

**SURVEILLANCE OF INVASIVE *VIBRIO* SPECIES IN DISCHARGED  
AQUEOUS EFFLUENTS OF WASTEWATER TREATMENT PLANTS IN  
THE EASTERN CAPE PROVINCE OF SOUTH AFRICA**

**BY**

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**2010**

**DECLARATION**

I, the undersigned, declare that this thesis submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Microbiology in the Faculty of Science and Agriculture, School of Biological and Environmental Sciences, and the work contained herein is my original work with exemption to the citations and that this work has not been submitted at any other University in partial or entirely for the award of any degree.

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#### COPYRIGHT

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## ***Dedication***

*I dedicate this work to my beloved parents, Professor Igho Benjamin Igbiosa and Mrs. Eunice Oghomwen Igbiosa “Thanks for guiding me through the dark paths towards knowledge”.*

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## **GENERAL ABSTRACT**

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## General Abstract

*Vibrio* infections remain a serious threat to public health. In the last decade, *Vibrio* disease outbreaks have created a painful awareness of the personal, economic, societal, and public health costs associated with the impact of contaminated water in the aquatic milieu. This study was therefore designed to assess the prevalence of *Vibrio* pathogens in the final effluents of wastewater treatment plants (WWTPs) in the Eastern Cape Province, as well as their abilities to survive the treatment processes of the activated sludge system either as free cells or as plankton-associated entities in relation to the physicochemical qualities of the effluents.

Three wastewater treatment facilities were selected to represent typical urban, sub-urban and rural communities, and samples were collected monthly from August 2007 to July 2008 from the final effluent, discharge point, 500 meter upstream and downstream of the discharge points and analysed for physicochemical parameters, *Vibrio* pathogens prevalence and their antibiogram characteristics using both culture based and molecular techniques.

Physicochemical parameters measured include pH, temperature, electrical conductivity, salinity, turbidity, total dissolved solid (TDS), dissolved oxygen (DO), chemical oxygen demand (COD), nitrate, nitrite and orthophosphate levels. Unacceptably high levels of the assayed parameters were observed in many cases for COD (<10 - 1180 mg/l), nitrate (0.08 - 13.14 mg NO<sub>3</sub><sup>-</sup> as N/l), nitrite (0.06 - 6.78 mg NO<sub>2</sub><sup>-</sup> as N/l), orthophosphate (0.07-4.81 mg PO<sub>4</sub><sup>3-</sup> as P/l), DO (1.24 - 11.22 mg/l) and turbidity (2.04 -159.06 NTU). Temperature, COD and nitrite varied significantly with season ( $P < 0.05$ ), while pH, EC, salinity, TDS, COD, and nitrate all varied significantly with sampling site ( $P < 0.01$ ;  $P < 0.05$ ).

In the rural wastewater treatment facility, free-living *Vibrio* densities varied from 0 to  $3.45 \times 10^1$  cfu ml<sup>-1</sup>, while the plankton-associated *Vibrio* densities vary with plankton sizes as follows: 180 µm (0 –  $4.50 \times 10^3$  cfu ml<sup>-1</sup>); 60 µm (0 –  $4.86 \times 10^3$  cfu ml<sup>-1</sup>); 20 µm (0 –  $1.9 \times 10^5$

cfu ml<sup>-1</sup>). The seasonal variations in the *Vibrio* densities in the 180 and 60 µm plankton size samples were significant ( $P < 0.05$ ), while the 20 µm plankton size and free-living vibrios densities were not. Molecular confirmation of the presumptive vibrios isolates revealed *V. fluvialis* (36.5 %), as the predominant species, followed by *V. vulnificus* (34.6 %), and *V. parahaemolyticus* (23.1%), and *V. metschnikovii* (5.8 %) (detected using only API 20 NE), suggesting high incidence of pathogenic *Vibrio* species in the final effluent of the wastewater facility. Correlation analysis suggested that the concentration of *Vibrio* species correlated negatively with salinity and temperature ( $P < 0.001$  and  $P < 0.002$  respectively) as well as with pH and turbidity ( $P < 0.001$ ), in the final effluent.

Population density of total *Vibrio* ranged from  $2.1 \times 10^1$  to  $4.36 \times 10^4$  cfu ml<sup>-1</sup> and from  $2.80 \times 10^1$  to  $1.80 \times 10^5$  cfu ml<sup>-1</sup> for the sub-urban and urban communities treatment facilities respectively. *Vibrio* species associated with 180 µm, 60 µm, and 20 µm plankton sizes, were observed at densities of 0 -  $1.36 \times 10^3$  cfu ml<sup>-1</sup>, 0 -  $8.40 \times 10^2$  cfu ml<sup>-1</sup> and 0 -  $6.80 \times 10^2$  cfu ml<sup>-1</sup> respectively at the sub-urban community's WWTP. In the urban community, counts of culturable vibrios ranged from 0 -  $2.80 \times 10^2$  cfu ml<sup>-1</sup> (180 µm); 0 -  $6.60 \times 10^2$  cfu ml<sup>-1</sup> (60 µm) and 0 -  $1.80 \times 10^3$  cfu ml<sup>-1</sup> (20 µm). Abundance of free-living *Vibrio* species varied between 0 and the orders of  $10^2$  and  $10^3$  cfu ml<sup>-1</sup> in the sub-urban and urban communities WWTPs respectively. Molecular confirmation of the presumptive vibrios isolates revealed the presence of *V. fluvialis* (41.38 %), *V. vulnificus* (34.48 %), and *V. parahaemolyticus* (24.14%) in the sub-urban community effluents. In the urban community *V. fluvialis* (40 %), *V. vulnificus* (36 %), and *V. parahaemolyticus* (24%) were detected. There was no significant correlation between *Vibrio* abundance and season, either as free-living or plankton-associated entities, while *Vibrio* species abundance correlated positively with temperature ( $r = 0.565$ ;  $P < 0.01$ ), salinity and dissolved oxygen ( $P < 0.05$ ). Turbidity and pH showed significant seasonal variation ( $P < 0.05$ ) in both locations.

The *Vibrio* strains showed the typical multi-antibiotic-resistance of an SXT element. They were resistant to sulfamethoxazole (Sul), trimethoprim (Tmp), cotrimoxazole (Cot), chloramphenicol (Chl) and streptomycin (Str), as well as other antibiotics such as ampicillin (Amp), penicillin (Pen), erythromycin (Ery), tetracycline (Tet), nalidixic acid (Nal), and gentamicin (Gen). The antibiotic resistance genes detected includes *dfr18* and *dfrA1* for trimethoprim; *tetA*, *strB*, *floR*, *sul2* *blaP1*, for tetracycline, streptomycin, chloramphenicol, sulfamethoxazole and  $\beta$ -lactams respectively. A number of these genes were only recently described from clinical isolates, demonstrating genetic exchange between clinical and environmental *Vibrio* species.

This study revealed that there was an adverse impact on the physicochemical characteristics of the receiving watershed as a result of the discharge of inadequately treated effluents from the wastewater treatment facilities. The occurrence of *Vibrio* species as plankton-associated entities confirms the role of plankton as potential reservoir for this pathogen. Also the treated final effluents are reservoirs of various antibiotics resistance genes. This could pose significant health and environmental risk to the biotic component of the environment including communities that rely on the receiving water for domestic purposes and may also affect the health status of the aquatic milieu in the receiving water. There is need for consistent monitoring programme by appropriate regulatory agencies to ensure compliance of the wastewater treatment facilities to regulatory effluent quality standards.

# **CHAPTER 1**

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## **GENERAL INTRODUCTION**

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# CHAPTER 1

## GENERAL INTRODUCTION

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## GENERAL INTRODUCTION

The fraction of treated sewage being discharged into rivers in developing countries has increased over time, and this has led to the deterioration of the qualities of major rivers in such nations including South African. Of the treated sewage discharges, few often meet the discharge standard (Arceivala, 1997; Igbinosa and Okoh, 2009), thus contributing to increased densities of disease causing bacteria in the receiving waterbodies and subsequently in increasing incidences of emerging pathogens. As these waterbodies are the major sources of potable water, their contamination has led to many water related disease outbreaks in the past (Mishra *et al.*, 2004; Nair *et al.*, 2004; Obi *et al.*, 2004). These water sources are used for bathing, and for other domestic activities, and water related rituals for cultural and religious purposes by millions of people, thus increasing the risks of the public to be exposed to biological hazards from sub-quality wastewater effluents. The existing international standards for biological qualities of treated sewage are applicable for beneficial uses like agriculture, bathing etc. However, in developing countries like South Africa, rivers play a pivotal role in the life of the people for social, cultural and religious reasons, and for these reasons, such dependent populace are directly exposed to prevailing poor quality of river water thus necessitating the need for the regulation of biological quality of both wastewater effluents and the receiving waterbodies.

Due to urbanization and population growth, the problem of water shortage is becoming more serious, especially in arid and semi-arid regions. Also, the rapid increases in urbanization and population result in the generation of more wastewater. Wastewater contains a wide range of pathogens and sometimes heavy metals and organic compounds that are hazardous to human health and the environment. Hence, the discharge of inadequately treated wastewater into the environment degrades not only the surrounding area but also the water quality.

Poor sanitation and lack of drinking water of high quality is estimated to be the main global cause of approximately 4,000 deaths per day (Bartram *et al.*, 2005). To be able to fulfill the millennium development goals, water and sanitation are of highest international priority. The global perspective on water and sanitation is very different from the South African situation with abundance of clean water and possibilities of surface water discharge. However, water shortage may temporarily occur as well as infections due to contaminated water.

In South Africa and other developed areas of the world, wastewater from cities is most often treated at municipal treatment plants. All collected water – stormwater from roofs, streets and other hard surfaces, blackwater from toilets, greywater from kitchen sinks, bathrooms and washing facilities and industrial wastewater; are transported to the treatment plant. Potentially more than 100 different types of pathogenic viruses, bacteria and parasites that may cause an immense range of diseases and clinical symptoms in humans such as diarrhoea, meningitis, myocarditis and hepatitis, can be present in faecally-polluted water (Pegram *et al.*, 1998; Obi *et al.*, 2002). Diseases can be transmitted to humans via water due to activities such as swimming or drinking contaminated water, and from food produced using contaminated water for irrigation. Run-off from agriculture land could also reintroduce pathogens from sludges and manures onto surface waters, etc. The circulation of pathogens in the society is to a large extent dependent on the water and wastewater systems that we build. How the systems are designed could also have direct bearings on the environmental transmission of pathogens.

In spite of the potential for domestic wastewater to contaminate the receiving milieu, approximately 90% of sewage in cities in developing countries are discharged untreated into waterbodies (Senzia *et al.*, 2003). According to Mara and Feachem (2001), deficiencies in water supply, sanitation and hygiene are, global, second only to malnutrition as the principal cause of death and disability-adjusted years lost. They went on to claim that in 2000, 18% of the world

population was without sufficient water supplies and about 40% was without adequate sanitation. Whilst figures indicate that an impressive number of people are receiving improved water supply and sanitation, due to population growth, the overall statistics are actually weakening. The situation in Africa is even worse than indicated by these global figures where morbidity and mortality, due to lack of adequate safe water supplies and sanitation, are still very high. Water related diseases kill an estimated 3 million Africans annually (Rose, 2002). At the turn of the 20<sup>th</sup> century it was estimated that around 21 million South Africans did not have adequate sanitation and approximately 45% of the rural population did not have an acceptable water supply (Rose, 2002). The South African Department of Water Affairs and Forestry (DWAF) recognize this in their management policy, where they state that improved access to water is necessary to “increase levels of health and general well being” (DWAF, 2002). They emphasize in their water quality management goal that while water resources need to be scientifically managed and conserved, they also need to meet the social and economic requirements of the country (DWAF, 2002). The South African Water Act of 1998 supports this goal by stipulating that future water resource developments should be environmentally sustainable and that a component of the natural flow of rivers should be reserved to ensure some level of ecological functioning (Hughes and Hannart, 2003). This challenge is exacerbated by the arid climate in South Africa. South Africa receives only half the world’s average rainfall and, due to high evaporation rates, only 8% of this rainfall is carried in the rivers, compared to the world mean of 31% (van Zyl, 2003). The combined effect of climate, rapid population growth and inefficient water infrastructure is increasing the pressure on the river ecosystems across the whole of Southern Africa.

This threat to natural water supplies is also manifest in the Eastern Cape, where the State of South Africa Population Report (2000) notes that only about 34% of households have access to sewage treatment facilities. The provision of sewage treatment facilities does not in itself

ensure satisfactory effluent water quality. In a study carry out by Mohale (2003), it was found that of the 190 treatment works listed in the Eastern Cape, only 98 (51.6%) were monitored by DWAF between 2002 and 2003. Of those that were monitored only 12% met all the set discharge limits. Of particular concern, is the high level of indicator organisms measured in some of the effluents. Some of the 'treated' effluents in this area showed faecal coliform counts of over  $10^4$  per 100ml, some 100 times the discharge limit (Antrobus, 2003). Since around 1982 cholera has been spreading through South Africa from its origin in KwaZulu-Natal and has recently become endemic in the Eastern Cape (Rural Development Services Network, RDSN, 2003). The RDSN (2003) attributes this largely to the discharge of untreated or partially treated sewage.

The attendant consequences of the impact of such inadequately treated wastewater effluents are the compromising of the primary health of people especially with death threatening diarrhoeal disease (Bourne and Coetzee, 1996), caused by invasive strains of *Vibrio* species especially in the age group 1-5 years (Mackintosh and Colvin, 2003), and immunocompromised individuals resulting in tens of thousands of deaths annually (Pegram *et al.*, 1998).

Among the pathogen distributed in water sources, enteric pathogens are the ones most frequently encountered. Enteric pathogens such as vibrios are usually transmitted to humans by ingestion of contaminated water and foods. This enteric bacterial pathogen is incriminated in cases of diarrhoea, which accounts for a substantial degree of morbidity and mortality in different age groups world wide (Obi *et al.*, 1998; 2004). The World Health Organization (WHO) estimated that about 1.1 billion people globally drink unsafe water and the vast majority of diarrhoea diseases in the world (88%) are attributed to unsafe water, sanitation and hygiene. Approximately 3.1% of annual deaths (1.7 million) and 3.7% of the annual health burden world-wide (54.2 million) are attributable to unsafe water, sanitation and hygiene (WHO, 2003).

*Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio fluvialis* are serious human pathogens (CDC, 1999; Finkelstein, 2002; Kothary *et al.*, 2003; Chakraborty *et al.*, 2006) mainly transmitted via water and food. They cause diarrhoea, but in ways that are entirely different. *V. vulnificus* and *V. parahaemolyticus* are invasive organisms affecting primarily the colon; *V. cholerae* is non-invasive, affecting the small intestine through secretion of an enterotoxin (Todar, 2005) and is the etiologic agent of cholera. The clinical symptoms of *V. fluvialis* gastroenteritis are similar to cholera with the additional manifestation of bloody stools which is suggestive of an invasive pathogen (Oliver and Kaper, 2001). Other *vibrios*, e.g., *Grimontia hollisae*, *V. alginolyticus*, *V. cincinnatiensis*, *V. fluvialis*, *V. furnisii*, *V. harveyi*, *V. metschnikovii*, and *V. mimicus*, have been sporadically found in human infections (Farmer and Hickman-Brenner, 1992; Abbott and Janda, 1994; Carnahan *et al.*, 1994). Large numbers of *Vibrio* ( $4.3 \times 10^6/\text{mm}^2$ ) attached to the external surface of plankton (zooplankton and phytoplankton) have been reported (Heidelberg *et al.*, 2002), thus suggesting a close partnership between these bacteria and planktons.

Studies have provided information about the health implications of pathogens living in association with planktons. Maugeri *et al.* (2004) establish evidence that the colonization of zooplankton by organism capable of causing human disease is a widespread fact. The survey assessed the occurrence of species of *Campylobacter*, *Vibrio* and other genera in Italy's coastal waters, together with comparison of free-living bacteria and those associated with zooplankton and plankton-bound organism with selected pathogen. The result revealed that not only *Vibrio* and *Aeromonas* spp. but also *Escherichia coli*, *Enterococci* spp., *Campylobacter* spp. and *Arcobacter* spp.; were found linked with zooplankton. It also shows that potentially pathogenic organisms living in close association with zooplankton have considerable epidemiological and ecological implications. The biofilm formation by *Vibrio* spp. on the exoskeletons of these

planktons and other marine organisms may in fact constitute a strategy to survive during starvation and/or other environmental stresses (Wai *et al.*, 1999 and Lipp *et al.*, 2002). In biofilms these bacteria can use trapped and absorbed nutrients, resist antibiotics, and establish favorable partnerships with other bacteria or hosts.

In South Africa, cholera has a seasonal pattern that appears to follow the ambient local patterns of rainfall and temperature. El Tor cholera was first detected in South Africa in 1974, when it was brought to the country by migrant mine workers. The outbreak remained confined to a small number of mines, but subsequent outbreaks have been more serious. In October 1980, the first case was detected in the KaNqwane area of the Eastern Transvaal, possibly from the same cause, and the ensuing epidemic continued until 1987. How the disease survives during inter-epidemic periods remains a point of speculation. About a decade ago South Africa experienced its worst cholera outbreak in twenty years with the first cases of the current epidemic appearing in August 2000. As of March 2000, almost 46,000 people had been infected, with more than 100 deaths. The disease has spread to all but one of the South Africa's nine provinces (Lee, 2001a).

Mackintosh *et al.* (2003) reported that drinking-water quality across South Africa is poor, partly due to failure of Water Services Authority to comply with compulsory national standards for portable water quality. That report also reveals that of all the local municipalities surveyed across the country, municipalities in the Eastern Cape Province recorded the lowest compliance levels (20%). It is no surprise therefore that frequent outbreaks of waterborne diseases are reported in South Africa almost on weekly basis (Simpson and Charles, 2000; DPLG, 2001; Mail and Guardian, 2005). The alarming state of water services in the Eastern Cape was further reaffirmed when a State media outfit (SABC 3) reported in one of her documentary programmes (Special Assignment) on the 27<sup>th</sup> of May, 2008, the death of 153 children in Ukhahlamba

municipal district (alone) between January and May, 2008, due to waterborne infections. More alarming is the fact that most of these children died after consuming supposedly treated waters.

The root cause of this outbreak was the local government authority's ceasing to provide free water to local residents. The new policy is a result of ongoing pressures on the government to reduce public spending as part of wider macroeconomic reforms. The policy includes introducing a charge to very poor people living in a squatter settlement near the town of Empangeni. The settlement is not unlike many others in South Africa and other African countries that form migrating populations seeking employment. Often times these informal settlements are located near local rivers used for both drinking water and sanitation. In South Africa about 80% of poor (largely black) people have no running water and higher proportion have no toilet (Sidley, 2001). With reduced access to clean water, and increased use of open river water, the cholera epidemic has spread rapidly.

Polluted water not only has the potential to cause human suffering, but also result in economic loss. An assessment of the cost of water-related enteric illness in developing regions, based on Indian condition estimated an average cost of approximately USD1700 per 100 people per annum, with associated estimate of approximately 1500 days lost per 100 people per annum (Verma and Srivastava, 1990). This implies that a very strong association exists between microbial contamination of water and human health risk, especially in communities like the Eastern Cape Province with a rather high HIV/AIDS statistic and poverty level. Hence, the need to ensure protection of the existing waterbodies in the province against contamination by *Vibrio* pathogens from wastewater treatment plants effluents becomes imperative.

The continuing challenges of *Vibrio* infections in the early twenty-first century are integrally linked to the nature of the global changes around us. Processes of globalisation, and how these processes are affecting the broader determinants of health including our social and

natural environments, are core factors in the way cholera-like infections have changed. Global technological, economic, political and environmental changes are invariably intertwined and cannot be separated from epidemiology of the disease. This raise clear implication on how we think about infectious disease control amidst globalisation, and the actions needed to put the protection of public health more central at the heart of global governance (Lee, 2001b).

This study is therefore designed to assess the prevalence of *Vibrio* pathogens in the final effluents of wastewater treatment plants in the Eastern Cape Province, as well as their abilities to survive the treatment processes of the activated sludge system either as free cells or as plankton-associated entities in relation to the physicochemical qualities of the effluents. The specific objectives of this study include:

- (1) To carry out an in-depth review of literature on *Vibrio* pathogens and their implication in public health.
- (2) To assess the impact of discharged wastewater effluents on the physicochemical qualities of receiving watershed in a representative community in the Eastern Cape Province.
- (3) To assess the prevalence and distribution of *Vibrio* pathogens in the final effluents of the wastewater treatment plants and the receiving watershed in the Eastern Cape Province, both as free living and as plankton-associated entities.
- (4) To assess the effect of season on the prevalence and distribution of *Vibrio* pathogens in the final effluents and the receiving watersheds.
- (5) To elucidate the antibiotic susceptibility profiles of the *Vibrio* pathogens as well as the distribution of relevant antibiotic resistance genes amongst the *Vibrio* pathogens.
- (6) Compare data on *Vibrio* pathogens obtained from typical urban, sub-urban and rural communities, and in relation to the physicochemical qualities of the effluents.

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## CHAPTER 2

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### **Emerging *Vibrio* species: an unending threat to public health in developing countries**

*(Published in Research in Microbiology)*

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## CHAPTER 2

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## CHAPTER 2

### **Emerging *Vibrio* species: an unending threat to public health in developing countries**

#### **Abstract**

Discharge of inadequately treated sewage effluents into the environment in developing countries have increased over the years leading to the deterioration of water qualities of major watersheds in developing nations and consequently in increased incidences of emerging pathogens such as *vibrio* species, the prevalence of which has been generally underestimated in developing nations. This review underscores the need for a proactive approach against risk factors of emerging *Vibrio* infections pursuant to establishing adequate prevention measures.

**Key words:** Wastewater; Receiving watershed; Public health; *Vibrio* species; Emergence

## 2.1 Introduction

In developing countries, the fraction of treated sewage being discharged in rivers has increased over time, and this has led to the deterioration of water quality of major rivers in developing nations like South Africa. Of the treated sewage discharged, few meet the discharge standard [1], and this has resulted in the high density of disease causing bacteria in the receiving waterbodies and subsequently in increasing incidences of emerging pathogens. As these waterbodies are the major sources of potable water, their contamination has led to many water related outbreaks in the past [56, 60]. These water sources are used for bathing and for, other domestic activities, and water related rituals for cultural and religious reasons by millions of people, thus increasing the risks of the public to exposure to biological hazards. The existing international standards for biological qualities of treated sewage are applicable for beneficial uses like agriculture, bathing etc. However, in developing countries like South Africa, rivers play a pivotal role in the life of the people for social, cultural and religious reasons. People at large are directly exposed to prevailing poor quality of river water which has necessitated the regulation of biological quality of both effluents and the receiving waterbodies.

Previous research has focused on cholera vibrios because of the severity of the disease they produce but over the last decade several studies have involved relatively minor *Vibrio* species of medical interest, some of which are described as emerging pathogen able to cause mild to severe human diseases. The term ‘emerging pathogen’ has been defined in various literatures as a microorganism whose ability to cause disease has only recently been identified [84], while Sharma et al [77] defined an emerging pathogen as any new re-emerging, or drug resistant infection whose incidence in humans has increased within the past two decades or whose incidence threatens to increase in the future. Emerging diseases are commonly associated with many specific factors such as ecological modifications, microbial adaptation, human

demographic changes and behavior, travel and commerce, technology and industry, breakdown in public health measures, and finally, the increasing number of susceptible people.

*Vibrio* consists of Gram-negative straight or curved rods, motile by means of a single polar flagellum. Vibrios are capable of both respiratory and fermentative metabolism. Oxygen is a universal electron acceptor; they do not denitrify. Vibrios are one of the most common organisms in surface waters of the world. They occur in both marine and freshwater habitats and in associations with aquatic animals [87].

Vibrios are widespread in the marine and estuarine environments and a few pathogenic species are known to be commonly associated with outbreaks of *Vibrio* infections due to the consumption of foods and water contaminated with human faeces or sewage, raw fish and seafood, or they are associated with the exposure of skin lesion, such as cuts, open wound and abrasions to aquatic environments and marine animals [50].

The genus *Vibrio* includes more than 60 species, mostly marine in origin, and its taxonomy is continuously updated due to the addition of new species. A number of *Vibrio* species, other than *V. cholerae*, may cause disease in man mainly by the ingestion of contaminated water. *V. parahaemolyticus*, *V. mimicus* and *V. vulnificus* are food poisoning bacteria which are normal inhabitants of estuarine and marine environments and are frequently isolated from seawater and seafood. *V. parahaemolyticus* and *V. vulnificus* is an invasive organism affecting primarily the colon; *V. cholerae* is noninvasive, affecting the small intestine through secretion of an enterotoxin. *Vibrio vulnificus* is an emerging pathogen of humans. This organism causes wound infections, gastroenteritis, or a syndrome known as "primary septicemia." [87]. Among halophilic vibrios, *V. alginolyticus*, *V. fluvialis* and *V. metschnikovii* are also pathogenic for humans, while *V. anguillarum* represents a pathogen for fishes and other marine animals [23].

The objective of the review is the evaluation of the risk of emerging *Vibrio* infections due to inappropriate wastewater treatment in developing countries, in order to develop adequate prevention measures.

## **2.2 *Vibrio* species**

The genus *Vibrio* is Gram-negative straight or curved rod-shaped bacteria, which are 1.4-2.6 µm wide, and motile by means of single polar flagellum in liquid media. They do not form endospores or microcysts and are facultative anaerobes capable of both fermentative and respiratory metabolism. Most vibrios are oxidase-positive (the only exception is *V. metschnikovii*). All of them utilize D-glucose as a sole or main source of carbon and energy [23]. Ammonium salts generally represent their source of nitrogen. Vibrios produce many extracellular enzymes including gelatinase, amylase, chitinase and Dnase. The main pathogens, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* decarboxylate lysine and ornithine, with no alkaline reaction from arginine. Most *vibrios* are sensitive to the vibrostatic agent 0129 (2, 4-diamino-6, 7-di-isopropylpteridine) which is used as diagnostic test [67].

*Vibrio* species are mainly halophilic and sodium ions stimulate the growth of all species and are an absolute requirement for most. The wide range of salt concentrations necessary for bacterial growth reflects the different ability of the species to live in aquatic environments with various salinities [84]. Vibrios grow well at neutral and alkaline pH values up to pH 9.0, therefore the pH values of both selective and enrichment media are generally 8-8.8 and all species are acid sensitive.

The genus *Vibrio* of the family Vibrionaceae has undergone numerous changes in recent years, with a number of less common species described or reclassified. It contains more than 60 species and its taxonomy is being continuously updated as new species are being detected by

molecular taxonomic techniques. Currently there are twelve pathogenic *Vibrio* species implicated in human infection [80]. Several species are also pathogenic to marine vertebrates and invertebrates [17, 55] and the species have significant diagnostic test (Table 2.1) that have been confirmed by molecular techniques [67]. Most *Vibrios* have relatively simple growth factor requirements and will grow in synthetic media with glucose as a sole source of carbon and energy. However, since *vibrios* are typically marine organisms, most species require 2-3% NaCl or a sea water base for optimal growth. *Vibrios* vary in their nutritional versatility, but some species will grow on more than 150 different organic compounds as carbon and energy sources, occupying the same level of metabolic versatility as *Pseudomonas* [55]. In liquid media *Vibrios* are motile by polar flagella that are enclosed in a sheath continuous with the outer membrane of the cell wall. On solid media they may synthesize numerous lateral flagella which are not sheathed [19, 87]. Some species are bioluminescent and live in mutualistic associations with fish and other marine life. Other species are pathogenic for fish, eels, and frogs, as well as other vertebrates and invertebrates [87, 21].

### **2.3 Occurrence of potentially pathogenic vibrios**

Disease outbreaks in marine organisms appear to be escalating worldwide [33] and a growing number of human bacterial infections have been associated with recreational and commercial uses of marine resources [82]. However, in light of heightened human dependence on marine environments for fisheries, aquaculture, waste disposal and recreation, the potential for pathogen emergence from ocean ecosystems remains a cause for concern. A surprising number of *Vibrio* species have been reported from marine environments and the probability of their transmission to humans is correlated to factors that affect their distribution. Both indigenous and introduced pathogens can be the cause of illness acquired from marine environments and their occurrence

depends on their ecology, source and survival [86]. To judge the risk from introduced pathogens, levels of indicator organisms are routinely monitored at coastal sites. However, methods targeting specific pathogens are increasingly used and are the only way to judge or predict risk associated with the occurrence of indigenous pathogen populations.

Interest in the occurrence of potentially pathogenic vibrios is high from an epidemiological and ecological point of view. Vibrios able to cause human disease include the cholera toxin-producing strains of *V. cholerae*, that are responsible for epidemic/pandemic cholera, thermostable direct hemolysin-producing strains of *V. parahaemolyticus*, a leading cause of gastro-enteritis and *V. vulnificus*, one of the most invasive and rapidly lethal human pathogens. These forms are characterized by wound oedema, erythema, cellulites and vesicle formation. Disease may progress to tissue necrosis accompanied by secondary sepsis [31, 84]. The transmission of *V. cholerae* strains from their environmental reservoir to humans through water sources or seafood has been demonstrated. Other non-epidemic *Vibrio* species, including *V. parahaemolyticus* and *V. vulnificus*, are usually associated with the consumption of raw or undercooked shellfish and seafood or exposure of skin wounds to seawater [87]. *V. alginolyticus*, *V. fluvialis*, *V. furnissii*, *V. hollisae* and *V. metschnikovii* are halophilic vibrios also involved in human diseases [84]. These species are present in estuarine and marine environments along with other pathogenic and non-pathogenic species. In Europe, the occurrence of pathogenic vibrios in the marine environment has been well documented by several authors [33, 34]. In Italy, the occurrence of potentially pathogenic vibrios in aquatic environments such as rivers [7, 75], brackish [55], estuarine [6] and coastal marine sites [7, 34] has been reported. The association of *Vibrio* spp. with marine plankton has also been demonstrated in the Mediterranean area for *V. alginolyticus*, *V. cholerae* non-O1, *V. vulnificus*, *V. parahaemolyticus* and *V. fluvialis* in different seasons and in different temperature and salinity conditions [37, 6, 55].

Table 2.1: Diagnostic test of human pathogenic *Vibrio* species.

	<i>V. alginolyticus</i>	<i>V. cholerae</i>	<i>V. cincinnatiensis</i>	<i>V. damsela</i>	<i>V. fluvialis</i>	<i>V. furnisii</i>	<i>V. hollisae</i>	<i>V. metchnikovii</i>	<i>V. mimicus</i>	<i>V. parahaemolyticus</i>	<i>V. vulnificus</i>
TCBS	Y	Y	Y	G	Y	Y	N	Y	G	G	G
mCPC	N	P	ND	N	N	N	N	N	N	N	Y
AGS	KA	Ka	ND	ND	KK	KK	Ka	KK	KA	KA	KA
Growth( 0% NaCl)	-	+	-	-	-	-	-	-	+	-	-
Growth (3% NaCl)	+	+	+	+	+	+	+	+	+	+	+
Growth (6% NaCl)	+	-	+	V	+	+	+	+	-	+	+
Growth (8% NaCl)	+	-	-	-	V	+	-	V	-	+	-
Growth (10% NaCl)	+	-	-	-	-	-	-	-	-	-	-
Growth at 42°C	+	+	-	-	V	-	ND	V	+	+	+
Sucrose	+	+	+	-	+	+	-	+	-	-	-
Cellobiose	-	-	+	+	+	-	-	-	-	V	+
Lactose	-	-	-	-	-	-	-	-	-	-	+
Abrabinose	-	-	+	-	+	+	+	-	-	+	-
Mannose	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	-	-	+	+	-	+	+	+	V
Oxidase	+	+	+	+	+	+	+	-	+	+	+
ONPG	-	+	+	-	+	+	-	+	+	-	+
Voges-Proskauer											
Arginine dihydrolase	-	-	-	+	+	+	-	+	-	-	-
Lysine decarboxylase	+	+	+	V	-	-	-	+	+	+	+
Ornithine decarboxylase	+	+	-	-	-	-	-	-	+	+	+
Sensitivity 0/129 (10µg)	R	S	R	S	R	R	ND	S	S	R	S
Sensitivity 0/129 (150µg)	S	S	S	S	S	S	ND	S	S	S	S
Gelatinase	+	+	-	+	+	+	-	+	+	+	+
Urease	-	-	-	+	-	-	-	-	-	V	-

Legend: TCBS-Thiosulfate-citrate-bile salts-sucrose; mCPC-modified cellobiose-polymyxin B-colistin; Y = yellow; N = no or poor growth; S = susceptible; ND = not determine ; G = green; V = variable among strains; R = resistant; P = purple ; KK = Slant alkaline / Butt alkaline; KA = Slant alkaline /Butt acidic; Ka = Slant alkaline/ Butt slightly acidic; += 80% (or more) of strains are positive; — = 80% (or less) of strains are negative Source: Poda [63].

