Investigating the occurrence and survival of *Vibrio cholerae* in selected surface water sources in the KwaZulu-Natal province of South Africa

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EXECUTIVE SUMMARY

In the rural communities of developing countries like South Africa, rivers play a pivotal role in the life of the population for social, cultural and religious reasons. The population in these communities is exposed to prevailing poor quality of river water, which may result in people contracting waterborne disease such as cholera. Cholera is an intestinal disease caused by the bacterium *Vibrio cholerae*, which colonises the human intestine and causes severe diarrhea, which may be fatal if not diagnosed or treated early. The transmission of the disease is mediated by water, with two main routes of transmission for cholera being reported in the literature. The primary transmission occurs from a natural reservoir of pathogens in the aquatic environment to the human host. The second transmission route is mediated by the ingestion of faecal contaminated water or food. Therefore, the infection is always caused by ingestion of water either contaminated by *V. cholerae* present in a natural reservoir (primary route) or contaminated by humans (secondary infection). The role of the aquatic environment is thus crucial for the transmission as well as for the spreading of the disease particularly where river water is used domestically without treatment. The *V. cholerae* is widely considered to be an environmental bacterial pathogen. The driving force influencing *V. cholerae's* survival in the environment is likely an integrated outcome of changes in physicochemical factors, availability of suspended substrates and abundance of biological hosts or reservoirs. This study was undertaken to determine the presence of *V. cholerae* in three rivers in the KwaZulu-Natal Province. The study assessed whether *V. cholerae* was present in the environment and to also better understand where and how the bacteria live in this aquatic environment. The specific aims of this study were to:

1. Detect *V. cholerae* using optimised real-time PCR culture based detection method;
2. Detect *V. cholerae* using optimised real-time PCR culture independent method;
3. Implement an internal process control to monitor the performance of *V. cholerae* real-time PCR assays; and
4. Using the newly validated methods, to determine the occurrence and distribution of *V. cholerae* from zooplankton, phytoplankton, amoeba, invertebrates, animal stools, sediments and water from the selected rivers in KwaZulu-Natal province.

Different niches that may facilitate survival of *V. cholerae* in rivers were identified and sampled. These included zooplankton, phytoplankton, amoeba, invertebrates, animal stools, sediments and the river water itself. Samples were collected in and around the Msunduzi, Umlazi and Isipingo Rivers once monthly for a period of fourteen months (October 2012- December 2013).The samples were also analysed for total coliform and *Escherichia coli* counts. Temperature, pH and conductivity of the samples were measured at the sample sites and turbidity and salinity were measured at the laboratory.

Culture dependent and culture independent real-time PCR methods were used for the detection of the bacterium *V. cholerae* from the selected samples. A culture dependent high resolution melt (HRM) real-time PCR method was performed at East Rand Water Care Company (ERWAT) laboratory in parallel to the real-

time PCR methods conducted at the Water and Health Research Centre (WHRC) to compare and validate PCR results.

A total of 124 water samples were analysed for *V. cholerae* using culture dependent and culture independent real-time PCR methods. The results indicated that 83/124 (67%) tested positive for the presence of non-toxigenic *V. cholerae*non-O1/O139 with the culture dependent real-time PCR method. In contrast, only 46/124 (37%) of water samples tested positive for the presence of non-toxigenic *V. cholerae* non-O1/non-O139 with culture independent real-time PCR method. The combination of filtration, enrichment, DNA extraction and m-PCR method provided a sensitive and specific method for the detection of *V. cholerae* in environmental water samples (culture dependent real-time PCR method). This method proved to be the most effective for detection and identification of selected *V. cholerae* when compared to the culture independent real-time PCR method.

The non-toxigenic *V. cholerae* (non-O1/O139) was isolated from 483 (71%) of 676 river water samples collected at all sites sampled for the three rivers studied. Non-toxigenic *V. cholerae* (non-O1/O139) was detected from all sample types except for amoeba and cow stool samples. Toxigenic and non-toxigenic *V. cholerae* O1 and/or O139 were not detected in any of the samples collected from the three rivers monitored. A statistically significant correlation between temperature, pH, turbidity, salinity, *E. coli* numbers and the isolation of non-toxigenic *cholerae* non-O1/0139 was observed.

The results from the study indicated that Msunduzi, Umlazi and Isipingo Rivers are frequently isolated with autochthonous non-toxigenic *V. cholerae* non-O1/0139, and may cause infections in sensitive population groups such as immune-compromised individuals. The non-toxigenic *V. cholerae* non-O1/0139 in the rivers was found in a free-living form or in association with planktons, invertebrates and the sediment compartment of the rivers.

The results of this study as well as from previous studies indicate that environmental *V. cholerae* strains are represented by the non-toxigenic strains. The study concluded that the environmental *V. cholerae* strains are well adapted to survive in the environment and one such adaptation is the ability to grow as a biofilm on a range of abiotic and biotic surfaces and in a viable but non-culturable state. *V. cholerae* bacteriophage could transfer genes to the non-toxigenic environmental *V. cholerae* strains, producing strain with different characteristics. Therefore, future studies should focus on the detection, identification and characterisation of *V. cholerae* bacteriophage that may by present in the Msunduzi, Umlazi and Isipingo rivers.

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ABBREVIATIONS

ATCC	American Type Culture Collection
APW	Alkaline peptone water
CFU	Colony forming unit
Ct	Threshold cycle
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide
EAEC	Entero-aggregative *E. coli*
EDTA	Ethylenediaminetetraacetic acid
EHEC	Entero-haemorrhagic *E. coli*
EIEC	Entero-invasive *E. coli*
EPEC	Entero-pathogenic *E. coli*
ETEC	Entero-toxigenic *E. coli*
ERWAT	East Rand Water Care Company
GuSCN	Guanidimthiocyanate
GPS	Global Positioning System
HCL	Hydrochloric acid
HRM	High resolution melt
IC	Internal control
KZN	KwaZulu-Natal
$MgCl_2$	Magnesium chloride
m-PCR	Multiplex polymerase chain reaction
NHLS	National Health laboratory Services
NCTC	National Collection of Type Cultures
OD	Optical density
PAS	Page's modified Neff's amoeba saline
PMA	Propidium monoazide
PCR	Polymerase Chain Reaction
Rpm	Revolutions per minute
TAE	Tris-acetate-EDTA
TCBS	Thiosulfate-citrate-bile salts-sucrose
Tm	Melting temperature
TRFLP	Terminal restriction fragment length polymorphism
UV	Ultraviolet
VBNC	Viable but non-culturable
WHO	World Health Organisation
WHRC	Water and Health Research Centre

CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

Vibrio cholerae is suggested to be an autochthonous member of the aquatic environment and the causative agent of cholera. Cholera has historically occurred in periodic epidemics with the most severe reported in India and Bangladesh and some African and South American countries (Sudhanandh et al. 2010). Nearly 200 *V. cholerae* serogroups have been identified to date, but only two serogroups, O1 and O139 are associated with epidemic cholera (Keimer et al. 2007). Although the majority of environmental isolates of *V. cholerae* are considered to be non-pathogenic, studies have confirmed that natural populations of *V. cholerae*, including *V. cholerae* non-O1/O139 isolates can serve as a precursor for new pathogenic or epidemic strains (Faruque et al. 2002). Because of this inherent risk, it is important to understand the mechanisms that affect the natural population of *V. cholerae* in the environment. Colwell (1996) reported that the presence of cholera in the Indian subcontinent and the re-emergence of cholera in other continents may be highly dependent on environmental factors. An understanding of the occurrence and survival of *V. cholerae* in the environment would contribute towards effectively monitoring water bodies for the presence of *V. cholerae*. This could assist in the early detection of cholera outbreaks giving local municipalities and water boards the opportunity to put measures in place to prepare and even prevent possible outbreaks.

1.2 PROJECT AIMS

The following were the aims of the project:

- Detection of *V. cholerae* using optimized real-time PCR culture based detection method;
- Detection of *V. cholerae* using optimized real-time PCR culture independent method;
- Implementation of an internal process control to monitor the performance of *V. cholerae* real-time PCR assays; and
- Using the newly validated methods, determine the occurrence and distribution of *V. cholerae* from zooplankton, phytoplankton, amoeba, invertebrates, animal stools, sediments and water from selected rivers in KwaZulu-Natal province.

CHAPTER 2: LITERATURE REVIEW

2.1 EPIDEMILOGY FEATURES OF *VIBRIO CHOLERAE*

The epidemiology of cholera has been dominated by its tendency to spread throughout the world in pandemics (Table 2.1) (Hunter, 1997). Since 1817, cholera has emerged from endemic areas in Asia in seven pandemic waves that have involved much of the world (Table 2.1) (Gaffga et al. 2007). Cholera is endemic in some regions of Asia and Africa, with a few cases in America and Australia. In these areas, it has been reported that water contaminated with human faeces or sewage is the main route of infection, although some cases are caused by fish and seafood consumption (Weise, 2001). Cholera is one of the first infections whose mode of transmission was understood and for which effective prevention measures, collectively referred to as 'the sanitary revolution" was developed and implemented. Because of these early observation and interventions, cholera has become vanishingly rare in the developed countries. However, neither the sanitary revolution nor the treatment revolution has been fully realized in Africa, where illness and mortality rates are soaring for lack of drinking water and sanitation infrastructure (Mintz et al. 2009).

Table 2.1.The cholera pandemics (Hunter, 1997)

Pandemic	Organism	Origin	Duration	Affected regions
First	O1-Classical	Bangladesh	1817-1823	India, SE Asia, Middle East, East Africa
Second	O1-Classical	Bangladesh	1826-1851	India, SE Asia, Middle East, Africa, Europe, Americas
Third	O1-Classical	Bangladesh	1852-1859	India, SE Asia, Middle East, Africa, Europe, Americas
Fourth	O1-Classical	Bangladesh	1863-1879	India, SE Asia, Middle East, Africa, Europe, Americas
Fifth	O1-Classical	Bangladesh	1881-1896	India, SE Asia, Middle East, Africa, Europe, Americas
Sixth	O1-Classical	Bangladesh	1899-1923	India, SE Asia, Middle East, Africa, Europe, Americas
Seventh	O1-El Tor	Indonesia	1961-	India, SE Asia, Middle East, Africa, Europe, Americas

Between January and February 2012, a cumulative total of 10 691 cases and 178 deaths were reported to the World Health Organization (WHO) from 13 countries in Africa which translates to over 90% of the total cases of cholera reported to the WHO (WHO, 2012). Africa, where cholera outbreaks have been reported at an increasing annual rate since 1990, has been described as the new homeland for cholera (Gaffga et al. 2007).

As early as 1971, South Africa was considered to be at risk of cholera due to its hot, humid summers, seaports, overcrowded communities with low standard of environmental sanitation and scanty, restricted and unprotected water supplies in certain areas (Igbinosa and Okoh, 2008). In 1973 a cholera surveillance programme was started for the mining industry (Mugero and Hoque, 2001). In 1974, mines in Eastern Transvaal, now Limpopo Province yielded cholera positive sewer pads (Küstner and Du Plessis, 1991). Two healthy carriers were identified at one of the mines (Isaäcson et al. 1974). In 1980 a case was diagnosed in open community at Shongwe hospital in KaNgwane (Küstner and Du Plessis, 1991). These people lived on farms in the Malelane area of the current Limpopo province where they drank water from the Malelane irrigation canal (Küstner et al. 1981). In October 1980 to July 1987, 25 251 cholera positive cases were identified near the border of Mozambique (Mugero and Hoque, 2001). An outbreak of cholera in KwaZulu Natal (KZN) was first reported from the rural area of Ndabayake in the district of Lower Umfolozi during August 2000 (Mendelsohn and Dawson, 2008). Thereafter, the epidemic quickly spread to five other districts of KZN and other provinces of South Africa namely Mpumalanga, Limpopo, Eastern Cape, Gauteng, and North West (five out of nine provinces) (Hoque and Worku, 2005). By July 2001, 106 389 people had been infected with 229 deaths reported (Mudzanani et al. 2003). However, epidemics subsequent to 2001 declined in magnitude and duration. Between August 2001 and December 2002 a total of 18 224 cases of cholera and 122 deaths were reported to the National Department of Health. By September 2003, a total of 3795 cases and 40 deaths had been reported to the National Department of Health (Mudzanani et al. 2003). As of 25 December 2008, a total of 26 497 cases, including 1 518 deaths due to cholera, were reported in Zimbabwe. The outbreak took on a sub-regional dimension with cases being reported from neighbouring countries like Botswana and South Africa. In South Africa as of 26 December 2008, 1 279 cumulative cases and 12 deaths were recorded, with the bulk of the cases (1 194) occurring in the Limpopo Province (WHO, 2008). The re-occurrence of cholera comes regardless of knowledge of the definite causative organism, modes of transmission, risk factors and seasonal tendencies (Mudzanani et al. 2003).

2.2 METHOD DETECTION

The conventional culture-based methods for *Vibrio cholerae* involve selective pre-enrichment of samples, plating onto selective solid media followed by morphological, biochemical and serological characterization (Igbinosa and Okoh, 2008). However, the isolation and identification of *V. cholerae* by culture methods is expensive, time-consuming, labour-intensive, and unable to precisely distinguish between the toxigenic and non-toxigenic *V. cholerae* forms (Park et al. 2013). The shortcomings of culture detection methods are further exacerbated by the ability of these microorganisms to enter a viable but non-culturable (VBNC) state (Vora et al. 2005). Bacteria in the VBNC state fail to grow on the routine bacteriological media, on which they would normally grow and develop into colonies, but are metabolically active and capable of renewed culturability (Oliver, 2005).

In contrast to culturing, molecular methods have several advantages including that they are rapid, sensitive, and highly selective and do not require extensive hands-on time (Le Roux and van Blerk, 2011). Within molecular methods, Polymerase Chain Reaction (PCR) has become the most extensively used molecular method (Chapela et al. 2010). PCR can be performed in combination with gel electrophoresis, probe

hybridization, or real-time fluorescence for detection (Liu et al. 2013). Real-time PCR has received increased use because of its ability to rapidly quantify bacterial and viral markers from the environment, identify contamination sources, and estimate the human health risk associated with contaminated water bodies (Green and Field, 2012). Multiplexing the PCR for the detection of multiple pathogens or targets is currently employed to enable the testing of samples for multiple pathogens in a single run (Woubit et al. 2012).

The ultimate goal of the development of PCR detection assays is their adoption and establishment as routine diagnostic tools. The routine use of PCR necessitates the use of reliable controls to verify the accuracy of the results obtained (Rodŕquez-Lazaro et al. 2004). The ability of laboratories to consistently produce accurate and precise results is not only essential, it is the core of quality assurance programs for testing laboratories (Espy et al. 2006).

To validate PCR results, both internal and external controls should be included with each assay to monitor assay performance (Murphy et al. 2007). External controls are amplified in parallel with the test template in a separate tube, while internal controls are co-amplified with the test template in the same tube (Ballagi-Pordány and Belák, 1996). Food and environmental matrices often have materials that are inhibitory to PCR and quantitative PCR, and could results in false negative reporting on a sample (Blackstone et al. 2007). A positive result from internal control target sequence indicates that the amplification reaction was not inhibited, thereby validating a negative result for the primary target (Nolte, 2004). When an internal control is introduced into an unprocessed water sample, it can also serve to monitor the reliability of the complete procedure of nucleic acid extraction, amplification and PCR detection (Rosenstraus et al. 1998). Several multiplex assays specific for the detection of *V. cholerae*, targeting two or more genes, have been reported (Lyon, 2001; Rivera et al. 2003; Gubala, 2006; Fykse et al. 2007; Huang et al. 2009; Tall et al. 2012). However, these assays do not contain internal controls to assess both bacterial DNA extraction and amplification.

