Prevalence and pathogenicity of vibrios in treated final effluents of selected wastewater treatment plants in the Amathole District Municipality of Eastern Cape Province of South Africa

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 of Science 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 exception of the citations and that this work has not been submitted at any other university, either in part or in its entirety, for the award of any degree.

Name: ____

Signature: ____

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LIST OF ABBREVIATIONS

CFU - Colony Forming Units

C_T - Contact time

DNA - Deoxyribonucleic acid

DP - Discharge Point

DWAF - Department of Water Affairs and Forestry

FDA - Food and Drug Administration

FE - Final Effluent

I - Intermediate

mg/L – Milligram per liter

MH - Mueller-Hinton agar

MIC - Minimum Inhibitory Concentration

mm - Millimeter

^{o}C - Degree Celsius

PCR - Polymerase Chain Reaction

R – Resistance

S – Susceptibility

s- seconds

TCBS – Thiosulphate Citrate Bile Salt

TDH - thermostable direct haemolysin

TDH - thermostable related haemolysin

UN - United Nations

USEPA - United States Environmental Protection Agency

UV - Ultraviolet

V. fluvialis - *Vibrio fluvialis*

V. parahaemolyticus - *Vibrio parahaemolyticus*

V. vulnificus - *Vibrio vulnificus*

WHO - World Health Organization

WWPT-Z - Wastewater Treatment Plant at Zwelitsha

WWTP - Wastewater Treatment Plant

WWTP-K - Wastewater Treatment Plant at Komga

WWTP-S - Wastewater Treatment Plant at Schornville

µg/ml - Microgram per Milliliter

µl – Microliter

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ABSTRACT

Waterborne diarrhoeal infections continue to be a major health setback in developing countries, especially in rural areas which lack adequate supply of portable water and sanitation facilities. Globally, waterborne diarrhoeal infections occur with an estimated mortality rate of 10–25 million deaths per year, 95% of which are children under the age of 5 years. The *Vibrio* species is one of the major groups of enteric pathogens that are responsible for diarrhoeal infections. Many strains of these bacterial species continue to cause epidemics of diarrhoea throughout the world. In this study, the prevalence of *Vibrio* pathogens in wastewater final effluents was assessed. Wastewater final effluent and discharge point samples were collected monthly between September 2012 and August 2013. All samples were collected aseptically using sterile 1 L Nalgene bottles containing 0.5 ml of sterile sodium thiosulphate solution and transported on ice to the laboratory for analyses within 6 h of collection. The membrane filtration method was used for enumeration of presumptive *Vibrio* densities on thiosulfate citrate bile salt (TCBS) agar plates. Polymerase chain reaction (PCR) was then used to confirm the identities of the presumptive *Vibrio* species using the species-specific primers. The confirmed isolates were further subjected to molecular characterization to confirm their respective pathotypes. Presumptive *Vibrio* densities varied from 0 to 2.11×10^2 cfu/100 ml. Out of 300 confirmed *Vibrio* isolates; 13.3% (40/300) were *Vibrio fluvialis*, 22% (66/300) were confirmed to be *Vibrio parahaemolyticus*, and 24.7% (74/300) proved to be *Vibrio vulnificus*, and 40% (120/300) were other *Vibrio* species which were not assessed for in this study. The strains of *Vibrio fluvialis* were found to exhibit 100% resistance to Polymixin and Tetracycline. However, Gentamicin was active against all the three *Vibrio* species selected for the purpose of this research. The recovery of *Vibrio* species in the discharged effluents throughout the sampling period even in adequately

disinfected effluents is not acceptable considering the fact that *Vibrio* is a pathogenic bacterium. The findings of this study underline the need for constant monitoring of the microbiological qualities of discharged effluents and might also be suggestive for a review of the disinfection methods used at the treatment works.

CHAPTER ONE : BACKGROUND AND INTRODUCTION

The importance of water in all aspects of life cannot be over emphasized, and so is its quality. Because of its centrality to life, many urban settlements were built near some source of water supply (Tarr *et al.*, 2007). It is shocking that in this, the 21st century, an estimated 1.1 billion people worldwide still do not have access to safe potable water. Mostly, these people are from the developing world, especially in the rural areas and low-income communities (Momba *et al*., 2006a). It is of paramount importance that communities be provided with safe and clean water, this is the most important aspect in improving the health of the people (WHO/UNICEF, 2006; WHO, 2007).

Water resources are scarce and extremely limited worldwide (WHO, 2004). To compensate for water scarcity, countries like Jordan, Kuwait, Israel, Spain, Australia, Namibia, Germany, United Kingdom, and the United States of America opted for reclaiming wastewater, and this has formed a crucial element of water demand management in these countries (IWA, 2008). The reuse of wastewater may well be an unavoidable prospect for South Africa in the near future as a viable alternative in the effort towards overcoming the trials of current and future water shortages (Alam *et al*., 2006a). And while this need to be done, sensible steps must be taken to ensure acceptable wastewater quality for re-use in order to safeguard both public health and the environment. Since 2004, it has been recorded that up to 70% of municipal waste treatment facilities in South Africa are failing where proper maintenance and extension are concerned (Alam *et al*., 2006b).

Researchers have shown that water is the primary source of various waterborne infectious diseases which affect a number of numerous communities, especially those in rural and indigenous geographical areas (Momba, 2009; Murcott, 2006; Venter, 2000). Estimates of five million people lose their lives as a consequence of water-related diseases each year (Pritchard *et al*., 2009 and Baumgartner *et al*., 2007).

Bacterial pathogens in water tend to cause gastrointestinal infections such as diarrhoea, dysentery, typhoid shigellosis and human enteritis (Okoh *et al*., 2007 and Venter, 2000).

Wastewater works discharge significant amounts of pathogenic microorganisms, this thus leads to a compromise in the quality of water (Simpson and Charles, 2000; Bahlaoui *et al*., 1997). Those in authority are not only obligated to provide safe drinking water, but also to address public health risks of polluted environmental water affecting the entire community, as well as to meet the terms of the stipulated standards. The poor operational state and inadequate maintenance of most of sewage treatment works result in major pollution problem and impacts on the quality of water resources, with marine water quality standards consequently not meeting regulatory standards (Momba *et al*., 2006a).

It is advised that there is need to reassess the operational efficacy of wastewater treatment plants to get rid of pathogens such as *Vibrio* from wastewater discharges, prior to their release into the receiving watersheds (WHO, 2004). Authorities also point out that the country's local experts are increasingly incapable of managing the constant demand for effective sewage treatment (Srinivasan *et al*., 2006). Recent studies on wastewater treatment plants have shown that small wastewater treatment plants are often situated periurban or rural areas, where technical and management capacity is hard to come by (Momba, 2009). Current wastewater treatment expertise utilized for reclamation is also increasingly failing to meet obligatory treatment levels (Liang *et al*., 2013).

In order to meet the water quality criteria for the aquatic environment, water recycling, reuse and advanced wastewater treatment technologies ought to be employed. This is also pivotal for the protection of the public health (USEPA, 2004). Given the severe limitations in financial and human resources that characterize developing countries, the activities of the wastewater manager are often limited to solving acute operational problems, such as pump failures and sewer blockages. As such, the actual failing performance of the wastewater system appears to the wastewater manager as one without solution.

Wastewater managers must, however, opt for a strategy that entails a gradual improvement in system performance (Zhang and DiGiano, 2002).

Various strategies may be used to curb water-related infections in communities such as those caused by *Vibrio* species. These include; the use of antimicrobials, health education, safe water provision and management of human waste disposal (Abuga *et al*., 2008). These strategies usually depend on the response of the communities to epidemics. Indeed, the socio-economic status of the community, the effectiveness of governance and quality of sanitation infrastructure play a paramount importance in curbing these infections (Olago *et al*., 2007).

This study focused on the prevalence of *Vibrio* pathogens and three wastewater treatment facilities were selected for the accomplishment of this objective. Members of the genus Vibrio belong to the Gammaproteobacteria. These species are found to be among the causative agents of mass deaths of marine animals (Cheng et al., 2004). In immature even adult bivalves, many bacterial diseases are provoked by Gram-negative bacteria, mostly the genus Vibrio (Paillard *et al.,* 2004). These organisms are widely distributed in effluent environments related to domestic sewage. Also, they are associated with aquatic living species and contain pathogens for aquatic animals and humans who ingest contaminated seafood or drinking water (Thompson *et al*., 2004).