## 2.4 Pathogenicity and virulence factors

The major features of the pathogenesis of vibrios are well established. Infection due to vibriosis begins with the ingestion of contaminated water or food. After passage through the acid barrier of the stomach, the organism colonizes the epithelium of the small intestine by means of toxin-coregulated pili [25] and possibly other colonization factors such as the different haemagglutinins, accessory colonization factor, and core-encoded pilus, all of which are thought to play a role. Cholera enterotoxin produced by the adherent vibrios is secreted across the bacterial outer membrane into the extracellular environment and disrupts ion transport by intestinal epithelial cells. The two main virulence factors of *V. cholerae*, cholera toxin (CT), which is encoded on the filamentous cholera toxin phage (CTX phage) and cause a profuse watery diarrhea, and the toxin co-regulated pilus (TCP), which is an essential intestinal colonization factor and the host receptor for CT, were both acquired by a subset of isolates by horizontal gene transfer (HGT) [46]. In the aquatic environment, *V. cholerae* has been found in association with zooplankton and phytoplankton, on the chitinous exoskeletons and moults of copepods (crustaceans) and in the mucilaginous sheaths of blue-green algae [43]. The mannose-sensitive haemagglutinin (MSHA), which is encoded by all the sequenced Vibrionaceae genomes, is involved in *V. cholerae* adherence to zooplankton [10]. Indeed, the majority of serogroups, with the exception of the O1 and O139 groups, do not encode CTX phage or TCP. Different ecotypes can exist within the same species and those isolates that carry CTX phage and TCP belong to one ecotype, which might preferentially associate with humans and crustaceans, and other ecotypes might carry traits that allow them to enter other states [64].

Intensive study of toxigenic strains of O1 and O139 yielded a set of virulence genes mostly belonging to TCP Pathogenicity Island, *tcpA*, *tcpI*, and *acfB* encoding colonization factor TCP, and CTX prophage *ctxA* encoding CT toxin [25, 32]. More genetic variants to the proteins

are surfacing as the bacteria continue to transfer genetic components [24, 25]. The emergence of *V. cholerae* O139 likely originated from a homologous recombination event of environmental strain 022 O-antigen specific genes, which becomes the O139 *wbf* region, with O1 El Tor strain. *V. cholerae* O139 and O1 El Tor strains share all their virulence factors and similar ribotypes, from analysis of highly conserved rRNA [24]. *V. cholerae* O139 strains are genetically highly diverse, with all combinations of nontoxicogenic strains with various virulence genes. Those with TCP+ genotypes may undergo toxigenic conversion by CTX phage [24]. Horizontally transferable conjugative transposons in *V. cholerae* or the presence of integrons converting antimicrobial resistance of other organisms into functional operons may explain the fluctuating antimicrobial character and genomic diversity of O139. Genomic instability probably contributes to the bacterium's evolution and success.

Studies examining the presence of strain specific DNA among pathogenic *V. cholerae* isolates have shown the acquisition of several regions that are specific to pandemic isolates [46]. The appearance of a novel epidemic *V. cholerae* O139 serogroup in 1992 resulted from the acquisition of O139 gene cluster, and a capsule polysaccharide and an integrative element [25]. Unlike *V. cholerae*, which causes secretory diarrhea by producing CT. *V. parahaemolyticus* can cause gastroenteritis associated with inflammatory diarrhea [18]. In recent years, outbreaks of *V. parahaemolyticus* infection have increased worldwide and in regions with high seafood consumption, *V. parahaemolyticus* causes over half of all food poisoning outbreak of bacterial origin [18]. One of the main *V. parahaemolyticus* virulence factors is the thermostable direct haemolysin (TDH) (Table 2). TDH is a pore-forming protein that might contribute to the invasiveness of the bacterium. The mechanism by which *V. vulnificus* is pathogenic to humans depends, to a large extent, on host susceptibility, and this bacteria is predominantly an opportunistic pathogen [31]. Human infections occur generally after consumption of

contaminated raw oyster or wound infection from seawater or contaminated fish [31]. Multiple virulence factors, including the capsular polysaccharide (CPS), and iron availability in the host, as well as a short generation time, contribute to the highly invasive nature of *V. vulnificus* [76]. Host susceptibility has a crucial role in *V. vulnificus* disease progression; hepatitis, haemochromatosis and an impaired immune system can result in lethal septicemia [31]. Among *V. cholerae* and *V. parahaemolyticus*, pathogenic isolates are clearly defined by the presence of CT and TDH, respectively, however, among *V. vulnificus* isolates host susceptibility seems to be a defining factor for virulence. Another pathogenicity factor in *V. cholerae* is neuraminidase, (Table 2.2) which is encoded by the *nanH* gene on *Vibrio* pathogenicity island-2 (VPI-2) [46]. Neuraminidase cleaves mucin from intestinal cells, unmasking GM1 gangliosides, and the receptors for CT, and releasing sialic acid, a carbon source [46]. *V. cholerae* isolates encoding neuraminidase could have a selective advantage source in their natural environment. The *nanH* gene has a limited distribution among the sequenced Vibrionaceae and has so far only been found in *V. cholerae* O1 serogroup strains [46]. Both CT encoded on CTX phage and TCP encoded on *Vibrio* pathogenicity Island-1 (VPI-1) were acquired by HGT, probably by transduction.

## **2.5 Ecology of *Vibrio* species**

The ecology of pathogenic Vibrios decreases the occurrence and epidemiology of human infections, which has spurred environmental research on these bacteria. The Vibrios may also be required for balanced ecosystem in the marine environment, and their association with higher organisms may provide a beneficial effect on salt retention by copepods and other organism which play vital roles in food chain [34]. Evidence from ecological studies on the role of *Vibrio* spp. in aquatic environments points out their importance in biodegradation, nutrient regeneration

and biogeochemical cycling [7]. A number of authors have reported some species common in freshwaters but they are being transported to saline environments through water flow [7]. *Vibrio* species show a different sensitivity to environmental conditions but it is difficult to assess the effect of a single parameter as some factors are interactive [7]. Water temperature is considered the most important factor governing the distribution and abundance of pathogenic vibrios. Their density generally remains rather low at temperatures below 20°C and the highest concentrations 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 environments is below 10 °C or exceeds 30°C. The direct relation between *Vibrio* species and water temperature determines a seasonal and geographical variation in bacterial distribution was observed in USA, Asia and Europe [3, 34]. The main factors influencing the occurrence and distribution of *Vibrio* in aquatic environments are temperature, salinity, nutrient availability and the association with marine organisms. In temperate regions vibrios can readily be cultured and detected from seawater and seafood in the spring-summer period while in general they are found in scant amounts in winter when the water temperature declines. At temperatures below 10°C most vibrios disappear from the water column but they may persist in sediments to reappear and multiply under favourable conditions [34, 35]. Due to environmental stress in natural ecosystems, some pathogenic vibrios such as *V. vulnificus* and *V. cholerae* enter into viable but nonculturable (VBNC) state rather than die when exposed to reduced nutrient levels [75]. In the viable but nonculturable state, bacteria are metabolically active but do not form colonies when plated onto conventional culture media. Under favourable conditions often season-dependent, they can revert to the vegetative state for their growth and multiplication [62].

Table 2.2: Potential pathogenicity and virulence factors of pathogenic *Vibrio* species.

Factors	Assay	Target system	Species	References
Flagellum	Motility	Gastrointestinal tract	<i>V. cholerae</i>	25, 24
Adhesins	Hydrophobicity, Erythrocytes agglutination	Gastrointestinal tract	<i>V. cholerae</i> , <i>V. parahaemolyticus</i>	25,18, 24
Serum resistance	Serumcidal activity	Blood	<i>V. vulnificus</i>	31, 76
Polysaccharides acidic	Ruthenium red staining	Blood	<i>V. vulnificus</i>	31, 76
Enterotoxin Cholera toxin	Rabbit ileal loop, Adrenal cells (mouse), Immunodiffusion, ELISA, gene probe	Gastrointestinal tract	<i>V. cholerae</i> O1, <i>V. cholerae</i> non-O1, <i>V. mimicus</i>	52,76, 64
Enterotoxin Labile toxin or heat-stable toxin	Suckling mouse	Gastrointestinal tract	<i>V. cholerae</i> O1, <i>V. cholerae</i> non-O1, <i>V. mimicus</i> , <i>V. fluvialis</i> , <i>V. hollisae</i>	32, 25,43
Cytolysin Labile toxin	Adrenal cells (mouse), Chinese hamster ovary cell, Erythrocytes, animal models	Wounds, GI tract	<i>V. vulnificus</i> , <i>V. fluvialis</i> , <i>V. damsela</i> , <i>V. cholerae</i> non-O1	31, 52, 43, 25
Cytotoxin Labile toxin	Chinese hamster ovary cell	Gastrointestinal tract	<i>V. fluvialis</i>	43, 76
Cytotoxin shiga	HeLa	Gastrointestinal tract	<i>V. cholerae</i> O1, <i>V. cholerae</i> non-O1, <i>V. parahaemolyticus</i>	24, 25, 18
Hemolysin (Thermostable direct hemolysin)	Wagatsuma agar, probe, ELISA, Elek	Gastrointestinal tract	<i>V. parahaemolyticus</i> , <i>V. hollisae</i>	18
Collagenase	Enzymatic	Wounds, cutaneous tissues	<i>V. alginolyticus</i> , <i>V. vulnificus</i>	31, 76, 64
Protease	Enzymatic	Cutaneous lesions	<i>V. vulnificus</i>	31, 76
Siderophore	Arrow, Csaky	Blood	<i>V. vulnificus</i>	31, 76
Mucinase	Enzymatic	Gastrointestinal tract	<i>V. cholerae</i> O1, <i>V. cholerae</i> non-O1, <i>V. mimicus</i> , <i>V. fluvialis</i> , <i>V. parahaemolyticus</i>	24, 25, 76, 18
Neuraminidase	Enzymatic	Gastrointestinal tract	<i>V. cholerae</i> O1	46

## 2.6 Environmental regulation of virulence expression

The vibrios have been found in association with a wide range of aquatic life, including cyanobacteria (*Anabaena variabilis*) [42], diatoms (*Skeletonema costatum*) [54], in freshwater filamentous green algae (*Rhizoclonium fontanum*) [42], oysters (*Crassostrea virginica*) [37], water hyacinths (*Eichornia crassipes*) [81], the arthropod *Gerris spinolae* [78], and blue crab (*Callinectes sapidus*) [39]. Vibrios produce a chitinase and are able to bind to chitin, a semitransparent material, predominantly mucopolysaccharide, that is the principal component of crustacean shells [12]. It colonizes the surfaces of copepods [82, 68], with preferential attachment to the oral region and the egg sac. *V. cholerae* O1 also attaches to *Volvox* sp., a colonial form of phytoplankton and the attachment appears as a “ring” pattern [11]. Chitinases and mucinases facilitate the attachment of vibrios to aquatic organisms, while algae surface films enhance the growth of the pathogen [22]. It has been suggested that vibrios can survive an interepidemic period and colonize the surfaces of algae, phytoplankton, and water hyacinth [43]. In an epidemic area like the Ganges river delta, copepods favour survival of *V. cholerae* because of the organism’s production of chitinase and ability to use chitin as a source of nutrient.

When present in the environment, vibrios may undergo a series of major physical and metabolic changes, and while the altered cells are more difficult, or even impossible, to grow using conventional techniques, they have been shown to retain their virulence factors. Colwell et al [15] suggested that *V. cholerae* O1 can assume a state of dormancy in response to nutrient deprivation, elevated salinity, and/or reduced temperature. This finding was supported by others, who reported strong linear correlations between *V. cholerae* non-O1 and temperature and salinity [97]. Nutritionally deprived *V. cholerae* shows an initial rapid decline in total lipids and carbohydrates and a more gradual decline in proteins and DNA [22].

Vibrios have been classified into two groups, 'cholera vibrios' and 'noncholera vibrios'. The cholera vibrios include isolates belonging to serogroup O1 and O139 of *V. Cholerae*, the noncholera consists of serogroups other than O1 and O139 and isolates belonging to other *Vibrio* species. The O1 and O139 strains may produce CT and other virulence factors; non-O1 and non O139 strains rarely possess these attributes. Laboratory studies have shown no loss of toxigenicity by *V. cholerae* O1 under conditions of low salinity, adverse pH, adverse water chemistry, low sodium, or long-term starvation, suggesting that toxin-producing ability is unlikely to be lost when the organism is exposed to environmental stress [97]. Survival of toxigenic *V. cholerae* O1 in water at different temperatures and with different values of salinity, pH, and cation concentration and composition also indicate its ability to survive for extended periods in warm water (25°C) containing no nutrients, with a salinity of 0.25–3.0% and a pH of around 8.0 [14]. Some strains have shown increased toxin production under certain conditions, such as when attached to various aquatic plants [42]. The numbers of *V. cholerae* suspended in water are generally low and usually approximately  $10^3$  cfu/l for non-O1s and less than 50 cfu/l for O1s. However, the organism can multiply rapidly in badly stored drinking water and may be found in large numbers associated with aquatic species such as cyanobacteria, algae, zooplankton, and crustacea (including commercial species such as crabs). While counts of free organisms in the water may be low, copepods found in the same water may have  $10^5$  organisms attached to their surface [35]. The continuing failure of attempts to isolate toxigenic *V. cholerae* O1 from natural aquatic environs remains unexplained. Although toxigenic *V. cholerae* has been isolated from surface waters, no study has yet demonstrated water as a reservoir of toxigenic *V. cholerae* in the absence of a person with cholera using that water. Organisms of the O1 serogroup have frequently been isolated from aquatic environs, but most of the environmental O1 isolates do not produce CT, the toxin to which the clinical state of cholera is principally

attributed. Even in a hyperendemic area like Calcutta, toxigenic *V. cholerae* O1 could not be isolated from several aquatic bodies examined for a year [60, 69].

Vibrios are often found in association with the guts and skin of marine animals, phytoplankton, sediments and suspended detritus. The association of pathogens with marine biota has been compared to vector-borne disease in terrestrial environments as variability in environmental conditions can affect both the vector distribution and the amplification of the pathogen within the host [51]. For example, algal and zooplankton blooms can promote proliferation of associated bacterial communities by providing microenvironments favouring growth and by exuding nutrients into the water [51]. Associations between zooplankton and pathogenic *Vibrio* species have been observed [14, 35]. Correlations of the dynamics of attached pathogenic *Vibrio* species to seasonal algal and zooplankton blooms have been established [14, 35] and may exist for additional algal and zooplankton associated pathogens. Incidence of epidemic cholera in Bangladesh has also been correlated to seasonal algal and zooplankton blooms, suggesting a link between the abundance of marine indigenous pathogens and outbreaks of human disease [14].

Environmental parameters such as salinity, temperature, nutrients, and solar radiation influence the survival and proliferation of *Vibrio* species directly by affecting their growth and death rates and indirectly through ecosystem interactions. The survival of contaminant *Vibrio* spp in water environments has been shown to decrease with elevated sunlight [30, 38]; high salinity [74] and increased temperature. However, elevated nutrients and particle associations have been shown to promote the survival of waterbodies' contaminants. There is increasing evidence that many pathogens found as pollutants in waterbodies environments can survive harsh environmental conditions for prolonged periods of time in a spore-like, "viable but non-culturable" (VBNC) state [65,66].

In contrast to microbial contaminants, waterbodies indigenous pathogens are adapted to prevalent environmental conditions and their proliferation may be triggered by specific factors. For example, warm water temperatures appear to have a positive effect on the abundance of human-invasive pathogens, which tend to have mesophilic growth optima. In temperate environments, the distribution of such pathogens is typically seasonal with peaks in both environmental abundance and human infection occurring during the warmer months. This has been demonstrated for pathogenic *Vibrio* species [8, 35, 86], including *V. cholerae* [44], *V. parahaemolyticus* [36], and *V. vulnificus* [96]. In addition, elevated sunlight can stimulate growth of waterbodies indigenous heterotrophic bacteria by increasing nutrient availability by photochemical breakdown of complex polymers to release organic metabolites [20]. Nutrient enrichment in seawater samples and sediments has been correlated to increases in the relative abundance of *Vibrio* populations [49]. It remains to be established whether stimulated growth of opportunistic invasive pathogens, in response to nutrient enrichment, is a general feature of seawater environments. Overall, the effect of salinity, temperature, and nutrients on the proliferation of marine *Vibrio* spp. will be determined by both the ecosystem interactions and the growth optimum of the pathogen population. To investigate whether rising water temperature, salinity changes, or coastal eutrophication may impact human exposure to pathogens in waterbodies' environments, ecological interactions influencing pathogen abundance and distribution must be better characterized.

## **2.7 Epidemiological features of vibrio**

As highlighted earlier, *Vibrio* species grow naturally in estuarine and marine environments worldwide and are able to survive and replicate in contaminated water with increased salinity. Asymptomatically infected humans can also be an important reservoir for this organism in areas

where *V.cholerae* disease is endemic. *Vibrio cholerae* O1 and O139 produce cholera toxin and are responsible for cholera [52]. Seven major pandemics of cholera have occurred since 1817. Currently cholera is endemic in some region 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 [91]. Developing countries where socio-economic conditions are poor, sanitary and water supply systems not sufficient, and the level of personal hygiene is very low, are the most severely affected by epidemic cholera. Cholera is mostly transmitted to humans by ingestion of contaminated water and food; hence for prevention it is of utmost importance to have sustainable wastewater treatment, and safe drinking water systems in place.

In 1991, an epidemic occurred in Bangladesh which was responsible for an estimated 8 000 deaths in a 12-week period [73]. Since 1991, the world has witnessed extension of the seventh pandemic into South America and South Africa, as well as the appearance of a previously unknown pathogenic serogroup of *V. cholerae* (O139) [94]. In 1992 to 1993, 21 countries in the Western Hemisphere, mostly in coastal areas, reported 800 000 cholera cases with more than 8 000 cases resulting in death [85]. In July 1994, 14 000 deaths from cholera were reported in refugee camps in Rwanda [73]. In April 1997, a total of 1 521 deaths were recorded during a cholera outbreak among 90 000 Rwandan refugees residing in temporary camps in Democratic Republic of Congo [59]. Recent cholera outbreaks started in February 2006 in Luanda, Angola. Further cases have been detected and confirmed in the provinces of Bengo, Benguela, Bie, Kuanza Norte, Kuanza Sul, Huambo, Huila, Malange, and Democratic Republic of Congo. As at 3rd May, 2006, 26 979 cumulative cases and 1 085 deaths had been registered by United Nations International Children's Education Foundation [88]. The underlying cause of the outbreak across the affected communities and municipalities in all affected provinces is the

cramped living conditions within the affected areas. Furthermore the appalling condition of sanitation, environmental conditions and inappropriate hygiene practices greatly exacerbate problems in the areas [88]. South Africa has not escaped the cholera onslaught, and recently suffered a major epidemic. From January 2000 to December 2003 there were close to 130,000 cases of cholera reported in South Africa, with a total of 396 deaths (communication from Department of Health, South Africa).

In 1974, the first epidemic of cholera in South Africa broke out in the western part of the Transvaal Province now Limpopo province [40, 48]. Between 1974 and 1980, the disease was repeatedly introduced into Limpopo province from the north but was kept under control by appropriate control measures coupled with good surveillance [40]. In 1980, however, the disease reached epidemic proportions, and since then cholera has been regarded as being endemic in Limpopo and KwaZulu Natal [41] with an annual epidemic occurring each summer. In October 2001, a cholera epidemic started in KwaZulu-Natal and between January and March 2002, the disease spread to other provinces. A total of 17 890 cases of cholera were reported compared to the 106 389 cases reported during the previous epidemic. Most of the cases and deaths were in KwaZulu-Natal and the Eastern Cape. The Eastern Cape is the second largest province in South Africa, covering an area of 169 580 square kilometres, but also one of the poorest of nine provinces. The Eastern Cape was the second most affected province where the epidemic started in the Oliver Tambo District and lasted for six months. The epidemic was mainly attributed to unsafe water as a result of untreated wastewater that was emptied into the Umtata River [61]. Mozambique was hit in August 1997 by an epidemic caused by *V. cholerae* O1 biotype El Tor with the number of cases totaling more than 10,000 by the end of 1997. The epidemic continued into 1998 and 1999, with *V. cholerae* O1 being introduced into South Africa in 1998 with migrant workers from Mozambique, in particular to the provinces of Gauteng and Mpumalanga,

the latter bordering Mozambique [4]. Although all the cases in Gauteng were identified in migrant labourers, many of those in Mpumalanga were acquired in South Africa through contamination of local water sources.

African countries have in recent years experienced more epidemics and cases of cholera than countries in South-East Asia [92, 93]. After the seventh pandemic caused by *Vibrio cholerae* O1 biotype El Tor reached and initially spread in West African countries in the 1970s, the majority of countries in the Eastern and Southern parts of Africa have experienced major cholera epidemics, occasionally with unusually high mortalities ranging from 2 to 20%, in rare cases [92,93]. A total of 293,121 cholera cases and 10,586 deaths were reported to the World Health Organization (WHO) in 1998, with Africa accounting for the largest part with 72% of the global total [92]. In contrast to the *V. cholerae* strains causing outbreaks in South-East Asia, which have been extensively characterized, little information is available about the characteristics of the recent epidemic strains implicated in cholera outbreaks in Africa.

Pathogenic non-cholera vibrios, a number of which are also defined as emerging cause of different disease either by exposure of open wounds, cut or abrasions to aquatic environments or following the consumption of fish or seafood both in developing and developed countries. Infection caused by non-cholera vibrios species have been reported in Asia, Australia, Europe, The Middle East, North and South America [93].

## **2.8 Antimicrobial resistance**

Historically, a number of large cholera epidemics have been associated with multiple-antibiotic-resistant (MAR) strains of *V. cholerae* [26]. The antibiotic resistance pattern of epidemic strains has also changed frequently, and the emergence of *V. cholerae* O1 or O139 with different antibiograms has been documented [27, 72]. Waldor et al [90] reported the presence of

a self transmissible element, sulfamethoxazole-trimethoprim (SXT), encoding resistance to sulfamethoxazole, trimethoprim, and streptomycin in *V. cholerae* O139. Since then, the SXT element appears to have spread naturally, and most *V. cholerae* O1 strains currently associated with epidemic cholera in Bangladesh carry the SXT element [28]. Recently, a MAR strain of *V. cholerae* O1 resistant to tetracycline and erythromycin in addition to sulfamethoxazole, trimethoprim, and streptomycin was isolated from patients in Bangladesh. Ciprofloxacin and norfloxacin are broad-spectrum fluoroquinolones and possess excellent activity against *Vibrio cholerae* O1 and O139 serogroups [98]. Strains isolated from an O139 outbreak in Bangladesh in 1997 were found to be mostly sensitive to SXT and streptomycin [29]. In keeping with the observation in Bangladesh, comparison of the antibiotic resistance patterns between the O139 strains isolated during 1992 and 1993 and those isolated in 1996 and 1997 in India also showed that the later strains were susceptible to SXT [57]. Analysis of genetic changes associated with the observed SXT sensitivity showed that sensitivity to SXT and streptomycin was associated with a deletion of a ~3.6-kb region of the SXT element in strains that were sensitive to SXT and streptomycin [29]. Since 1997, the O139 strains isolated in India also showed an increased trend of resistance to ampicillin and neomycin and susceptibility to chloramphenicol and streptomycin [58]. This pattern of rapid shift in antimicrobial resistance is consistent with previous reports indicating substantial mobility of genetic elements, which confers resistance to antimicrobials, a phenomenon which has also been observed in *V. cholerae* O1 strains [27]. A multiple antibiotic-resistance plasmid belonging to incompatibility group C has also been associated with drug resistance of *V. cholerae* O139 [99]. Between March 1994 and December 1996, 80 to 100% of *V. cholerae* O1 isolates in Kenya and South Sudan and 65 to 90% of isolates in Somalia were sensitive to tetracycline [53], whereas all isolates in Tanzania and Rwanda were resistant. In Kenya and Somalia, the percentage of isolates resistant to chloramphenicol and cotrimoxazole

markedly increased from 15% in 1994 to more than 90% in 1996 [53]. The O139 serogroup of *V. cholerae* which emerged during 1992 to 1993 was sensitive to tetracycline [16].

## **2.9 Diagnosis and detection of *Vibrio* species**

The detection of *Vibrio* species is based on the traditional phenotyping techniques as well as more recent molecular tools [83, 89]. Generally, conventional culture-based methods involve a selective pre-enrichment of samples, plating onto selective solid media followed by morphological, biochemical and serological characterization. Standard operating procedures optimized for the detection and identification of *V. cholerae* and enumeration of *V. parahaemolyticus* and *V. vulnificus* [84, 13], include an inoculation of the test portion into the selective enrichment medium APW (alkaline peptone water) and incubation at optimum temperatures, streaking onto the selective solid medium thiosulphate citrate bile salt agar (TCBS). Presumptive colonies, sub-cultured on TSA (trypticase soya agar), are subjected to microscopic and biochemical analysis, such as Gram staining, motility, oxidase, arginine dihydrolase, lysine decarboxylase, ortho-nitrophenil-galactopyranoside (ONPG), acid–gas from glucose, saccharose, cellobiose, and halophilic characteristics according to Bergey’s Manual of Systematic Bacteriology [23]. Many species of *Vibrio* may be isolated with different solid media such as cellobiose polymyxin B colistin (CPC), blood agar flooded with oxidase reagent after incubation, or mannitol–maltose agar [23]. The Food and Drug Administration procedure includes the evaluation of *V. parahaemolyticus* pathogenicity by detection of the Kanagawa phenomenon using Wagatsuma agar, containing high-salt blood to detect the haemolytic activity of *V. parahaemolyticus* thermostable direct haemolysin (TDH)-positive strains only. Therefore, rapid methods are necessary to improve the detection and diagnosis of *Vibrio* infections. Serological methods have been used successfully for the identification of *Vibrio* species in food

and the aquatic environment. *Vibrio* species may be serotyped according to both somatic ‘O’ and capsular polysaccharide ‘K’ antigens. The identity of *V. vulnificus* strains may be confirmed by immunological test, such as enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody species-specific for an intracellular antigen. Among the immunological tests, ELISA-based system is commercially available for the detection of *V. parahaemolyticus* thermostable direct haemolysin [84].

Traditional standard microbiological techniques for the detection of *Vibrio* spp. are, however, slow, laborious and often require several days to be performed. Moreover, the conventional phenotypic assays, characterized by a low sensitivity, may fail to detect strains of bacteria present in the samples at low levels and with unusual phenotypic profiles [23]. Molecular methods for the identification of *Vibrio* species have increased lately, especially the use of Polymerase Chain Reaction (PCR)-based techniques to amplify specific DNA sequences, as well as digestion of these fragments with restriction enzymes. The most frequently used molecular methods to identify *Vibrio* species have been Amplified Fragment Length Polymorphism (AFLP), Fluorescence *In Situ* Hybridization, Microarrays, Multilocus Enzyme Electrophoresis (MLEE), Multilocus Sequence Typing (MLST), Real-Time PCR, Restriction Fragment Length Polymorphism (RFLP) and Ribotyping [2]. Recent advances in molecular technology have caused a shift from conventional phenotypic methods for the identification of micro-organisms to molecular methods, constituting more sensitive and specific tools for the detection of both low numbers of bacteria and ‘viable but not culturable micro-organisms’ [25, 29]. Different genomic approaches have been used for the identification of *Vibrio* spp. both in clinical samples, and food or environmental ones. An important diagnostic marker for prokaryote identification is the 16S rRNA gene analysis. Ribosomal targets for identification purposes are an appropriate choice for routine analysis, related to the presence of a sufficient number of

phylogenetically informative positions to characterize many prokaryotic agents. The study of the inter- and intrgeneric relationships based on 16S rRNA sequences of ten representative *Vibrio* species has singled out the presence of variable regions, which could be used as target sites for genus- and species-specific oligonucleotide probes and polymerase chain reaction (PCR) primers for molecular identification [2]. In addition to 16S rDNA, many other molecular targets have been used to identify the presence of *Vibrio* spp. and determine their phylogenetic and taxonomic relationships. A molecular test based on the detection of the *tdh* and/or *trh* genes, encoding for thermostable direct haemolysin and thermostable-related haemolysin respectively has been applied to identify *V. parahaemolyticus* [89, 71].

Molecular targets for routine identification of pathogens and rapid characterization of bacteria with particular growth requirements or unusual biochemical patterns is a valid alternative approach to culture-based methods [2, 27]. Considering the increasing importance of *Vibrio* spp., the molecular approach is an adequate and reliable alternative procedure which has proven to be highly rapid, sensitive and specific for routine microbial screening and monitoring of environmental and food samples, although the PCR-based assays are not able to discriminate viable from nonviable cells. The molecular approach also offers new interesting perspectives for epidemiological and phylogenetic studies. In addition, molecular methods seem to be particularly interesting to use in the discrimination and comparison of toxigenic and non-toxigenic bacterial strains. Finally, the introduction of molecular tools for bacteriological analysis and the disclosure of newly sequenced and informative molecular targets associated to conventional phenotypic methods will be useful for characterization of isolates. They can particularly be relied on to study ‘viable but not culturable micro-organisms’ and bacteria that are difficult to identify by traditional means because of variations in morphology, metabolic status and individual interpretation [84].

## **2.10 Public Health Implication**

### *2.10.1 Improvements in Public Health*

Transmission of cholera occurs through a combination of factors: seasonal bloom of bacteria, contaminated water supply, inadequate sanitation, and contaminated seafoods. Water filtration is an effective prevention step for cholera as the bacteria inhabit copepods at an infectious dose of  $10^5$  cells [14]. A low cost filtration of folded sari cloth was introduced in a cholera-endemic area with dramatic 50% reductions in seasonal cases [15]. Rehydration products, various ORS, are commercially available and widely used. Vaccines continue to be developed, with the latest targeting identified virulent proteins, but vaccination is limited to 6 months effectiveness and is therefore not practical for populations continually exposed to the evolving bacterium. Fluorescence detection techniques are available for pre-infectious levels of *V. cholerae*. In the 1900s the US was successful in curbing cholera cases through the provision of safe municipal water, especially in the New York area. Cholera is still endemic to areas in the southwestern United States. There are seasonal outbreaks in those areas, especially when the surrounding waters are compromised by pollution [45].

## **2.11 Future Concerns**

Concerns about the potential health risk from *Vibrio* species especially with regards to their association with wastewater will continue into the foreseeable future. Over the last decade, at least one new *Vibrio* sp. per year that could be transmitted through the environment has been recognized as new public health threat [95]. This is due to a number of factors including (i) changes in the way we treat our wastewater; (ii) advances in molecular biology, which allows us to identify new pathogens and trace their source; (iii) the evolution of pathogens; and (iv)

application of microbial risk assessment to quantify risks from environmentally transmitted pathogens.

The application of microbial risk assessment demand better data on the survival and transport of specific pathogens during the discharge of wastewater into the watershed. Information is needed on the effects of treatment process and survival of emerging and newly recognized pathogens; the concentration of pathogens in wastewater; and the potential for the regrowth of pathogens after treatment. Only with this information can we be certain that we are using the best management practices for environmental sustainability to meet with the United Nations Millennium Development Goals (MDGs).

## **2.12 Conclusion**

*Vibrio* species remains among those infectious disease agents posing a potentially serious threat to public health. To understand disease emergence, it is important to investigate the disease agent as well as its interactions with its environmental reservoir, vector and other animal host. Knowledge of the complete life history of disease-causing organisms, what Skelly and Weinstein [79] call “pathogen survival trajectories”, will improve our understanding and approach to disease prevention, control and surveillance. This ‘eco-environmental’ approach models the pathogen population as it moves through those environments that define its life history and includes human disease surveillance, epidemiological case-control studies and microbiological and ecological investigations. The relationship between the organism and environmental conditions continues to be a subject of keen debate especially in relation to the qualities of final effluents of wastewater treatment facilities in developing countries, which is a subject of intensive investigation in our laboratory.