Despite the advantages of PCR-based technologies it is important to be mindful of their limitations. One of the most significant obstacles is that PCR-based methods do not readily distinguish between viable and non-viable organisms since all fractions contain DNA. To preferentially detect DNA from live cells, treatment of microbial samples with propidium monoazide (PMA) has become an increasingly applied method (Nocker et al. 2010). Due to the increased membrane permeability of dead cells, PMA diffuses into dead cells where it intercalates with DNA. Upon light activation, PMA covalently binds to DNA and inhibits subsequent PCR amplification of this DNA (Schmidlin et al. 2010). The treatment of samples with PMA for preferential detection of live cells with intact cell membrane has been used in combination with a number of different downstream analysis tools employing DNA amplification. These include quantification PCR (PMA-qPCR) [Nocker et al. 2007], terminal restriction fragment length polymorphism (PMA-TRFLP) [Rogers et al. 2008], Denaturing Gradient Gel Electrophoresis (PMA-DGGE) [Rieder et al. 2008] and microarray analysis (PMA-microarray) [Nocker et al. 2009]. Qualitative PCR with PMA modification has been successfully used in a number of studies for different microorganisms (Pan and Breidt, 2007; Cawthorn et al. 2008; Vesper et al. 2008; Kobayashi et al. 2009) and in complex environmental matrices (Nocker et al. 2007; Wagner et al. 2008; Bae and Wuertz, 2009; Varma et al. 2009; Lin et al. 2011).

2.3 ECOLOGY

Although scientist had long thought that *V. cholerae* survived outside the mammalian intestines only for brief period, research in the past few decades has indicated that *V. cholerae* are an abundant, naturally occurring component of aquatic systems (Islam et al. 1993; Cottingham et al. 2003) and have often been isolated from aquatic environments such as bays, rivers, canals, ditches and ground water (Borroto, 1997). The capability to survive in a wide range of environmental niches is largely due to the evolution of a range of adaptive responses that allow *V. cholerae* to survive stressors such as nutrient deprivation, fluctuations in salinity and temperature and to resist predation by heterotrophic protists and bacteriophage (Lutz et al. 2013).

Previous research has revealed the existence of a dormant state into which *V. cholerae* enters, in response to extreme temperature and salinity as well as nutrient deprivation known as the viable but non-culturable state (VBNC) (Thomas et al. 2006). *Vibrio cholerae* may have survival advantage if they are able to enter into a VBNC (Halpern et al. 2006). Bacteria in the VBNC state fail to grow on the routine bacteriological media, on which they would normally grow and develop into colonies, but are metabolically active and capable of renewed culturability under favourable conditions (Oliver, 2005). Animal models have demonstrated that such non-culturable cells can regain culturability and remain pathogenic (Colwell et al. 1985; Alam et al. 2007).

Vibrio cholerae have been reported to attach to a wide variety of aquatic organisms, including phytoplankton, zooplankton, macrophytes, benthos, fish, aquatic plants and sediments (Huq et al. 1983; Turner et al. 2009; du Preez et al. 2010; Collin and Rehnstam, 2011). The ability for *V. cholerae* to live in association with aquatic organisms is because it secretes chitinase, an enzyme that enables it to digest chitin and use it as a source of nutrients (Borroto, 1997). Pruzzo et al. (2008) reported that *V. cholerae* association with chitin provide the microorganism with a number of advantages, including food availability, adaptation to environmental factors such as nutrient gradients, change in salinity and temperature and protection from predators.

Once attached to either a living host or detrital aggregates, *V. cholerae* forms “matrix-enclosed, surface-associated communities” or biofilms (Yildiz and Visick, 2009). Biofilm formation is enhanced through the action of type IV pili, flagella and production of the biofilm matrix and Vibrio polysaccharide (Watnick and Kolter, 1999). Vibrio polysaccharide is involved in cell immobilization, microcolony formation and biofilm maturation (Watnick et al. 2001). Compared to their planktonic or free-swimming counterparts, biofilm-associated bacteria are less susceptible to Ultraviolet (UV) irradiation, host defence and the toxic effects of antimicrobial agents (Karatan et al. 2005). The ability of *V. cholerae* to form biofilm is hypothesized to confer a survival advantage both in the environment and within a host (Fong et al. 2010).

Table 2.2 gives a broad overview of *V. cholerae* temperature and salinity ranges in various experimental settings. The salinity most favourable for *V. cholerae* is between 2 and 14 g/ℓ (Colwell, 2004). Water temperature is a strong predictor for the presence of Vibrio species in the environment (Johnson et al. 2012). The *V. cholerae* densities are reported to remain low at temperatures below 20°C and the highest concentration occur when the water temperature is between 20 and 30°C. Vibrios of clinical interest are less

frequently isolated when the temperature of natural aquatic environment is below 10°C or exceeds 30°C (Heath et al. 2002). The combined temperature and salinity conditions – both of which display seasonal patterns - predict the presence of *V. cholerae* with an accuracy ranging from 75.5 to 88.5% (Louis et al. 2003).

Table 2.2. Range of temperature and salinity tolerance of *V cholerae* in various experimental settings (Colwell, 2004)

Experimental setting	Temp (°C) (Study range)	Salinity (g/ℓ) (Study range)
Laboratory microcosm		5, 10
Laboratory microcosm		15
Laboratory microcosm	20, 25	15, 25
Chesapeake bay (estuary)	15-20	4-12
Chesapeake bay (estuary)	>17[a]	4-17
Southern California (coastal areas)	No preference	1-10
Louisiana (coastal areas0	dependent on salinity (18-30)	<1
Florida (estuary) and laboratory microcosm	20-35	12-25, 10-25
England (river and marsh ditch)	>9[b]	3-12
Japan (rivers and coastal areas)	ca. 21[c]	0.4-32[d]

[a]*V. cholerae* was detected at temperature above 10°C. [b] Highest temperature occurred in August. [c]*V. cholerae* was detected at temperature above 7°C. [d]The detection range was 0.4-32.5 g/ℓ.

Grazing by phagotrophic protists, especially bacterivorous nanoflagellates, has been identified as a significant factor that modifies bacterial populations in aquatic ecosystems including control of bacterial mortality (Cole, 1999). However, in some habitats and seasons, metazoan grazing (e.g. by daphnids), or lysis by phages may play a more important role (Hahn and Höfle, 2001). The control of *V. cholerae* numbers by heterotrophic protist was demonstrated by Worden et al. (2006), where *V. cholerae* growth rates of up to 2.5 doubling per day were countered by heavy grazing mortality by heterotrophic nanoflagellates. Bacteria have evolved different defence strategies towards protozoan grazing including general avoidance (e.g. motility) and direct consumer effects (e.g. digestional resistance, toxin production) (Matz and Kjelleberg, 2005). Furthermore, *V. cholerae* has been shown to exhibit an increase in biofilm and/or micro-colony formation in response to protozoan grazing and that these biofilms are grazing resistant, while planktonic cells are rapidly eliminated (Matz et al. 2005). Biofilm formation and aggregation has been suggested to be important for survival of most microorganisms and contributes to the environmental persistence of *V. cholerae* (Sun et al. 2013).

In addition to predation pressure by protozoa, phages (bacterial viruses) also affect the abundance of *V. cholerae* in the environment. Abundant phage present in the environment has been found to inversely correlate with the abundance of toxigenic *V. cholerae* in water samples and the subsequent incidence rates of cholera (Faruque et al. 2005). This control of *V. cholerae* by phage is also supported by continuous culture experiments, which suggest that *V. cholerae* populations may be influenced by phage to a larger extent than by nutrient limitation (Wei et al. 2011).

2.4 CONCLUSION

For many bacterial pathogens including *V. cholerae*, appropriate culture methods for valid identification are lacking. Target bacteria might be in biofilm form and not be accessible to culture methods. Thus, after exposure to water, bacteria might enter a VBNC state, in which they cannot be detected by culture (Brettar and Höfle, 2008). Therefore, a state-of-the-art detection method has to be considered for detection and identification of bacteria in the water, including the attached biofilm and the VBNC form. The approach currently pursued is to analyse nucleic acids, extracted directly out of drinking water with PCR.

Although an increase in publications reporting PCR-based methods for detection of water-borne pathogens has attracted the attention of end user laboratories, the lack of standardization and internationally accepted PCR-based methods has hampered implementation in end user laboratories. Also, while commercial PCR kits are available, it is important that end users have access to open-formula, non-commercial, and non-proprietary PCRs in which the information on target gene and reagent is fully available. The prerequisites for a PCR published in scientific literature to be adopted as a standard are that it has to be non-proprietary and has to have been validated through multi-centre collaborative trials (Hoorfar et al. 2003). It therefore follows that fast, accurate and sensitive method for the detection of *V. cholerae* are required as these will facilitate more rapid diagnosis and can be used to obtain greater understanding of the distribution of *V. cholerae* in the environment.

Huq et al. (1996) reported that cholera is a waterborne disease with the infectious agent *V. cholerae* being transmitted via water. As *V. cholerae* is a common inhabitant of many aquatic habitats and is most likely because it has evolved a range of strategies to enable its persistence in the natural environment. People in the rural communities of most developing countries like South Africa are still dependent on surface water sources for all their water needs and are therefore at risk of cholera infection where the *V. cholerae* proliferation is favoured by environmental conditions. It is therefore important to determine the occurrence and distribution of toxigenic *V. cholerae* in these surface water sources. This study aims to determine the occurrence and distribution of toxigenic *V. cholerae* in selected river water in KZN where there has been cholera outbreaks previously reported. The findings of the study may contribute towards interventions required to prevent rural communities of KZN from possible cholera outbreak.

CHAPTER 3: METHODOLOGY

3.1 MATERIAL

All chemicals used in the analysis were of molecular grade and were occurred from commercial suppliers and used without any further purification.

3.2 METHODS

3.2.1 Bacterial strains

The bacterial strains used for this study (Table 3.1) were obtained from the National Health Laboratory Services (NHLS, S.A), American Type Culture Collection (ATCC, U.S) and National Collection of Type Cultures (NCTC, U.K). All bacterial strains were stored at –70°C in Microbank™_cryovials (Pro-Lab Diagnostics, Ontario, Canada). Strains were grown on nutrient agar (Oxoid®, U.K) or in nutrient broth (Oxoid®, U.K) at 37°C.

Table 3.1. Bacterial strains used for all experimental work

Micro-organisms	Source	Micro-organisms	Source
Escherichia coli (commensal)	NHLS	*Shigelladysenteriae*type 1	NHLS
Enterohaemorrhgic*E. coli*	NHLS	*Shigellaflexneri*	NHLS
Enterotoxigenic*E. coli*	NHLS	*Shigelladysenteriae*type 2	NHLS
Enteroaggregative*E. coli*	NHLS	*Shigellaboydii* serotype B	NHLS
Enteroinvasive*E. coli*	NHLS	*Shigellasonnei*	NHLS
Enteropathogenic*E. coli*	NHLS	*Salmonellatyphimurium*	NHLS
Aeromonasveronii	ATCC	*Vibrio fluvialis*	NCTC
Pseudomonas aeruginosa	NHLS	*Salmonella enteritidis*	NHLS
Klebsiella pneumonia	NHLS	*Salmonella typhi*	NHLS
Bacillus cereus	NHLS	*Vibrio mimicus*	NHLS
Bacillus subtilis	NHLS	*Vibrio cholerae*(Ogawa)	NHLS
Enterococcus faecium	NHLS	*E. coli* BW31004	Yale University
Enterococcus faecalis	NHLS	*Vibriofurnissii*	NHLS
Morganella morganni	NHLS	*Salmonella gallanarum*	NHLS
Vibrio cholerae non-O1	NHLS	*Vibrio cholerae* O1	NCTC
Vibrio cholerae O139	NHLS	*Vibrio parahaemolyticus*	NHLS

3.2.2 DNA extraction methods for the isolation of bacterial DNA

DNA extractions were performed following two methods; the modified method reported by Boom et al. (1990) using the spin columns as described by Borodina et al. (2003); and the rapid boil method.

3.2.2.1 Column preparation

The columns used for DNA extractions were prepared as described by Borodina et al. (2003) where the cap of a 0.5 mℓ microfuge tube (Sigma-Aldrich, Germany) was severed leaving a small "tail" (Figure 3.1). Two holes were punctured in the bottom of the tube with a red-hot inoculation needle. Silica membranes were cut out from GF/F brosilica glass fibre paper (Whatman, England) and two membranes were inserted tightly into the tube. For loading and washing the column was placed inside a reusable 2 mℓ microfuge tube (Sigma-Aldrich, Germany) and for DNA elution it was placed inside a 1.5 mℓ microfuge tube (Sigma-Aldrich, Germany).

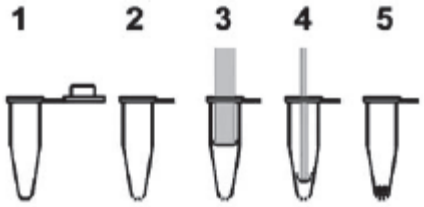

Figure 3.1.Preparation of Homemade spin columns
Original tube (1) used for the experiments; severing the cap leaving the small tail and making the holes (2); sequential steps of the filter insertion (3-5) (Borodina et al. 2003).

3.2.2.2 Preparation of chemicals and buffers for DNA extractions

This DNA extraction method made use of prepared celite solution, lysis buffer and wash buffer. The celite solution was prepared by adding 50 mℓ of distilled water and 500 µℓ of 32% (w/v) HCl to 10 g of celite (Supelco, U.S). The suspension was aliquoted into aluminium foil wrapped small glass bottles (100 ml) followed by sterilisation at 121°C for 15 minutes. After sterilisation, the glass bottles were refrigerated at 4°C. This suspension can be used for up to 6 months stored at room temperature.

The lysis buffer was prepared by dissolving 120 g of guanidiumthiocyanate (GuSCN) (Sigma-Aldrich, Germany) in 100 mℓ of a 0.1M Tris hydrochloride buffer (pH 6.4; Promega, U.S)followed by addition of 22 mℓ of a 0.2 M EDTA (Saarchem, U.S) solution (pH 8) and 2.6 g of Triton X-100 (Sigma-Aldrich, Germany) prior to homogenization.

The washing buffer was prepared by dissolving 120 g of GuSCN in 100 mℓ of a 0.1 M Tris hydrochloride buffer (pH 6.4, Promega, U.S). Dissolving of the GuSCN was facilitated by heating the solution in a water bath at 65°C under continuous shaking until completely dissolved. To remove any possible contaminating DNA, 5 mℓ celite suspension was added to 50 mℓ of each of the buffers (lysis and wash buffer). The suspensions were mixed and left at room temperature for at least an hour (mixing sporadically) after which the suspensions were centrifuged using Neofuge 15R centrifuge (Shanghai Lishen Scientific Equipment Co.

Ltd, China) at 2000 rpm for 5 min. The supernatant was then transferred to clean sterile 50 mℓ polypropylene tubes (Greiner Bio-one, Germany). The polypropylene tubes were wrapped in aluminium foil and stored at 4°C. The buffers were stable for at least 6 months at room temperature in the dark. A commercial elution buffer (AE buffer) was obtained from Qiagen.

3.2.2.3 DNA extraction procedure

An adapted version of the protocol described by Boom et al. (1990) was used for the extraction of DNA from bacterial isolates. This method entails the centrifugation at 13300 rpm for 60 sec of 2 mℓ of enrichment broth or a single bacterial colony re-suspended in 1 mℓ sterile distilled water using Spectrafuge 24D centrifuge (Labnet International, Inc; USA). The resulting bacterial pellet was re-suspended in 700 µℓ of lysis buffer followed by incubation at 70°C for 10 min. A volume of 250 µℓ 100% (v/v) ethanol was added to this mixture and further incubated at 56°C for 10 min. After incubation, 50 µℓ of the celite solution was added followed by incubation at room temperature for 10 min (with occasional mixing of the mixture). A clean, sterile spin column (Figure 3.1.) was then placed inside a sterile 2 mℓ microfuge tube. Approximately 500 µℓ of the lysis mixture was loaded into the column followed by centrifugation at 13300 rpm (Spectrafuge 24D centrifuge, Labnet International, Inc; USA) for 30 seconds to separate the buffer from the celite. This was repeated until all of the lysis mixture was loaded into the column. The column was washed twice by adding 400 µℓ wash buffer followed by centrifugation at 13300 rpm for 30 sec (Spectrafuge 24D centrifuge, Labnet International, Inc; USA). This was followed by two more wash steps using 400 µℓ of a 70% (v/v) ethanol solution followed by a last centrifugation step at 13300 rpm for 2 min (Spectrafuge 24D centrifuge, Labnet International, Inc; USA) to ensure that all ethanol was removed from the column. The column was then transferred into a clean, sterile 1.5 mℓ microfuge tube and 100 µℓ elution buffer (AE buffer; Qiagen, Germany) was added to the column followed by incubation at 56°C for 2 min. To elute the DNA from the celite, the column was centrifuged for 2 min at 13300 rpm (Spectrafuge 24D centrifuge, Labnet International, Inc; USA) after which the column was discarded. The AE buffer containing DNA was collected in the 1.5 mℓ microfuge tube.

