V. fluvialis*, while 28% were *V. vulnificus* and 11.6 % were *V. parahaemolyticus* (Table 4.2). The remaining isolates (31.8%) belonged to other species that were not assessed in this study. The same target gene for *V. parahaemolyticus* and *V. fluvialis* was used, but targeting different regions that are highly specific for each species. Gel picture of the PCR products of the species differentiation experiments are as shown in Figures 4.3-4.8.

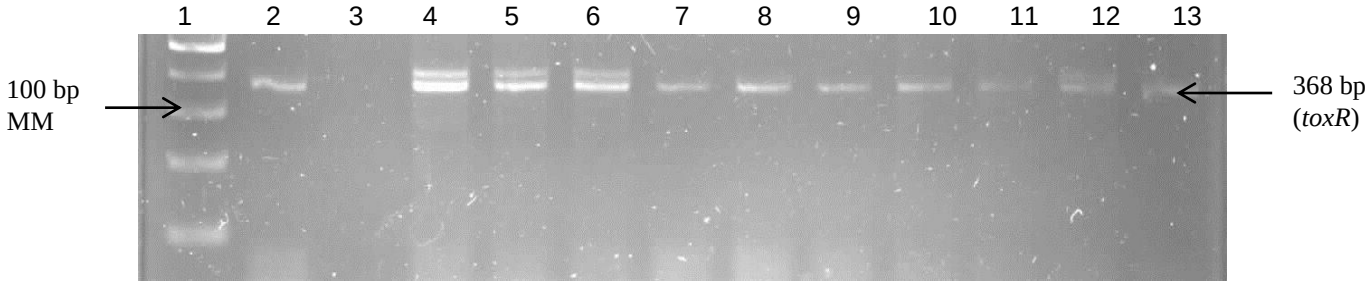


Figure 4.3: PCR products of confirmed *V.parahaemolyticus*

Lane 1: Molecular Marker Thermo scientific (100 bp); Lane 2: Positive control *V.parahaemolyticus* DSM 11058*)*; Lane 3: Negative control; Lane 4-13: Samples.

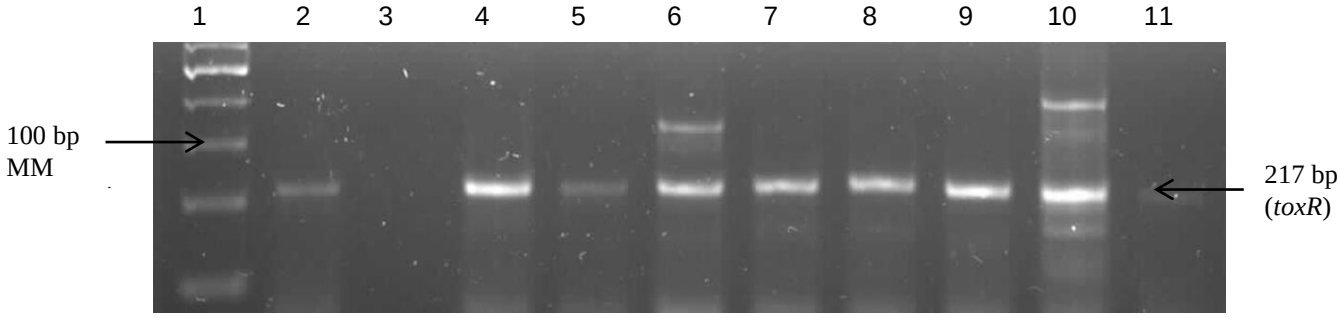


Figure 4.4: PCR products of some of the confirmed *V.fluvialis*

Lane 1: Molecular Marker Thermo scientific (100bp); Lane 2: Positive control (*V.fluvialis* DSM 19283); Lane 3: Negative control; Lane 4-13: Samples.