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## CHAPTER 3

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# **Toxigenic *Vibrio cholerae* strains and their associated malaises**

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## CHAPTER 3

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**Figure 3.1:** Classification of *Vibrio cholerae* serogroups with toxigenic and non-toxigenic group

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## CHAPTER 3

### Toxigenic *Vibrio cholerae* strains and their associated malaises

#### Abstract

Toxigenic strains of *Vibrio cholerae* belonging to the O1 and O139 serogroups cause cholera, a severe diarrhoeal disease that occurs frequently as epidemic in many developing countries. Although *V. cholerae* is known to be a human pathogen, the bacteria constitute part of the normal aquatic flora in the ecosystem, which includes both epidemic and non epidemic strains that vary in their virulence gene content. *V. cholerae* O1 and O139 strains are commonly known to carry a set of virulence genes necessary for pathogenesis in human. The major virulence factors of *V. cholerae* include cholera toxin (CT), which is responsible for the profuse watery diarrhoea, and a pilus colonization factor known as toxin coregulated pilus (TCP). The presence of virulence-associated genes in the environmental strains provides interesting possibility to understand the pathogenicity of the disease. The emergence of toxigenic *V. cholerae* strains has provided an opportunity to study the co-evolution of different serogroups of epidemic *V. cholerae* strains, apparently driven by competition for survival and thereby attaining enhanced fitness. This review attempts to bring together some of the important researches in recent times that have gone into understanding the genetic, epidemiology, and evolution of toxigenic *V. cholerae*.

**Keywords:** *Vibrio cholerae* O1 and O139; wastewater; virulence-associated factors; environmental strains; mediating gene transfer

### 3.1 Introduction

Toxigenic strains of *V. cholerae* belonging to the O1 and O139 serogroups cause cholera, a severe diarrhoea disease that occurs frequently as epidemics and pandemic which is of public health concern in many developing countries. Cholera is thus categorized as one of the “emerging and re-emerging” infections (Satcher, 1995). The disease is an acute dehydrating diarrhoea caused principally by the potent enterotoxin, cholera toxin (CT), produced by these organisms during pathogenesis (Kaper et al., 1995). In Southern Asia, parts of Africa and Latin America, cholera is endemic with seasonal epidemics occurring widely, and is particularly associated with poverty and poor sanitation. Cholera is a waterborne disease, and the importance of water ecology is suggested by the close association of *V. cholerae* with water. Although *V. cholerae* is known to be a human pathogen, the bacteria constitute part of the normal aquatic flora in estuarine and brackish waters and are able to persist in the absence of human host (Colwell and Huq, 1994; Colwell and Spira, 1992). This novel feature of the disease raises a question regarding the genetic similarity or diversity of the different toxigenic clones. Strains belonging to other serogroups, collectively referred to as non-O1, non-O139, have also been implicated as etiological agents of moderate to severe human gastroenteritis (Morris and Black, 1985; Janda et al., 1988). Although, the vast majority of the non-O1, non-O139 strains are presumed to be non-pathogenic bacteria constituting part of the normal aquatic flora. *V. cholerae* O1 and O139 strains are commonly known to carry a set of virulence genes necessary for pathogenesis in humans (Kaper et al., 1995; Faruque et al., 1998a, 2004a).

The major virulence factors of *V. cholerae* include cholera toxin (CT), which is responsible for the profuse diarrhoea, and a pilus colonization factor known as toxin coregulated pilus (TCP). In addition to CT and TCP, cholera pathogenesis is presumed to depend on the synergistic effect of a number of putative accessory virulence-associated factors. These factors

include the mannose-sensitive haemagglutinin (MSHA) pilus, the RTX toxin, hemolysins, as well as a few other accessory toxins (Kaper et al., 1995; Faruque et al., 1998a, 2004; Faruque and Mekalanos, 2003a). However, the roles of the accessory virulence factors in cholera pathogenesis are not well established, and recent studies are beginning to reveal that at least some of these factors also play a role in environmental fitness of the pathogen (Watnick et al., 1999; Chiavelli et al., 2001). The pathogenic strains of *V. cholerae* have evolved from nonpathogenic environmental strains, and horizontal transfer of virulence-related gene clusters play a major role in the process (Faruque et al., 1998b; Faruque and Mekalanos, 2003a). The *ctxAB* genes encoding CT reside in the genome of a lysogenic filamentous phage, CTX $\Phi$  (Waldor and Mekalanos, 1996), whereas genes encoding the major colonization factor, TCP, are part of a large cluster of genes also referred to as the TCP pathogenicity island (Kovach et al., 1996; Faruque and Mekalanos, 2003a).

To track the evolutionary events in the origin of pathogenic *V. cholerae* from their non-pathogenic progenitors, it is important to identify intermediate strains that are likely to carry some of the virulence-related genes, but fall short of the complete set of genes required for pathogenesis and epidemic spread. For example, occasionally, environmental non-O1, non-O139 vibrios have been found to carry one or a few of the virulence-associated genes or their homologs (Mukhopadhyay et al., 2001; Faruque et al., 2004a). Understanding the evolution of bacterial pathogens from their non-pathogenic progenitors is challenging. Toxigenic *V. cholerae*, the etiologic agent of cholera, represents an paradigm for this process in that this organism evolved from environmental non-pathogenic *V. cholerae* by acquisition of virulence genes. The aquatic environment of a cholera endemic area harbors strains with various virulence gene profiles, and thus constitutes a reservoir of diverse virulence genes (Chakraborty et al., 2000). The ecological settings presumably favours extensive genetic interactions among *V. cholerae*

mediated by phages and mobile genetic elements (Faruque and Mekalanos, 2003a) as well as selection of pathogenic clones leading to clustering of a critical combination of genes required for the emergence of an epidemiologically thriving pathogenic strain. However, the general prevalence of virulence-associated genes among non-O1, non-O139 serogroups of *V. cholerae*, and the selection pressures for environmental *V. cholerae* carrying putative virulence genes are not clear. Studies have also identified new putative virulence related gene clusters in *V. cholerae* including genes for a type III secretion system (TTSS) (Dziejman et al., 2005), and the Vibrio seventh pandemic islands (VSP-1 and VSP-2) (Dziejman et al., 2002), the distribution of which in environmental *V. cholerae* strains, is largely unknown. In agreement with these recent findings from genetic analysis of toxigenic *V. cholerae*, molecular epidemiological surveillance of cholera in areas of endemic infection has also revealed temporal changes in the properties of toxigenic *V. cholerae* and a continual emergence of new epidemic clones which often replace existing clones (Faruque et al., 1993, 1994, 1995, 1997a, 1997b; Mitra et al., 1996). The probable functions of virulence genes or their homologues in the environment, and the ecology of toxigenic *V. cholerae* which support emergence of new epidemic clones and maintains the seasonal pattern of cholera epidemics, have not been adequately explained.

In this paper we attempt to review available information on the possible role on ecology and epidemiology of toxigenic *Vibrio cholerae* as well as the significance of emerging clonal diversity within the serogroup and their virulence genes in the environment.

### **3.2 Classification scheme of toxigenic *V. cholerae***

*Vibrio cholerae*, a non-invasive Gram-negative bacterium (Chatterjee et al., 1998), is classified on the basis of its somatic antigens (O antigens) into serovars or serogroups, and there are at least 200 known serogroups (Baumann et al., 1984; Shimada et al., 1993, 1994). Until 1992, the only

serogroup known to cause epidemic cholera was O1. Strains belonging to the O1 serogroup were further classified into two biotypes, namely the classical and El Tor, and these can be differentiated by different phenotypic traits and more recently, by precise genetic markers (Kaper et al., 1995). Seven recorded pandemics of cholera have occurred globally and there is firm evidence that at least the fifth and sixth were caused by the classical biotype O1 strains (Blake, 1994). The El Tor biotype is responsible for the ongoing seventh pandemic (Blake, 1994). In 1992, another serogroup, namely O139 started causing outbreaks of cholera in India and Bangladesh (Ramamurthy et al., 1993).

Currently, the classical biotype O1 and the EL Tor biotype are associated with endemic and epidemic cholera, while the other *V. cholerae* serogroups not associated with epidemics or pandemics are collectively referred to as non-O1, non-O139 *V. cholerae* or also as non-epidemic serogroups (Figure 3.1). Serogrouping is carried out using specific absorbed antisera or monoclonal antibodies against the 'O' antigen component of the bacterial lipopolysaccharide (Shimada et al., 1994). In addition to this, *V. cholerae* O1 is classified into three serotypes, namely Ogawa, Inaba, and Hikojima, the last of which is a rare and inadequately described serotype (Shimada et al., 1994). These serotypes are divided into A, B and C antigens. 'A' antigen made of 3-deoxy-L-glycerotetronic acid, B and C antigen has not been characterized yet. The O139 Bengal strain and the O1 serogroup outbreak strains of both the classical and El Tor biotypes show many similarities, but their major differences are significant. The O139 strain is capsulated unlike the O1 strains and has significant dissimilarities in the 'O' antigen component of the bacterial lipopolysaccharide (Johnson et al., 1994).

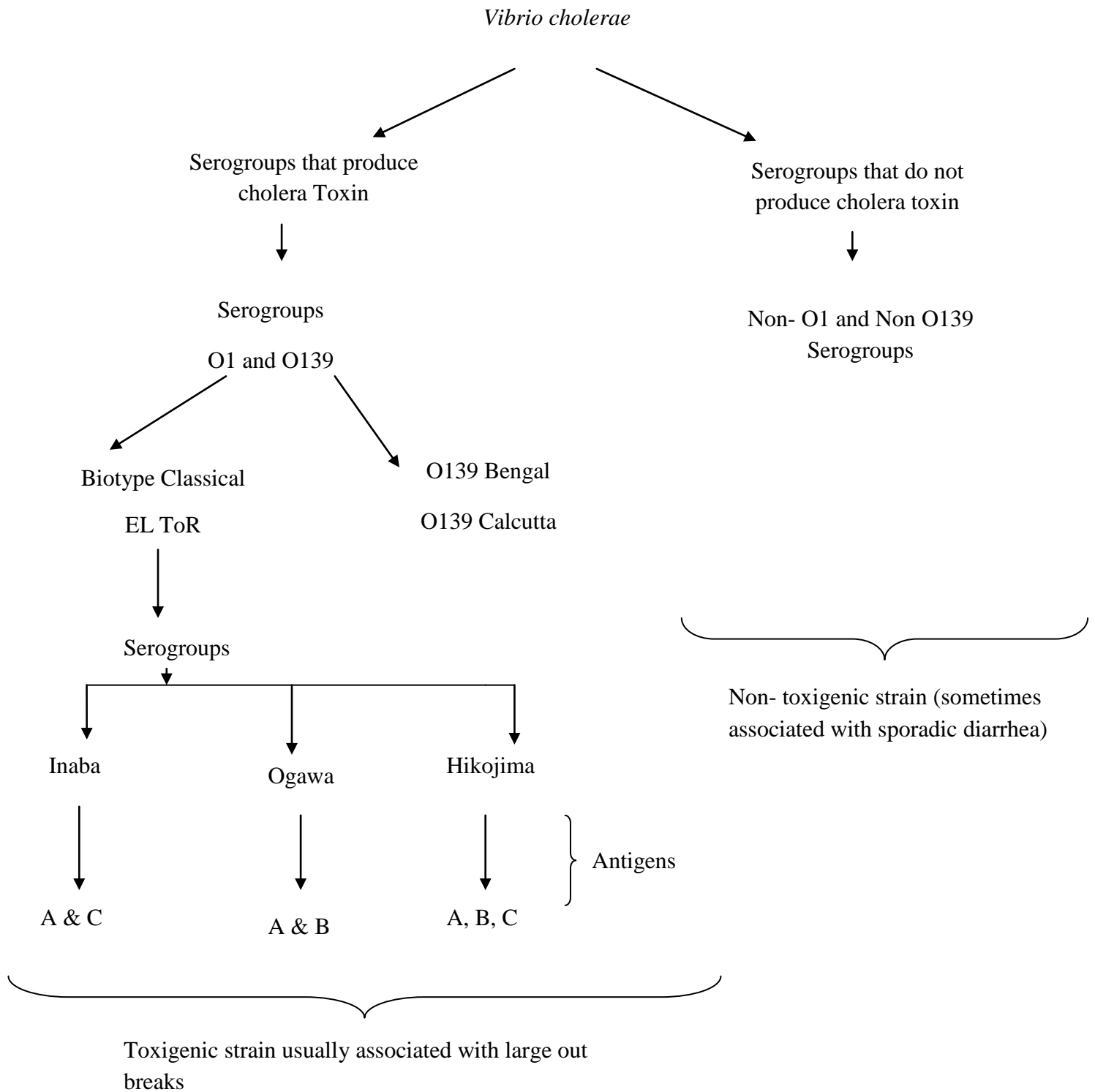


Figure 3.1: Classification of *Vibrio cholerae* serogroups with toxigenic and non-toxicogenic group. (Source: Uma et al. (2003).

### 3.3 Sources of infection

Microbial contamination of water is the largest and most immediate health hazard. Surface water quality is subjected to frequent dramatic changes in microbial quality as a result of the variety of activities on the watershed (Okoh et al., 2005). These changes are caused by discharges of municipal raw waters or treated effluent at a specific point-source into the receiving waters (Okoh et al., 2007). Okoh and co-workers (2007) reported that effluent from treatment plant discharge significant amount of pollution-indicator and pathogenic microorganism, leading to the deterioration in the quality of water.

All infectious agent causing diarrhoea are efficiently spread by the fecal-oral route (Okoh and Osode, 2008). According to epidemiologic investigation, feacally contaminated water and food are the most common vehicles for cholera infection. As highlighted earlier cholera is a highly epidemic diarrhoeal disease which continues to devastate many developing countries where socio-economic conditions and poor, sanitary systems and public hygiene are rudimentary, and safe drinking water is not available (Igbinsosa and Okoh, 2008). Okoh et al. (1996) reported the water component along with other wastewaters of the flow stations may be channeled into the saver pits prior to discharge into the environment, usually water bodies such as streams or rivers are potential source of this infection. In rural and sub-urban settings of most developing nations, the use of sewage and wastewater is often the only source of water for irrigation in these areas, eating fruit and vegetables that have been irrigated with contaminated water and eaten raw is one likely way that toxigenic *V. cholerae* can be ingested (Okoh et al., 2007; Igbinsosa and Okoh, 2008). The practice of direct discharge of effluent into receiving water bodies is of major concern as it could result amongst other things in the substantial increase in organic load and consequently depletion of the dissolved oxygen content of the receiving water body (Okoh et al., 1996, 2006, 2007; Okoh and Trejo-Hernandez, 2006).

### 3.4 Ecology of toxigenic strains

*V. cholerae* has been regarded as a member of a group of organisms whose major habitats are aquatic ecosystems (Hughes et al., 1994). The physicochemical conditions for the survival of toxigenic *V. cholerae* have been studied, and the possibility of survival of the organism in an estuarine environment, brackish waters and treated effluents is widely established (Colwell and Spira, 1992; Colwell and Huq, 1994, Okoh et al., 2007, Igbiosa and Okoh, 2009). However, the nature of the survival and persistence of toxigenic *V. cholerae* O1 or O139 in aquatic milieu and the factors involved in the conservation of the CTX element and other pathogenic genes in the aquatic environment are not clear (Colwell and Huq, 1994). The survival may be dependent on several factors: - occurrence of particular physicochemical conditions, a specific association of the bacteria with aquatic plants or animals, and/or the existence of specific ecological associations involving several components of the aquatic environment. It has been stated that under stress conditions, such as the vibrios are converted to a viable but non-culturable (VBNC) form that cannot be recovered by standard culture techniques and that such VBNC forms are able to cause infection and can revert to the culturable form (Colwell and Huq, 1994).

In areas of endemic infection, cholera epidemics occur in a regular seasonal pattern. It is not clear what determines the seasonal emergence of epidemic *V. cholerae* strains and outbreaks of cholera (Islam et al., 1994). Although, it has been suggested that during interepidemic periods toxigenic *V. cholerae* exists in an unexplained ecological association with aquatic organisms, possibly in the VBNC form, until the next epidemic season, when environmental factors trigger the dormant bacteria to multiply and lead to cholera outbreaks (Islam et al., 1994). However, differences in genetic or phenotypic properties have been often noticed among *V. cholerae* O1 and O139 strains isolated during different epidemics (Mukhopadhyay et al., 1998; Siddique et al., 1989; Nakasome et al., 1987). Analysis of restriction fragment length polymorphisms in cholera

toxin and rRNA gene restriction patterns of *V. cholerae* strains has also shown clonal diversity among epidemic strains (Faruque et al., 1993, 1994, 1995; Mitra et al., 1996). These events have raised questions about whether seasonal epidemics are caused by periodic appearances of the same strains of *V. cholerae* or are due to a continual emergence of new toxigenic clones from non-toxigenic progenitors. In addition to the O1 and O139 serogroups of *V. cholerae* which normally carry a set of virulence genes, certain environmental isolates belonging to diverse serogroups have also been found to possess virulence genes or their homologues (Chakraborty et al., 2000; Mukhopadhyay et al., 2001). This raises question whether the factors which have been described as virulence factors in the context of human infection with *V. cholerae* have other important roles when the bacterium resides in its environmental habitat.

The concept of an aquatic reservoir of *V. cholerae* O1 or O139 implies not only that the vibrios survive, in whatever form, but also that they form an essential component of the ecosystem. Several studies have illustrated the ability of toxigenic *V. cholerae* to associate with a variety of zooplankton, phytoplankton, and algae (Islam et al., 1994). The associations prolong survival, and presumably the vibrios gain nutrients from the host. However, these studies did not explain whether such association is specific for the epidemic serogroups of toxigenic *V. cholerae* or is a general phenomenon for all other *V. cholerae* serogroups and thus does not seem to be a mechanism for selective enrichment of toxigenic *V. cholerae* (Faruque et al., 1997a, 1997b). Moreover, the benefit imparted to the pathogen by possessing and maintaining the virulence-associated genes with respect to its survival and persistence in the environment is not clear. The role of extracellular enzymes including CT in the environmental micro-ecology of toxigenic *V. cholerae* is uncertain, although it has been suggested that in freshwater systems, local ionic micro-environment can be controlled by toxigenic *V. cholerae* by use of toxin acting on other living cells. However, such hypothesis has yet to be proven with specific experimental data, and

hence further studies are required to understand the more definitive roles of the virulence-associated factors and environmental selection pressures for toxigenic *V. cholerae* (Chakraborty et al., 2000; Mukhopadhyay et al., 2001).

Studies so far suggest that causation of cholera in humans is also linked to a natural process of enrichment of toxigenic *V. cholerae* and partly explains the benefit imparted to the pathogen during the disease in humans (Faruque et al., 1998b, 1998c). However, to understand the general epidemiological behaviour of *V. cholerae*, which includes mechanisms leading to seasonal pattern of epidemics, transient appearance and disappearance of different clones, and emergence of new epidemic clones, it is important to study the interactions among the bacteria, genetic elements mediating the transfer of virulence genes, the human host, and possible environmental factors (Faruque and Nair, 2002).

### **3.5 Potential for the emergence of new toxigenic strains**

The major pathogenic genes in *V. cholerae* are clustered in several regions of the *V. cholerae* chromosome and the structure of these pathogenic gene clusters indicates that they are capable of being propagated horizontally (Harkey et al., 1994; Karaolis et al., 1999; Kovach et al., 1996; Ogierman et al., 1993; Waldor et al., 1996; 1997). This could be attributed to the possibility that environmental strains of *V. cholerae* may have developed the ability to adapt to the intestinal environment through acquisition of virulence genes (Mukhopadhyay et al., 2001). The demonstration of the existence of environmental strains of *V. cholerae* which carry one or more virulence gene or their homologues further supports the possibility of an environmental origin for pathogenic *V. cholerae* (Chakraborty et al., 2000; Mukhopadhyay et al., 2001). These environmental strains may constitute reservoirs of virulence genes, and participate in gene transfer events leading to emergence of strains carrying a crucial combination of virulence genes.

Since acquisition of virulence genes appear to provide increased fitness to the bacteria, the ecosystem for *V. cholerae*, which includes the aquatic milieu as well as the host compartment, should support the origin of pathogenic clones (Faruque and Nair, 2003b). Contributing to the apparent dichotomy between toxigenic and non-toxigenic strains is the issue of acquisition of virulence genes in the environment, as most environmental isolates harbor neither *tcp* nor *ctxAB* (Singh et al., 2001). Recombination and acquisition of foreign DNA appear to be common features among vibrios and *V. cholerae* (Heidelberg et al., 2000). Genes for both TCP and CTX can be readily transduced into recipient strains via temperate phages. This is a key issue in the emergence of toxigenic strains, how this occurs in the environment is still under study.

In a recent study by Rahman et al. (2008), 10 environmental strains of *V. cholerae* non-O1, non-O139 that were positive for both TTSS and the TCP island genes, and eight of these strains were infected by CTX $\Phi$  in the intestine of infant mice were identified. Previous studies showed that the efficiency of CTX $\Phi$  infection was considerably higher *in vivo*, and this was attributed to more adequate expression of the phage receptor TCP *in vivo* than under laboratory conditions (Waldor and Mekalanos, 1996; Faruque et al., 1998b). Rahman and co-worker, (2008) did not detect any Km<sup>R</sup> transductants of the environmental strains in the *in vitro* assays, and infection with CTX $\Phi$  was detectable only in the infant mouse assay. They suggested that infection of these *V. cholerae* strains by CTX $\Phi$  was possibly TCP dependent. Hence, these TTSS-positive environmental strains of *V. cholerae* carry TCP island genes, and these genes are functional and capable of producing TCP pili; thus, these strains are potentially susceptible to CTX $\Phi$  infection under natural conditions, that is, in the human host or other environments where TCP is functionally expressed. The authors propose that these strains are intermediates in the evolution of a group of pathogenic *V. cholerae* strains that carry TTSS in addition to producing TCP and CT. They assume that it further supported by previous study in which clinical TCP<sup>+</sup> and

CT<sup>+</sup> strains belonging to O141 serogroup have been found positive for the TTSS (Dziejman et al., 2005).

It has been shown that naturally occurring strains of toxigenic *V. cholerae* O1 and O139 are inducible lysogens of CTX $\Phi$ . The phage can be induced *in vitro*, but the induction is not normally associated with cholera pathogenesis in humans (Faruque et al., 1998c). It seems possible that in the natural ecological settings, unidentified environmental factors induce lysogenic CTX $\Phi$  in toxigenic *V. cholerae*, resulting in the release of extracellular CTX $\Phi$  particles into the aquatic environment. The cell-free phage particles participate in the emergence of novel toxigenic strains of *V. cholerae* through interactions with non-toxigenic strains which exist in the environment and in the human population that consumes the environmental waters. CTX $\Phi$  uses TCP as its receptor, and hence the phage can infect only *V. cholerae* cells expressing TCP. The TCP genes, which are part of a greater genetic element referred to as the TCP pathogenicity island, appear to be the initial genetic factors required for the origination of epidemic strains. Analysis of the structure of the pathogenicity island suggests that this could be of phage origin, or may be transferred by transducing phages (Chakraborty et al., 2000; Karaolis et al., 1998; Kovach et al., 1996; Faruque et al., 1998c). Since genes responsible for the production of TCP are carried mostly by *V. cholerae* O1 or O139, while other serotypes of *V. cholerae* usually do not carry genes for TCP, it is obvious that the CTX element is also found mostly in the O1 and O139 vibrios, whereas most non-O1 vibrios are usually non-toxigenic. This further supports the assumption that in natural settings, CTX $\Phi$  probably plays an important role in the origination of new toxigenic strains of *V. cholerae*. Since *V. cholerae* strains which are TCP positive but CTX negative are not frequently isolated during environmental sampling, it is possible that such strains are normally present in very small numbers in the environment, but following conversion by CTX $\Phi$  to toxigenicity, the strains are enriched in the gastrointestinal

environment and later become detectable as new strains of toxigenic *V. cholerae*. Subsequent increases in the concentration of toxigenic *V. cholerae* in the aquatic environment may lead to epidemic outbreaks of cholera. It has been demonstrated that CTX $\Phi$  infects recipient *V. cholerae* strains more efficiently in the intestinal environment, where virulence factors such as TCP are adequately expressed (Faruque et al., 1998b; Waldor and Mekalanos, 1996). While the conversion of non-toxigenic *V. cholerae* is favoured within the gastrointestinal tract of the mammalian host, the natural selection and persistence of the novel toxigenic strains may involve both intestinal and environment factors, the immune status of the host population, and antigenic properties of the new pathogenic strain. The induction of CTX $\Phi$  lysogens is probably controlled by precise environmental signals such as optimum temperature, sunlight, and osmotic conditions and this may account for the observed seasonal outbreaks of cholera in regions of endemic infection (Faruque et al., 1998a).

### **3.6 Molecular epidemiology of toxigenic *V. Cholerae***

Epidemiological surveillance of cholera was limited before the 1970s by the lack of suitable typing systems (Faruque et al., 1998a). Epidemiological information on the emergence and prevalence of toxigenic *V. cholerae* O139 and its coexistence with the O1 El Tor strains are available primarily from Bangladesh and India through systematic surveillance studies. In the Ganges Delta region of India and Bangladesh, epidemics of cholera occur with a regular seasonality, but temporal variation in the prevalence of the two epidemic serogroups O1 and O139 have been observed (Faruque et al., 1995, 1997b; Basu et al., 2000). The emergence of *V. cholerae* O139 initially caused a complete displacement of the El Tor biotype strains in these countries. However, *V. cholerae* O139 was again displaced in 1994 by a new genetic variant of the O1 strain, and this variant strain dominated until 1996 in India (Faruque et al., 1997a; Basu et

al., 2000). In August, 1996, a new variant of the O139 strain emerged, and cholera caused by the new O139 genetic variant dominated for a year, until September, 1997 in Calcutta. Similarly in neighboring Bangladesh, during 1994 and till the middle of 1995, in most northern and central areas of the country, the O139 vibrios were replaced by a new clone of *V. cholerae* O1 of the El Tor biotype, whereas in the southern coastal regions, the O139 vibrios continued to exist (Faruque et al., 1997b, 1999; Basu et al., 2000). By late 1995 and through 1996, cases of cholera caused by both *V. cholerae* O1 and O139 were again detected in various regions of Bangladesh. However, since 1996, cholera in Bangladesh was caused mostly by *V. cholerae* O1 of the El Tor biotype, whereas only a few cases were caused by strains of the O139 serogroup.

Recent developments in DNA analysis techniques have introduced several new typing methods and have permitted studies of the epidemiology of toxigenic *V. cholerae* on a larger global perspective (Chen et al., 1991; Cook et al., 1983; Faruque et al., 1992, 1993, 1994, 1995; Wachsmuth et al., 1993, 1994; Waldor and Mekalanos, 1996; Basu et al., 2000). These techniques include the analysis of restriction fragment length polymorphisms (RFLPs) in different genes. The use of gene probes to study RFLPs in the *ctxAB* genes and their flanking DNA sequences, which are part of a larger genetic element (CTX element), indicated that U.S Gulf Coast isolates of toxigenic *V. cholerae* are clonal and that they are different from other seventh-pandemic isolates (Kaper et al., 1982). RFLPs in conserved rRNA genes have also been used to differentiate *V. cholerae* strains into different ribotypes. Analysis of isolates from the Latin American epidemic in 1991 showed that they were related to the seventh-pandemic isolates from other parts of the world and that the Latin American cholera epidemic was an extension of the seventh pandemic (Faruque et al., 1992; Wachsmuth et al., 1993; 1994). Analysis of toxigenic El Tor strains by multi locus enzyme electrophoresis has also been used to group the El

Tor strains into major clonal groups. The clones seem to reflect broad geographical and epidemiological associations.

Comparative analysis of the El Tor strains of *V. cholerae* O1 and the epidemic O139 strains suggested that the O139 strains are related to El Tor strains and were derived from them by possible genetic changes in the serotype-specific gene clusters (Faruque et al., 1994; Wachsmuth et al., 1994; Basu et al., 2000). Numerical analysis of ribotype patterns (Faruque et al., 1995) has also revealed that *V. cholerae* strains belonging to the non-O1 non-O139 serogroups diverge widely from the O1 and O139 *V. cholerae* strains. Molecular analysis of *V. cholerae* strains isolated during the epidemics period of 1961 and 1996 in Bangladesh revealed clonal diversity among strains isolated during different epidemics (Faruque et al., 1993, 1994, 1995, 1997a; Basu et al., 2000). These studies demonstrated the transient appearance and disappearance of more than six ribotypes of classical vibrios, at least five ribotypes of El Tor vibrios, and three different ribotypes of *V. cholerae* O139. Different ribotypes often showed different CTX genotypes resulting from differences in the copy number of the CTX element and variations in the integration site of the CTX element in the chromosome (Faruque et al., 1995, 1997a; Basu et al., 2000). These studies indicated that there had been a continual emergence of new clones of toxigenic *V. cholerae* which replaced existing clones, possibly through natural selection involving unidentified environmental factors and immunity of the host population (Basu et al., 2000; Faruque et al., 2003b).

### **3.7 Virulence associated toxigenic factors in *V. cholerae***

The pathogenesis of cholera is a complex process and involves a number of factors which aid the pathogen to reach and colonize the epithelium of the small intestine and produce the enterotoxin that disrupts ion transport by intestinal epithelial cells. Although production of CT, encoded by

the *ctxAB* genes, is directly responsible for the manifestation of diarrhoea, cholera pathogenesis relies on the synergistic action of a number of other genes, including the genes for one or more colonization factors (Kaper et al., 1995). Several bacterial pathogens have acquired clusters of virulence genes that display a typical base composition, and these pathogenicity Islands are not present in related non-pathogenic species (Basu et al., 2000).

In *V. cholerae*, the major virulence genes appear to exist in clusters, and there are at least two regions of the *V. cholerae* chromosome in which genes encoding virulence factors are clustered (Everiss et al., 1994; Harkey et al., 1994; Ogierman et al., 1993; Pearson et al., 1993; Trucksis et al., 1993). These include the CTX element, which has now been shown to be the genome of a filamentous bacteriophage, and the TCP-accessory colonization factor (ACF) gene cluster, referred to as the TCP pathogenicity island (Waldor and Mekalanos, 1996; Kovach et al., 1996). The pathogenicity island shares several characteristics with those of other species of pathogenic bacteria. These include the presence of groups of virulence genes, a regulator of virulence genes, a transposable gene, specific attachment sites flanking each end of the island, and an integrase with homology to a phage integrase gene (Karaolis et al., 1998; Kovach et al., 1996). Thus, the TCP pathogenicity island appears to have a phage origin but may now be defective (Kovach et al., 1996).

Since colonization is a requirement to establishing a productive infection by *V. cholerae*, the existences of other possible factors responsible for colonization are investigated. This includes the mannose-fructose-resistant cell-associated hemagglutinin (MFRHA) which has been implicated as a virulence determinant but its exact role in colonization is not clear (Franzon et al., 1993). The mannose-sensitive hemagglutinin (MSHA), which is expressed mostly by strains of the El Tor biotype, is a flexible pilus composed of subunits (Jonson et al., 1991). Antibodies to some purified outer membrane proteins (OMPs) of *V. cholerae* have been shown to inhibit

intestinal colonization in the infant mouse model (Sengupta et al., 1992). The role of other OMPs in virulence has not been established in either animal or human studies (Sengupta et al., 1992). Other factors that have been examined for possible roles in virulence include the core-encoded pilus, which is encoded by the *cep* gene located within the CTX genetic element (Pearson et al., 1993), and several possible adhesions such as the lipopolysaccharide (Chitnis et al., 1982) and a slime agglutinin present on the flagellum (Attridge et al., 1983). Although some of these factors including MFRHA, MSHA, and OMPs are suspected to play a role in enhancing adhesion and colonization, possibly in association with other factors, when tested in animal models, their exact role in the virulence of *V. cholerae* in humans is still uncertain.

Studies by Faruque and co-workers (2003b) have shown that the major virulence genes of *V. cholerae* required for pathogenesis in humans and animal models are the genes involved in the production of TCP and CT. The structures of the TCP pathogenicity island and the CTX genetic element are suggestive of horizontal transfer of these gene clusters as a possible mechanism for the origination of new pathogenic clones of *V. cholerae*. It seems possible that the acquisition of the TCP pathogenicity island and the CTX element has allowed specific strains of *V. cholerae* to become adapted to the human intestinal environment (Faruque et al., 1998a).