3.2.2.4 Rapid boiling method

One millilitre of overnight culture was transferred into a clean, sterile 1.5 mℓ microfuge followed by centrifugation at 13300 rpm for 2 min (Spectrafuge 24D centrifuge, Labnet International, Inc; USA). After centrifugation, the supernatant was discarded and the pellet was re-suspended in 100 µℓ PCR grade water. The bacterial cells were lysed by heating the suspension for 10 min at 100°C followed by centrifugation at 13300 rpm for 5 min to remove cellular debris. The supernatant containing the bacterial DNA was immediately used in subsequent PCR reactions, or was stored at -20°C for later use in PCR reactions.

3.2.3 Detection and identification methods

3.2.3.1 Internal process control for the real-time m-PCR assays

An *E. coli* strain (BW31004) carrying a single genomic copy of the green fluorescent protein (*gfp*) gene from *Aequorea victoria* was used as a positive process control. The GFP-expressing strain (designated *E. coli*-GFP) was obtained from the *E. coli* Genetic Stock Center, MCDB Department, Yale University. The *E coli*-

GFP was grown to log phase (OD520nm between 0.15 and 0.2) in nutrient broth. The number of log phase *E. coli*-GFP cells were estimated by serially diluting the enriched broth followed by spread plating (in triplicate) onto nutrient agar. The colonies were counted after overnight incubation at 37°C. The quantified *E. coli*-GFP was stored at -20°C until further use. When analysing water samples with the real-time multiplex PCR (m-PCR) assay, *E. coli*-GFP was added to 1 mℓ alkaline peptone water (APW) enrichment before nucleic acid extraction. The *E. coli*-GFP was added to the 1 mℓ enriched APW at final concentration of 10^4 CFU (Ntema and Barnard, 2013).

3.2.3.2 Multiplex real-time PCR (m-PCR) assay

Two duplex real-time PCR assays were optimised for the detection of toxigenic and non-toxigenic *V. cholerae* (both O1 and O139 serotypes). The first duplex real-time PCR assay targeted the *ctxA* (cholera toxin) and *hlyA* (hemolysin, *V. cholerae* species specific) genes while the second duplex real-time PCR assay targeted the O1-*rfb* (*V. cholerae* O1) and O139-*rfb* (*V. cholerae*O139) genes. The two duplex real-time PCR assays were each multi-plexed with the *gfp* assay, the latter serving to detect the *E. coli*-GFP added as a positive process internal control. The primers and TaqMan probes for the two real-time PCR were obtained from Integrated DNA Technologies (U.S). According to the HANDS (Homo-Tag Assisted Non-Dimer System) principle, all of the primers had a common tag sequence at their 5' ends that served as a universal primer binding site, and the tag was used as the universal primer (Huang et al. 2009). To enable simultaneous detection, each of the TaqMan probes was labelled with a different fluorophore as shown in Table 3.2.

Table 3.2. Primers and probes used in the multiplex real-time PCR detection assays

Primers	Sequence (5' – 3')	Conc. (µM)	size (bp)
ctxA-F	Tag-TCCGGAGCATAGAGCTTGGA	0.3	
ctxA-R	Tag-TCGATGATCTTGGAGCATTCC	0.3	120
ctxA-P	FAM -AGCCGTGGATTCATCATGCACCGCCGGG – IOWA BLACK FQ	0.05	
hlyA-F	Tag-CGCTTTATTGTTCGATGCGTTA	0.3	
hlyA-R	Tag-ACTCGGTTATCGTCAGTTTGG	0.3	141
hlyA-P	TYE 665- CGATAATCTTGGGCAATCGCATCGGTTGACC - IOWA BLACK RQ	0.05	
gfp-F	Tag-CCTGTCCTTTTACCAGACAACCA	0.05	
gfp-R	Tag-GGTCTCTCTTTTCGTTGGGATCT	0.05	77
gfp-P	HEX- TACCTGTCCACACAATCTGCCCTTTCG - IOWA BLACK FQ	0.05	
O1rfb-F	Tag-CCAGATTGTAAAGCAGGATGGA	0.3	
O1rfb-R	Tag-GGTCATCTGTAAGTACAAC	0.9	203
O1*rfb*-P	TYE 705 - TGAGTTTGTAAGCCCACTACCGCATTCATATCC – IOWA BLACK RQ	0.05	
O139*rfb*-F	Tag-CATACCAACGCCCTTATCCATT	0.1	
O139*rfb*-R	Tag-GCATGACTGGCATCCCAAAAT	0.1	160
0139*rfb*-P	TYE 665- CGGGTGTTATTGCTGTCTTTTCTCACGAGGG - IOWA BLACK RQ	0.05	
Hands tag	GCAAGCCCTCACGTAGCGAA	1.2	

Real-time PCR reactions were performed using a Rotor-Gene Q thermal cycler (Qiagen, Germany) in a total reaction volume of 20 µℓ. For both the multiplex real-time PCR, each reaction consisted of 1X TaqMan Environmental Master mix 2.0 (Applied Biosystems, USA), 1.2 µM universal primer identical to the common tag sequence (Table 3.2), 0.1–0.9 µM primer pairs specific for each gene target ,0.05 µM of each of the five differently labelled probes (Table 3.2) and 2 µℓ of template DNA. Reaction conditions consisted of an enzyme activation step at 95°C for 10 min; 10 cycles of 95°C for 10 s, 58°C for 20 s (with a 1°C decrease for each

cycle) and 72°C for 15 s; and 45 cycles of 95°C for 10 s, 56°C for 20 s and 72°C for 15 s. Positive, negative and no-template controls were used in every experiment.

Fluorescence was measured following the annealing step during the last 45 cycles. PCR products were detected by monitoring the increase in fluorescence of the reporter dye after each PCR cycle. Using the Rotor Gene Q software, the normalized fluorescence emitted from the relevant reporter dyes were plotted against the number of amplification cycles. The threshold cycle (Ct) values were determined, i.e. the PCR cycle number at which fluorescence increases above a defined threshold level. For the *V. cholerae* target genes (*ctxA*, *hlyA*, O1-*rfb* and O139-*rfb*) Ct values greater than 40 were regarded as negative PCR detection while Ct values less than 40 were regarded as positive PCR detection. For the *gfp* component of the m-PCR assays, a mean Ct value of less than 32 was interpreted as no PCR inhibition (calculated from the mean Ct value obtained when adding 10^4 CFU *E. coli*-GFP cells to 1 mℓ APW broth), a mean Ct value of greater than 40 cycles, as total inhibition of the PCR reaction and a mean Ct value of between 32.1 and 40 as a loss of reaction sensitivity (Ntema and Barnard, 2013).

3.2.4 Study area

Samples were collected monthly from Msunduzi River, Umlazi River and Isipingo River from October 2012 to December 2013 except for January 2013. Msunduzi River (Figure 3.2) is a river in Msunduzi local municipality of Umgungundlovu District Municipality, KwaZulu-Natal, South Africa. Msunduzi local municipality encompasses the city of Pietermaritzburg, which is the capital of the KwaZulu-Natal province and the main economic hub of Umgungundlovu District Municipality. A portion of the Msunduzi River within the city of Pietermaritzburg has been dammed by weirs, and is used for canoeing and rowing practice. The Dusi Canoe Marathon is an annual canoe marathon from Pietermaritzburg to Durban, which starts on the Msunduzi River, and ends on the Mngeni River. The race attracts around 2000 paddlers, and receives national media coverage in South Africa. Umlazi and Isipingo rivers pass through Umlazi Township (Figure 3.3). Umlazi is a township on the east coast of KwaZulu-Natal, South Africa. The township is located south-west of Durban. Umlazi Township is under the eThekwini municipality. About 15% of homes in Umlazi are informal settlements. During the time of the study most of these informal settlements were been demolished and replaced with brand new homes and roads that are part of the residential development project. The rivers monitored in this study are used for recreational, traditional and religious purposes by populations in the two municipalities. The Global Positioning System coordinates for all sample points are shown in appendixes (APPENDIX D: GLOBAL POSITIONING SYSTEM COORDINATES OF SAMPLING SITES)

3.2.4.1 Zooplankton and Phytoplankton samples

Zooplankton and phytoplankton samples were collected by filtering 100 ℓ of river water through a plankton net (mesh size 64µm for zooplankton and 20µm for phytoplankton). During the filtration process, 200 mℓ of filtrate from the 20 µm mesh net was collected to be analysed for planktonic (unattached, free-living) bacteria (Alam et al. 2006). Triplicate portions of each plankton sample (2 mℓ) were homogenized using a tissue disruptor (Disruptor gene, Scientific Industries Inc, USA) in 2 mℓ microfuge tubes containing glass beads (0.1 mm, BioSpecProducts, USA). The homogenates were combined and enriched in alkaline peptone water.

Bacteria from water samples were concentrated by filtration (0.45 µm, Millipore, U.S) followed by enrichment in APW.Part of the plankton samples (64 µm and 20 µm fractions) were transferred to 100 mℓ brown (to limit the penetration of light) plastic bottles containing 2 mℓ of formaldehyde (for preservation). These preserved plankton samples were used for zooplankton and phytoplankton identification purposes using standard procedures (Standard methods, 2005).

Figure 3.2. Map of the Msunduzi municipality showing Msunduzi River sampling points.

1. Emaswazini, 2. Magwenyane, 3. Kobongwaneni, 4. Shange Bridge, 5. Smero, 6. Herwood Bridge, 7. Plessislaer, 8. Makro, 9. Promed Road, 10. Grimthorpe.

Figure 3.3. Map of Umlazi Township showing Umlazi River and Isipingo River sampling points.

1. Msebe primary (Umlazi River), 2. How long park, 3. Vumokuhle primary school, 4. Maphumephethe. Sampling points 2-4 are for Isipingo River.

3.2.4.2 Zooplankton and Phytoplankton samples

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3.2.4.3 Sediments samples

Sediments samples were collected using a core sampler and transported to the laboratory in sterile 250 mℓ glass bottles. One hundred millilitres of sterile, distilled water were added to one gram of sediment sample. The samples were mixed and allowed to settle. A 10 mℓ aliquot of the slurry was centrifuged at 2000 rpm for 8 min (Neofuge 15R centrifuge, Shanghai Lishen Scientific equipment co. Ltd, China) at room temperature to remove particulate matter. A 2 mℓ aliquot of the slurry was enriched in APW.

3.2.4.4 Macro invertebrates samples

Macro invertebrates were sampled from stones and aquatic vegetation biotopes of the rivers monitored. The stone biotope was sampled by rolling the stones over and bumping against each other to dislodge any organisms. A sampling net (mesh size 1 mm) was placed near stones to be rolled over, in a position where the current would carry the dislodged organisms downstream into the net. Aquatic vegetation was sampled by sweeping the sampling net within the aquatic vegetation and pushing against and through the vegetation to dislodge any organisms. A combination of short lateral sweeps with vertical lifts aided in dislodging and catching the suspended organisms. After sampling, invertebrates were transferred into sampling bottles for identification and analysis of *V. cholerae*. A portion of the sampled organisms were preserved in 70% ethanol for taxonomical identification following standard procedures (Standard methods, 2005).

3.2.4.5 Animal stool samples

Animal stool samples were collected aseptically from ground surface areas and stored in sterile urine containers before transportation (at 4°C) to the laboratory. Analyses were performed on the day samples were collected. Approximately one gram of each of the stool samples was suspended in 10 mℓ phosphate buffered saline and homogenised by vortexing. Any debris not dissolve was removed by centrifugation at 13000 rpm for 30 seconds (Spectrafuge 24D, Labnet International Inc, USA) after which 500 µℓ of the supernatant was used for direct plating onto TCBS media as well as enrichment in APW (Keshav et al. 2010).

3.2.5 Detection of Amoeba resistant *V. cholerae*

3.2.5.1 Amoebal enrichment

For amoebal enrichment, a colony of *E. coli* (ATCC 25922) was inoculated onto a nutrient agar plate and incubated at 37°C. Once growth was obtained, the plate was stored (at 4°C) and sub-cultured onto nutrient agar every two weeks. When needed, the *E. coli* culture was recovered with a sterile swab, suspended in sterile, distilled water and heat killed by boiling. Three drops of the heat killed *E. coli* suspension was inoculated onto a non-nutrient agar plate and spread evenly across the whole surface with a sterile swab. Two nutrient agar plates were also inoculated with this suspension and incubated overnight at 37°C to ensure that no organisms were viable in the suspension used to prepare the non-nutrient agar-*E coli* plates.

3.2.5.2 Detection of V. cholerae from cultured amoebae

Water samples from the Msunduzi, Umlazi and Isipingo rivers were collected aseptically in clean, sterile one litre plastic bottles and held at <10°C during transit to the laboratory. All samples were analysed on the day of collection (Standard method, 2005). One hundred millilitres of each water sample was filtered onto a nitrocellulose membrane filter (0.45 µm pore size, 47 mm diameter; Millipore Corporation, U.S). The filters were placed upside down onto fresh non-nutrient agar-*E.coli* plates. Three drops of Page's modified Neff's amoeba saline (PAS) were added to the non-nutrient agar-*E.coli* plates to assist the growth of amoebae present in the samples. The plates were left at room temperature for 10 minutes to settle and were subsequently incubated at 33°C (aerobically) in plastic bags to avoid desiccation. The plates were checked daily using a light microscope with a 10x objective (Olympus, U.S) for the morphological appearance of amoebal trophozoites and cysts. Plates without growth were re-incubated for at least 3 weeks before reporting a negative result.

Plates containing amoebae were sub-cultured by aseptically cutting small agar plugs from the areas on the plate where active amoebae were observed. The agar plugs were placed upside down onto fresh non-nutrient agar-*E.coli* plates with 3 drops of PAS. The plates were left at room temperature for 10 minutes to settle, then again incubated at 33°C. After one to three sub-cultures, the amoebae present in the samples were harvested by gently scraping the agar surface, followed by suspension in 2 mℓ PAS and subsequent wash steps (three times) by centrifugation at 3300 rpm (Spectrafuge 24D, Labnet International Inc, USA) for 15 minutes. A volume of 100 µℓ of the resulting pellet was inoculated into a 24 well, flat bottomed microtitre plate (Nunc, U.S) with 1 mℓ PAS and incubated at 33°C for 48 hours. The wells were examined using an inverted microscope with a 40x objective (Leica, U.S) for the morphological appearance of amoebal trophozoites and cysts containing intracellular bacteria, or alternatively for wells containing disintegrated amoebal cells.

Wells containing intracellular bacteria or disrupted amoebal cells were considered presumptively positive for amoeba resistant bacteria. The PAS solution from these wells was removed, the amoebae were gently removed from the bottom and sides of the wells, washed three times by centrifugation at 1000 x g for 10 minutes to remove extra cellular bacteria and gram stained using standard procedures. This was to detect

intracellular, gram negative bacteria with typical *V. cholerae* morphology. Specimens were inoculated into alkaline peptone water, incubated at 37°C overnight and the top portion of the growth was inoculated onto TCBS agar, followed by overnight incubation at 37°C. Typical *V. cholerae* colonies were confirmed by multiplex real-time PCR.