These gram negative, motile rods are mesophilic and have a facultative fermentative metabolism (Thompson *et al*., 2004). Some of them are human pathogens and are part of the natural flora of bacteria in seawater and in the gut of many seawater organisms. They cause gastrointestinal illnesses in humans which include diarrhoea (Oliver and Kaper, 2001).

Vibrio species are broadly clustered into two groups namely, the cholera and non-cholera groups (Brooks *et al*., 1998). Among the *Vibrio* species that can cause human infections are *Vibrio cholerae, V. vulnificus, V. parahaemolyticus, V. fluvialis, V. mimicus, V. furnissii, V. hollisae, V. alginolyticus, V. damsel, V. carchariae and V. cincinnatiensis*. However, the main focus of this study was on three *Vibrio* species; *V.*

fluvialis, V. parahaemolyticus as well as *V. vulnificus. Vibrio vulnificus* and *V. parahaemolyticus* are invasive organisms which affect the human colon (Kothary *et al*., 2003). *Vibrio parahaemolyticus* is often accompanied with abdominal cramping, vomiting, fever and chills; *V. vulnificus* causes bacteremia, skin and soft tissue infections while the watery diarrhoea caused by *V. fluvialis* is associated with wound infection, septicemia and gastroenteritis (Chiang and Chuang, 2003).

This study was to characterize the three water-borne *Vibrio* species isolated from three selected wastewater treatment plants' effluents in both the Amathole District Municipality of the Eastern Cape Province of South Africa.

Justification of the study

South Africa is a country that has a high population of the immunocompromised especially in the poor Eastern Cape Province. The Eastern Cape Province is the second largest and third most populated province in South Africa (Census, 2011; NRA, 2007). Also, it is a mostly non-urban, underprivileged and is without adequate infrastructure. A significant proportion of the rural communities lack pipe-born water, and as such depend on streams, rivers, groundwater and other available waterbodies for drinking and domestic purposes (Momba *et al*., 2006b).

A major constraint in the Eastern Cape Province is the scarcity of safe drinking water supplies. This region has an overabundance of socio-economic problems whose effects gush down to the environmental degradation, mostly the pollution of surface water resources. In this region water is utilized for multi-purposes such as drinking, recreation, domestic and shellfish harvesting. Rivers and dams are negatively impacted by untreated or inadequately treated wastewater effluents from municipal wastewater treatment plants that is subsequently discharged into them (Igbinosa *et al*., 2009). This impact compromises the primary health of people surrounding the affected water resource, especially the elderly, the very young and the immunocompromised; who are more susceptible to infections mainly caused by the microbial pathogens such as *Vibrio s*pecies present in contaminated waters (Kwok *et al*., 2002).

In a study done by Okoh *et al*. (2007) it was shown that the wastewater treatment facilities in the Eastern Cape Province of South Africa are a veritable source of pathogens in aquatic environments of the study area and negatively impact the quality of receiving watershed (Igbinosa and Okoh, 2010). Therefore, it is highly possible that they might also be a source of *Vibrio* species in the water environment.

The socio-economic status of the community, the effectiveness of governance and quality of sanitation infrastructure play a paramount importance in curbing vibrio infections. The information obtained from this study may be useful to the health institutions and local municipalities of the Eastern Cape Province so that they become more proactive rather than reactive in containing *vibrio* epidemics in future.

Hypothesis

The working hypothesis of this study was that wastewater treatment plants of the Amathole District Municipality in the Eastern Cape Province of South Africa are veritable sources of Vibrio pathogens in the receiving watershed.

Aims and objectives

The overall aim of this study was to assess the prevalence of pathogenic *Vibrio* species in the final effluents of three wastewater treatment plants located in the in the Eastern Cape Province, South Africa.

The specific objectives of this study include:

(1) To assess the occurrence of *Vibrio* pathogens in the final effluents and discharge points water samples

(2) To confirm the identities of the isolates, and to confirm the pathotypes of the Vibrios using conventional PCR.

(3) To determine the antibiotic susceptibility profiles of the *Vibrio* pathogens.

CHAPTER TWO：LITERATURE REVIEW

2.1 An overview of water related challenges worldwide

Water is a critically scarce resource globally, the expansion of the economy and the population increase play a pivotal role in the escalation of stress where this resource is concerned. The world's economic growth is also dependant on its ability to meet the wide-ranging needs for water (Gieldereich, 1990). According to Vasiliu and Jelev (2008) and Cosbuc (2010), the share of freshwater in the total volume of obtainable water on earth is 2.7% while the rest (97.3%) is sea water. Jelev *et al.* (2011) evidenced that only 0.46% is usable freshwater, whereas the remaining 99.45% is found in various forms in nature; with ice constituting 77.2%; groundwater 22.41%, and water vapour and water lakes constituting 0.35%. They then gathered that the total of available 'freshwater' is represented by 0.009% of all the water available on earth. Seeing the prominence and worth of water for humans, Duca (2009) stated that it is a threatened resource that is tremendously treasured, given that lesser and lesser people have drinking water owing to the rapid increase in population size. In this viewpoint, Safriel (2011) stated that 40% of humans are competing with nature for water.

It is predicted that by the year 2025, about 60% of the population worldwide might undergo physical distress owing to water scarcity (Rijsberman, 2006; Pimentel *et al*., 1999). To compensate for water pollution and shortages, most countries opted for wastewater recycling, and this has helped with the design of a crucial element of water demand management (IWA, 2008). The reuse of wastewater may well be an unavoidable prospect for South Africa in the near future as a viable alternative in the effort towards overcoming the trials of current and future water shortages (Grau *et al*., 2005). Wastewater reclamation reduces contamination levels while generating new sources of water provisions (Wade Miller, 2006). However, functional steps ought to be taken to ensure acceptable wastewater quality for re-use in order to safeguard both public health and the environment. Wastewater recycling has dual benefits; the decrease in the discharged effluent to the milieu as well as a potential decrease of the pressure on the present water resources (Toze, 2006). Nonetheless, there are risks associated with wastewater recycling; this method might endanger people's lives by exposing them either to pathogens, heavy metals or even pharmaceutically active compounds like antibiotics. Wastewater recycling must therefore be treated cautiously until the understanding of the potentially adverse concerns for the reuse of water containing low concentrations of these emerging contaminants is reached. Studies by Kinney *et al*. (2006a, 2006b) have proven the sustained presence of pathogens in soils irrigated with reclaimed water. These researchers have also shown that even at low concentrations, emerging pollutants can have detrimental effects.

The main objective of wastewater treatment is to reduce the risks to public health by the removal of as many of the disease-causing agents and chemical pollutants as possible (Akpor *et al*., 2008; Shaler and Klecka, 1986). One of the many causes of disruptions in water facilities is scanty supervision (Momba *et al*., 2006). A compromise in drinking water standards increases the consumers' risk of waterborne diseases even from treated water supplies (Momba *et al*., 2005; Momba *et al*., 2006; MacKintosh and Colvin, 2003). The operational state and the upkeep of most of sewage treatment works are rather very poor and inadequate in most wastewater treatment works in South Africa, especially in rural areas. This results in a major pollution problems and impacts on the quality of water resources (Momba *et al*., 2006a).

Existing wastewater treatment plants (WWTPs) are not explicitly intended to eradicate micropollutants (Koivunen *et al*., 2003) but depending on the type of treatment method and the disinfection system, they may eliminate up to 99.96% of bacteria (Okoh *et al*., 2007; Guardabassi *et al*., 2002; Johnson and Stell, 2000). The most commonly used disinfectant in many countries is chlorine (Hijnen *et al*., 2006). Its advantages include availability, low cost, ease of dosing control and moderate to average time for the effluent to pass through the disinfection stage. It can be added to effluent in a gaseous form, but can also be applied as a liquid or solid (Okoh *et al*., 2007). However, even after treatment, numerous bacteria are able to pass through wastewater treatment methods by virtue of their persistency. Safeguarding and monitoring arrangements for micropollutants have not been well established in many WWTPs (Bolong *et al*., 2009). Subsequently, many of these organisms end up in the water milieu, thus posing threats to aquatic life and predicting concern for drinking water industry. To date, discharge guidelines and standards do not exist for most micropollutants (Fent *et al*., 2006; Pruden *et al*., 2006).