### 3.7.1 *Vibrio Pathogenicity Island (VPI)*

An interesting aspect of toxigenic *V. cholerae* is that the receptor for CTX $\Phi$  entry into the bacterium is the TCP, which is involved in the colonization of the bacteria on the human gut epithelium as well, and the ACF are among the genetic modules that have been acquired by the *V. cholerae* genome from other bacterial donors by horizontal gene transfer (Karaolis et al., 1998). TCP and ACF are borne on the genetic island designated as the *Vibrio Pathogenicity Island (VPI)* a characteristic of epidemic and pandemic *V. cholerae* strains.

DiRita et al. (1991) investigated the coregulation of CT and TCP by the ToxR regulatory system, which includes the ToxT protein. The genes encoding ToxT and TCP are located in the same chromosomal region (Brown et al., 1995), together with other ToxR-regulated genes including those for ACF (Everiss et al., 1994; Kovach et al., 1994). Molecular study has revealed that although the major subunit of TCP is encoded by the *tcpA* gene, the formation and function of the pilus assembly require the products of a number of other genes located on the chromosome adjacent to the *tcpA* gene, and that these constitute the *tcp* gene cluster (Oigerman et al., 1993). At least 15 open reading frames (ORFs) are found in the *tcp* cluster, which is located immediately downstream of the *tagD* gene. The *tcpH* and *tcpI* genes are two ToxR-regulated genes that influence TcpA synthesis. Inactivation of *tcpH* results in decreased pilin synthesis, whereas inactivation of *tcpI* leads to increased synthesis of TcpA (Faruque et al., 1998a). Harkey et al. (1994) suggested that regulators such as TcpI, which acts downstream of ToxR and ToxT, may function to fine-tune the expression of the TCP virulent determinant throughout the pathogenic cycle of *V. cholerae*. Hase and Mekalanos (1998) showed that TcpP and TcpH constitute homologues of ToxR and ToxS and cooperate with ToxR and ToxS in the transcriptional activation of the ToxT promoter. Adjacent to and downstream of the *tcp* cluster is located the *acf* gene cluster. The precise nature of the colonization factor is not clear, but *acfD*, one of the four ORFs (*acfABCD*), encodes a lipoprotein (Parsot and Mekalanos, 1991).

Karaolis et al. (1998) study the relationship between pathogenic and non-pathogenic strains of *V. cholerae* revealed that a pathogenicity island (PAI) is present in the toxigenic strains alone. Karaolis and co-workers (1998) found that ~40 kb VPI has a low GC content of 35%, suggesting acquisition from another source by horizontal gene transfer. Putative integrase and transposase genes and flanking *att* sites were present in VPI and could possibly be of phage origin. Polymerase chain reaction (PCR) and Southern hybridization assays revealed that this

gene cluster was absent in non-toxigenic environmental strains of *V. cholerae*, but were invariably present in all epidemic and pandemic strains investigated (Vital Brazil et al., 2002). Boyd et al. (2000) established that the VPI is a necessary element for epidemic and pandemic strains, it was postulated that the evolution of toxigenic strains from non-toxigenic ones must be a multi-step process, the initial step of which would be the acquisition of the VPI. This would lead to the expression of the *tcp*, which would in turn facilitate the acquisition of the CTX $\Phi$ , thus providing the genes for cholera toxin. The extent of transfer of these virulence-related elements was demonstrated convincingly when Chakraborty et al. (2000) reported the presence of the toxin co-regulated pilus as well as the cholera toxin genes in environmental isolates that contained neither the O1 nor the O139 antigen of *V. cholerae*. This study proved that virulence genes were not exclusively associated with clinical strains and also set the tone for the hypothesis that environmental strains could act as reservoirs for virulence genes. This track of thought was highlight in a later study by Mukhopadhyay et al. (2001) where intact or nearly intact VPI islands were found in a set of environmental isolates investigated using PCR assays. The studies also reported the finding of three putative unreported *rstR* repressor genes, as well as a new *tcpA* allele. TcpA variants in toxigenic non-O1, non-O139 serogroup isolates have been studied by (Boyd et al., 2002). While TcpA is involved in interaction with the immune system of the host, selection favours diversity at the exposed regions of the protein. This is reflected in the sequence diversity of various *tcpA* variants at the carboxyl region. An assessment of the diverse variants has, however, revealed that they are all capable of functioning as colonization factors (Uma et al., 2003). It appears that the TCP pathogenicity island is the initial genetic element required for the origination of epidemic strains, since CTX $\Phi$  uses TCP as its receptor (Waldor and Mekalanos, 1996). Also, the role of TCP as an essential colonization factor inside the host intestine is well recognized (Attridge et al., 1993; Hase and Mekalanos, 1998).

### 3.7.2 Cholera toxin and CTX phage

Toxigenic *V. cholerae* carries one or more copies of cholera toxin (CT) genes encoded by the genes *ctxA* and *ctxB* (Zhang et al., 1995; Waldor et al., 1996). The A and B subunits of CT are encoded by two separate but overlapping open reading frames (ORFs). *V. cholerae* also produces a putative toxin known as zonula occludens toxin (Zot), which increases the permeability of the small intestinal mucosa by affecting the structure of intercellular tight junction, or zonula occludens (Fasano et al., 1991; Baudry et al., 1992). A third toxin that has been described is accessory cholera enterotoxin (Ace) which is capable of inducing fluid accumulation in rabbit ligated ileal loops (Trucksis et al., 1993). In toxigenic *V. cholerae*, CT is encoded by filamentous bacteriophage designated CTX $\Phi$ , which exists as a prophage in the bacterial chromosome. CTX $\Phi$  is unusual among filamentous phages because the phage genome encodes the functions necessary for a site specific integration system and thus can integrate into the *V. cholerae* chromosome at a specific attachment site known as *attRS*, forming stable lysogens (Waldor and Mekalanos, 1996; Waldor et al., 1997).

The phage genome consists of two regions, RS2 and core; the RS2 represents a site-specific recombination system that allows the lysogenic phage to integrate at specific sites on the host chromosome, and the core consists of a retinue of genes, including the *ctxA*, *ctxB*, *zot*, *ace*, *psh*, *cep* and *orfU* (Waldor and Makalanos, 1996; Waldor et al., 1997). The RS2 region consists of the *rstR*, *rstA* and *rstB* genes. The *rstA* gene product is responsible for phage DNA replication, *rstB* is involved in site-specific integration of the phage and *rstR* is a repressor of *rstA* function (Waldor et al., 1997). The *rstR* gene is transcribed in a direction opposite to that of other genes of the phage. The *rstR* gene is flanked by two intergenic regions, ig-1 and ig-2. The ig-2 region carries the *rstA* promoter. It has been experimentally inferred that the *rstR* proteins are biotype specific, that is classical *rstA* can be repressed by classical *rstR* but not by El Tor *rstR* (Kimsey

and Waldor, 1997). The *rstR* genes exhibit the phenomenon of heteroimmunity, which means that El Tor *V. cholerae* resist superinfection with another El Tor derived CTX $\Phi$ , but classical *V. cholerae* will allow the integration of an El Tor-derived CTX $\Phi$  (Kimsey and Waldor, 1997). The RS2 module of the phage is preceded immediately by a similar element (Waldor et al., 1997; Campos et al., 1998), the RS1, which has three genes identical in sequence to the corresponding genes in the RS2 module, these are the *rstR1*, *rstA1*, *rstB1*. There is one gene in RS1 that has no counterpart in the RS2 module: this is the *rstC* gene (Waldor et al., 1997; Campos et al., 1998). The RS1 was experimentally proven to be a satellite phage that could exploit the morphogenesis genes of the CTX $\Phi$  to exist as a phage itself (Faruque et al., 2002). The function of RstC was elucidated to be that of an antirepressor which could counteract the activity of the *rstR* gene product. RstC was also shown to induce *ctxAB* expression, thus contributing to virulence in *V. cholerae* (Davis et al., 2002).

It has been showed that under suitable conditions toxigenic *V. cholerae* strains can be induced to produce extracellular CTX $\Phi$  particles (Waldor and Makalanos, 1996; Faruque et al., 1998a). The phage can be propagated in recipient *V. cholerae* strains in which the CTX $\Phi$  genome either integrates chromosomally at a specific site forming stable lysogens or maintained extrachromosomally as a replicative form of phage DNA (Faruque et al., 1998a). Studies have established that some naturally occurring non-toxigenic strains of *V. cholerae* are infected by CTX $\Phi$  and converted to toxigenic strain with epidemic potential (Faruque et al., 1998b). The bacteriophage uses the TCP as a receptor, and therefore expression of TCP by the bacterium is a requirement for its susceptibility to the phage. Consequently, a virulence factor of the bacterium in humans also serves as a receptor for CTX $\Phi$ , demonstrating a co-evolution of genetic elements mediating the transfer of virulence genes with the pathogenic bacterial species they infect (Faruque et al., 2002).

### 3.8 Expression and regulation of virulence genes in toxigenic *V. cholerae*

The regulation of virulence-associated genes in *V. cholerae* involves multiple systems. Expression of several critical virulence genes in *V. cholerae* is coordinately regulated so that multiple genes respond in a similar fashion to environmental conditions (DiRita et al., 1991; Skorupski and Taylor, 1997). Coordinate expression of virulence genes results from the activity of a cascading system of regulatory factors. The *ctxAB* is a coordinated multi-step process that requires the elaboration of a number of virulence factors (Peterson and Mekalanos, 1988). One such fundamental step is the successful colonization of the host epithelium by toxigenic bacteria, and this is mediated by their filamentous surface structures: the pili or fimbriae. The toxin co-regulated pilus is a well-characterized surface organelle of *V. cholerae*. It is a bundle-forming pilus which is coordinately expressed with CT from which it derives the name toxin coregulated pilus. TCP expression is regulated by ToxR, a transmembrane component that activates CT in response to appropriate environmental signals (Miller and Mekalanos, 1984). TcpA, the protein that constitutes the pilus structure, bears homology to bacterial type-IV pilins involved in colonization (Shaw and Taylor, 1990). The other genes in the cluster are involved in the biogenesis, secretion and export of TcpA (Uma et al., 2003).

Another potential colonization factor of *V. cholerae* designated as *acf* for Accessory Colonization Factor, also regulated by ToxR, is present immediately downstream of the TCP group of genes; this also includes a cluster of genes (Brown and Taylor, 1995). The *ctx* module is synchronized in its function by a regulatory arrangement designated as the cascade system. The regulatory cascade induces the *ctx* module in response to environmental stimuli, and thus serves to control virulence in response to environmental conditions like temperature, pH, oxygen concentration, etc. The regulation involves the expression of the toxin genes, the toxin co-regulated pilus and metabolic genes like *aldA* and *tag*. The *tox* genes (*toxT*, *toxS* and *toxR*) are

part of the cascade. ToxS is a 'sensory' membranous protein that activates ToxR, also a membrane-spanning protein. ToxT is present in the cytoplasmic matrix and is induced by ToxR; ToxT regulates the expression of the *ctx* and *tcp* genes (Miller and Mekalanos, 1984; Peterson and Mekalanos, 1988; Shaw and Taylor, 1990).

ToxR, a transmembrane protein, is the master regulator and is itself regulated by environmental signals. The ToxR protein binds to a tandemly repeated 7-bp DNA sequence found upstream of the *ctxAB* structural gene and increases the transcription of *ctxAB*, resulting in higher levels of CT expression (DiRita et al., 1991; Miller and Mekalanos, 1984). The activity of ToxR is enhanced by another transmembrane protein, ToxS, which interacts with ToxR. ToxS serves to assemble or stabilize ToxR monomers into the dimeric form (Miller and Mekalanos, 1988). ToxR regulates not only the expression of *ctxAB* but also that of at least 17 distinct genes, which constitute the ToxR regulon. These include the TCP colonization factor (Taylor et al., 1987), the accessory colonization factor (Peterson and Mekalanos, 1988), the OMPs OmpT and OmpU (Miller and Mekalanos, 1988), and three other lipoproteins (Parsot et al., 1991). Except for the *ctxAB* genes, other genes in the ToxR regulon are controlled through another regulatory factor called ToxT. ToxR controls the transcription of the *toxT* gene, which encodes a member of the AraC family of bacterial transcription activators (Higgins and DiRita, 1996). The resulting increased expression of the ToxT protein then leads to activation of other genes in the ToxR regulon. Thus, ToxR is at the top of the regulatory cascade that controls the expression of CT and other important virulence factors in *V. cholerae*, while the expression of ToxR itself remains under the control of environmental factors (Skorupski and Taylor, 1997). It has also been recognized that *V. cholerae* has ToxT-dependent and ToxT-independent branches of the ToxR regulon (Champion et al., 1997).

It has also been reported that expression of CT from the RF of CTX $\Phi$  is independent of ToxR. This indicates that phage induction may provide another mechanism for the regulation of CT production (Lazar and Waldor, 1998). The coordinate regulation of virulence genes through the ToxR regulon demonstrates that the organism has developed a mechanism of sampling and responding to its environment. *V. cholerae* also possesses a system for expression of genes in response to temperature variation (Parsot and Mekalanos, 1990). Immediately upstream of the *toxR* gene is the *htpG* gene, which encodes a heat shock protein (Parsot and Mekalanos, 1990). The *toxR* and *htpG* genes, which are transcribed in opposite directions, have their promoters so close that only one RNA polymerase can bind in the intergenic region. This mechanism work in such a way that the normal  $\sigma$ -70 RNA polymerase binds to the *toxR* promoter and transcribes the *toxR* gene only at low temperatures. At elevated temperatures,  $\sigma$ -32, the RNA polymerase sigma subunit involved in the transcription of heat shock genes, binds to the *htpG* promoter, thus repressing the *toxR* promoter. Expression of certain genes in response to low iron concentration is another distinct regulatory system in *V. cholerae*, that controls additional putative virulence genes including hemolysins and several outer membrane protein (OMP) that are not expressed when cells are grown in iron-rich media (Sigel and Payne, 1982). Sciortino and Finkelstein (1983) have suggested that the intestinal site for growth of *V. cholerae* is a low-iron environment which triggers the expression of these iron-regulated genes *in vivo*. Different regulatory systems in *V. cholerae* apparently allow the bacterium to vary the expression of its genes to optimize survival in different environments, which include the human intestine and the estuarine environment.

### 3.9 Conclusion

In spite of efforts to control cholera, the disease continues to occur as a major public health problem in many developing countries (Igbinosa and Okoh, 2008). Numerous studies over more than a century have made advances in our understanding of the disease and ways of treating patients, but the mechanism of emergence of new epidemic strains, and the ecology supporting the regular epidemics, remain mysterious and challenging to researchers in the field. Recent studies of the pathogenicity island, the discovery of bacteriophage encoding CT, and the characteristic system for acquisition of different genes by toxigenic *V. cholerae* have provided an impetus for further study of the organism to understand the molecular basis for the emergence of pathogenesis and natural fact controlling the conservation of particular genetic traits. The emergence of toxigenic *V. cholerae* strains has provided an opportunity to study the coevolution of different serogroups of epidemic *V. cholerae* strains, apparently driven by competition for survival and thereby attaining enhanced fitness. Toxigenic *V. cholerae* provides a natural system to study the coevolution of bacteria and the virulence-associated genetic elements, and the mutual benefits imparted to each other in terms of attaining greater evolutionary fitness (Faruque et al., 1998a). Therefore, future challenge for researcher investigating the ecology of toxigenic *V. cholerae* strains should be aimed at explaining more about the emergence of pathogenic strains and factors involved in the natural selection of the species to ensure its continued existence. Also the interaction between regulators and the virulence genes in the pathogenesis of toxigenic *Vibrio* especially in relation to point source pollution such as inadequate treated effluents from wastewater treatment facilities in developing nations should be of interest, and these are subjects of intensive investigation in our laboratory.

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## CHAPTER 4

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### **Impact of discharge wastewater effluents on the physico-chemical qualities of a receiving watershed in a typical rural community**

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## CHAPTER 4

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## CHAPTER 4

### **Impact of discharge wastewater effluents on the physico-chemical qualities of a receiving watershed in a typical rural community**

#### **Abstract**

The qualities of the treated final effluents of a wastewater treatment plant located in a rural community of the Eastern Cape Province of South Africa were assessed over the duration of 12 months. Parameters measured include pH, temperature, electrical conductivity, salinity, turbidity, total dissolved solid (TDS), dissolved oxygen (DO), chemical oxygen demand (COD), nitrate, nitrite and orthophosphate levels, and these were simultaneously monitored in the treated final effluents and the receiving watersheds using standard methods. Unacceptably high levels of the assayed parameters were observed in many cases for COD (7.5-248.5 mg/L), nitrate (1.82-13.14 mg/L), nitrite (0.09-1.3 mg/L), orthophosphate (0.07-4.81 mg/L), DO (4.15-11.22 mg/L) and turbidity (3.68-159.06 NTU) during the study period, and are severally outside the compliance levels of the South African guidelines and World Health Organization tolerance limits for effluents intended for discharge through public sewers into receiving watersheds. The study has revealed that there was an adverse impact on the physico-chemical characteristics of the receiving watershed as a result of the discharge of inadequately treated effluents from the wastewater treatment facility. This poses a health risk to several rural communities who rely on the receiving water bodies primarily as their sources of domestic water. There is need for the intervention of appropriate regulatory agencies to ensure production of high quality treated final effluents by wastewater treatment facilities in rural communities of South Africa.

**Keywords:** Orthophosphate, nitrate, chemical oxygen demand, dissolved oxygen, conductivity, total dissolved solid

#### **4.1 Introduction**

Sewage discharge is one of the problems presently facing South Africa, and several efforts are being vigorously pursued to control it. Water contaminated by effluents from various sources is associated with heavy disease burden (Okoh *et al.*, 2007) and this could influence the current shorter life expectancy in the developing countries when compared with developed nations (WHO, 2002). In developing countries, most of which have huge debt burdens, population explosion and moderate to rapid urbanization, people rely heavily on water sources of doubtful quality in the absence of better alternatives, or due to economic and technological constraints to adequately treat the available water before use (Calamari and Naeve, 1994; Aina and Adedipe, 1996). The scarcity of clean water and pollution of fresh water has therefore led to a situation in which one-fifth of the urban dwellers in developing countries and three quarters of their rural dwelling population do not have access to reasonably safe water supplies (Lloyd and Helmer, 1992).

Assessment of water and wastewater is very crucial to safeguard public health and the environment (Okoh *et al.*, 2005; 2007). However, water quality data on fresh and marine waters in South Africa are still sparse and uncoordinated. Therefore, monitoring these parameters is important for safety assessment of the environment and human public health in particular (Okoh *et al.*, 1996; 2007). Morrison *et al.* (2001) reported the impact of Keiskammahoek Sewage Treatment Plant on the Keiskamma River water quality while Fatoki *et al.* (2003) have recently evaluated the physicochemical quality of Keiskamma River and in the impoundment

downstream in Eastern Cape, South Africa and concluded that the level of electrical conductivity, nitrate, orthophosphate and oxygen-demanding substance were above the South Africa guideline values.

Sewage discharges are a major component of water pollution, contributing to oxygen demand and nutrient loading of the waterbodies; promoting toxic; algal blooms and leading to a destabilized aquatic ecosystem (DWAF and WRC, 1995; WRC, 2000; Morrison *et al.*, 2001). The problem is compounded in areas where wastewater treatment systems are simple and not efficient, as is the case in most rural communities in the Eastern Cape Province of South Africa. In this study, we evaluate the impact of the treated final effluents of a rural wastewater treatment facility has been evaluated in the Eastern Cape Province of South Africa on the physicochemical quality of the receiving watershed.

## **4.2 Materials and Methods**

### *4.2.1 Plant description and study site*

The Wastewater treatment facility is situated at geographical coordinates of 32°50'36"S, 26°55'00"E and approximately 1 km East of Alice town in the Eastern Cape Province of South Africa. The plant which has a design capacity of 2000 m<sup>3</sup>/day receives domestic sewage, some light industrial wastewater as well as run-off water, and treatment is based on the activated sludge system. The treated final effluent is discharged into the Tyume River.

#### *4.2.2 Sampling*

Water samples were collected from the treated final effluent, discharge point, 500 m downstream and upstream of the discharge point. Samples were collected monthly between August 2007 and July 2008. Samples were collected in glass containers, pre-cleaned by washing with non-ionic detergents, rinsed in tap water, 1:1 hydrochloric acid and finally with deionised water. Before sampling, the bottles were rinsed three times with sample water before being filled with the sample. The actual samplings were done midstream by dipping each sample bottle at approximately 20-30 cm below the water surface, projecting the mouth of the container against the flow direction. The samples were then transported in cooler boxes containing ice to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, Alice for analyses within 2 to 4 h after collection.

#### *4.2.3 Physicochemical analysis*

All field meters and equipment were checked and calibrated according to the manufactures specification. The pH, temperature, electrical conductivity, total dissolved solids (TDS), salinity and dissolved oxygen (DO) of the samples were determined onsite using a multiparameter ion specific meter (Hanna instruments, version HI9828). The turbidity was measured onsite using a microprocessor turbidimeter (HACH Company, model 2100P). The concentrations of orthophosphate as P, nitrate, nitrite and chemical oxygen demand (COD) were determined in the laboratory by the standard photometric method (DWAF, 1999) using the spectroquant NOVA 60 photometer (Merck Pty Ltd). Samples for COD analysis were digested with a Thermo reactor Model TR 300 (Merck Pty Ltd) and then analyzed by the spectroquant NOVA 60 photometer (Merck Pty Ltd). Blank determinations were performed for COD, nitrate, nitrite and orthophosphate and results were adjusted for blank measurement in the presented results. New standards were created for each parameter during every measuring month.

#### 4.2.4 Statistical analysis

The data obtained were subjected to descriptive statistical analysis (95% confident limit). The general linearized model (GLM) of SAS was used to generate analysis of variance (ANOVA), means, standard error and range. Duncan Multiple Range Test (DMRT) was used to test differences among all possible pairs of treatments. Correlation was performed using Proc Corr procedure of SAS (SAS version 8, SAS Institute, Cary, NC).

### 4.5 Results and Discussion

The results of the physicochemical qualities of samples from the different points are as shown in Table 4.1. The pH regimes vary significantly ( $P < 0.05$ ) in the sample points throughout the study period and ranged from 6.40 to 7.70 during the autumn; 7.03 to 7.63 during the summer; 6.10 to 7.85 during the winter; and 6.70 to 7.92 during the spring. In particular, pH of the treated final effluent varies between 6.10 and 7.03. Generally the pH values obtained fall within the World Health Organization standard of 7.0 to 8.5 and the water quality ranges of 6.5 to 8.5 for drinking water and water meant for full contact recreation, respectively (WHO, 1984; DWAF, 1996b). The EU also sets pH protection limits of 6.0 to 9.0 for fisheries and aquatic life (Chapman, 1996). The neutral to alkaline pH values obtained in most sampling points is similar to that reported elsewhere (Morrison *et al.*, 2001).

The temperature profile of the treated final effluent and receiving waterbody vary significantly ( $P < 0.05$ ) and ranged from 17.79 to 19.82 °C during the autumn; 22.98 to 24.73 °C during the summer; 12.97 to 15.24 °C during winter; and 17.48 to 20.98 °C during the spring. The treated final effluents in particular had temperature ranges of 15.24 to 24.73 and were below 25°C, which is the recommended limit for no risk according to the South African Water Quality

Guidelines for Domestic Use (DWAF, 1993). Based on these guidelines, the temperature of the effluent does not appear to pose any threat to the homeostatic balance of the receiving water bodies, in conformity with the report of Jaji *et al.* (2007).

The electrical conductivities of the water samples generally varied significantly ( $P < 0.05$ ) and ranged from 225.53 to 490.80  $\mu\text{Scm}^{-1}$  throughout the study period with the treated final effluent samples ranging between 268.33 and 298.50  $\mu\text{Scm}^{-1}$  (Table 4.1). Higher conductivities were observed upstream and downstream of the discharged points in winter and spring seasons, suggesting that there could be other point sources pollution entering into the receiving waterbody that resulted in the high values. The South African guideline for conductivity in effluent that could be discharged into the receiving waterbodies is 250  $\mu\text{Scm}^{-1}$  (Government Gazette, 1984), and based on this guideline the effluent quality does not appear to be compliant with the regulation for electrical conductivity. The South African acceptable limit for conductivity in domestic water supply is 70  $\mu\text{Scm}^{-1}$  (DWAF, 1996a). This limit was exceeded in the receiving waterbody. Thus the parameter does give concern and it could make the water unsuitable for direct domestic use. The conductivity values obtained in this study is similar to the findings of previous study on the nearby Keiskamma River (Fatoki *et al.*, 2003).

The turbidity profile vary significantly ( $P < 0.05$ ) amongst the sample points throughout the study period and ranged from 6.25 to 84.08 NTU during the autumn; 9.64 to 159.06 NTU during the summer; 3.81 to 7.06 NTU during the winter and 3.68 to 7.92 NTU during the spring season (Table 4.1). In particular, turbidity of the treated final effluent varies between 3.68 and 9.64 NTU, but there is no standard for turbidity for effluent discharge in South Africa (Government Gazette, 1984). The turbidity values obtained from the stations in all seasons was higher than WHO standard of 5 NTU (World Health Organization, 2004). None of the receiving waterbody met the South African guideline of 0 to 1 NTU for turbidities in water for domestic

use (DWAF, 1998). These values are grossly exceeded in the water samples and it disqualifies the receiving waterbody for direct domestic use. Also, the excessive turbidity in water can cause problem with water purification processes such as flocculation and filtration, which may increase treatment cost (DWAF, 1998). There may be a tendency for an increase in trihalomethane (THM) precursors, where highly turbid waters are chlorinated. High turbid waters are often associated with the possibility of microbiological contamination, as high turbidity makes it difficult to disinfect water properly (DWAF, 1998). However the turbidity values were generally lower during the winter and spring seasons compared to summer and autumn. The increase in values during the summer and autumn season could be attributed to surface runoff and erosion carrying soil/silt (Morokov, 1987). The turbidity values obtained in this study were higher than those reported for Umtata River and Keiskamma River in South Africa (Fatoki *et al.*, 2001; 2003).

The salinity of the water samples generally varied from 0.11 to 0.26 psu throughout the study period with treated final effluent samples ranging between 0.13 and 0.15 psu (Table 4.1). Although there are no set standard for salinity level for effluent discharge into the aquatic ecosystems in South Africa. The water quality criteria for South African coastal zones (SACOR, 1984) put the acceptable range of salinity in marine ecosystem for all biological activity at 33-36 psu, while Whitfield and Bate (2007) gave a multipurpose limit of ~0 psu for freshwater and ~35 psu for marine waters. Some of the impacts of excess salinisation on water resources include reduced crop yield, increases formation of scale and added corrosion in domestic and increased requirements for pretreatment of water for selected industrial use such as boiler feed water (DEAT, 2000).

The total dissolved solid (TDS) profile of the treated final effluent and receiving waterbody samples vary significantly ( $P < 0.05$ ) and ranged from 118.16 to 149.50 mg/L during

the autumn season; 112.80 to 133.26 mg/L during the summer season; 144.77 to 233.44 mg/L during the winter season and 168.40 to 245.60 mg/L during the spring season (Table 4.1). The treated final effluents in particular had TDS ranges of 133.26 to 168.40 mg/L and these fell within the allowed limits of 0 to 450 mg/L (DWAF, 1996d). These TDS concentrations automatically influenced the quality of the received waterbody. Elevated TDS can be toxic to freshwater animals by causing osmotic stress and affecting the osmoregulatory capability of the organisms (McCulloch *et al.*, 1993).

The dissolved oxygen (DO) profile throughout the seasons varied significantly ( $P < 0.05$ ) and ranged from 4.15 to 6.26 mg/L during autumn; 4.99 to 5.38 mg/L during summer; 4.85 to 11.22 mg/L during winter and 4.96 to 6.69 mg/L during the spring season. In general, the treated final effluent varies between 4.15 and 5.38 mg/L. The DO content in treated final effluent which was observed to deplete faster than DO from the receiving waterbody could be attributed to the presence of degradable organic matter which resulted in a tendency to be more oxygen demanding. The DO values obtained from this study are similar to those reported elsewhere (Fatoki *et al.*, 2003; Obire *et al.*, 2005; Jaji *et al.*, 2007). Dissolved oxygen is an important factor used for water quality control. The effect of waste discharge on a surface water source is largely determined by the oxygen balance of the system and its presence is essential in maintaining biological life within a system (DFID, 1999). Dissolved oxygen concentrations in unpolluted water normally range between 8 and 10 mg/L and concentrations below 5 mg/L adversely affect aquatic life (DFID, 1999; Rao, 2005). DO standard for drinking purpose is 6 mg/L whereas for sustaining fish and aquatic life is 4-5 mg/L (Rao, 2005). The DO value from this study fell short of the recommended standard. For water quality variable such as dissolved oxygen, water quality criteria are set at the minimum acceptable concentration to ensure the maintenance of biological function.

Table 4.1: Seasonal and annual variation in the concentrations for physicochemical qualities.

