Cholera Monitoring and Response Guidelines

Report presented to the
Water Research Commission

by

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WRC Report No 2432/1/18
ISBN 978-0-6392-0063-7

November 2018

WATER
RESEARCH
COMMISSION

Obtainable from
Water Research Commission
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This report form part of a series of two reports. The other report is *A Manual for the Monitoring of Cholera and Non-Cholera causing Vibrio Pathogens in Water, Vegetables and Aquatic Animals* (WRC Report No. TT 773/18).

EXECUTIVE SUMMARY

Water has been recognised as one of the critical elements for sustainable socio-economic development and set at the core of sustainable development (Saravanan *et al*., 2009). It is directed towards ensuring the improvement of health and living conditions, and, in turn, sustaining the use of natural resources, with the goal of providing a better life for all. Thus, regulating and sustaining this resource is of utmost importance. However, about 1.1 billion people lack access to safe drinking water worldwide (Harshfield *et al*., 2009), and as such depends on available freshwater resources for drinking and domestic purposes. Unfortunately, the qualities of many of these freshwater resources are often compromised by industrial- and treated municipal wastewater effluents, thus subjecting such communities that directly rely on such freshwater resources including rivers, streams, wells, dams, pond water sources to the risk of contracting waterborne diseases such as cholera (District *et al*., 2014). Additionally, these water resources are major abstraction water sources for potable water production and their contamination could influence the cost of treatment to potable water status. Among the pathogens found in water resources, enteric pathogens such as *Vibrio* species are often encountered. Many studies have indicated that untreated and treated wastewater is a major source of pollution of water resources. Inadequately treated wastewater effluents from wastewater treatment plants have been associated with death threatening diarrheal diseases (Igbinosa, 2010). In South Africa, it has been reported that many municipal wastewater treatment plants are not discharging effluents of acceptable quality (Okoh *et al*., 2014; Mema, 2004), causing increased densities of pathogens in the receiving waterbodies. This becomes a public health threat as many communities directly rely on these receiving waterbodies for their daily activities. There are guidelines established for the qualities of wastewater effluents destined for discharge into their receiving waterbodies. However, there is evidence that suggest that the organisms causing illnesses or even outbreaks are not necessarily the ones that are routinely assayed for. Studies have indicated the need to monitor not only the classical pollution indicators, i.e. culturable total, or faecal coliforms; but also viral pathogens, toxigenic *E. coli*, and highly infectious bacterial pathogens such as Vibrio spp. (Griffith *et al*., 2006; Wyn-Jones *et al*., 2011; CDC, 2012) and further justifies the subject of this current study on *Vibrio* species pursuant to developing a cholera monitoring and response guidelines with particular emphasis on the Eastern Cape Province. From the height of the outbreak that occurred in the Eastern Cape in 2004, in which 738 people were diagnosed with cholera and four recorded deaths, it is still the country's most impoverished province, identified as one of the worse off provinces in relation to resources, socio-economic status and burden of disease. It also has the lowest proportion of people with access to potable water supplies and notably a significant number of infant and child deaths under the age of five, just to mention a few (BLACKSASH, 2010). Increasing amounts of sewage being discharged into rivers, treated or untreated, in recent times, has not helped matters in the Eastern Cape Province, leading to the deterioration of the quality of major rivers. There is, however, paucity of information on the epidemiology of vibrios and ecology of the same in the aquatic milieu of the Eastern Cape Province, South Africa. This is despite the fact that over the

past few years, the region encompassing South Africa has been plagued by outbreaks of *Vibrio*-related waterborne infections that are suspected to be linked to inefficiently treated effluents discharge from wastewater treatment facilities (Igbinosa *et al*., 2011). If the scourge of cholera is to be put under control, this gap needs to be urgently closed through research targeted at understanding the ecology and dynamics of vibrios both in the aquatic resources in the Province and final effluents of wastewater treatment plants.

PROJECT AIMS

The overall aim of this research is the development of a cholera monitoring and response guidelines for inclusion in the water resource monitoring programme. To achieve this, the following specific aims are set:

1. To assess the various types of wastewater treatment processes that are used in the Eastern Cape Province (ECP);
2. To evaluate the laboratories used for sample analyses and whether these are accredited or not;
3. To verify the analytical methods used in the laboratories in line with the green-drop reporting system;
4. To monitor the compliance of the wastewater treatment plants (WWTP) in the Eastern Cape Province to operational standards and determine the rate and reason(s) of failure of the treatment technologies;
5. To evaluate the implemented measures put in place to ensure that wastewater treatment occurs during failure;
6. To assess the prevalence of Vibrio pathogens in discharged final effluents of WWTP and rural waters in the ECP and make recommendations on the reason(s) contributing to the prevalence of these pathogens in rural waters.
7. To ascertain the strains of Vibrio pathotypes that are common in the aquatic milieu of the Eastern Cape Province as well as their epidemiology with a view to tackling the recurring scourge of cholera (and cholera-like diarrhoea) outbreaks in South Africa.
8. To evaluate aquatic animals (including shrimp, fish, crab, crayfish) and vegetables as potential reservoirs of Vibrio pathogens.
9. To compile and submit report on the findings to the WRC and other relevant stakeholders.

CHANGES MADE TO THE AIMS

The Reference Group meeting suggested that pursuant to achieving aims 6 to 9 listed above it is important for the team to first carry out a cholera hotspots survey of the Eastern Cape Province and subsequently select the identified hotspots for the detailed study.

METHODOLOGY

Administration of questionnaires (aims 1, 2, 3, 4 and 5)

The questionnaires as presented in Deliverable 2A were administered to the following wastewater treatment plants: Adelaide WWTP, Craddock WWTP, Alice WWTP, Aliwal North WWTP, Sterkspruit WWTP, Maclear WWTP, Ugie WWTP, Mthatha WWTP, Butterwort WWTP, Mayfield WWTP, Belmont valley WWTP.

Cholera hotspot survey (pursuant to aims 6-9)

Rivers

The cholera hotspot survey commenced with the Tyhume and Kat River catchments both of which are important freshwater resources within the Amathole District Municipalities in the Eastern Cape Province. Subsequently, water samples were collected from Great fish river, Tsitsa river, Mthatha river, Mbashe river, Qunu river, Kowie river, Boesman river, Buffalo river, Mqanduli river, Ngqeleni river, Kubusi river, Tsomo river, Broukrans, Ngcorgora river, White kei river, Ooskleinemonde, Weskleinemonde, Swartkops, Mzimvubu, Quamanco, Mgwali, Ngonyama, Xuka, Gonube river, Nahoon river, Kwelera, Langkloof, Karnmelkspruit, Orange river, Kraai river, Saalboomspruit river, Mooi river; all in other District Municipalities covered in this project. Sites along the rivers that have close proximities to human settlements and farming communities (and that are accessible) were prioritized.

Wastewater Treatment Plants

Wastewater treatment plants investigated during the hotspot survey included Adelaide WWTP, Craddock WWTP, Alice WWTP, Aliwal North WWTP, Sterkspruit WWTP, Maclear WWTP, Ugie WWTP, Mthatha WWTP, Butterwort WWTP, Mayfield WWTP, Belmont valley WWTP.

Sample collection and processing

Rivers

Water sample collection was carried out from identified sampling points along the stretch of the Great fish river, Tsitsa river, Mthatha river, Mbashe river, Qunu river, Kowie river, Boesman river, Buffalo river, Mqanduli river and Ngqeleni river, Kubusi river, Tsomo river, Broukrans, Ngcorgora river, White Kei river, Ooskleinemonde, Weskleinemonde, Swartkops, Mzimvubu, Quamanco, Mgwali, Ngonyama, Xuka, Gonube river, Nahoon river, Kwelera, Langkloof, Karnmelkspruit, Orange river, Kraai river, Saalboomspruit river, Mooi river between September 2015 and October 2016 using sterile 1 L bottles. All water samples were transported on ice from the sampling sites to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, Alice for analyses within 6 hours of collection.

Wastewater treatment plants

Final effluent samples were collected from the Adelaide WWTP, Craddock WWTP, Alice WWTP, Aliwal North WWTP, Sterkspruit WWTP, Maclear WWTP, Ugie WWTP, Mthatha WWTP, Butterwort WWTP, Mayfield WWTP, Belmont valley WWTP in the different municipalities in the Eastern Cape Province. Samples were also collected from the abstraction point (those accessible) and their receiving waterbodies (rivers) 500 m up- and downstream from abstraction point. All samples were collected in sterile 1 L bottles containing 1.7 ml sterile 3% (w/v) sodium thiosulpate solution to de-chlorinate the sample. Samples were either transported on ice from the sampling sites to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, Alice for analyses within 6 hours of collection; or analysed on site and isolates transported to the laboratory on ice for further analysis.

Detection of *Vibrio* species in the water samples

Water samples collected from the different sampling sites were assessed for the presence of *Vibrio* species. The water samples were firstly concentrated by membrane filtration technique as recommended by APHA (1998). A volume of 100 mL of each water sample was filtered through a sterile polycarbonate membrane filter (0.45 µm pore size, 47 mm diameter) and the filtration process was facilitated using a vacuum/pressure pump. Alkaline peptone water (APW) was used for enrichment of the samples followed by incubation at 36°C for 18-24 h. At the end of the incubation period, total genomic DNA was extracted from the enrichment cultures. The enriched cultures were also streaked out for isolation on TCBS agar and distinct colonies further purified on nutrient agar and subjected to molecular identification.

DNA extraction was done using the method described by Maugeri *et al. (*2006) with slight modification. About 1 mL of the enriched culture was transferred into sterile 2 mL microfuge tubes. The microfuge tubes were then centrifuged at 11000 × *g* for 10 min, the supernatant was discarded, and pellet suspended in 200 µl of sterile nuclease free water and vortexed. Thereafter, they were boiled using a heating block for 10 min at 100°C. The cell debris was removed by centrifugation at 11,000 × g for 10 min using a Mini-Spin micro-centrifuge (LASEC, RSA). The supernatant (10 µl) was transferred into new sterile microfuge tubes and then used as template DNA in the Polymerase Chain Reaction (PCR) assays. All genomic DNA extracted were screened for the presence of the genomes of *Vibrio* genus and *Vibrio cholerae* using specific primers. The PCR conditions are as reported elsewhere (Nongogo and Okoh, 2014).

Detailed vibriology study (to finalize aims 6-9)

Based on the findings of the hotspot survey the following WWTPs and Rivers were selected for the detailed vibriology study**:**

- **WWTPs:** Butterworth WWTP, Adelaide WWTP, Belmont Valley WWTP, Mayfield WWTP, Mthatha WWTP and Craddock WWTP
- **Rivers:** Kat river, Keiskamma river, Qunu river, Mthatha river, Tsitsa river, Great Fish river, Kubusi river, Tsomo river and Kowie river

The choice for these study sites was based on the high prevalence of *V. cholerae* from the metagenomic DNA recovered from the water samples from the sites and the fact that some residents along the river courses use the resources for drinking, agricultural, recreation and other domestic needs. Furthermore, a couple of WWTPs discharge their final effluents into these rivers. For the WWTPs the high prevalence of at least one *Vibrio* species was used as an index for selection. The comprehensive vibriology study also included selected vegetables and aquatic animals as per the aims and objectives of the project, and methods are similar to those used for the hotspots survey with the addition of antibiogram, virulence genes and speciation component following after standard methods described in detail in the report. A summarised stand-alone cholera monitoring and response guideline was also compiled.

SUMMARY OF MAJOR FINDINGS

The major findings of this study are as summarised below:

- Reports from WWTPs managers as inferred from the questionnaires suggest that the wastewater effluent compliance of these wastewater treatment plants surveyed had complications as none of the wastewater treatment plant operators had any clue on their level of compliance to set Greendrop guidelines.
- On average, 88% of the WWTP complied with process control and maintenance and management skill requirements; 59% carried out wastewater monitoring; 76% submits their final effluent for analysis; 65% had proof of a documented wastewater incident management protocol, responding to wastewater quality failures; 70% had documentation of authorisation detailing effluent quality standards; 53% had a microbiological compliance of greater than 90%; and 47% were functioning within their design capacity.
- Feedback from the questionnaire survey for the microbiological analysis carried out by approved laboratory reveals that the scope of microbiological examination rely only on culture based presumptive identification with no confirmation through the more specific molecular identification techniques. Also the target organisms for the water samples from the WWTPs do not include potentially pathogenic *Vibrio* species which has great health significance to humans.

Hotspots survey

Rivers

A total of 141 water samples were collected from the 37 rivers. All the rivers were positive for the Vibrio genus and over 80% of them were positive for *Vibrio cholerae*. With respect to other

pathogenic Vibrio species none were detected in Saalboomspruit, Kraai, Orange, Karnmelkspruit, Langkloof, Broukrans, Kubusi, Keiskamma and Kat rivers. The proportions of the rivers that harboured the other pathogens follow the order: *V. paraheamolyticus* (61%); *V. fluvialis* (35%); and *V. vulnificus* (26%). However, no toxigenic strains of the *V. cholerae* was detected suggesting that cholera appears to have been significantly controlled in the Eastern Cape Province at least within the precinct of its aquatic environment, but the presence of other diarrheogenic strains is a cause for concern.

Wastewater Treatment Plants (WWTPs)

A total of 14 WWTPs were sampled and 215 presumptive Vibrio isolates were recovered from the effluents. About 39% of the presumptive Vibrio species were confirmed as belonging to the Vibrio genus and in only one case (Mayield WWTP) was *V. cholerae* detected. The detected *V. cholerae* was not a toxigenic strain which corroborates our earlier report on the rivers. Also, *Vibrio parahaemolyticas*, *Vibrio fluvialis* and *Vibrio vulnificus* constituted 7%, 8% and 15% of the confirmed Vibrio species respectively.

Detailed vibriology study

Rivers

A total number of 328 confirmed isolates were identified from River samples collected from 10 rivers. About 24% were confirmed as *V. cholerae*, 3% as *V. vulnificus*, 5% as *V. alginoliticus*, 3% as *V. fluvialis*, 10% as *V. paraheamoliticus* and 29% as *V. mimicus*. The antibiotic resistance profile of the Vibrio species revealed multiple antibiotic resistance against a panel of 18 antibiotics. All the *V. cholerae* isolates exhibited resistance against polymyxin B, while 82%, 3% and 8% of this species were resistant against nitrofurantoin, ofloxacin and azithromycin respectively. *V. mimicus* exhibited significant resistance against some of the antibiotics in the following proportion: polymyxin B (94%), ampicillin (91%), nitrofurantoin (88%), gentamycin (6%) and ofloxacin (17%). *V. fluvialis* showed the highest resistance against ampicillin (100%) and nalidix acid (75%). The lowest resistance frequency was against gentamycin (8%), amikacin (8%) and meropenem (8%). *V. paraheamolyticus* showed a high resistance frequency against polymyxin (97%); and no resistance against ofloxacin. All the *V. vulnificus* isolates were resistant against ampicillin while 80% and 90% of the isolates were resistant against nitrofurantoin and polymyxin B respectively. *V. alginolyticus* was highly resistant against ampicillin (94%), kanamycin (72%) and nitrofurantoin (72%). The least resistance frequency was against the remaining antibiotics and ranged between 0 to 22% of the isolates.

Wastewater Treatment Plants (WWTPs) and receiving waterbodies

A total of 6 WWTPs were sampled and 148 confirmed Vibrio isolates were recovered from the final effluents. Of these, 25% were identified as *V. cholerae*, 9% as *V. vulnificus*, 8% as *V. alginoliticus*, 9% as *V. fluvialis*, 45% as *V. paraheamoliticus* and 6% as *V. mimicus*. The others confirmed Vibrio species made up 14%. Multiple antibiotic resistance was exhibited by the Vibrio species against a panel of 18 antibiotics. *V. cholerae* exhibited resistance against 17 of the 18 antibiotics at frequescies of 6.4% against azithromycin to 93.5% against ampicillin. *V.*

mimicus isolates displayed some level of resistance against all the antibiotics; with the highest resistance against polymyxin B (by 94.1% of the isolates). The *V. vulnificus* isolates exhibited resistance against only 8 antibiotics with all the species resistant against ampicillin and nalidixic acid. Resistance in *V. parahaemolyticus* was against 17 of the 18 antibiotics with the highest resistance against ampicillin. Remarkable resistance patterns were observed also with *V. fluvialis*, *V. alginolyticus* and other uncharacterized Vibrio species.

Vegetables

A total of 64 confirmed Vibrio isolates were recovered from vegetable samples purchased from wet markets and supermarkets. Of the confirmed isolates, 34.4%, 21.9%, 1.7%, 3.1% and 1.7% were *V. cholerae, V. parahaemolyticus, V. fluvialis, V. mimicus* and *V. alginolyticus* respectively. The frequency of antimicrobial resistance of the Vibrio species recovered from vegetables was observed to be highest against polymyxin B with a frequency of 92.2% followed by 90.6% against amoxicillin-clavulanic acid and 81.2% against imipenem. The targeted species (*V. cholerae, V. mimicus, V. parahaemolyticus*, *V. alginolyticus* and *V. fluvialis*) exhibited resistance against 9 to 16 antibiotics. The presence of pathogenic Vibrio species in the vegetables and the high rate of antibiotic resistance displayed are worrisome. This highlight public health concerns about vegetable as a source of antibiotic resistance Vibrio species in humans.

Aquatic animals

The prevalence of *V. cholerae*, *V. mimicus, V. fluvialis, V. alginolyticus* and *V. parahaemolyticus* in the confirmed Vibrio spp. (n= 189) recovered from the aquatic animals were 26%, 9%, 4%, 23% and 38%, respectively. About 50% and above of all the pathogenic *Vibrio* species (*V. cholerae, V. mimicus, V. fluvialis, V. alginolyticus* and *V. parahaemolyticus*) recovered from the aquatic animals exhibited resistance against ampicillin. About 50% and above of the *V. cholerae* and *V. mimicus* isolates exhibited resistance against Polymyxin B, while 50% and above of the *V. fluvialis* isolates were resistant against kanamycin, nalidixic acid, azithromycin, nitrofurantoin and polymyxin B, and varying degrees of resistance by the targeted *Vibrio* pathogens against the remaining antibiotics used in this study was observed. All the isolates were susceptible to ofloxacin.

RECOMMENDATIONS FOR FUTURE INTERVENTIONS

- Previous reports and this present study has shown the survival of pathogenic Vibrio species in wastewater treatment plants effluent and the implications of the dissemination of the pathogens into receiving watershed and consequently infecting humans. There is therefore the need to include pathogenic Vibrio species in routine microbial quality assessment of water and wastewater final effluents. This will help in checkmating the spread of cholera and non-cholera causing vibrio infections in human population.

- There is a need for the engagement of more qualified personnel and re-training of existing staff working in wastewater treatment facilities pursuant to ensuring efficiency of the wastewater treatment processes towards meeting recommended standards. Also, detailed response strategy documents should be available in wastewater treatment plants in case *Vibrio* pathogens are detected. This should include protocols to follow for further analyses and identification of the pathogens; to indicate potential for outbreak or risk to human health; and alerting relevant authorities and stakeholders about the detections and what to do.
- There is the need for the development and integration of innovative methods for the removal of nucleic acids (and consequently antibiotic resistance genes) in wastewater effluents before discharge into the environment so as the curb the spread of antibiotic resistance determinants.
- The Green Drop initiative should encourage managers to ensure strict compliance to its standards towards improving the operational strategies of wastewater treatment plants in a manner that enhances good performance of the plants.
- There is a necessity for regular monitoring of vegetables, aquatic animals and freshwater resources used for irrigation, drinking, and domestic purposes for the presence of Vibrio pathogens. This will be a significant tool in monitoring the dynamics of this pathogen in the environment and foster proactive alertness that could be instrumental in averting cholera and non-cholera *Vibrio* infections and outbreaks.

CAPACITY-BUILDING

A total of 11 postgraduate students, consisting of 5 Master's and 5 doctoral students, have developed capacity in microbial water quality assessment techniques including culture-based and molecular diagnostic techniques based on the highly sensitive and reliable polymerase chain reaction (PCR) procedures and other microbiological procedures. The Master's students have graduated in 2017 while the doctoral students are scheduled to graduate May 2018. These students will form an important critical mass in water quality and infectious diseases sectors with particular emphasis on vibriology competencies. The list of all the students involved in this project is as follows:

- **MSc Microbiology:**

Ms S Gaqavu (Graduated in 2017)
Ms S Chisi (Graduated in 2017)
Ms D September (Graduated in 2017)
Ms CE Iheanacho (Graduated in 2017)

- **PhD Microbiology:**

Ms N Nontongana (Graduating in May 2018)
Mr. TO Fadare (Graduating in May 2018)
Mr. OE Abioye (Graduating in May 2018)
Ms A Okeyo (Graduating in May 2018)
Mr. AC Osunla (Graduating in May 2018)
Ms O Gcilitshana (In view)

ACKNOWLEDGEMENTS

The project team wishes to thank the following people for their contributions to the project.

Reference Group	**Affiliation**
Dr J Molwantwa	Water Research Commission (Chairperson)
Dr E Ubomba-Jaswa	Water Research Commission (Chairperson)
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Mr G Sery	Amathole District Municipality
Mr V Ntema	Magalies Water
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ACRONYMS

AEMREG	Applied Environmental Microbiology Research Group
CFU	Colony Form Unit
DNA	Deoxyribonucleic Acid
DWAF	Department of Water Affairs
ECP	Eastern Cape Province
PCR	Polymerase Chain Reaction
TCBS	Thiosulfate Citrate Bile Salt Sucrose
WHO	World Health Organisation
WRC	Water Research Commission
WWTP	Wastewater Treatment Plant

PREAMBLE

This document represents the final report on this project as the 6th deliverable. Five deliverables have earlier been presented and included deliverable 2A (Detailed literature review and study plan), deliverable 2B (Report on treatment processes and laboratories), deliverable 3 (Progress Report), deliverable 4 (Bursaries) and deliverable 5 (Progress report pursuant to deliverable 5 as it was work in progress then). This final report therefore presents a comprehensive overview of the project in line with the set aims and objectives.

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CHAPTER 1

1.1 INTRODUCTION

The Eastern Cape Province straddles two worlds: a severe underdeveloped rural world and a modern, growing urban base. By many measures, it is still the country's most impoverished province, having inherited the apartheid government's deliberate underdevelopment of the 'homeland' areas (ECDC, 2007). This province has been identified as one of the worst off provinces in relation to resources, socio-economic status and burden of disease. Its major indicators include; the lowest proportion of people with access to potable water supplies, electricity and sanitation out of all nine provinces, the highest level of poverty out of all nine provinces, the highest number of infant deaths, and the second highest number of child deaths under the age of five (BLACKSASH, 2010). As of 1996, 45.1% of households in the Eastern Cape still used water from streams, rivers, boreholes, springs and dams/pools. Although this proportion dropped to 22.2% in 2011 (ECSECC, 2011), it still represents significantly higher pockets of the Eastern Cape population if considered by districts. In OR Tambo District for example, 36% of the population had no access to piped water as of 1997, and 28% made use of a river or stream, 2% made use of a spring, 2% of a borehole and 2% of a pool or dam where water is stagnant (National Road Agency, 1997). Unfortunately, the picture obtained in OR Tambo District in 1997 did not significantly change from the one obtained in 2011, as shown by the 2011 National Census results (ECSECC, 2011). Increasing amounts of sewage being discharged into rivers, treated or untreated, in recent times, has not helped matters in the Eastern Cape Province either, as this has led to the deterioration of the qualities of major rivers, same of which are used for drinking and other purposes by the rural people. Wastewater contains a wide range of pathogens and, of the treated sewage discharges, few often meet the set final effluents standards (Arceivala, 1997; Igbinosa and Okoh, 2009), thus contributing to increased densities of disease causing bacteria in the receiving waterbodies and subsequently in increasing incidences of emerging pathogens. As these waterbodies are the major sources of potable water, their contamination has led to many water related disease outbreaks in the past (Mishra *et al.,* 2004; Nair *et al.,* 2004; Obi *et al.,* 2004). In August 2000, South Africa experienced the start of one of the worst cholera epidemics nationwide in recent history. By July 2001, 106 389 people had been infected and 232 had died. The wrath of the epidemic hit KwaZulu-Natal, and Gauteng, Mpumalanga and the Northern Province were also affected. In 2004, 1773 cases were reported in Mpumalanga's Nkomazi region, which borders Mozambique in which 29 people died. During the same year, 738 people were diagnosed with cholera in the Eastern Cape, four of which died. In the North West Province, 260 cholera cases were reported, and two people died. A year earlier, South Africa had suffered a cholera outbreak in which 3 901 cases were reported in Mpumalanga, the Eastern Cape and KwaZulu-Natal and a total of 45 deaths were recorded (News24.com, 2008). In 2007, a malfunctioning and decaying water reticulation and purification system resulted in 80 diarrhoea-related child deaths in the greater Barkly East area, Ukhahlamba District Municipality, Eastern Cape Province (Bateman, 2008). In 2009, the South African

Health Ministry spokesperson confirmed that cholera is endemic in South African water resources (Staff Reporter, Mail and Guardian, 2009). Similarly, on the 24th of January 2014, Fort Beaufort and its surrounding communities had an outbreak of diarrhoea with mortality and many hospitalizations as was reported in the SABC news and residents attributed the poor quality of municipal supplied water as the cause of the malady (SABC; https://www.SABCNewsOnline/posts/10152142053906543). Typical report includes the outbreak in Bloemhof reported in 31st of May 2014, and another in Upington with infant mortalities on 30th of May 2014 (SABC news: http://article.wn.com/view/2014/06/04/Diarrhoea_Outbreak_Hits_Chinhoyi/). In our previous studies, we reported on the incidences of Vibrio species in three wastewater treatment systems in the Eastern Cape Province ((Igbinosa *et al*., 2009; 2011; 2012; Okoh and Igbinosa, 2010). In our other study (WRC Project K5/2145) we report high counts of Vibrio species in the final effluents of some wastewater treatment plants, and in some cases in the presence of high concentration of chlorine disinfectant, suggesting that the pathogens are developing resistance to the regular recommended dose of chlorine disinfectant. While it can be assumed that the presence of these Vibrio pathogens in final effluents in the receiving watersheds is a major problem, no effort was made to investigate the epidemiology of the Vibrio pathogens in the rural surface waters, which along with a detailed microbial source tracking data are key ingredients in ascertaining the impacts of the wastewater treatment plants as sources of Vibrio pathogens in the aquatic milieu. Also, sometimes a cholera outbreak is reported without any clear linkage of index case to neighbouring countries or travel to affected areas. This usually raises the question on where the cholera causing bacteria could have originated from. We hypothesise that such outbreaks could be related to either the persistence of organisms as free-living, altered or adapted forms capable of reverting to a pathogenic variant or to continuous year round transmission by sub-clinical cases or a combination of both, and we therefore propose the inclusion of *Vibrio* pathogens monitoring in wastewater final effluents so as to ascertain the dynamics of this pathogen in the province's population and aquatic environment and to be able to contain the pathogen before it results in disease outbreak.