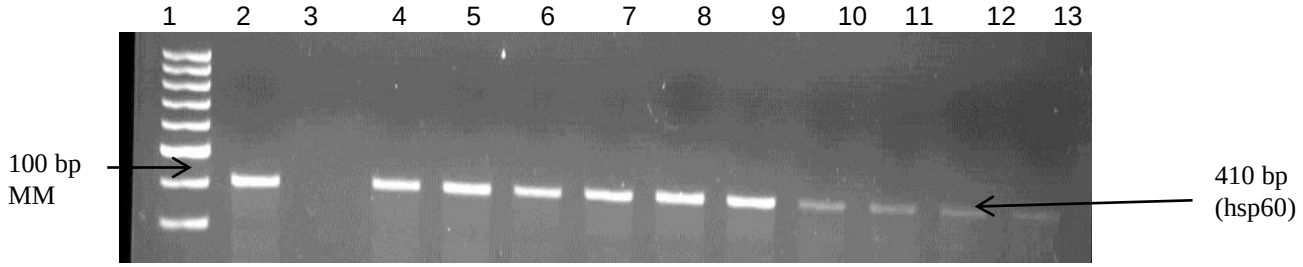


Figure 4.5: Gel electrophoresis of PCR products of some of the confirmed *V. vulnificus*

Lane 1: Molecular Marker Thermo scientific (100bp); Lane 2: Positive (+ve) control *V. vulnificus* (410bp); Lane 3: Negative control; Lane 4-13: Samples

Table 4.2: Prevalence of Vibrio species

Species	***V. parahaemolyticus***	***V. fluvialis***	***V. vulnificus***	**Other *Vibrio***
Prevalence	35(11.6%)	86(28.6%)	84(28%)	95(31.8%)

4.4 Antibiogram of the confirmed isolates

All the isolates belonging to the 3 species assessed i.e. *V. parahaemolyticus*, *V. vulnificus* and *V. fluvialis* were susceptible to imipenem, gentamicin and meropenem and resistant to tetracycline. Resistance varied against other antibiotics with 60-100% of the V. parahaemolyticus isolates showing phenotypic resistance to Polymixin B, Sulfamethazole, Erythromycin, Penicillin G, Chloramphenicol, Trimethroprim and Trimethroprim- Sulfamethazole. Between 7.1% to 100% of the *V. vulnificus*, and 2.5 to 100% of the *V. fluvialis* showed resistance to the same panel of antibiotics (Table 4.3).

Table 4.3: Antibiotic susceptibilities of the characterized species

Antibiotic		***V. parahaemolyticus*** **N=35**	***V. vulnificus*** **N=84**	***V. fluvialis*** **N=86**
Imipenem	R	0%(0)	0%(0)	0%(0)
	I	0	0	0
	S	100%(35)	100%(84)	100%(86)
Nalidic acid	R	20%(7)	14.1%(12)	10%(9)
	I	40%(14)	14%(12)	0
	S	40%(14)	71%(60)	90%(77)
Erythromycin	R	90%(32)	71%(60)	100%(86)
	I	0	14%(12)	0
	S	10%(3)	14.2%(12)	0
Sulfamethazole	R	100%(35)	85.7%(72)	87.5%(75)
	I	0	0	12.5%(11)
	S	0	14.2(12)	0
Cefuroxime	R	40%(14)	28.5%(24)	25%(21)
	I	0	7.1%(6)	0
	S	60%(21)	64%(54)	75%(65)
Penicillin G	R	90%(32)	100%(84)	87.5%(75)
	I	10%(3)	0	0
	S	0	0	12.5%(11)
Chloramphenicol	R	90%(32)	7.1%(6)	2.5 %(21)
	I	0	0	0
	S	10%(3)	92%(78)	75%(65)

Polymixin B	R	100%(35)	100%(84)	87.5%(75)
	I	0	0	0
	S	0	0	12.5%(11)
Trimethroprim &	R	60%(21)	10%(8)	0
Sulfamethazole	I	0	0	0
	S	40%(14)	90%(78)	100%(86)
Tetracycline	R	100%(35)	100%(84)	100%(86)
	I	0	0	0
	S	0	0	0
Gentamicin	R	0	0	0
	I	0	0	0
	S	100%(35)	100%(84)	100%(86)
Meropenem	R	0	0	0
	I	0	0	0
	S	100%(35)	100%(84)	100%(86)
Trimethoprim	R	90%(32)	90%(76)	100%(86)
	I	0	0	0
	S	10%(3)	10%(8)	0

4.5 Proportion of *Vibrio spp*. carrying antibiotic resistance genes

V. fluvialis, *V. parahaemolyticus* and *V. vulnificus* were screened for four antibiotic resistance genes (SXT, *dfR A, floR, sul2*). The *floR* gene was not detected in any of the species whereas SXT was only detected in 9% of the *V. paraheamolyticus* isolates. Identification of the *dfRA* gene cut across the three species ranging from 6% (5) in *V. fluvialis* to 100 %(35) in *V. parahaemolyticus*. Similarly, *Sul2* gene ranged from 1% in *V. fluvialis* to 4% in *V. vulnificus* as shown in Table 4.4. Gel pictures of the PCR products of the amplified genes are shown in the appendix section.