3.2.6 Analysis of environmental samples

Environmental samples were analysed following two approaches as illustrated in Figure 3.4. These approached included culture dependent and culture independent real-time PCR detection assays. One of the culture dependent PCR assays employed high resolution melt (HRM) curve analysis (Le Roux and Van Blerk, 2011) for amplicon differentiation and identification and was used to compare and validate the results obtained from the probe-based PCR assays used in this study. The HRM real-time PCR assay was performed at East Rand Water Care Company (ERWAT) laboratory facility in parallel to the real-time PCR methods conducted at the Water and Health Research Centre (WHRC), University of Johannesburg. All water samples were analysed using both the culture dependent and independent PCR assays while all the other sample types were analysed using the culture dependent PCR assays only. The different culture dependent and independent real-time PCR assays are discussed below.

3.2.6.1 Culture based real-time m-PCR detection (WHRC)

One hundred millilitres of each water sample were filtered onto a nitrocellulose membrane filter (0.45 µm pore size, 47 mm diameter; Millipore Corporation, U.S) and the membrane enriched in 100 mℓ APW for 18 hours at 36°C according to manufacturer's instructions (Oxoid®, U.K). Two millilitre of the enriched APW broth culture was taken from the surface (aerobic interface) and DNA extracted following the procedure as described in section3.2.2.4. The extracted DNA was used as a template for the multiplex real-time PCR assays as described in section 3.2.3.2.

3.2.6.2 Culture based HRM real-time PCR detection (ERWAT)

A high resolution melt (HRM) real-time PCR assay for the detection of toxigenic *Vibrio cholerae* was used as described by le Roux and van Blerk, (2011). These analyses were performed by the ERWAT PCR laboratory. Primers used in the HRM real-time PCR assay specifically targeted the *ompW* (*V. cholerae* species-specific gene) and *ctxAB* (cholera toxin) genes (Nandi et al. 2000; Goel et al. 2005). Primer nucleotide sequences with their expected amplicon sizes and melting temperatures (Tm) are shown in Table 3.3. Real-time PCR reactions were performed in either 0.2 mℓ or 0.1 mℓ thin walled tubes in a total reaction volume of 25 µℓ. Amplification was performed in a RotorGene® 6000 2-plex rotary thermal cycler with HRM capability (Qiagen, Germany). Each reaction consisted of 1X SensiMix HRM (Bioline, UK) reaction buffer containing dNTPs, $MgCl_2$, a heat activated DNA polymerase and EvaGreen dye, to which 0.2 µM of each primer, nuclease free water (Applied Biosystems, USA) and 5 µℓ genomic DNA serving as template was added. Cycling conditions were as follow; polymerase activation step at 95°C for 10 min followed by 45 cycles of DNA denaturation at 95°C for 30 s, annealing at 64°C for 30 s and extension at 72°C for 30 s. A final extension step was performed at 72°C for five minutes following cycling. For the differentiation and

identification of the resulting amplification products, HRM curve analysis was performed by lowering the temperature to 60°C for 5 minutes and a subsequent increase to 90°C in increments of 0.1°C per second. Fluorescence was measured continuously and the Tm peaks were calculated based on the initial fluorescence curve (F/T) by plotting the negative derivative of fluorescence over temperature versus temperature (-dF/dT versus T). Positive, no template and reagent blank controls were used in every experiment.

Table 3.3. Oligonucleotide primers used for the HRM real-time PCR assay

Primer	Sequence (5' – 3')	Conc. (µM)	size (bp)
ompW	CACCAAGAAGGTGATTTTATTGTG	0.2	588
	GAACTTATAACCACCCGCG	0.2	
ctxAB	CCTGTCCTTTTACCAGACAACCA	0.2	564
	GGTCTCTCTTTTCGTTGGGATCT	0.2	

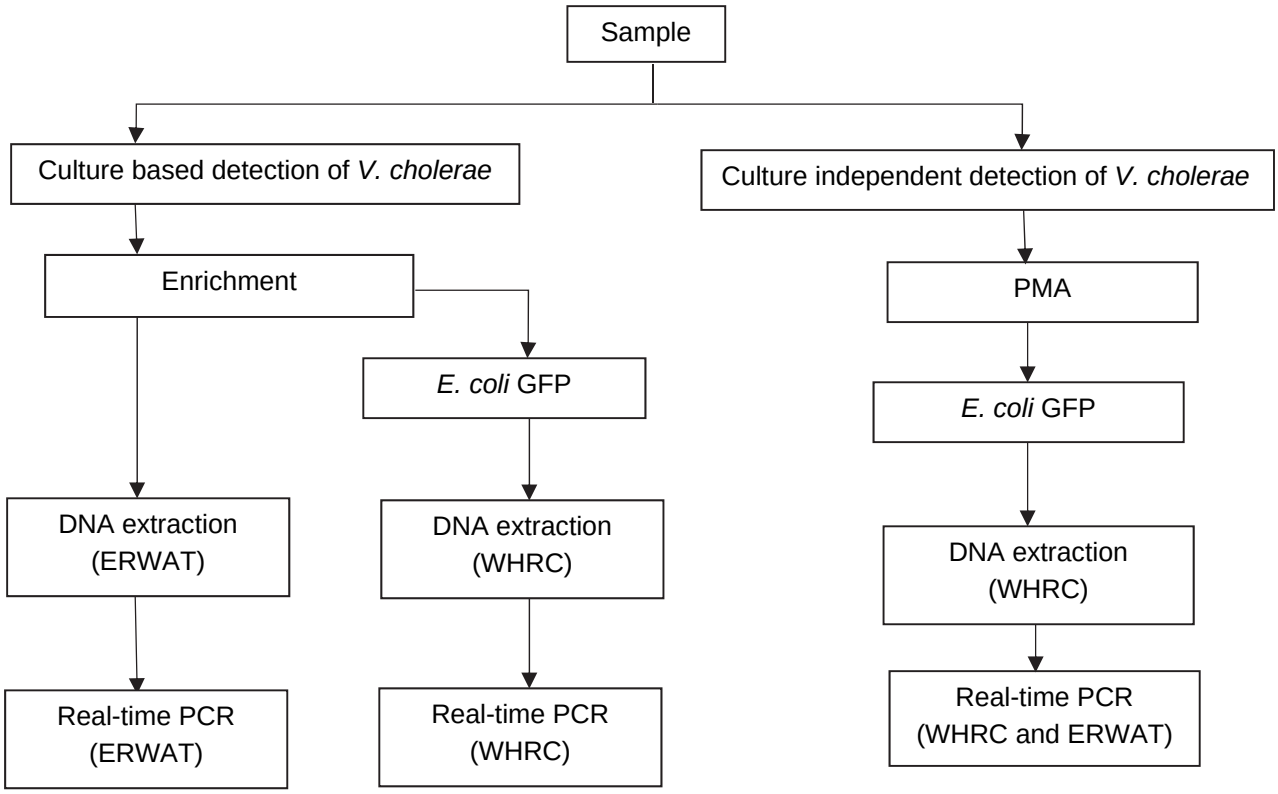

Figure 3.4. Flow diagram illustrating sample analyses procedures

Genomic DNA was extracted from bacteria using the InstaGene™ matrix commercial kit (Bio Rad, U.S.) following the manufacturer's instructions. Briefly, 1 mℓ of enriched bacteria (APW) were concentrated by centrifugation at 13 000 rpm (Spectrafuge 24D, Labnet International Inc, USA) for 5 min. The resulting pellet was re-suspended in 100 µℓ of InstaGene™ matrix and incubated at 56°C for 25 minutes. Following incubation, the suspension was vortexed at high speed for 10 seconds and further incubated at 100°C for 10 min. After incubation, the suspension was vortexed at high speed for 10 seconds and centrifuged for 2 min at 12000 rpm (Spectrafuge 24D, Labnet International Inc, USA). Five microliters of the resulting supernatant was used as template in subsequent HRM real-time PCR analyses.

3.2.6.3 Culture independent real-time PCR detection

One hundred millilitres of each water sample were centrifuged at 8000 rpm for 8 minutes using Neofuge 15R centrifuge. After centrifuging, supernatant was discarded and the pellet resuspended in 1 mℓ PCR grade water. The bacteria suspended in PCR grade water was treated with propidium monoazide (PMA) as described in section 3.2.6.4. After PMA treatment samples were spiked with *E. coli*-GFP and bacterial DNA extracted as described in section 3.2.2.3. Extracted DNA was used as template in the m-PCR analyses as described in section 3.2.2.3. Bacterial DNA extracted using this method was also used in the HRM real-time PCR assays as employed by the ERWAT PCR laboratory.

3.2.6.4 PMA treatment

Treatment of bacterial cells was followed as described by Nocker et al. (2007). Light transparent 1.5 mℓ microcentrifuge tubes were used for PMA treatment. Phenanthridium, 3-amino-8-azide-5-[3-(diethylmethylammonio)propyl]-6-phyenyl dichlororide (PMA, Biotium, US) was dissolved in 20% dimethyl sulfoxide to create a stock concentration of 20 mM, protected from light and stored at -20°C. A volume of 5 µℓ of PMA was added to 1 mℓ culture aliquots to final concentration of 50 µM. Following an incubation period of 5 min in the dark with occasional mixing, samples were light exposed for 5 min using a 650W halogen light source. Samples tubes were laid horizontally on ice (to avoid excessive heating), placed about 20 cm from the light source. Occasional shaking was performed to promote homogeneous light exposure. After photo-induced cross-linking (light exposure), cells were centrifuged at 13300 for 5 min (Spectrafuge 24D, Labnet International Inc, USA) followed by DNA extraction as described in section 3.2.2.3.

3.2.7 Enteric bacteria identification and enumeration

In addition to detecting *V. cholerae* in the samples collected, *Escherichia coli* and total coliforms were simultaneously enumerated using the Colilert® and Quanti-tray®/2000 commercial kit (IDEXX, U.S). The manufacturer's instructions were followed promptly and Quanti-tray®/2000 wells turning yellow were regarded as positive for total coliforms while fluorescent wells under UV (366nm) exposure were regarded as *E. coli* positive. *Escherichia coli* from positive Quanti-tray®/2000 wells were evaluated for the presence of pathogenic *E. coli* strains using a m-PCR assay (Omar and Barnard, unpublished). Briefly, 2 mℓ of media from Quanti-tray®/2000 wells positive for *E. coli* was removed using a sterile 1 mℓ Neomedic disposable syringe with mounted needle (Kendon Medical Supplies, USA) and aliquoted into 2 mℓ sterile microfuge tubes. Bacterial DNA was extracted following an adapted version of the Boom et al. (1990) protocol as described in section 3.2.2.3. The extracted DNA was used as a template in a m-PCR assay as described below (section 3.2.7.1).

3.2.7.1 M-PCR assay for the detection of pathogenic E. coli

A multiplex PCR assay for the detection of entero-pathogenic (EPEC), entero-haemorrhagic (EHEC), entero-invasive (EIEC), entero-toxigenic (ETEC) and entero-aggregative *E. coli* (EAEC) was used to evaluate *E. coli* positive Quanti-tray's. Information regarding the primers used is listed in Table 3.4.

Table 3.4. Oligonucleotide primers used for the *E. coli* m-PCR assay

Pathogen	Primer	Sequence(5'-3')	Size (bp)	Conc. µM	Reference
E .coli	*mdh (*F)	GGTATGGATCGTTCCGACCT	304	0.1	Tarr et al. (2002)
	mdh(R)	GGCAGAATGGTAACACCAGAG T			
EIEC	*ial (F)*	GGTATGATGATGATGAGTGGC	650	0.2	López-Saucedo et al. (2003)
	ial (R)	GGAGGCCAACAATTATTTCC			
EHEC/ Atypical EPEC	*eaeA(F)*	CTGAACGGCGATTAC GCGAA	917	0.3	Aranda et al. (2004)
	eaeA(R)	GACGATACGATCCAG			
Typical EPEC	*bfp(F)*	AATGGTGCTTGCGCTTGCTGC	550	0.3	Aranda et al.
	bfp(R)	TATTAACACCGTAGCCTTTCGCTGAAG			From this study
EAEC	*eagg(F)*	AGACTCTGGCGAAAGACTGTATC	194	0.2	Pass et al. (2000)
	eagg(R)	ATGGCTGTCTGTAATAGATGAGAAC			
EHEC	*stx1(F)*	ACACTGGATGATCTCAGTGG	614	0.5	Moses et al. (2006)
	stx1(R)	CTGAATCCCCCTCCATTATG			
	stx2(F)	CCATGACAACGGACAGCAGTT	779	0.3	Moses et al. (2006)
	stx2(R)	CCTGTCAACTGAGCACTTTG			
ETEC	*lt(F)*	GGCGACAGATTATACCGTGC	360	0.1	Pass et al. (2000)
	lt(R)	CGG TCT CTA TAT TCC CTG TT			
	st(F)	TTTCCCCTCTTTTAGTCAGTCAACTG	160	0.5	Pass et al. (2000)
	st(R)	GGCAGGATTACAACAAAGTTCACA			
E. coli toxin	*asta(F)*	GCCATCAACACAGTATATCC	106	0.3	Kimata et al. (2005)
	asta(R)	GAGTGACGGCTTTGTAGTC			
Internal control	*gapdh(F)*	GAGTCAACGGATTTGGTCGT	238	0.3	Mbene et al. (2009)

Targeted genes were amplified in a 20 µℓ reaction mixture containing 2 x Qiagen® m-PCR master mix (HotstartTaq DNA polymerase, 10 x buffer, 2 mM MgCl2 and dNTP mix), 5X Q-solution, PCR grade water, $MgCl_2$, template DNA and primers. All PCR reactions were performed under the following conditions: enzyme activation at 95°C for 15 min followed by 35 cycles of DNA denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec, extension at 68°C for 2 min with a final elongation step at 72°C for 5 min. PCR reactions were performed in a Biorad MycyclerTM Thermal cycler. For the no template control reaction template DNA was replaced with PCR grade water and the positive control contained DNA from *E. coli* reference strains (EPEC, EHEC, EIEC, ETEC and EAEC).

Following PCR amplification, DNA was analysed on a horizontal agarose slab gel [2% (w/v)] with ethidium bromide (0.5 µg/mℓ) in TAE buffer (40 mM Tris acetate; 2 mM EDTA, pH 8.3). Electrophoresis was performed for 1 to 2 hours in an electric field strength of 80 V and the DNA was visualized under UV light (Gene Genius Imaging system, Vacutec®). The relative sizes of the DNA fragments were estimated by

comparing their electrophoretic mobility with that of standards run [100 bp markers (Fermentas®)] with the samples on each gel.

3.2.8 Physico-chemical properties

The temperature, pH and conductivity of water samples were measured on site during sampling using a HI 98129 waterproof tester (Hanna Instruments Inc, U.S). The salinity of water samples was determined using the obtained temperature and conductivity values. The turbidity of water samples was measured in the laboratory using a Eutech TN-100 turbidity meter (Eutech Instruments, Singapore).

3.3 STATISTICAL ANALYSIS

The degree of linear dependence between physico-chemical parameters, total coliforms and *E. coli* was measured using the Pearson product-moment correlation coefficient. Bivariate analysis of individual predictors and individual dependent variables was conducted; difference in frequency of *Vibrio cholerae* isolation for individual abiotic and biotic parameters was measured using the independent-samples t-test. Multivariate analysis of multiple predictors and an individual dependent variable was conducted; logistic regression was used to test the relationship between a set of abiotic and biotic predictor variables and the frequency of *Vibrio cholerae* isolation. Statistical analyses were performed using SPSS version 21 software. Statistical analysis where done by Statistical Consultation Services from the University of Johannesburg (Pallant, 2007).