1.2 RATIONALE

Cholera infection continues to be a substantial health burden in developing countries, especially in Africa and Asia, due to lack of proper hygiene and sanitation infrastructure. Strains of the causative agent *V. cholerae* are classified into 206 serogroups (O1-O206). The O1 serogroup, which has been implicated in many cholera epidemics, is further classified into two biotypes, namely, classical and El Tor (Naha *et al*., 2013). Serogroup O139 is restricted to some parts of Asia whereas serogroup O1 is found worldwide (Marin *et al*., 2013). Africa is endemic for cholera and frequently affected by outbreaks and epidemics (Marin *et al*., 2013). Healthy carriers of *V. Cholerae* excrete vibrios intermittently with the duration of pathogen discharge being relatively short, averaging 6 to 15 days with a maximum period between 30 to 40 days. Chronic convalescent carriers have been observed to shed vibrios intermittently for periods of 4 to 15

months (Nevondo and Cloete, 2001). The survival of vibrios in the aquatic environment relates sharply to various chemical, biological and physical characteristics of a given waterbody. Also the viability of *V. cholerae* in surface waters has been observed to vary from 1h to 13 days and although cholera vibrios may persist for only a short while in grossly polluted aquatic environment, faecal contamination from victims of epidemics and the carriers may continue to reinforce their population in water (Nevondo and Cloete, 2001). In South Africa and much of the developing world, people in some rural areas use surface water sources for domestic activities, bathing, cultural and religious purposes, thus increasing the risks of exposure to such biological hazards from sub-quality water sources, especially those impacted with sewage effluents. For these reasons, there is need for the regulation of biological quality of both wastewater effluents and the receiving waterbodies. Also, though some studies have been carried out on the occurrence of *Vibrio* pathogens in the Eastern Cape Province (Igbinosa *et al*., 2011; Okoh and Igbinosa, 2010; Igbinosa *et al*., 2009), the studies were restricted to the final effluents of a few wastewater treatment plants, which is grossly inadequate to represent the aquatic milieu of the Eastern Cape Province. Our previous studies show that some Vibrio species appear to survive the activated sludge based wastewater treatment plants as free cell and as plankton associated entities (Igbinosa *et al.,* 2011; 2009). This suggests that the provision of wastewater treatment facilities does not in itself ensure production of effluents of satisfactory quality. In a study carried out by Mohale (2003), it was found that of the 190 treatment works listed in the Eastern Cape, only 98 (51.6%) were monitored by DWAF between 2002 and 2003. Of those that were monitored, only 12% met all the set discharge limits. Of particular concern is the high level of indicator organisms measured in some of the effluents with some recording faecal coliform counts of over 10^4 CFU/100 ml, which is 100 times the prescribed discharge limit (Antrobus, 2003). The attendant consequences of the impact of such inadequately treated wastewater effluents are the compromising of the primary health of people especially with death threatening diarrhoeal disease (Bourne and Coetzee, 1996) including those caused by pathogenic strains of Vibrio species especially in the age group 1-5 years (Mackintosh and Colvin, 2003), and immunocompromised individuals resulting in tens of thousands of deaths annually (Pegram *et al.,* 1998).

Vibrio species are incriminated in cases of diarrhoea, which accounts for a substantial degree of morbidity and mortality in different age groups worldwide (Obi *et al.,* 2004). *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio fluvialis* are serious human pathogens (CDC, 1999; Finkelstein *et al.,* 2002; Kothary *et al.,* 2003; Chakraborty *et al.,* 2006) mainly transmitted via water and food. They cause diarrhoea, but in ways that are entirely different. *V. vulnificus* and *V. parahaemolyticus* are invasive organisms affecting primarily the colon; *V. cholerae* is non-invasive, affecting the small intestine through secretion of an enterotoxin (Todar, 2005) and is the etiologic agent of cholera. The clinical symptoms of *V. fluvialis* gastroenteritis are similar to cholera with the additional manifestation of bloody stools which is suggestive of an invasive pathogen (Oliver and Kaper, 2001). Other vibrios, e.g.

Grimontia hollisae, *V. alginolyticus*, *V. cincinnatiensis*, *V. fluvialis, V. furnisii*, *V. harveyi*, *V. metschnikovii*, and *V. mimicus*, have been sporadically found in human infections (Farmer and Hickman-Brenner, 1992; Abbott and Janda, 1994; Carnahan *et al*., 1994). Large numbers of *Vibrio* ($4.3 \times 10^6/mm^2$) attached to the external surface of plankton (zooplankton and phytoplankton) have been reported (Heidelberg *et al*., 2002), thus suggesting a close partnership between these bacteria and planktons, which have also been implicated in municipal wastewaters (Ahmadi *et al.,* 2005; Chindah *et al.,* 2007; Mukhopadhyay *et al*., 2007). *Vibrio fluvialis* in particular, has been identified as an important cause of cholera-like bloody diarrhoea and primary septicaemia in immunocompromised individuals, especially in underdeveloped countries with poor sanitation (Igbinosa and Okoh, 2010). This organism has been isolated from treated wastewater effluent system in South Africa (Igbinosa *et al*., 2009**)** and there are reports linking it to food poisoning (Kobayashi and Ohnaka, 1989), especially due to consumption of raw shellfish (Levine and Griffin, 1993). However, of all the Vibrio species which have been associated with illness in humans, the most important are *V. cholerae* subgroups O1 and O139, the causative agent of epidemic cholera (Heymann, 2008). The debilitating disease caused by *V. cholerae* is the result of an enterotoxin known as choleragen. *V. cholerae* O1 occurs in 3 serotypes namely Ogawa, Inaba and Hikojima, and is further characterised into two biotypes – El Tor and classic (Heymann, 2008). However, infection with non-O1 *V. cholerae* causes milder clinical manifestations because this subgroup of *V. cholerae* lacks the cholera-toxin-producing gene.

Although antimicrobials are prescribed for the management of severe cases, to shorten the duration of illness and reduce the volume of rehydration solution required, *V. cholerae* strains are resistant to a number of antimicrobials including tetracycline, co-trimoxazole, trimethoprim and sulfamethoxazole. While adequate and timely rehydration therapy remains the gold-standard treatment for cholera (Heymann, 2008), knowledge of the antimicrobial susceptibility profile of local strains is important for the management of complicated cases. During a cholera outbreak in South Africa in 2009, the Enteric Diseases Research Unit (EDRU) at the National Institute for Communicable Diseases (NICD) processed 570 *V. cholerae* O1 isolates associated with the outbreak. Further laboratory characterisation showed that 98% of the isolates were serotype Ogawa and 2% were serotype Inaba; all were biotype El Tor and 99.5% of the isolates were positive for the cholera toxin. The 2008/2009 outbreak isolates were all resistant against co-trimoxazole, nalidixic acid, while the frequencies of resistance against other antibiotics follow the trend; chloramphenicol (48%), tetracycline (3%) and erythromycin (39%). Although all the isolates were resistant against nalidixic acid, none of the isolates associated with this outbreak was resistant against ciprofloxacin (Keddy, 2010a). In another outbreak in 2008, reported from Shebagold Mine in the Ehlanzeni district of Mpumalanga, 31 isolates were submitted for analysis to the EDRU. All were biotype El Tor and displayed resistance against ampicillin, amoxycillin-clavulanate, sulfamethoxazole, trimethoprim, chloramphenicol, nalidixic acid, kanamycin, streptomycin and tetracycline, which was initially the antimicrobial agent of choice in the treatment of cholera in Africa. Although the isolates exhibited resistance against nalidixic acid,

they were susceptible to ciprofloxacin and imipenem. Further resistance to third-generation cephalosporins ceftriaxone and ceftazidime was observed, indicating extended-spectrum β-lactamase (ESBL) activity (Crowther-Gibson *et al*., 2011; Keddy, 2010b). In the Eastern Cape Province, *Vibrio fluvialis* isolates recovered from rural-based wastewater treatment plants showed frequency of resistance against trimethoprim (100%), cephalothin (92%), penicillin (90%), cotrimoxazole (70%) and streptomycin (80%) (Okoh and Igbinosa, 2010). In that study, several Vibrio species were isolated and tested for the presence of antibiotic resistance genes and the SXT element and results showed that *Vibrio fluvialis* harboured most of the antibiotic resistance genes and SXT element, positioning it as an emerging pathogen in the Eastern Cape Province of South Africa.

Antimicrobial resistance has become a major medical and public health problem as it has direct links with disease management and containment (Okoh and Igbinosa, 2010), 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). Such knowledge is invaluable, especially in efforts to prevent and/or contain cholera epidemics in a given area. There is, however, paucity of information on the epidemiology of vibrios and ecology of the same in the aquatic milieu of the Eastern Cape Province of South Africa. This is despite the fact that over the past few years, the region encompassing South Africa has been plagued by outbreaks of *Vibrio*-related waterborne infections that are suspected to be linked to inefficiently treated effluents discharge from wastewater treatment facilities (Igbinosa *et al*., 2011). If the scourge of cholera is to be put under control, this gap needs to be urgently closed through research targeted at understanding the ecology and dynamics of vibrios both in the aquatic milieu and the human host.

Impacts of cholera

Social impact

Cholera transmission is closely linked to inadequate environmental management. Typical at-risk areas include peri-urban slums, where basic infrastructure is not available, as well as camps for internally displaced people or refugees, where minimum requirements of clean water and sanitation are not met. The consequences of a disaster, such as disruption of water and sanitation systems, or the displacement of populations to inadequate and overcrowded camps, can increase the risk of cholera transmission should the bacteria be present or introduced (WHO, 2014).

Economic impact

Polluted water not only has the potential to cause human suffering, but also result in economic loss. An assessment of the cost of water-related enteric illness in developing regions, based on Indian conditions estimated an average cost of approximately USD1700 per 100 people per annum, with an associated estimate of approximately 1500 days lost per 100 people per annum (Verma and Srivastava, 1990). This implies that a very strong association exists between

microbial contamination of water and human health risk, especially in communities like the Eastern Cape Province with a rather high HIV/AIDS statistic and poverty level. Hence, the need to ensure protection of the existing waterbodies in the province against contamination by *Vibrio* pathogens from wastewater treatment plants effluents becomes imperative. In addition to human suffering caused by cholera, its outbreaks bring panic, disrupt the social and economic structure and can impede development in the affected communities. Unjustified panic-induced reactions by other countries include curtailing or restricting travel to and from countries where a cholera outbreak is occurring, or import restrictions on certain foods. For example, the cholera outbreak in Peru in 1991 cost the country US$ 770 million due to food trade embargoes and adverse effects on tourism.

Health impact

Cholera remains a global threat to public health and a key indicator of lack of social development. The re-emergence of cholera occurs in parallel with the ever-increasing size of vulnerable populations living in unsanitary conditions. The number of cholera cases reported to WHO continues to rise. In 2011 alone, a total of 589 854 cases were notified from 58 countries, including 7816 deaths. Many more cases were unaccounted for due to limitations in surveillance systems and fear of trade and travel sanctions. The true burden of the disease is estimated to be 3-5 million cases and 100 000-120 000 deaths annually (WHO, 2014).

Environmental impact

It is recognized that *V. cholerae* is a component of coastal, estuarine and riverine microbial ecosystems, with the copepod species of zooplankton that comprise the aquatic fauna of rivers, bays, estuaries and the open ocean serving as host for the bacteria (Islam *et al*., 1994). *V. cholerae* can be found attached to the carapace and in the gut of copepods in large numbers, the copepod essentially serving as a vector for this human pathogen (Rawlings *et al*., 2007). A single copepod, for example, can contain as many as 10^3-10^5 *V. cholerae* cells (Colwel and Spira, 1992). Because a concentration of 10^9 ml^{-1} *V. cholerae* comprises an infective dose, ingestion of untreated water containing a relatively small number of copepods carrying *V. cholerae* can initiate the disease. Therefore, conditions favorable for multiplication of copepods and related chitinous zooplankton species for which *V. cholerae* is commensal or symbiotic will result in an increase in the number of *V. cholerae*. The importance of copepods in cholera transmission was demonstrated in a study showing that the number of cholera cases in Bangladeshi villages was significantly reduced when a simple filtration method that effectively removed the plankton and particulate matter was used to treat drinking water (Colwell *et al.,* 2003). This association of cholera vibrios with zooplanktonic substrate exposes the public to risk of cholera illness in instances where people rely on river water for daily use.

1.3 AIMS AND OBJECTIVES

The overall aim of this research is the development of a cholera monitoring programme for inclusion in the water resource monitoring programme. To achieve this, the following specific aims are set:

1. To assess the various types of wastewater treatment processes that is used in the Eastern Cape Province (ECP);
2. To evaluate the laboratories used for sample analyses and whether these are accredited or not;
3. To verify the analytical methods used in the laboratories in line with the green-drop reporting system;
4. To monitor the compliance of the wastewater treatment plants (WWTP) in the Eastern Cape Province to operational standards and determine the rate and reason(s) of failure of the treatment technologies;
5. To evaluate the implemented measures put in place to ensure that wastewater treatment occurs during failure;
6. To assess the prevalence of Vibrio pathogens in discharged final effluents of WWTP and rural waters in the ECP and make recommendations on the reason(s) contributing to the prevalence of these pathogens in rural waters.
7. To ascertain the strains of Vibrio pathotypes that are common in the aquatic milieu of the Eastern Cape Province as well as their epidemiology with a view to tackling the recurring scourge of cholera (and cholera-like diarrhoea) outbreaks in South Africa.
8. To evaluate aquatic animals (including shrimp, fish, crab, crayfish) and vegetables as potential reservoirs of Vibrio pathogens.
9. To compile and submit report on the findings to the WRC and other relevant stakeholders.

CHAPTER TWO

2.1 LITERATURE REVIEW

The genus *Vibrio* belongs to the family *Vibrionaeceae* which includes opportunistic pathogens of humans and animals (Daniels *et al.,* 2000; Thompson *et al.,* 2004). *Vibrio* species are Gram-negative, facultative anaerobes that test positive for oxidase and are typically found in aquatic habitat. All members of the genus are motile and they have polar flagella (Colwell, 1989). The species are amongst those enteric pathogens which are threats to human health and have been mostly known for causing cholera. *Vibrios* 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 newly discovered species. The roles of *Vibrio* species in the aquatic milieus have been shown to include biodegradation, nutrient regeneration and biogeochemical cycling (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 organisms and include many important pathogens of aquatic animals and humans who ingest contaminated seafood or polluted drinking water (Thompson *et al*., 2004). They are present in the environment either as free-living, or associated 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). A great number of *Vibrio* species 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 cholera* because of the severity of the disease it produces (Kaper *et al.,* 1982; Nair *et al.,* 1994; Mishra *et al.,* 2004); but in the course of the last decade, several studies have revolved around relatively minor *Vibrio* species of medical interest (Daniels *et al.,* 2000), some of which have been described as emerging pathogens with the potential to cause mild to severe human diseases (Igbinosa *et al.,* 2008).

The interest in *Vibrio* species worldwide stems from the history and epidemiology of cholera which originated from Asia thousands of years ago (Eyisi *et al.,* 2013). *Vibrio* species are autochthonous to marine and estuarine environments (Cantet *et al.,* 2013) and can survive in some aquatic environments for months to years, in association with zooplankton and other aquatic organisms (Akoachere and Mbuntcha, 2014). Individual *Vibrio* species respond differently to environmental factors, but typically water temperature and salinity are the most important in determining population concentration or distribution (Froelich *et al.,* 2013; Eja *et al.,* 2008). Thirteen different species have been associated with human pathogenesis. Among the pathogenic Vibrio species, *V. alginolyticus, V. cholerae, V. costicola, V. mimicus, V. cincinnatiensis, V. hollisae, V. furnissii, V. parahaemolyticus, V. vulnificus, V. carchariae* and *V. metschnikovii* are clinically important as they cause different types of vibriosis (Ramamurthy

et al., 2014). The majority of human *Vibrio* infections are associated mainly with *Vibrio cholerae, Vibrio parahaemolyticus,* and *Vibrio vulnificus* (Garrido-Maestu *et al.,* 2014). *Vibrio* species are responsible for the various cases of diarrhoea, accounting for a high degree of morbidity and mortality in different age groups worldwide (Okoh *et al.,* 2014). Also, pathogenic *Vibrio* species are not only a major economic threat to aquaculture, but are also a public health concern as they are easily transmitted to humans through the consumption of contaminated aquatic organisms (Costa *et al.,* 2010).

2.2 HUMAN PATHOGENIC VIBRIO SPECIES

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 to cause three major syndromes of clinical illness namely wound infections, gastroenteritis and septicaemia (Daniels and Shafaie, 2000).

V. cholerae

Based on the somatic O antigen, *V. cholera* is classified into more than 200 O-antigen serogroup. Only two serogroups O1 and O139 are recognized to cause cholera. *V. cholera* O1 serogroup is further divided into two biotypes, namely classical and EI Tor, each of which has three serotypes, Ogawa, Inaba and Hikojima (Tabatabaei and Khorashad, 2015). Toxigenic *V. cholera* produce an enterotoxin called cholera toxin (CT) which causes the manifestation of the disease cholera (Mandal *et al.,* 2011). *V. cholera* belonging to other serogroups that are not associated with cholera are called non-O1 and non-O139. Some non-O1 and non-O139 *Vibrio cholera* are pathogenic and have been associated with gastroenteritis and extra-intestinal infections in humans (Dziejman *et al.,* 2005).

Cholera is an enteric disease that is described by profuse watery diarrhoea and vomiting which results in dehydration, electrolytes loss and eventually hypovolemic shock and renal failure (Akoachere and Mbuntcha, 2014; Alam *et al.,* 2006 ; Banerjee *et al.,* 2014 ; Chakraborty *et al.,* 2000). According to the World Health Organization (WHO) estimates, about 3-5 million people around the world are infected with cholera and 100,000-120,000 people die from this infectious disease annually (Samson, 2014). Cholera is estimated to cause 120, 000 deaths globally every year (Shittu *et al.,* 2010). It is a disease of poor sanitation and poor living conditions and, in South Africa, was most prevalent in fairly densely populated rural communities of low socio-economic status (Kustner & Du Plessis, 1991). It also is a diarrhoeal disease with epidemic and pandemic tendency (Akoachere and Mbuntcha, 2014).

One of the first epidemics of cholera occurred in 1817 along the coastal region near the mouth of the Ganges River and after that it spread rapidly across the world (Mandal *et al.,* 2011).

From 1961 seven major cholera pandemics affecting millions of people living in South America, Africa, Europe, and Asia have been documented (Mandal *et al.,* 2011; Igbinosa & Okoh, 2008).

In 1991, an epidemic that took place in Bangladesh led to about 8 000 casualties within three months period (Siddique *et al.,* 1995). The world experienced seventh pandemic in South America and South Africa, as well as the emergence of previously pathogenic serogroup of *V. cholerae* (O139) in the same year (WHO, 2001). With interest now focused on scourge of cholera, in 1992 through 1993, 21 nations of the earth, mostly in coastal areas, reported 800 000 cholera cases with more than 8 000 casualties (Tauxe *et al.,* 1994). Likewise in July 1994, 14 000 casualties were recorded from an outbreak of cholera in refugee camps in Rwanda (Siddique *et al.,* 1995). In April 1997, a total of 1 521 casualties resulted from a cholera outbreak among 90 000 Rwandan refugees living in temporary camps in Democratic Republic of Congo (Nabeth *et al.,* 1997). Other cases recorded are in the provinces of Bengo, Benguela, Bie, Kuanza Norte, Kuanza Sul, Huambo, Huila, Malange, and Democratic Republic of Congo. As of 3rd May, 2006, 26 979 cumulative cases and 1 085 casualties had been recorded by UNICEF (UNICEF, 2006). The underlying reason for the epidemics occurrence in the affected communities and municipalities is as a result of poverty, the cramped living conditions and poor sanitation and inappropriate hygiene practices greatly exacerbate problems in the affected areas (UNICEF, 2006). Massive outbreak of O1 El Tor *V. cholera* mediated cholera took place in the South American continent and caused over 750 000 cases and 6500 deaths (Swerdlow and Isaacson, 1994). In 2013, 47 countries reported a total of 129 064 cases of cholera including 2102 deaths (WHO, 2014).

In 1974, South Africa experienced the first epidemic of cholera in a part of the Transvaal Province now known as Limpopo province (Isaäcson *et al.,* 1974; Isaäcson & Koornhof, 1981; Küstner *et al.,* 1981). From 1974 to 1980, the epidemic re-occurred in the northern part of Limpopo province, but was controlled early as a result of good surveillance (Küstner *et al.,* 1981). The year 1980, however, witnessed annual epidemic occurring each summer, Limpopo and KwaZulu-Natal was regarded as epidemic zone (Isaäcson *et al.,* 1981). In October 2001, a cholera epidemic started in KwaZulu-Natal and between January and March 2002, the disease extended to other provinces. A total of 17, 890 cases of cholera were recorded compared to the 106, 389 cases reported during the previous epidemic. It was more brutal in KwaZulu-Natal and the Eastern Cape. On the 24th of January 2014, Fort Beaufort and its surrounding communities had an outbreak of diarrhoea and residents attributed the poor quality of municipal supplied water as the cause of the malady (http://article.wn.com/view/2014/01/27/ 45 hospitalisedin_diarrhoea_outbreak/).

The Eastern Cape, the poorest of nine provinces, is the second largest province in South Africa, covering an area of 169 580 square kilometres. The second most disastrous cholera epidemic in Eastern Cape occurred in Oliver Tambo District and lasted six months. Untreated wastewater

emptied into the Umthatha River was reported to be the cause of epidemic in this area (National Department of Health, 2003).

Developed countries, as known today, rarely witness cholera cases and outbreaks are typically travel-associated (CDC, 2012). Yet, infections other than cholera can be caused by the non-epidemic *V. cholera* serogroups that, collectively, are tagged as non-O1/non-O139 and are generally acquired through raw or inadequately cooked seafood consumption. *V. cholerae* non-O1/non-O139 infections are continuously reported worldwide (Schirmeister *et al.,* 2014; Trubiano *et al.,* 2014), emphasizing their clinical significance. Although *V. cholerae* non-O1/non-O139 strains typically doesn't produce cholera toxin, other virulence factors contribute to the expressed pathogenicity, including hemolysin *hly*A (Figueroa-Arrendondo *et al.,* 2001), protease *hap*A (Wu and Magnusson, 2000), cytotoxic actin cross-linking repeats in toxin *rtx*A (Lin *et al.,* 1999), sialidase *nan*H (Almagro-Moreno and Boyd, 2009), heat stable toxin NAG-ST (Morris *et al*., 1990), a type 6 secretion system (T6SS) (Unterweger *et al.,* 2012), and a type 3 secretion system (T3SS) (Shin *et al.,* 2011). Occasionally, cholera toxin *ctx*A and toxin- co-regulated-pilus-associated genes *tcp*A and *tcp*I are reported to be present in *V. cholerae* non-O1/non-O139 isolates (Hasan *et al.,* 2013; Li *et al.,* 2014).

Vibrio paraheamolyticus

Vibrio paraheamolyticus is a halophile which is very common in both estuarine and marine environment (Letchumanan *et al.,* 2014). It can be subtyped based on its somatic (O) and capsular (K) antigen patterns. The review by Drake *et al. (*2007) on *V. parahaemolyticus* shows that it has about 12 O (somatic) antigens and over 70 K (capsular) antigens. The organism is classified by serotyping using antibodies specific for O and K antigens and such typing yielded 76 different serovars while many strains cannot be typed (Drake *et al.,* 2007; Kaysner and DePaola, 2001). It has been reported that serovars O3:K6, O4:K68 and O1: K (untypeable) are associated with various *V. para*h*aemolyticus* pandemic (Osawa *et al.,* 2002; Iida *et al.,* 2001; Chang *et al.,* 2002). The organism has also been classified based on production of hemolysin gene as Thermostable Direct Hemolysin (TDH), Thermostable Related Hemolysin (TRH) or Thermolabile Hemolysin *V. para*h*aemolyticus* (Drake *et al.,* 2007).

This organism is identified as a major cause of gastroenteritis especially in regions where consumption of seafood is high such, as Southeast Asia (Joseph *et al.,* 1982). It is generally undetectable in marine water below 19ºC but may grow in culture at temperatures as low as 5ºC and on food at 10ºC (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). 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 has the ability of causing life-threatening septicaemia in people

with underlying health conditions, such as iver disease or immune disorders (Su and Liu, 2007). *Vibrio parahaemolyticus* is a common cause of gastroenteritis and the leading cause of foodborne illness in Japan (Lee *et al.,* 2001).

Vibrio vulnificus

Vibrio vulnificus is considered an emerging pathogen of humans (Todar, 2009). It was first identified as an agent of disease in 1976 (Reichelt *et al.,* 1976). The bacteria thrive in warm seawater and are part of a group of vibrio species 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 (CDC, 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 septicaemia (Iwamoto *et al.,* 2010; Oliver, 2005). Also, persons who are immune-compromised, especially those with chronic liver disease, or hepatitis B or C are at 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). *V. vulnificus* outbreaks commonly occur in warm climates and small, generally lethal, outbreaks occur regularly. A typical example is an outbreak that occurred in New Orleans after Hurricane Katrina and several lethal cases occur most years in Florida (Jabulecki *et al.,* 2005). *V. vulnificus* accounted for about 34% of wound-associated infections in Texas, Louisiana, Mississippi, Alabama, and Florida but accounted for only 17% of cases nationwide. The increased report of *Vibrio* wound infections in the residents of Gulf Coast states could most likely be linked with the exposure of skin and soft-tissue injuries to the contaminated floodwaters (CDC, 2005). In 2007, about 95 cases of *V. vulnificus* contagion were reported to centres for disease control and prevention (CDC), most isolates were recovered from blood (64%) and wound (29%) cultures.

Vibrio fluvialis

Vibrio fluvialis associated illness is defined by nausea, loss of appetite, vomiting, gastroenteritis, watery bloody diarrhoea with abdominal cramps and fever. Moderate to severe hypokalaemia, metabolic acidosis, dehydration, and occasionally, hypovolemic shock can occur in 4 to 12 hours if fluid losses are not replaced. Stools are colourless, with small flecks of mucus and contain high concentrations of sodium, potassium, chloride, and bicarbonate. In the wound infection (cellulitis) that is caused by direct inoculation of bacteria into the skin or exposure of a wound to contaminated water (FDA, 2006).