Table 4.4: Proportion of Vibrio species carrying resistance genes

Antibiotic Resistance Gene	***V. fluvialis*** **N=86**	***V. parahaemolyticus*** **N=35**	***V. vulnificus*** **N=84**
SXT	0	9 % (3)	0
dfR A	6% (5)	100%(35)	7 %(6)
FloR	0	0	0
Sul2	1% (1)	3% (1)	4% (3)

CHAPTER 5: DISCUSSION

Vibrio outbreaks have been reported worldwide in the past, and presently is still a major threat globally (Hendriksen *et al*., 2011; Potasman *et al*., 2002; Materu *et al*., 1997). According to WHO (2014), almost every developing country has faced a cholera outbreak or the threat of a cholera epidemic. However, this remains a challenge to countries where access to safe drinking water and adequate sanitation cannot be guaranteed. Other species of this genus apart from *V.cholerea* have also gained popularity as potential threats. In this study, Vibrio species were isolated through the use of TCBS agar. TCBS is one of the recommended media for the selective isolation of Vibrio species (Ottaviani *et al*., 2003). The selectivity of this medium may, however, vary (Farmer and Hickman-Brenner, 1991) and it is important that the identity of the putative *Vibrio* isolates be verified. Although it has been recommended, Joseph *et al*. (1982) has reported that other bacteria other than *Vibrio* such as *Enterobacteriaceae*, *Proteus*, *Aeromonas* and *Staphylococci* may also grow on TCBS but are distinguishable from Vibro species by their morphology.

Vibrio densities for the twelve- month study period ranged between 1-1.48× 10^4 CFU/100 ml (Table 4.1). Although Vibrio species naturally occur in marine environments, the isolation of *Vibrio* in these final effluents has shown that this pathogen can be widely distributed in effluents associated with domestic sewage (Igbinosa and Okoh 2009; Gugliandolo *et al*., 2005; Maugeri *et al*., 2000). Normally, they are associated with aquatic living species (Maugeri *et al*., 2004) but in wastewater effluents, survive as free-living forms or biofilms attaching to the surface of the tanks.

Of all the five WWTPs, two had extremely high Vibrio counts; Queenstown WWTP in Chris Hani D.M with 1.48×10^4 CFU/100ml in November 2012 which was categorized under the critical condition in the Greendrop report of 2012.East Bank WWTP in Amathole D.M

emerged with 1.28×10^4 CFU/100ml in the month of May 2013. The Queenstown (Chris Hani D.M) WWTP had challenges with the pipeline system and as a result upgrading of the plant was done between the months of September 2012-May 2013. As shown in Table 4.1, these months tend to have the highest counts of Vibrio species. That is why it becomes imperative to properly maintain and monitor wastewater treatment systems in order to identify and solve operational problems before they advance, thus reducing the amount of time it also takes to address these problems. However, after the refurbishment of Queenstown was completed, the plant had major improvements and experienced counts as low as 1 CFU/100 ml in July 2013 to 0 CFU/100 ml in August 2013. Reduction in the Vibrio densities could have also been influenced by the winter season which normally starts from June to August characterized by low temperatures. Similar studies by Igbinosa *et al*. (2009) showed that the abundance of Vibrio species in the final effluent has been linked to temperature, while its relationship to salinity is less clear.