CHAPTER 4: RESULTS AND DISCUSSION

4.1 INTERNAL CONTROL AND *V. CHOLERAE* MULTIPLEX REAL-TIME PCR

Four genes, *ctxA* (cholera toxin), *hlyA* (*V. cholerae* hemolysin A), O1-*rfb* (*V. cholerae* O1 serotype) and O139-*rfb* (*V. cholerae* O139 serotype) were selected as *V. cholerae* gene targets (Huang et al. 2009). An *Escherichia coli* strain carrying a single genomic copy of the green fluorescent protein (*gfp*) gene from *Aequorea victoria* was used as a positive process internal control (IC). The five assays were combined to form two multiplex real-time PCR assays. The first multiplex detection assay targeted the cholera toxin (*ctxA*), hemolysin gene (*hlyA*) and green fluorescent protein gene (*gfp*), while the second assay targeted the green fluorescent protein gene (*gfp*), O1-specific *rfb*, and O139-specific *rfb* loci (Ntema and Barnard, 2013).

The two real-time PCR assays can robustly detect all *V. cholerae* serogroups of interest. The *V. cholerae* species-specific *hlyA* gene provides species information for taxonomic identification. The *ctxA* gene, encoding cholera toxin, is the most important determinant of the toxigenic potential of a *V. cholerae* strain. Together with the O1/O139-rfb targets, the four genes proved to be a suitable combination for differentiation of the two major outbreak serogroups (O1 and O139) from other types of *V. cholerae* and for toxigenic potential determination (Huang et al. 2009). The incorporation of a positive process internal control in the two multiplex real-time PCR assays enables the identification of PCR inhibition by the sample matrix and reduces the likelihood of false negative results (Murphy et al. 2007).

The specificity and sensitivity of the two multiplex real-time PCR assays have been previously demonstrated by Ntema and Barnard, (2013). The authors reported a minimum level of detection of 20 CFU per reaction for all four *V. cholerae* target genes (*hlyA*, *ctxA*, O1-*rfb*, O139-*rfb*) using the two multiplex real-time PCR assays (data not shown). Sensitivity testing was performed in triplicate and concordant results were obtained (Ntema and Barnard, 2013). The process IC described here was used in such a way that it allowed the evaluation of the entire PCR procedure from DNA extraction to PCR amplification and PCR product detection. This was achieved by spiking 1 ml of pre-enriched environmental water samples (APW, Oxoid®, U.K) with *E. coli*-GFP strain (BW31004) and detecting the *gfp* gene using real-time PCR. The use of a viable strain as IC has the advantage of being more representative of the pathogen being targeted by the PCR assay. At a concentration of 10^4 CFU per tube the positive process control is present at approximately 200 CFU per reaction after DNA extraction, assuming 100% extraction efficiency. Prior to template preparation, when added to the enriched APW broth at a cell concentration of 10^4 CFU *E. coli*-GFP mean Ct values between 29 and 31 cycles could be obtained. This concentration of the positive process IC DNA did not affect the sensitivity of the two multiplex real-time PCR assays.

4.2 COMPARISON OF CULTURE DEPENDENT AND CULTURE INDEPENT REAL-TIME PCR DETECTION ASSAY

A total of 109 river water samples (Msunduzi, Isipingo and Umlazi Rivers) were collected and tested for the presence of *V. cholerae* using culture dependent real-time PCR detection assay as employed by the Water and Health Research Centre (WHRC) laboratory, University of Johannesburg, culture dependent real-time PCR detection assay with High Resolution Melt (HRM) curve analysis as employed by ERWAT laboratory and culture independent real-time PCR assay as employed by the WHRC.

As indicated in Table 4.1culture dependent real-time PCR detection assays used by both the WHRC and ERWAT laboratories detected planktonic *V. cholerae* non-O1/O139 with higher percentages compared to the culture independent real-time PCR assay. The process internal control (WHRC assay) showed no inhibition or loss of sensitivity of the culture dependent real-time PCR assay. The use of an IC ensured credibility of negative results. Reaction inhibition was observed for the culture independent PCR assay as *V. cholerae* was detected in fewer water samples when compared with the culture dependent PCR assays.

Table 4.1. Detection of *V. cholerae* non-O1/O139 using culture dependent and culture independent PCR assays. Percentage in brackets

Method	N (Water samples)	No. of samples positive (*V. cholerae*)
Culture dependent real-time PCR detection (WHRC)	109	74 (68)
Culture dependent real-time PCR detection (ERWAT)	109	74 (68)
Culture independent real-time PCR detection (WHRC)	109	28 (26)

The results presented in Table 4.1indicate that enrichment in APW increased the sensitivity of the detection assays. This may be attributed to the fact that enrichment allowed for the multiplication of *Vibrio cholerae* bacteria present in the water samples, increasing their numbers and subsequently the ability to detect them. As the 2 mℓ of enriched APW broth was collected at the surface of the broth (i.e. the aerobic inter-phase) it is assumed that the *Vibrio cholerae* detected by PCR was able to reach the broth surface and therefore is viable. These results are consistent with results reported by Chomvarin et al. (2007). The authors reported increased detection efficiency of PCR assays when samples were filtered and enriched before PCR as opposed to samples filtered without enrichment before PCR.

4.3 BIOTIC AND ABIOTIC PARAMETERS OF THE RIVERS STUDIED

Environmental factors such as pH, temperature, salinity and nutrient concentration are important for Vibrio survival. Water temperature of the three rivers under study varied from 6°C to 28°C (mean of 19±4°C) during the study period. The pH fluctuated between 6.6 and 8.8 with a mean of 7.6±0.3. Salinity of the water

appeared very low ranging between 0.04 psu and 0.38 psu with a mean of 0.14±0.77 psu. Turbidity oscillated between 0.34 NTU and 760 NTU with a mean of 55±127 NTU. Conductivity ranged between 60 µs/cm and 647 µs/cm with a mean of 253±149 µs/cm. Total coliform counts fluctuated between 600 and 1.3x107 CFU/100ml with a mean of 2.7x105±1.0x106 CFU/100ml and that of *E. coli* varied between 0 CFU/100ml to 1.1x106 CFU/100ml with a mean of 4.8x104±1.4x105 CFU/100ml.

Correlations between the physicochemical parameters observed and enteric bacteria detected were studied and results presented in Table 4.2. Except for conductivity all the physicochemical parameters strongly correlated with total coliform and *E. coli* counts ($p < 0.01$). Conductivity only correlated with total coliform and *E. coli* counts ($p < 0.05$). There was a strong correlation between pH and temperature ($p < 0.01$) and salinity strongly correlated with conductivity ($p < 0.01$). Total coliform and *E. coli* counts were also strongly correlated ($p < 0.01$). Because of the high correlation between salinity and conductivity as well as total coliform and *E. coli* counts, it was decided to exclude conductivity and total coliform from the logistic regression analyses as presented in Section 4.4 below.

Table 4.2. Correlation coefficient *r* for the physicochemical parameters and enteric bacteria observed for the study period

	pH	**Temperature**	**Salinity**	**Turbidity**	**Conductivity**	**Total coliform**	***E. coli***
pH	1	0.52 ***	0.11 NS	0.05 NS	0.18 **	0.31 ***	0.25 ***
Temperature		1	0.13 NS	0.15 NS	0.28 ***	0.39 ***	0.26 ***
Salinity			1	-0.24 ***	0.98 ***	0.61 ***	0.65 ***
Turbidity				1	-0.20 **	0.22 **	0.18 **
Conductivity					1	0.65 ***	0.68 ***
Total coliform						1	0.84 ***
E. coli							1

** = correlation is significant at P< 0.05 level (2-tailed); *** = correlation is significant at P< 0.01 level (2-tailed); NS = Not significant.

A total number of seventeen phytoplankton species were found in samples from the Msunduzi, Isipingo and Umlazi Rivers (Table 4.3). Zooplankton species found in the river samples included *Copepods, Diptera, Ephemerop, Hyacarina, Nematoda, Ostracoda* and *Rotatoria.* Various species of invertebrates were detected and identified in all the samples from rivers monitored in this study. The invertebrate species identified included *Baetidae*, *Hydropsychidae*, *Turbellaria*, *Coenagrionidae*, *Pleidae*, *Atyidae*,

Notonectidae,*Chironomidae, Caenidae, Trichorythidae, Lymnaeidae, Leptophlebidae, Liptoceridae, Culicidae pupae*, *Naucoridae, Corixidae.*

Table 4.3. Phytoplankton species detected in the Msunduzi, Umlazi and Isipingo rivers

Bacillariophyceae	Chlorophyceae	Cyanophyceae	Dinophyceae
Diatoms	*Choococus*	*Cyclotella*	*Ceratium*
Coscinodiscus	*Closterium*	*Cymbella*	*Peridinium*
Melosira	*Cocconeis*	*Oscillatoria*	
Synedra	*Pediastrusm*		
Biddulphia	*Scenedesmus*		
Stephanodiscus			
Ditylum			

4.4 ISOLATION OF *VIBRIO CHOLERAE* FROM RIVER WATER SAMPLES

Non-toxigenic *V. cholerae* (non-O1/O139) was isolated from 483 (71%) of 676 river water samples collected at all three rivers studied (Table 4.4). Non-toxigenic *V. cholerae* (non-O1/O139) was detected from all sample types except for amoeba and cow stool samples. Toxigenic and non-toxigenic *V. cholerae* O1 and/or O139 were not detected in any of the samples collected from the three rivers monitored. The Umlazi River showed the highest number of samples positive for *V. cholerae* (91%) followed by the Isipingo River (90%) and the Msunduzi River (65%). Table 4.4 indicate that non-toxigenic *V. cholerae* (non-O1/O139) was mostly isolated from the 63 µM and 20 µM net plankton samples as compared to the other sample types with 77% and 76% of the samples being positive for non-toxigenic *V. cholerae* non-O1/O139 respectively. Compared to all other sample types, macro invertebrates showed the lowest presence of non-toxigenic *V. cholerae* (63%).

Table 4.4. Prevalence of *V. cholerae* non-O1/O139 in the rivers studied. Percentage are giving in brackets

	Msunduzi River	Isipingo River	Umlazi River	Total
Free-floating *V. cholerae*	84/140 (60%)	36/42 (86%)	10/12 (83%)	130/194 (67%)
Sediment attached *V. cholerae*	78/120 (65%)	30/36 (83%)	9/11 (82%)	117/167 (70%)
20 µM net *V. cholerae*	66/100 (66%)	30/30 (100%)	10/10 (100%)	106/140 (76%)
63 µM *net V. cholerae*	70/100 (70%)	29/30 (97%)	9/10 (90%)	108/140 (77%)
Macro invertebrates *V. cholerae*	17/28 (61%)	4/6 (67%)	1/1 (100%)	22 /35 (63%)
Total	315/488 (65%)	129/144 (90%)	39/43 (91%)	483/676 (71%)

It has been reported that *Vibrio cholerae* can be found either as non-symbiotic 'free-living' organisms in the water column or attached to phytoplankton, zooplankton and other aquatic organisms (Neogi et al. 2012). Surface attachment provides a selective advantage through access to nutrients that accumulate at the liquid-surface interface (Lutz et al. 2013). The surface of planktonic organisms provide resource-rich microhabitats since *V. cholerae* can metabolize chitin, surface material of crustacean zooplankton and mucilage from the outer surface of some phytoplankton (Cottingham et al. 2003). Living plankton also supplies nutrients to bacteria through excretion or exudates (Islam et al. 1994), while host movement may prevent the depletion of nutrients in the surrounding water (Threlkeld et al. 1994). Besides being free-living and plankton

associated *V. cholerae* was also isolated from sediments samples (Table 4.4) which is in agreement with findings by previous studies (Sudhanandh et al. 2010; Nandini and Somashekar, 1999). Sudhanandh et al. (2010) reported higher *Vibrio* counts in sediments than in the water column. The same was also reported by Nandini and Somashekar (1999) who stated that sediments and adsorption of the microorganism to sand and clay particles culminated in the increase in the density of bacteria at the bottom zone. The sediment is by far the most important ecosystem component for microbial life in the aquatic environment as it provides both biotic and abiotic surfaces for bacterial development. The concentration of organic matter in sediments is 10 000 to 100 000-fold higher than in the overlaying water column.

The results as presented in Table 4.5 indicate that *V. cholerae* non-O1/O139 was mostly detected during autumn. As expected, winter showed the lowest occurrence of *V. cholerae*. The lower detection rate during winter could be attributed to lower temperatures associated with this season. The minimum and maximum temperatures measured during autumn ranged between 11°C and 24°C with a mean temperature of 17±4°C. Compared to other seasons, the *V. cholerae* detected during autumn was mainly attached to plankton and sediment suggesting different seasonal behaviour (Table 4.5). Previous studies showed that the occurrence of *Vibrio* species followed a seasonal pattern that is largely dictated by temperature (Louis et al. 2003; Binsztein et al. 2004). Results from this study however showed that change in temperature or seasons had little effect on the overall presence of *V. cholerae* in the rivers studied. The prevalence of *V. cholerae* in the rivers studied could be due to the attachment to biotic and abiotic surfaces. The association of *V. cholerae* with plankton has been reported to provide a number of advantages including nutrient availability and protection against change in salinity, temperature and protection from predators (Pruzzo et al. 2008).

Table 4.5. Seasonal abundance of *V. cholerae* non-O1/O139 in the rivers studied. Percentage in brackets

	Autumn	Winter	Spring	Summer
Free-floating *V. cholerae*	28/42 (67%)	26/42 (62%)	45/69 (65%)	31/41 (76%)
Sediment attached *V. cholerae*	34/42 (81%)	28/42 (67%)	25/42 (60%)	30/41 (73%)
20 µM net *V. cholerae*	35/42 (83%)	31/42 (74%)	30/42 (71%)	10/14 (71%)
63 µM *net V. cholerae*	42/42 (100%)	27/42 (64%)	30/42 (71)	9/14 (64)
Macro invertebrates *V. cholerae*	7/9 (78%)	2/6 (33)	6/9 (67)	7/11 (64)
Total	146/177 (82%)	114/174 (66%)	136/204 (67%)	87/121 (72%)

An independent-sample t-test was conducted to establish whether there is an individual change in biotic and abiotic parameters when *V. cholerae* non-O1/O139 is present or absent as (a) free-floating bacteria, (b) sediment attached, (c) 20 µM net plankton attached, (d) 63 µM net plankton attached and (e) macro invertebrates attached cells.

An independent-sample t-test results shows that there was a statistically significant difference in the presence or absence of *V. cholerae* with changes in salinity, total coliform and *E. coli* counts for water samples (planktonic *V. cholerae*), 20µM net plankton samples and 63µM net plankton samples (see APPENDIX B: INDEPENDENT SAMPLE T-TEST). Changes in turbidity also had a significant difference on *V. cholerae* presence and absence in water samples (planktonic *V. cholerae*) and macro invertebrate samples (see APPENDIX B: INDEPENDENT SAMPLE T-TEST). Temperature variations showed a

significant difference in *V. cholerae* presence and absence scores for 63µM net plankton samples and free-floating bacterial samples. Direct logistic regression was performed to assess the impact of biotic and abiotic parameters on the presence and absence of *V. cholerae* in (a) free-floating bacterial samples [water samples], (b) sediments samples, (c) 20µM net plankton samples, (d) 63µM net plankton samples and (e) macro invertebrate's samples. The model contained five independent variables (pH, temperature, salinity, turbidity and *E. coli*). Logistic regression analyses indicated that all five independent variables made a statistically significant contribution to the model with different sample types (see APPENDIX C: LOGISTIC REGRESSION ANALYSIS). Among the independent variables, salinity was the strongest predictor of reporting the presence of free-floating and attached *V. cholerae*. The relationship between *V. cholerae* occurrence and salinity appears to be variable, with some studies reporting a significant correlation (Singleton et al. 1982; Johnson et al. 2010), while others demonstrate a lack of correlation between the occurrence of the organism and salinity (Johnson et al. 2012). In water *Vibrio* abundance is related to temperature, salinity, concentration of organic matter and the presence of chitinous organisms (e.g. zooplankton), which are in turn controlled by larger-scale climate variability (Lipp et al. 2002). Knowledge on factors controlling *Vibrio* occurrence in the environment led to the development of conceptual, dynamics and predictive modelling to estimate the role of climate and ecological variables on *Vibrio* population (Lobitz et al. 2000; Bertuzzo et al. 2010), thus greatly improving our current approaches to the epidemiology of *Vibrio*-related diseases.