Developing countries like South Africa rely on rivers for social, cultural and religious purposes (Gieldereich, 1990). Obtainable universal standards for the microbiological quality of treated sewage have been put in place and are relevant for valuable use such as agriculture, bathing, has among others and have compelled regulations of microbiological quality of both effluents and receiving water bodies (Igbinosa and Okoh, 2008). This results from the fact that a portion of treated sewage being discharged into rivers has increased over time thus leading to deterioration of the quality of water of the major rivers. It is apparent that the treated sewage discharged, is in most instances inadequately treated and fail to comply with the discharge standards (Arceivala, 1997), this has caused a high density of disease causing bacteria in the receiving water bodies, and subsequently, in an increasing incidence of emerging pathogens.

Not only is water a source of life, it is also the basis of countless waterborne communicable infections which affect many communities and most victims are the rural residents (Momba, 2009; Murcott, 2006; Venter, 2000). Subsequently, five million lives are lost owing to water-related infections each year (Pritchard *et al*., 2009; Baumgartner *et al*., 2007). Water sources are exposed to recurrent microbial and chemical quality changes, this being a consequence of the many events on the watershed. These variations are caused by discharges of municipal raw waters or treated effluent at a definite point-source into the receiving waterbodies such as streams, rivers, lakes and ponds (Altekruse *et al*., 2000; Gieldereich, 1990).

2.2 *Vibrio* species

Vibrios are affiliates of the family Vibrionaceae and play a pivotal role in causing diarrheal diseases globally (Kaper *et al*., 1995). They are Gram-negative bacteria that attain a curved rod form and inhabit marine environments (Blackwell and Oliver, 2008; Eiler *et al*., 2007; Wright *et al*., 1996; Oliver *et al*., 1983; Tamplin *et al*., 1982). These bacteria have a single polar flagellum which they utilize for locomotion in liquid media. Also, they are oxidase-positive, facultative anaerobic microorganisms which do not form endospores or microcysts (Farmer III and Brenner, 1992).

More than 60 species are included in the genus *Vibrio*, and its taxonomy is endlessly restructured owing to the accumulation of new species (Igbinosa *et al*., 2009). These organisms are pervasive and extensively distributed in marine environments (Thompson and Polz, 2006; Urakawa and Rivera, 2006). A number of species within the genus *Vibrio* are recognized as human pathogens (Tarr *et al*., 2007). Amongst these, *Vibrio cholerae*, *V. vulnificus*, and *V. parahaemolyticus* are considered the utmost significant pathogenic *Vibrio* species. *Vibrio cholerae* and *V. parahaemolyticus* are generally contracted via the ingestion of polluted seafood and/or seawater, and are responsible for causing gastroenteritis while *V. vulnificus* are responsible for triggering septicemia through contact of a wound to seawater or intake of tainted seafood (McLaughlin *et al*., 2005; Morris, 2003).

The most favourable temperatures for the growth of most vibrios are between 15 and 30°C, depending on the strain under analysis. Most of these organisms are able to endure the freeze drying process well. Ironically, they are problematic to cultivate on any culture media (Biosca *et al*., 1997). Different *Vibrio* spp. respond inversely to environmental influences, water temperature and salinity are the most pivotal factors in defining population concentration or distribution (Tantillo *et al*., 2004). During winter, culturable cells are either reduced or absent but omnipresent throughout summer (Tantillo *et al*., 2004). Surveys based on selective cultivation using thiosulphate citrate bile salt sucrose (TCBS) agar have been employed by most researchers followed by the characterization of isolates using traditional biochemical tests (Hervio-Heath *et al*., 2002; Diggles *et al*., 2000). The shortcomings of these methods are that cells in the viable but not culturable (VBNC) state escape detection. Numerous *Vibrio* spp. preserve a viable state in marine environments (Thompson *et al*., 2004). Many *Vibrio* spp. enter the dormancy state when exposed to unfavourable growth conditions, in which state the cells are still viable although not culturable (Ramaiah *et al*., 2002; Oliver, 1995; Roszak and Colwell, 1987). Frequently, nutrient inadequacy is the most common environmental stress that is encountered by bacteria in an ecosystem. Nevertheless, the *Vibrio* spp. is able to adapt to the stressful environment by means of sequential changes in physiology of their cells and ongoing changes in morphology (Morita, 1993; Albertson *et al*., 1990). When organisms are in the unculturable state, they represent reservoirs for *Vibrio* distribution and infection. Most vibrios require a minimum of salinity for growth and persistence, and display maximum salt tolerance (Baker-Austin *et al*., 2012; Froelich and Oliver, 2013; Gomez –Gil and Roque, 2006; Thompson and Polz, 2006).

Furthermore, in developed countries, use of treated wastewater for domestic, industrial and agricultural purposes is recognized as the most important method of reusing wastewater. This fact can lead to an increased risk of human infection for emerging pathogens such as *Vibrio* (Fremaux *et al*., 2009; Igbinosa and Okoh, 2008). The faecal indicator bacteria levels in water or seafood unfortunately do not link with the presence or absence of pathogenic *Vibrio* spp. (Campbell and Wright, 2003). Currently, there is a general agreement on the need of both monitoring the existence of vibrios in the environment and studying their pathogenicity potential in order to suitably protect human health (Jones and Oliver 2009; Baffone *et al*., 2003).

Even though humans are frequently exposed to various environmental microbes, only a small quantity of these bacteria, *Vibrio* included, are proficient of interacting with the host such that contamination and subsequently illnesses result (Igbinosa, 2010). *Vibrio* (viz. *V. parahaemolyticus, V. anguillarum, V. vulnificus, V. alginolyticus and V. salmonicida*) is the causative agent for Vibriosis (Silva-Aciares *et al*., 2013; Almedia *et al*., 2009). In Asia, Europe and the United States, the occurrence of the infections deviates expressively. In the Asian cluster, *Vibrio* spp. have been documented as the most prominent cause of foodborne outbreaks in many countries including China (Yang *et al*., 2008), India (Chakraborty *et al*., 2008) and Malaysia (Noorlis *et al*., 2011).

2.2.1 *Vibrio vulnificus*

Vibrio vulnificus belongs to the Gammaproteobacteria group, in the family Vibrionaceae. It is a Gram-negative halophilic bacterium, and it lives and thrives in estuarine and coastal waters (Broza *et al*., 2009; Harwood *et al*., 2004). *Vibrio vulnificus* is a food-borne and wound associated human pathogen that is capable of causing an array of infections including gastroenteritis, necrotizing fasciitis, and septicemia (Baker-Austin *et al*., 2010; Oliver, 2006; Oliver, 1989). The infections caused by this bacterium often call for hospitalization and have tremendously high fatality rates, even with aggressive antibiotic treatment (Oliver, 2006; Centers for Disease Control, Prevention, 2009). This bacterium accounts for 95% of seafood-borne deaths, and these deaths are mainly from the ingestion of raw or undercooked oysters (Oliver, 2006). It is also able to endure under adverse environmental conditions, adjusting to different ecological habitats (Ramirez *et al*., 2009; Jones *et al*., 2008). Grau *et al*. (2005) described a phenotype of *V. vulnificus* that is capable of forming biofilms that enable the bacteria to tolerate unfavourable environments. Furthermore, Wong and Liu (2008) have shown the ability of *V. vulnificus* to adjust to low salinities when the bacteria is in a medium rich in amino acids, and wastewaters are very rich in organic compounds.