Looking back at the history of the plant with respect to the Green drop performance, its 2011 scores were average (56.5%) although other wastewater treatment plants under the Chris Hani Municipality were in critical risk condition (DWAF, 2011). The same report also highlighted that, certain components such as the aeration section have problems due to dysfunctional motors and aerators at the time of the report (Green drop Report, 2011). In 2012, the risk rating of the plant went up to 100%, resulting in a critical risk rating of the plant which was due to the failure by the municipality to provide information on the assessment of the plant as a whole (Green drop report, 2012). Now, with the results from this study, improvement on the upcoming Green drop certification is expected, if the results obtained after the refurbishment are maintained and continue to improve. This also applies to the East Bank wastewater treatment plant which also had an upgrade of the chlorine dosing plant unit since it had been identified in the Green drop Report 2011 to have an ineffective disinfection due to the questionable contact time during disinfection.

On the contrary, Reeston WWTP had very low counts throughout the months; with majority of the counts being zero. This is an indication that the plant is at its best compared to the other plants that were assessed in this study.

During some months of the sampling period, the final effluent was observed to be bluish in colour with a strong odour of a chemical which we speculated to be chlorine. This could result to killing of aquatic animals and also a potential threat to the health of the people exposed to the water. Previous studies have discovered chlorine to react with organic matter and form a wide range of substances known as disinfection by-products (DBPs) in wastewater treatment plants (Watson *et al*., 2012). This reaction occurs naturally when carbon compounds such as decayed vegetation, fish, or aquatic organisms disintegrate (Xie, 2004). Some of these DBPs have been reported to cause cancer and others acute health problems such as liver damages (Bhardwaj, 2004). The discovery of DBPs and their adverse health effects highlights the necessity for better understanding of the disinfection process and striking a balance between prevention of waterborne diseases and the health effects the DBPs cause (Bhardwaj, 2004).

The remaining WWTPs i.e. Mdantsane and Whittlesea were also characterised by high *Vibrio* densities. The Mdantsane WWTP had no consistency as *Vibrio* densities kept fluctuating throughout the sampling period. The major challenge in this plant was that the chlorination point and the discharge point took place at the same area resulting in inadequate time for contact between the disinfectant and pathogens. No infrastructure problems were reported in Whittlesea WWTP but still *Vibrio* escaped the disinfection system.

Seasonal distribution of Vibrio species in each treatment plant varied (Figure 4.1). There was a similar trend in three of the WWTPs viz. Eastbank WWTP, Mdantsane WWTP and Whittlesea WWTP where the highest mean counts were obtained during spring and the lowest mean counts in winter. These findings were similar to that of Lin and Schwarz. (2003) where no *V. vulnificus* was detected during the winter months whereas isolation in the spring months was abundant. The same reasons such as refurbishment of the plants and insufficient contact time between disinfectant and pathogen which were explained for the occurrence of vibrios also could explain the high counts obtained in spring and other seasons. The low counts in winter could be explained by the effect of low temperatures on pathogens. A study by Igbinosa *et al*. (2009) reported high levels of *Vibrio.spp* during summer than during winter and favoured temperatures between 17 and 27°C, indicating a strong dependence of the culturable forms of the pathogens on temperature. Maugeri *et al*. (2004) also confirmed that the distribution of pathogenic *Vibrio spp.* in aquatic environments is greatly influenced by temperature. At the East bank WWTP there was a significant difference in *Vibrio* densities obtained in spring and summer ($p \leq 0.024$). Similarly Mdantsane WWTP also had a significantly higher *Vibrio* counts in spring as compared to other seasons ($p \leq 0.05$). *Vibrio* densities obtained at the Whittlesea WWTP were significantly different from one season to the other, for all the four seasons ($p \leq 0.05$). The Reeston and Queenstown WWTPs had a different trend compared to the three discussed above. Compared to each other, however, they had similar trends, with both plants recording their highest mean *Vibrio* densities in autumn. The lowest mean counts were obtained in winter for the Queenstown WWTP and in spring and summer for the Reeston WWTP. Statistically, when Vibrio densities were compared by season, significant differences were observed between spring and autumn ($p \leq 0.029$), and autumn and winter ($p \leq 0.010$) for the Queenstown WWTP whereas the Reeston WWTP had significant differences in *Vibrio* densities obtained in autumn compared to spring ($p \leq 0.000$); summer ($p \leq 0.000$) and winter ($p \leq 0.001$).