4.5 TOTAL COLIFORMS AND *E. COLI*

Total coliforms and *E. coli* were enumerated using Colilert® Quanti-Tray/2000 (IDEXX, US). Bacterial genomic DNA was extracted from *E. coli* positive wells and used as a template in multiplex real-time PCR assays targeting genes specific to entero-pathogenic (EPEC), entero-haemorrhagic (EHEC), entero-invasive (EIEC), entero-toxigenic (ETEC) and entero-aggregative E. coli (EAEC) as described by Omar et al. (2010).

Total coliforms and *E. coli* were detected with varying counts from all the samples (See APPENDIX A: TOTAL COLIFORM AND *E. COLI* COUNTS). Total coliform and *E. coli* counts varied during the study period. High *E. coli* and total coliform counts could be attributed to raw sewage seeping into the rivers studied due to blocked sewage mains (Figure 4.1). The *E. coli* counts were found to be higher than the total coliform counts on two occasions; Herwood bridge sampling point for November 2012 and How long park for December 2012 sampling (See APPENDIX A: TOTAL COLIFORM AND *E. COLI* COUNTS). The Colilert® Quanti-Tray results of both sampling occasion had more wells that fluoresced without being yellow. No samples were taking at all sampling site for January 2013.There was no sampling for Msebe primary for October and December 2012. Guidelines published by the WRC and DWAF (1998) state that total coliform levels of 10-100 CFU/100ml could lead to clinical infections in sensitive population groups. Total coliform values higher than 100CFU/100ml increased risk of clinical infections that become more common. Table 4.6shows the mean, minimum and maximum values of the *E. coli* and total coliform counts of the study. Using the WRC/DWF guideline (1998) the *E. coli* and total coliform counts obtained for all the water samples would pose a serious health impact on the humans.

Although *E. coli* has been used as an indicator of faecal pollution the group consists of both commensal and diarrhoegenic strains which constitute a serious human health risk (Omar et al. 2010). PCR results indicated the presence of the diarrhoegenic *E. coli* in the river water samples as shown in Table 4.7. Diarrheagenic *E. coli* are the most common of bacterial pathogens implicated in diarrhoea worldwide (Cabral, 2010). While it is reported that EPEC, EIEC, ETEC and EAEC has high infectious doses EHEC has a low infectious dose with outbreaks reported by doses of only 102 organisms (Hunter, 2003).

Figure 4.1. A blocked sewage main draining into Msunduzi River

Table 4.6. Minimum, maximum and mean *E. coli* and total coliform counts observed

Sample name	Total coliform (CFU/100ml)				*E. coli* (CFU/100ml)			
	Mean	Min	Max	SD	Mean	Min	Max	SD
Emaswazini	8174	3020	38300	12284	1159	100	33990	9383
Grimthorpe	107221	8700	13019800	3322957	8739	870	198600	61339
Herwood Bridge	30217	14100	64200	13336	4088	2200	7600	1682
Kobongwaneni	15573	4035	184680	50607	2804	400	59610	18479
Magwenyane	11475	600	307600	76715	3336	500	93300	23245
Makro	129579	18600	980400	340349	14960	2300	228100	63420
Plessislaer	92545	37200	727000	172266	12173	2000	72065	21007
Promed Road	103145	40800	338580	74834	7463	1800	27100	6753
Shange Bridge	8353	1300	155300	42109	2010	200	31335	8315
Smero	18372	5200	307600	75667	2773	600	140180	35496
Vumokuhle	475574	57940	2622000	945460	73985	11500	1119900	372323
Maphumephethe	237314	10000	1732900	630929	64654	9513	365400	108871
How long Park	200629	31000	2419600	662102	67587	3550	727000	231609
Msebe Primary	142086	25400	727000	177848	16295	3190	62010	19580

Table 4.7.PCR results obtained for the detection of pathogenic *E. coli*. The percentages are given in brackets

Sample Name	N	EIEC	EPEC	EAEC	EHEC	ETEC
Emaswazini	10	0 (0)	7 (70)	3 (30)	4 (40)	0 (O)
Grimthorpe	10	1 (10)	7 (70)	3 (30)	3 (30)	1 (10)
Herwood Bridge	10	2 (20)	8 (80)	6 (60)	5 (50)	3 (30)
Kobongwaneni	10	2 (20)	10 (100)	0 (0)	4 (40)	3 (30)
Magwenyane	10	0 (0)	9 (90)	0 (0)	6 (60)	2 (20)
Makro	10	1 (10)	7 (70)	5 (50)	3 (30)	4 (40)
Plessislaer	10	1 (10)	8 (80)	4 (40)	5(50)	2 (20)
Promed Road	10	1 (10)	7 (70)	3 (30)	3 (30)	3 (30)
Shange Bridge	10	0 (0)	7 (70)	2 (20)	6 (60)	2 (20)
Smero	10	1 (10)	7 (70)	1 (10)	2 (20)	3 (30)
Vumokuhle Primary	10	0 (0)	8 (80)	6 (60)	6 (60)	5 (50)
Maphumephethe	10	0 (0)	5 (50)	4 (40)	7 (70)	5 (50)
How long Park	10	0 (0)	8 (80)	7 (70)	4 (40)	4 (40)
Msebe Primary	10	0 (0)	8 (80)	5 (50)	5 (50)	3 (30)

CHAPTER 5: CONCLUSIONS & RECOMMENDATIONS

5.1 CONCLUSIONS

Considering the results presented in this study it can be concluded that the *E. coli*-GFP strain described here provides an easily applicable positive process internal control capable of improving the credibility of real-time PCR test assays. The positive process internal control showed no inhibition or loss of sensitivity in the APW enriched river water samples analysed with the two multiplex *V. cholerae* real-time PCR assays. This study demonstrated that the multiplex real-time PCR assays, when combined with the *E. coli*-GFP internal process control, provides a greater degree of confidence, speed, specificity and sensitivity for the detection of toxigenic and non-toxigenic strains of *V. cholerae* in APW enriched water samples.

The combination of filtration, enrichment, DNA extraction and m-PCR provided a sensitive, specific and easy method for the detection of *V. cholerae* in environmental water samples. The culture dependent PCR assays proved to be the most effective for detection and identification of *V. cholerae* compared to the culture independent real-time PCR detection assay. The inclusion of an enrichment step allowed for the detection of culturable bacteria which is crucial as PCR detection does not provide information on the viability of the detected organism.

Results from this study showed that *Vibrio cholerae* non-O1/O139 was frequently isolated from the Msunduzi, Umlazi and Isipingo rivers either in a free-living (planktonic) phase or in association with planktons, invertebrates and the sediment compartment of the rivers. A statistically significant correlation between temperature, pH, turbidity, salinity, *E. coli* numbers and the isolation of *V. cholerae* non-O1/0139 was observed.

Although non-O1/O139 *V. cholerae* strains are believed to be non-toxigenic, they have been associated with sporadic cases of gastroenteritis, septicaemia and extra-intestinal infections (Fraga et al. 2007). It has been reported that these strains encode some putative virulence factors such as NAG-ST enterotoxin, hemolysins and proteases (Bidinost et al. 2004) that have not been investigated during this study. Thus, the non-toxigenic *V. cholerae* strains detected and identified using the methods described in this study may have the ability to cause infections in sensitive population groups (e.g. immune-compromised individuals).

5.2 RECOMMENDATIONS FOR FUTURE STUDIES

- The results of this study as well as from previous studies indicate that environmental *V. cholerae* strains are represented by non-toxigenic strains. Environmental *V. cholerae* strains are well adapted to survive in the environment. One such adaptation is the ability to grow as a biofilm on a range of abiotic and biotic surfaces. Future studies should therefore focus on *V. cholerae* in the viable but

non-culturable state in both a free-living phase as well as in the form of biofilms on abiotic and biotic surfaces.

- *Vibrio cholerae* bacteriophage could transfer genes to the non-toxigenic environmental *V. cholerae* strains, producing strain with different characteristics. Therefore, future studies should also focus on the detection, identification and characterisation of*V. cholerae* bacteriophage that may by present in the Msunduzi, Umlazi and Isipingo Rivers.

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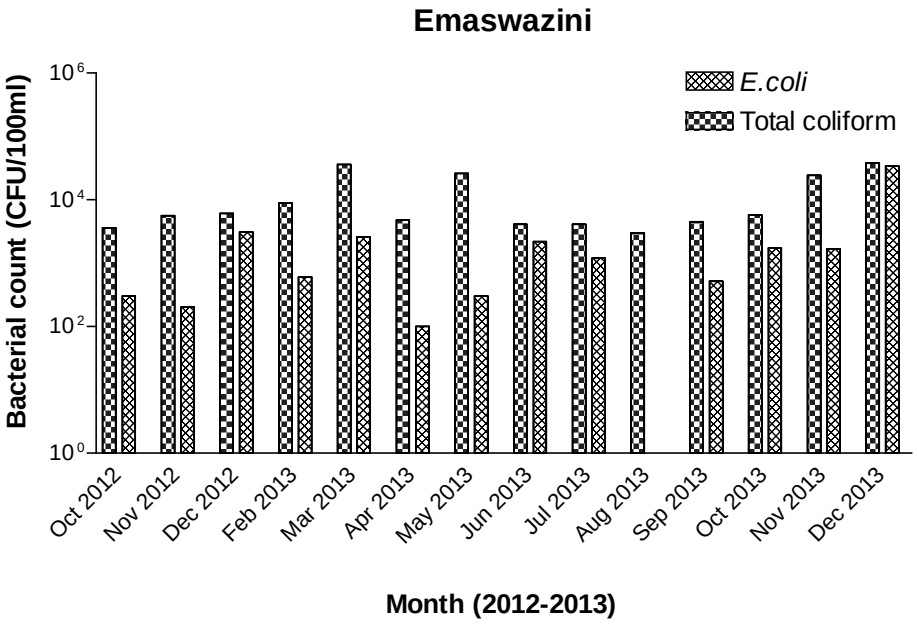
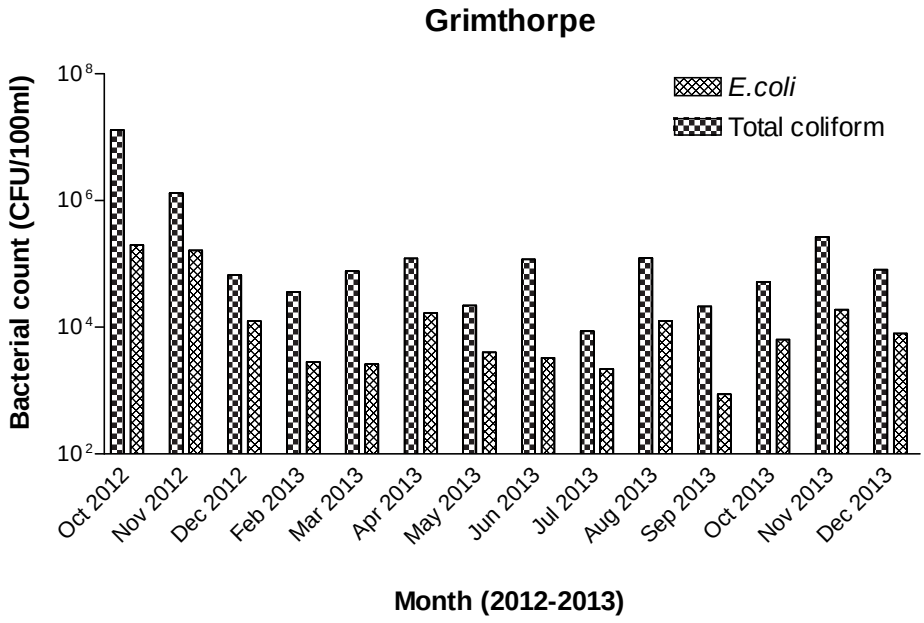
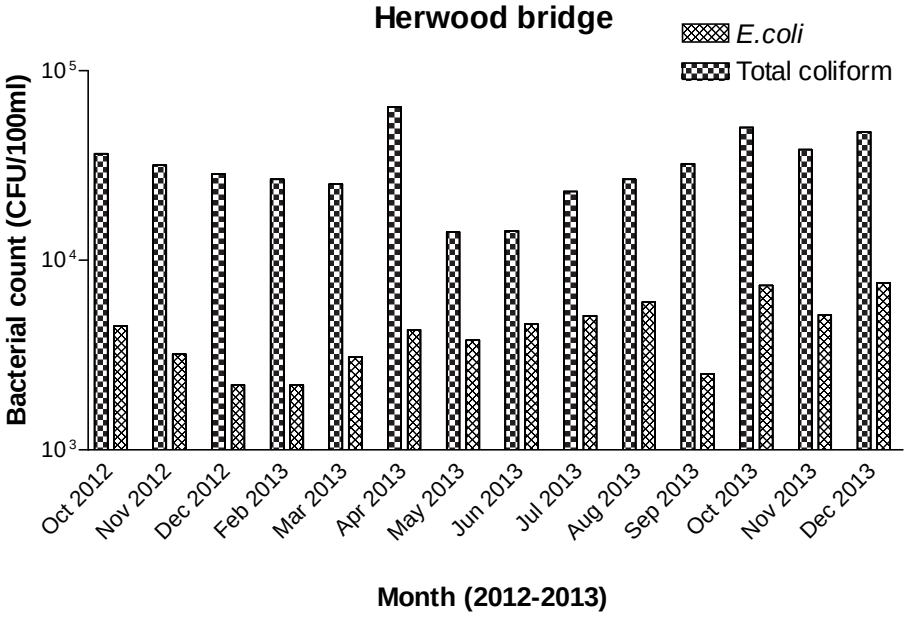
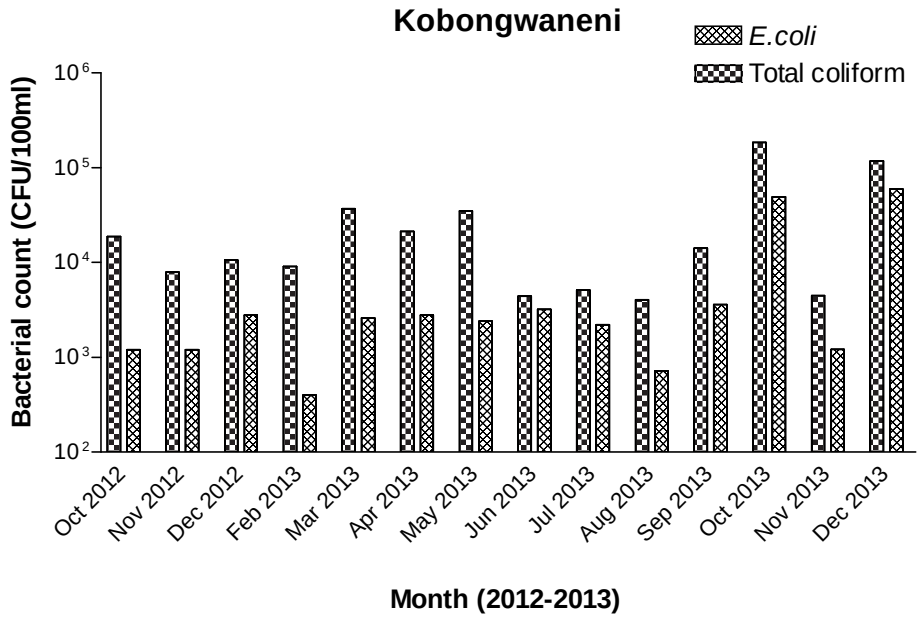
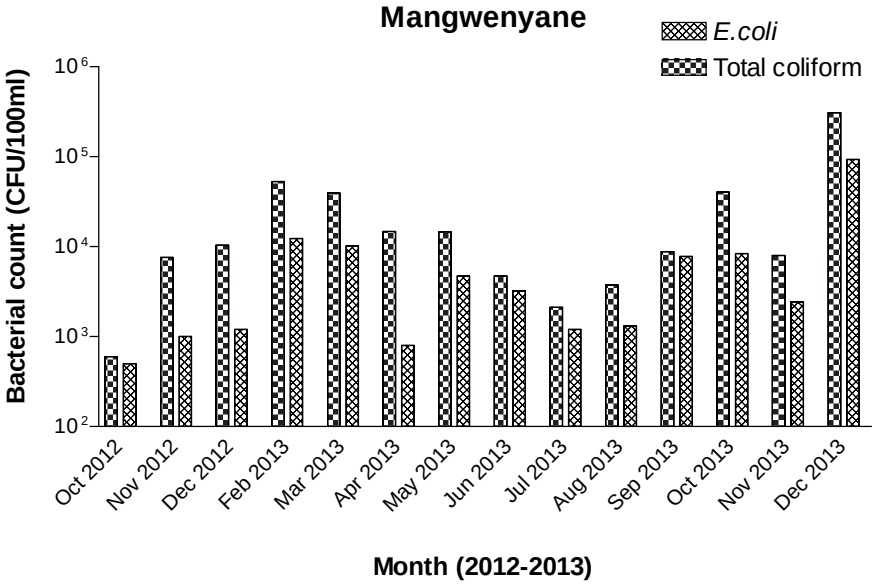
APPENDIX A: TOTAL COLIFORM AND *E. COLI* COUNTS