Variables	Seasons	Stations (Mean $\pm$ SD)				F-Value	Pr > F
		Treated final effluent	Discharge point	500 m Downstream discharge point	500 m Upstream discharge point		
pH	Autumn	6.40 $\pm$ 0.29 <sup>C</sup>	6.41 $\pm$ 0.01 <sup>C</sup>	7.56 $\pm$ 0.43 <sup>B</sup>	7.70 $\pm$ 0.20 <sup>A</sup>	1390.56	< .0001
	Summer	7.03 $\pm$ 1.31 <sup>C</sup>	7.18 $\pm$ 0.01 <sup>B</sup>	7.63 $\pm$ 0.14 <sup>A</sup>	7.59 $\pm$ 0.12 <sup>A</sup>	463.19	< .0001
	Winter	6.10 $\pm$ 0.58 <sup>D</sup>	6.52 $\pm$ 0.00 <sup>C</sup>	7.59 $\pm$ 0.43 <sup>B</sup>	7.85 $\pm$ 0.43 <sup>A</sup>	2346.40	< .0001
	Spring	6.70 $\pm$ 0.34 <sup>C</sup>	6.76 $\pm$ 0.00 <sup>C</sup>	7.82 $\pm$ 0.01 <sup>B</sup>	7.92 $\pm$ 0.12 <sup>A</sup>	1788.38	< .0001
Temperature (°C)	Autumn	19.82 $\pm$ 3.01 <sup>A</sup>	19.77 $\pm$ 0.06 <sup>A</sup>	17.79 $\pm$ 2.58 <sup>B</sup>	17.96 $\pm$ 2.44 <sup>B</sup>	773.74	< .0001
	Summer	24.73 $\pm$ 2.28 <sup>B</sup>	25.25 $\pm$ 0.08 <sup>A</sup>	23.33 $\pm$ 2.09 <sup>C</sup>	22.98 $\pm$ 2.22 <sup>D</sup>	154.03	< .0001
	Winter	15.24 $\pm$ 2.00 <sup>A</sup>	15.35 $\pm$ 0.04 <sup>A</sup>	13.25 $\pm$ 2.17 <sup>B</sup>	12.97 $\pm$ 2.57 <sup>C</sup>	740.15	< .0001
	Spring	20.98 $\pm$ 0.98 <sup>A</sup>	20.54 $\pm$ 0.18 <sup>B</sup>	17.95 $\pm$ 1.21 <sup>C</sup>	17.48 $\pm$ 0.84 <sup>C</sup>	40.00	< .0001
Conductivity ( $\mu\text{Scm}^{-1}$ )	Autumn	298.50 $\pm$ 0.54 <sup>A</sup>	295.16 $\pm$ 1.91 <sup>A</sup>	248.83 $\pm$ 61.02 <sup>B</sup>	235.50 $\pm$ 64.57 <sup>C</sup>	297.98	< .0001
	Summer	268.33 $\pm$ 14.35 <sup>A</sup>	265.33 $\pm$ 4.91 <sup>A</sup>	225.53 $\pm$ 127.51 <sup>B</sup>	225.93 $\pm$ 134.95 <sup>B</sup>	86.42	< .0001
	Winter	289.33 $\pm$ 22.03 <sup>B</sup>	280.33 $\pm$ 2.73 <sup>C</sup>	467.00 $\pm$ 61.39 <sup>A</sup>	467.77 $\pm$ 80.99 <sup>A</sup>	451.96	< .0001
	Spring	297.80 $\pm$ 11.62 <sup>C</sup>	300.33 $\pm$ 3.47 <sup>C</sup>	459.60 $\pm$ 78.68 <sup>B</sup>	490.80 $\pm$ 99.10 <sup>A</sup>	363.49	< .0001
Turbidity (NTU)	Autumn	6.25 $\pm$ 4.86 <sup>C</sup>	6.14 $\pm$ 0.31 <sup>C</sup>	84.08 $\pm$ 74.40 <sup>A</sup>	16.45 $\pm$ 0.28 <sup>B</sup>	10114.0	< .0001
	Summer	9.64 $\pm$ 7.32 <sup>C</sup>	6.37 $\pm$ 2.20 <sup>C</sup>	31.44 $\pm$ 11.39 <sup>B</sup>	159.06 $\pm$ 271.76 <sup>A</sup>	1084.18	< .0001

Salinity (psu)	Winter	$3.81 \pm 0.93^C$	$4.22 \pm 0.22^C$	$7.06 \pm 1.08^A$	$4.95 \pm 0.66^B$	11.45	< .0001
	Spring	$3.68 \pm 2.24^D$	$4.89 \pm 0.24^C$	$7.92 \pm 6.57^A$	$6.89 \pm 5.53^B$	152.03	< .0001
	Autumn	$0.16 \pm 0.00^A$	$0.15 \pm 0.00^B$	$0.13 \pm 0.03^C$	$0.12 \pm 0.03^C$	126.14	< .0001
	Summer	$0.13 \pm 0.00^A$	$0.13 \pm 0.00^A$	$0.11 \pm 0.06^C$	$0.12 \pm 0.09^B$	905.77	< .0001
TDS (mg/L)	Winter	$0.15 \pm 0.01^B$	$0.15 \pm 0.00^B$	$0.25 \pm 0.03^A$	$0.25 \pm 0.04^A$	381.95	< .0001
	Spring	$0.15 \pm 0.00^C$	$0.15 \pm 0.00^C$	$0.24 \pm 0.04^B$	$0.26 \pm 0.05^A$	311.47	< .0001
	Autumn	$149.50 \pm 0.54^A$	$147.50 \pm 2.91^A$	$119.33 \pm 37.78^B$	$118.16 \pm 32.73^B$	37.94	< .0001
	Summer	$133.26 \pm 6.80^A$	$132.66 \pm 2.53^A$	$112.80 \pm 63.62^B$	$113.00 \pm 67.81^B$	81.20	< .0001
DO (mg/L)	Winter	$144.77 \pm 10.68^B$	$146.22 \pm 1.38^B$	$233.44 \pm 30.66^A$	$233.11 \pm 41.31^A$	406.18	< .0001
	Spring	$168.40 \pm 42.48^B$	$150.50 \pm 8.67^B$	$230.00 \pm 39.26^A$	$245.60 \pm 49.50^A$	13.27	< .0001
	Autumn	$4.15 \pm 0.90^C$	$5.61 \pm 0.12^B$	$6.26 \pm 0.82^A$	$6.12 \pm 0.34^A$	29.04	< .0001
	Summer	$5.38 \pm 2.73^A$	$5.01 \pm 0.07^B$	$5.11 \pm 0.20^B$	$4.99 \pm 0.37^B$	83.98	< .0001
COD (mg/L)	Winter	$4.85 \pm 1.25^C$	$8.11 \pm 0.11^B$	$7.81 \pm 1.40^B$	$11.22 \pm 5.90^A$	362.13	< .0001
	Spring	$4.96 \pm 1.56^B$	$5.49 \pm 0.14^B$	$6.56 \pm 0.29^A$	$6.69 \pm 1.03^A$	24.23	< .0001
	Autumn	$46.00 \pm 41.69^A$	$48.75 \pm 1.41^A$	$7.50 \pm 3.78^C$	$16.00 \pm 6.92^B$	215.65	< .0001
	Summer	$238.00 \pm 333.71^A$	$140.63 \pm 37.57^A$	$248.50 \pm 334.88^A$	$238.00 \pm 174.35^A$	10.58	< .0001
	Winter	$49.00 \pm 26.92^{A,B}$	$38.00 \pm 4.28^{C,B}$	$55.50 \pm 16.76^A$	$29.50 \pm 18.93^C$	6.85	0.0016

	Spring	34.82 ± 17.98 <sup>B</sup>	54.50±1.11 <sup>A</sup>	31.17 ± 27.10 <sup>B</sup>	18.52 ± 11.57 <sup>C</sup>	100.47	< .0001
Nitrate (mg/L)	Autumn	11.75 ± 8.14 <sup>A</sup>	11.52±0.15 <sup>A</sup>	2.25 ± 0.12 <sup>B</sup>	1.82 ± 0.22 <sup>B</sup>	989.58	< .0001
	Summer	8.73 ± 2.08 <sup>A</sup>	8.86±0.32 <sup>A</sup>	3.77 ± 1.62 <sup>B</sup>	3.30 ± 1.11 <sup>B</sup>	21.30	< .0001
	Winter	13.10 ± 0.95 <sup>A</sup>	13.28±0.15 <sup>A</sup>	5.20 ± 0.31 <sup>B</sup>	1.85 ± 0.23 <sup>C</sup>	328.26	<.0001
	Spring	7.96 ± 5.22 <sup>A</sup>	12.80±0.76 <sup>A</sup>	2.50 ± 2.09 <sup>B</sup>	2.48 ± 2.27 <sup>B</sup>	16.95	0.0006
Nitrite (mg/L)	Autumn	0.12 ± 0.07 <sup>B</sup>	0.17±0.00 <sup>A</sup>	0.10 ± 0.04 <sup>B</sup> <sup>C</sup>	0.09 ± 0.03 <sup>C</sup>	25.18	0.0002
	Summer	0.19 ± 0.08 <sup>A</sup> <sup>B</sup>	0.23±0.01 <sup>A</sup>	0.17 ± 0.09 <sup>B</sup>	0.14 ± 0.08 <sup>B</sup>	6.44	0.0001
	Winter	0.21 ± 0.15 <sup>B</sup>	0.21± 0.00 <sup>B</sup>	0.27 ± 0.14 <sup>A</sup>	0.16 ± 0.09 <sup>C</sup>	127.17	<.0001
	Spring	1.30 ± 1.85 <sup>A</sup>	0.38±0.00 <sup>B</sup>	0.18 ± 0.06 <sup>C</sup>	0.22 ± 0.22 <sup>D</sup>	1190.18	< .0001
Orthophosphate (mg/L)	Autumn	0.33 ± 0.18 <sup>A</sup>	0.33±0.03 <sup>A</sup>	0.08 ± 0.04 <sup>B</sup>	0.07 ± 0.01 <sup>B</sup>	13.39	0.0028
	Summer	4.81 ± 0.58 <sup>A</sup>	1.67±0.07 <sup>A</sup>	3.13 ± 0.76 <sup>B</sup>	2.93 ± 0.88 <sup>B</sup>	23.67	< .0001
	Winter	2.16 ± 1.71 <sup>A</sup>	2.12±0.10 <sup>A</sup>	1.48 ± 1.48 <sup>B</sup>	0.70 ± 0.31 <sup>C</sup>	65.29	<.0001
	Spring	3.98 ± 0.13 <sup>A</sup>	4.09±0.03 <sup>A</sup>	0.67 ± 0.45 <sup>B</sup>	0.28 ± 0.23 <sup>C</sup>	1134.51	< .0001

Values are means of triplicates ± Standard deviations (SD); Means with the same letter are not significantly different ( $P > 0.005$ ).

The chemical oxygen demand (COD) of the water samples generally varied from 18.52 to 248.50 mg/L throughout the study period with the treated final effluent samples ranging between 34.82 and 238.00 mg/L (Table 4.1). The values obtained in all seasons were higher than the South African guideline for COD in effluents to be discharged into the receiving waterbody which is 30 mg/L (Government Gazette, 1984). Higher levels of COD were observed upstream and downstream of the discharge points in summer. The increased of COD concentrations during summer season could be attributed to run-off washed into waterbody. This is undesirable since continuous discharge of effluent has impacted the receiving waterbody to some extent, and this may have negative effects on the quality of the freshwater and subsequently cause harm to the aquatic life especially fish, downstream (Morrison *et al.*, 2001). When this present result was compared with results of COD of the treated final effluent and receiving waterbodies from developed countries, it was observed that the concentrations of COD differ as reported by UNEP (1993). According to Ogunfowokan *et al.* (2005) this increase in COD could be attributed to an increase in the addition of both organic and inorganic substance from the environment as well as organic contaminant entering the systems from the municipal sewage treatment plants. In the same light, one observation agrees with the previous works of Fatoki *et al.* (2003) and Morrison *et al.* (2001) who reported that the contribution of COD to the effluent and receiving waterbodies in South Africa appears to be significant.

Nitrate, the most highly oxidized form of nitrogen compounds is commonly present in surface and groundwater because it is the end product of aerobic decomposition of organic nitrogenous matter. Unpolluted natural waters usually contain only minute amounts of nitrate (Jaji *et al.*, 2007). The mean nitrate concentrations in each season are shown in Table 4.1. In this study, the nitrate-N concentrations ranged between 1.83 and 11.75 mg/L during the autumn season; 3.30 and 8.73 mg/L during summer; 1.85 and 13.10 mg/L during winter and 2.48 and

7.96 mg/L during the spring season. In the treated final effluent it varied from 7.96 and 13.10 mg/L (Table 4.1) and differ significantly ( $P < 0.05$ ). The South African guideline for nitrate in sewage effluent is 1.5 mg/L  $\text{NO}_3^-$  as N (Government Gazette, 1984). The effluents did not meet this standard. It is important to note that nitrate level in the treated final effluent could be a source of eutrophication for receiving water as the values obtained exceeded the recommended limit. The effluent from the treatment works could be said to be a source of nitrate into the receiving waterbody. The high nutrient levels in the upstream discharge point of the receiving water may be as a result of diffuse sources from settlement and agricultural runoff.

The nitrite profile of the water samples generally varied from 0.09 to 1.30 mg/L throughout the study period with the treated final effluent samples ranging between 0.12 and 1.30 mg/L (Table 4.1) and differ significantly ( $P < 0.05$ ), high nitrite levels were found in effluent zone than in receiving waterbody. The total nitrite levels obtained during the study period exceeded the regulatory limits, and thus nitrite is considered to pose a problem to communities when the receiving waterbody are used for domestic purposes. This may give rise to methaemoglobinemia (Fatoki *et al.*, 2003). However, it is important to note that the nitrite from the treated final effluents could be a source of eutrophication for the receiving water bodies as the values obtained from the wastewater treatment plant exceeded the recommended limits for no risk of 0 to 0.5 mg/L as N (DWAF, 1996d).

The orthophosphate  $-P$  contents varied from 0.07 to 0.33 mg/L during the autumn season; 2.93 to 4.81 mg/L during summer; 0.70 to 2.16 mg/L during winter and 0.28 to 3.98 mg/L during the spring season. The treated final effluent particularly had orthophosphate ranges between 0.33 and 4.81 mg/L (Table 4.1) and differ significantly ( $P < 0.05$ ). High phosphate levels were found in effluent zone than in receiving waterbody. The possible reason could be a consequence of dilution effect. However, the level of phosphate in water systems that will

reduce the likelihood of algal and other plant growth is  $5\mu\text{g/L}$  (DWAF, 1996c). Other investigators have pointed out that eutrophication-related problems in temperate zones of aquatic systems begin to increase at ambient total P concentrations exceeding  $0.035\text{ mg P}^{-1}$ . In warm-water systems, the values range between  $0.34$  and  $0.70\text{ mg P}^{-1}$  (Rast and Thornton, 1996). These represent nutrient threshold levels beyond which there will be a corresponding increase in the risk and intensity of plant-related water quality problems (OECD, 1982). Generally, the phosphate-P values were higher during summer season compared to other seasons, for the downstream and upstream of the discharge points. This could be attributed to phosphorus in runoff from domestic, municipal and agricultural waste (non-point sources) flowing into rivers as well as washing along the riverside with detergent (Correll, 1998). Rapin *et al.* (1989) reported that introduction of tertiary wastewater treatment and ban on phosphate in detergent in Switzerland led to a decline in phosphorus concentration in Lake Lemman. Comparison of the result obtained in this present study from the receiving watershed with some receiving waterbodies e.g. Keiskamma river ( $0.03$  to  $2\text{ mg/L}$ ) (Morrison *et al.*, 2001), Osun river ( $0.064\text{ mg/L}$ ) (Olajire and Imeokparia, 2001) and Mukuvisi river ( $0.9$ - $11.7\text{ mg/L}$ ) (Mathuthu *et al.*, 1993) showed higher phosphate concentrations than that obtained in this study.

In water quality studies, nitrogen and phosphorus are the nutrients most commonly identified as pollutants. Nitrogen in the form of ammonia ( $\text{NH}_3$ ) and nitrates ( $\text{NO}_3^-$ ) and phosphorus are essential nutrients to plant life, but when found in excessive quantities; they can stimulate excessive and undesirable plant growth such as algal blooms. Eutrophication could adversely affect the use of rivers and dams for recreation purposes as the covering of large areas by macrophytes could prevent access to waterways and could cause unsightly and malodorous scum which could lead to the growth of blue-green algae, and release toxic substances (cyanotoxins) into the water systems. These substances are well known to cause the death of

farm livestock (Holdsworth, 1991) and this must be a matter of concern in the Eastern Cape as these receiving waterbody are used for drinking by the farm livestock. Moreover, it is well known that eutrophication could increase the treatment cost of drinking water through filter clogging in water treatment works (Murray *et al.*, 2000).

The correlations among the physicochemical properties were studied and results presented in Table 4.2. There was no significant correlation observed between pH and changes in temperature. But pH with conductivity, salinity, TDS and DO exhibited a significant positive correlation ( $r = 0.241, 0.231, 0.234, 0.384$  at  $P < 0.05$ , respectively). pH with  $\text{NO}_3^-$  indicated a negative correlation ( $r = -0.674, P < 0.01$ ). Temperature and DO were negatively significantly related ( $r = -0.216, P < 0.05$ ). Also conductivity exhibited negative significant correlation with turbidity ( $r = -0.351, P < 0.01$ ), and positive significant correlation with salinity TDS and DO ( $r = 0.987, 0.987$  and  $0.391$  at  $P < 0.01$ , respectively). This will help to understand the nature of these physicochemical variables and their species speciation in the effluent and receiving watershed.

It is generally known that an increase in concentration of pollutants will occur during low flows when point sources dominate. This was observed in few cases in the downstream of discharge point during winter and autumn seasons. It was also observed that non-point sources of pollution contributed to the upstream discharge point of the receiving water across the seasons. The results obtained in this study also suggest that some of the measured pollutants have localized impacts which then get diluted downstream. Other pollutants such as turbidity and pH appeared cumulative during the summer and spring seasons. While rainfall may dilute and weaken the effects of point source pollution, it also increases the contribution of non-point sources or diffuse pollution through land runoff from agricultural fields and leachates from refuse dumps (Jaji *et al.*, 2007). However, a close inspection of the overall data indicates that the

classical dilution of “point sources” during high flows did not occur. The results of the analyses for most parameters did not show the expected trends in water quality from upstream to downstream stations on the receiving water. It is expected that the concentration of most downstream discharge point should be higher than that of upstream discharge point. This is not the case in this study.

#### **4.6 Conclusion**

This study was carried out to evaluate the impact of the treated final effluents of a typical wastewater treatment plant in a rural community of the Eastern Cape on the receiving watershed. Our finding revealed that the treatment plant exhibited effluent qualities that meet acceptable standard in some parameters, like pH and total dissolved solid (TDS). We also observed that the effluents fell short of standard requirements that are critical to the provision of clean and safe water such as organic waste (measured as turbidity DO, COD, orthophosphate, nitrate and nitrite). The results of this study therefore show that the effluent could pose significant healthy and environmental risk to rural communities who rely on the receiving water as their source of domestic water purpose without treatment, and may also affect the health status of the aquatic milieu in the receiving water. The study showed a need for a continuous pollution monitoring programme of the surface waters in rural setting in Eastern Cape Province of South Africa. In addition the provincial government and all agencies concerned with environmental matter in South Africa should evolve measures to check and ensure that discharge effluents comply with laid down rules and regulations. Overall, the study has revealed that there was an adverse impact on the physico-chemical characteristics of the receiving watershed as a result of the discharge of inadequately treated effluents from the wastewater treatment plant.

Table 4.2: Correlation coefficient  $r$  for the different physicochemical variables from the study period.

Variables	pH	Temperature	Conductivity	Turbidity	Salinity	TDS	DO	COD	NO <sub>3</sub> <sup>-</sup>	NO <sub>2</sub> <sup>-</sup>	PO <sub>4</sub> <sup>3-</sup>
<b>pH</b>	1.000	-0.041	0.241	0.069	0.231	0.234	0.384	0.040	-0.674	-0.073	-0.177
		ns	**	ns	**	**	***	ns	***	ns	ns
<b>Temperature</b>		1.000	-0.177	0.031	-0.181	-0.172	-0.216	0.043	0.017	-0.007	-0.090
			ns	ns	ns	ns	**	ns	ns	ns	ns
<b>Conductivity</b>			1.000	-0.351	0.989	0.987	0.391	-0.092	-0.001	-0.029	-0.049
				***	***	***	***	ns	ns	ns	ns
<b>Turbidity</b>				1.000	-0.333	-0.349	-0.078	0.032	-0.162	-0.020	0.055
					***	***	ns	ns	ns	ns	ns
<b>Salinity</b>					1.000	0.976	0.384	-0.079	-0.006	-0.032	-0.055
						***	***	ns	ns	ns	ns
<b>TDS</b>						1.000	0.390	-0.093	0.024	-0.026	-0.011
							***	ns	ns	ns	ns
<b>DO</b>							1.000	-0.090	-0.409	0.078	-0.055
								ns	***	**	ns
<b>COD</b>								1.000	0.018	-0.067	-0.078
									ns	ns	ns
<b>NO<sub>3</sub><sup>-</sup></b>									1.000	-0.139	-0.037
										ns	ns
<b>NO<sub>2</sub><sup>-</sup></b>										1.000	0.526
											***
<b>PO<sub>4</sub><sup>3-</sup></b>											1.000

#### **4.7 Acknowledgement**

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## CHAPTER 5

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**Occurrence of potentially pathogenic vibrios in the final  
effluents of a wastewater treatment facility in a rural  
community of the Eastern Cape Province of South Africa**

*(Published in the Research in Microbiology)*

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## CHAPTER 5

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## CHAPTER 5

### **Occurrence of potentially pathogenic vibrios in the final effluents of a wastewater treatment facility in a rural community of the Eastern Cape Province of South Africa**

#### **Abstract**

A number of *Vibrio* species are known human and/or animal pathogens usually associated with aquatic environment mostly in developing countries. We assessed the occurrence of these pathogens in the final effluents of a rural wastewater treatment facility in the Eastern Cape Province of South Africa as free or plankton-associated (180  $\mu\text{m}$ , 60  $\mu\text{m}$  and 20  $\mu\text{m}$  plankton sizes) entities using standard culture-based and molecular techniques. The free-living vibrios densities varied from 0 to  $3.45 \times 10^1$  cfu ml<sup>-1</sup>, while the plankton-associated *Vibrio* densities vary with plankton sizes as follows: 180  $\mu\text{m}$  (0 –  $4.50 \times 10^3$  cfu ml<sup>-1</sup>); 60  $\mu\text{m}$  (0 –  $4.86 \times 10^3$  cfu ml<sup>-1</sup>); 20  $\mu\text{m}$  (0 –  $1.9 \times 10^5$  cfu ml<sup>-1</sup>). The seasonal variations in the *Vibrio* densities in the 180 and 60  $\mu\text{m}$  plankton size samples were significant ( $P < 0.05$ ), while the 20  $\mu\text{m}$  plankton size and free-living vibrios densities were not. Molecular confirmation of the presumptive vibrios isolates revealed *V. fluvialis* (36.5 %), as the predominant species, followed by *V. vulnificus* (34.6 %), and *V. parahaemolyticus* (23.1%), only API 20NE was employ to detect *V. metschnikovii* (5.8 %), suggesting high incidence of pathogenic *Vibrio* species in the final effluent of the rural wastewater facility. Correlation analysis suggested that the concentration of *Vibrio* species correlated negatively with salinity and temperature ( $P < 0.001$  and  $P < 0.002$  respectively) as well as with pH and turbidity ( $P < 0.001$ ), in the final effluent. The occurrence of *Vibrio* species as plankton-associated entities confirms the role of plankton as potential reservoir for this

pathogen. We conclude that rural wastewater treatment facilities in the Eastern Cape Province of South Africa are potential sources of *Vibrio* pathogens in the aquatic environment of the communities.

**Keywords:** *Vibrio*, free-living, plankton-associated, wastewater effluent discharge.

## 5.1 Introduction

The genus *Vibrio* is formed by Gram-negative straight or curved rod-shaped bacteria, which are 1.4-2.6 µm wide, and motile in liquid media by means of single polar flagellum. They do not form endospores or microcysts and are facultative anaerobes capable of both fermentative and respiratory metabolism. Most vibrios are oxidase-positive (the only exception is *V. metschnikovii*) [10]. The genus *Vibrio* includes more than 60 species, mostly marine in origin, and its taxonomy is continuously updated due to the addition of new species. A number of *Vibrio* species, other than *V. cholerae*, may cause disease in man mainly by ingestion of contaminated water. *V. parahaemolyticus*, *V. mimicus* and *V. vulnificus* are food poisoning bacteria which are normal inhabitants of estuarine and marine environments and are frequently isolated from seawater and seafood. *V. parahaemolyticus* and *V. vulnificus* are invasive organisms affecting primarily the colon; *V. cholerae* is noninvasive, affecting the small intestine through secretion of an enterotoxin. *Vibrio vulnificus* is an emerging pathogen of humans. This organism causes wound infections, gastroenteritis, or a syndrome known as "primary septicemia" [37]. Among the halophilic Vibrios, *V. alginolyticus*, *V. fluvialis* and *V. metschnikovii* are also pathogenic for humans [10].

In Europe, the occurrence of pathogenic vibrios in the marine environment has been well documented by several authors [13, 14]. In Italy, the occurrence of potentially pathogenic vibrios in aquatic environments such as rivers [6, 33], brackish [25], estuarine [3] and coastal marine sites [6, 14] has been reported. The association of *Vibrio* species with marine plankton has also been demonstrated in the Mediterranean area for *V. alginolyticus*, *V. cholerae* non-O1, *V. vulnificus*, *V. parahaemolyticus* and *V. fluvialis* in different seasons and in different temperature and salinity conditions [16, 3, 25].

Vibrios are widely distributed in effluents environments associated with domestic sewage. They are commonly associated with aquatic living species and include many important pathogens for aquatic animals and humans who consume contaminated seafood or polluted drinking water [36]. They are present in the environment as free-living and associated with different biofilms [34] which enable them to survive in the natural environment longer than free-living forms, by means of adhesive strategies, thus improving their adaptability to adverse conditions [4]. Enormous numbers of vibrios are associated with zooplankton [17], thus suggesting that *Vibrio* species have a competitive advantage when chitinous zooplankton is present [15].

The interest in vibrios abundance is therefore of epidemiological and ecological importance. Vibrios possess some virulence factors which include the cholera toxin-producing strains of *V. cholerae* that are responsible for epidemic/pandemic cholera; thermostable direct hemolysis-producing strains of *V. parahaemolyticus*, a leading cause of gastro-enteritis; and *V. vulnificus*, which can cause sepsis and serious wound infections [29].

Over the past few years South Africa has experienced outbreaks of waterborne diseases [18] resulting in tremendous attention from the media and placing immense pressure on regulatory agencies to prevent future cases. Discharge of wastewater treatment plant effluents

into the environment is likely to enrich the primary habitat with vibrios [18]. In this paper, we report the prevalence and distribution of *Vibrio* pathogens as free-living and plankton associated entities, in the final effluents of a rural wastewater treatment facility in the Eastern Cape Province of South Africa as well their relationships with some physico-chemical parameters.

## 5.2 Material and Methods

### 5.2.1 Study site

The Wastewater treatment facility is situated at geographical coordinates of 32°50'36"S, 26°55'00"E and approximately 1 km East of Alice town in the Eastern Cape Province of South Africa. The plant which has a design capacity of 2000 m<sup>3</sup>/day receives domestic sewage, some light industrial wastewater as well as run-off water, and treatment is based on the activated sludge system. The final effluent is discharged into the nearby Tyume River.

### 5.2.2 Treatments of samples

All samples were collected aseptically using sterile 1000 ml Nalgene bottles and transported on ice from the sampling site to the laboratory for analyses. Water samples from the final effluents were dechlorinated by adding 0.5 ml of sterile concentrated sodium thiosulphate solution to give a final concentration of 100 mg l<sup>-1</sup>. Samples were stored at 4°C until analyses were complete. All samples were processed after 24 h of collection.

As described by Alam et al. [1] one liter of wastewater was filtered successively through 180 µm, 60 µm and 20 µm nylon nets (Millipore Corp., Bedford, MA), sequentially arranged in that order to a collection base. After filtration, each nylon nets and its content were suspended in

25 ml physiological-buffered saline containing sterile glass beads (0.1 mm BioSpec Products) and homogenized for 2 min in a glass homogenizer at  $3,000 \times g$  to dislodge the attached bacteria, and the homogenates used for direct plating.

### *5.2.3 Physicochemical analyses*

The measurement of sample pH, temperature, turbidity and salinity has been described elsewhere [19]. The concentrations of free chlorine residual in the final effluents were determined using a multi-parameter ion-specific meter (Hanna BDH-laboratory) and analysis was carried out in triplicate.

### *5.2.4 Estimation of Vibrio densities*

For direct plate count analyses of plankton free samples, the samples were subjected to 10-fold dilution prior to pour plating using thiosulphate-citrate-bile-salts-sucrose (TCBS) agar and incubated at 37 °C for 24 h. For the plankton associated samples, the abundance of *Vibrio* species was obtained by inoculating 0.5 µl aliquots of concentrated homogenates onto thiosulfate-citrate–bile salt-sucrose (TCBS) agar and incubating at 37 °C for 24 h in accordance with the description of Alam et al. [1]. Yellow and green colonies were considered total presumptive *Vibrio* colonies and counted as described elsewhere [2].

### *5.2.5 Isolation and biochemical identification of Vibrio species*

Aliquots of the plankton free and plankton associated samples were inoculated into alkaline peptone water (APW) and incubated aerobically at 37 °C for 18-24 h. Turbid cultures were streaked onto TCBS agar incubated at 37 °C for 24 h. Five to ten isolated colonies per plate were

randomly picked from each sample and subsequently subcultured on fresh TCBS agar plates. The pure isolates were subjected to Gram staining and oxidase test. Only Gram-negative, oxidase-positive isolates were selected for biochemical identification using API 20 NE kit. The strips were then read and the final identification was secured using API lab plus software (BioMerieux, Marcy l'Etoile, France). Only excellent identification reports were accepted.

#### 5.2.6 Molecular identification

Polymerase chain reaction (PCR) was used to confirm the identities of the *Vibrio* species using the specific primers described in Table 5.1. DNA extraction and PCR were carried out as described by Maugeri et al. [27] with little modification. Single colonies of presumptive vibrio strains grown overnight at 37 °C on TCBS agar plates were picked, suspended in 200 µl of filtered distilled water and bacterial cells were collected by centrifugation at 11 000 × g for 10 min at 4 °C. The pellet was suspended in 100 µl of filtered and autoclaved water and boiled for 10 min. The cell lysates (10 µl) were used as template in the PCR assays immediately after extraction or following storage at -20 °C. The thermal cycling profile was as follows: a 15 min denaturation at 93 °C followed by 35 cycles at 92 °C for 40 s, 57 °C for 1 min and 72 °C for 1.5 min and final extension at 72 °C for 7 min. The amplified products were held at 4 °C after completion of the cycles. For *Vibrio fluvialis* the amplification conditions were initial denaturation at 94 °C for 5 min, followed by 30 cycles consisting of denaturation at 94 °C for 40 s, annealing at 65 °C for 40 s and extension at 72 °C for 1 min. The PCR products were electrophoresed in 1.5 % agarose gel containing 0.5 mg/l ethidium bromide for 1 h at 100 V and then visualized using a UV transilluminator.

Table 5.1: List of primers used in this study.

Target species	Primer	Sequences (5'- 3')	Target gene	Amplicon size (bp)	Reference
All <i>Vibrio</i> spp	V. 16S-700F	CGG TGA AAT GCG TAG AGA T	16S rRNA	663	[22]
	V. 16S-1325R	TTA CTA GCG ATT CCG AGT TC			
<i>V. cholerae</i>	<i>Vc. sodB</i> -F	AAG ACC TCA ACT GGC GGT A	<i>sodB</i>	248	[35]
	<i>Vc. sodB</i> -R	GAA GTG TTA GTG ATC GCC AGA GT			
<i>V. parahaemolyticus</i>	<i>Vp. flaE</i> -79F	GCA GCT GAT CAA AAC GTT GAG T	<i>flaE</i>	897	[35]
	<i>Vp. flaE</i> -934R	ATT ATC GAT CGT GCC ACT CAC			
<i>V. vulnificus</i>	<i>Vv. hsp</i> -326F	GTC TTA AAG CGG TTG CTG C	<i>hsp60</i>	410	[39]
	<i>Vv. hsp</i> -697R	CGC TTC AAG TGC TGG TAG AAG			
<i>V. fluvialis</i>	<i>Vf- toxR</i> F	GAC CAG GGC TTT GAG GTG GAC GAC	<i>toxR</i>	217	[7, 31]
	<i>Vf- toxR</i> R	AGG ATA CGG CAC TTG AGT AAG ACTC			

### 5.2.7 Statistical analysis

To understand the relationship between *Vibrio* species abundance in the final effluent and physico-chemical parameters of the environment, linear regression was performed on the collected data. The *Vibrio* species abundance was natural-log transformed to achieve normal distribution before use as the dependent variable. All other measured environmental factors were used as independent variables in the regression analysis. The relationship between independent variables was examined by analysis of variance (ANOVA). All statistical analyses were performed using SAS (SAS version 8, SAS Institute, Cary, NC).

## 5.3 Results

### 5.3.1 Physico-chemical parameters

In our previous study [19] we reported the temperature of the final effluents to range from 13.04 °C to 27.21 °C, while the pH, turbidity and salinity ranged between 5.53 to 9.38; 1.89 to 22.47 NTU; and 0.13 to 0.17 psu respectively (Table 5.2). Also, the temperatures of the effluent samples were highest in January and lowest in July. An increase in temperature was associated with increased *Vibrio* densities. However, other factors also had significant effects on *Vibrio* species abundance. High pH value was observed in the month of January while lower values were obtained in November. There was marginal evidence of an association between  $\log_{10}$  *Vibrio* count and pH. Also, increasing turbidity was associated with increasing *Vibrio* abundance suggesting a positive association. Salinity negatively correlated with abundance of *Vibrio* species ( $r = -0.632$ ;  $P < 0.001$ ) over the entire sampling period. However, at different salinity levels, the relationship did not follow the overall trend.

Table 5.2: Physico-chemical qualities of the final effluents\*.

<b>Variables</b>	<b>Seasons</b>	<b>Treated final effluent</b>	<b>F-Value</b>	<b>Pr &gt; F</b>
pH	Autumn	6.40 ± 0.29 <sup>C</sup>	28.76	0.0001
	Summer	7.03 ± 1.31 <sup>C</sup>	1064.10	<0.0001
	Winter	6.10 ± 0.58 <sup>D</sup>	3716.95	<0.0001
	Spring	6.70 ± 0.34 <sup>C</sup>	737.29	<0.0001
Temperature (°C)	Autumn	19.82 ± 3.01 <sup>A</sup>	5996.47	<0.0001
	Summer	24.73 ± 2.28 <sup>B</sup>	604.69	<0.0001
	Winter	15.24 ± 2.00 <sup>A</sup>	3527.67	<0.0001
	Spring	20.98 ± 0.98 <sup>A</sup>	96.56	<0.0001
Turbidity (NTU)	Autumn	6.25 ± 4.86 <sup>C</sup>	14842.8	<0.0001
	Summer	9.64 ± 7.32 <sup>C</sup>	1193.01	<0.0001
	Winter	3.81 ± 0.93 <sup>C</sup>	1.83	0.1847
	Spring	3.68 ± 2.24 <sup>D</sup>	905.16	<0.0001
Salinity (psu)	Autumn	0.16 ± 0.00 <sup>A</sup>	486.96	<0.0001
	Summer	0.13 ± 0.00 <sup>A</sup>	2769.97	<0.0001
	Winter	0.15 ± 0.01 <sup>B</sup>	284.15	<0.0001
	Spring	0.15 ± 0.00 <sup>C</sup>	578.82	<0.0001

Values are means of triplicates ± Standard deviations (SD); Means with the same letter are not significantly different ( $P > 0.005$ ).