In South East Queensland, Australia, *V. fluvialis* was isolated more frequently from river waters next to *V. cholerae* (Myatt and Davis, 1989). The presence of *V. fluvialis* with other potential pathogens has been reported in several locations of Varanasi, India (De *et al.,* 1993). *V. fluvialis* has also been isolated from natural waters in Myanmar (Oo *et al.,* 1993) and in a wide range of coastal environments of Japan (Uchiyama, 2000). Compared to other vibrio species, the recovery of *V. fluvialis* has been high from wastewater effluents from suburban communities in South Africa. However, their occurrence was not associated with any season or plankton blooms, but had a positive correlation with salinity, temperature, and dissolved oxygen (Igbinosa *et al.,* 2011a).

V. fluvialis has emerged as potential enteropathogens in Bangladesh and outbreaks that link the pathogen to diarrhoeal diseases have been reported (Huq *et al.,* 1980; Tanabe *et al.,* 1999). Also, *V. fluvialis* has been frequently isolated from estuarine and marine environment (Seidler *et al.,* 1980; 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; 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, but reports from the US implicated the organisms in gastroenteritis among infants (Hickman-Brenner *et al.,* 1984; Bellet *et al.,* 1989; Kolb *et al.,* 1997). Since 1979, *V. fluvialis* was isolated as one of the important pathogens in Tenri Hospital, Japan (Aihara *et al.,* 1991). The prevalence of the organism among children with diarrhoea was very low during 1988 in Calcutta (now Kolkata), India (Chatterjee *et al.,* 1989). In the same region, progressive increase of the pathogen among hospitalized acute diarrhoeal patients has been reported (Chowdhury *et al.,* 2012). During 1996-1998, prevalence of *V. fluvialis* was 9.4% among hospitalized diarrhoeal patients in North Jakarta (Lesmana *et al.,* 2002), and in Zhejiang Province, China, the pathogen was identified as the second most prevalent pathogen among acute diarrhoeal cases next to *V. parahaemolyticus* (Jiang, 1991). In many instances, *V. fluvialis* was found to be associated with cholera-like diarrhoea (Allton *et al.,* 2006).

Vibrio alginolyticus

It is a halophilic vibrio species which has been isolated from coastal water and sediments from various parts of the world and considered as part of marine microflora like the other pathogenic Vibrio species (Chen *et al.,* 2011). It has been associated with infections such as gastroenteritis, otitis media and externa, wound infection and endophthalmitis (Li *et al.,* 2009; Gomez *et al.,* 2003; Reina, 1993). This species is considered mildly virulent in that most infections caused by it are self-limiting and opportunistic in nature (Tantillo *et al.,* 2004). The organism has the ability to survive in nutrient deficient sea water without losing its virulence (Ben Kahla-Nakbi *et al.,* 2007). *V. alginolyticus* has been considered to have the ability to harbour several virulence

determinants found in other *Vibrio* species (Mustapha *et al.,* 2013), and the organism has been reported to be resistant against antibiotics such as penicillin and vancomycin (Ben kahla Nakbi *et al.,* 2006; Joseph *et al.,* 1978). The presence of hemolysin gene in some strains of this vibrio species suggest that the organism has pandemic potential (Guardiola, 2015; Mustapha *et al.,* 2013).

V. mimicus

V. mimicus like some other *Vibrio* species is a motile Gram-negative bacteria with polar flagellum and the ability to produce oxidase and catalase but is Voges-Proskaure negative. The organism was once described as atypical *Vibrio cholera*. The ability of the organism to produce toxins, hemolysins, hemagglutinins, proteases and siderophores has been reported (Hasan *et al.,* 2010; Farmer, 2005; Davis *et al.,* 1981). The organism can survive at a temperature as low as -30°C and can withstand up to 6% sodium chloride (Wong *et al.,* 1994; Chowdhury, 1989). The bacteria have been linked to disease conditions in countries such as Nigeria, Bangladesh, Brazil, Mexico, United States, Venezuela and Thailand. The organism produces several toxic factors with the most common being a haemolytic toxin called *V. mimicus* hemolysin (Guardiola, 2015). A CDC report in June 2010, showed the ability of some strains of the bacteria to carry cholera toxin gene (CDC, 2010). This bacteria was reported to cause diarrhoea in individuals who consumed raw turtle eggs in Costa Rica (Campos *et al.,* 1996).

2.3 PREVALENCE OF VIBRIO SPECIES IN FINAL EFFLUENTS AND WATER RESOURCES

Communities across the world have one thing in common; they produce wastewater (WHO, 1996). 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 (W_2RAP, 2011). Hence, in South Africa, such programs as the Green Drop certification have been introduced which 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). However, contamination of waterbodies by effluents from treatment plants leading to outbreaks of diseases in some areas of Eastern Cape Province was reported in 2002 (Cottle, 2002), and recent reports by Nongogo and Okoh (2014) and Okoh *et al. (*2014) suggests that such problems still persist in the Eastern Cape Province and jeopardizes public health in the use of the receiving watersheds for recreational, agricultural and domestic purposes.

Vibrio species are amongst the widely distributed pathogens in effluent environments associated with domestic sewage (Igbinosa *et al.,* 2009; Naidoo and Olaniran, 2013) which pose a major threat to public health worldwide (Zhou and Smith, 2002). Several studies have reported the occurrence of Vibrio species in different surface waters in the Gauteng (Giorgis *et al.,* 2015),

Mpumalanga (Madoroba and Momba, 2010) and Limpopo Provinces (Bessong *et al.,* 2009) posing a threat to the end users of the water.

V. cholerae has been isolated from surface water (Fraga *et al.,* 2007; Percival *et al.,* 2004) and the occurrence of *V. cholerae* in water sources can be linked to faecal pollution (Cox *et al.,* 2005). Domestic and farm animals have been shown to be carriers of *V. cholerae* strains, contributing to their sustained presence in the environment (Visser *et al.,* 1999; Sanyal *et al.,* 1974). Though, both toxigenic and non-toxigenic *V. cholerae* reside in the aquatic ecosystem, the non-toxigenic strains are more dominant in the environment (Singh *et al.,* 2001; Faruque *et al.,* 2004). Some of the identified sources of microbial contamination of waterbodies which could present risk to aquatic animals are indiscriminate dumping of refuses, using rivers for domestic purposes (e.g. bathing and washing) especially in rural areas, poorly treated effluents from wastewater treatment plants, run-offs from livestock farms and farm soil manure, defecating and urinating in water body by humans and grazing animals (Nevondo and Cloete, 2001; USEPA, 2001; WHO, 2010; Abakpa *et al.,* 2013). Contamination due to the aforementioned sources could be more pronounced in the Eastern Cape Province since most of her waterbodies are under low or no protection (Sanlam, 2013).

2.4 POTENTIAL RESERVOIRS OF VIBRIO SPECIES

Vegetables

Fresh vegetables are perceived to be healthy and nutritious foods owing to the array of scientifically proven and documented health benefits (Maslow *et al.,* 2010). Nevertheless, some foodborne outbreaks throughout the world have been intensely linked to consumption of fresh fruits, vegetables, and unpasteurized juices (Gorny, 2006) affecting mostly immune-compromised people, pregnant women and children (CDC, 2006). Meldrum *et al. (*2009) reported that health problems could arise from consumption of contaminated vegetables which have been demonstrated to be vehicles for transmission of a range of microorganisms (Erdogrul and Sener, 2005). The presence of *Escherichia coli*, *Vibrio* spp. and *Salmonella* spp. in raw vegetables harvested from soils irrigated with contaminated streams in Nigeria has been reported (Okafo *et al.,* 2003). Also, the prevalence of *Vibrio parahaemolyticus* in raw vegetables in retail outlets in Malaysia has been reported (Tunung *et al.,* 2011), hence, cholera pandemic and vibrio related food diseases can manifest in human population through consumption of fresh vegetables contaminated by *Vibrio* species.

Aquatic animals

Aquatic animals are essential components of food for ample sections of the world population (Varadharajan *et al.,* 2013; Ozcan *et al.,* 2013; Trivedi *et al.,* 2012; Sakthivel and Fernando, 2012; Bark *et al.,* 2011) and act as important vehicles of transmission of foodborne pathogens. Although *Vibrio* exists in the marine environment as common heterotrophic bacteria, a small percentage of the environmental isolates carry the genetic determinants for human pathogenesis

(Chakraborty *et al.,* 2000). Seafood includes mollusc, finfish, marine mammals, fish eggs, and crustacean (Iwamoto *et al.,* 2010). A variety of Vibrio species have been isolated from aquatic animals. For example, Maheshwari *et al. (*2011) reported the presence of *V. cholera* in fish, crab and shrimp collected from local fish markets. In another study (Adeleye *et al.,* 2010), *V. cholerae* was isolated from shrimps, crabs and cuttle fish. Seafood such as finfish and crustaceans have been reported by Arunagiri *et al. (*2013) to harbour *Vibrio* species in India. It has been suggested that cockle (*Anadaraa granosa*) which is used in Malaysia for preparation of several local delicacies could be a reservoir of *Vibrio* species since they are cultured in coastal waters, kept after harvesting and sold in local markets without depuration. The isolation of *V. cholera* O1 biotype El Tor from aquatic birds such as the great blue herons in Colorado and Utah but not from water samples collected from the birds' habitat shows its potential as reservoir of toxigenic vibrio species (Ogg *et al.,* 1989).

Epidemiologic studies have established a relationship between the prevalence of cholera and the consumption of fish and other raw or undercooked seafood (Halpern and Izhaki, 2017). Seafood such as finfishes and crustaceans have been reported by Arunagiri *et al. (*2013) to harbour *Vibrio* species in India.

2.5 SURVIVAL STRATEGIES AND MODE OF TRANSMISSION OF VIBRIO PATHOGENS

The ability of *Vibrio cholerae* to adhere strongly to the gut of shellfish such that removal by rinsing or depuration is almost impossible has been recognized as one of the factors that aid its ability to cause infection via consumption of contaminated seafood (Garate-Lizarraga, 2006). The hyper-infectivity of *Vibrio cholerae* shed by humans has been linked to aggregate form which the pathogen enters into under *in vivo* stress conditions. This aggregate form has been confirmed to be hyper-infective when compare to corresponding planktonic cells (Suzita *et al.,* 2009; Garate-Lizarraga, 2006) suggesting the epidemiological importance of the phenotypic nature of vibrio pathogen released into the aquatic environment and the possibility of transferring vibrio infection to healthy human. Vibrio pathogens especially *V. cholerae* have been reported to have the ability to secrete an amorphous exopolysaccharide which enhance biofilm formation when co-dwelling in shellfish (Watnick and Kolter, 1999). The symbiotic relationship between vibrio pathogens and chitinous exoskeletons of some aquatic animals provide the bacteria with abundant carbon and nitrogen sources thereby enhancing the ability of the microorganisms to thrive in the aquatic milieu and their ability to cause infection in man via consumption of seafood (Tarsi and Pruzzo, 1999). Changes in the Vibrio community may be correlated with shifts in the abundance or composition of Vibrio reservoirs such as plankton, sediment and shellfish (Maugeri *et al.,* 2004; Thompson *et al.,* 2004). Aquatic animals, especially filter feeders have the ability to concentrate Vibrio pathogens in their guts at a concentration that is 100-fold higher than those in the environment (Morris, 2003; Wright, 1996). An epidemiologically and clinically significant finding of Ndip *et al. (*2002) that shrimps are potential reservoirs for *Vibrio* species in

the coastal area of South West Cameroon supports the report on the ability of the aquatic animals to concentrate vibrio species in its gut. The presence of *Vibrio* species in marine animals has been reported as possible sources of human infections (Hinz *et al.,* 1999). The change in atmospheric conditions due to greenhouse effects worldwide has been suggested to be a factor that could affect the ecology and abundance of agents of infections such as pathogenic Vibrio species in the aquatic environment. Climatic changes that support rapid growth of aquatic plants, filamentous green algae, copepods, crustaceans, plankton's bloom and associated bacterial proliferation increases population and persistence of pathogens in aquatic environment (Landsberg, 2002). The ability of both cholera and non-cholera causing *Vibrio* species to form a three-dimensional biofilm on surfaces of aquatic animals, plants and planktons in a microenvironment which facilitates the persistence of the pathogens in aquatic environment during inter-epidemic periods have been severally reported. Cholera has thus evolved from an infection of oral-faecal transmission linear model involving waterborne pathogens and humans to a more composite ecological framework of infectious diseases (Koelle *et al.,* 2005; Lobitz *et al.,* 2000; Pascual *et al.,* 2000). It has been reported that vibrio pathogens are mainly affected by temperature and pH in aquatic environment such that they enter into viable-but-non-culturable (VBNC) state during unfavourable conditions such as temperature, osmolarity and nutrient deprivation. It is also known that *Vibrio cholera* exists in viable but non culturable form in association with zooplanktons and algae which serve as natural refuge for the pathogen (Morris, 2003). Studies have shown that there is correlation between algae bloom and cholera outbreaks but there are no empirical evidences that confirm the enrichment of *Vibrio cholera* population by algae. This thus suggests that phyto- and zooplanktons may serves as reservoirs for cholera bacteria (Tamplin, 2001; Islam *et al.,* 1999; Colwell, 1996; Islam *et al.,* 1994; Epstein, 1993). At this state, they lose their flagellum, become smaller and spherical in spore-like stage but still infectious (McDougald, 1998; Xu, 1982). This dormant state helps the pathogens to survive changes in salinity, temperature and availability of organic matter that is detrimental to them (Colwell, 1996). In their aquatic habitats, these bacteria are found mostly attached to the exoskeletons of phyto-planktons and zooplanktons to improve their adaptation in aquatic habitat. *Vibrio* species has different ways of colonizing the surface which depends on the presence and characters of both conserved and variable genes (Mueller *et al.,* 2007). In the aquatic environment, diverse surfaces are available for colonization (biofilm formation), including suspended mineral particulates (of which the negatively charged silicates are a major component), plants whose surfaces include organic polymers such as cellulose, and the exoskeletons of crustaceans (including zooplankton organisms), which are composed primarily of chitin (Watnick *et al.,* 1999).

Cholera is generally referred to as the classic waterborne disease due to its association with aquatic environment. However this description underrates the transmission of *V. cholerae,* because the bacterium has been reported to also be transmitted by contaminated food. In developed countries, it has been established that contaminated food (especially undercooked

seafood) is the usual means for transmission, and contaminated water has been implicated in developing countries (Sack *et al.,* 2004; Shapiro *et al.,* 1999). Also, cholera has pronounced seasonality as normally experienced in countries like Bangledash and Peru where outbreaks usually occur in the temperate seasons and after monsoon rains (Tauxe *et al.,* 1995). This has been linked to rapid growth of Vibrio species in warm temperatures. The common belief that cholera can only be transmitted by infected people to other vulnerable individuals through faecal contamination of water and food and that mass global movement of populations accounted for the global movement of the disease. Recent studies have investigated the aquatic environment and reported that *V. cholera* including the O1 and O139 serogroups are natural inhabitants of surface water especially brackish waters, where they grow and thrive in association with zooplankton and phytoplankton without coexisting with infected human beings (Morris, 2011). Although a cholera patient excretes vibrio cells for a few days, the carrier particularly in the case of El Tor cholera poses the greatest threat in the communication of the disease in or between bordering nations.

The ratio of serious cases to trivial or unapparent infections (carriers) is 1:5 to 1:10 for classical cholera, and can be as high as 1:100 for El Tor cholera (Finkelstein, 1996). The presence of relatively large numbers of unknown carriers makes wide spreading of the Vibrio cells possible within a short period. Toxigenic *V. cholera* O1 biotype El Tor has been isolated from shrimps and crabs in Louisiana of United States (Blake *et al.,* 1980). Hood *et al. (*1981) also reported the isolation of non-toxigenic strains of serogroup O1 biotype El Tor from oysters harvested from estuarine waters in Florida. The presence of *V. cholera* O1 has also been reported in fish and molluscs harvested in the coastal waters of Lima, Peru (Tamplin and Parodi, 1991) which further supports chitinous surface of crustaceans as enabling environment for reproduction the pathogenic vibrio species.

2.6 SOCIO-ECONOMIC IMPACTS OF CHOLERA AND NON-CHOLERA CAUSING VIBRIO OUTBREAKS

The scourge of pathogenic *Vibrio* species is not peculiar to man because it also causes a great deal of loses in aquaculture farms leading to colossal death of freshwater prawns cultured in Taiwan (Bonami and Widada, 2011). Unfortunately, mild heat enough to open the shells of shellfish is not enough to expose the internal organs of the shellfish to high temperatures capable of killing *Vibrio* pathogens and proper cooking does not guarantee protection against Vibrio infections illness in cases of recontaminations (Suzita *et al.,* 2009; Millard *et al.,* 1987). Typical example to support this ascertain is one of the largest cholera outbreaks in USA in 1978 where cooked shrimps were transferred into boxes where raw shrimp had been shipped and were held at warm temperature before serving in Louisiana (Suzita *et al.,* 2009). Many of the outbreaks in Thailand were associated with consumption of mussels containing *Vibrio cholera* smuggled into the country and ten outbreaks in US between 1973 and 1992 were associated with vibrio contaminated seafood such as crab harvested from Gulf Coast (Todd, 2006; Rabbani and

Greenough, 1999). Also, an outbreak among travellers was linked to consumption of contaminated seafood served in an international aircraft (Eberhart-Phillips *et al.,* 1996).

The Eastern Cape Province coastline is about 821 km in length and complex in nature, being characterized by estuaries, sandy beaches, rocky shores and offshore reefs (Britz *et al.,* 2001). The sub-tidal zone of rocky shores is usually dominated by algal beds, and invertebrate resources such as oysters, mussels, rock lobster and abalone (Coastal & Environment Services, 2004). The province has been known to have a vast array of natural marine resources, and the possibility of developing a strong marine and freshwater aquaculture sector. The aquaculture sector is anticipated to add more socio-economic diversification that will address the issue of job creation, rural development, food security, poverty relief and the development of technology and skills in the poverty stricken ECP (Elsenburg, 2005; Hinrichsen, 2008).

The South African Government generates revenue from exportation of the aquatic animals (e.g. squid, Snoek, Cape lobster, abalone, calamari, octopus, oysters, shrimp, crab and mussels) which serve as raw food or food ingredient (Gosling, 2013). Indeed the squid industry in Eastern Cape Province contributes about R500 million to approximately R3.5 billion revenue annually from exportation of seafood (Morné du Plessis, 2011**;** SAinfo reporter, 2014). The recent license issued to twelve South African seafood companies to export seafood to Russia starting from 2015 is expected to boost revenue generation from this sector (SAinfo reporter, 2014). In 2009, an estimate of R340 million was expected from aquaculture production with abalone contributing about 93.9 % oysters, 2.8 % mussels, 2.4 % and 0.7% finfish and prawns combined (DAFF, 2010).

The involvement of aquatic animals used as food in recreational fishing could add values to tourism in South Africa (DAFF, 2014). Subsistent fish farming serves as source of livelihood in terms of employment and food for rural people of the ECP where poverty is rated very high (Perret and Kirsten, 2000; Kibirige, 2013) hence the Eastern Cape Development Cooperation (ECDC) embarked on a R250 Million aquaculture project in 2013 in an attempt to explore the aquaculture potential of the province for the purpose of poverty alleviation (http://www.getnews.co.za). The absence of proper monitoring and surveillance of Cholera could really affect international trade, which normally happens when cholera-free countries impose prohibition on merchandises normally imported from cholera infected countries. Cholera-free countries also may ban their ships from visiting cholera-infected countries, thus reducing both imports and exports. For example, during the 1965 epidemic in Iran, Switzerland refused to accept airmail originating from Iran, and Russia stopped chromate ore importations from Iran. These extreme measures injure the economy of the countries affected, particularly if the embargoed material is perishable. It is extremely difficult to calculate this type of loss and its magnitude is intentionally minimized by infected countries, but depends on the type of goods, the value of the trading that is interrupted, and the duration of the embargo, amongst others.

Taiwan recorded a loss of $6,000,000 as a result of neighbouring countries closing their seaports during the 1962 epidemic. Low inflow of tourist into cholera infected countries is another negative economic loss that cholera infected countries suffers especially where this industry is an important factor in the economy.

Although there is no reliable data on the impact of cholera on the productive segment of the population in countries previously invaded with cholera outbreaks, the cost to the economy will be greater in those countries due to low productivity as a consequence of illness and death in the labour force. Most of the people affected by outbreaks of cholera are mainly lower socioeconomic groups living under unhygienic environments, and the costs of treatment in relation to the small monetary incomes of the individuals and the community are such that treatment places a heavy burden on them. The burden of disease such as gastrointestinal disorder caused by these pathogens in developing countries is massive and estimated as greater than 26 billion dollars per year (Payment and Riley, 2002). Khouadja *et al. (*2013) reported isolation of *V. parahaemolyticus* from sea bass (*Dicentrarchus labrax*) during a diseases outbreak in fish farms in Tunisia. Also, there were reports on the isolation of Vibrio species from marine and freshwater aquatic animals in Burkina Faso, Namibia, and Nigeria (Adebayo-Tayo *et al.,* 2011; Shikongo-Nambabi *et al.,* 2012; Eyisi, 2013, Traoré *et al.,* 2014). Also, the Peru government, during cholera outbreak in 1991, lost US$ 770 million due to food trade embargoes and this had adverse effects on tourism according to the World Health Organisation.

The problem of the contamination of vegetables may lead to a suspension of exports to the EU and USA, leading in turn to lost markets, reduction of foreign exchange earnings and job losses. This should be prevented from happening because South Africa's local and export trade in fresh and processed fruit and vegetables is steadily growing (Census of commercial agriculture, 2002). Furthermore, consumption by South Africans of vegetables contaminated with foodborne pathogens might lead to outbreaks of foodborne illnesses, bearing in mind that a large proportion (i.e. more than 7 million) of the citizens have immune system compromised diseases (Suarez, 2009).

Since vegetables are often consumed raw, the occurrence of potentially pathogenic *V.* species in uncooked vegetables could be a threat to public health. The Eastern Cape Province has seven district municipalities. There is lack of information on the molecular epidemiology of Vibrio pathogens in vegetables marketed along river basins in the provinces of South Africa. The presence of such pathogens may compound the worrisome cases of Immune-compromised people, elderly people, pregnant women and children who are reported to be the most vulnerable to foodborne diseases (CDC, 2006).

2.7 VIRULENCE EXPRESSION OF VIBRIO SPECIES

The initiation of *V. cholerae* infections in humans starts with the contamination of water or food. The pathogenicity of *Vibrio cholera*e is established after successful passage of the acid barrier in the stomach and this is exhibited through their ability to ravage a mammalian host and produce a type IV bundle-forming pilus, known as the toxin co-regulated pilus (TCP) (Taylor *et al.,* 1987; Herrington *et al.,* 1988), and possibly other colonization factors like haemagglutinins, accessory colonization factor, and core-encoded pilus, all of which are believed to play a role in enhancing adhesion and intestinal colonization (Faruque *et al.,* 1998). The cholera toxin is produced by pili formation and the resulting colonization of *V. cholerae*, which induce diarrhoea (Lee *et al.,* 1999). The *Vibrio cholera*e establish infection by making the toxin to attach to the plasma membrane of epithelial cells and secretes an enzymatically active subunit which causes a rises in cyclic adenosine 5'- monophosphate (cAMP) production and results in massive discharge of electrolytes and water into intestinal lumen, eventually leading to loss of about 20 litres of fluid daily. This eventually leads to death if not treated early. The importance of cholera toxin is seen in the disease conditions of cholera because the secretion of cholera toxin is essential in the ability of a serogroup to cause epidemics. Such evidence is seen in the emergence of *V. cholerae* O139. The pathogenic infections of the non-cholera strains of *Vibrio cholera* are different from the toxigenic *V. cholerae* (Singh *et al.,* 2001). Some of these strains possess the CTX and TCP genes (Faruque *et al.,* 2004) while other strains produce a toxin known as RTX, and some encode a heat-stable enterotoxin (nag-ST) (Dalsgaard *et al.,* 1995; Lin *et al.,* 1999). Even though CTX_TCP_ clone of the pathogenic non-cholera vibrio strains are widely distributed globally (Dalsgaard *et al.,* 2001), most strains are not capable of causing endemic or pandemic disease as clones, and are highly discrete (Dalsgaard *et al.,* 1998; Sharma *et al.,* 1998). When sequenced, most of the genome of a non-cholera vibrio strains were found to carry a type III secretion system (TTSS) (Lee, 1997) and these include *Vibrio parahaemolyticus, Vibrio vulnificus* and *Vibrio fluvialis* TTSS gene cluster (Park *et al.,* 2004).

Several reports have shown that TTSS is available in many environmental and clinical non-cholera Vibrio strains. The presence of TTSS in some pathogenic non-cholera Vibrio strains might be contributing to the virulence and environmental fitness of these strains. Type III secretion systems (TTSSs) is an example of virulence factor of many pathogenic bacteria such as Salmonella, Pseudomonas, Yersinia, Shigella, and enteropathogenic and enterohemorrhagic Escherichia coli (Mattoo *et al.,* 2007; Galan, 2009; Kim *et al.,* 2010). *V. parahaemolyticus* produces diarrhoea, but in a way that is entirely different from *V. cholerae* (Todar, 2005) posing a risk of false diagnosis. Also in *V. vulnificus*, diarrhoeal cases, vomiting and abdominal pains have been reported in healthy people who have ingested *V. vulnificus* (Todar, 2009) though the infections are acute and have no long-term consequences. 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).

2.8 ANTIBIOTIC RESISTANCE IN VIBRIO SPECIES

Resistance of bacteria against antimicrobials has become an important public health problem that directly relates to disease management and control (Ansari and Raissy, 2010). Bacteria such as Vibrio species have been reported to be resistant against common antibiotics such as doxycycline, tetracycline, streptomycin and erythromycin used for the treatment of different bacterial diseases, (Lima, 2001; Ahmed *et al.,* 2004; Ceccarelli *et al.,* 2006; Ansari and Raissy, 2010). Recently, higher frequency of drug-resistant *Vibrio* has been reported (Ansari and Raissy, 2010, Okoh and Igbinosa, 2010). Contamination of food with antibiotic-resistant bacteria has become a major threat to public health, because of the implication of the transfer of antibiotic resistance determinants to other bacteria of human clinical significance (Chiu *et al.,* 2002). The recent decades has witnessed the increase in prevalence of antimicrobial resistance among food-borne pathogens, (Chiu *et al.,* 2002; Boonmar *et al.,* 1998; Threlfall *et al.,* 1999) which could be due to selection pressure caused by the indiscriminate use and misuse of antimicrobials in aquaculture, food production and animal farms (Teuber, 2001; Angulo *et al.,* 2000; Bywater *et al.,* 2004). Certain antibiotics are critical to human medicine because the target drugs available to treat human infections caused by multi-drug resistant pathogens are limited or because available alternative therapies are less effective or are associated with grave after-use effects. *V. cholerae* may have found a way around the susceptibility to commonly used antimicrobials in farm animals as it's resistance to these is increasing in the farms and the public health sectors, thus has emerged as a global problem.