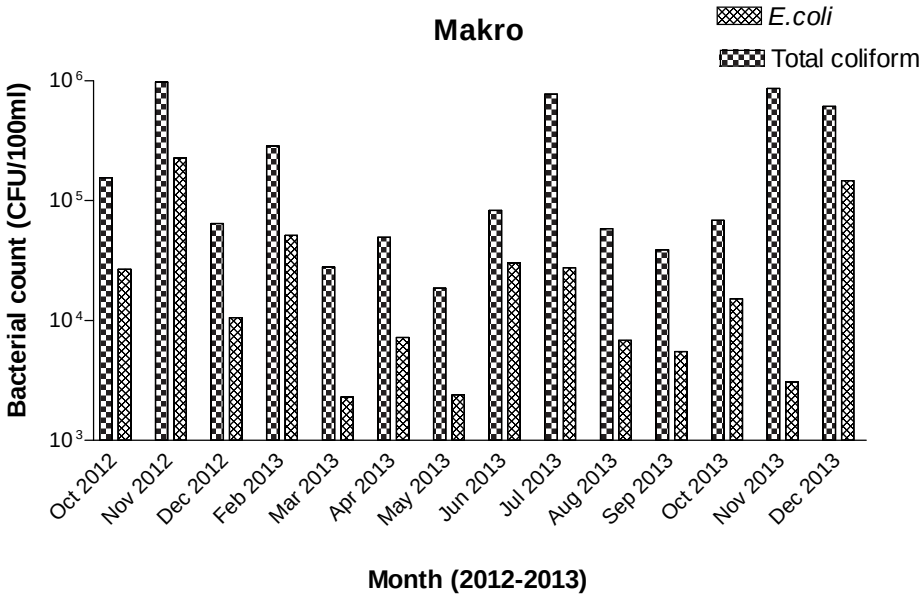


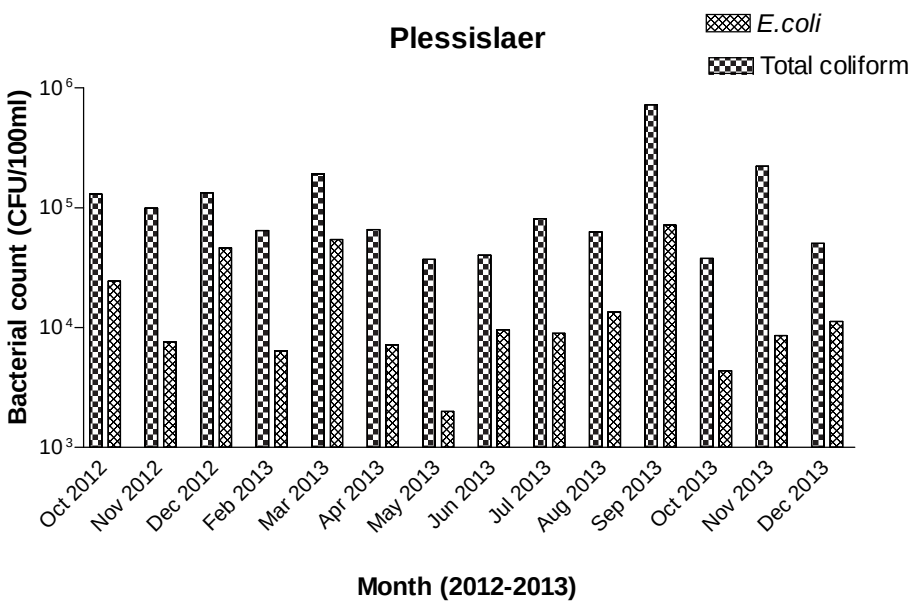


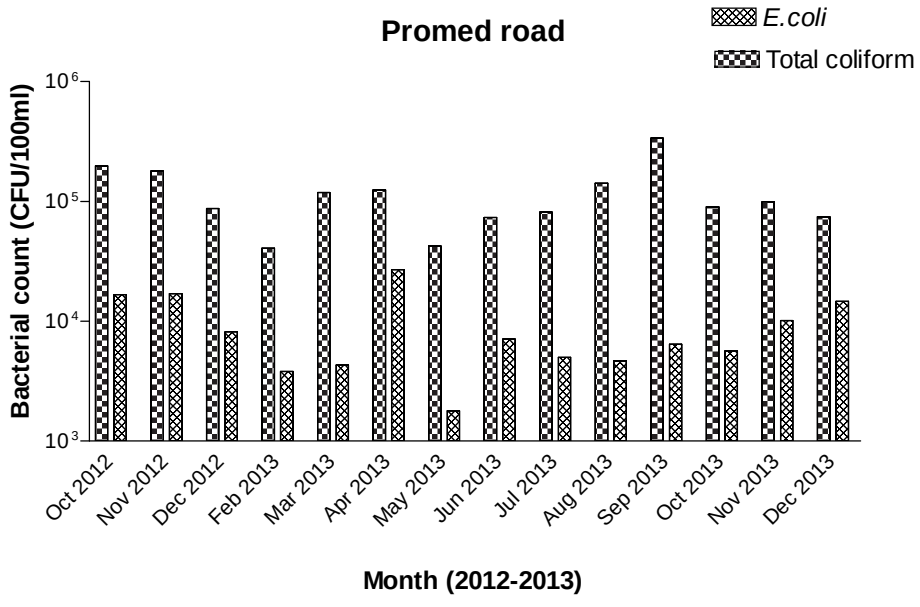

Herwood bridge
E.coli
Total coliform
Bacterial count (CFU/100ml)
10⁵
10⁴
10³
Oct 2012
Nov 2012
Dec 2012
Feb 2013
Mar 2013
Apr 2013
May 2013
Jun 2013
Jul 2013
Aug 2013
Sep 2013
Oct 2013
Nov 2013
Dec 2013
Month (2012-2013)

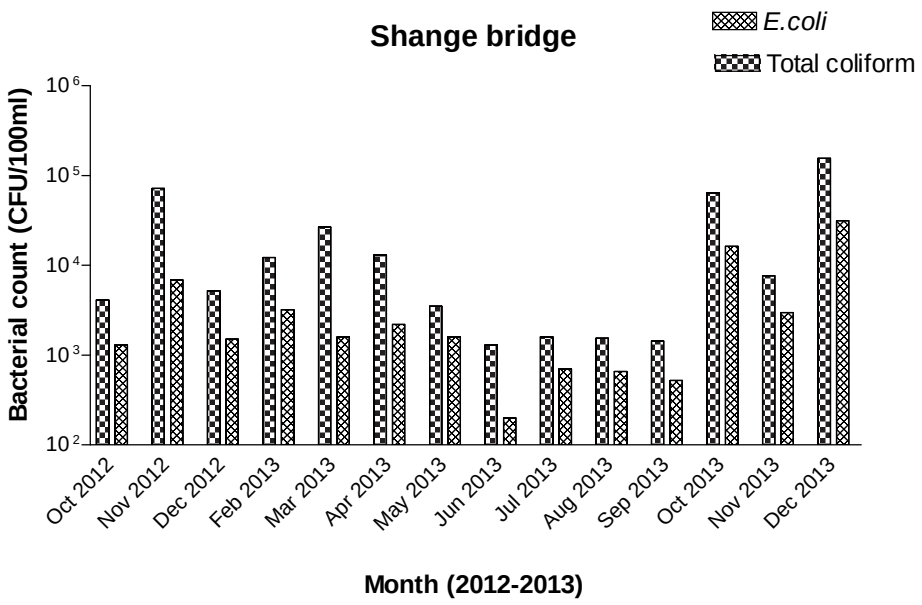

Kobongwaneni
E.coli
Total coliform
Bacterial count (CFU/100ml)
10⁶
10⁵
10⁴
10³
10²
Oct 2012
Nov 2012
Dec 2012
Feb 2013
Mar 2013
Apr 2013
May 2013
Jun 2013
Jul 2013
Aug 2013
Sep 2013
Oct 2013
Nov 2013
Dec 2013
Month (2012-2013)

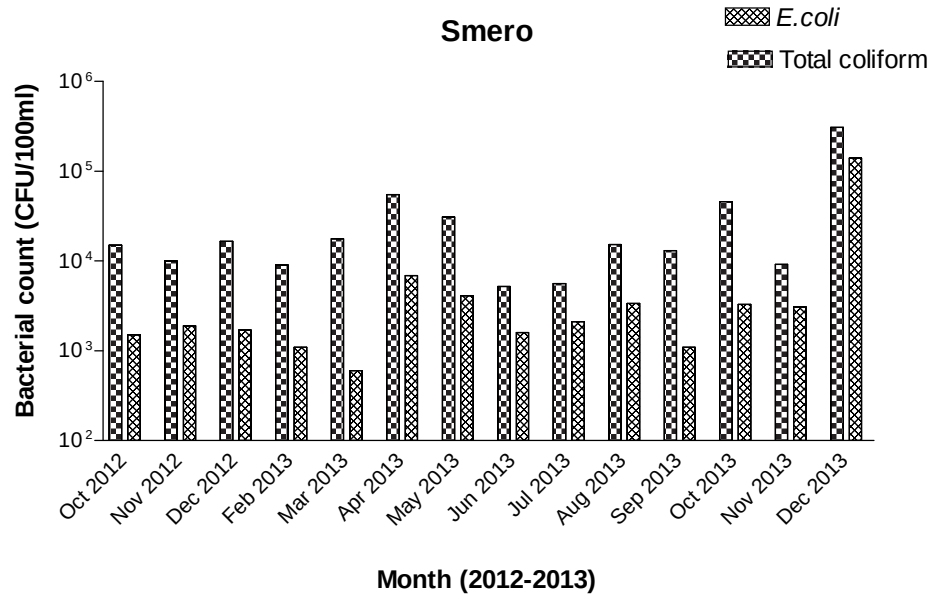

Mangwenyane
E.coli
Total coliform
Bacterial count (CFU/100ml)
10⁶
10⁵
10⁴
10³
10²
Oct 2012
Nov 2012
Dec 2012
Feb 2013
Mar 2013
Apr 2013
May 2013
Jun 2013
Jul 2013
Aug 2013
Sep 2013
Oct 2013
Nov 2013
Dec 2013
Month (2012-2013)

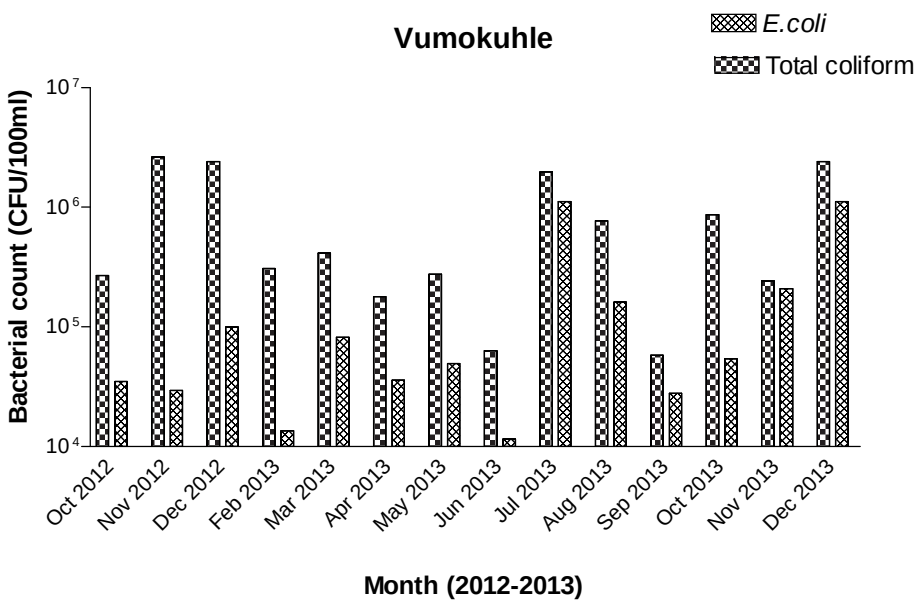

Makro
E.coli
Total coliform
Bacterial count (CFU/100ml)
10⁶
10⁵
10⁴
10³
Oct 2012
Nov 2012
Dec 2012
Feb 2013
Mar 2013
Apr 2013
May 2013
Jun 2013
Jul 2013
Aug 2013
Sep 2013
Oct 2013
Nov 2013
Dec 2013
Month (2012-2013)

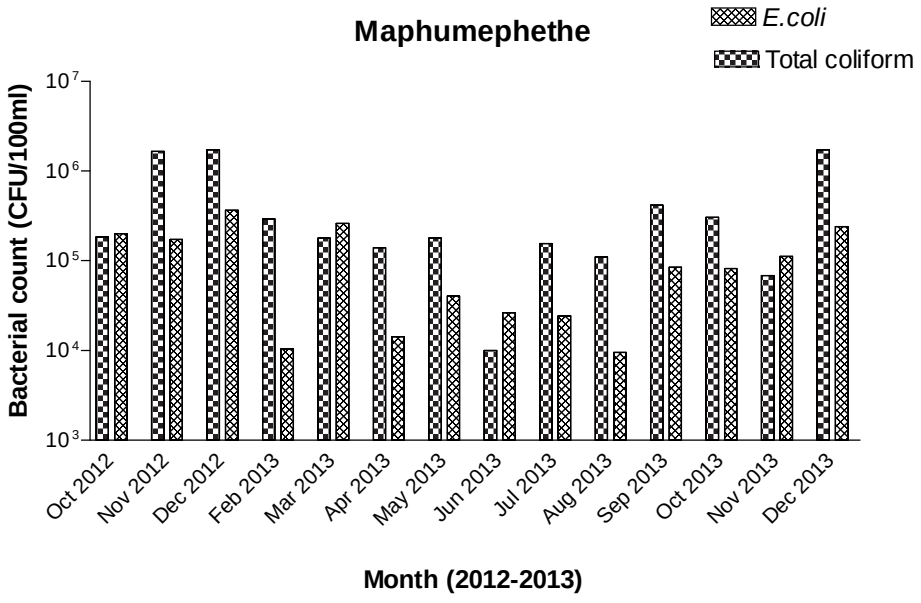

Plessislaer
E.coli
Total coliform
Bacterial count (CFU/100ml)
10^6
10^5
10^4
10^3
Oct 2012
Nov 2012
Dec 2012
Feb 2013
Mar 2013
Apr 2013
May 2013
Jun 2013
Jul 2013
Aug 2013
Sep 2013
Oct 2013
Nov 2013
Dec 2013
Month (2012-2013)