The bacteria is known to yield virulence factors, such as the cytolytic pore-forming toxin (PFT) *Vibrio vulnificus* hemolysin/cytolysin (VVH), a product of the *vvhA* gene (Jones and Oliver, 2009). The pathological processes of *V. vulnificus* are related to the availability of iron (siderophore production) (Litwin *et al*., 1996), capsule production (Simpson *et al*., 1987), the productions of metalloprotease (Miyoshi *et al.*, 1994), as well as the production of a large number of proteases (elastase, collagenase, neuraminidase, and lipase) (Miyoshi *et al*., 1994; Miyoshi *et al*., 1993; Simpson *et al*., 1987; Kreger and Lockwood, 1981). The strains of *V. vulnificus* differ both phenotypically and genetically and are usually characterized into 3 biotypes based on biochemical characteristics. Biotype 1 (BT1) strains are accountable for most of human infections. Biotype 2 (BT2) strains are often linked with disease in eels (Sanjuan *et al*., 2009; Bisharat *et al*., 1999); and biotype 3 (BT3) strains have different phenotypic and molecular patterns which are responsible for the indication of the existence of a recent genome hybridization event between BT1 and BT2 (Bisharat *et al*., 2007, Bisharat et al., 2005, Bisharat et al., 1999).

Vibrio vulnificus is the foremost source of mortalities from seafood in the United States, with about 40 cases each year (Bross *et al*., 2007). Outbreaks of *V. vulnificus* have also been reported in Europe and Asia (Lin *et al*., 2006; Frank *et al*., 2006; Lewis *et* al., 2005). According to the CDC (2009) report, it accounts for 95% of seafood related mortalities in the USA every year, with a fatality rate beyond 50% in cases that have been brought forward (CDC, 2009; Mead *et al*., 1999). In 2009, a total of 825 vibriosis were testified in the US, from which 107 (13%) were linked with *V. vulnificus*. Out of these, 90% of the subjects were hospitalized while 32% died (CDC, 2009). Europe and Asia have also experienced epidemics of *V. vulnificus* (Canigral *et al*., 2010; Jones and Oliver, 2009; Harwood *et al*., 2004; DePaola *et al*., 2003).

Death caused by this organism can transpire within a day or two after contact of the host (Harwood *et al*., 2004). Moreover, this organism may cause serious wound infection normally from the contact of open wounds to water contaminated with this bacterium. The wound infections advance hastily and may possibly lead to necrotizing fasciitis at the infection site. The death rate for wound infections is, however, lesser than that of a systemic disease. Gastroenteritis initiated by *V. vulnificus* is normally not very serious, even the symptoms are mild and hardly ever warrant medical attention, such cases usually go unreported (Drake *et al*., 2007).

2.2.2 *Vibrio fluvialis*

Vibrio fluvialis is a halophilic pathogen normally found in marine environments or marine products. Currently, *V. fluvialis* is recognized as an organism that is liable for serious infections and has clinical symptoms of gastroenteritis making it similar to the clinical manifestations of *V. cholerae*, the causative agent of cholera (Kothary *et al*., 2003). *Vibrio fluvialis* flourishes in halophilic marine environments. It is a Gram-negative, oxidase-producing organism which has been associated with causing mainly gastroenteritis with diarrhoea (Tacket *et al*., 1982). This human pathogen has been linked with numerous life-threatening conditions, most stemming from wound infections which develop septicaemia (Mouzopoulos *et al*., 2008).

Furniss and co-workers (1977) were the first researchers to isolate *V. fluvialis*; this organism was later on termed by Lee *et al*. (1981). In 2000, about 10% of *Vibrio* gastroenteritis cases in a US survey were blamed upon the bacterium *V. fluvialis* (Chakraborty *et al*., 2006). It is different from other *Vibrio* species which have frequently been stated to cause extraintestinal infections, because it is exclusively linked with gastroenteritis, with only a few cases of extraintestinal infections (hemorrhagic cellulitis with cerebritis, bacteremia, and peritonitis) (Lai *et al*., 2006; Huang and Hsu, 2005; Ratnaraja *et al*., 2005; Tacket *et al*., 1982).

Vibrio fluvialis has been reported as an emerging pathogen with prevalent potential and therefore it is a public health concern (Singh *et al*., 2012; Chowdhury *et al*., 2011; Igbinosa and Okoh, 2010; Ahmed *et al*., 2005; Ahmed et al., 2004). Severe gastroenteritis by way of diarrhoea is the utmost common medical demonstration of an infection instigated by *V. fluvialis*. In a study done in the United States, this bacterium was held responsible for 10% of gastroenteritis cases (Chakraborty *et al*., 2006). Moreover, this organism has been stated to cause extraenteric infections, such as necrotizing fasciitis and bacteremia, these were linked with minor trauma and exposure to fish, raw oysters, shellfish, crabs and seawater (Tsai *et al*., 2004; Morris, 2003).

The spreading of *V. fluvialis* is a universal occurrence, according to many researchers' reports, this organism was only isolated from human diarrhoeal cases (McNicol *et al*., 1980). However, the findings of a study done by Igbinosa *et al*. (2009) showed that *V. fluvialis* can also be present in aquatic environments. This bacterium has been linked with extra-intestinal diseases, generally, and its clinical manifestations include dehydration, vomiting, fever, abdominal pain and diarrhoea (Seidler *et al*., 1980). There is paucity of information available on the virulence factors associated with its infectious diseases, as well as on the mechanism of the organisms' pathogenicity.

2.2.3 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus, a Gram-negative, halophilic, mesophilic, rod that possess a single curve to its shape was discovered in 1950 by Tsunesaburo Fujino from the Osaka University (Shinoda, 2011). This bacterium is a nautical microbe that exists as either a swimmer cell with a single polar flagellum, or a swarmer cell concealed in lateral flagella. Also, it is dependent on environmental settings. *V. parahaemolyticus* can produce a capsule and has over 70 different K antigens detected in various strains (Nair *et al*., 2007). It causes acute gastroenteritis by means of the ingestion of tainted raw or undercooked seafood. In the course of infection, this microorganism uses a broad selection of virulence factors, including adhesins, toxins and type III secretion systems, these factors play role in causing both cytotoxicity in cultured cells (Zhang and Orth, 2013).

During wintertime when water temperatures are unfavorable, *V. parahaemolyticus* may perhaps be untraceable. It has been suggested that the bacterium usually endures in sea sediment, and is restored to the water column when temperatures increase. However, in situations where water temperatures do not decrease lower than 15ºC, the bacterium may be isolated throughout the year (Su and Liu, 2007).

Vibrio parahaemolyticus is considered as the utmost significant seafood-borne pathogen prompting gastrointestinal sicknesses to humans. The incidences of eruptions of *V. parahaemolyticus* infections in countless countries across all the continents have raised a noteworthy public concern of seafood safety (Vugia *et al*., 2009; Nair *et al*., 2007).

Though this species is frequently present in seafood; most of its isolates are non-pathogenic to humans (Nishibuchi and Kaper, 1995). Nonetheless, the isolates bearing *tdh* and *trh* genes are known to be pathogenic to humans. These two genes encode the haemolysins thermostable direct haemolysin (TDH) and the TDH-related haemolysin (TRH), respectively (Zhang and Austin, 2005; Nishibuchi and Kaper, 1995), which are considered as major virulence factors of this organism (Zhang and Austin, 2005; Kaysner and DePaola, 2001). To date, as many as 12 pathogenic serotypes of *V. parahaemolyticus* have been identified (Gonzalez-Escalona *et al*., 2011). A lot of strains have been sequenced, comprising the O3:K6 serotype strains RimD 2210633 and AQ3810; the O4:K12 strain Vp10329; and the O4:K68 serotype strains AN-5034, K5030 and Peru-466 (Gonzalez-Escalona *et al*., 2011; Boyd *et al*., 2008; Makino *et al*., 2004). Most medical and ecological strains deprived of a sequence have been employed to comprehend the ecology of *V. parahaemolyticus* (Zhou *et al*., 2009; Yeung and Boor, 2004; Bej *et al*., 1999). The first completely sequenced and explained genome was the RimD 2210633 strain (Makino *et al*., 2004) and is consequently the emphasis of most studies.

2.3 Antibiotic resistance profiles of *Vibrio* species

The reasons of antibiotic resistance are complex and comprise the behaviour of humans at numerous heights of humanity; everyone in the world is affected by the concerns. The diminishing efficiency of antibiotics in treating infections has accelerated lately, also with the presence of not curable strains of carbapenem resistant Enterobacteriaceae; we are at an arising post-antibiotic era (Gahrn-Hansen and Hornstrup, 1994). In developed countries, a swing to pricey and more broad-spectrum antibiotics has been forced by constant high rates of antibiotic use in hospitals. Communities and agriculture have contributed to selection pressure that has sustained resistant strains (Honda and Iida, 1993).