Pathogenic bacteria and antimicrobial resistance genes are often released with wastewater discharges into aquatic environments (Baquero *et al.,* 2008). Naturally occurring bacteria produce antibiotics in the environment for signalling and regulatory purposes in microbial communities (Martinez, 2008). However, bacteria protect themselves from the toxicity of these antibiotics by acquiring and expressing antibiotic resistance genes (Wright, 2007). As a result, naturally occurring aquatic bacteria are capable of serving as reservoirs of antibiotic resistance genes and these, coupled with the introduction and accumulation of antimicrobial agents, detergents, disinfectants, and residues from industrial processes, could play important roles in the evolution and spread of antibiotic resistance in aquatic environments (Baquero *et al.,* 2008).

Antibiotic-resistant bacteria have been found in a surprisingly diverse range of environments, including human clinics, animal husbandry, orchards, aquaculture, food, sewage, chlorinated, and unchlorinated water supplies (Chopra, 2001). *Vibrio* species in the estuarine-marine environment are of particular concern for human health and may be increasing in pathogenicity and abundance (Baker-Austin *et al.,* 2010), with *Vibrio vulnificus* and *Vibrio parahaemolyticus* being two of the three most commonly reported sources of Vibrio infection (Newton *et al.,* 2012). Antibiotics used against these vibrio infections are also prone to indiscriminate and inappropriate use like all other antibiotics, thus posing a potential threat to human health due to the presence of individual and multiple antibiotic resistance strains among both human and non-

human pathogenic vibrio species. Exchange of resistance genes among *Vibrio* species was also shown in studies by Garg *et al. (*2000) where dissipation of some of the resistant patterns commonly found among clinical strains of *V. cholerae* non-O1, non-O139 or O1 serogroups to the O139 serogroup and vice versa was observed during succeeding years from 1992 to 1997 (Garg *et al.,* 2000).

Antibiotics such as tetracycline, doxycycline, norfloxacin, ciprofloxacin and streptomycin may be used as adjuncts in rehydration therapy and are critical in the treatment of septicaemia patient (Lima *et al.,* 2001; Chiang *et al.,* 2003). Resistance against many of these drugs which have emerged in Vibrio pathogens is a matter of major concern, particularly in the case of *Vibrio vulnificus, Vibrio cholerae*, *Vibrio fluvialis* and *Vibrio parahaemolyticus* (Rowe-Magnus *et al.,* 2006; Chiang, 2003).

Disease outbreak is an important constraint to the development of aquaculture (Defoirdt *et al.,* 2011). Using antibiotics to treat bacterial infections and incorporation of sub-therapeutic doses of antibiotics into feeds for cultured organism contributed to the global increase in antibiotic resistance among pathogenic bacteria via resistance plasmids (Redondo *et al.,* 2004; Vivekanandhan *et al.,* 2002). These plasmids are found in both Gram-negative and Gram-positive bacterial species (Actis *et al.,* 1999), and their carriage by strains of different *Vibrio* species such as *V. fluvialis*, and *V. furnissii* have also been reported (Redondo *et al.,* 2004; Vaseeharan *et al.,* 2008).

Vibrio species are known to be highly susceptible to most clinically used antibiotics (Mala *et al.,* 2014; Shaw *et al.,* 2014). However, over the years, antibiotic resistant strains have emerged in the environment due to the excessive use of antibiotics and other chemotherapeutic agents in human, agriculture, and aquaculture fields (Cabello *et al.,* 2013). As reported by Baquero *et al. (*2008) the study of antibiotic resistance in indigenous aquatic organisms is important, as it might indicate the extent of alteration of water ecosystems by human action. In the aquaculture field, antimicrobials are used not only to promote growth but also to prevent and treat bacterial infections in fish and other invertebrates (Cabello *et al.,* 2013). Oxytetracycline, tetracycline, quinolone, sulphonamides, and trimethoprim are among the antibiotics allowed and used for example in the Asian aquaculture industry to ensure continuous production of seafood (Rico *et al.,* 2012; Yano *et al.,* 2014). Also, Vibrio species have the ability to acquire resistance when exposed to antibiotics and at the same time transfer antibiotic resistance determinates via conjugation, transformation, transduction, mobile DNA materials and plasmid within their population (Serrano, 2005). Ability of Vibrio species to transfer antibiotic resistance determinants is also enhanced by the presence of R-factors in their population. These R-factors increase the chance of sharing resistance determinates within vibrio populations and among non-vibrio microbes that co-habit the same environment through horizontal and vertical DNA transfer (Madigan *et al.,* 2003). The possibility of multiple antibiotic resistance gene transfer by the

aforementioned raises a big concern. Also, the indiscriminate use of antibiotics in aquaculture farms has been reported to lead to development of antibiotic resistant bacterial in such farms. Resistance determinants like *PenA, TEM-1*, cati-iv and floR, tatA-E and tatG-Z that codes resistance against β-lactam penicillin, chloramphenicol and tetracycline respectively have been found to be common in the environment especially aquaculture farms where they are commonly used (Macauley *et al.,* 2007; Dang *et al.,* 2007, 2008; Zhang *et al.,* 2009; Kim *et al.,* 2013). *Vibrio* species that are resistant against antibiotics such as tetracyclines and oxytetracycline have also been frequently reported (Park *et al.,* 1994). These have become a major concern to human health since with the frequent isolation of *Vibrio* species particularly *V. parahaemolyticus* that are resistant against clinically used antibiotics from the environment (Srinivasan *et al.,* 2005; Zhang *et al.,* 2009). There have been reports on isolation of antibiotic resistant *V. parahaemolyticus* which harbour antibiotic resistance determinants from shrimps in Thailand, Malaysia and China (Peng *et al.,* 2010; Al-Othrubi *et al.,* 2011; Sani *et al.,* 2013; Xu *et al.,* 2014, Yano *et al.,* 2014). *V. parahaemolyticus* isolated from Ponnani water in India exhibited multiple resistance against nalidixic acid, trimethoprim, ampicillin, tetracycline and ciprofloxacin while multiple antibiotic resistance was observed in isolates from three states in Peninsular, Malaysia against vancomycin, streptomycin, chloramphenicol, ciprofloxacin, ceftriaxone, enrofloxacin and norfloxacin (Reyhanath and Kutty, 2014; Sahilah *et al.,* 2014). *V. cholera* has been shown to be resistant against tetracycline in endemic and epidemic cholera areas while chloramphenicol resistant strains have been reported in Chile, Western Mediterranean and France (Mirand and Zemelman, 2002; Michel *et al.,* 2003; Chelossi *et al.,* 2003). *Vibrio cholerae* strains isolated during 2008 outbreak in Shebagold Mine, Ehlanzeni district of Mpumalanga Province in South Africa were reported by Enteric Diseases References Unit (EDRU) to exhibit multiple antibiotic resistance against antibiotics of choice for cholera treatment in Africa (Keddy, 2010; Crowther Gibson *et al.,* 2011). *V. fluvialis* has also been reported to exhibit multiple resistance against antibiotics such as sulfadiazine-trimehtoprim, ampicillin, kanamycin, cefalothin and some reports from South Africa have it that some treated effluents from treatment plants harbour multiple antibiotic resistant *V. fluvialis*. Report from China also showed that much of *V. fluvialis* isolated in the country exhibited multiple resistance against antibiotics such as β-lactams, azithromycin, and sulfamethoxazole (Laganà *et al.,* 2011; Liang *et al.,* 2013). *V. fluvialis* recovered from clinical specimen of diarrhoeal patients in Kolkata were also reported to be resistant against β-lactam and fluoroquinolones antibiotics. Also, *V. parahaemolyticus* isolated from Ponnani water in India exhibited multiple resistance against nalidixic acid, trimethoprim, ampicillin, tetracycline and ciprofloxacin while multiple antibiotic resistance was reported against vancomycin, streptomycin, chloramphenicol, ciprofloxacin, ceftriaxone, enrofloxacin and norfloxacin (Pilakka & Ranjeet, 2014; Sahilah *et al.,* 2014). In some isolates recovered from three states in Peninsular, Malaysia. *V. vulnificus* strains have also been reported to be resistant against quinolones, fluoroquinolones, penicillin class and cefoxitin (Shaw *et al.,* 2014).

2.9 RECONNAISSANCE AND DISEASE DISCOVERY

Reliable and evidence based data on cholera is essential for outbreak management. Such data which should be forwarded to WHO is expected to involve geographical locations of cholera incidences or outbreaks, time of cholera incidence or outbreaks and status of people who are affected by the cholera incidence or outbreak. This data is thus expected to be published by WHO in her *Weekly Epidemiological Record.* Unfortunately, some countries with high incidence of cholera hoard or give incomplete data to WHO because of the fear of stigmatization and economic damages they could suffer should the world know about their cholera problem. Official reports to WHO indicates only a segment of real cases and some countries do not report at all.

WHO does not declare outbreaks of cholera except after laboratory confirmation of the presence of *V. cholera* in clinical samples taken from first set of reported cases of acute watery diarrhoea. Also, WHO guidelines approve the use of thiosulfate citrate bile salt sucrose (TCBS) which is a selective agar for the laboratory (WHO, 1999). After the confirmation of the outbreak, the guide line further recommends periodic sampling and verification in other to monitor the antibiotic susceptibility profile of isolated strains of *V. cholera* to safeguard the re-emerging of cholera cases. This is very difficult and challenging in remote areas lacking laboratory facilities. However, some quick and advanced diagnostic kits have been developed to solve this problem. Also the use of mobile laboratories for isolation and confirmation of the isolated strains at any place and time has been suggested (Ouedraogo *et al.,* 2008).

2.10 PREVENTION, DIAGNOSIS AND TREATMENT OF CHOLERA AND CHOLERA-LIKE INFECTION

The need for a quick medical diagnosis and treatment of cholera cases boils out of the fact that one out of three persons who have severe cholera infection are always at the verge of death (WHO, 2013). The choice of treatment for cholera infection to replace lost body electrolytes caused by diarrhoea is oral rehydration therapy (ORT) in non-severe cases and intravenous fluid replacement therapy (IFRT) in severe cases. This is often couple with antibiotics to prevent shedding of *V. cholera* to the environment and limit period of infection in infected individual (WHO, 2013). Zinc supplemented with vitamin A which has been proven to reduce period and complication of diarrhoea is now being administered to children to reduce impact of the diseases and death due to the infection (WHO, 2013). Hypoglycemia, hypovolemic shock, septic shock, hypothermia, and acute renal failure are the most common complications of cholera. Management of cases and minimizing transmission are critical steps in early stage of cholera outbreaks while effective treatment regimen and further prevention of transmission via monitoring and intervention programmes are critical steps to take in the course of the epidemics. It is also important to evaluate the effectiveness of the intervention programmes such as administration of oral cholera vaccines (OCV). Curtailing the scourge of cholera and cholera-like infections also involves institutional, community and personal actions. Stakeholders at the

national and local levels are to ensure a lasting policy on provision of effective waste disposal system and potable water for all members of the community. Most cases of cholera respond to simple treatment especially when basic health facilities are within the grasp of patients. Therefore, the need for both government and non-governmental organizations on ensuring that primary health care centres are well equipped to proffer basic health services such as ORS and antibiotics (which should only be given in severe dehydration cases) to patients especially at the local primary health care centres becomes imperative. Effective system that monitor the antibiotic susceptibilities of *V. cholera* and by extension other pathogenic *Vibrio* species as well as updating the epidemiological profile of cholera and cholera-risk area needs be continuously updated by relevant government authorities. This system should ensure standardized and uniform definition and reporting of cases by employing PAHO/WHO cholera definition and reporting methods. The effective management of outbreaks also calls for time-to-time refresher training for medical teams and non-medical personnel that are expected to take part in outbreaks management. Proper awareness on safe disposal of human waste and its importance should be created via health education to the general populace by the national and local government.

The prevention of cholera and effective management in cases of outbreak also has a lot to do with individuals' hygiene. Individuals are expected to drink potable water only or boil their water when the integrity of water is in doubt before drinking. Foods, especially seafood should be prepared such that adequate heat that could inactive pathogens is achieved within the food matrix and contact between cooked food and raw food needs be avoided as well. It is also advisable that raw food be avoided as much as possible especially in cholera or cholera-risk area. In cases where raw foods such as vegetables and fruits that cannot be peeled are unavoidable, such should be properly rinsed with potable water before consumption. Hands should also be carefully washed with soap after using toilet and before preparing food. A simple pit of dimension 0.3 m × 0.3 m × 0.3 m can be situated at least 30 m away from water resources (e.g. well) in emergency cases before proper latrine or toilet which should be situated below water sources in non-swampy area is built. Bathing water of doubtful integrity needs be avoided as much as possible and antibiotics should not be used unless prescribed by certified health workers. All cases of cholera and cholera-like infections should be reported to the appropriate authority.

ENVIRONMENTAL INTERVENTIONS AND RESPONSES

Sanitary and potable water Interventions

Good hygiene practises have been known to reduce or even prevent cholera outbreaks but hygiene is impossible in the absence of sanitary facilities and/or potable water. Cholera and cholera-like infections are contracted and transmitted via faecal-oral route, i.e. by ingesting faecal contaminated water or food material infected with *Vibrio cholera* and other pathogenic Vibrio species. Thus, provision of both sanitary and potable water which will enhance and

improve good sanitation and hygiene practises are critical to interrupting the cycle of cholera and cholera-like outbreaks.

Hygiene and sanitation interventions

Anthropogenic activities such as indiscriminate dumping of refuse, urinating, defecating and improper waste disposal are activities which can trigger the cycle of cholera and cholera-like outbreaks. Therefore, well monitored sanitation programs and practises which will separate human wastes especially faeces from human contact is expected to substantially increase the health of human (Brown *et al.,* 2002). In other to achieve a successful sanitation programme, public toilets/latrines that are well managed by communities needs be made available in places like markets and schools. It has been estimated that an average of one latrine to 20 people will be okay for crowded places like refugee camps and slum. Since it is practically difficult to build latrines or solid waste disposal system in acute cholera outbreak conditions (Brown *et al.,* 2002), it is important that preventive measures such as effective sanitation programs which should involve building of public toilets/latrines should be one of the paramount goal of every local community.

Oral cholera vaccine (OCV)

The recent inclusion of OCV in cholera prevention and control kits is to allow for synergy between the earlier known interventions and OCV. The most common OCV in developing countries is Shanchol which reduces the ability of *V. cholera* to colonise intestinal tract of humans thus reducing the spread of the infection by human. The concern on possible toxic side effects of thiomersal which is a mercury containing compound used as preservative for the vaccine had made the WHO to advice the production of Shanchol to be without the compound. Non-mercury containing compounds are to be used as preservatives which will thus make the vaccine devoid of any known side effect (WHO, 2004; Sur *et al.,* 2009). The dosing regimen of the vaccine is usually two weeks interval. Despite its promise, OCV is not widely promoted among public health agencies. There have been reservations by some organisations involved in cholera prevention and control on the effects of promoting OCV on traditional interventions. The reservation is on the possibility of channelling the limited resources used in traditional interventions towards OCV alone. Nevertheless, the possible synergy between the traditional interventions and OCV cannot be denied and should be explored as recommended by WHO in 2010.

Community interventions

The ultimate success of various cholera prevention and control programs also lies with the readiness of various local communities to be ready to key in into the programs. The community leaders are the first to be educated on the benefit of environmental changes that will benefit the prevention and control of cholera especially when such will affect community cultural believes and practises. This is expected to be followed up with creation of necessary awareness on

anticipated personal and joint contributions/actions towards cholera prevention and control in the community. It has been shown that participatory approach in promoting hygiene is more efficient than message-based approach that only gives information on cholera prevention and control. Also, members of the community must not just be informed but must be technically mobilized to adhere to safe hygiene practices since it will involve behavioural changes. Techniques that could be employed include social media, peer connections and participatory training.

Monitoring and surveillance of V. cholerae

Cholera is the most devastating waterborne disease in the history of mankind and strikes so suddenly with speedy fatality in many cases. Since cholera is caused by ingesting water and food contaminated by the faeces of cholera patient, casual contact with contaminated wares, bedding, water and even food might be all that is needed to contract cholera. The disease can be so quick and rapid to the extent that it can kill within 12 and 48 hours. Cholera continues to be an important cause of illness and death worldwide with an estimation of more than 100,000 death tolls each year ((http://www.who.int/mediacentre/factsheets/fs107/en/index.html accessed on 11/12/2017). The good news is that it was through earlier occurrences of cholera outbreaks that the epidemiologist discovered the link between sanitation and public health, which led to the discovery of modern day water and sewage system. Despite efforts to control cholera, the global frequency of occurrences is on the rise. It has been reported that there exist a correlation between socio-economic factors such as population density, poverty and poor sanitation and hygiene and lack of sanitary and potable water supply (WHO, 2010). The various ways of improving water quality are well known to include boiling, chlorination, and filtration. These methods are not economical for most rural and urban dwellers, e.g. the cost of paraffin for boiling water makes boiling method uncommon especially in poverty stricken communities; and sanitation which could have made up for this, is still very poor in many rural or peri-urban communities of developing countries especially in Asia and Africa (Borroto and Martines-Piedra, 2000; Soussan, 2003; United Nations, 2009).

In rural areas, people depend mostly on water sources such as surface and ground water for drinking and domestic use. In a situation where the water source is contaminated, diseases can be transmitted to the communities through which they flow. Cholera outbreaks have often been traced to faulty or dysfunctional wastewater treatment plants, pipes and contaminated water supplies in municipal systems. Such was the case of broad street episode in the Soho district of London, England in the year 1854 that led to 616 deaths in the space of three months (Parkes, 2013). WHO has reported that about 78% of Third World population lack access to sanitary and potable water while up to 85% of those people also lack access to proper sewage treatment and this makes cholera an ongoing concern for the entire world.

America has successfully controlled and prevented cholera using operational interferences for decades. Unfortunately, cholera re-emerged in Haiti that had been free of the disease for many

decades following major earthquake disaster in 2010. This led to more than 600,000 cases and 7,000 deaths (Barzilay *et al.,* 2013). The interferences include effective and early disease detection and diagnosis, treatment with ORS and antibiotics when necessary, provision of sanitary and potable water, provision of proper sewage treatment plants and advocacy for improved personal hygiene (Waldman *et al.,* 2013). Unfortunately, implementation often takes years as was the case in Latin America during the 1990s. To address this issue, some organizations such as Bill and Melinda Gates Foundation are developing a comprehensive and effective global cholera control and prevention strategies through funding The Task Force for Global Health and Harvard Medical School/Partners in Health.

A national cholera prevention and control plan is of importance to any country since it has the potential of giving direction to controlling the disease. This plan is expected to involve key players such as public health agencies, local and national laboratories for cholera monitoring, relevant government bodies, media, community leaders, environmental agencies and any other relevant bodies. National guidelines and recommendation on best practises for cholera prevention and control will be more helpful if they are regularly updated with new research findings.

Advocacy for food guidelines

About 1.8 million childhood deaths have been attributed to acute diarrhoeal illnesses in developing countries while the burden of this illness is also enormous in developed countries (Scallan *et al.,* 2005). However, the estimation of foodborne diseases global burden has not been accurate over the years due to various factors, which include lack of uniformity in defining acute diarrhoeal illness in studies, unreported and/or incomplete data on diarrhoeal illness to public health authorities, and difficulty in linking illnesses to food. There is need for a research based guidelines on appropriate ways of linking gastroenteritis to food which is an important medium for transmitting pathogens of significant public health importance, although not all gastroenteritis is foodborne and *vice versa* (Flint *et al.,* 2005). The proportion of individuals susceptible (e.g. elderly and immunosuppressed) to severe foodborne illnesses are growing in many countries. Also, the globalization of food supply can contribute to the spread of pathogens to new geographical locations as was the case of discharge of ballast water contaminated with *Vibrio cholera*e in the Americas in 1991 if strict guidelines for exportation and importation of food products are not followed. Tourists, refugees and immigrants are likely to be exposed to unacquainted foodborne hazards in their new locations. The ability of pathogens to evolve into more virulent variants by developing new or modified virulence determinants and by developing resistance against antibiotics as well as the increasing number of individuals exposed to poor hygiene in commercial food settings especially in developing countries contribute to foodborne illness burden. This suggests the need for the preparedness of public health officers towards continuous development of improved methods to combat these threats in a changing environment.

Unrecognized, unreported and uninvestigated cases of foodborne outbreaks are common especially in developing countries where most available resources to investigate foodborne diseases outbreaks are not adequate. This thus calls for the need to develop introductory monitoring guidelines on identification and investigation of pathogens involves in foodborne outbreaks. The guidelines should be developed based on the peculiarity of each country such that investigation techniques could be modify to suite unique characteristics of resident foodborne pathogen(s) in local communities. The effective control and prevention of foodborne outbreaks depends on the efficiency of the public health workers as well as the implementation of well-functioning and integrated food control systems. It is therefore imperative for stakeholders (food law and regulations bodies, food control management, inspection services, epidemiological and food monitoring-laboratory services and health education department) in food control systems to collaborate with consumers to solve the malady of foodborne outbreaks.

2.11 MONITORING AND RESPONSE GUIDELINES

Monitoring the environment is one of the means employ by epidemiologist to understand the patterns of diseases in a population and has been defined as systematic collection, analysis and dissemination of information about the level (e.g. occurrence, incidence, prevalence) of infections or diseases that are known to occur in a specified population (WHO, 2001). Monitoring focuses on understanding the pattern/progress of a known disease in a population while surveillance focuses on ascertaining the presence of a disease that has not been reported in a particular environment (Angus, 2002). In both cases, guidelines are necessary for quality assurance and a guideline is one of the three components (standard, specifications and guidelines) of Microbial Criteria. A microbial guideline is a Microbial Criteria applied at any stage of microbiological investigation which aids in identifying situation(s)/area(s) that require actions/responses (Huss, 2003). An effective public health protection relies on a generation of representative data obtain from classification and microbiological monitoring programs (EC, 2012). Generation of representative data and interpretations of data depends on several factors such as sampling size, sites, time, sampling duration and anthropogenic activities in sampling environment. As an example, monitoring programme developed by EC (2012) for microbiological classification and monitoring of bivalve mollusc production using *E. coli* as indicator organism lay emphases on factors earlier mentioned and the inherent pathogenicity and virulence factors in *E. coli* as imperative factors to take into consideration especially when such monitoring will be used for both immediate responses and future health risk forecasting in a particular environment. The need for multifactorial risk based surveillance and monitoring programmes which will inform reactive and/or proactive responses in epidemiological studies/analyses have been proposed (Oidtmann *et al.,* 2013). This kind of approach to monitoring involves continuous sampling of a chosen biological indicator(s) for a long period of time in order to determine seasonal trend of such indicators in a sampling area which will thus inform the possibility of pollution and thus call for tracing source of such pollution using Microbial Source Tracking in case of microbial analysis (Oidtmann, 2013). The European Union

in one of her reports advocated for consideration of Vibrio pathogens that are of less significance to be included among bacteria to be monitored in the environment on the basis of pathogenicity risk assessment. The advocacy was based on the fact that the so called pathogenicity irrelevant species of vibrio (*V. fluvialis, V. hollisae and V. mimicus*) were found out to have been associated with significant number of infections arising from contaminated seafood (EU, 2001). Development of a multifactorial risk based model for forecasting health risk levels based on prevalence of vibrio species in an environment has been attempted but the main challenge was dearth of data to test such model (Oidtmann *et al.,* 2013). Despite this shortcoming, risk base monitoring program which is more cost effective (Oidtmann *et al.,* 2013; FAO, 2011; Huss, 2007; FAO, 2002; FAO, 2001) can still be employed to plan monitoring program for cholera and cholera related infections in the ECP prior to development of a risk analysis model for vibrio. The level of risk in an environment will be based on anthropogenic activities captured by questionnaires and microbial analysis such as virulence determinants analysis and antibiogram analysis of recovered vibrio species in the ECP environment.

CHAPTER THREE
CHOLERA HOTSPOTS SURVEY

This chapter reports on the cholera hotspots survey and administration of questionnaires.

3.1 METHODOLOGY

3.1.1 Administration of questionnaires (aims 2, 3, 4 and 5)

Questionnaires as approved in Deliverable 2A were administered to the WWTP managers in Amathole District Municipality, Joe Gqabi District municipality, OR Tambo Metropolitan Municipality, Sarah Baartman District Municipality and Buffalo City Municipality.

3.1.2 Cholera hotspot survey

The cholera hotspot survey commenced with the Tyhume and Kat River catchments both of which are important freshwater resources within the Amathole District Municipality in the Eastern Cape Province. The catchment of the cholera hotspot survey was subsequently expanded and freshwater samples were collected from other rivers important to the Eastern Cape Province including Great fish river, Tsitsa river, Mthatha river, Mbashe river, Qunu river, Kowie river, Boesman river, Buffalo river, Mqanduli river and Ngqeleni river, Kubusi river, Tsomo river, Broukrans, Ngcorgora river, White Kei river, Ooskleinemonde, Weskleinemonde, Swartkops, Mzimvubu, Quamanco, Mgwali, Ngonyama, Xuka, Gonube river, Nahoon river, Kwelera, Langkloof, Karnmelkspruit, Orange river, Kraai river, Saalboomspruit river and Mooi river. Sites along the rivers that have close proximity to human settlements and farming communities, and that were accessible, were prioritized for sampling between September 2015 and October 2016. Subsequently the survey was also expanded to cover some WWTPs within the time frame of the survey as stated above.

3.1.2.1 Description of study area

A) RIVERS

Tyhume River

The Tyhume River is a freshwater resource in Nkonkobe local municipality. It is significant to the inhabitants of the rural settlements through which it flows as it is used extensively for different domestic purposes, as well as for irrigation, fishing, livestock watering, recreation and a source of potable water. The river takes its source in the Amathole Mountains flowing through the lower coastal steep slope in different rural settlements down to Alice, from where it flows to Manqulweni community forming a confluence with the Keiskamma River. Six sampling sites viz Drayini, Manqulweni (Amadama and Njwaxa), Hala, Khayalethu, Melani and Alice were selected following after our previous report (K5/1968).

Kat River

The sampling points covers of the Kat River catchment and its tributaries, including the Kat River Dam in Seymour and the river in Balfour through to Fort Beaufort. The Kat River is 150 kilometres long, and supplies the Kat River Valley with irrigation water for large citrus orchards, and supplies Seymour and Fort Beaufort with domestic water (Motteux, 2001). Fort Beaufort is a town situated in the Eastern Cape Province. Commercial farming is taking place mainly in the Middle Kat and Lower Kat, whereas small holders and emerging farmers mostly practice agriculture in the Upper catchment. The catchment is home to ≈178,000 people, of whom about 10% reside in Fort Beaufort, the only urban centre in the Kat River region. The rest of the people live in rural, remote villages, where only a few have access to potable water and where they work as farm labourers (Obi *et al.,* 2011).