\*Extracted from Igbinsosa and Okoh [19] with kind permission from the journal editor.

### 5.3.2 Concentration of free chlorine residual in the final effluent

The chlorine residual of the final effluent varied significantly ( $r = 0.525$ ;  $P < 0.001$ ) from  $0.09 \text{ mg l}^{-1}$  in the month of November to the highest level of  $1.4 \text{ mg l}^{-1}$  in the months of February (Figure 5.1). There is no legislation that regulates the concentration of chlorine residual in treated final effluent in South Africa at present, and as such we considered those for domestic water supplies which recommend ranges of  $0.3$  to  $0.6 \text{ mg l}^{-1}$  [28].

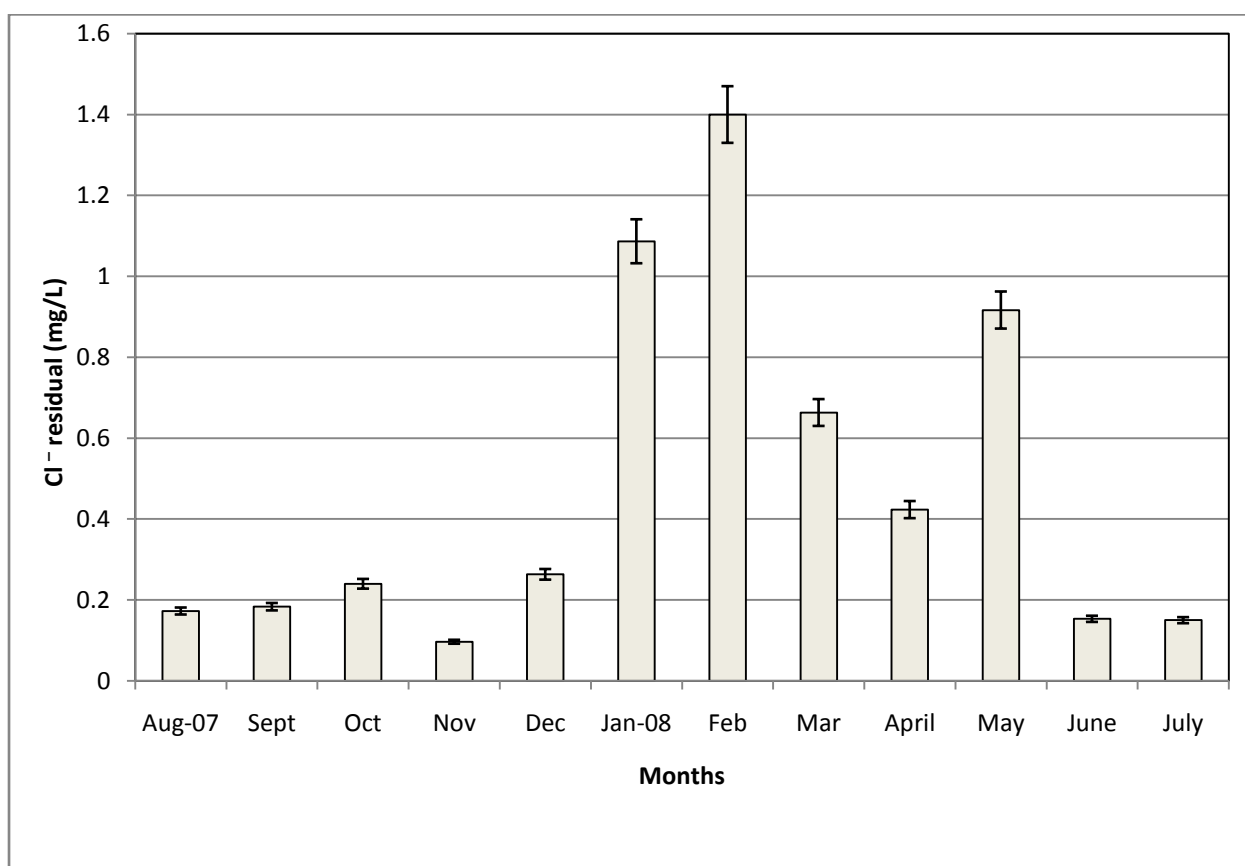


Figure 5.1. Concentrations of free chlorine residual (mg/L) in the final effluents during the sampling period.

### 5.3.3 Seasonal abundance of *Vibrio* species

The densities of *Vibrio* pathogens in the final effluent varied appreciably among plankton sizes. *Vibrios* associated with  $180 \mu\text{m}$  plankton size samples ranged from  $0 - 4.50 \times 10^3 \text{ cfu ml}^{-1}$  and

their maximum value was observed in September while low values were observed in April, June and July (Figure 5.2). In the 60  $\mu\text{m}$  plankton size category *Vibrio* density ranged between 0 and  $4.86 \times 10^3$  cfu ml<sup>-1</sup> being highest in September and low in the months of June and July. Also in 20  $\mu\text{m}$  plankton size category the vibrios densities ranged from 0 to  $1.9 \times 10^5$  cfu ml<sup>-1</sup> with the highest count observed in February, and the lower counts in May to July (Figure 5.2). The free-living vibrios densities varied between 0 and  $3.45 \times 10^1$  cfu ml<sup>-1</sup> (Figure 5.2). The seasonal variations in the *Vibrio* densities in the 180 and 60  $\mu\text{m}$  plankton size samples were significant ( $P < 0.05$ ), while the 20  $\mu\text{m}$  plankton size and free-living vibrios densities were not. Of the total of 56 isolated presumptive vibrios, 27 were identified as *Vibrio* species by API 20 NE system, while PCR assay was more sensitive and confirmed 52 of the isolates to belong to the *Vibrio* genus. Detection of target *Vibrio* species by PCR revealed the presence of *V. fluvialis* (36.5 %), *V. vulnificus* (34.6 %), and *V. parahaemolyticus* (23.1%). The presence of *V. metschnikovii* (5.8 %) was revealed only by API 20 NE identification. *Vibrio cholerae* was not detected neither by API 20 NE nor by PCR methods. The results of identification by both methods were combined to determine the total prevalence of the target species apart from *V. metschnikovii* which was only detected by API 20NE (Table 5.3).

#### 5.3.4 Statistical analysis of vibrios abundance and physico-chemical parameters

Using the natural log transformed *Vibrio* species abundance (lnVA) as the dependent variable and all other physicochemical factors as predictors, ANOVA yielded an F value of 12.037, indicating that all predictors were acting independently.

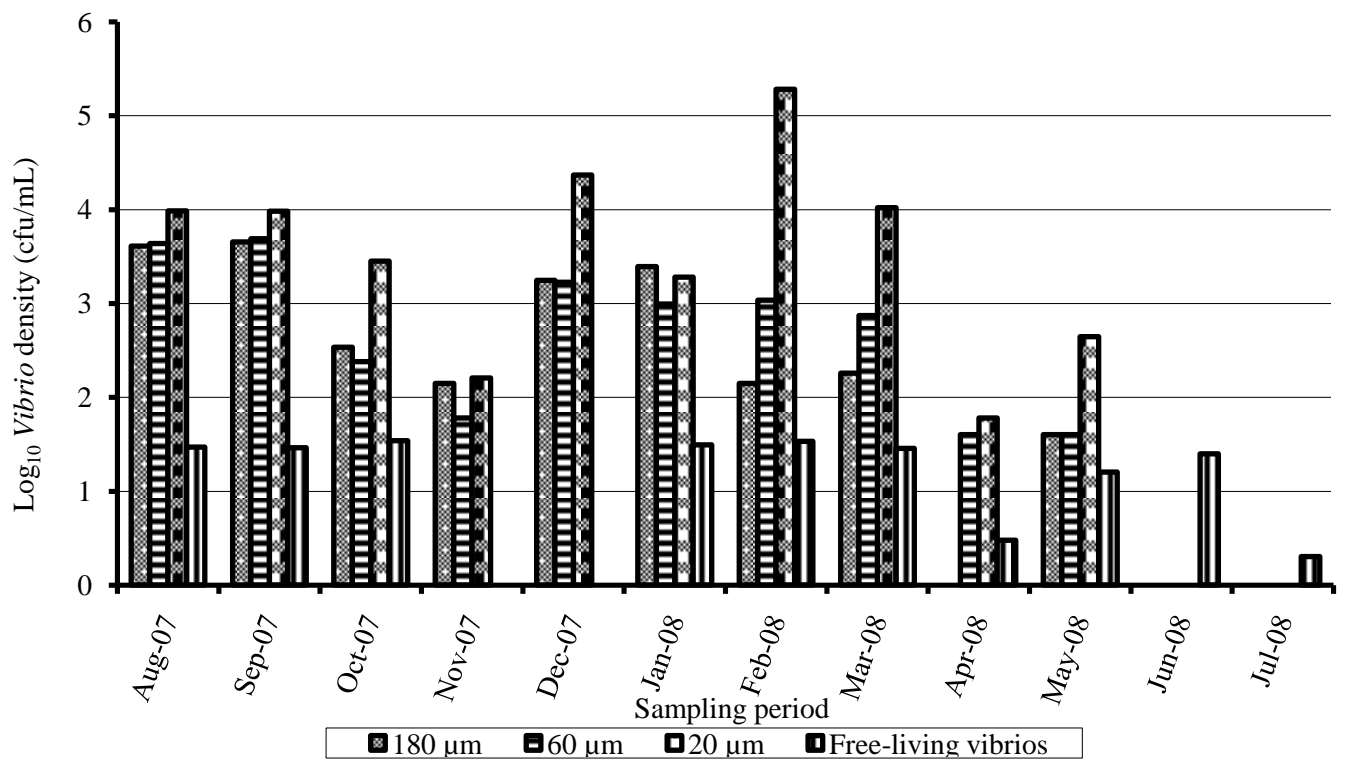


Figure 5.2: Abundance of total vibrios in the final effluents of the wastewater treatment plant.

The results of regression using the (InVA) as dependent and all other factors as independent showed that vibrios negatively correlated with salinity and temperature and positively correlated with pH and turbidity ( $P < 0.001$ ;  $P < 0.007$  respectively) (Table 5.4). Free-living vibrios abundance was found directly correlated with temperature, while vibrios associated with 20 µm plankton size showed a negative relationship with temperature, indicating a different seasonal activity, There was a positive relationship between densities of 180 and 60 µm plankton sizes, thus strongly suggesting that *Vibrio* species in these habitats survive the wastewater treatment process as plankton-associated biofilms.

Table 5.3: Prevalence of *Vibrio* pathogens in final effluents of the wastewater treatment plant.

Target <i>Vibrio</i> species	Plankton associated				Total prevalence (%)
	180 µm	60 µm	20 µm	Free-living	
<i>V. cholerae</i>	-	-	-	-	-
<i>V. parahaemolyticus</i>	5/13 (38.4 %)	3/13 (23.1 %)	2/13 (15.4 %)	2/13 (15.4 %)	12/52 (23.1%)
<i>V. vulnificus</i>	6/13 (46.2 %)	5/13 (38.5 %)	4/13 (30.8 %)	3/13(23.1 %)	18/52 (34.6 %)
<i>V. fluvialis</i>	2/13 (15.4 %)	5/13 (38.5 %)	7/13 (53.8 %)	5/13 (38.5 %)	19/52 (36.5 %)
<i>V. metschnikovii</i>	-	-	-	3/13 (23.1 %)	3/52 (5.8 %)

Table 5.4: Linear regression coefficients of *Vibrio* species and physicochemical parameters.

<b>Independent Variable</b>	<b>Regression coefficient</b>	<b>Standardized coefficients</b>	<b>Significant value (P-value)</b>
Temperature	-0.203	-0.978	0.002
pH	0.464	0.996	0.001
Salinity	-0.918	-1.000	0.001
Turbidity	0.050	0.664	0.007
Chlorine residual	0.475	0.667	0.003
Log <sub>10</sub> 180 µm	0.666	0.908	0.010
Log <sub>10</sub> 60 µm	0.533	0.466	0.004
Log <sub>10</sub> 20 µm	-0.333	-0.275	0.012
Log <sub>10</sub> free-living vibrios	0.325	0.407	0.024

## 5.4 Discussion

Vibrios are halophilic bacteria found in effluent environments associated with domestic sewage. Several studies have reported its presence in the treated effluents [12, 25, 26]. In the herein presented study we could show, that the investigated area is subject to a wide spatial fluctuation of *Vibrio* pathogens contamination probably as a consequence of inefficiently treated effluent.

Abundances of free-living vibrios, small and large plankton associated *Vibrio* species show higher seasonal fluctuation rate.

The wide spatial distribution of *Vibrio* species found in this study is comparable to the report by Gugliandolo et al. [12]. Free-living vibrios abundance was found directly correlated with temperature, while vibrios associated with 20 µm plankton showed a negative relationship with temperature, indicating a different seasonal behaviour. Also, the statistically significant difference between the densities of free-living and plankton-associated vibrios suggests that *Vibrio* species in the final effluent habitat are of independent existence.

According to White [38] the most prevalent practice of disinfection is the use of free chlorine which dissociate into molecular hypochlorite and the hypochlorite ion. Although the 1996 South African Water Quality Guidelines do not specify any standard for free chlorine residual in treated effluent. This study considered those for domestic water supplies which recommended a range of 0.3 to 0.6 mg l<sup>-1</sup> as ideal free chlorine residual concentration and 0.6-0.8 mg l<sup>-1</sup> as good free chlorine residual concentration with insignificant risk of health effects [28]. Based on this concentration, the free chlorine residual in the effluents complied with the regulatory standard, but failed to eliminate the *Vibrio* species (Figure 2). Previous studies have reported the potentials of bacterial growth in the treated water with chlorine residual concentrations up to 1 mg/l [30, 23]. Hence, there is need for a more efficient disinfection procedure to eliminate the *Vibrio* pathogens from the final effluents.

The abundance of *Vibrio* species in the final effluent has been linked to temperature, while its relationship to salinity is less clear. The level of vibrios is higher during summer than during winter. In this study, *Vibrio* species appear to be more abundant at a temperature range between 17.07 and 27.15°C, indicating a strong dependence of the culturable forms of the

pathogens on temperature. Similarly, the salinity profile observed in this study was consistently lower than the maximum requirement for optimal growth of vibrios [20]. Depending on the range of salinities encountered, some previous studies [8, 9] have found a significant relationship between salinity and the abundance of total *Vibrio* species. One study [41] has also reported a positive correlation between *Vibrio* species and turbidity, which is also consistent with the observation reported by Watkins and Cabelli [40] that found *V. parahaemolyticus* levels in water to be strongly correlated with turbidity during summer, when water temperatures are relatively constant. It has been hypothesized that higher nutrient level associated with highly turbid and polluted waters may stimulate growth of vibrios. [41]. Our results also appear consistent with those of Watkins and Cabelli [40] who found poor correlation between *V. parahaemolyticus* levels and pH of the water.

Large numbers of plankton organisms are known to rise toward the surface of the water at night and sink downward during the day [42, 43, 44]. Light has been the main environmental factor and the primary stimulus that produces diurnal migration of zooplankton [45], such that when light fades, zooplankton moves towards the surface to feed on bacteria and when intensity of light increases (daybreak) zooplankton moves downward. However, Gilbert and Hampton [46] reported that the zooplankton *Polyarthra remata* were most abundant near the surface during the day. Reference materials are limited and though it may seem reasonable, it was not possible for us to investigate this parameter in this current study.

The occurrence of *Vibrio* species as plankton-associated entities confirms the role of plankton as a potential reservoir of *Vibrio* pathogens [12, 34] and is corroborated by previous findings on the association of pathogenic bacteria with planktonic organisms in the Mediterranean environment [5, 11, 26]. The major free-living *Vibrio* species identified in final effluent during the study regime was *V. vulnificus* which is considered a pathogenic *Vibrio*

species, particularly of wound infections, gastroenteritis, or a syndrome known as primary septicemia in individuals exposed to marine environment contaminated with effluents [12]. Currently, *V. fluvialis* has been shown to produce an enterotoxin and is known to cause serious infections because its clinical symptoms of gastroenteritis are very similar to those caused by *V. cholerae* O1 and non-O1 strains.

### **5.5 Conclusion**

This study has shown that *Vibrio* pathogens very easily survived the treatment processes of the rural wastewater treatment facility either as free-living organisms or as plankton associated entities. Moreover the *Vibrio* pathogens appear to be resistant to chlorine disinfection at normal recommended concentrations, thus posing a potential health risk to the rural communities who depend on the receiving watershed for domestic and recreational purpose. There is need for the intervention of the appropriate regulatory agencies to ensure compliance of the wastewater treatment facilities to regulatory effluent quality standards.

### **5.6 Acknowledgement**

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## CHAPTER 6

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**Seasonal abundance and distribution of total *Vibrio* species  
in final effluents of wastewater treatment facilities in typical  
sub-urban and urban communities of the Eastern Cape  
Province, South Africa**

*This chapter has been submitted in this format for publication in Applied and Environmental  
Microbiology*

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## CHAPTER 6

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## CHAPTER 6

### **Seasonal abundance and distribution of total *Vibrio* species in final effluents of wastewater treatment facilities in typical sub-urban and urban communities of the Eastern Cape Province, South Africa**

#### **Abstract**

We assessed the seasonal abundance and distribution of total *Vibrio* species in the final effluents of some wastewater treatment plants (WWTP) located in typical sub-urban and urban communities of the Eastern Cape Province, South Africa, as well as some selected environmental parameters. Population density of total *Vibrio* ranged from  $2.1 \times 10^1$  to  $4.36 \times 10^4$  cfu/ml and from  $2.80 \times 10^1$  to  $1.80 \times 10^5$  cfu/ml for the sub-urban and urban communities respectively. *Vibrio* species associated with 180  $\mu\text{m}$ , 60  $\mu\text{m}$ , 20  $\mu\text{m}$  plankton sizes, were observed at densities of 0 -  $1.36 \times 10^3$  cfu/ml, 0 -  $8.40 \times 10^2$  cfu/ml and 0 -  $6.80 \times 10^2$  cfu/ml respectively at the sub-urban community's WWTP. Also, in the urban community, counts of culturable vibrios ranged from 0 -  $2.80 \times 10^2$  cfu/ml (180  $\mu\text{m}$ ); 0 -  $6.60 \times 10^2$  cfu/ml (60  $\mu\text{m}$ ) and 0 -  $1.80 \times 10^3$  cfu/ml (20  $\mu\text{m}$ ). Abundance of free-living *Vibrio* species varied between 0 and the orders of  $10^2$  and  $10^3$  cfu/ml in the sub-urban and urban communities' WWTPs respectively. Molecular confirmation of the presumptive vibrios isolates revealed the presence of *V. fluvialis* (41.38 %), *V. vulnificus* (34.48 %), and *V. parahaemolyticus* (24.14%) in the sub-urban community effluents. Also, in the urban community *V. fluvialis* (40 %), *V. vulnificus* (36 %), and *V. parahaemolyticus* (24%) were observed. There was no significant correlation between *Vibrio* abundance and season, either as free-living or plankton-associated entities, while *Vibrio* species abundance correlated positively

with temperature ( $r = 0.565$ ;  $P < 0.01$ ), salinity and dissolved oxygen ( $P < 0.05$ ). Turbidity and pH showed significant seasonal variation ( $P < 0.05$ ) across the seasons in both locations. This study underscores the potentials of waste treatment plants as sources of vibrios pathogens in the watershed of sub-urban and urban communities of South Africa.

## 6.1 Introduction

*Vibrio* species a halophilic Gram-negative, bacterium that occur naturally in aquatic environments and is transmitted to humans primarily through consumption of contaminated waters, raw or mishandled seafood (4, 32). Gastroenteritis which usually is associated with *Vibrio* infections causes bloody diarrhoea, but primary septicemia can occur in individuals with underlying chronic illness (4). A number of *Vibrio* infection outbreaks have been reported in many developed and developing countries thus igniting interests in this pathogen and its ecology (8, 34, 15).

Although they require a host for growth, vibrios are common in natural waters, such as streams, rivers and lakes which have been impacted by inefficiently treated effluents discharge from wastewater treatment plants, runoffs from agriculture lands and direct contamination by faeces of wild birds and animals (10, 15, 29). Once *Vibrio* species are introduced into the environment, the survival or persistence of these organisms depends on many factors such as oxygen content, the presence of nutrients, temperature, and pH (8, 15, 34). Previous studies have demonstrated strong relationship between *Vibrio* abundance and environmental conditions such as salinity, temperature and attachment to planktonic organisms (11, 24, 40). Additionally, *Vibrio* species may enter a viable but non-culturable state (where organisms are not able to grow in culture but remain metabolically active), as first described by Rollins and Colwell (37). The

viable but non-culturable state has been considered to play a role in prolonging vibrios species survival in the environment (13).

*Vibrio* pathogens are distributed throughout the world, but reported densities in the environment and treated effluents vary greatly according to season, location, sample type and analytical methods employed (6, 26, 36). Most Vibriosis cases in the world occur in the summer and there are declines in the fall and winter (4, 38). It has been suggested that changes in food handling, water supplies and consumption in the summer months may lead to this predictable seasonality (38). The seasonal cycles of *V. parahaemolyticus*, in sediment, water and plankton in the US was first reported by Kaneko and Colwell (16, 17) in the Chesapeake Bay and *V. parahaemolyticus* was detected in sediment during the winter months. In the Pacific Northwest, it was reported that *V. parahaemolyticus* was detectable only during the summer months, when water temperatures ranges from 15-22 °C (18, 19). Duan and Su (9), reported that 15 % of oysters, 20 % of seawater, and 48 % of sediment samples collected bi-weekly from two Oregon oyster-growing areas over one year period were positive for total *V. parahaemolyticus*. Densities of *V. parahaemolyticus* in both growing areas were found to be positively correlated with water temperature, and higher densities of *V. parahaemolyticus* vary considerably even at optimal temperatures, and other possible links between this variability and other environmental factors remain uncertain (35, 42). Turbidity and pH levels have been previously hypothesized to correlate with vibrios densities (12, 23, 42).

Over the past few years, the Southern African region has been plagued by outbreaks of vibrios related waterborne infections suspected to be linked to inefficiently treated effluents discharge from wastewater treatment facilities. In this paper, we report the seasonal abundance of total *Vibrio* species in the final effluents of two wastewater treatment plants in the Eastern

Cape Province of South Africa as well as their relationships with some environmental parameters.

## **6.2 Material and Methods**

### *6.2.1 Study site*

The wastewater treatment plants are located in Dimbaza [sub-urban community] and East London [urban community] with the geographical coordinates of 32°51'28"S, 27°35'29"E and 33°02'07"S, 28° 16'17"E respectively in the Eastern Cape Province of South Africa. The Dimbaza plant is designed to treat an average of dry weather flow of 7 000 m<sup>3</sup>/day and an average wet weather flow of 21 000 m<sup>3</sup>/day, and receives domestic sewage, industrial wastewater and run-off water. It discharges its final effluents into a stream that empties into the Tembisa River. The East London wastewater treatment plant have a higher average daily inflow of 36 200 m<sup>3</sup>/day due to its higher number of industries and high population of residents. It discharges its final effluents into the Indian Ocean between Nahoon and Eastern Beach at Bats Cave. Both treatment facilities are based on the activated sludge system.

### *6.2.2 Treatments of samples*

Wastewater final effluent samples were collected monthly between August 2007 and July 2008. All samples were collected aseptically using sterile 1 L Nalgene bottles containing 0.5 ml of sterile concentrated sodium thiosulphate solution to give a final concentration of 100 mg/l, and transported on ice to the laboratory for analyses. Samples were stored at 4 °C until analyses were completed. All samples were processed within 24 h of collection.

As described by Alam et al. (1) one liter of wastewater was filtered successively through 180  $\mu\text{m}$ , 60  $\mu\text{m}$  and 20  $\mu\text{m}$  nylon nets (Millipore Corp., Bedford, MA), sequentially arranged in that order to sterile collection bottles. The water that flowed through the 20  $\mu\text{m}$  pore size nylon nets were collected in sterile containers for free-living *Vibrio* cells analyses. After filtration, each of the nylon nets and their content were suspended in 25 ml physiological-buffered saline containing sterile glass beads (0.1 mm BioSpec Products) and homogenized for 2 min in a glass homogenizer at  $3,000 \times g$  to dislodge the attached bacteria, and the homogenates used for direct plating.

### 6.2.3 Physicochemical analyses

The temperature, salinity, turbidity, dissolved oxygen and pH of the final effluent samples were determined as described elsewhere (14), while the free residual chlorine were estimated using a multi-parameter ion-specific meter (Hanna BDH-laboratory). All analyses were carried out in triplicate.

### 6.2.4 Estimation of total *Vibrio* species densities

For direct plate count analyses of plankton free samples, the samples were serially diluted and aliquots pour-plated using thiosulphate-citrate-bile-salts-sucrose (TCBS) agar and incubated at 37 °C for 24 h. For the plankton associated samples, the abundance of *Vibrio* spp. was obtained by inoculating aliquots of concentrated homogenates (0.5  $\mu\text{l}$ ) onto thiosulfate-citrate–bile salt-sucrose (TCBS) agar and incubating at 37 °C for 24 h in accordance with the description of Alam et al. (1). Yellow and green colonies were considered total presumptive *Vibrio* colonies and counted as described elsewhere (2).

### 6.2.5 Isolation and biochemical identification of *Vibrio* species

Aliquots of the plankton free and plankton associated samples were inoculated into alkaline peptone water (APW) and incubated aerobically at 37 °C for 18-24 h. Turbid cultures were streaked onto TCBS agar incubated at 37 °C for 24 h. Five to ten isolated colonies per plate were randomly picked from each samples and subsequently purified on fresh TCBS agar plates. The pure isolates were subjected to Gram staining and oxidase test. Only Gram-negative, oxidase-positive isolates were selected for biochemical identification using API 20 NE kit. The strips were then read and the final identification was secured using API lab plus software (bioMerieux, Marcy l'Etoile, France).

### 6.2.6 Molecular identification

Polymerase chain reaction (PCR) was used to confirm the identities of the *Vibrio* species using the species-specific primers described in Table 6.1. Deoxyribonucleic acid (DNA) extraction and PCR were carried out as described by Maugeri et al. (25) with little modification. Single colonies of presumptive vibrios strains grown overnight at 37 °C on TCBS agar plates were picked, suspended in 200 µl of filtered distilled water and bacterial cells were collected by centrifugation at  $11,000 \times g$  for 10 min at 4 °C. The pellet was suspended in 100 µl of filtered and autoclaved water and boiled for 10 min. The cell lysates (10 µl) were used as template in the PCR assays immediately after extraction or following storage at -20 °C. The thermal cycling profile for *Vibrio vulnificus*, and *Vibrio parahaemolyticus* was as follows: a 15 min denaturation at 93 °C followed by 35 cycles at 92 °C for 40 s, 57 °C for 1 min and 72 °C for 1.5 min and final extension at 72 °C for 7 min. The amplified products were held at 4 °C after completion of the cycles. For *Vibrio fluvialis* the amplification conditions were initial denaturation at 94 °C for 5

Table 6.1: Details of primers used in this study.

Target species	Primer name	Primer sequences (5' - 3')	Target gene	Amplicon size (bp)	Reference
All <i>Vibrio</i> spp.	V. 16S-700F	5'-CGG TGA AAT GCG TAG AGA T-3'	16S rRNA	663	(21)
	V. 16S-1325R	5'-TTA CTA GCG ATT CCG AGT TC-3'			
<i>V. parahaemolyticus</i>	Vp. <i>flaE</i> -79F	5'-GCA GCT GAT CAA AAC GTT GAG T-3'	<i>flaE</i>	897	(41)
	Vp. <i>flaE</i> -934R	5'-ATT ATC GAT CGT GCC ACT CAC-3'			
<i>V. vulnificus</i>	Vv. <i>hsp</i> -326F	5'-GTC TTA AAG CGG TTG CTG C-3'	<i>hsp60</i>	410	(43)
	Vv. <i>hsp</i> -697R	5'-CGC TTC AAG TGC TGG TAG AAG-3'			
<i>V. fluvialis</i>	Vf- <i>toxR</i> F	5'-GAC CAG GGC TTT GAG GTG GAC GAC--3'	<i>toxR</i>	217	(5, 33)
	Vf- <i>toxR</i> R	5'-AGG ATA CGG CAC TTG AGT AAG ACTC-3'			

min, followed by 30 cycles consisting of denaturation at 94 °C for 40 s, annealing at 65 °C for 40 s and extension at 72 °C for 1 min. The PCR products were electrophoresed in 1.5 % agarose containing 0.5 mg/l ethidium bromide for 1 h at 100 V and then visualized using a UV transilluminator.

### *6.2.7 Statistical analysis*

Total *Vibrio* species counts were analyzed by regression of the mean log<sub>10</sub> density of replicate samples against environmental factors (temperature, turbidity, pH, salinity and dissolved oxygen). For seasonal analyses, seasons were defined as follows: summer (November to March); autumn (April to May); winter (June to August); spring (September to October) according to South African Weather Service. A test of significance of observed differences in *Vibrio* species abundance and environmental parameter across the two sampling sites was conducted using one-way analysis of variance (ANOVA). All statistical analyses were performed using Statistical Analysis System version 8 (SAS Institute, Cary, NC).

## **6.3 Results**

### *6.3.1 Effects of environmental parameters on the abundance and distribution of Vibrio species*

#### *6.3.1.1 Temperature*

Total *Vibrio* species counts in the final effluents were compared with environmental parameters. Overall, the temperature of the final effluents ranged from 14.4 °C (July - 08) to 26.5 °C (March - 08). Temperature of the effluents from the sub-urban community treatment plant varied between 14.5-23.3 °C, while that situated in the urban community ranged from 17.8 to 26.5 °C respectively (Fig. 6.1A). Regression analysis indicated a significant positive association between

*Vibrio* abundance and temperature in sub-urban ( $P < 0.001$ ; Table 6.2). The abundance of total *Vibrio* species in urban location was seasonal and correlated ( $r = 0.58$ ,  $P < 0.001$ ; Table 6.2) with temperature.

#### 6.3.1.2 Turbidity

The turbidities of the effluents ranged from 2.16 NTU (January - 08) to 17.5 NTU (August - 08) (Fig. 6.1B), and varied significantly ( $P < 0.05$ ) with season at the two study locations. *Vibrio* species counts significantly correlated with turbidity ( $P < 0.0001$ ; Table 6.2).

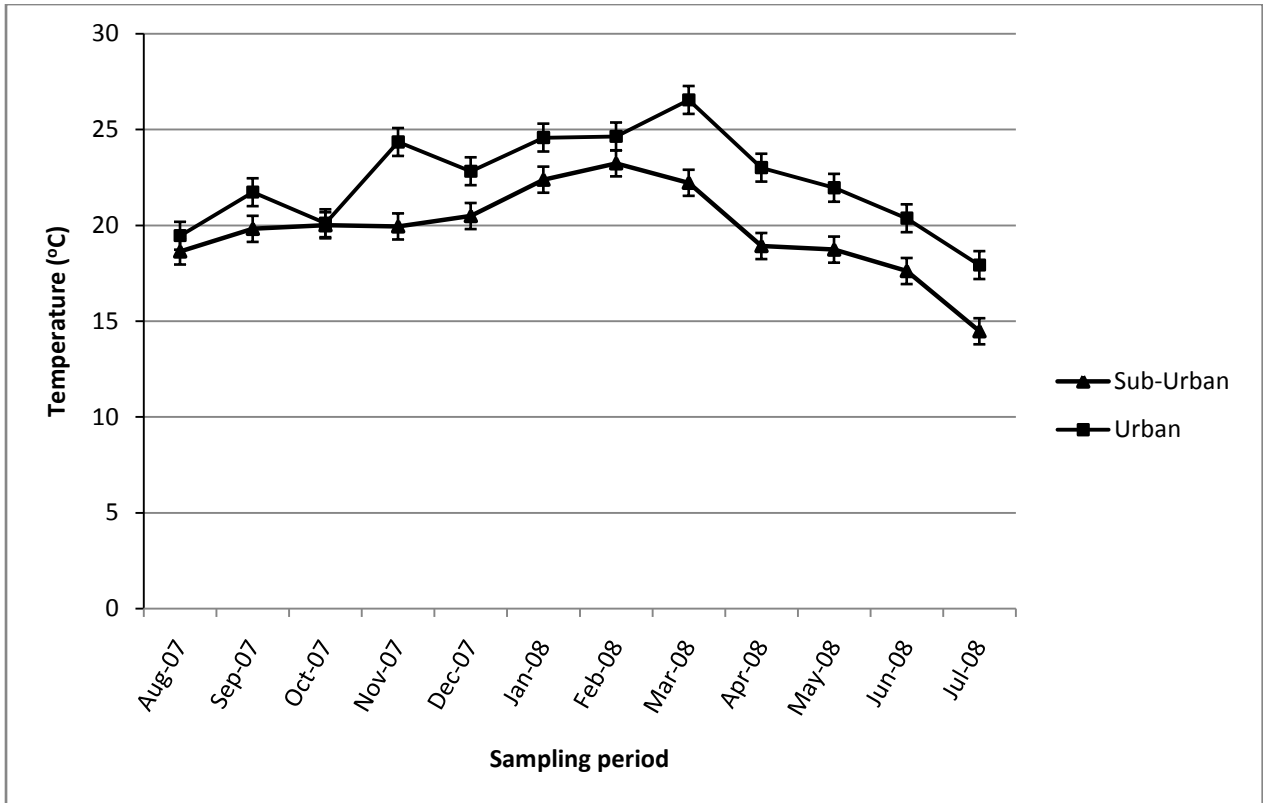
#### 6.3.1.3 pH

The pH values of the final effluents ranged from 6.63 (July - 08) to 7.74 (August - 07) and vary significantly with season ( $P < 0.05$ ). The annual mean final effluent pH was 7.04 and 7.14 for sub-urban and urban respectively (Fig. 6.2). There was marginal evidence of an association between *Vibrio* species counts and pH.