Bacterial identification of isolates as Vibrio species from all the study sites revealed the presence of potentially pathogenic strains for humans and animals. Confirmation of all Vibrio species was done by targeting the 16S rRNA gene. In the process, three target species were detected viz *V. parahaemolyticus*, *V. vulnificus* and *V. fluvialis.* It is known that among vibrios, these three species can adapt themselves to adverse conditions e.g organic matter limitation by means of survival strategies such as adhering to different substrata (Tamplin *et al*., 1990; Maugeri *et al*., 2004; Gugliandolo *et al*., 2005), hence survival of these pathogenic vibios in wastewater treatment plants is possible. The identities of *V. fluvialis and V. parahaemolyticus* were confirmed by use of species-specific primers targeting the *toxR* gene. The same gene was used to target the two species, but in reality they target different regions within the same gene. *toxR* is an ancestral gene of the family *Vibrionaceae* which encodes a transcriptional activation domain (TAD), a transmembrane domain (TMD) and a periplasmic domain (PD) (Osorio and Klose, 2000). Among Vibrio species, high level of homology within the TAD of the ToxR proteins and relatively conserved homology in the TMD and PD have been reported (Osorio and Klose, 2000). Interestingly, there is essentially no homology within the region between TAD and TMD. This region connects the TAD to the cytoplasmic membrane and it was therefore named the membrane tether region and is highly specifc for *V. fluvialis.* *toxR*-based species-specific identification has also been developed for *Vibrio parahaemolyticus* (Kim *et al*., 1999), *Vibrio hollisae* (Vuddhakul *et al.*, 2000) and for *V. fluvialis* (Chakraborty *et al*., 2006).

The most prevalent species detected was *V. fluvialis* followed by *V. vulnificus* and *V. parahaemolyticus* in that order (Table 4.2). The same pattern was reported by Igbinosa *et al*. (2009) where they assessed the occurrence of potentially pathogenic Vibrio in the final

effluents of a wastewater treatment facility in a rural community of the Eastern Cape Province of South Africa, specifically Alice town, and found that *V. fluvialis* was the most abundant. Conversely, Gugliandolo *et al*. (2005) found *V. vulnificus* as a major species in the marine environments in Italy. The dangers which come with the abundance of this pathogen (*V. fluvialis)* is the production of an enterotoxin known to cause a serious infection, as its clinical symptoms of gastroenteritis are very similar to those caused by *V. cholerae* O1 and non-O1 strains (Huq *et al*., 1980). According to a recent study by Liang *et al*. (2013), *Vibrio fluvialis* has been considered to be an emerging foodborne pathogen and has been becoming a high human public health hazard all over the world, especially in coastal areas of developing countries and regions with poor sanitation (Liang *et al*., 2013). Isolation of *V. fluvialis* in large numbers can pose a significant economic threat for aquaculture in areas where seafood consumption is high, making a cycle back to faecal waste and final effluents (Tall *et al*., 2003). This would apply to wastewater treatment plants like East Bank in Amathole D.M which discharges the contaminated final effluents directly to the sea and perhaps could be the reason why this plant had the highest density of Vibrio. Similarly, both *V. vulnificus* and *V. parahaemolyticus* are also foodborne pathogens which are associated with raw seafood causing 3 major syndromes of clinical illness, i.e., gastroenteritis, wound infections, and septicaemia (Daniels and Shafaie, 2000). *V. parahaemolyticus* has been often isolated from seafood, including shrimp, in markets in South East Asian countries (Elhadi *et al*., 2004) and previous studies at markets in China have shown *V. vulnificus* as dominant in cultured shrimps (Ji *et al*., 2011; Yano *et al*., 2004). The remaining unidentified species found meant that final effluents are reservoirs for more than the 3 species that were targeted in this study. The presence of *Vibrio* spp. as mixed pathogens in final effluents indicates that the mode of transmission in the food chain is via contaminated water or food (Chowdhury *et al*., 2012)