Keiskamma River

The Keiskamma River is a freshwater resource in Amathole District Municipality in the central part of the Eastern Cape Province. It springs in the forested mountains of the Amatola Mountains, above Keiskammahoek, and runs down the Keiskamma river valley, and flows through the eastern verge of the small towns of Middle drift, Bomapass, Lower and upper Ngqumeya, bordering Fort Cox College compounds. It was dammed shortly after Keiskammahoek as Sandile Dam at (32º42'29.6"S 27º06'08.8"E), as a water storage reservoir for Keiskammahoek transitional local council area, the rural settlements, the entire Middle drift District and farms in the area for irrigational purpose. Its main tributary is the Tyhume River. The river flows into the Indian Ocean in the Keiskamma estuary, located by Hamburg Nature Reserve, near Hamburg, midway between East London and Port Alfred (Fatoki *et al.,* 2003).

Qunu River

Qunu river is an important freshwater resources in Eastern Cape, located in Qunu a small village of King Sabata Dalindyebo Municipality, O.R Tambo District in the Transkei. It is situated at coordinate 31º47’S28º37’E, 32 km south-west in between Mthatha and Buttherworth road. The Qunu river serves the trio purposes of irrigation, drinking and recreational to the community. It is worthy of note that even with the existence of tap water facility in some households within the area of the community, person-to-person interrogation in the locality during a reconnaissance visit to identify potential sample points shows that some people still prefer drinking from the river mostly due to its assumed therapeutic potentials. The observation of anthropogenic impacted run-offs from the community that empties into the river coupled with the heavy presence of excreta suspected to be from grazing animals as well as lots of subsistence farming located around the banks of the river makes it a good choice for inclusion in the hotspot survey. Five sampling points which were 500 m apart were chosen for this study along the stretch of the community.

Mbhashe River

Mbhashe River flows from the banks of Ngcobo through Dutywa, Gatyana (Willowmore) and Xhora (Elliotdale), the three towns of Mbhashe, into the sea at Mbhashe Point, close to the Haven. Mbhashe River remains the only feasible source of bulk water supply for the southern portions of the Mbhashe Municipality. It is also believed to be a recipient for wastewater effluent disposal from Mbhashe Municipality's major towns of Idutywa, Gatyana, Xhora and numerous rural settlements. Also, the river is prone to various agricultural runoffs from farms around the river sides. The dependence of communities along the Mbahshe river on the river for drinking and domestic use in spite of its obvious microbiological state made it to be considered in this hotspot survey. Five points were considered along the stretch of the river in Mbashe community.

Tsitsa River

The Tsitsa River is a river in the Eastern Cape Province, South Africa. It is a tributary of the Mzimvubu River and belongs to the Mzimvubu to Keiskamma Water Management Area. It is located in OR Tambo District Municipality, Eastern Cape, South Africa and has a length of 23.27 kilometres (Liquid Journal, 2008). Tsitsa River is used for irrigation, domestic and industrial purposes. Sampling points were selected along the course of the river.

Mthatha River

Mthatha River is a river in the Eastern Cape Province which is about 250 km long and has its source in Baziya mountain range at Langeni forests and its mouth in the Indian Ocean at an estuary located near Coffee Bay. The major primary user of water from the Mthatha/ River is the Mthatha Municipality which supplies all other secondary users in the catchment with treated water abstracted from the river. Mthatha dam supplies Mthatha and surrounding towns with domestic water, and also serves as storage for the hydropower balancing dams (DWAF, 2004). Traditional healers (sangomas) use the river water and also churches use the river water for baptisms. A large population of Transkei, most of which is rural uses water from Mthatha River for various domestic purposes like brick making, irrigation, cultural purposes and forestry related industries among others. The state of the water quality of the River is very poor, due for example to raw wastewater effluents discharge, storm water runoff from the town of Mthatha and other dense informal settlements downstream, uncontrolled and excessive removal of wood and plants from the riparian zone, rubbish dumping and improper sanitation practices. Inadequate treatment, overloaded sewage works and poor management of the sewage systems result in the discharge of untreated sewage effluent into the river which in turn causes eutrophication, potential toxic algal blooms and microbial contamination which is a health hazard to water users. Soil erosion due to human activities such as removal of vegetation and cattle crossing, is also evident, thus increasing sediment which has a negative impact on the river channel. The poor water quality is a major problem in the Mthatha River as communities live alongside the riverbanks, particularly in the region immediately downstream of the city. These communities rely on the Mthatha River as a source of domestic water supply without any form of treatment. Three points were randomly

selected for sampling in the Mthatha dam and another two points along the river in the centre of the town close to a major wastewater works (Fatoki *et al.,* 2001).

Boesmans River

Boesmans River is a river in the Eastern Cape, South Africa. It originates from north of Kirkwood and runs east past Alicedale, before it turns and twists south and east to Kenton on Sea, where it mouths into the Indian Ocean through a tidal estuary only 1.7 km to the South West of the mouth of the Kariega River (http://mapio.net/o/864362/). Several informal and low-cost housing settlements are situated on the banks of Boesmans River that depend solely on the river for domestic purposes and communal irrigation. There have been reported cases of leakages from pipes that transfer sewages to the treatment plant. The spills run through several farms where farmers used the water to irrigate pastures and drinking water for dairy herds. The presence of excess nutrient has resulted in eutrophication and thereby altering the flow of the boesmans River. Offensive odour and Eutrophication was observed during visit which corroborates the report of Midland news. Two sampling points were selected along the course of the river Alicedale and Kwanonzwakazi.

Kowie River

Kowie River is one of the important rivers in the Eastern Cape Province, which takes its source in the hills of Grahamstown. The river flows east-ward of its source and empties into the Indian Ocean at Port Alfred via an estuary at coordinate 33º 36'06''S26º53'58''E. The river is locally called "Ecawa" and it is the longest tidal boat navigable river in South Africa. Its major tributaries include Bloukrans river (which receiving watershed for final effluent of wastewater treatment plants in Grahamstown), Bak River, Lushington or (Torrens) River and several other unnamed small rivers including Little Kowie River which enters into Kowie river's estuarine about 14 km from its mouth. The river is approximately 70 km^2 long and the area of the river catchment has been estimated to range between 576 km^2 to 769 km^2. Two of the major cities that the river passes through (Bathrust and Port Alfred) are found in Ndlambe local municipality while the third city (Grahamstown) is located in Makana local municipality of Cacadu District municipality, Eastern Cape, South Africa. The river is one of the Fish to Tsitsikamma Water Management Areas. Agriculture products supported by this river includes Pineapples, citrus, chicory, fodder crops, beef and goats while aquatic animals found in the freshwater side of the river include mullet, Eels while crabs and mussel are common at the bank of its estuarine. The river is used for irrigation of fields used for dairy production but the low quality of the water as indicated by various water quality indicators measured in 2001 raised a lot of public health concerns. In 2013, Mngxitama-Diko reported that water-borne diseases (e.g. diarrhoea and cholera) outbreak that will be linked to Kowie river as a result of the discharge of unacceptable final effluent from wastewater treatment plants into some of the river's tributaries was looming. Also, it was observed that several anthropogenic activities were ongoing at the mouth of the river

during our reconnaissance visit. Thus, this river was included as one of the hotspots during our survey and a water sample was taken at the mouth of the river and analyzed.

Buffalo River

The Buffalo River is one of the important freshwater resources in South Africa with its course passing through two provinces (KwaZulu-Natal and Eastern Cape) of the country. While the part of the river in the Eastern Cape Province is called Cwenqgcwe in Xhosa and the part in KwaZulu-Natal called uMzinyathi in Zulu, Afrikaans speakers generally call it Buffelsrivier irrespective of its location. The river takes its source in the seeps and sponges of the Amatola Mountains at an altitude of 1200 m and the river is flanked by rock cliffs up to 120 m high, runs a length of 126 km through the Amatola indigenous forest in a deeply-incised channel and empty into Indian Ocean at East London Harbour. The river is divided into three reaches and provides water for city/town it flows through. The river has a catchment area of 1287 km^2, supports about 570,000 people while its major tributaries include Cwenqgcwe, Izele, Mgqakwebe, Ngqokweni and Yellowwoods. The major city/town which serves a passage to the river includes Bisho, East London, King Williams Town, Mdantsane and Zwelitsha. The river serves recreational, transportation, domestic and agricultural (irrigation) purposes as agricultural practices are widespread in the middle reaches of the river catchment area. The major dams in the river catchment are bridle, laing, maden and rooikrantz. The quality of domestic and industrial effluents discharge into the rivers has been questioned while factors such as eutrophication, algae bloom, faecal contamination due to broken sewers, have been reported to impact the river negatively. In the recent past, the quality of the river has raised public health concerns which need immediate attention as physicochemical parameters and microbial analysis shows that the water body serves a significant health hazard. The report of our laboratory on the presence of non-cholera causing Vibrio pathogen in the final effluent of some wastewater treatment plants in Amathole District Municipality amidst other factors has earlier indicated that the river could potentially harbour vibrio pathogens. Thus the river was included in our hotspot survey and five samples were taken from different sampling point along the course of the river for vibriology study (Chigor *et al.,* 2013).

Mqanduli River and Ngqeleni River

Mqanduli river and Ngqeleni river are important rivers to the communities of Mqanduli and Ngqeleni in the Eastern Cape Province as they are used extensively as a source of drinking, agriculture and recreational purposes. The history of frequent contamination of these waterbodies by sewage works is the reason for selection in this hotspot survey.

Great Kei River

The Great Kei River (Afrikaans: Groot-Keirivier) is a river in the Eastern Cape Province. It is formed by the confluence of the Black Kei River (Afrikaans: Swart-Keirivier) and White Kei River (Afrikaans: Wit-Keirivier), northeast of Cathcart. It flows for 320 km (199 miles) (South

Africa Estuarine land: Great kei catchment, undated) and ends in the Great Kei Estuary at the Indian Ocean with the small town Kei Mouth on the west bank. Historically the Great Kei River formed the southwestern border of the Transkei region. It can be estimated that the Great Kei Basin occupies the area of approximately 20,480 square kilometres. Major tributaries of the Great Kei River are the Tsomo, Kubusi, Gcuwa and Tyityaba rivers; the latter being inaccessible due to its location in a gorge area with a rough and steep terrain. The Great Kei River and its tributaries flow through confined valleys with gentle slopes hence meandering. The Kei River catchment receives its rainfall mainly in the summer. Considering its location in the driest part of the country, numerous dams and barriers had been built to meet the dire need of water supply in the rural and urban settlement in the Great kei catchment area. Major reservoirs include the following: Lubisi Dam with the capacity of 158 million cubic meters, Ncora Dam with about 150 million cubic meters, Xonxa of about 97.5 million cubic meters and Wriggleswage Dam of about 91.5 million cubic meters. Observed in the rural and urban settlement of great Kei Catchment area are markedly different units of land use activities which includes the following: Livestock farming (Beef, dairy, sheep, poultry and goats), Subsistence farming (maize and vegetables), Game farming (Builder beast, antelope, bush pig or warthog and kudu), Commercial farming lands occur mostly on the western side of the catchment (mainly Lucerne), where extensive irrigation occurs. It is believed that most of the land use activities could impact the quality of the River (DWAF, 2009).

Mgwali River

The Mgwali River (Latitude: -32°31'8.54", Longitude: 27°39'51.66") located in the Eastern Cape, is one of the 4 main tributaries for Mbhashe River (Main tributaries: Xuka River, Mgwali River, Dutywa River and the Mnyolo River). The River is used for recreational fishing, irrigation and at some points along its course used for drinking.

Xuka River

The Xuka River (Latitude: -31°42'26.25", Longitude: 28°19'41.3") is also a major tributary of Mbashe River located in the Eastern Cape. The River is used for various Agricultural purposes.

Swart kei River

The Swart Kei River (Latitude: -32°9'6.12", Longitude: 27°15'55.08") part of the Mzimvubu to Keiskama Water Management Area is located in the Chris Hani District Municipality in the Eastern Cape Province. It is part of the confluence that forms the Great Kei River. It is documented to originate from the North of Queenstown, beginning its course as the Grootvleispruit River; and eventually joining the Black Kei River, to form the Great Kei River. Xonxa dam is situated on it and is used mainly for irrigation purposes.

Gonubie River/ Gqunube River

The Gonubie River (Latitude: -32°55'59.99", Longitude: 28°1'59.99") is located in the Eastern Cape Province, Buffalo City Municipality. It is used majorly for recreational purposes; passing through the Lomardy Private Nature Reserve and Gonubie Estuary.

Ngonyama River

Ngonyama is situated in Eastern Cape Province, with geographical coordinates 31° 52' 0" South, 27° 41' 0" East. The community use the river for recreational purpose and to irrigate their farms, for this reason the river was targeted in this hotspot studies. Two sampling points along the river flow were chosen for this survey.

Kubusi River

The Kubusi River is an important freshwater resources near Stutterheim a town in Amahlathi local Municipality in the Eastern Cape Province of South Africa. It is one of the major tributaries of Great Kei river in the Ciskei region. It is significant to the inhabitants of the rural settlements through which it flows as it is used extensively for different purposes such as irrigation, fishing, livestock watering, recreation and a source of potable water. Also, the river is prone to various agricultural runoffs from commercial farms around the river banks. During the reconnaissance visit, we observed run off from rural and small villages settlements located on the banks of the river makes it a good choice of inclusion in the hotspot survey.

Tsomo River

The Tsomo River is a river in the Eastern Cape Province. It is a tributary of the Great Kei River. It originates about 10 km to the north west of the town Elliot and flows southward to meet the right-hand bank of the Great Kei River. It is situated at coordinates 32°22'60" S and 27°49'60" E. Towns lying on the banks of the Tsomo River include: Tsomo, Cala and Ncora. Tsomo River supplies all other secondary users in the catchment with water abstracted from the river. Ncora dam supplies Tsomo, cala and Ncora and surrounding towns with domestic water. Ncora dam also serves as a means of irrigation to all neighbouring commercial farms. The current state of the water quality of the river is very poor, owing to indiscriminate disposal of inadequately treated wastewater effluents, storm water and anthropogenic run off from rural and small villages situated on the banks of the river made it to be considered for the hotspot survey.

White Kei River

The White Kei River or Wit-Kei River is a river in the Eastern Cape, South Africa. It originates from north of Queenstown, beginning its course as the Grootvleispruit river and eventually joining the Black Kei River, to form the Great Kei River. It is situated at coordinates 32°9'0" S and 27°24'0" E. The Xonxa Dam is located in the White Kei River. The dam serves mainly for irrigation purposes and its hazard potential has been ranked high. Presently this river is part of

the Mzimvubu to Keiskama Water Management Area. White Kei River is used for irrigation, domestic and industrial purposes. Sampling points were selected along the path of the river.

Bloukrans River (Grahamstown)

The Bloukrans River (Grahamstown) is a tributary of the Kowie River, and is situated near Grahamstown in the Eastern Cape Province of South Africa. It is situated at coordinates 33°25′S 26°39′ E. The river runs through the residential areas of Grahamstown, and is subject to various sources of pollution, such as overflowing sewage drains and litter. As the stream leaves Grahamstown, effluent from the Grahamstown Wastewater Treatment Plant is released into the stream, which substantially increases flow. The stream runs through a short section of irrigated pastures for dairy farming and irrigated agriculture. The stream also runs into a pool within the Blaauwkranz Nature Reserve approximately 40 km downstream, and it is also used for religious and spiritual ceremonies by the local communities. The water quality within the Bloukrans Stream affects the water quality of the pool downstream, as well as the receiving Kowie River, and therefore the quality of the Bloukrans Stream is of ecological and social importance.

Kraai River

The Kraai River ("Crow River") is a tributary of the Orange or Gariep River that flows near Barkly East in the Eastern Cape Province. The Kraai River originates in the mountains south of Lesotho and flows westward from the confluence of the Bell River and the Sterkspruit at Moshesh's Ford at 30°51′09″S 27°46′40″E all the way to Aliwal North, where it joins the Orange River at 30°40′02″S 26°45′06″E. The Kraai is used for fishing and irrigation. The huge dependence on the river for a host of agricultural and recreational activities justifies its inclusion in this hotspot survey.

Saalboomspruit

Saalboomspruit is a major tributary of the Kraai River and is used extensively for irrigation inclined agricultural practice. Two points were sampled from this river.

Mooi River

The Mooi River is about 160 km from Durban and 64 km from Pietermaritzburg. Mooi River is used by the farming and textile centre in the area. This river was originally named Lawrenceville after the Irish farmer who formalised its settlement during the 1800s. This river is asloused for trout fishing and irrigation.

Qumanco River

The Qumancu River is a stream in the Eastern Cape Province. It is located at an elevation of 817 meters above sea level. The coordinates are 31°43'60" S and 28°1'0". Samples were collected from two points along the stretch of the river

Mzimvubu River

Mzimvubu or Umzimvubu River is one of the most important rivers in the South Africa, and it is located in the Eastern Cape Province. Mzimvubu River starts from an altitude of about 2 700 m above sea level on the Drakensberg escarpment to the Indian Ocean over a distance of approximately 300 km. The mainstream has four tributaries; the Tsitsa, Tina, Kinira and the Mzintlava rivers. Major landuse practices observed on Mzimvubu are agriculture which includes commercial agriculture with farm dams, irrigation schemes, crop production and animal husbandry as well as subsistence agriculture which is mainly maize fields, vegetable gardens and livestock. Also observed, is that farmers and people in the surrounding communities use this river water for domestic, drinking and recreational purposes.

Swartkops River

Swartkops Rivers originates from the Groot Winterhoek Mountains, and meanders seaward for about 155 km passing the semi-urban areas of Despatch, Uitenhage and Motherwell before joining the estuary 19 km lower down close to Bethelsdorp before joining and discharging into the sea.

This river is used by fish farmers around Swartkops area and Uitenhage mostly for fishing. The townships of KwaZakhele and Motherwell are located further from the river but induce an indirect effect on the system through pollution. Industrial activities such as Fish water flats sewage works saltpans, sand/clay mining, brickworks and power stations, motor industry, wool industry, tanneries, aquaculture, railway yards and deports, with limited agricultural activities take place along the river route (Elizabeth, 2009). Nelson Mandela Bay is a major seaport and automotive manufacturing centre and the powerhouse of the Eastern Cape located on the South East of the Province with many people migrating there from rural areas for employment purposes thus the extension of informal settlements on the area. It is surrounded by urban and township areas of Port Elizabeth, Motherwell, KwaNobuhle, Despatch and Bethelsdorp to name a few were informal settlements predominates, with 87% of the households having flushing toilets connected to the sewage and 74.10% with access to piped water inside their dwelling place. The Swartkops, which remains the river of choice is the closest but unfortunately is also polluted. A toxicology study of the Swartkops has now become common knowledge and the results are simply shocking. The consumption of fish from this river should be avoided at all costs as harmful heavy metals are present in the flesh of these fish and over time will cause many health complications (Cape estuaries, 2009)

Ooskleinemonde and Weskleinemonde River

The Kleinemonde rivers are important twin coastal rivers in the Eastern Cape that serve as a source of recreation and fishing to communities where they span through. The vast amount of human activities observed along the course of the river informed the reason for the inclusion in the hotspot survey.

Ngcongcolora River

Ngcongcolora is a stream in Eastern Cape Province. It is located at an elevation of 854 meters above sea level. It is situated at coordinates 32°4'0" S and 27°46'0" E, and is an important to the residents of the rural settlements through which it flows as it is used extensively for different purposes including irrigation, fishing and a source of drinking water. Also, the river is prone to various agricultural runoffs from agricultural projects that are taking place along its course

B) WASTEWATER TREATMENT PLANTS

Final effluents from WWTPs described in Table 3.1 below were sampled during the Hotspot survey.

Table 3.1: Description of wastewater treatment plants used in this hotspots survey.

WWTPs	Domicile District/Metropolitan Municipality	Technology used	Design Capacity (Ml/d)
Adelaide WWTP	Amathole	Activated sludge and sludge drying beds	0.5
Idutywa	Amathole	Oxidation ponds	1.1
Craddock WWTP	Chris Hani	Activated sludge and sludge lagoons	4.2
Alice WWTP	Amathole	NA	NA
Aliwal North WWTP	Joe Gqabi	Activated sludge and sludge lagoons	5.5
Sterkspruit WWTP	Joe Gqabi	Oxidation ponds	0.2
Maclear WWTP	Joe Gqabi	Activated sludge	1.4
Ugie WWTP	Joe Gqabi	Oxidation ponds	0.5
Mthatha WWTP	OR Tambo	Biofilters	12.0
Butterworth WWTP	Amathole	Biofilters, anaerobic digestion and sludge drying beds	12.6
Mayfield WWTP	Sarah Baartman	Activated sludge, aerobic digestion	2.5
Belmont valley WWTP	Sarah Baartman	Biofilters, anaerobic digestion	5.4

NA= Not applicable

3.1.2.2 Sampling and analytical procedures

Sample collection

Water samples were collected from the above-listed rivers between September 2015 and October 2016 using sterile 1 L bottles. All water samples were transported on ice from the sampling sites to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, Alice for analyses within 6 hours of collection.

Detection of *Vibrio* species in genomic DNA of the water samples

Water samples collected from the different sampling sites were assessed for the presence of *Vibrio* species. The water samples were firstly concentrated by membrane filtration technique as recommended by APHA (1998). A volume of 100 mL of each water sample was filtered through a sterile polycarbonate membrane filter (0.45 µm pore size, 47 mm diameter; Whatman) and the filtration process was facilitated using PALL vacuum/pressure pump; after which the filtration unit was disassembled carefully. Alkaline peptone water (APW) was used for enrichment of the filtered concentrates followed by incubation at 36°C for 18-24 h. At the end of the incubation period, total genomic DNA was extracted from the enrichment cultures. DNA extraction method was carried out as described by Maugeri *et al. (*2006) with slight modification. About 1 mL of the enriched culture was transferred into sterile 2 mL Eppendorf tubes. The tubes were then centrifuged at 11000 × *g* for 10 min, the supernatant discarded, and the pellet suspended in 200 µl of sterile nuclease-free water and vortexed. Thereafter, they were boiled using an AccuBlock (Digital dry bath, Labnet) for 10 min at 100°C. The cell debris was removed by centrifugation at 11,000×g for 10 min using a Mini-Spin micro-centrifuge (LASEC, RSA). The supernatant (10 µl) was transferred into new sterile Eppendorf tubes and then used as template DNA in the Polymerase chain reaction (PCR) assays. All genomic DNAs extracted were screened for the presence of the genomes of *Vibrio* genus and *Vibrio cholerae* using specific primers listed in Table 3.2. The PCR conditions are as reported elsewhere (Nongogo and Okoh, 2014),

Detection of Vibrio species among pure culture isolates from the water samples

After alkaline peptone water (APW) enrichment of the membrane filtered samples followed by incubation at 37°C for 18-24 h, a loopfull of the enriched broth was streaked onto thiosulphate citrate bile salts sucrose (TCBS) agar plates and incubated at 37°C for up to 48 h. At the end of the incubation period, typical yellow and green colonies were considered as presumptive Vibrio species. Five to ten colonies per plate were then randomly picked and subsequently subcultured on sterile TCBS and nutrient agar plates for purity and molecular identification (Huq *et al.,* 2012).

Table 3.2: Primers used for Vibrio genus and *Vibrio* species detection.

Genus/Specie	Primer	Sequence	Size
Vibrio genus	V16S-700F	CGG TGA AAT GCG TAG AGA T	663bp
	V16S-1325R	TTA CTA GCG ATT CCG AGT TC	
	ompW-F	CACCAAGAAGGTGACTTTATTGTG	883 bp
V. cholerae	ompW-R	GAACTTATAACCACCCGCG	
	Vp. flaE-79F	GCA GCT GAT CAA AAC GTT GAG T	897 bp
V. parahaemolyticus	Vp. flaE-934R	ATT ATC GAT CGT GCC ACT CAC	
	Vv. hsp-326F	GTC TTA AAG CGG TTG CTG C	410 bp
V. vulnificus	Vv. hsp-697R	CGC TTC AAG TGC TGG TAG AAG	
	Vf toxR-F	GAC CAG GGC TTT GAG GTG GAC	217 bp
V. fluvialis	Vf toxR-R	AGG ATA CGG CAC TTG AGT AAG ACTC	
ctxA *V. cholerae* toxin gene	*ctx*A (F)	CTC AGA CGG GAT TGT TAG GCA CG	302 bp
		TCT ATC TCT GTA GCC CCT ATT ACG	
	*ctx*A (R)		

3.2 RESULTS

3.2.1 Questionnaires administration

Table 3.3 summarizes the different responses from the questionnaires distributed in some of the wastewater treatment plants in Amathole District Municipality, Buffalo City Metropolitan Municipality, OR Tambo District Municipality, Joe Gqabi District Municipality, Sarah Baartman District Municipality and Nelson Mandela Metropolitan Municipality while Table 3.4 is summarized responses from a survey used to assess the accreditation of laboratories (*on-site* and external labs) used for analyses of the qualities of the WWTP samples.

According to the Greendrop document for wastewater quality assessment, a compliant WWTP has to concur with the following:

1. In terms of it process control maintenance and management skills; a plant must have a certified copy of registration certificate of works, copies of registration certificates of process controllers and supervisors, proof of a maintenance team used in their overall maintenance; and proof of site specific operation and maintenance manual.
2. Wastewater quality monitoring. This focuses on the details of the sampling sites, determinants and frequencies of operational monitoring as well as compliance monitoring.
3. Wastewater submission for analysis; a WWTP must be able to provide proof and name of laboratory used and compliance to quality assurance as prescribed in standard methods. Results obtained from monitoring analysis are used to amend process controlling in the WWTP.

4. The WWTP must have an authorisation document detailing effluent quality standards/limits used to calculate compliance; in line with effluent quality categories.
5. There must be proof of a documented wastewater incident and management protocol employed.
6. A WWTP must have a documented design capacity of the facility, medium to long term planning ensuring sufficient capacity for treatment systems and to ensure effluent quality compliance. A WWTP must operate with its recommended capacity.
7. The WWTP effluents must have microbiological compliance of 90% required for wastewater quality standards.

Table 3.3 shows the level of compliance in comparison with the Greendrop guidelines for the various WWTP. On the affirmative, 88% of the WWTP complied with process control and maintenance and management skill requirements; 59% carried out wastewater monitoring; 76% submits their final effluent for analysis; 65% had proof of a documented wastewater incident management protocol, responding to wastewater quality failures; 70% had documentation of authorisation detailing effluent quality standards; 53% had a microbiological compliance of greater than 90%; and 47% were functioning within their design capacity. According to Greendrop report for WWTP, internal and external laboratories need to be duly accredited to perform specific methods that checks for water quality determinants.