#### 6.3.1.4 Salinity

The salinity of the effluents ranged from 0.11 psu (January -08) to 0.82 psu (April -08) (Fig. 6.3A). The annual mean salinity was 0.13 psu and 0.42 psu at sub-urban and urban communities' plants respectively. Also, the mean salinity at the sub-urban location, which had the lowest incidence of *Vibrio* species, was found to be significantly lower than that of the urban community. However, with regression analysis, salinity was found to be only marginally associated with  $\log_{10}$  total *Vibrio* species counts ( $P < 0.05$ ; Table 6.2).

A



B

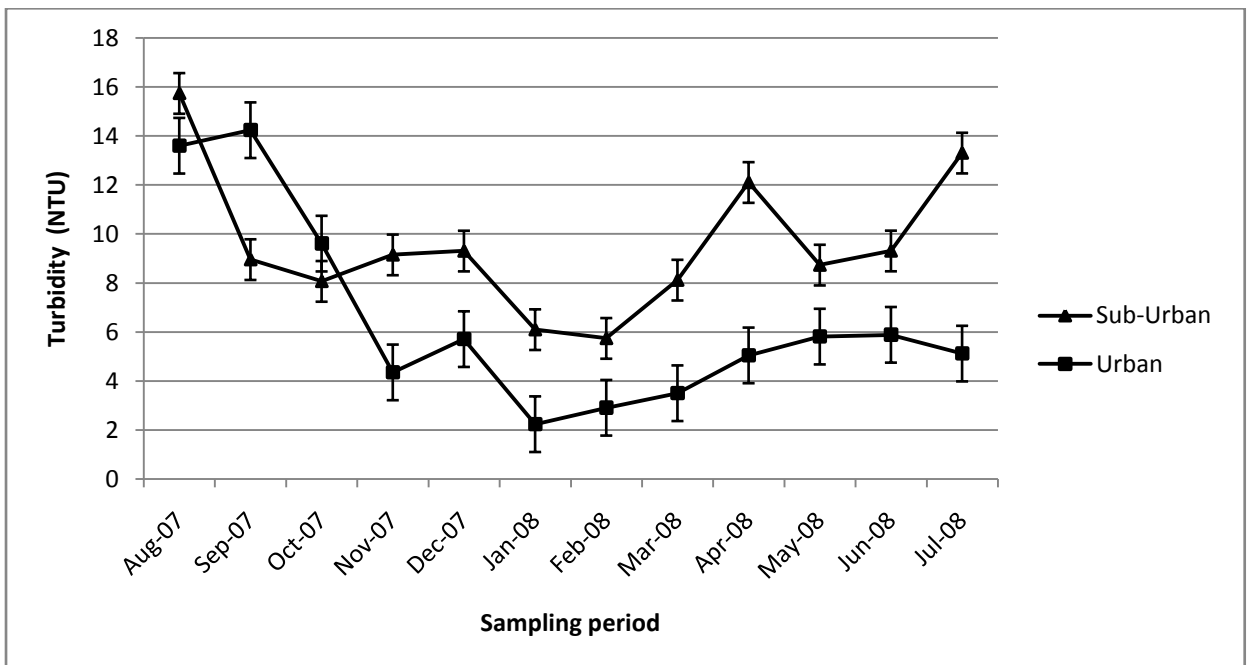


Figure 6.1: Profile of (A) temperature and (B) turbidity of the final effluents of the sub-urban and urban communities' wastewater treatment plants.

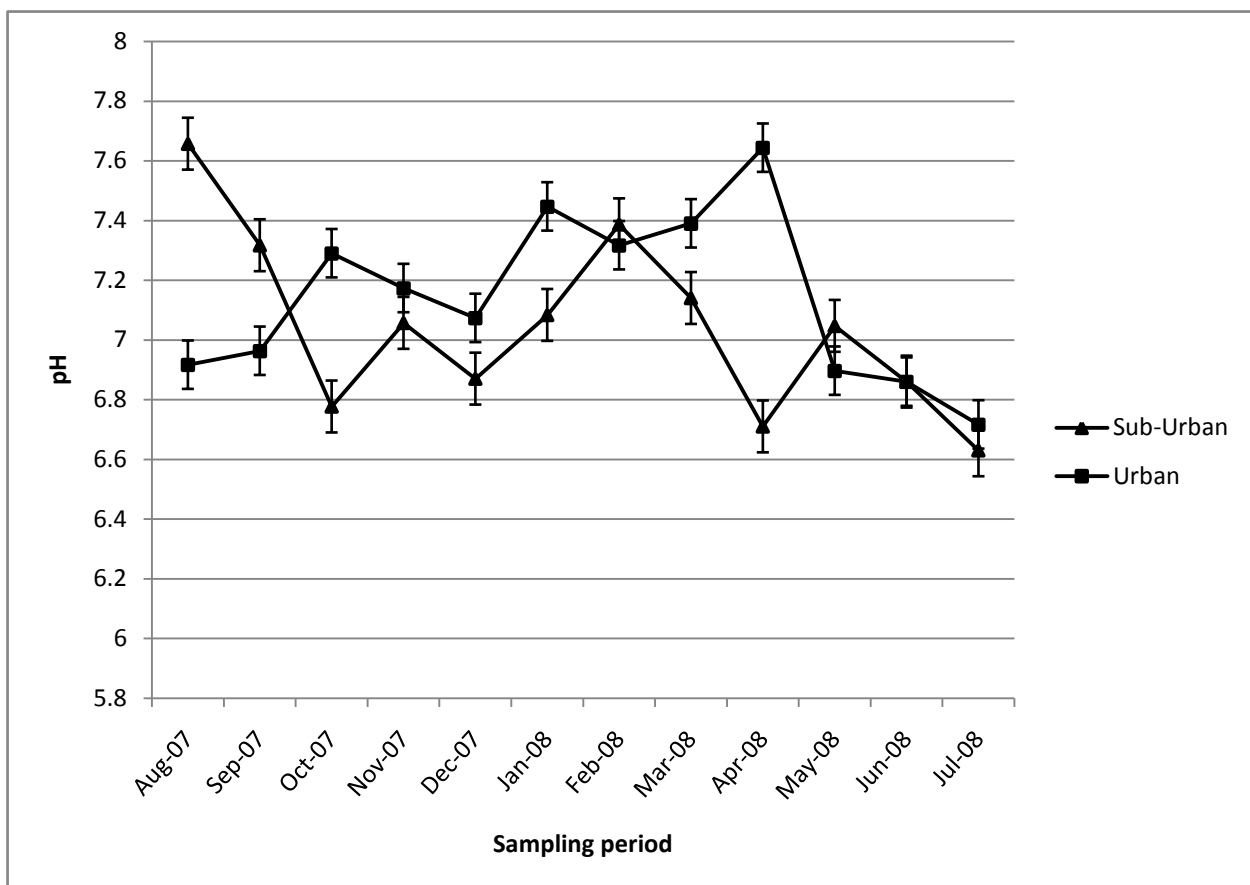
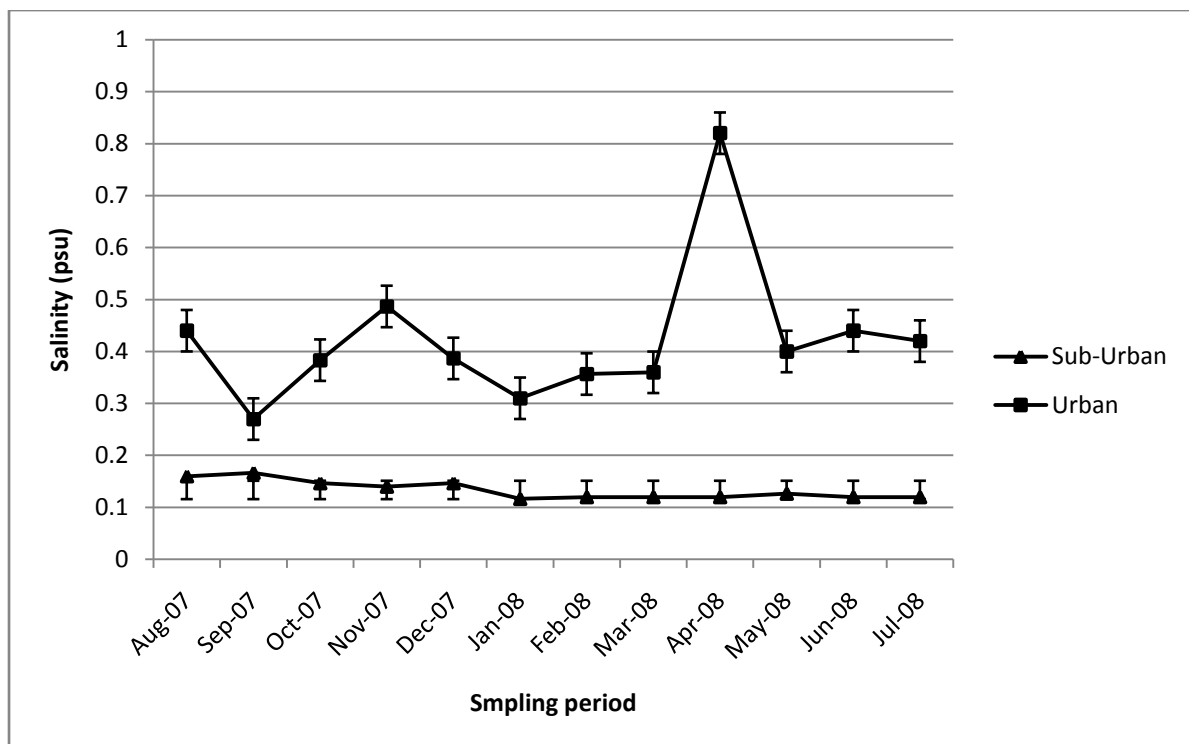


Figure 6.2: Profile of pH of the final effluents of the sub-urban and urban communities' wastewater treatment plants.

A



B

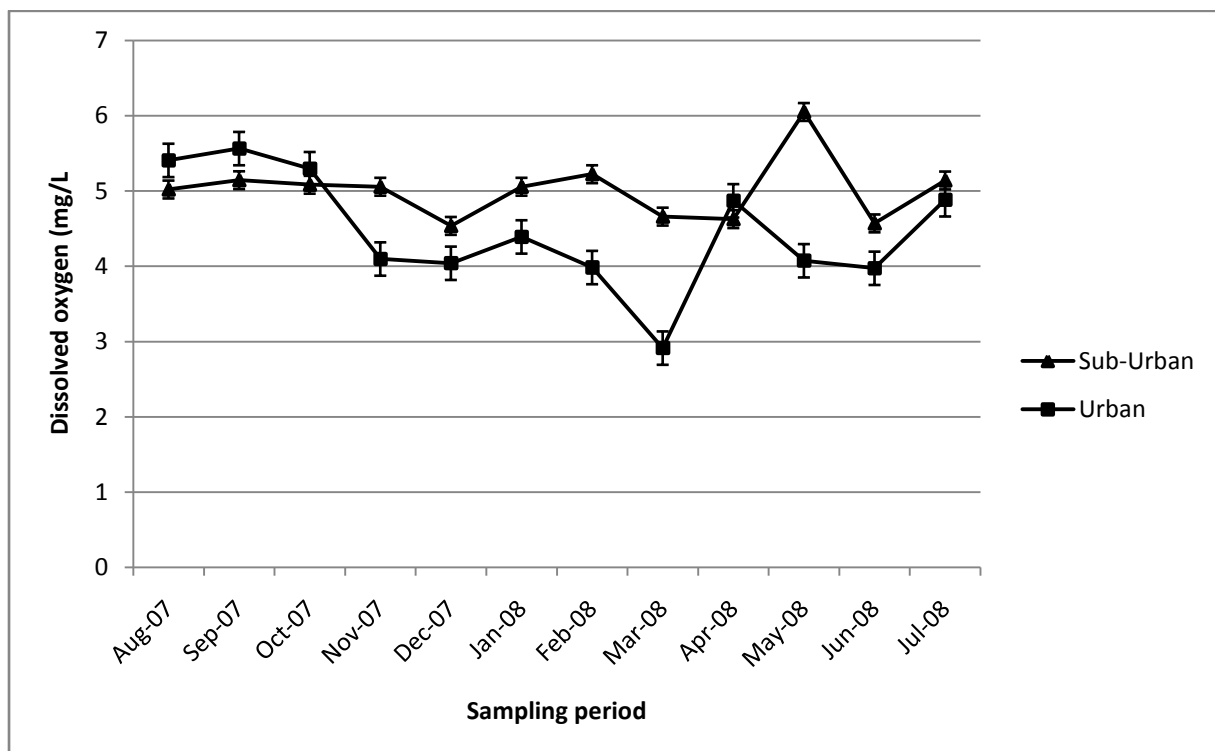


Figure 6.3: Profile of (A) salinity and (B) dissolved oxygen of the final effluents of the sub-urban and urban communities' wastewater treatment plants.

### 6.3.1.5 Dissolved oxygen

The dissolved oxygen (DO) concentration varied significantly ( $P < 0.0001$ ) with season and ranged between 2.38 mg/l (March - 08) and 6.78 mg/l (May - 08) (Fig. 6.3B), with annual means of 5.01 mg/l and 4.45 mg/l for the sub-urban and urban communities' plants effluents respectively. It is also positively associated with *Vibrio* species abundance ( $P < 0.05$ ), and correlated inversely with effluents temperature ( $r = - 0.76$ ,  $P < 0.001$ ).

Table 6.2: Linear regression analysis of  $\log_{10}$  total *Vibrio* species versus environmental variables.

<b>Independent Variable</b>	<b>Regression coefficient</b>	<b>Standardized coefficients</b>	<b>Significant value (P-value)</b>
Temperature	0.6728	1.1964	0.001
pH	0.0581	0.682	0.05
Turbidity	0.2335	0.9884	0.0001
Salinity	0.0459	0.9264	0.012
Dissolved oxygen	0.5897	0.8926	0.05
Chlorine residual	0.034	0.742	0.091
Total vibrios counts	0.6941	0.8687	0.001

### 6.3.2 Concentration of free chlorine residual in the final effluents

The chlorine residual of the final effluent varied significantly ( $r = 0.840$ ;  $P < 0.001$ ) across the seasons and among the plants ranging from 0.07 mg/l in the month of September to the highest concentration of 3.85 mg/l in the month of October (Fig. 6.4). There is no legislation that regulates the concentration of chlorine residual in treated final effluent in South Africa at present, and as such we considered those for domestic water supplies which recommend ranges of 0.3 to 0.6 mg/l (27).

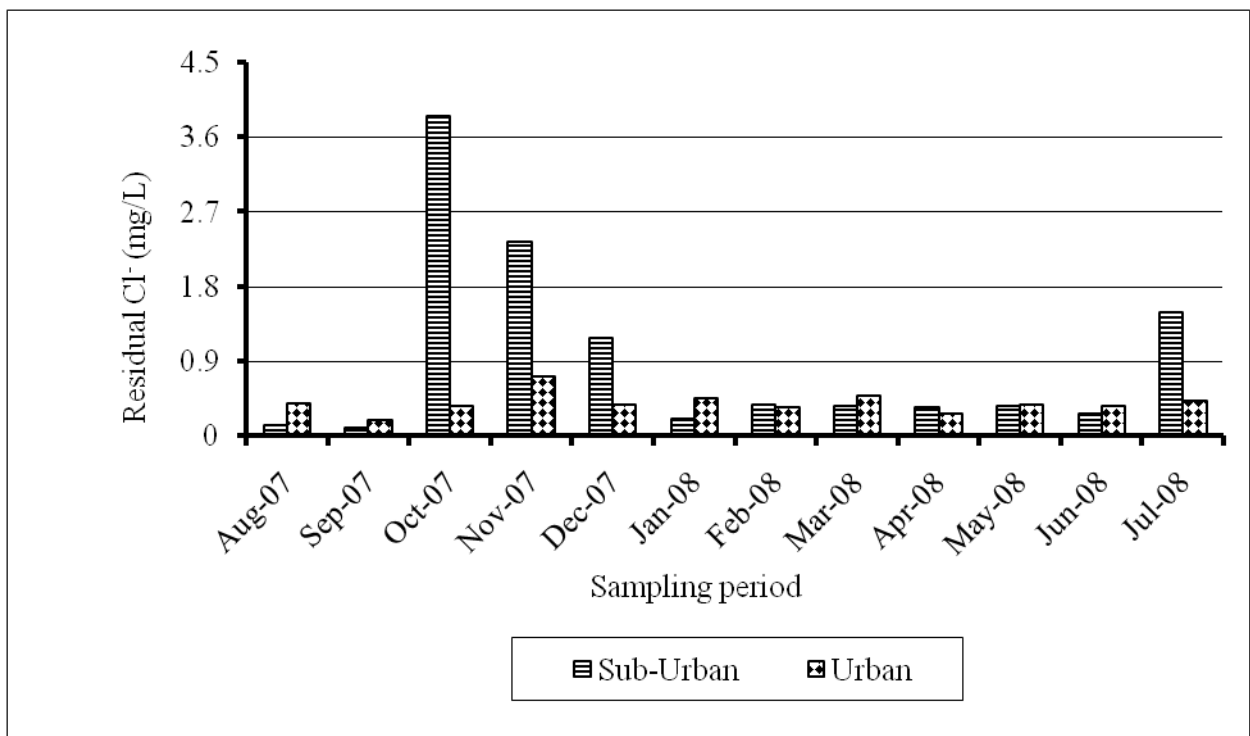


Figure 6.4: Concentrations of free chlorine residual (mg/L) in the final effluents of sub-urban and urban communities' wastewater treatment plants.

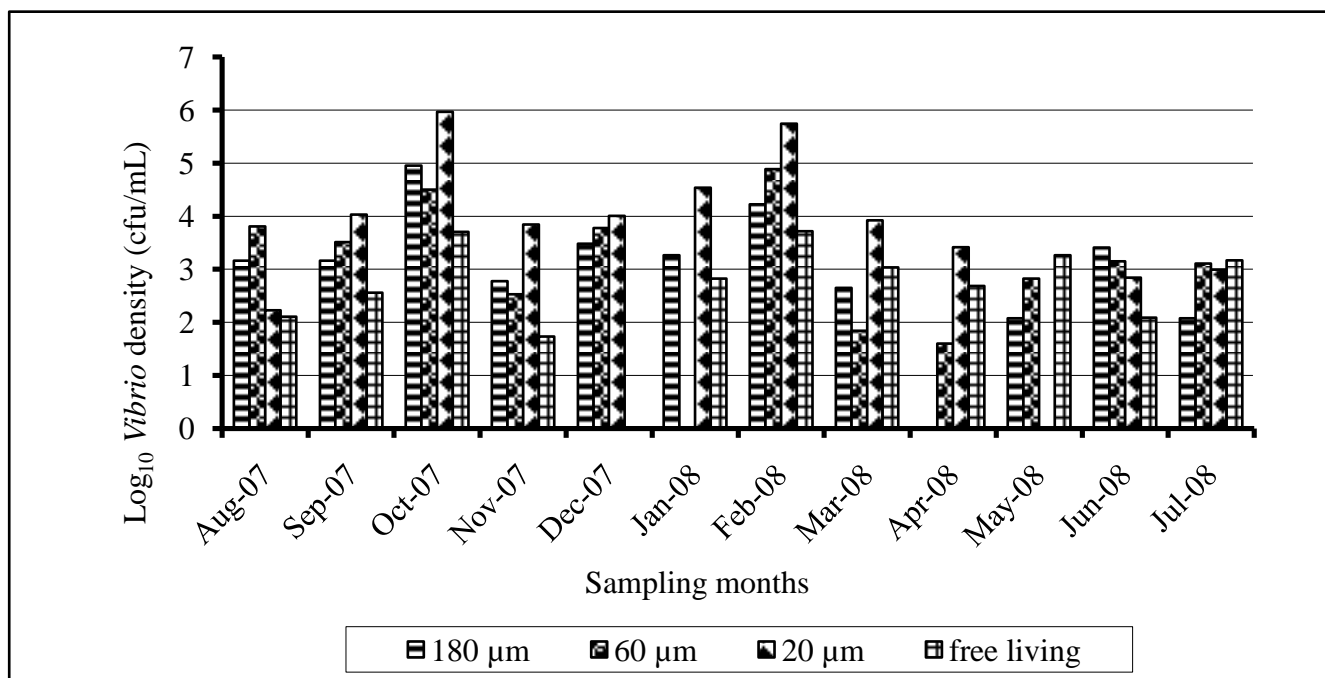
### 6.3.3 Seasonal abundance and distribution of total *Vibrio* species

Similar seasonal trends in total vibrios abundance at the two sampling sites are as shown in Figs. 6.5A & B. Total *Vibrio* counts ranged from  $2.1 \times 10^1$  to  $4.36 \times 10^4$  cfu/ml and from  $2.80 \times 10^1$  to

$1.80 \times 10^5$  cfu/ml for sub-urban and urban communities' plants respectively, although these variations were not significant. The lowest count was observed during the winter in the month of May 2008 while the highest count was observed in summer month of October 2007 at the sub-urban plant. For the urban community plant, the lowest *Vibrio* density was observed during the winter month of June 2008, while the highest was observed in summer month of February 2008. Abundance of free-living *Vibrio* species varied between 0 and  $8.0 \times 10^2$  cfu/ml and 0 and  $1.52 \times 10^3$  cfu/ml for the sub-urban and urban plants' effluents respectively. *Vibrio* species associated with 180  $\mu$ m, 60  $\mu$ m, 20  $\mu$ m plankton sizes, were observed at densities of 0 -  $1.36 \times 10^3$  cfu/ml, 0 -  $8.40 \times 10^2$  cfu/ml and 0 -  $6.80 \times 10^2$  cfu/ml respectively at the sub-urban community plant. Also in the urban location, counts of culturable vibrios ranged from 0 -  $2.80 \times 10^2$  cfu/ml (180  $\mu$ m); 0 -  $6.60 \times 10^2$  cfu/ml (60  $\mu$ m) and 0 -  $1.80 \times 10^3$  cfu/ml (20  $\mu$ m). There was no significant correlation between *Vibrio* abundance and season either as free-living or plankton-associated species. The counts at both sites followed a seasonal trend, with higher *Vibrio* counts in the warmer months.

Of a total of 66 and 60 isolated presumptive vibrios isolated from the sub-urban and urban locations respectively, 31 (sub-urban) and 23 (urban) were identified as *Vibrio* species by API 20 NE system, while PCR assay confirmed 58 (sub-urban) 50 (urban) of the isolates to belong to *Vibrio* genus. Detection of the target *Vibrio* species by PCR revealed the presence of *V. fluvialis* (41.38 %), *V. vulnificus* (34.48 %), and *V. parahaemolyticus* (24.14%), in the sub-urban location. Also, in the urban location, PCR revealed *V. fluvialis* (40 %), *V. vulnificus* (36 %), and *V. parahaemolyticus* (24%). *V. fluvialis* is the most predominant species isolated from both sites. The results of identification by both methods were pooled to determine the total occurrence of the target species (Table 6.3).

### A. Sub-urban



### B. Urban

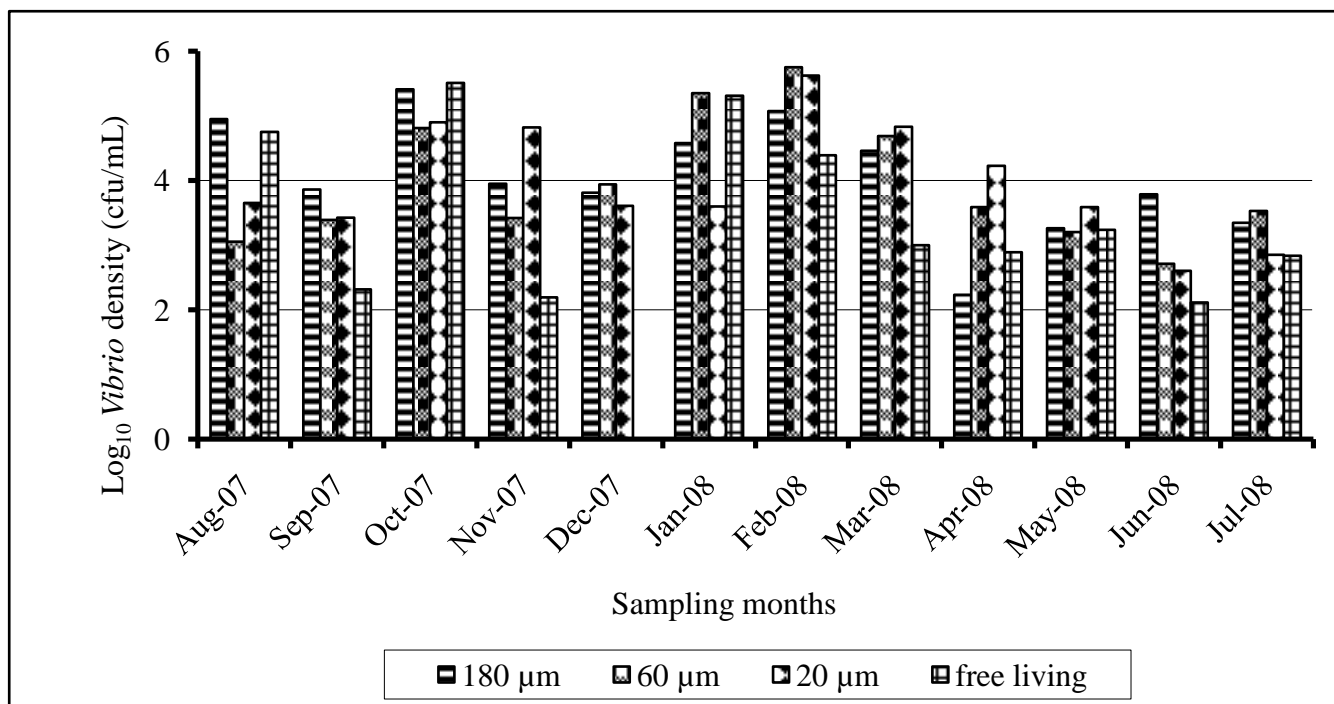


Figure 6.5: Densities of total vibrio species from the final effluents of wastewater facilities in sub-urban (A) and urban communities (B).

Table 6.3: Prevalence of *Vibrio* pathogens in final effluents of the wastewater treatment facilities in sub-urban and urban communities.

Target <i>Vibrio</i> species	Sub-urban site					Urban site				
	Plankton associated				Total frequency (%)	Plankton associated				Total frequency (%)
	180 µm	60 µm	20 µm	Free-living		180 µm	60 µm	20 µm	Free-living	
<i>V. fluvialis</i>	6/14 (42.86 %)	7/15 (46.67 %)	5/15 (33.33 %)	6/14 (42.86 %)	24/58 (41.38 %)	5/12 (41.67 %)	4/13 (30.76 %)	5/11 (45.45 %)	6/14 (42.85 %)	20/50 (40 %)
<i>V. vulnificus</i>	4/14 (28.57 %)	5/15 (33.33 %)	6/15 (40 %)	5/14 (35.71 %)	20/58 (34.48 %)	3/12 (25 %)	6/13 (46.15 %)	4/11 (36.36 %)	5/14 (35.71 %)	18/50 (36 %)
<i>V. parahaemolyticus</i>	4/14 (28.57 %)	3/15 (20 %)	4/15 (26.67 %)	3/14 (21.43 %)	14/58 (24.14 %)	4/12 (33.33 %)	3/13 (23.07 %)	2/11 (18.18 %)	3/14 (21.43 %)	12/50 (24 %)

## 6.4 Discussion

The abundance of total *Vibrio* species in the final effluents of the two wastewater treatment facilities in Eastern Cape appeared to be influenced by season in agreement with previous reports (7, 8, 6, 9, 15). The abundance of *Vibrio* species appear to be generally affected by environmental variables, although on some occasion's vibrios abundance appeared to fluctuate independently of temperature. The correlation between temperature and total vibrios counts in this study corroborates our previous studies (15), and other authors have equally reported similar trends from Atlantic, Pacific and Gulf Coasts, as well as from Japan and Germany (6, 8, 9, 22). It was not surprising to detect a positive correlation between total vibrios counts and temperature in this study because a similar relationship was found in our earlier study conducted in a rural community of the Eastern Cape Province of South Africa (15).

Turbidity was relatively higher at sub-urban than at the urban location, and analysis indicated a positive association between turbidity and vibrios densities at the sub-urban site. Lower levels and less variability in turbidity may have obscured the effects of turbidity at the urban site. The association between turbidity and total vibrios densities at the sub-urban was generally consistent and statistically significant ( $P < 0.0001$ ). Increase in turbidity was associated with increasing *Vibrio* species level from the study. Recent studies (44, 15) have also reported a positive correlation between *Vibrio* species and turbidity. This is also consistent with the observations obtained by Watkins and Cabelli (42) that found *V. parahaemolyticus* levels in water to be strongly correlated with turbidity during the summer, when water temperatures are relatively constant. These authors hypothesized that higher nutrient levels associated with more turbid and polluted waters may have stimulated *V. parahaemolyticus* growth.

Watkins and Cabelli (42) reported *V. parahaemolyticus* levels in water to be strongly correlated with dissolved oxygen, which is in agreement, with the present study. However, the

Watkins and Cabelli (42) study was restricted to summer season, when water temperatures were relatively constant and during which the influence of other environmental parameters may likely increase.

Differences in salinity levels between the two sampling sites during the study period may account for some of the discrepancies in *Vibrio* species densities. A significant correlation between salinity and total vibrios densities in effluent was identified at the urban site, and the opposite trend observed at the sub-urban plant is likely a consequence of the lower and narrow range of salinity observed therein. Previous studies indicated that *V. parahaemolyticus* densities decrease as salinity increases (3, 7, 8). In the present study, the densities of total *Vibrio* species were generally positively associated with increasing salinity. Depending on the range of salinities encountered, some previous studies (6, 8) have found a significant relationship between salinity and the abundance of total *V. parahaemolyticus* which other studies (7, 36) have not. These differences between studies are likely a consequence of the difference in the range of variation of salinities and the sample sizes of the studies. In general, wide ranges (5.6-34 ppt) of salinities were observed in the Cook et al. (6) study compared to other studies where no significant salinity effect was identified.

Most South African wastewater treatment plants disinfect wastewater by chlorination prior to discharge into receiving waterbodies. The aim is to eliminate pathogens from wastewater. To attain this aim, residual chlorine is maintained at sufficient levels and in contact with the microbial community in the chlorination tank. There is no recommended standard for residual chlorine concentration for wastewater effluents in South Africa at the moment. This study considered those for domestic water supplies which recommended a range of 0.3 to 0.6 mg/l as ideal free residual chlorine concentration and 0.6 - 0.8 mg/l as good free residual chlorine concentration with insignificant risk of health effects (27). Based on this concentration, the free

residual chlorine in the effluents complied with the regulatory standard, but failed to eliminate the *Vibrio* species (Figure 6.4). Even when there was over dose in October and November 2007 at the sub-urban location plant, high densities of *Vibrio* pathogens were observed. Similar ranges have been reported for residual chlorine concentration in South African water works (30, 31, 15), suggesting that some South African water works do not comply with stipulated standards with reference to free residual chlorine concentration. Residual chlorine concentration did not significantly affect the abundance of *Vibrio* species in this study ( $P > 0.05$ ; Table 6.2).