To counteract the pattern of an unreasonable use of antibiotics amongst the public and the health professionals, antibiotic stewardship programmes need to be put in place, and these should pay attention on the proper use of antibiotics and on safeguarding the sustainability of behavioural change and reorientation of public customs (Sumpradit *et al*., 2012; WHO, 2002). The emergence of antibiotic resistant bacteria is expected in any setting where antibiotics are released. The manifestation of antibiotic resistant bacteria is growing in marine environments (Al-Bahry *et al*., 2009; Lima-Bittercourt *et al*., 2007).

Chemicals and antibiotics are extensively used to inhibit or treat infections. Nonetheless, Nogueira-Lima *et al.* (2006) discovered that assessing the risks associated with the use of chemicals in aquaculture is problematic owing to the lack of quantitative data from most countries involved in this activity. Antibiotic susceptibilities vary among species but nearly all strains are sensitive to chloramphenicol, tetracycline, aminoglycosides, quinolones and -lactams. Some strains of *V. alginolyticus* and *V. parahaemolyticus* are from time to time resistant to antibiotics comprising chloramphenicol, tetracycline and cefotaxime (Radu *et al.*, 1998). The role of antibiotics in managing human diseases resulting from Vibrio species is not clear yet; antimicrobial resistance could however be an imperative problem for therapy directed against these organisms (Radu *et al*., 1998, Liu *et al*., 1997; Arai *et al*., 1985).

Antibiotics are the solitary effective treatment for food-borne infections (Mao *et al*, 2007). In recent times, a lot of aerobic and anaerobic microorganisms were told to show antibiotic resistance (Yong *et al*., 2004). Occurrence of antibiotic resistance strains of *V. parahaemolyticus* has become a severe danger in the seafood industries (Tendencia and Pena, 2001). The findings of Abraham *et al*. (1997) and Bhattacharya *et al*. (2000) revealed that the strains of *V. parahaemolyticus* showed resistance to erythromycin, streptomycin, penicillin and ampicillin. The level of resistance to ampicillin and penicillin was witnessed in 100% of the analyzed isolates. The family of penicillins are bactericidal and highly efficacious against susceptible bacteria, this has however been restricted by the emergence of resistant bacterial strains in recent years (Miller *et al*., 2002).

CHAPTER THREE：METHODOLOGY

3.1 Description of study site and samples collection

The Eastern Cape is a provice within South Africa. It is the second largest province and mainly consists of rural settlements with little or no adequate hygienic services. This study was conducted in one of the district municipalities of this region, known as the Amathole District Municipality. Three sites were selected for the purpose of the study, and these were the Komga, Schornville, and Zwelitsha wastewater treatment plants (WWTPs). The geographical coordinates for the study sites are S32º37.34’ E27º31.20, S 32°.89’71 E 27°.39’81, and S32°55′E 27°25′, respectively.

Wastewater samples were collected from the treated final effluent and the discharge point of three wastewater treatment plants (WWTP-K, WWETP-S and WWTP-Z). Some characteristics of the plants are as described in Table 3.1 below. Samples were collected monthly between September 2012 and August 2013. Samples were collected aseptically in sterile 1000 ml Nalgene bottles. Water samples from the final effluents were dechlorinated by adding 0.5 ml of sterile concentrated sodium thiosulphate solution to give a final concentration of 100 mg $l^{-1.}$ Samples were stored at 4 °C until all analyses were complete. All samples were processed after 24 h of collection (Igbinosa *et al*., 2009).

Table 3.1: **List and characteristics of WWTPs selected for this study (Green Drop Report, 2012).**

WWTP	Zwelitsha	Schornville	Zwelitsha
Technology	Biofilters, anaerobic digestion and sludge drying beds	Activated sludge and BNR, biofilters, anaerobic digestion and sludge drying beds	Biofilters, anaerobic digestion and sludge drying beds
Design Capacity (Ml/d)	9.3	4.8	9.3
Operational % in relation to Design Capacity	84.90%	133.3	84.90%
Microbiological Compliance	16.00%	0.00%	16.00%
Chemical Compliance	61.70%	64.70%	61.70%
Physical Compliance	76.30%	84.30%	76.30%
Critical Risk Area	Poor effluent compliance	Poor effluent compliance, operating capacity exceeds design capacity	Poor effluent compliance

3.2 Estimation of *Vibrio* counts

For direct plate count of *Vibrio* species the samples were appropriately diluted and 100 ml of filtered through sterile 0.45 µm millipore filters, this was repeated three times, and the filters were placed onto thiosulphate-citrate-bile-salts-sucrose (TCBS) agar plates and incubated at 37^{o}C for 24 h in accordance with the description of Alam *et al.* (2006a). After the incubation period, the characteristic *Vibrio* species colonies were counted and recorded. Yellow and green Alam *et al.*, (2006b). Randomly selected colonies were purified and stored in 20% glycerol stock at -80^{o}C for further analyses

3.3 Molecular characterization

3.3.1 Isolation of genomic DNA

Genomic DNA of the bacterial isolates was extracted following a modified scheme of Maugeri *et al.*, (2006). Single colonies of *Vibrio* species isolates grown overnight at 37 ^{o}C on TCBS agar plates were picked, suspended in 200 µl of sterile Milli-Q PCR grade water (Merck, SA) and the cells were lysed using Dri-block DB.2A (Techne, SA) for 15 min at 100 ^{o}C. The cell debris was removed by centrifugation at 11, 000 × g for 2 min using a MiniSpin micro centrifuge (Merck, SA). The cell lysates (10 µl) were used as template in the PCR assays immediately after extraction placed on ice for 5 min or following storage at -80 ^{o}C. Sterile Milli-Q PCR grade water (Merck, SA) was included in each PCR assay as a negative control.

3.3.2 PCR amplification assay

Polymerase chain reaction (PCR) was used to confirm the identities of the *Vibrio* species using the specific primers described in Table 3.2. DNA extraction and PCR were carried out as described by Maugeri *et al*., (2006) with little modification. Single colonies of presumptive *Vibrio* strains grown overnight at 37^{o}C on TCBS agar plates were picked, suspended in 200 µl of sterile distilled water and bacterial cells were collected by centrifugation at 11 000 × g for 10 min at 4^{o}C. The pellet was suspended in 100 µl of sterile distilled water and boiled for 10 min, cooled on ice and centrifugation at 11 000 × g for 10 min at 4 ^{o}C to pellet cell debris. The supernatant which contained DNA was carefully transferred into another sterile vial and used as DNA template in the PCR assays or stored at -20 ^{o}C until ready for use. The thermal cycling profile was as follows: a 15 min denaturation at 93^{o}C followed by 35 cycles at 92^{o}C for 40 s, 57°C for 1 min and 72°C for 1.5 min and final extension at 72°C for 7 min. The amplified products were held at 4°C after completion of the cycles. The PCR conditions used for the delineation of the confirmed *Vibrio* isolates into the *Vibrio fluvialis*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* are as described in Table 3.2. For *Vibrio fluvialis* the amplification conditions were initial denaturation at 94ºC for 5 min, followed by 30 cycles consisting of denaturation at 94ºC for 40 s and extension at 72ºC for 1 minute. The PCR products were electrophoresed in 1.5% agarose gel containing 0.5mg/l ethidium bromide for 1h at 100V and then visualized using a UV transilluminator.