Table 3.4 shows the accredited laboratories reported in the survey. The East London Laboratory (external Laboratory) that is central to receiving WWTP samples from around the Eastern Cape Province is not accredited. However it is ISO 17025 certified and suffices to quality management requirements. The laboratory carries out both physicochemical (pH, conductivity, free chlorine, ammonia, nitrate, nitrite, phosphate and COD) and microbiological analysis (*E. coli*, heterotrophic plate count, faecal coliform and total coliform). The Laboratory is able to carry out laboratory presumptive identification of indictor bacteria; and technical management like calibration of machines and staff training. The Butterworth laboratory is *on site*. It has been accredited and also ISO 17025 certified. It also concurs with the quality management requirements, but only carries out physicochemical (pH, conductivity, free chlorine, ammonia, nitrate, nitrite, phosphate and COD) analyses. The Laboratory is able to carry out technical management like calibration of machines and staff training.

Table 3.3: Summary of different responses from the questionnaires distributed in some of the wastewater treatment plants in Amathole District Municipality, Joe Gqabi District Municipality and Buffalo City Municipality.

WWTP /DM	Process control (Registration compliance)	Wastewater monitoring	Wastewater submission for analysis	Authorisation document detailing effluent quality standards	Wastewater quality risk management	Wastewater treatment capacity (> design capacity)	Microbiological compliance (> 90%)
Komga/Amathole	Yes	No	Yes	No	Yes	Yes	Yes
Alice/Amathole	No	No	No	No	No	No	No
Middledrift/Amathole	Yes	No	Yes	No	Yes	Yes	Yes
Idutywa/Amathole	No	No	Yes	No	No	No	No
Butterworth/ Amathole	Yes	No	Yes	Yes	No	No	Yes
Adelaide/Amathole	Yes	Yes	Yes	Yes	Yes	Yes	-
Seymour/Amathole	Yes	No	Yes	Yes	Yes	Yes	Yes
Postdam/BCM	Yes	Yes	Yes	Yes	Yes	No	-
Mdantsane/BCM	Yes	Yes	Yes	Yes	Yes	No	-
Sterkspruit/Joe Gqabi	Yes	No	No	No	No	No	-
Maclear/Joe Gqabi	Yes	Yes	Yes	Yes	No	No	-
Belmont valley/ Sarah Baartman	Yes	Yes	No	Yes	Yes	Yes	Yes
Mayfield/Sarah Baartman	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Central Treatment Works/Buffalo City	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Stutterheim WWTP/Amathole	Yes	Yes	Yes	Yes	Yes	No	Yes

Fort Beaufort/Amathole	Yes	Yes	-	Yes	No	Yes	No
Fish Water Flats/Nelson Mandela	Yes	Yes	Yes	Yes	Yes	No	Yes
% Affirmative	88	59	76	70	65	47	53

- = Not Answered; DM = District Municipality.

Table 3.4: Summarized responses from a survey used to assess the accreditation of laboratories (On-site and External labs) used for analysis of WWTP samples in the Eastern Cape.

Criteria	East London laboratory (External), BCM	Butterworth Laboratory (On-site), Amathole
Laboratory accreditation	No	Yes
ISO 17025 assessment	Yes	Yes
Quality management requirements (Sampling, handling and transportation practices)	Yes	Yes
Physicochemical parameters evaluated	PH, conductivity, free chlorine, ammonia, nitrate, nitrite, phosphate and COD	PH, conductivity, free chlorine, ammonia, nitrate, nitrite, phosphate and COD
Microbiological analysis	E. coli, heterotrophic plate count, faecal coliform and total coliform	No
LABORATORY CAPABILITIES		
Presumptive identification	Yes	No
Molecular confirmation	No	No
Cholera monitoring	No	No
TECHNICAL MANAGEMENT		
Calibration of machine	Yes	Yes
Staff training	Yes	Yes
Technical experts on ISO 17025	Yes	Yes

3.2.2 Vibriology

A total of 141 water samples were collected from the 37 rivers listed in Table 3.5 below. All the rivers were positive for the Vibrio genus suggesting that at least one species of Vibrio bacteria are domiciled in all the rivers sampled. Also, *Vibrio cholerae* was detected in some of the rivers in varying degrees relative to the number of samples tested per river. However, no toxigenic strains of the *V. cholerae* was detected suggesting that there are no cholera causing Vibrio pathogens in the rivers, thus implying that cholera appears to have been significantly controlled in the Eastern Cape Province at least within the precinct of its aquatic environment.

Table 3.5: Qualitative molecular detection of Vibrio genus and *V. cholerae* in metagenomics DNA recovered from the rivers.

River	# of samples	% positive relative to number of samples		
		Vibrio genus	*V. cholerae*	Toxigenic *V. cholerae*
Kat river	13	100	7.7	0
Tyhume river	11	89	25	0
Keiskamma river	16	87	7.1	0
Mbahshe river	7	100	42.8	0
Qunu river	9	100	66.6	0
Mthatha river	2	100	50	0
Tsitsa river	4	100	100	0
Boesmans river	2	100	50	0
Kowie river	1	100	100	0
Buffalo river	5	100	80	0
Ngqeleni river	1	100	100	0
Mqanduli	1	100	100	0
Great Fish river	8	100	100	0
Great Kei river	1	100	100	0
Kubusi	4	100	75	0
Tsomo	5	100	100	0
Broukrans	3	100	100	0
Ngcorgcora	1	100	100	0
White Kei	1	100	100	0
Ooskleinemonde	1	100	0	0
Weskleinemonde	1	100	100	0
Swartkops	3	100	0	0
Mzimvubu	4	100	0	0
Qumanco	2	100	100	0
Mgwali	4	100	100	0
Ngonyama	2	100	100	0
Xuka	2	100	0	0
Swart Kei	1	100	100	0
Gonube	2	100	0	0
Nahoon	3	100	0	0
Kwelera	2	100	100	0
Langkloof	1	100	0	0
Karnmelkspruit	2	100	0	0
Orange	1	100	0	0
Kraai	3	100	0	0
Saalboomspruit	2	100	0	0
Mooi	2	100	0	0

Table 3.6 summarizes the prevalence of some key pathogenic Vibrio species in 31 rivers in the Eastern Cape Province based on culture and isolation data. The numbers of presumptive Vibrio species used for the assays ranged between 1 and 106 for all the rivers, and of these between 10 and 100% of isolates recovered from each of the rivers were confirmed to belong to the Vibrio genus. With respect to the other key pathogenic Vibrio species, none were detected in Saalboomspruit, Kraai, Orange, Karnmelkspruit, Langkloof, Broukrans, Kubusi, Keiskamma and Kat rivers, while the proportions of the rivers that harboured the other pathogens follow the order: *V. paraheamolyticus* (61%); *V. fluvialis* (35%); and *V. vulnificus* (26%). Also, in corroboration of our earlier observation with respect to the incidence of toxigenic Vibrio based on direct detection from metagenomics DNA samples, our culture and isolation data also revealed the absence of toxigenic *V. cholerae* strains thus supporting our earlier remark to the effect that cholera appears to have been significantly controlled in the Eastern Cape Province.

Table 3.6: Prevalence of selected key pathogenic Vibrio species in some rivers in the Eastern Cape Province.

Rivers	# of presumptive Vibrio sp.	Prevalence relative to the # of confirmed Vibrio genus (%)						
		Confirmed Vibrio genus	***V. cholerae***	**Toxigenic *V. cholerae***	***V. parahaemolyticus***	***V. fluvialis***	***V. vulnifius***	**Uncharacterized vibrio species**
Kat	60	95	7	0	0	0	0	93
Keiskamma	74	77	88	0	0	0	0	12
Mbahshe	24	100	13	0	0	0	8	79
Qunu	56	95	6	0	2	6	4	83
Mthatha	35	86	30	0	0	27	0	43
Tsitsa	49	96	49	0	ND	ND	ND	ND
Buffalo	106	82	0	0	1	0	0	99
Ngqeleni	16	100	13	0	0	31	0	56
Great Fish	95	95	26	0	6	0	9	60
Kubusi	37	92	94	0	0	0	0	6
Tsomo	60	100	8	0	0	10	0	82
Broukrans	25	92	96	0	0	0	0	4
Ngcorgcora	20	100	40	0	0	5	0	55
White Kei	16	100	63	0	0	19	0	19

Rivers	# of presumptive Vibrio sp.	Prevalence relative to the # of confirmed Vibrio genus (%)						
		Confirmed Vibrio genus	***V. cholerae***	**Toxigenic *V. cholerae***	***V. parahaemolyticus***	***V. fluvialis***	***V. vulnifius***	**Uncharacterized vibrio species**
Ooskleinemonde	2	100	0	0	0	50	0	50
WesKleinemonde	4	50	50	0	0	0	0	50
Swartkops	50	10	0	0	14	0	0	86
Mzimvubu	76	74	0	0	14	0	0	86
Qumanco	8	100	50	0	13	0	0	38
Mgwali	14	100	43	0	0	43	0	14
Ngonyama	21	100	5	0	0	14	5	76
Xuka	19	100	0	0	0	11	11	79
Swart Kei	8	100	75	0	0	0	0	25
Gonube	23	78	0	0	0	0	11	89
Nahoon	17	88	0	0	0	13	7	80
Kwelera	30	73	14	0	0	0	18	68
Langkloof	12	67	0	0	0	0	0	100
Karnmelkspruit	1	100	0	0	0	0	0	100
Orange	8	100	0	0	0	0	0	100
Kraai	4	100	0	0	0	0	0	100
Saalboomspruit	8	88	0	0	0	0	0	100
Detection rate (%)			61	0	19	35	26	NA

ND = Not determined; NA = Not applicable.

Table 3.7 summarizes the prevalence of the Vibrio genus, *Vibrio. cholerae*, *Vibrio parahaemolyticas*, *Vibrio fluvialis* and *Vibrio vulnificus* in the final effluents of some wastewater treatment plants in the Eastern Cape Province. A total of 14 WWTPs were sampled, and 215 presumptive Vibrio isolates were recovered from the effluents in the proportions ranging between 4 and 29 per plant. About 39% of the presumptive Vibrio species were confirmed as belonging to the Vibrio genus and in only one case (Mayfield River) was *V. cholerae* detected. Even then, the detected *V. cholerae* is not a toxigenic strain. Analysis for *Vibrio parahaemolyticas*, *Vibrio fluvialis* and *Vibrio vulnificus* shows that they constituted 7%, 8% and 15% of the confirmed Vibrio species respectively.

3.3 DISCUSSION

The development of the questionnaires for wastewater treatment plants was based on the Greendrop requirements of 2013/2014/2015 (DWAF, 2013, 2015). According to the requirements, different aspects considered in the evaluation are scored and each score depends on the significance of each parameter/aspect in the process of wastewater treatment. In the requirements, the areas which are considered include: 1) Process control, maintenance and management skills (10%); 2) Wastewater monitoring (15%); 3) Submission of data to DWA (5%); 4) Wastewater Effluent Quality Compliance (30%); 5) Wastewater Quality Risk Management (15%); 6) Bylaws (5%); 7) Wastewater treatment capacity (5%); and 8) Wastewater asset management (15%). However, according to the weight of the scores, wastewater effluent quality compliance carries the highest score. Having that in mind, the wastewater effluent compliance of these wastewater treatment plants surveyed had complications. None of the wastewater treatment plant operators knew if the compliance was above or equal to 90% and what it was. As much as documentation of authorisation detailing effluent quality standards was available, most plants (47%) did not monitor any microbiological compliance as stipulated in the Greendrop requirements. In the surveys, they reported that microbiological and chemical compliance is determined by their external laboratory in East London under Buffalo City Municipality (Scientific services). In the case of the evaluated wastewater treatment plants in Amathole, they reported that none of them have "On-site" laboratories with the exception of the Butterworth WWTP which in any case their *on-site* lab is not functional.

All the WWTPs reported that they rely on the East London Laboratory for microbiological and chemical analyses. It was obvious that the East London Laboratory has too much responsibility as it is the only one serving the Amathole and Buffalo City Municipality. It is important to note that the scope of microbiological examination in the East London laboratory as observed rely only on colonial presumptive identification with no confirmation through the more specific molecular procedures such as polymerase chain reaction (PCR) techniques. Also, the target organisms for the water samples from the WWTPs do not include potentially pathogenic microorganisms such as *V. cholerae*.

The results for the qualitative molecular detection of vibrio genus and *Vibrio cholerae* in metagenomic DNA recovered from the rivers confirms the presence of the species in the freshwater resources in the Eastern Cape Province (Table 3.5). The incidences of *Vibrio cholerae* ranged from 7.1-100%. All the *V. cholerae* isolates were screened for the cholera toxin gene but all were negative implying that they are not cholera causing strains (O1 and O139 pathotypes), suggesting that diarrhoeal disease caused by these strains may be less severe. However, the presence of non-cholera causing vibrio pathogens (non-O1 and non-O139 *V. cholerae*) in the aquatic environment suggests that environmental conditions in the freshwater resources are supportive of their growth and could also support the growth cholera causing *V. cholerae*. This

serves as a strong motivation towards the importance of ongoing monitoring of these freshwaterbodies in the province. Further confirmation of key human pathogenic species mainly *V. fluvialis, V. vulnificus and V. parahaemolyticus*, suggest that freshwater resources are important reservoirs of pathogenic Vibrio species and a potential source of their accompanying infections in the case of exposure (Table 3.6).

Table 3.7: Confirmation of the prevalence of Vibrio genus, *V. cholerae*, *V. parahaemolyticas*, *V. fluvialis* and *V. vulnificus* in final effluents of the selected WWTPs.

WWTPs	# of presumptive Vibrio isolates	Number of confirmed isolates of the Vibrio genus	Proportion of pathogenic Vibrio species detected.			
			Vibrio cholerae	*Vibrio parahaemolyticas*	*Vibrio fluvialis*	*Vibrio vulnificus*
Adelaide	18	14 (77%)	0	1(7%)	0	0
Craddock	14	14 (100%)	0	0	6(43%)	0
Alice	14	12 (85%)	0	0	0	1(8%)
Aliwal North	17	13 (76%)	0	0	0	0
Sterkspruit	04	0	0	0	0	0
Maclear	07	02 (28%)	0	0	0	0
Ugie	08	0	0	0	0	0
Mthatha	21	10 (47%)	0	0	0	4(40%)
Butterworth	29	11 (37%)	0	2(18%)	0	3(27%)
Mayfield	15	09 (60%)	1[#] (11%)	3(33%)	0	1(11%)
Belmont valley	08	03 (37%)	0	0	0	2(67%)
Tsomo	23	23(100%)	0	0	0	0
Whittle Sea	12	12 (100%)	0	0	0	0
Fort Beaufort	10	5(50%)	0	0	1(10%)	2(20%)
Total	215	85(39%)	1(1%)	6(7%)	7(8%)	13(15%)

[#]=Not positive for ctx gene

The implication of WWTP effluents in the pollution of receiving waterbodies have been documented worldwide; with the presence of pathogenic organisms from such pollutions playing significant roles in water related disease outbreaks. The Vibrio genus and human pathogenic species; *V. cholera*, *V. fluvialis, V. vulnificus and V. parahaemolyticus*, detected in the final effluents of the WWTPs (Table 3.7) highlights the impact that these plants may have on their receiving watersheds, environment and the community as a whole.

3.4 CONCLUSION

All selected water resources in the Hotspot survey were positive for *Vibrio* genus; with some sites positive for *V. cholerae*, *V. fluvialis, V. vulnificus* and *V. parahaemolyticus*. It is apparent that the water resources selected in this study could pose significant health and environmental risk to communities who rely on them for their daily activities. Contamination of water resources used as major sources of drinking water have resulted in several waterborne disease outbreaks in the past; hence the need for a continuous pollution monitoring programmes of water resources especially in rural communities of the Eastern Cape. In addition, the provincial government and other relevant authorities concerned with environmental matters in the province should be involved. The PCR technique used in this survey for microbial identification and surveillance has high sensitivity and specificity and could be employed in routine microbial water quality assessments. The findings of this hotspots survey dictated the choices of sites of concentrations for the detailed comprehensive vibriology as concluding parts of aims 6-9 of this project and driven by the project topics listed in Table 3.8 below for the doctoral students.

Table 3.8: Project topics of the doctoral students as part of the detailed and comprehensive vibriology which concludes the aims 6.9 of this study.

Student name	Project title
Nontongana N	Appraisal of wastewater final effluents and surface waters as reservoirs of *Vibrio* pathogens: A case study of the Mthatha and Butterworth WWTP as well as the Mthatha and Qunu river
Okeyo A	Assessment of the prevalence of non-cholera causing *Vibrio* pathogens in some wastewater final effluents and their receiving waterbodies, in the Eastern Cape Province: Sarah Baartman municipality as a case study.
Osunla AO	The Prevalence and public health significance of pathogenic *Vibrio* species in selected freshwater resources and treated final effluents of wastewater treatment plants in Chris Hani district municipalities in the Eastern Cape, South Africa.
Abioye OE	Molecular studies on cholera and non-cholera causing *Vibrio* pathogens isolated from selected aquatic animals and water resources in Amathole, Sarah Baartman, Nelson Mandela and Buffalo city municipalities in Eastern Cape, South Africa.
Fadare TO	Evaluation of cholera-causing and non-cholera causing *Vibrio* species in some freshwater resources used for vegetable irrigation, irrigated vegetables and raw vegetables marketed in the Amathole, Chris Hani, OR Tambo and Buffalo City District municipalities of Eastern Cape, South Africa.
Gcilitshana O	Prevalence of *Vibrio* Pathogens in Rivers and Wastewater treatment plants of the Eastern Cape Province using Fort Beaufort and Adelaide regions as case studies.

CHAPTER 4

DETAILED STUDY OF THE VIBRIOLOGY OF THE SELECTED SITES

Based on the findings of the hotspot survey the following WWTPs and Rivers were selected for the detailed vibriology study**:**

- **WWTPs:** Butterworth WWTP, Adelaide WWTP, Belmont Valley WWTP, Mayfield WWTP, Mthatha WWTP and Craddock WWTP
- **Rivers:** Kat river, Keiskamma river, Qunu river, Mthatha river, Tsitsa river, Great Fish river, Kubusi river, Tsomo river and Kowie river

The choice for these study sites was based on the high prevalence of *V. cholerae* from the metagenomic DNA recovered from the water samples from the sites and the fact that some residents along the river courses use the resources for drinking, agricultural, recreation and other domestic needs. Furthermore, a couple of WWTPs discharge their final effluents into these rivers. For the WWTPs, the high prevalence of at least one *Vibrio* species was used as an index for selection. The comprehensive vibriology study also included selected vegetables and aquatic animals as per the aims and objectives of the project, and methods are similar to those used for the hotspots survey with the inclusion of antibiogram, virulence genes and speciation components following after standard methods described in detail in the report. A summarized stand-alone manual for the monitoring of cholera and non-cholera causing vibrio pathogens in water, vegetables and aquatic animals was also compiled. Maps showing areas covered in the detailed vibriology is presented in Figures 4.1-4.4.

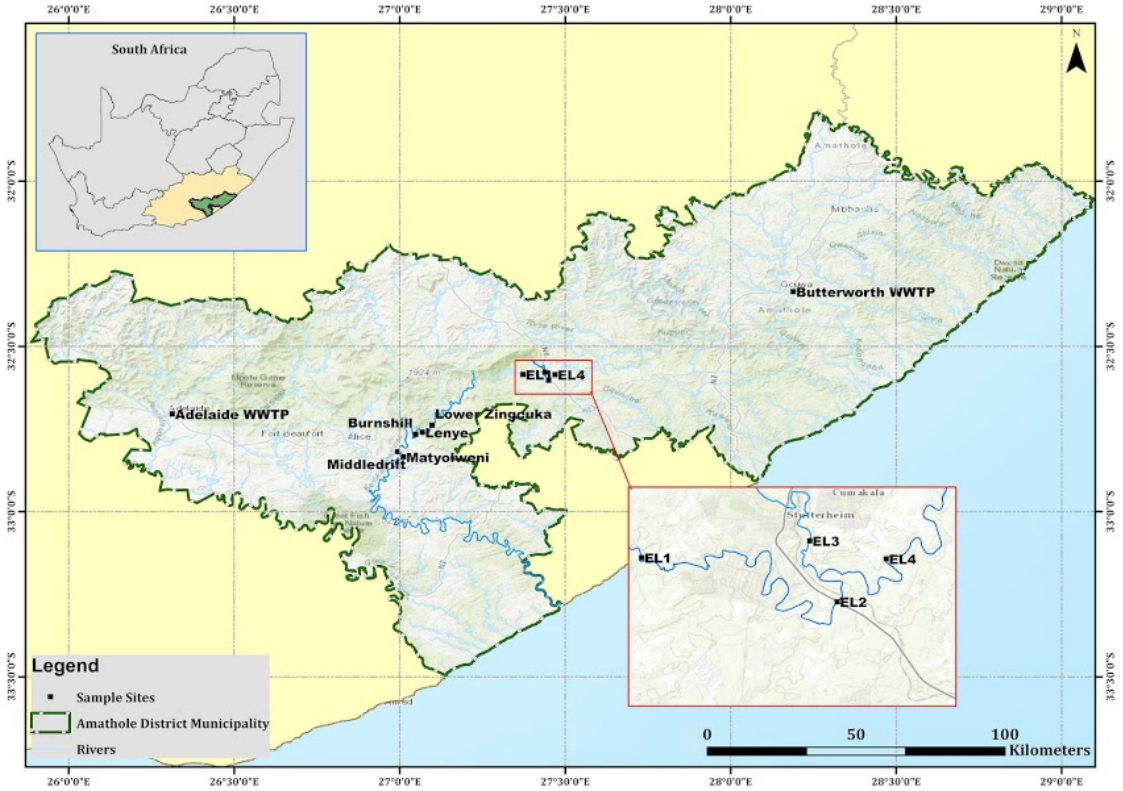

Figure 4.1: Map showing sampling points in the Amathole district municipality.

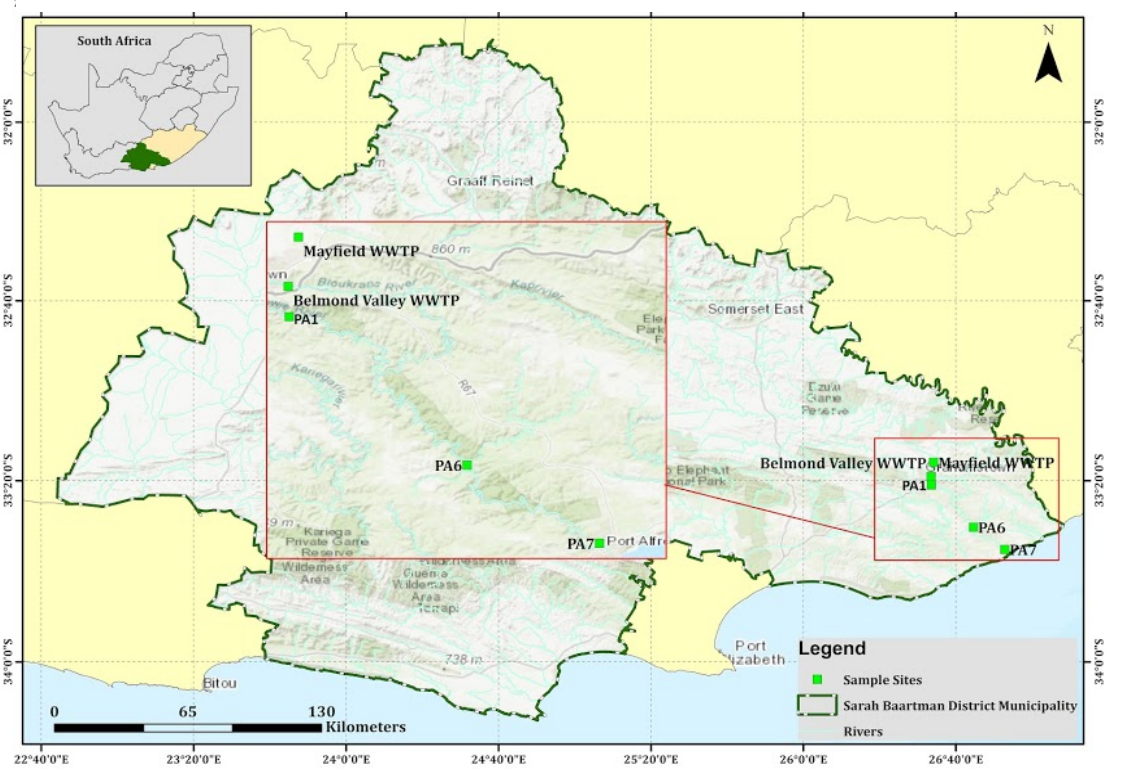

Figure 4.2: Map showing sampling catchments in Sarah Baartman district municipality.

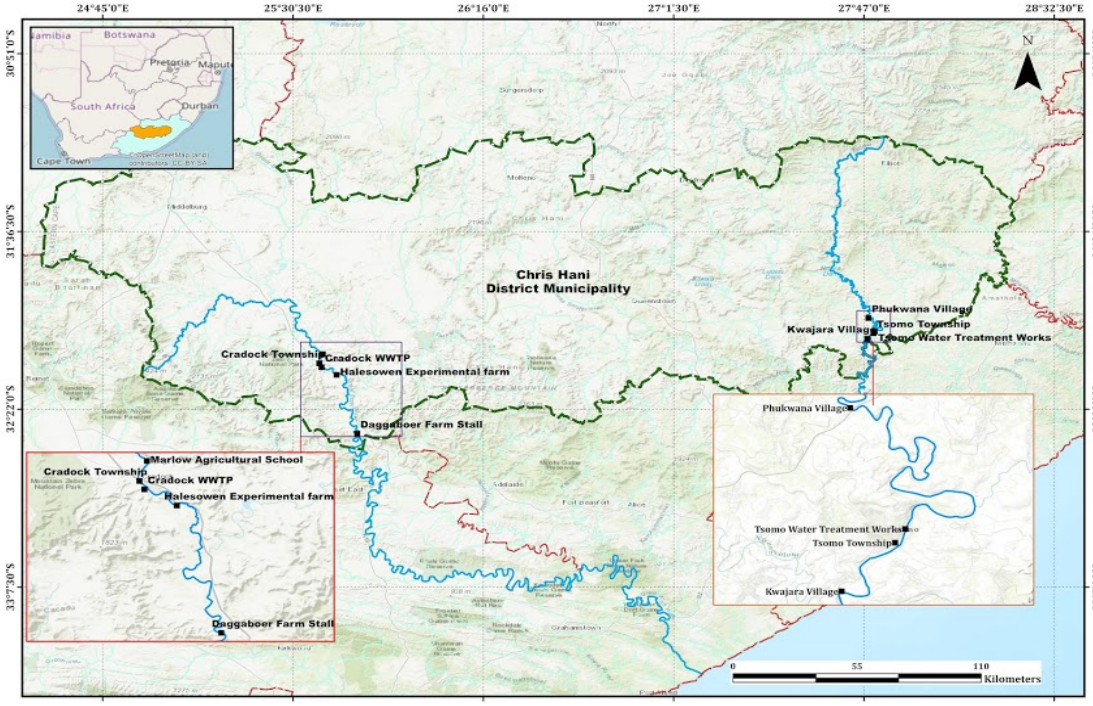

Figure 4.3: Map showing the sampling sites in Chris Hani district municipality.