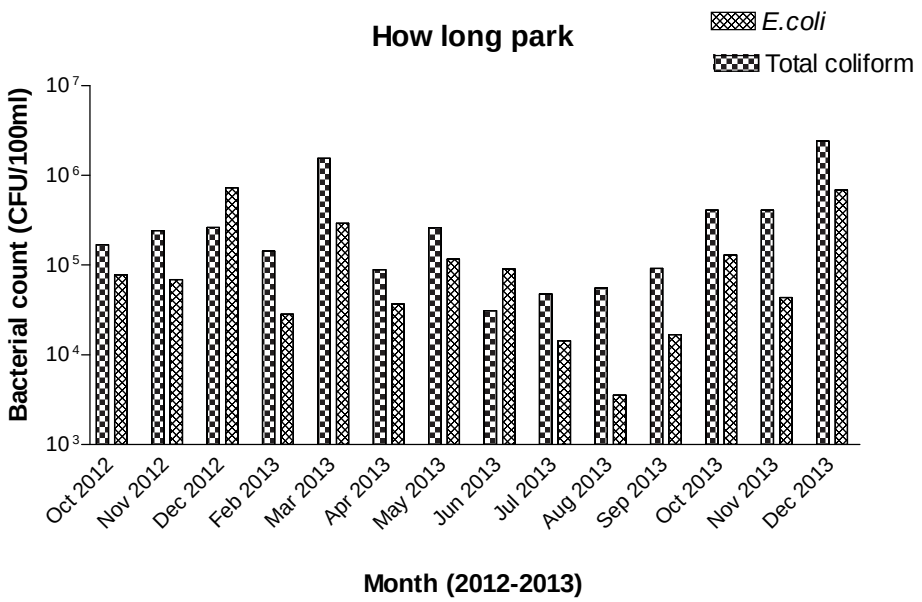

Promed road
E.coli
Total coliform
Bacterial count (CFU/100ml)
10^6
10^5
10^4
10^3
Oct 2012
Nov 2012
Dec 2012
Feb 2013
Mar 2013
Apr 2013
May 2013
Jun 2013
Jul 2013
Aug 2013
Sep 2013
Oct 2013
Nov 2013
Dec 2013
Month (2012-2013)

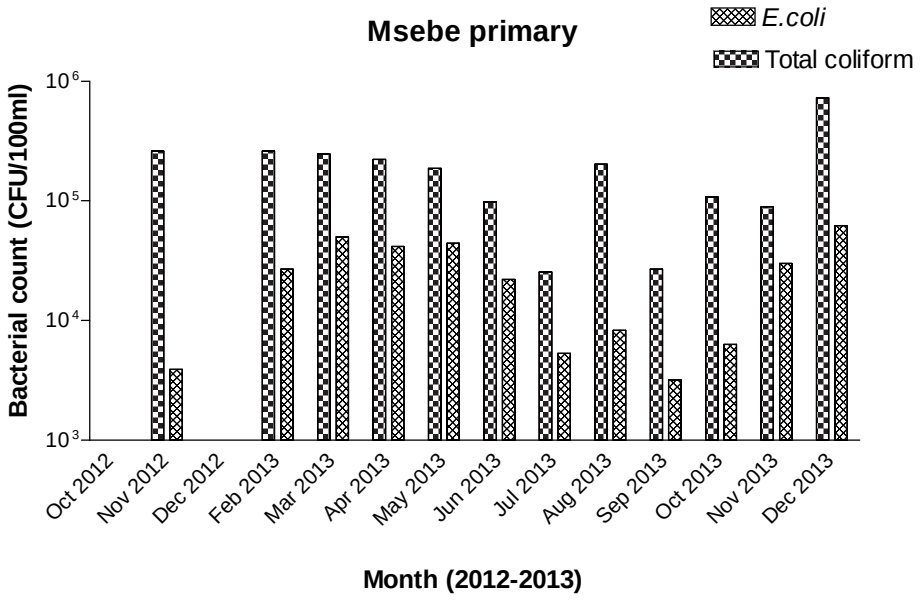

Shange bridge
E.coli
Total coliform
Bacterial count (CFU/100ml)
10⁶
10⁵
10⁴
10³
10²
Oct 2012
Nov 2012
Dec 2012
Feb 2013
Mar 2013
Apr 2013
May 2013
Jun 2013
Jul 2013
Aug 2013
Sep 2013
Oct 2013
Nov 2013
Dec 2013
Month (2012-2013)


Smero
E.coli
Total coliform
Bacterial count (CFU/100ml)
10⁶
10⁵
10⁴
10³
10²
Oct 2012
Nov 2012
Dec 2012
Feb 2013
Mar 2013
Apr 2013
May 2013
Jun 2013
Jul 2013
Aug 2013
Sep 2013
Oct 2013
Nov 2013
Dec 2013
Month (2012-2013)


Vumokuhle
E.coli
Total coliform
Bacterial count (CFU/100ml)
10^7
10^6
10^5
10^4
Oct 2012
Nov 2012
Dec 2012
Feb 2013
Mar 2013
Apr 2013
May 2013
Jun 2013
Jul 2013
Aug 2013
Sep 2013
Oct 2013
Nov 2013
Dec 2013
Month (2012-2013)


Maphumephethe
E.coli
Total coliform
Bacterial count (CFU/100ml)
10^7
10^6
10^5
10^4
10^3
Oct 2012
Nov 2012
Dec 2012
Feb 2013
Mar 2013
Apr 2013
May 2013
Jun 2013
Jul 2013
Aug 2013
Sep 2013
Oct 2013
Nov 2013
Dec 2013
Month (2012-2013)


How long park
E.coli
Total coliform
Bacterial count (CFU/100ml)
10^7
10^6
10^5
10^4
10^3
Oct 2012
Nov 2012
Dec 2012
Feb 2013
Mar 2013
Apr 2013
May 2013
Jun 2013
Jul 2013
Aug 2013
Sep 2013
Oct 2013
Nov 2013
Dec 2013
Month (2012-2013)


Msebe primary
E.coli
Total coliform
Bacterial count (CFU/100ml)
10^6
10^5
10^4
10^3
Oct 2012
Nov 2012
Dec 2012
Feb 2013
Mar 2013
Apr 2013
May 2013
Jun 2013
Jul 2013
Aug 2013
Sep 2013
Oct 2013
Nov 2013
Dec 2013
Month (2012-2013)

APPENDIX B: INDEPENDENT SAMPLE T-TEST

A) Free-floating *V. cholerae* non-O1/O139		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
pH	Equal variances assumed	2.27	0.13	-0.92	192	0.36	-0.05	0.05	-0.15	0.05
Temperature	Equal variances not assumed			-2.82	91.58	0.01	-2.01	0.71	-3.42	-0.59
Salinity	Equal variances not assumed			-4.93	157.19	0.00	-0.05	0.01	-0.07	-0.03
Turbidity	Equal variances not assumed			-0.18	190.99	0.86	-0.02	0.09	-0.18	0.15
Total coliforms	Equal variances assumed			-5.78	192	0.00	-0.63	0.11	-0.84	-0.41
E. coli	Equal variances assumed	3.083	0.08	-4.49	191	0.00	-0.52	0.12	-0.74	-0.29

B) Sediments attached *V. cholerae* non-O1/O139		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
pH	Equal variances assumed	0.00	0.95	0.56	95	0.58	0.05	0.08	-0.12	0.21
Temperature	Equal variances not assumed			-1.44	30.49	0.16	-2.06	1.43	-4.99	0.86
Salinity	Equal variances not assumed			-1.22	52.90	0.23	-0.02	0.02	-0.05	0.01
Turbidity	Equal variances assumed	1.263	0.26	-2.51	95	0.02	-0.28	0.11	-0.51	-0.06
Total coliforms	Equal variances not assumed			-1.33	30.63	0.19	-0.26	0.19	-0.65	0.14
E. coli	Equal variances assumed	0.15	0.70	-0.87	95	0.39	-0.16	0.18	-0.52	0.20

C) 20 µM net plankton attached *V. cholerae* non-O1/O139		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
pH	Equal variances assumed	0.88	0.35	1.23	68	0.22	0.14	0.11	-0.09	0.36
Temperature	Equal variances assumed	0.03	0.87	-1.9	68	0.06	-2.49	1.30	-5.09	0.11
Salinity	Equal variances not assumed			-4.44	36.89	0.00	-0.07	0.02	-0.10	-0.04
Turbidity	Equal variances not assumed			-1.34	23.46	0.19	-0.16	0.12	-0.41	0.09
Total coliforms	Equal variances assumed	0.50	0.48	-2.80	68	0.01	-0.57	0.20	-0.98	-0.16
E. coli	Equal variances assumed	3.15	0.08	-2.83	68	0.01	-0.64	0.23	-1.09	-0.19

D) 63 µM net plankton attached *V. cholerae* non-O1/O139		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
pH	Equal variances not assumed			-1.24	10.61	0.24	-0.19	0.15	-0.52	0.15
Temperature	Equal variances not assumed			-8.68	21.66	0.00	-6.72	0.77	-8.3	-5.1
Salinity	Equal variances not assumed			-2.65	18.43	0.02	-0.05	0.02	-0.09	-0.01
Turbidity	Equal variances not assumed			-4.19	34.88	0.00	-0.38	0.09	-0.56	-0.19
Total coliforms	Equal variances assumed	0.41	0.52	-4.69	68	0.00	-0.95	0.20	-1.35	-0.55
E. coli	Equal variances assumed	2.92	0.09	-2.48	68	0.02	-0.61	0.25	-1.10	-0.12

E) Macro invertebrates attached *V. cholerae* non-O1/O139		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
pH	Equal variances assumed	0.70	0.41	1.38	28	0.18	0.21	0.15	-0.10	0.52
Temperature	Equal variances not assumed			-1.01	8.96	0.34	-2.50	2.48	-8.10	3.10
Salinity	Equal variances assumed	0.16	0.691	0.36	28	0.72	0.01	0.03	-0.05	0.07
Turbidity	Equal variances assumed	0.62	0.44	-4.52	28	0.00	-0.65	0.14	-0.95	-0.36
Total coliforms	Equal variances assumed	0.05	0.83	-0.75	28	0.46	-0.21	0.28	-0.80	0.37
E. coli	Equal variances assumed	0.50	0.49	0.07	28	0.94	0.02	0.31	-0.60	0.65

APPENDIX C: LOGISTIC REGRESSION ANALYSIS

A) Free-floating *V. cholerae* non-O1/O139	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B)	
							Lower	Upper
pH	-0.90	0.61	2.18	1	0.14	0.41	0.12	1.35
Temperature	0.11	0.05	4.96	1	0.03	1.11	1.01	1.22
Salinity	7.59	3.16	5.79	1	0.02	1984.00	4.09	963114.36
Turbidity	0.18	0.29	0.37	1	0.54	1.19	0.68	2.10
E. coli	0.53	0.29	3.25	1	0.07	1.70	0.96	3.01
Constant	2.52	4.26	0.35	1	0.55	12.44		

B) Sediments attached *V. cholerae* non-O1/O139	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B)	
							Lower	Upper
pH	-0.80	0.65	1.53	1	0.21	0.47	0.13	1.60
Temperature	0.09	0.05	3.17	1	0.08	1.09	0.99	1.20
Salinity	11.47	3.60	10.18	1	0.00	96085.32	83.50	110563258.353.00
Turbidity	0.71	0.36	3.97	1	0.05	2.04	1.01	4.11
E. coli	-0.09	0.31	0.09	1	0.77	0.91	0.50	1.68
Constant	3.59	4.60	0.61	1	0.45	36.26		

C) 20 µM net plankton attached *V. cholerae* non-O1/O139	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B)	
							Lower	Upper
pH	-2.21	0.90	5.97	1	0.01	0.11	0.02	0.65
Temperature	0.14	0.07	3.57	1	0.06	1.15	1.00	1.33
Salinity	20.33	6.08	11.20	1	0.00	6.78E+08	4563.19	1.0E+14
Turbidity	0.61	0.46	1.71	1	0.19	1.83	0.74	4.53
E. coli	0.37	0.42	0.80	1	0.37	1.45	0.64	3.29
Constant	11.32	6.25	3.28	1	0.07	82522.10		

D) 63 µM net plankton attached *V. cholerae* non-O1/O139	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B)	
							Lower	Upper
pH	-0.66	0.79	0.71	1	0.40	0.52	0.11	2.40
Temperature	0.10	0.07	2.19	1	0.14	1.10	0.97	1.26
Salinity	11.81	4.67	6.38	1	0.01	1.34E+05	14.10	1.28E+09
Turbidity	0.35	0.44	0.65	1	0.42	1.42	0.60	3.37
E. coli	0.38	0.40	0.92	1	0.34	1.47	0.67	3.22
Constant	1.37	5.51	0.06	1	0.81	3.93		

E) Macro invertebrates attached *V. cholerae* non-O1/O139	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B)	
							Lower	Upper
pH	-14.02	7.74	3.28	1	0.07	0.00	0.00	3.17
Temperature	1.00	0.52	3.72	1	0.05	2.73	0.98	7.560
Salinity	50.14	27.10	3.42	1	0.06	5.98E+21	0.05	7.04E+44
Turbidity	9.36	4.48	4.37	1	0.04	11609.72	1.78	75546397.919
E. coli	-2.98	1.54	3.74	1	0.05	0.05	0.00	1.04
Constant	85.07	51.34	2.75	1	0.10	8.82E+36		

APPENDIX D: GLOBAL POSITIONING SYSTEM COORDINATES OF SAMPLING SITES

a) Msunduzi River sampling points

Sample point name	GPS coordinates	
	First Geographical coordinate	Second Geographical coordinate
Emaswazini	S29°39.395'	E030°05.963
Grimthorpe	S29°39.094'	E030°26.814
Herwood Bridge	S29°38.543'	E030°19.400
Kobongwaneni	S29°40.937'	E030°10.814
Magwenyane	S29°41.214'	E030°10.171
Makro	S29°37.323'	E030°22.613
Plessislaer	S29°38.029'	E030°20.361
Promed Road	S29°36.122'	E030°24.782
Shange Bridge	S29°39.943'	E030°13.429
Smero	S29°39.853'	E030°17.522

b) Isipingo and Umlazi sampling points

Sample point name	GPS coordinates	
	First Geographical coordinate	Second Geographical coordinate
How long park	S29°37.094'	E030°26.814
Maphumephethe	S29°58.463'	E030°52.042
Vumokuhle primary	S29°56.436'	E030°51.768
Msebe primary	S29°56.550'	E030°52.622

APPENDIX E: CAPACITY BUILDING AND CONFERENCES

A) Capacity building

Doctorate (University of Johannesburg):

Mr. Vusi Ntema
Title: *Investigating the occurrence and survival of Vibrio cholerae in surface water sources in the KwaZulu-Natal province of South Africa.*

National Diploma (University of Johannesburg):

Ms. Helen Lufuluabo-Kantu
Mr. Tshediso Radebe
Mr. Sphiwe Ntuli

B) Conference Presentation

VM Ntema and TG Barnard. Molecular detection methods for cholera in fresh, environmental and wastewater. Cholera Symposium, Earth Observation and Environmental modelling for mitigation of Health Risks, 14-16 February 2013. Mpumalanga, South Africa.

VM Ntema, TG Barnard, N Potgieter and N van Blerk. Occurrence and survival of *V. cholerae* in the Msunduzi-, Umlazi- and Isipingo rivers in the KwaZulu-Natal province of South Africa. Water and Sanitation conference, 27-30 May 2013. Polokwane, South Africa.

VM Ntema, TG Barnard, N Potgieter and N van Blerk. Occurrence and survival of *V. cholerae* in the Msunduzi-, Umlazi- and Isipingo rivers in the KwaZulu-Natal province of South Africa. 3rd Regional conference of the Southern African Young Water Professionals, 16-18 July 2013. Western Cape, South Africa.

VM Ntema, TG Barnard, N Potgieter and N van Blerk. Occurrence and survival of *V. cholerae* in the Msunduzi-, Umlazi- and Isipingo rivers in the KwaZulu-Natal province of South Africa. Water and Health Conference: Where Science Meet Policy, 14-18 October 2013. North Carolina, United States of America.