Table 3.2: **List of primers used in this study.**

Target species	Primer	Sequences (5'- 3')	Target gene	Amplicon size (bp)	Reference
All Vibrio spp	V. 16S-700F	CGG TGA AAT GCG TAG AGA T	16S rRNA	663	Kwok et al., 2002
	V. 16S-1325R	TTA CTA GCG ATT CCG AGT TC			
V. parahaemolyticus	Vp. flaE-79F	GCA GCT GAT CAA AAC GTT GAG T	flaE	897	Tarr et al., 2007
	Vp. flaE-934R	ATT ATC GAT CGT GCC ACT CAC			
V. vulnificus	Vv. hsp-326F	GTC TTA AAG CGG TTG CTG C	hsp60	410	Wong & Chow, 2002
	Vv. hsp-697R	CGC TTC AAG TGC TGG TAG AAG			
V. fluvialis	Vf- toxR F	GAC CAG GGC TTT GAG GTG GAC GAC	toxR	217	Chakraborty et al., 2006;
	Vf- toxR R	AGG ATA CGG CAC TTG AGT AAG ACTC			Osorio & Klose, 2000

3.4 Antibiotic susceptibility test

Bacterial susceptibility to antimicrobial agent was performed by disk diffusion method using guidelines established by Bauer *et al*. (1996) using commercial antimicrobial discs. A total of 10 antibiotic discs (Mast Diagnostics, Merseyside, United Kingdom) which includes cefuroxime (30 µg), erythromycin (15 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), tetracycline (30 µg), trimethoprim (30 µg), sulfamethoxazole (25 µg), gentamicin (10 µg), penicillin G (10 unit), and cefuroxime (30 µg) were employed. The resistance or susceptibility profile of the isolates were determined by measuring inhibitory zone and then compared with the interpretative chart to determine the sensitivity of the isolates to the antibiotics.

3.5 Statistical analysis

ANOVA F-test to compare the statistical differences of the vibrio counts across the sampling months per site and among the sampling points per sampling month.

CHAPTER FOUR : RESULTS

The results obtained by culturing methods using selective media (TCBS) indicated that effluents from all three wastewater treatment plants in this study were contaminated with presumptive *Vibrio* species. The findings of this study showed that the presumptive *Vibrio* densities varied from 0 to 2.11×10^2 cfu/100 mL for the WWTP-K (as shown in Figure 4.1). In the WWTP-S, presumptive *Vibrio* isolates were isolated in densities ranging from 0 to 1.84×10^2 cfu/100 mL (as shown in Figure 4.2). Figures 4.3 and 4.4 show results for the WWTP-Z (final effluent (FE)) and WWTP-Z (discharge point (DP)). Differences in the number of colonies were observed for the months of February 2013 (1.07×10^2 cfu/100 mL (FE) and 1.34×10^2 cfu/100 mL (DP)), April 2013 (9.2×10^1 cfu/100 mL (FE) and 1.07×10^2 cfu/100 mL (DP)).

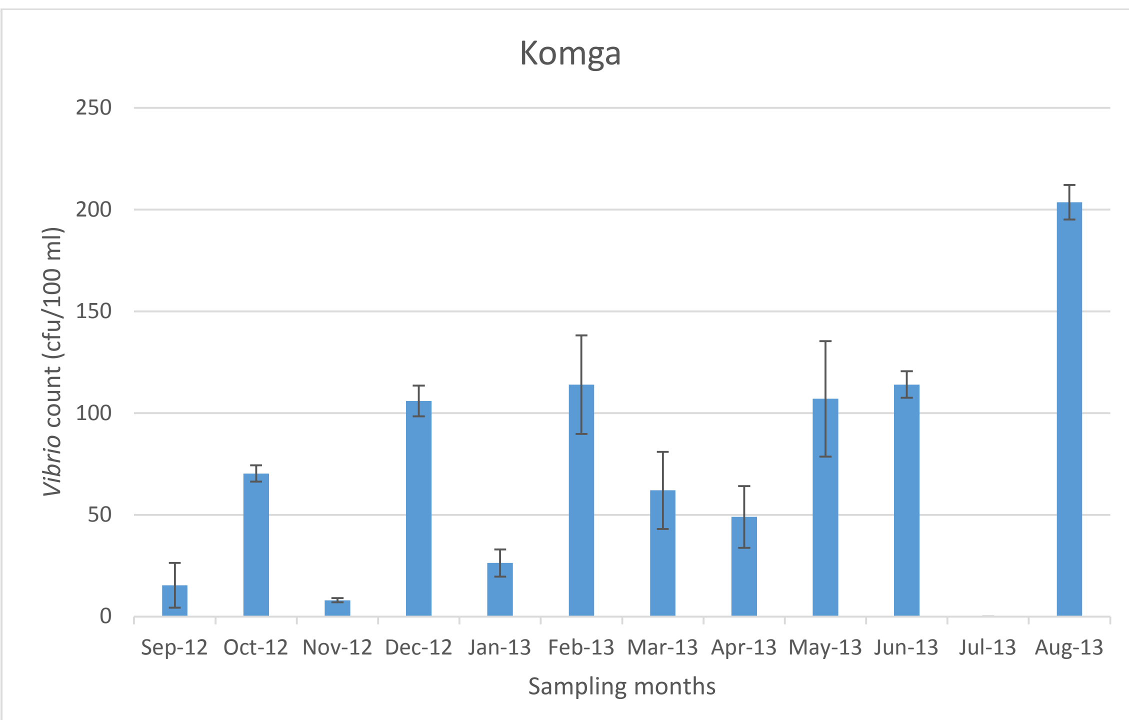


Figure 4.1: **Presumptive *Vibrio* counts for the WWTP-K between September 2012 and August 2013.**

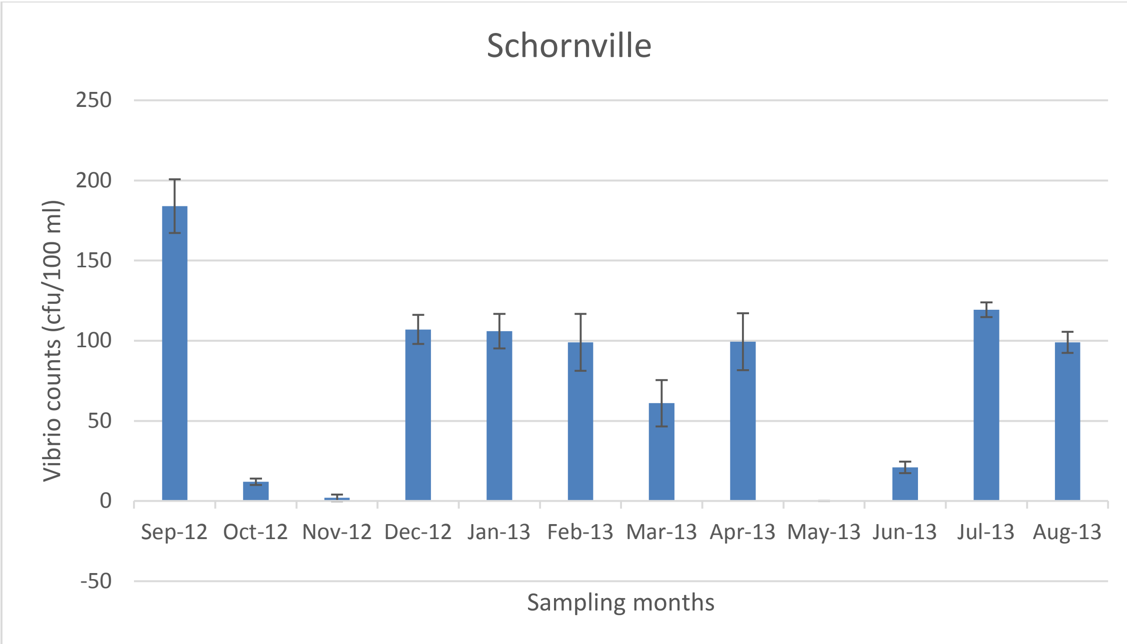


Figure 4.2: **Presumptive *Vibrio* counts for the WWTP-S between September 2012 and August 2013.**