The antibiotic susceptibility profiles of *V. parahaemolyticus*; *V. fluvialis*; *V. vulnificus* against 13 different antibiotics is represented in (Table 4.3). All (100%) of the isolates were susceptible to imipenem, gentamicin and meropenem and showed resistance to only tetracycline. Tetracycline (TC) is one of the most popular antibiotics that is used in aquaculture for its effectiveness over a broad spectrum of pathogens and has a low cost (Neela *et al*., 2007). However, in this study it was not effective against any of the test organisms. It is also one of the drugs of choice for treating cholera, and Bhattachrya *et al*. (2003) has reported increased resistance among pathogens. Incidence of resistant genes usually would explain the concept of resistance, although spontaneous resistance is also known to occur (Walsh, 2003). There are two predominant mechanisms for TC resistance: efflux and ribosomal protection (Roberts, 2005), which move among the bacterial community. However, recent reports revealed that some determinants of TC resistance have been detected among a variety of species, and that there is some distribution within species (Kim *et al*., 2004), suggesting the presence of non-transferable *tet* gene(s).

Resistance varied against erythromycin, penicillin G, chloramphenicol and trimethroprim as well as the combination of trimethroprim and sulfamethazole in all the 3 species used in this study (Table 4.3). All the species seemed to have a similar pattern of multiple-drug resistance against the antimicrobials used. The antimicrobial resistance pattern is generally consistent with the previous studies from environmental isolates (Baker-Austin *et al*., 2008). In Korea antimicrobial resistance profile of *V. parahaemolyticus* (Oh *et al*., 2011) in farmed fish was similar to the results obtained in this study with the exception of the trend shown against tetracycline. Though antibiotics such as tetracycline, doxycycline, norfloxacin, ciprofloxacin and streptomycin have been used as an adjunct in rehydration therapy and are critical in the treatment of septicemia patients (Bhattacharya, 2001; Chiang *et al*., 2003),

resistance to many of these drugs in many pathogens including *Vibrio* pathogens such as *V. vulnificus*, *V. cholerae*, *V. fluvialis* and *V. parahaemolyticus* have been documented

Antibiotic resistance is mainly known to be caused by antibiotic resistance genes. SXT, *dfr*A, *flo*R and *sul*2 resistance genes were determined as represented in Table 4.4. The intensive use of antibiotics for human, veterinary and agricultural purposes, results in their continuous release into the environment (Brown *et al*., 2006; Díaz-Cruz *et al*., 2003; Kümmerer, 2009). In wastewater treatment plants, resistance could be promoted by a number of factors such as medical waste. Sewage from hospitals and pharmaceutical plants has been reported to be make- up wastewater and a possible factor for the development of these resistance genes (Guardabassi *et al*., 2002). Household products such as toothpastes, cement and paints which normally have antibacterial ingredients like triclosan, quartenary ammonium compounds, alcohol, and bleach can contribute to onto microbial resistance. Microbes resistant to each of these compounds have been documented in nature and in some human pathogens (Callahan, 2011). About 24 million pounds of antibiotics are fed to animals every year (WHO, 1996). Due to this practice, antibiotic resistance in foods has become a health concern. It is also possible that our normal gut microbiota have gained antibiotic resistance from antibiotic-exposed food animals. A popular theory is that vancomycin resistant strains of the bacterium Enterococcus (VRE), a major cause of postsurgical infections, have arisen in Europe due to the use of the antibiotic avoparcin as an animal growth promoter (Meade-Callahan, 2004). The use of oxytetracycline in aquaculture has been shown to cause a seasonal shift in bacterial species towards Enterobacteriaceae and is associated with increased antibiotic resistance. This could be the same case with other species including *Vibrio* spp.

CONCLUSION

The presence of *Vibrio* species in the final effluents continues to be a potential public threat in the Eastern Cape following isolation of *V.parahaemolyticus*, *V.fluvialis* and *V.vulnificus* in this study. The isolation of these pathogens seemed to be frequent in treatment plants under refurbishment, which indicates a direct effect on the efficiency of treatment plants to eliminated pathogens. All plants not functioning well during this study experienced high Vibrio counts. Three pathotypes were identified for the purpose of this study but other species within the genus could also be harbored in these treatment plants. Although resistance genes were detected in some of the isolates, not all the species were positive for them. This suggest that though phenotypic resistance to antibiotics was observed, other factors besides genetics also mediate the phenotypic resistance to antibiotics.