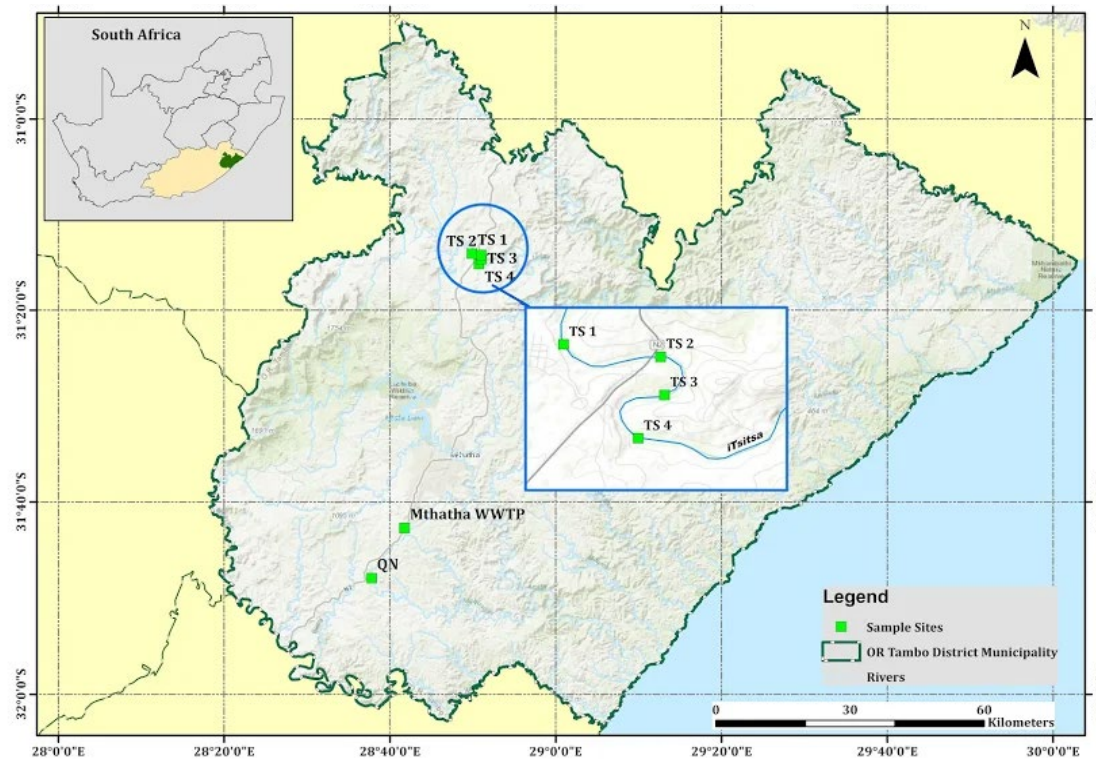

Figure 4.4: Map showing sampling sites in the OR Tambo district municipality.

4.1 METHODOLOGY

The methodology was adopted and modified from the hotspots survey. In addition the antibiogram and virulence determinants components were also conducted.

4.1.1 Analysis of antibiotic resistance/susceptibility profile

Antimicrobial susceptibility testing was done using the standard disc diffusion method recommended by the Clinical and Laboratory Standards Institute (Jorgensen *et al*., 2015). Briefly, fresh cultures (~22 hours) were suspended in 5 ml sterile normal saline and the turbidity of the suspension adjusted to 0.5 McFarland standards. Sterile swabs were then dipped into the bacterial suspensions and used to inoculate the Mueller Hinton agar plates by spreading uniformly on the surface of the agar, after which the antibiotic discs placed on the bacterial lawn and the plates incubated at 35 ± 2°C for 18 to 24 hrs. At the end of the incubation period, the plates were examined for zones of inhibition which were then measured and interpreted using the zone diameter breakpoints (Jorgensen *et al*., 2015). The antibiotics were used in this study are listed in Table 4.1 below and are those used for the treatment of cholera and other *Vibrio* spp. infections.

Table 4.1: Antimicrobial agents used to test level of susceptibility.

Antimicrobial class	Antimicrobial agent	Disk content (µg)	Zone diameter breakpoints (mm)		
			S	I	R
Penicillins	Ampicillin	10	≥17	14-16	≤13
	Augmentin	20/10	≥18	14-17	≤13
Cephems	Cefotaxime	30	≥26	23-25	≤22
	Cefuroxime	30	≥18	15-17	≤14
Carbapenems	Imipenem	10	≥23	20-22	≤19
	Meropenem	10	≥23	20-22	≤19
Aminiglycocides	Amikacin	30	≥17	15-16	≤14
	Gentamycine	10	≥15	13-14	≤12
	Trimethoprim	10	≥15	12-14	≤11
	Kanamycin	30	≥18	14-17	≤13
	Nitrofurantoin	300	≥17	15-16	≤14
Fluroquinolones	Ciprofloxacin	5	≥21	16-20	≤15
	Ofloxacin	5	≥16	13-15	≤12
	Nalidixic acid	30	≥21	16-20	≤15
Folate pathway inhibitors	Trimethoprim-sulfamethoxazole	1.25/23.75	≥16	11-15	≤10
Phenicols	Chloramphenicol	30	≥18	13-17	≤12
Lipopeptides	Polymixin B	300	≥14	11-13	≤12

(*Source: CLSI, 2015)

4.1.2 Virulence genes analysis

PCR assays were used to reveal the prevalence of the relevant virulence genes in the pathogens using specific primers listed in Table 4.2. The PCR was performed in a mixture consisting of 12.5 µl of OneTaq Master Mix with Standard Buffer, 1 µl (10 pmol) of each of the primers (reverse and forward), 5.5 µl of nuclease free water and 5 µl of the DNA template. The amplification conditions were the following: 15 min initial denaturation at 93°C; followed by 35 cycles of 92°C for 40 secs denaturation, 1 min annealing and 72°C for 1.5 min of extension and final extension at 72°C for 7 min. The PCR products were electrophoresed in 1.5% agarose gel with a 100-bp DNA ladder as molecular size marker (100 bp DNA ladder). The gels were stained with ethidium bromide and visualized using a UV trans-illuminator (ALLIANCE 4.7).

Table 4.2: Primer sequence for virulence determinants of *Vibrio* spp.

Target gene	Primer sequence (5'-3')	Annealing Temp (°C)	Amplicon Size(bp)
Tdh	(F) GTA AAG GTC TCT GAC TTT TGG AC (R) TGG AAT AGA ACC TTC ATC TTC ACC	48	269
Trh	(F) TTG GCT TCG ATA TTT TCA GTA TCT (R) CAT AAC AAA CAT ATG CCC ATT TCC G	58	500
Tlh	(F) AAAGCGGATTATGCAGAAGCACTG (R) GCTACTTTCTAGCATTTTCTCTGC	55	450
VPI	(F) GCA ATT TAG GGG CGC GAC GT (R) CCG CTC TTT CTT GAT CTG GTA G	52	680
tcpA	(F) CACGATAAGAAAACCGGTC AAGAG (R)-Classical TTACCAAATGCAACGCCGA ATG (R)-El Tor CGAAAGCACCTTCTTTCACA CGTTG	55	 620 453
Zot	(F) TCGCTTAACGATGGCGCGT TTT (R) AACCCCGTTTCACTTCTACC CA	60	947
hylA	(F) GAG CCG GCA TTC ATC TGA AT (R) CTC AGC GGG CTA ATA CGG TTT A	60	738
ompU	(F) ACGCTGACGGAATCAACCA AAG (R) GCGGAAGTTTGGCTTGAAG TAG	54	869
toxR	(F) CCTTCGATCCCCTAAGCAA TAC (R) AGGGTTAGCAACGATGCG TAAG	54	779

(Source: Xie *et al*., 2005)

4.2 RESULTS AND DISCUSSION

4.2.1 Wastewater

The wastewater treatment plants (WWTPs) and their receiving waterbodies are located in the Sarah Baartman, OR Tambo, Amathole and Chris Hani District Municipalities. The sampling points included the final effluents of the in the WWTPs and 500 m upstream and downstream their discharge points. Samples positive for the Vibrio genus were randomly selected from each site to represent isolates from each district municipality and used for the determination of the prevalence of the key Vibrio species targeted in this study which includes *V. cholerae*, *V. vulnificus*, *V. alginolyticus*, *V. fluvialis*, *V. parahaemolyticus* and *V. mimicus*. These species have been documented to be predominant in Vibrio related food and waterborne infections (Tantillo *et al*., 2004; Oliver, 2005; De and Mathur, 2011). The antibiotic susceptibility profile and the incidence of selected virulence genes were also determined.

4.2.2 Prevalence of Vibrio pathogens in selected wastewater effluents and receiving waterbodies

The WWTPs in the Sarah Baartman District Municipality included Mayfield and Belmond Valley WWTPs. Their receiving waterbodies are Mayfield River and Kowie River respectively. Five key pathogenic Vibrio species including *V. cholerae, V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus* AND *V. fluvialis* were detected in this study site in the following order of prevalence: Mayfield WWTPs: *V. cholerae* (3.5%), *V. alginolyticus* (2.3%) and *V. parahaemolyticus* (1.2%) from the final effluents; and *V. cholerae* (1.2%) and *V. parahaemolyticus* (2.3%) from downstream the WWTP; Belmond Valley WWTP: *V. cholera* (14%), *V. vulnificus* (3.5%), *V. alginolyticus* (3.5%), *V. fluvialis* (2.3%) and *V. parahaemolyticus* (3.5%) from the final effluent; and *V. cholerae* (3.5%) and *V. alginolyticus* (3.5%) upstream the plant; and *V. cholera* (3.5%), *V. vulnificus* (1.2%), *V. alginolyticus* (1.2%), *V. fluvialis* (1.2%) and *V. parahaemolyticus* (1.2%) downstream the plant (Table 4.2).

The Vibrio species composition in the final effluents samples from the Mthatha WWTP was as follows: *V. vulnificus* (7%), *V. alginolyticus* (2.3%) and *V. parahaemolyticus* (39.5%). Although downstream the Mthatha WWTP discharge point was inaccessible due to the dangerous terrain, species identified from upstream the discharge point followed the order: *V. fluvialis* (18.6%), *V. parahaemolyticus* (30.4%), *V. vulnificus* (4.7%) and *V. alginolyticus* (4.7%). In the Amathole District Municipality, Butterworth WWTP final effluents samples had Vibrio species compositions in the order: *V. cholera*e (4.7%), *V. vulnificus* (9.3%), *V. fluvialis* (7%) and *V. parahaemolyticus* (14%) (Table 4.2). Its receiving waterbody was in accessible.

The Cradock and Adelaide WWTPs effluents and their receiving waterbodies (Great Fish River and Koonap River respectively) are located in the Chris Hani District Municipality. From the Cradock WWTP effluents, the composition of the *Vibrio* species is as follows: *V. cholerae* (5%), *V. parahaemolyticus* (1.7%) and *V. mimicus* (1.7%). Vibrio species identified from upstream the discharge point (Great Fish River) were *V. cholerae* (3.3%), *V.*

parahaemolyticus (1.7%) and *V. mimicus* (3.3%); and *V. cholerae* (5%). *V. parahaemolyticus* (1.7%) and *V. mimicus* (3.3%) were the only two detected in the downstream samples. With respect to the Adelaide WWTP effluents, upstream and downstream its receiving waterbody (Koonap River) were 6.7%, 5% and 3% *V. cholera*; 6.7%, 5% and 3% *V. parahaemolyticus*; and 1.7%, 1.7% and 1.7% *V. mimicus* respectively (Table 4.2).

Vibrio pathogens are common inhabitants of marine coastal ecosystems and adapts well to the changing environments such as changes in temperature, algal blooms, etc. Their adaptability to adverse conditions may also be responsible for their presence in other environments like sewage (Lobitz *et al*., 2000; Worden *et al*., 2006; Dungeni *et al*., 2010). Studies have shown the association and resilience of the Vibrio genus in wastewater, and being able to survive and thrive in the harsh conditions that is characteristics of wastewater effluents (Igbinosa *et al*., 2009; 2011; Okoh *et al*., 2015). The identified Vibrio species are etiologic agents of some diseases in humans mainly contracted through ingestion of contaminated water or food. The most comprehensively studied Vibrio pathogen, known for its effect on the small intestines through the release of enterotoxins; causes infections in humans mostly through the ingestion of contaminated water and food is *V. cholerae. V. parahaemolyticus* may less commonly cause acute gastroenteritis, diarrhoea and abdominal pain in individuals who eat contaminated seafood (usually raw or undercooked sea food like oysters) or wound infections when exposed to sea water. *Vibrio vulnificus* on the other hand is known to be extremely virulent and can cause 3 types of infection: 1) acute gastroenteritis from eating raw or undercooked seafood; 2) necrotizing wound infections when injured skin is exposed to contaminated marine water for instance; and 3) invasive septicaemia when the bacteria invades the blood stream (80 times more likely in immunocompromised individuals) (Tantillo *et al*., 2004; Oliver, 2005; De and Mathur, 2011). *V. fluvialis* causes cholera-like bloody diarrhoea, wound infection and primary septicaemia in immunocompromised individuals (Igbinosa and Okoh, 2010). *Vibrio alginolyticus* is also medically important; it causes otitis media (ear infection) and wound infection caused from practices such as seaweed wound dressing with unsterilized sea weed (Reilly *et al*., 2011). *Vibrio mimicus* is a Vibrio species that mimics *V. cholerae*. It has been recognized to also cause gastroenteritis transmitted from eating raw sea food. Although in very rare instances whereby *V. mimicus* is harbouring the genes encoding cholera toxin, it can cause severe watery diarrhoea (Hasan *et al*., 2010).

4.2.3 Antibiogram of Vibrio species recovered from WWTPs effluents

Pools of isolates belonging to the 6 key Vibrio pathogens targeted in this study, i.e. *V. parahaemolyticus, V. vulnificus, V. cholerae, V. mimicus, V. alginolyticus, V. fluvialis* and other *Vibrio* species exhibited varying degrees of antimicrobial resistance in this study. Resistance patterns varied from one species to another. The trend of resistance against the different classes of antibiotics is presented in Figure 4.5. *V. cholerae* exhibited resistance against 17 of the 18 antibiotics with frequencies of between 6.4% against azithromycin and 93.5% against ampicillin. *V. mimicus* isolates displayed some level of resistance against all the antibiotics; the highest frequency however was against polymyxin B at 94.1%. The

V. vulnificus isolates exhibited resistance against only 8 antibiotics with all the isolates being resistant against ampicillin and nalidixic acid. Antimicrobial resistance in *V. parahaemolyticus* was against 17 of the 18 antibiotics with the highest frequency observed against ampicillin. Remarkable resistance patterns were observed also with *V. fluvialis, V. alginolyticus* and other uncharacterized *Vibrio* species. The antibiotic sensitivity patterns of the confirmed *Vibrio* isolates recovered from wastewater effluents reveals that a larger percentage of the *Vibrio* species were resistant to one or more of the antimicrobial agents tested. Antibiotic resistance of *Vibrio* species against some antibiotics from treated effluents has been reported by previous studies (Okoh and Igbinosa, 2010; Mandal *et al.,* 2012). In a similar study, Imziln and Hassani (1994) reported resistance against used antibiotics except for streptomycin which was not included in our study. More recently, a study carried out in Tanzania was in accordance with our findings where virtually all the *Vibrio* species recovered from wastewater effluents were resistant to ampicillin but quite susceptible to phenicol or quinolone (Hounmanou *et al.,* 2016). The finding confirms that WWTPs are reservoirs of multi antibiotic resistant *Vibrio* species.

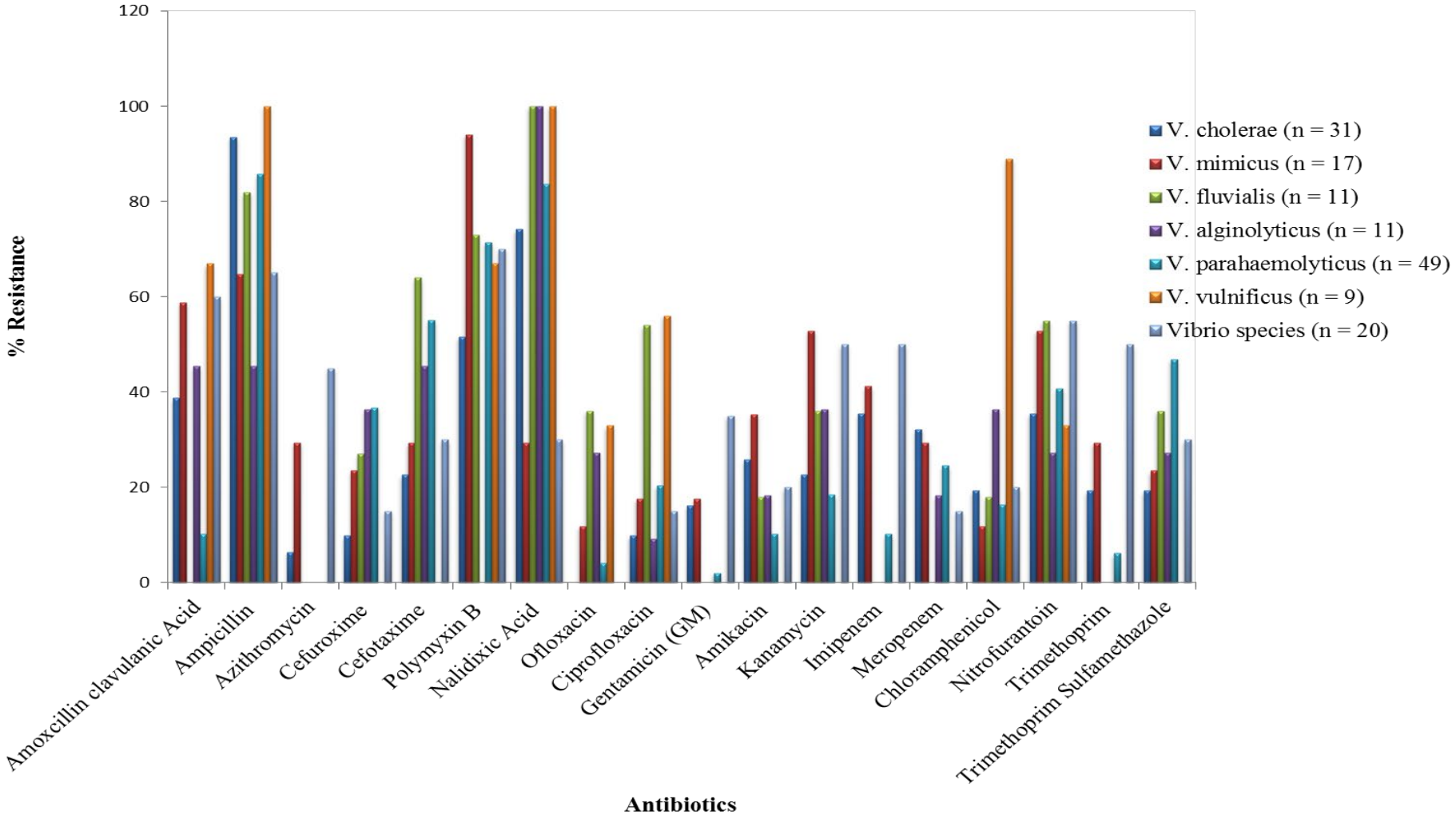

Figure 4.5: Antibiotic resistance profile of Vibrio species recovered from some wastewater treatment plants effluents.

Table 4.3: Abundance of Vibrio species in WWTP effluents and receiving waterbodies.

Sarah Baartman District Municipality (n = 86)							
Water sample source	***V. cholera***	***V. vulnificus***	***V. alginolyticus***	***V. fluvialis***	***V. parahaemolyticus***	***V. mimicus***	Other (Vibrio genus)
Mayfield	3 (3.5%)	_	2 (2.3%)	_	1 (1.2%)	_	47 (54.7%)
Mayfield river (upstream)	_	_	_	_	_	_	
Mayfield river (downstream)	1 (1.2%)	_	_	_	2 (2.3%)	_	
Bbelmond valley	7 (14%)	3 (3.5%)	3 (3.5%)	2 (2.3%)	3 (3.5%)	_	
Kowie river (upstream)	3 (3.5%)	_	3 (3.5%)	_	_	_	
Kowie river (downstream)	3 (3.5%)	1 (1.2%)	1 (1.2%)	1 (1.2%)	1 (1.2%)	_	
OR Tambo District Municipality (n = 43)							
Mthatha	_	3 (7%)	1 (2.3%)	_	17 (39.5%)	_	22 (51.1%)
Mthatha river (upstream)	_	2 (4.7%)	2 (4.7%)	8 (18.6%)	14 (30.4%)	_	
Mthatha river (downstream)	N/a	N/a	N/a	N/a	N/a	N/a	
Amathole District Municipality (n = 43)							
Butterworth	2 (4.7%)	4 (9.3%)	_	3 (7%)	6 (14%)	_	28 (65.1)
Butterworth receiving waterbody (up and downstream)	N/a	N/a	N/a	N/a	N/a	N/a	

Chris Hani District Municipality (n = 60)							
Water sample source	***V. cholera***	***V. vulnificus***	***V. alginolyticus***	***V. fluvialis***	***V. parahaemolyticus***	***V. mimicus***	Other (Vibrio genus)
Great Fish river (upstream)	2 (3.3%)	_	_	_	1 (1.7%)	2 (3.3%)	20 (33.3%)
Cradock	3 (5%)	_	_	_	1 (1.7%)	2 (3.3%)	
Great Fish river (downstream)	3 (5%)	_	_	_	1 (1.7%)	2 (3.3%)	
Koonap river (upstream)	3 (5%)	_	_	_	3 (5%)	1 (1.7%)	
Adelaide	4 (6.7%)	_	_	_	4 (6.7%)	1 (1.7%)	
Koonap river (downstream)	3 (5%)	_	_	_	3 (5%)	1 (1.7%)	

4.2.4 Prevalence of Vibrio pathogens in selected rivers

Table 4.4 summarizes the prevalence of some key pathogenic Vibrio species in 10 rivers in the Eastern Cape Province. The freshwater source of interest in the Sarah Baartman District Municipality was Kowie River with the targeted species showing a prevalence of *V. cholerae* (4%), *V. alginolyticus* (25%) and *V. mimicus* (3%). For the OR Tambo District Municipality, the Qunu River and Tsitsa River were sampled. Species identified for Qunu River were: *V. cholerae* (4%), *V. vulnificus* (60%) and *V. fluvialis* (56%). Tsitsa River showed a prevalence of *V. cholerae* (13%), *V. vulnificus* (3%), *V. fluvialis* (11%) and *V. mimicus* (24%). Four rivers including Kubusie, Tyhume, Kat and Keiskamma rivers, were selected in the Amathole District Municipality and *V. cholerae* was detected in all 4 rivers, with a prevalence of 4%, 14%, 19%, and 13%, respectively. *V. vulnificus* was detected only in the Kat river, while the frequencies of detection of the other pathogens were in the following orders: *V. alginolyticus* Kubusie river (25%) and Kat river (13%); *V. fluvialis* – Tyhume (22%) and Kat river (11%); *V. parahaemolyticus* – Kat river (45%), Tyhume (24%) and Keiskamma river (6%); *V. mimicus* – Tyhume river (10%), Kat river (8%), and Keiskamma river (36%). The Freshwater resources in the Chris Hani District Municipality covered in this study included Tsomo River, Great Fish River and Klipplaat River. None of the target pathogens were detected in Klipplaat River. *V. cholerae* and *V. mimicus* were detected in Tsomo River while both organisms and *V. parahaemolyticus* were detected in Great Fish River. The presence of these pathogens in these freshwater resources is a cause of concern to the environment and public health. The detected pathogens and their presence suggest that these water resources are significant reservoirs of the Vibrio pathogens.

Table 4.4: Prevalence of Vibrio species recovered from the study rivers.

Rivers	*V. cholerae*	*V. vulnificus*	*V. alginolyticus*	*V. fluvialis*	*V. parahaemolyticus*	*V. mimicus*
Sarah Baartman District Municipality						
Kowie river	3 (4%)	_	4 (25%)	_	-	3 (3%)
OR Tambo District Municipality						
Qunu river	3 (4%)	3 (60%)	2 (13%)	5 (56%)	-	-
Tsitsa river	10 (13%)	3 (60%)	4 (25%)	1 (11%)	-	23 (24%)
Amathole District Municipality						
Kubusie river	3 (4%)	-	4 (25%)	-	-	-
Tyhume river	11 (14%)	-	-	2 (22%)	8 25%	10 (10%)
Kat river	15 (19%)	4 (80%)	2 (13%)	1 (11%)	15 (47%)	8 (8%)
Keiskamma river	10 (13%)	-	-	-	2 (6%)	35 (36%)
Chris Hani District Municipality						
Tsomo river	10 (13%)	-	-	-	-	5 (5%)
Great Fsh river	15 (19%)	-	-	-	8 (25%)	12 (13%)
Klipplaat river	-	-	-	-	-	-
Total # of isolates	80 (100%)	10 (100%)	16 (100%)	9 (100%)	33 (100%)	96 (100%)

Key: Other – Vibrio species positive for Vibrio genus; n – total number of positive isolates from each municipality

This is a health risk and a possibility of contracting cholera and other Vibrio infections is inevitable. Our findings suggest that the freshwater resources investigated in this study could pose a significant health and environmental risk to the communities who rely on them for several activities such as irrigation, recreation, domestic and spiritual activities. Safe water in rural areas of most developing countries is limited and in some areas non-existent and as a result affected communities rely on available surface waters for drinking and other domestic uses. Thus, this is a major public health risk especially in the rural communities of the Eastern Cape Province where 36 % of the population still drink water directly from rivers.

4.2.5 Antibiogram of *Vibrio* species recovered from rivers

The antibiotic susceptibility profiles of representatives of some confirmed *Vibrio* species recovered from the rivers are summarized in Fig. 4.2. All the *V. cholerae* isolates were

resistant against Polymyxin B. Also, high resistance frequencies were exhibited against nitrofurantoin (82%) and nalidixic acid (73%). The least resistance frequency was however observed against ofloxacin (3%). For the *V. parahaemolyticus* isolates, varying degrees of resistance frequencies against 17 of the 18 test antibiotics was observed. The highest resistance frequency was observed against polymyxin B (97%) while the least resistance was observed against meropenem. *V. fluvialis* isolates exhibited varying degree of resistances against all of the test antibiotics. The highest resistance frequency was observed against ampicillin (100%) while the least (8%) was against gentamicin, amikacin and meropenem. The *V. mimicus* isolates were resistant notably against polymyxin B (94%), followed by ampicillin (91%). The least resistance response was observed against gentamicin (6%). *V. alginolyticus* isolates on the other hand exhibited resistance mostly against ampicillin (94%). The least resistance frequency of these species was against nalidixic acid (22%). Also, *V. vulnificus* isolates were all resistant against ampicillin while the least resistance was observed against cefuroxime (30%).