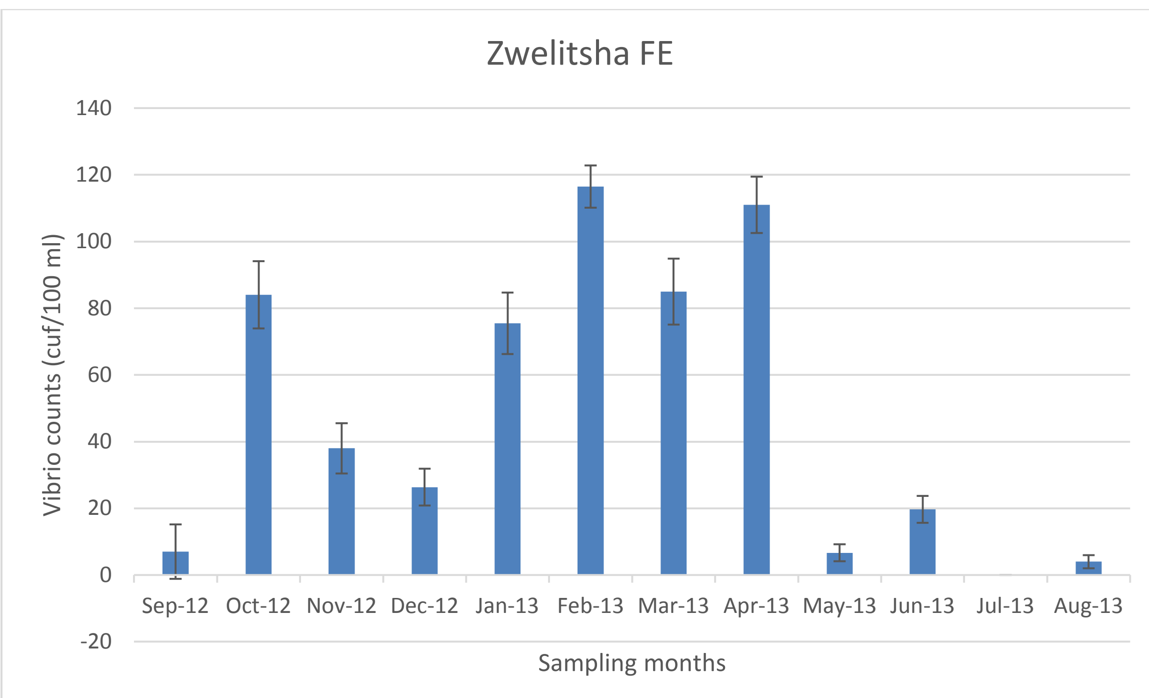


Figure 4.3: **Presumptive *Vibrio* counts for the final effluent of the WWTP-Z between September 2012 and August 2013.**

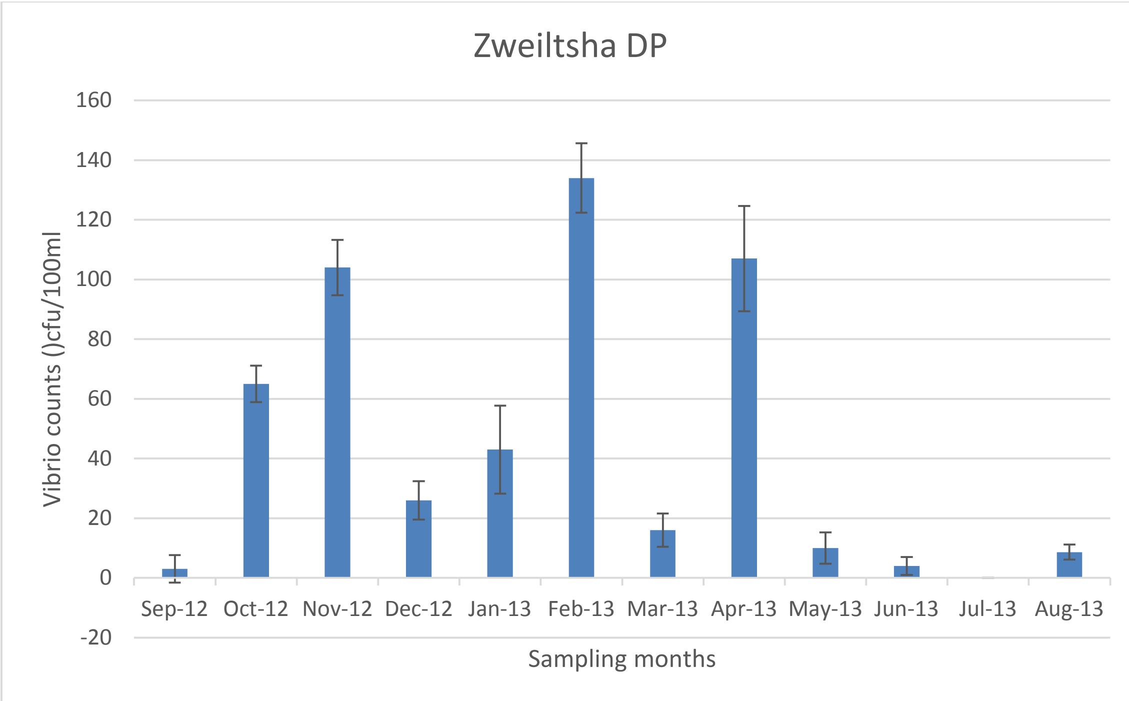
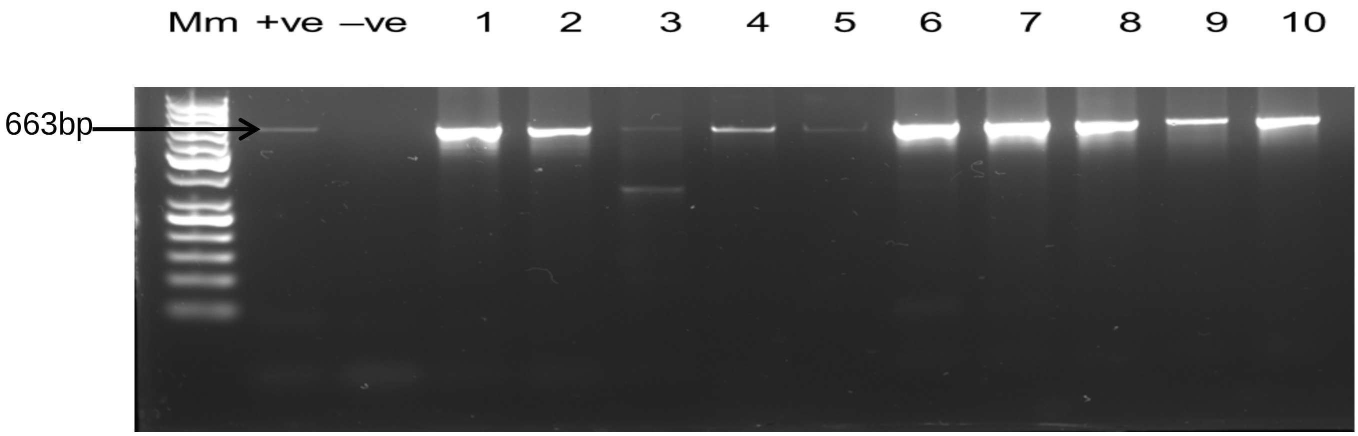


Figure 4.4: **Presumptive *Vibrio* counts for the discharge point of the WWTP-Z between September 2012 and August 2013.**

4. 2 Confirmation of *Vibrio* isolates

Out of 500 presumptive *Vibrio* isolates, 300 were confirmed to be *Vibrio* species using the polymerase chain reaction analysis. In this analysis the *16S rRNA (*663bp) specific primers confirmed that a genetic region homologous in size to the *Vibrio* 16S *rRNA* gene, including the regulatory region, was present in these *Vibrio* isolates. Figure 4.5 is a gel picture showing bands for some of the positive *Vibrio* isolates.



Legend: lane1: Molecular weight marker; lane 2: positive control; lane3: negative control; lanes 4 to 10 *Vibrio* isolates.

Figure 4.5: **Agarose gel electrophoresis of *Vibrio* species responding to the *16S rRNA* gene.**

4.3 Pathotyping

Three hundred confirmed *Vibrio* spp. isolates were further subjected to molecular characterization to confirm their respective pathotypes. Only 13.3% (40/300) of the isolates tested positive for the presence of *toxR* gene which confirmed them to be *Vibrio fluvialis*, 22% (66/300) were confirmed to be *Vibrio parahaemolyticus* using the *flaE* gene, and 24.7% (74/300) proved to be *Vibrio vulnificus* which matched with the *toxR* gene which was used as a control strain, and the rest were the *Vibrio* species which were not included in this study.

Table 4.1: *Vibrio* pathotypes detection frequency.

Percentage species detected (n =300)			
V. fluvialis	*V. parahaemolyticus*	*V. vulnificus*	Other Vibrio spp.
13.3% (40/300)	22% (66/300)	24.7% (74/300)	40% (120/300)

4.4 Antibiogram reporting

Vibrio fluvialis showed 100% resistance to Polymixin, Gentamicin and Tetracycline. All three species showed 100% susceptibility to Gentamicin, also these organisms were all 100% resistant to Tetracycline. The susceptibility profiles are presented in Table 4.2.