Recommendations

Betterment and improvement of the final effluent mostly depends on the refurbishment and upgrade of wastewater treatment plants to ensure efficient removal of possible pathogens. We recommend that this be done on a monthly period to counter-act problems before they advance. In most of the plants, the workers were not well trained as a result they do not even know the recommended guidelines set for the final effluents by the Department of water Affairs; it is therefore imperative that focus should be on the training or hiring workers who are qualified. We also recommend the introduction of other methods which will routinely isolate other pathogens other than *E.coli* and feacal coliforms complementing the existing monitoring methods. We also advise that more microbiological laboratories be involved in

routine sample analysis, rather than having all the wastewater treatment plants in the Eastern Cape dependent in one laboratory located in the East London.

Limitations and Potential Future Developments

During the course of the study there were limitations based on the fact that there were still more treatment plants in the Eastern Cape not included as part of the study. This was mainly because of the travelling distances from the different parts of the Eastern Cape to the University as samples needed to be analyzed within 24 hours. Potential future developments would involve the used of municipality Laboratories as a platform to conduct some of the research within their facilities for comparative and developmental purposes.

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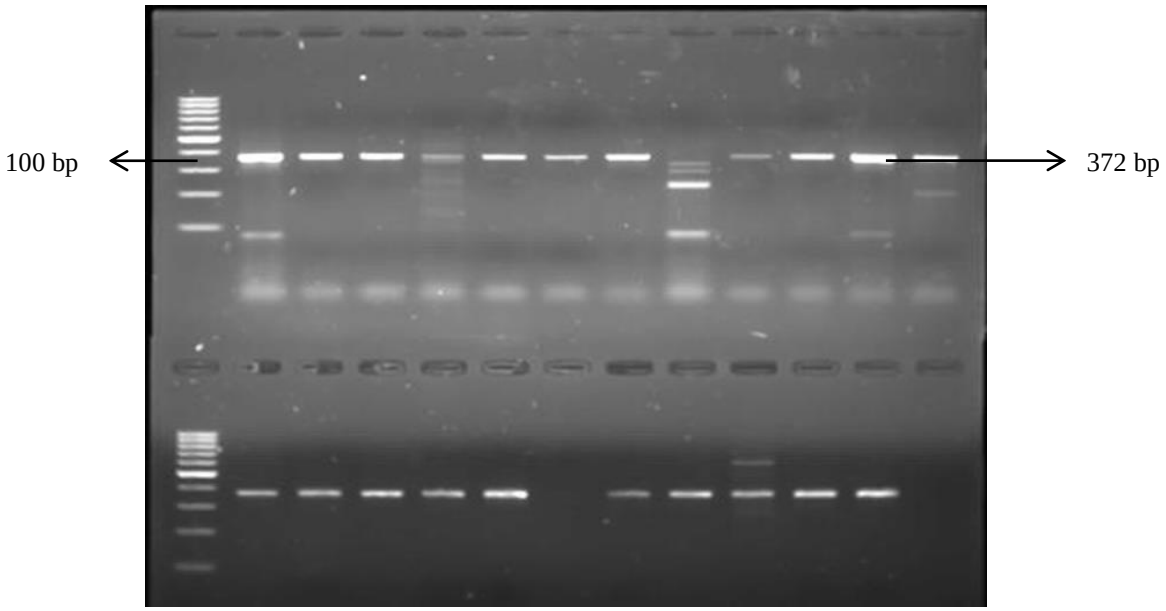
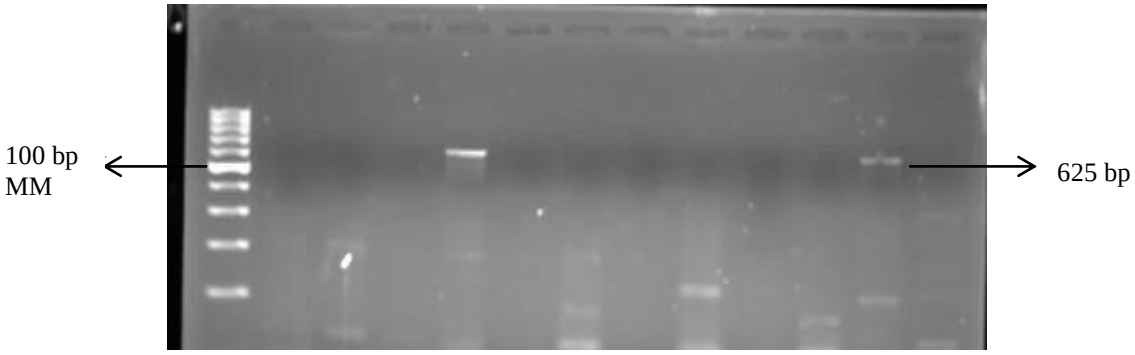
Appendix

V.parahaemolyticus, *V.fluvialis* and *V.vulnificus* were screened for the presence of the *dfrA* gene. Some of the isolates possessed this gene and are represented in Figure 1.