The antibiogram (Fig. 4.2) indicate that the *Vibrio* species recovered in this study expressed high levels of resistance against antimicrobials that are commonly used in clinical medicine. The resistance exhibited against these specific antimicrobials is sometimes encoded by plasmids, which may distribute resistance in susceptible bacteria through horizontal gene transfer (Hall and Barlow, 2004; Sayah, 2005). This could contribute to the spread and persistence of antimicrobial resistant bacteria and resistance determinants in humans and the environment. One of the most important factors contributing to the spread of antimicrobial resistance in bacteria has been attributed to the fact that in most developing countries, diarrheal diseases are treated with an inadequate regimen of antimicrobials and often without first identifying the pathogen (Ram *et al.,* 2007). Use of antibiotics in animal husbandry as growth promoters could be another factor contributing to the recovery of resistant bacteria in these water sources as the gut microbial flora of these animals end up developing resistance against these antimicrobials, and passing the same to autochthonous bacteria surface water systems. The dissemination of antimicrobial resistance in these *Vibrio* pathogens may have potential negative clinical implications for therapeutic advancement.

4.3 AQUATIC ANIMALS AND VEGETABLES

Fish samples were purchased from two retail shops; one in Buffalo City Metropolitan and the other in Sarah Baartman District Municipality. Fish samples harvested from two dams in Alice, Amathole District Municipality were also purchased from fishermen for vibriology study. Crab, mussel, mud prawn and limpet that were harvested from the Buffalo River (Buffalo City Metropolitan Municipality), Sunday River (Nelson Mandela Bay Municipality), Swartkops River (Nelson Mandela Bay Municipality) were purchased from fishermen at the river and analysed for Vibrio species. A variety of vegetables were also purchased from wet markets and supermarkets located in the Chris Hani, Amathole and Buffalo City District Municipalities.

4.3.1 Prevalence of Vibrio pathogens in selected aquatic animals and vegetables

The prevalence of the targeted Vibrio species in the aquatic animals samples are as summarized in Table 4.5.

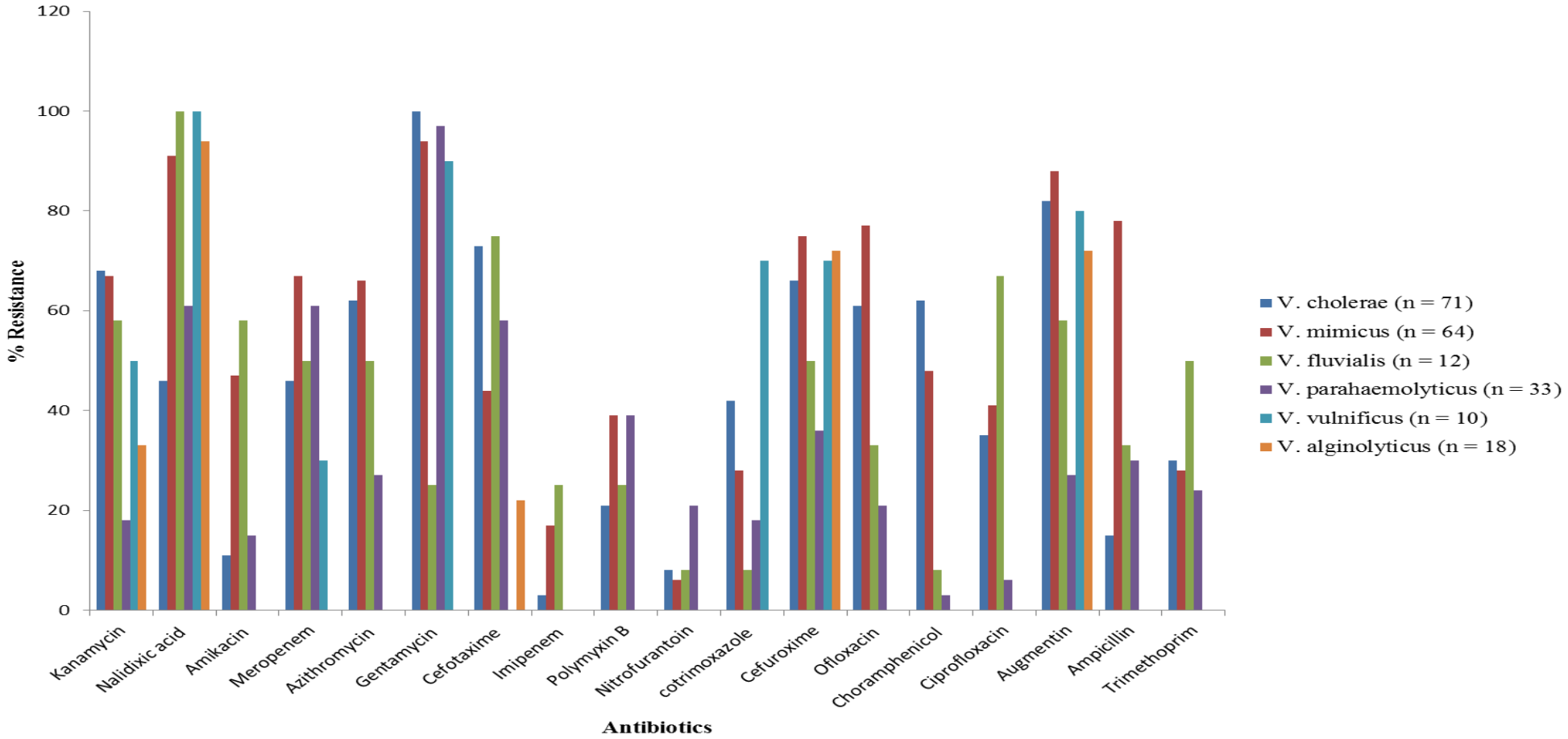

Figure 4.6: Antibiotic profile of *Vibrio* species recovered from rivers

Table 4.5: Prevalence of target *Vibrio* species recovered from aquatic animals.

Aquatic animals	Presumptive *Vibrio* isolates	*Vibrio* genus (%)	VC (%)	VM (%)	VF (%)	VA (%)	VP (%)	Other *vibrio*
Mollusc	80	71 (89)	0 (0)	0 (0)	0 (0)	20 (28)	18 (25)	33 (46)
Crustacean	70	49 (70)	9 (18)	2 (4)	0 (0)	10 (20)	11 (22)	17 (35)
Fish	80	69 (86)	9 (13)	4(6)	3 (4)	10 (14)	14 (20)	30 (43)
Total	230	189 (79)	18 (26)	6 (9)	3 (4)	40 (23)	43 (38)	110 (58)

Key: VA= *V. alginolyticus*, VC= *V. cholerae*, VF= *V. fluvialis*, VM= *V. mimicus*, VP= *V.* parahaemolyticus

The prevalence of confirmed *Vibrio* genus in each of the aquatic animals ranged from 70% to 89%. About 42% of the confirmed *Vibrio* isolate (genus) belong to the five Vibrio species targeted in this study while about 58% belong to other unidentified *Vibrio* species. The result presented in Table 4.5 above also shows that *V. alginolyticus* and *V. parahaemolyticus* are the most prevalent of the five targeted pathogenic *Vibrio* spp. This observation is in line with the reports of Kriem *et al.* (2015); Jones *et al.* (2014) and Mus *et al.* (2014) and could explain while the *Vibrio* infections involving the two pathogens are more common in cases of foodborne illnesses (CDC-COVIS 2009-2014).

From the vegetables samples purchased from supermarkets and wet markets, a total of 100 presumptive *Vibrio* isolates were recovered. Of these, 64% were positive for the Vibrio genus. The result of delineation of the confirmed *Vibrio* isolates into some key pathogenic *Vibrio* species showed that 34.4%, 21.9%, 1.7%, 3.1%, 1.7% of the isolates were *V. cholerae*, *V. parahaemolyticus, V. fluvialis, V. mimicus and V. alginolyticus* respectively. The remaining 24 isolates belong to other *Vibrio* species not targeted in this study. The recovery of pathogenic *Vibrio* species in vegetables has a very serious public health importance particularly with those vegetables eaten with little or no further processing. The occurrence of pathogenic *Vibrio* species have long been associated with contaminated seafood but this study has shown that vegetables can also be a potent vehicle of dissemination for *Vibrio* associated infections.

4.3.2 Antibiotic susceptibility pattern of Vibrio species Isolated from aquatic animals

The antibiogram of the targeted *Vibrio* species isolated from fish, crustacean and mollusc are as presented in Table 4.6. For the fish samples, about 50% and above of all the isolates were resistant against ampicillin while the same percentage of *V. cholera* exhibited resistance against nalidixic acid, polymyxin B and trimethoprim. Less than 50% of the *V. cholera* isolates were resistant against kanamycin, meropenem, azithromycin, cefotaxime, imipenem, nitrofurantoin, chloramphenicol, ciprofloxacin and augmentin. None of the *V. cholerae* isolates demonstrated resistance against the remaining four antibiotics. Also, about 50% and

above of the *V. mimicus* isolates showed resistance against azithromycin, polymyxin B and nitrofurantoin while 25% of the same species were resistant against kanamycin, nalidixic acid, meropenem, cefotaxime, cotrimoxazole, cefuroxime, augmentin and trimethoprim. Also, over half the *V. fluvialis* isolates were resistant against 80% of the antibiotics used in this study, while 33% of the isolates were resistant against cefotaxime. None of the *V. fluvialis* isolates showed resistance against the remaining six antibiotics. However, about 56% of the *V. alginolyticus* showed resistance against polymyxin B while 11% to 33% of the same organism showed resistance against nalidixic acid, azithromycin, cefotaxime, imipenem, nitrofurantoin, cotrimoxazole, cefuroxime, cotrimoxazole and trimethoprim. None of the *V. alginolyticus* were resistant against the remaining six antibiotics, but about 14% and above of the *V. parahaemolyticus* were resistant against polymyxin, nitrofurantoin and cefuroxime, while 7% of the same organism showed resistance against amikacin, azithromycin, gentamycin, imipenem, cotrimoxazole, chloramphenicol and augmentin. None of the *V. parahaemolyticus* showed resistance against the remaining six antibiotics.

The frequencies of *V. cholerae* isolates from crustacean that showed resistance against imipenem, polymyxin B, nitrofurantoin, cotrimoxazole, augmentine, ampicillin and trimethoprim ranged between 11% and 56%, while none of the isolates were resistant against the remaining 11 (61%) antibiotics used in this study. Also, about 50% of the *V. mimicus* recovered from crustacean showed resistance against nalidixic acid and meropenem while none of the isolates were resistant against the remaining 16 antibiotics used. Also, about 33% and 67% of *V. alginolyticus* isolates recovered from crustacean showed resistance against polymyxin B and ampicillin respectively. None of the *V. alginolyticus* showed resistance against the remaining 16 antibiotics. The range of *V. parahaemolyticus* isolated from crustacean that exhibited resistance against kanamycin, cefotaxime, polymyxin B, augmentin, ampicillin and trimethoprim was 33% to 67%. Only 17% of the *V. parahaemolyticus* recovered from crustacean demonstrated resistance against imipenem and cefuroxime, and none of the *V. parahemolyticus* showed resistance against the remaining antibiotics used for the antibiogram profiling of isolates tested.

About 50% and above of *V. parahaemolyticus* and *V. alginolyticus* recovered from mollusc showed resistance against ampicillin and while about 25% of the *V. alginolyticus* isolates showed resistance against kanamycin, cefotaxime and cotrimoxazole, the range of the *V. parahaemolyticus* that showed resistance against meropenem, cefotaxime, polymyxin B, cotrimoxazole, cefuroxime, chloramphenicol and augmentine was 33% to 67%.

The occurrence and prevalence of key *Vibrio* pathogens in aquatic animals especially those that are used as seafood have been reported from several geographical locations on the globe (Kim *et al.,* 2017; Parthasarathy *et al.,* 2017; Tan *et al.,* 2017; Xie *et al*., 2017). Furthermore, findings on the antibiogram patterns exhibited by these pathogens against antibiotics that are commonly used for the management of *Vibrio* infections and/or for epidemiological survey have been reported (Kang *et al.,* 2017; Igbinosa *et al.,* 2015; Okoh and Igbinosa, 2010).

The findings of the current study corroborate the findings of the earlier reports. These findings as articulated in Tables 4.5 to 4.7 suggest that the aquatic animals are carriers of pathogenic *Vibrio* species some of which demonstrated multiple antibiotic resistance phenotypes. The antibiogram fingerprints of the pathogenic Vibrio spp. suggest that these pathogens could be reservoir of antibiotic resistance determinants which could be transferred to other bacteria via horizontal gene transfer and consequently to humans. The devastating health effects of these pathogens especially those carrying virulence genes, have been reported over the years (Baker-Austin *et al.,* 2017; Ho, 2017; Gonzalez-Escalona *et al.,* 2016; CDC-COVIS 2009-2014; Harth *et al.,* 2009).

4.3.3 Antibiogram of *Vibrio* species recovered from vegetables

The antibiotic susceptibility profiles of representatives of confirmed *Vibrio* species recovered from the vegetable samples are summarized in Table 4.7. The frequency of resistance of the *Vibrio* species was observed to be highest against polymyxin B with a frequency of 92.2% followed by 90.6% with amoxicillin-clavulanic acid and 81.2% with imipenem. The least frequency of resistance was 6.2% against ofloxacin. The identified pathogenic species were susceptible to two or more antibiotics which varied from one species to another. *V. cholerae* isolates exhibited resistance against 15 of the 18 test antibiotics. The highest resistance frequency (100%) was exhibited against amoxicillin-clavulanic acid and the least resistance frequency was 14.3% each against trimethoprim sulfamethazole and kanamycin. *V. parahaemolyticus* from vegetables were resistant against 16 of the 18 test antibiotics with the highest resistance frequency against polymyxin B (100%) while the least resistance frequency was observed against trimethoprim-sulfamethazole (14.3%). *V. fluvialis* exhibited resistance against 15 of the 18 test antibiotics at a frequency of 100%. The *V. mimicus* isolates were all resistant against amoxicillin-clavulanic acid, ampicillin, azithromycin, polymyxin B, ciprofloxacin, imipenem, chloramphenicol, nitrofurantoin and trimethoprim. The *V. alginolyticus* isolates on the other hand exhibited resistance against amoxicillin-clavulanic acid, ampicillin, cefuroxime, nalidixic acid, azithromycin, polymyxin B, ciprofloxacin, imipenem, meropenem, chloramphenicol, nitrofurantoin, and trimethoprime.

Table 4.6: Resistance profile of *Vibrio* species isolated form aquatic animal samples.

Antibiotics	Resistance profile (%)											Frequency of resistance
	Mollusc		Crustacean				Fish					
	Va	Vp	Vc	Vm	Va	Vp	Vc	Vm	Vf	Va	Vp	
	(n = 4)	(n = 6)	(n = 9)	(n = 2)	(n = 3)	(n = 6)	(n= 9)	(n = 4)	(n= 3)	(n = 9)	(n= 14)	(n=69)
Kanamycin (30 µg)	01(25%)	-	-	-	-	04(67%)	01(11%)	01(25%)	02(67%)	-	-	09(13%)
Nalidixic acid (30 µg)	-	-	-	01(50%)	-	-	06(67%)	01(25%)	02(67%)	03(33%)	-	13(19%)
Amikacin (30 µg)	-	-	-	-	-	-	-	-	-	-	01(7%)	1(1%)
Meropenem (10 µg)	-	1(17%)	-	01(50%)	-	-	01(11%)	01(25%)	-	-	-	4(6%)
Azithromycin (15 µg)	-	-	-	-	-	-	03(33%)	02(50%)	02(67%)	02(22%)	01(7%)	10(14%)
Gentamycin (10 µg)	-	-	-	-	-	-	-	-	-	-	01(7%)	1(1%)
Cefotaxime (30 µg)	01(25%)	2(33%)	-	-	-	02(33%)	02(22%)	01(25%)	01(33%)	02(22%)	01(7%)	12(17%)
Imipenem (10 µg)	-	-	02(22%)	-	-	01(17%)	01(11%)	-	-	02(22%)	-	6(9%)
Polymyxin B(300 units)	02(50%)	2(33%)	07(78%)	-	-	02(33%)	06(67%)	03(75%)	-	05(55%)	04(29%)	32(46)
Nitrofurantoin (300 µg)	-	-	01(11%)	-	-	-	03(33%)	03(75%)	02(67%)	03(33%)	02(14%)	14(20%)
cotrimoxazole (25 µg)	01(25%)	1(17%)	01(11%)	-	-	-	07(78%)	01(25%)	02(67%)	01(11%)	01(7%)	15(22%)
Cefuroxime (30 µg)	-	1(17%)	-	-	-	01(17%)	-	01(25%)	02(67%)	02(22%)	02(14%)	9(13%)
Ofloxacin (5 µg)	-	-	-	-	-	-	-	-	-	-	-	-
Choramphenicol (30 µg)	-	1(17%)	-	-	-	-	04(44%)	-	02(67%)	02(22%)	01(7%)	10(14%)
Ciprofloxacin (15 µg)	-	-	-	-	-	-	01(11%)	-	01(33%)	-	-	2(3%)
Augmentin (15 µg)	-	1(17%)	05(56%)	-	-	02(33%)	01(11%)	01(25%)	02(67%)	02(22%)	01(7%)	15(22%)
Ampicillin (10 µg)	04(100%)	5(83%)	04(44%)	-	02(67%)	04(67%)	07(78%)	02(50%)	02(67%)	07(78%)	14(100)	51(74%)
Trimethoprim (5 µg)	-	-	01(11%)	-	-	02(33%)	05(56%)	01(25%)	02(67%)	02(22%)	01(7%)	14(20%)

Key: Va= *V. alginolyticus*, Vc= *V. cholerae*, Vf= *V. fluvialis*, Vm= *V. mimicus*, Vp= *V. parahaemolyticus*

Table 4.7: Antibiotic susceptibility profiles of Vibrio isolates recovered from vegetable samples.

Antibiotic	**Resistance profile (%)**						
	V. cholerae	***V. parahaemolyticus***	***V. fluvialis***	***V. mimicus***	***V. alginolyticus***	**Other *Vibrio* species**	**Frequency of resistance**
	(n = 22)	**(n = 14)**	**(n = 1)**	**(n = 2)**	**(n = 1)**	**(n = 24)**	**(n = 64)**
Amoxcillin clavulanic acid (AUG) 30 µg	22 (100%)	10 (71.4%)	01 (100%)	02 (100%)	01 (100%)	22 (91.6%)	58 (90.6%)
Ampicillin (AP) 10 µg	12 (54.5%)	12 (85.7%)	01 (100%)	02 (100%)	01 (100%)	21 (87.5%)	49 (76.6%)
Azithromycin (ATH) 15 µg	13 (59.1%)	06 (42.8%)	01 (100%)	02 (100%)	01 (100%)	20 (83.3%)	43 (67.2%)
Cefuroxime (CXM) 30 µg	10 (45.4%)	09 (64.3%)	01 (100%)	-	01 (100%)	19 (79.2%)	40 (62.5%)
Cefotaxime (CTX) 30µg	07 (31.8%)	01 (7.1%)	01 (100%)	-	-	20 (83.3%)	29 (45.3%)
Polymyxin B (PB) 300c	21 (95.4%)	14 (100%)	01 (100%)	02 (100%)	01 (100%)	20 (83.3%)	59 (92.2%)
Nalidixic acid (NA) 30 µg	19 (86.4%)	05 (35.7%)	01 (100%)	-	01 (100%)	15 (62.5%)	41 (64.1%)
Ofloxacin (OFX) 5 µg	-	-	01 (100%)	-	-	03 (12.5%)	04 (6.2%)
Ciprofloxacin (CIP) 5 µg	08 (36.4%)	-	-	02 (100%)	01 (100%)	07 (29.2%)	17 (26.6%)
Gentamicin (GM) 10 µg	-	03 (21.4%)	-	-	-	03 (12.5%)	06 (9.4%)
Amikacin (AK) 30 µg	-	11 (78.6%)	-	-	-	07 (29.2%)	18 (28.1%)
Kanamycin (K) 30 µg	03 (13.6%)	10 (71.4%)	01 (100%)	-	-	23 (95.8%)	37 (57.8%)
Imipenem (IMI) 10 µg	19 (86.4%)	07 (50%)	01 (100%)	02 (100%)	01 (100%)	22 (91.6%)	52 (81.2%)
Meropenem (MEM) 10 µg	10 (45.4%)	06 (42.8%)	01	-	01 (100%)	22 (91.6%)	40 (62.5%)

			(100%)				
Chloramphenicol (C) 30 µg	07 (31.8%)	03 (21.4%)	01 (100%)	02 (100%)	01 (100%)	16 (66.7%)	30 (46.9%)
Nitrofurantoin (NI) 300 µg	10 (45.4%)	12 (85.7%)	01 (100%)	02 (100%)	01 (100%)	23 (95.8%)	49 (76.6%)
Trimethoprim (TM) 5 µg	08 (36.4%)	12 (85.7%)	01 (100%)	02 (100%)	01 (100%)	24 (100%)	48 (75%)
Trimethoprim sulfamethazole (TS) 25µg/23.75 µg	03 (13.6%)	02 (14.3%)	01 (100%)	-	-	09 (37.5%)	15 (23.4%)

4.4 DISTRIBUTION OF VIRULENCE DETERMINANTS IN VIBRIO SPECIES ISOLATED FROM THE ENVIRONMENT.

Tables 4.9 to 4.13 below shows the detection of several virulence determinants peculiar to the *Vibrio* species in isolates recovered from the rivers, WWTPs effluents, vegetables and aquatic animals samples.. The presence of these virulence determinants further supports our other findings suggesting the ubiquity of the pathogens in the study environment and sample types and corroborates similar observations reported elsewhere (Mukhopadhyay *et al*., 2001; Faruque *et al*., 2003). Also *Vibrio* species from vegetables, and animal components in the aquatic milieu explored for food have been shown to possess key virulence determinants that define their pathogenicity (Baffone *et al*., 2000; Tunung *et al*., 2011). The roles of virulence-associated factors in the environment and the selection pressures for environmental *V. cholerae* and other Vibrio species harbouring these genes is not clear, but it is possible that these strains may be precursors of pathogenic strains or may participate in gene transfer events leading to the origination of pathogenic strains. Although, some *V. mimicus* recovered in this study possessed pathogenic determinants that are associated with *V. cholerae* the mechanism of its pathogenesis remains unclear. These findings reinforce the connection between environmental reservoirs and human infection and supports the value of monitoring the *Vibrio* species within the context of public health risks.

Table 4.8: Representative virulence genes detected in the non-O1/non-O139 *V. cholerae* isolates.

Genotype	Sources	# of isolates
toxR, ompU, zot, tcp, vpI, hylA, rtx	Vegetables, rivers, WWTPs	63
OmpU, zot, tcp, rtx	Rivers, vegetables	23
toxR, hylA	Crabs, rivers, WWTPs, vegetables	14
toxR, hylA, tcp	Crabs, rivers, WWTPs, vegetables	11
vpI, toxR, hylA,	Mud prawns, vegetables, crab, WWTPs	7
toxR, ompU, hylA,	WWTPs, rivers, fish fin	10
tcp, hylA	Crabs, rivers	10
ompU, tcp, toxR, vpI, zot	WWTPs	3
Ompu	Vegetables, rivers, WWTPs	8

Table 4.9: Representative virulence genes detected in *V. mimicus* isolates.

Genotype	Sources	# of isolates
toxR, ompU, tcp, hylA	Vegetables, rivers, WWTPs	24
ctx, hylA, vpI	Rivers, vegetables	32
hylA	Vegetables, rivers, WWTPs	17
vpI, ompU	Fish, crab, rivers	20
vpI	Crabs	2
toxR	Fish gill	1

Table 4.10: Representative virulence genes detected in *V. parahaemolyticus* isolates.

Genotype	Sources	# of isolates
tlh, vpI	Vegetables, rivers, WWTPs, mussels, crabs, mud prawns, fish gills	42
tlh, vpI, toxR	Rivers, vegetables	14
Toxr	Rivers, WWTPs, vegetables	23
Tlh	Crabs, rivers, WWTPs, vegetables, fish fins, fish intestines, fish flesh, fish gill	31

Table 4.11: Representative virulence genes detected in *V. alginolyticus* isolates.

Genotype	Sources	# of isolates
tlh, vppC, toxR	Vegetables, rivers	15
tlh, vppC	Rivers, vegetables	12
toxR, tlh	Rivers, WWTPs, vegetables	13
vpI, tlh	Fish intestine	1
Tlh	Crabs, rivers, WWTPs, vegetables, fish fins, fish intestines, fish gills	23

Table 4.12: Representative virulence genes detected in *V. vulnificus* isolates.

Genotype	Sources	# of isolates
viub, vcgc, vcge	Rivers, WWTPs	13

4.5 CONCLUSION

This study has shown that freshwater resources, wastewater effluents, aquatic animals and vegetables are potential reservoirs of pathogenic *Vibrio* species. The presence of pathogenic *Vibrio* species in water and food components as observed in this study is a major public health concern. The occurrence of antibiotic resistant *Vibrio* species in wastewater effluents increases public health risk concerns especially when discharged into receiving watersheds which are directly or indirectly utilized by humans. This may contribute to the

already serious scourge of antibiotic resistance and reduced efficacy of antibiotic treatment against human diseases caused by *Vibrio* species. We thus conclude that the WWTPs covered in this study are not efficient in the removal of pathogenic *Vibrio* species and the release of these pathogenic *Vibrio* species into the receiving watershed can be a source of disease when the water is used for drinking, recreational or irrigational activities. Also the conclusion is reached that using untreated water from the study rivers could pose a high risk of infections to humans as a result of the presence of pathogenic *Vibrio* species. Consumption of pathogenic *Vibrio* contaminated vegetables and seafood could be a major source of foodborne *Vibrio* related infections. It is however noted some relief that the cholera causing *V. cholerae* pathotypes appears to be under check in the Eastern Cape aquatic environment. Nevertheless, the antibiotic resistant *Vibrio* isolates could be transmitted through the food chain to humans and therefore constitutes a risk to public health. A proposal is hereby made for the inclusion of pathogenic Vibrio species in the regular microbiological quality monitoring of wastewater effluents, drinking and environmental water samples.

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