Table 4.2: **Antibiotic susceptibility profile for the three selected *Vibrio* spp.**

Antibiotic	***V. fluvialis* (n = 40)**			***V. parahaemolyticus* (n = 66)**			***V. vulnificus* (n = 74)**		
	S (%)	**I (%)**	**R (%)**	**S (%)**	**I (%)**	**R (%)**	**S (%)**	**I (%)**	**R (%)**
Cefuroxime	56	0	44	75	0	25	64	7.1	28.5
Chloramphenicol	10	0	90	75	0	25	92	0	7.1
Erythromycin	10	0	90	0	0	100	15	14	71
Gentamicin	100	0	0	100	0	0	100	0	0
Nalidixic acid	40	40	20	90	0	10	71	14	14.1
Penicillin g	0	10	90	10	0	90	0	0	100
Polymixin b	0	0	100	10	0	90	0	0	100
Sulfamethazole	0	0	100	0	20	80	14.2	0	85.7
Tetracycline	0	0	100	0	0	100	0	0	100
Trimethoprim	14	0	86	0	0	100	10	0	90

CHAPTER FIVE：DISCUSSION AND CONCLUSION

In South Africa, one of the most employed and economical method for disinfecting wastewater is chlorination (White, 1992). Between 2002 and 2003, Mohale (2003) discovered that of the 190 wastewater treatment plants recorded in the Eastern Cape, only 98 (51.6%) were supervised by DWAF. Even on the wastewater works that were monitored by DWAF only 12% met all the set discharge limits.

In this study, *Vibrios* were recovered from wastewater final effluents. Even after disinfection, these organisms still persist in the effluents in corroboration of previous reports (Gugliandolo *et al.*, 2005; Tampin *et al*., 1990). The South African Water Act of 1998 established requirements for treated wastewater to be released into surface water sources (National Water Act, 1998). This act was meant to ensure the compliance of treated wastewater effluents with set limits before their release into receiving water bodies.

All three wastewater treatment facilities in this study chlorinated their effluents. Certain standards have been put in place for the management of the free residual chlorine levels in South Africa (South African Water Quality Guidelines, 1996). It is expected that if these regulations are properly adhered to, pathogenic microorganisms will be eliminated or at the most, significantly reduced, from treated wastewater effluents. However, the outcome of this study revealed that although the free residual chlorine (Okoh *et al*., 2009), in the effluents of WWTP-K, WWTP-S and WWTP-Z conformed to the regulatory standards; they were unsuccessful in eradicating *Vibrio* species. In Figure 4.1, it was observed that the presumptive *Vibrio* counts varied from 0 to 2.11×10^2 cfu/100 mL and 0 to 1.84 × 102 cfu/100 mL for WWTP-K and WWTP-S, respectively. Differences in counts was witnessed for the months of February 2013

(1.07×10^2 cfu/100 mL (FE) and 1.34×10^2 cfu/100 mL (DP)), April 2013 (9.2×10^1 cfu/100 mL (FE) and 1.07×10^2 cfu/100 mL (DP)) for the WWTP-Z (final effluent (FE) and discharge point (DP)). This was contrary to expected results since the final effluent is expected to have higher counts than the discharge point owing to increased contact time (C_T) of the wastewater and disinfectant before it is finally discharged. It is well established that environmental factors such as seasonal variations, global climate change and natural disasters such as floods play a crucial role in the resurgence and dynamics of infectious diseases (Epstein, 2001; Senior, 2007), and the findings of this study are in agreement with these reports. Warm water temperatures seem to have a positive outcome on the abundance of human-invasive pathogens. It has thus been confirmed for pathogenic *Vibrio* species including *V. parahaemolyticus* and *V. vulnificus* (Heidelberg *et al*., 2002; Wright *et al*., 1996).

Also, the findings of this study proved this because in all the three sampling points, between December 2012 and February 2013, the Summer season, very high *Vibrio* counts were observed owing to high water temperature during this season, and low counts were observed during the cooler season (between April 2013 and July 2013). In a study done by Igbinosa *et al*. (2009), *Vibrio* species seem to be abundant at a temperature range between 17 and 27 ºC, demonstrating a steady dependency of the culturable forms of the pathogens on temperature. In September 2012 and July 2013, across the sampling points the counts obtained at the WWTP-S are significantly higher than those obtained from other points ($P \leq 0.05$). During the summer season the Vibrio counts are generally high for all the months in all three study sites, and this could be the influence of the warm water temperatures which are somewhat helpful for the survival of these organisms. But the high counts observed in July which is not so warm, could have been due to low concentrations of chlorine that was added for disinfection. At the WWTP-K a significant variance in *Vibrio* counts (very high counts) ($p \leq 0.05$) was witnessed in August owing to the information that this wastewater facility was faulty in the month of July. The WWTP-Z (discharge point) showed significantly high counts in February 2013 in comparison to the WWTP-Z (final effluent), theoretically the inverse of this was expected, however, the reason for the higher *Vibrio* counts seen at the point of ejection could have be because the disinfectant was still concentrated at the final effluent and there wasn't enough time for the disinfectant and the wastewater to mix properly.

In order to effectively characterize *V. fluvialis*, *V. parahaemolyticus* and *V. vulnificus*, molecular methods were used. Culture based methods are mainly useful in the preliminary identification of organisms but they lack sensitivity and reproducibility and hence they are not suitable for unequivocal identification of microorganisms. It has been proposed that molecular methods offer the advantages of accuracy, speed, sensitivity, and reproducibility. These methods are usually based on DNA or RNA-based identification of microorganisms (Appelbaum *et al*., 1984). Molecular techniques were successfully employed to confirm the presumptive *Vibrio* isolates. Confirmed Vibrio species were further subjected to molecular characterization to confirm their respective pathotypes.

Antimicrobial resistance has turned out to be one of the most important medical and public health dilemma as it has direct links with disease management (Ramamurthy, 2008). Numerous pathogens including *V. fluvialis*, *V. parahaemolyticus* and *V. vulnificus* are notorious for resistance to many antibiotics (Rowe-Magnus *et al*., 2006; Ahmed *et al*., 2004). This study employed the disk diffusion method for the characterization of the antibiogram of the isolates.

Over 30 antibiotics have been reported in the wastewater influent and effluent samples (Jones and Oliver, 2009). Even small traces of antibiotics may adversely impact the environment and public health due to their natural bioactivity (Ceccarelli *et al*., 2006) and the risk of encouraging a selection pressure for resistant organisms. *Vibrio fluvialis* showed 100% resistance to Polymixin, Sulfamethazole and Tetracycline, 90% to Penicillin g and Erythromycin, and 86% to Trimethoprim. *Vibrio parahaemolyticus* exhibited 100% resistance to Erythromycin, Tetracycline, and Trimethoprim; 90% resistance to Polymixin b and Penicillin g were also observed for this microorganism. Gentamicin was 100% active against all the test *Vibrio* strains, also, all the organisms exhibited 100% resistance to Tetracycline. Antimicrobial resistance (AMR) was observed in all the three *Vibrio* species. In a similar study (Igbinosa *et al*. 2010), treated effluents were reported to be the reservoir for *V. fluvialis* strains which are resistant to Ampicillin, Penicillin-G, Streptomycin, Sulfamethoxazole, Trimethoprim, Chloramphenicol, Erythromycin, Ciprofloxacin, and Polymyxin B (Igbinosa *et al*., 2011). Liang *et al*. (2013) reported that most strains of the same species were resistant to sulfamethoxazole. Efflux systems responsible for Nalidixic acid resistance have been reported in many *V. fluvialis* strains (Srinivasan *et al*., 2006). From the findings of this research, it can be deduced that treated wastewater effluents are prospective reservoirs of a variety of antibiotic resistant genes.

5.1 CONCLUSION

Based on the findings of this research, it can be concluded that the wastewater treatment processes of the treatment facilities under this study are not capable of significantly reducing *Vibrio* pathogens from their final effluents. This is a major concern especially for the health purposes for the downstream users. The information obtained may be useful to the health institutions and local municipalities of the Eastern Cape Province in curbing *Vibrio* epidemics in future.

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