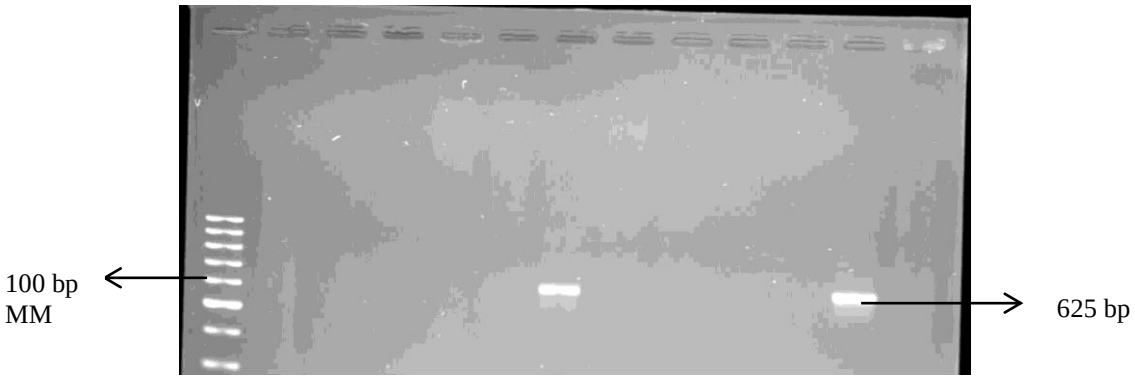
Lane 1: Thermo scientific Gene ruler (100bp); Lane 2 - 23: combines *Vibrio* spp with *dfR A gene;*

Figure 1: PCR products of *V. parahaemolyticus*, *V. vulnificus* and *V. fluvialis* species with *dfR A* gene



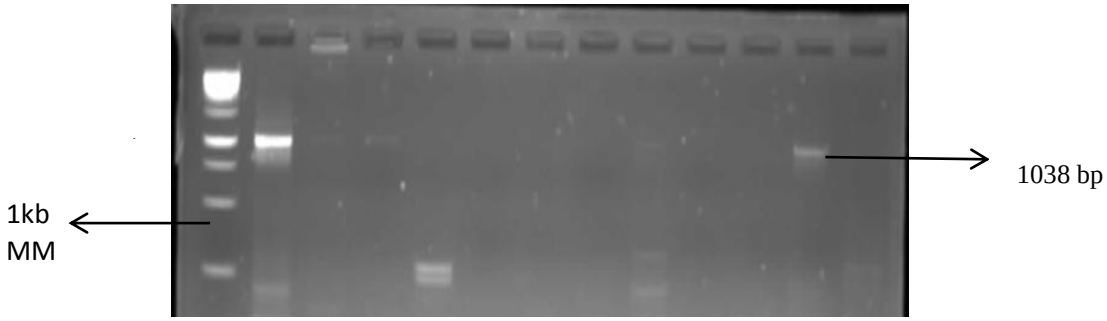
Lane 1: Thermo scientific Gene ruler (100bp); Lane 2: *Vibrio* spp identified with *sul 2* gene

Figure 2: PCR products of *V. vulnificus* species with *sul2* gene



Lane 1: Thermo scientific Gene ruler (100bp); Lane 2: *Vibrio* spp identified with *sul 2* gene

Figure 3: Gel electrophoresis of *V. parahaemolyticus* and *V. fluvialis* species with *sul2* gene



Lane 1: Thermo scientific Gene ruler (1kb); Lane 2: Vibrio spp identified with *SXT* gene

Figure 4: Gel electrophoresis of *V. parahaemolyticus* species with *SXT* gene