In this present study, *Vibrio* species were isolated more frequently and huge densities occurred during the summer months, which are consistent with the trends for clinical cases of *Vibrio* infections (34), but did not correspond with the low survival rates reported for summer temperatures *in vitro* (44). Some previous studies reported higher levels of *V. parahaemolyticus* in oysters (plankton) during the warmer months and detectable or lower levels of this bacterium during winter months (6, 8, 9). In Southern African region, the maximum number of clinical cases of *Vibrio* related infections also occurs in the summer months (28), when precipitation tends to be high. These results differ from the results of other environmental studies, wherein the detection of vibrios peaked in late autumn and winter months (8). However, not all strains of *V. parahaemolyticus* are pathogenic. It has been demonstrated that the Kanagawa phenomenon, a beta-hemolysis in high-salt blood agar (Wagatsuma agar), is associated with most clinical strains but with very few environmental strains (39). Pathogenic *V. parahaemolyticus* generally produces a thermostable direct hemolysin (TDH), the product of the *tdh* gene (8), and *V. vulnificus* causes primary septicemia and wound infections in humans (32). Primary septicemia usually occurs through ingestion of contaminated seafood and water, by persons who are predisposed to infection by increased serum iron level or who are immunocompromised. In this study we observed that *V. fluvialis* was the most predominant species in both sampling locations and this is alarming given that *V. fluvialis* is an emerging strain which has been shown to

produce enterotoxin and clinical symptoms of gastroenteritis similar to those of *Vibrio cholerae* O1 and non-O1 strains, more so with the recent characterization of an enterotoxigenic El Tor-like haemolysin in *V. fluvialis*, which represents one of the virulence factors of *V. cholerae* (20). Our study suggest that most of the pathogenic *Vibrio* strains isolated from the final effluents also possess potential virulence traits, and these data should be valuable for assessment of human health risk due to consumption of water from the receiving watershed impacted by inadequately treated final effluents.

## 6.5 Acknowledgement

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## CHAPTER 7

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**Antibiotic susceptibility profiles of some *Vibrio* strains  
isolated from wastewater final effluents in a rural  
community of the Eastern Cape Province of South Africa**

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## CHAPTER 7

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## CHAPTER 7

### Antibiotic susceptibility profiles of some *Vibrio* strains isolated from wastewater final effluents in a rural community of the Eastern Cape Province of South Africa

#### Abstract

**Objective:** To evaluate the antibiograms and antibiotic resistance genes of some *Vibrio* strains isolated from treated wastewater final effluents in a rural community of South Africa.

**Methods:** *V. vulnificus* (18), *V. metschnikovii* (3), *V. fluvialis* (19) and *V. parahaemolyticus* (12) strains were isolated from treated final effluents of a wastewater treatment plant (WWTP) in a rural community of South Africa. The disk diffusion method was used for the characterization of the antibiograms of the isolates. Polymerase chain reaction (PCR) was employed to evaluate the presence of established antibiotic resistance genes using specific primer sets.

**Results:** The *Vibrio* strains showed the typical multidrug-resistance phenotype of an SXT element. They were resistant to sulfamethoxazole (Sul), trimethoprim (Tmp), cotrimoxazole (Cot), chloramphenicol (Chl) and streptomycin (Str), in addition to other antibiotics such as ampicillin (Amp), tetracycline (Tet), nalidixic acid (Nal), and gentamicin (Gen). The antibiotic resistance genes detected includes *dfr18* and *dfrA1* for trimethoprim; *floR*, *tetA*, *strB*, *sul2* for chloramphenicol, tetracycline, streptomycin and sulfamethoxazole respectively. Some of these genes were only recently described from clinical isolates, demonstrating genetic exchange between clinical and environmental *Vibrio* species.

**Conclusions:** These results demonstrate that final effluents from wastewater plants are potential reservoir of various antibiotics resistance genes. Moreover, detection of resistance genes in *Vibrio* strains obtained from the wastewater final effluents suggests that these resistance determinants might be further disseminated in habitats downstream of the sewage plant, thus constituting a serious health risk to the community.

**Keywords:** SXT element, multidrug resistance, *Vibrio* species, antibiotic resistance genes

## 7.1 Introduction

The occurrence of antibiotic-resistant bacteria is of great medical concern with regards to the treatment of infectious diseases. Due to the widespread application of antibiotics, an increase in resistant bacteria has been observed in wastewater effluents.<sup>1</sup> These bacteria may be released directly into wastewater systems from hospitalised and non-hospitalised patients that are medicated with antibiotics. Antibiotic-resistant bacteria have been found in a surprisingly diverse range of environments, including human clinics, animal husbandry, orchards, aquaculture, food, sewage, chlorinated, and unchlorinated water supplies.<sup>2</sup> Moreover, antibiotic agents enter the environment from a variety of other anthropogenic sources.<sup>1, 3, 4</sup> The occurrence of drug-resistant bacteria can increase through increased selective pressure by antibiotics deposited in the environment; however, drug-resistant bacteria have also been detected in relatively pristine environments, suggesting that drug resistance in microbes commonly occur in nature.<sup>5</sup> From the perspective of public health, even if disinfection could eliminate the potential hazards of drug-resistant bacteria, drug resistance genes can still be retained in the environment.<sup>5</sup> This suggests that the natural environment can be a significant reservoir of harmful drug resistance genes that can spread to a wide range of human environments.

Antibiotic-resistant bacteria of wastewater treatment plants (WWTPs) are the focus of the present study. WWTPs are connected to private households and hospitals where antibiotics are used and resistances in bacteria might arise. Once antibiotic-resistant bacteria reach WWTPs, they potentially can disseminate their resistance freight among members of the endogenous microbial community. Evidence for horizontal transfer of resistance elements in sewage habitats has been obtained for model systems.<sup>6, 7</sup> Because of the favourable growth conditions they provide for many microorganisms, WWTPs have to be considered as hot-spots for horizontal transfer of genetic material, e.g. by means of conjugation.<sup>8, 9</sup> In addition, contamination of sewage with antibiotics might cause a selective advantage for resistant bacteria.<sup>10-12</sup>

Report of drug-resistant *V. cholerae* strains are appearing with increasing frequency.<sup>14</sup> Emergence of microbial resistance to multiple drugs is a serious clinical problem in the treatment and containment of the cholera-like diarrhoea disease, as reflected by the increase in the fatality rate from 1% to 5.3% after the emergence of drug-resistant strains in Guinea-Bissau during the 1996-1997 epidemic of cholera.<sup>15</sup>

Antimicrobial resistance has become a major medical and public health problem as it has direct link with disease management.<sup>16</sup> Antibiotics such as tetracycline, doxycycline, norfloxacin, ciprofloxacin and streptomycin may be used as an adjunct to rehydration therapy and are critical in the treatment of septicemia patient.<sup>17-19</sup> Resistance to many of these drugs which have emerged in these *Vibrio* pathogens is a matter of major concern, particularly in the case of *V. vulnificus*, *V. cholerae*, *V. fluvialis* and *V. parahaemolyticus*.<sup>20-22</sup> Multiple antibiotics resistance gene cluster with the same genetic locus (Resistance Island) can be transferred to other organisms. Spread of antibiotic resistance in microbes has been attributed to the mobilization of drug resistance marker by a variety of agents like plasmid, transposons and integrons.<sup>23, 24</sup> In *Vibrio* species, antibiotics resistance determinant have also been detected on integrons, plasmid and novel conjugative transposable element designated SXT.<sup>25, 21, 57</sup>

As the SXT genetic element plays a role in the acquisition of antibiotic resistance, it is important to also assess the presence of *sul2* (encoding sulfamethoxazole resistance), *dfrA1* (O1-specific trimethoprim resistance), *dfr18* (O139 and non-O139 trimethoprim resistance)<sup>26, 58</sup> and *strB* (streptomycin B resistance) gene in *V. cholerae* strains.<sup>27, 28, 29</sup> However, the antibiotic susceptibilities of organisms fluctuate spatially and temporally.<sup>30</sup> These susceptibilities have to be examined in order to better understand the organisms' epidemiological features.<sup>30</sup>

To the best of our knowledge, no antibiotic resistance gene profile has been investigated in *Vibrio* species isolated from treated wastewater final effluents in the rural communities of South Africa. South Africa is currently facing increasing pressure of water pollution from both domestic sewage and industrial wastewater, thus posing a potential threat to the public health of humans and ecological diversity of marine animals. As part of our ongoing surveillance study of *Vibrio* species, these *Vibrio* pathogens were isolated in our previous studies.<sup>31</sup> In this paper, we report the antibiotic susceptibility patterns of the *Vibrio* isolates as well as the distribution of antibiotic resistance genes in the isolates.

## **7.2 Material and Methods**

### *7.2.1 Study site*

The Wastewater treatment facility is situated at geographical coordinates of 32°50'36"S, 26°55'00"E and approximately 1 km East of Alice town in the Eastern Cape Province of South Africa. The plant which has a design capacity of 2000 m<sup>3</sup>/day receives domestic sewage, some light industrial wastewater as well as run-off water, and treatment is based on the activated sludge system. The final effluent is discharged into the nearby Tyume River.

### 7.2.2 Isolation and biochemical identification of *Vibrio* species

Sample collection and treatments of samples has been described in our previous work.<sup>31</sup> Aliquots of the plankton free and plankton associated samples were inoculated into alkaline peptone water (APW, Pronadisa) and incubated aerobically at 37 °C for 18-24 h. Turbid cultures were streaked onto thiosulphate citrate bile salts sucrose (TCBS, Pronadisa) agar and incubated at 37 °C for 24 h. Five to ten isolated colonies per plate were randomly picked from each sample and subsequently subcultured on fresh TCBS agar plates. The pure isolates were subjected to Gram staining and oxidase test. Only Gram-negative, oxidase-positive isolates were selected for biochemical identification using API 20 NE kit. The strips were then read and the final identification was made using API lab plus software (bioMerieux, Marcy l'Etoile, France).

### 7.2.3 Molecular identification

Polymerase chain reaction (PCR) was used to confirm the identities of the *Vibrio* species using the species-specific primers described in our previous study.<sup>31</sup>

### 7.2.4 Bacterial strains

A total of 52 strains of *Vibrio* species were included in this study. Of these, 12 were *V. parahaemolyticus*, 18 were *V. vulnificus*, 19 were *V. fluvialis* and 3 were *V. metschnikovii*. These *Vibrio* species were isolated in our previous study from the final effluent of a rural wastewater treatment plant in the Eastern Cape Province of South Africa.<sup>31</sup> *V. parahaemolyticus* strain SABS PM ATCC Vbr 1, *V. vulnificus* DSM 10143, *V. fluvialis* DSM 19283 were used as the PCR positive control for *sul2*, *dfrA1*, *strB*, *floR*, *dfr18*, *tetA*, and SXT. All strains were maintained in tryptic soy broth supplemented with 30% glycerol and stored at -80°C.

### 7.2.5 Antibiotic susceptibility test

Bacterial susceptibility to antimicrobial agent was performed by disk diffusion method using guidelines established by Bauer et al.<sup>32</sup> and recommended by Clinical and Laboratory Standards Institute<sup>33</sup> using commercial antimicrobial discs. A total of 21 antibiotic discs (Mast Diagnostics, Merseyside, United Kingdom) which includes ampicillin (25 µg), cotrimoxazole (25 µg), amikacin (30 µg), imipenem (10 µg), erythromycin (15 µg), meropenem (10 µg), streptomycin (25 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), cephalothin (30 µg), nalidixic acid (30 µg), tetracycline (30 µg), trimethoprim (30 µg), norfloxacin (10 µg), sulfamethoxazole (25 µg), gentamicin (10 µg), neomycin (30 µg), penicillin G (10 unit), nitrofurantoin (200 µg), polymyxin B (300 units) and cefuroxime (30 µg) were employed. The resistance or susceptibility profile of the isolates was determined by measuring inhibitory zone and then compared with the interpretative chart to determine the sensitivity of the isolates to the antibiotics.

### 7.2.6 Multiple antibiotic resistances (MAR) index

The multiple antibiotic resistance (MAR) index, when applied to a single isolate, is defined as  $[a/b]$ , where  $[a]$  represents the number of antibiotics to which the isolates was resistant and  $[b]$  represent the number of antibiotics to which the isolate was exposed. MAR index higher than 0.2 identifies organisms that originate from high-risk sources of contamination, where antibiotics are often used.<sup>34</sup>

### 7.2.7 Isolation of genomic DNA

Genomic DNA was extracted following a modified scheme of Maugeri et al.<sup>35</sup> Single colonies of *Vibrio* species strains grown overnight at 37 °C on TCBS agar plates were picked, suspended in 200 µl of sterile Milli-Q PCR grade water (Merck, SA) and the cells were lysed using Dri-block DB.2A (Techne, SA) for 15 min at 100 °C. The cell debris was removed by centrifugation at

11, 000 × g for 2 min using a MiniSpin micro centrifuge (Merck, SA). The cell lysates (10 µl) were used as template in the PCR assays immediately after extraction placed on ice for 5 min or following storage at -80 °C. Sterile Milli-Q PCR grade water (Merck, SA) was included in each PCR assay as a negative control.

### 7.2.8 PCR amplification assay

Polymerase chain reaction (PCR) was used to detect antibiotic resistant genes in the *Vibrio* species using the specific primer pairs and PCR conditions for detection of the SXT, *floR*, *strB*, *sul2*, *dfrA18*, *tetA* and *dfrA1* are listed in Table 7.1. All reactions were set in 50 µl volume of reaction buffer containing 0.05 unit/µl *Taq* polymerase as directed by the manufacturer (Fermentas Life Sciences). Cycling conditions (Bio-Rad My Cycler™ Thermal Cycler) were as follows; initial denaturation at 94 °C for 2 min was followed by 35 cycles of 94 °C for 1 min, 60.5 °C for 1 min and 72 °C for 1 min with a final extension at 72 °C for 10 min and cooling to 4 °C. Electrophoresis of amplicons was performed with 1% agarose gel (Hispanagar, Spain) containing 0.5 mg/L Ethidium Bromide (EtBr) (Merck, SA) for 1 h at 100 V in 0.5 × TAE buffer (40 mM Tris-HCl, 20 mM Na-acetate, 1 mM EDTA, pH 8.5) and visualized under an UV transilluminator (BioDoc-It System, UVP Upland, CA 91786, USA).

## 7.3 Results

### 7.3.1 Antibigram profile

The susceptibility against 21 different antibiotics by *V. vulnificus* (18 strains); *V. parahaemolyticus* (12 strains); *V. fluvialis* (19 strains) and *V. metschnikovii* (3 strains) were examined. The results from 52 total isolates of *Vibrio* strains are shown in Table 7.2. Data on the antibiotics resistance zone indicate that all the 52 isolates of *Vibrio* species were 100% resistant to ampicillin and sulfamethoxazole, and that 100% of isolates were sensitive to imipenem,

Table 7.1: Sequence of primers used for detection of antibiotic resistance gene and the SXT element.

Primer	Sequence (5' to 3')	Target gene	Amplicon size (bp)	Reference
SXT-F	ATGGCGTTATCAGTTAGCTGGC	SXT	1035	(27)
SXT-R	GCGAAGATCATGCATAGACC			
SUL2-F	AGGGGGCAGATGTGATCGC	<i>sul2</i>	625	(29)
SUL2-B	TGTGCGGATGAAGTCAGCTCC			
FLOR-F	TTATCTCCCTGTCGTTCCAGCG	<i>floR</i>	526	(54)
FLOR-2	CCTATGAGCACACGGGGAGC			
TMP-F	TGGGTAAGACACTCGTCATGGG	<i>dfr18</i>	389	(29)
TMP-B	ACTGCCGTTTTTCGATAATGTGG			
TetA-F	GTA ATT CTG AGC ACT GTC GC	<i>TetA</i>	950	(55)
TetA-R	CTG CCT GGA CAA CAT TGC TT			
strB-F	GGCACCCATAAGCGTACGCC	<i>strB</i>	470	(56)
strB-R	TGCCGAGCACGGCGACTACC			
dfr1-F	CGAAGAATGGAGTTATCGGG	<i>dfrA1</i>	372	(54)
dfr1-B	TGCTGGGGATTTTCAGGAAAG			

meropenem and norfloxacin. *Vibrio fluvialis* showed 100%, 90%, 70% and 80% resistance to trimethoprim, penicillin, cotrimoxazole and streptomycin, respectively. And 92%, 82% 90% and 100% of cephalothin resistance were exhibited by *V. vulnificus*, *V. parahaemolyticus*, *V. fluvialis* and *V. metschnikovii* respectively (Table 7.2).

Table 7.2: *In vitro* antibiotics susceptibility profile of the *Vibrio* strains isolated from final effluent of wastewater treatment plant.

Antibiotics		Percentage (%) Response to antibiogram profile			
		<i>V. vulnificus</i> (n=18)	<i>V. parahaemolyticus</i> (n=12)	<i>V. fluvialis</i> (n=19)	<i>V. metschnikovii</i> (n=3)
Ampicillin (25 µg)	R	100	100	100	100
	I	0	0	0	0
	S	0	0	0	0
Cotrimoxazole (25 µg)	R	30	20	70	40
	I	56	80	30	60
	S	14	0	0	0
Amikacin (30 µg)	R	42	40	67	40
	I	40	40	33	60
	S	15	20	0	0
Imipenem (10 µg)	R	0	0	0	0
	I	0	0	0	0
	S	100	100	100	100
Erythromycin (15 µg)	R	0	10	40	10
	I	30	50	20	57
	S	70	40	40	33
Meropenem (10 µg)	R	0	0	0	0
	I	0	0	0	0
	S	100	100	100	100
Streptomycin (25 µg)	R	60	20	80	20
	I	40	80	20	80
	S	0	0	0	0
Chloramphenicol (30 µg)	R	44	40	75	40
	I	10	60	25	60
	S	46	0	0	0
Ciprofloxacin (5 µg)	R	10	0	5	0
	I	0	30	10	0
	S	90	70	85	100
Cephalothin (30 µg)	R	92	82	90	100
	I	0	0	0	0
	S	8	18	10	0
Nalidixic acid (30 µg)	R	43	20	43	20
	I	57	60	43	80
	S	0	20	14	0
Tetracycline (30 µg)	R	29	20	0	40
	I	71	60	100	60
	S	0	20	0	0
Trimethoprim (30 µg)	R	50	46	100	100
	I	10	0	0	0
	S	40	54	0	0
Norfloxacin (10 µg)	R	0	0	0	0
	I	0	0	0	0

Sulfamethoxazole (25 µg)	S	100	100	100	100
	R	100	100	100	100
	I	0	0	0	0
Gentamicin (10 µg)	S	0	0	0	0
	R	70	66	60	50
	I	30	20	30	40
Neomycin (30 µg)	S	0	14	10	10
	R	57	80	67	60
	I	43	20	33	40
Penicillin G (10 unit)	S	0	0	0	0
	R	70	80	90	100
	I	20	20	10	0
Nitrofurantoin (200 µg)	S	10	0	0	0
	R	40	45	65	0
	I	10	20	35	100
Polymyxin B (300 units)	S	50	35	0	0
	R	29	40	30	20
	I	57	60	70	60
Cefuroxime (30 µg)	S	14	0	0	20
	R	50	10	55	40
	I	50	50	36	60
	S	0	40	9	0

Legend: R- Resistant; I- Intermediate; S- Susceptible

Overall, the multi-drug resistant of the *Vibrio* species were exemplified by resistance to five and up to ten antibiotics tested with multiple antibiotics resistance index (MAR) ranging from 0.30 - 0.47 (Table 7.3) thus suggesting resistance genes are genetically linked.

### 7.3.2 The antibiotic resistance gene cluster and SXT element of *Vibrio* species strains

To date, there have been no reports on the antibiotic resistance genes in *V. vulnificus*, *V. metschnikovii*, *V. fluvialis* and *V. parahaemolyticus* isolated from treated wastewater final effluents in rural communities of South Africa. The PCR result showed the presence of SXT elements in the *Vibrio* strains with an amplicon size of 1035 bp (Table 7.3).

Table 7.3: Phenotypic and genotypic characterization of *Vibrio* species containing the SXT element and antibiotics resistance gene.

Isolate Code	Name of species	MAR	Antibiotic resistance pattern	Strain(s) showing presence of gene encoding							
				<i>floR</i>	<i>dfr18</i>	<i>tetA</i>	<i>strB</i>	<i>dfrA1</i>	<i>sul2</i>	SXT	
AL028	<i>Vibrio vulnificus</i>	0.30	AMP, COT, CHL, NAL, ERY, CXM	+	-	-	-	-	-	-	+
AL 010	<i>Vibrio vulnificus</i>	0.30	AMP, CIP, TET, AK, ERY, CHL	+	-	+	-	-	-	-	-
AL016	<i>Vibrio vulnificus</i>	0.30	AMP, COT, AK, ERY, NAL, PEN	-	-	-	-	-	-	-	+
AL024	<i>Vibrio vulnificus</i>	0.30	AMP, TET, CIP, ERY, CXM, PB	-	-	+	-	-	-	-	-
AL026	<i>Vibrio vulnificus</i>	0.30	AMP, ERY, CHL, NIT, GEN, TMP	+	+	-	+	-	-	-	+
AL001	<i>Vibrio vulnificus</i>	0.30	AMP, CIP, AK, ERY, NAL, GEN	+	-	-	+	-	-	-	+
ALO15	<i>Vibrio vulnificus</i>	0.30	AMP, TET, CHL, COT, TMP, SUL	+	+	+	+	-	+	+	+
AL029	<i>Vibrio vulnificus</i>	0.30	AMP, TMP, NAL, STR, PB, SUL	-	+	-	+	-	+	+	+
AL038	<i>Vibrio vulnificus</i>	0.30	AMP, NEO, PB, AK, NOR, NIT	-	+	-	+	-	-	-	-
AL018	<i>Vibrio vulnificus</i>	0.30	AMP, CIP, COT, NAL, NEO, TMP	-	+	-	-	-	-	-	+
AL039	<i>Vibrio vulnificus</i>	0.30	AMP, NAL, CXM, SUL, TMP, PB	+	-	-	-	+	+	+	+
AL041	<i>Vibrio vulnificus</i>	0.33	AMP, ERY, CHL, CXM, PB, STR, SUL	+	+	+	+	-	+	+	+
AL044	<i>Vibrio vulnificus</i>	0.30	AMP, COT, TMP, ERY, CHL, CXM	+	+	-	-	-	-	-	+
AL054	<i>Vibrio vulnificus</i>	0.33	AMP, CEP, TET, NAL, ERY, CHL, NEO	+	-	+	+	+	-	-	-
AL042	<i>Vibrio vulnificus</i>	0.33	AMP, TET, NAL, ERY, CHL, TMP, STR	+	-	+	+	+	-	-	+
AL048	<i>Vibrio vulnificus</i>	0.30	AMP, ERY, NAL, CXM, STR, SUL	-	-	-	+	-	+	+	+
AL011	<i>Vibrio vulnificus</i>	0.30	AMP, COT, TMP, NAL, NEO, PB	-	+	-	-	-	-	-	+
AL056	<i>Vibrio vulnificus</i>	0.30	AMP, TET, AK, ERY, NAL, STR	-	-	+	+	-	-	-	-
AL012	<i>Vibrio metschnikovii</i>	0.30	AMP, GEN, PB, CEP, TMP, SUL	-	-	-	-	-	+	+	+
AL016	<i>Vibrio metschnikovii</i>	0.42	AMP, AK, TMP, ERY, CHL, CXM, CEP, STR, NEO	+	+	-	+	-	+	+	+
AL023	<i>Vibrio metschnikovii</i>	0.33	AMP, COT, TMP, NAL, TET, STR, GEN	+	-	+	+	+	+	+	+
AL014	<i>Vibrio fluvialis</i>	0.38	AMP, COT, CHL, NAL, GEN, SUL, TMP, STR	+	+	-	+	-	+	+	+

AL002	<i>Vibrio fluvialis</i>	0.33	AMP, NAL, NEO, PB, STR, SUL, TMP	-	+	-	+	+	+	+
AL025	<i>Vibrio fluvialis</i>	0.30	AMP, PB, GEN, STR, PEN, CHL	+	+	-	+	+	+	+
AL037	<i>Vibrio fluvialis</i>	0.30	AMP, NAL, GEN, NEO, STR, COT	-	+	-	+	+	+	+
AL033	<i>Vibrio fluvialis</i>	0.38	AMP, AK, NEO, PB CHL, PEN, SUL, TMP	+	+	-	-	+	+	+
AL013	<i>Vibrio fluvialis</i>	0.42	AMP, COT, PB, NAL, ERY, TMP, SUL, CHL, STR,	+	+	-	+	+	+	+
AL024	<i>Vibrio fluvialis</i>	0.33	AMP, PB, NAL, TMP, SUL, COT, CHL	+	+	-	-	+	+	+
AL027	<i>Vibrio fluvialis</i>	0.33	AMP, PB, NAL, NEO, COT, SUL, STR	-	+	-	+	+	+	+
AL029	<i>Vibrio fluvialis</i>	0.42	AMP, TET, ERY, TMP, SUL, STR, CHL, CXM, PB	+	+	+	+	+	+	+
AL034	<i>Vibrio fluvialis</i>	0.38	AMP, CIP, TMP, ERY, CHL, NEO STR, SUL	+	+	-	+	+	+	+
AL036	<i>Vibrio fluvialis</i>	0.42	AMP, COT, CIP, AK, TMP, NAL, CHL, SUL, STR	+	+	-	+	+	+	+
AL040	<i>Vibrio fluvialis</i>	0.42	AMP, COT, CEP, AK, TMP, NAL, CHL, STR, SUL	+	+	-	+	+	+	+
AL051	<i>Vibrio fluvialis</i>	0.42	AMP, COT, CEP, CXM, TMP, SUL, STR, PB, NEO	-	+	-	+	+	+	+
AL053	<i>Vibrio fluvialis</i>	0.42	AMP, COT, AK, ERY, TMP, SUL, STR, TET, CHL	+	+	+	+	+	+	+
AL019	<i>Vibrio fluvialis</i>	0.33	AMP, COT, TMP, CHL, STR, SUL, PB	+	+	-	+	+	+	+
AL004	<i>Vibrio fluvialis</i>	0.38	AMP, AK, NAL, ERY, PB, TMP, SUL, STR	-	+	-	+	+	+	+
AL006	<i>Vibrio fluvialis</i>	0.33	AMP, TET, PB STR, TMP, PEN, PB	-	+	+	-	+	-	+
AL022	<i>Vibrio fluvialis</i>	0.38	AMP, TMP, ERY PEN, NAL, STR, SUL, CHL	+	+	-	+	+	+	+
AL031	<i>Vibrio fluvialis</i>	0.30	AMP, GEN, PEN, COT, TMP, CHL	+	+	-	-	+	-	+
AL003	<i>Vibrio parahaemolyticus</i>	0.30	AMP, AK, PB TMP, COT, STR	-	-	-	+	+	-	+
AL008	<i>Vibrio parahaemolyticus</i>	0.30	AMP, ERY, PB, TMP, COT, SUL	-	+	-	-	-	+	+
AL014	<i>Vibrio parahaemolyticus</i>	0.30	AMP, ERY, NEO, TMP, STR, SUL	-	+	-	+	-	+	+
AL017	<i>Vibrio parahaemolyticus</i>	0.30	AMP, PB, CHL, TMP, COT, CIP	+	+	-	-	-	-	+

AL028	<i>Vibrio parahaemolyticus</i>	0.30	AMP, CHL, TMP, SUL, STR	+	-	-	+	+	+	+
AL009	<i>Vibrio parahaemolyticus</i>	0.33	AMP, PEN, PB, CXM, CEP, NOR, CIP	-	+	-	+	-	-	-
AL030	<i>Vibrio parahaemolyticus</i>	0.38	AMP, NAL, CIP, PEN, TMP, GEN, SUL, STR	+	+	-	+	-	+	+
AL032	<i>Vibrio parahaemolyticus</i>	0.38	AMP, AK, ERY, PEN, TMP, SUL, STR, CHL	+	-	-	+	+	+	+
AL043	<i>Vibrio parahaemolyticus</i>	0.33	AMP, AK, ERY, TMP, SUL, STR, TET	-	+	+	+	-	+	+
AL045	<i>Vibrio parahaemolyticus</i>	0.33	AMP, AK, PEN, TMP, SUL, STR, CHL	+	-	-	+	+	+	+
AL049	<i>Vibrio parahaemolyticus</i>	0.30	AMP, PB, PEN, TMP, SUL, NEO	-	+	-	-	-	+	+
AL055	<i>Vibrio parahaemolyticus</i>	0.47	AMP, TET, PEN, CIP, NOR, SUL, STR, COT, NEO, PB	-	-	+	+	-	-	+
Positive controls	<i>V. vulnificus</i> DSM 10143	0.38	AMP, NAL, STR, TMP, SUL, COT, PB, NEO	+	+	-	+	+	+	+
	<i>V. fluvialis</i> DSM 19283	0.57	AMP, CIP, GEN, STR, SUL, TMP, NEO, NAL, NOR, CHL, COT, PB	+	+	-	+	+	+	+
	<i>V. parahaemolyticus</i> strain SABS PM ATCC Vbr 1	0.33	AMP, TMP, STR, SUL, COT, NEO, PB	+	+	-	+	+	+	+

AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; NAL, nalidixic acid; NEO, neomycin; NOR, norfloxacin; STR, streptomycin; SUL, sulfamethoxazole; COT, cotrimoxazole; TET, tetracycline; TMP, trimethoprim; ERY, erythromycin; CEP, cephalothin; CXM, cefuroxime; AK, amikacin; AUG, augmentin; PB, polymyxin B; PEN, penicillin G; MAR, Multiple antibiotics resistance index.

The SXT element encodes different types of antibiotic resistance genes, *floR* (526 bp), *sul2* (625 bp), and *strB* (470 bp), (Table 7.3) which confer resistance to chloramphenicol (Chl), sulfamethoxazole (Sul), and streptomycin (Str), respectively. Trimethoprim (Tmp) resistance genes were detected with the amplification of a 372 and 389 bp fragment of *dfrA1* and *dfr18* (Table 7.3). The molecular analysis of these genes has been carried out in *V. cholerae* O1 and O139.<sup>29</sup> In an attempt to find a relationship between the multidrug-resistance phenotypes of *V. vulnificus*, *V. metschnikovii*, *V. fluvialis* and *V. parahaemolyticus* and the presence of the SXT element, PCR was carried out using specific primers for the SXT antibiotic resistance genes (Table 7.1). Results revealed that some *V. vulnificus*, *V. metschnikovii*, *V. fluvialis* and *V. parahaemolyticus* contained one to six of the antibiotic resistance genes of SXT element (Table 7.3). The most abundant strain that harbour most of the antibiotic resistance genes and SXT element is *V. fluvialis*.

SXT element is responsible for resistance to cotrimoxazole, trimethoprim and streptomycin in *V. cholerae*.<sup>26</sup> However, inspite of antibiotics resistance against cotrimoxazole, trimethoprim and streptomycin among the *Vibrio* isolates viz AL024; AL038; AL054; AL056 and AL009 did not harbour the SXT element during this study.

#### **7.4 Discussion**

An increase in the emergence of multi-antibiotics resistant bacteria in recent years is worrisome and the presence of antibiotics resistance genes on bacterial plasmids has further helped in the transmission and spread of drug resistance among pathogenic bacteria.<sup>36</sup> The growing problems with antimicrobial drug resistance are beginning to erode our antibiotic abilities to combat antibiotic resistance and thus limiting therapeutic options to present-day clinicians.<sup>36</sup>

Most study on the antimicrobial susceptibility profiles of *Vibrio* species focus almost exclusively on clinical and/or food isolates with little information in the literature on antibiotic susceptibility profiles for *Vibrio* strains isolated from environmental sources such as treated

municipal wastewater effluents. To our knowledge, this is the first study that specifically evaluated the antimicrobial susceptibility profile and detection of multiple antibiotics resistance genes of *Vibrio* strains isolated from treated municipal wastewater effluent in South Africa.

The results reveal the high individual and multiple resistances to antibiotics among 52 *Vibrio* species strains from the final effluent (Table 7.2 & 7.3). Data on antibiotic resistant zones indicates that all 52 strains of *Vibrio* species were 100% resistant to ampicillin and sulfamethoxazole and more than 50% resistant to cotrimoxazole, erythromycin, chloramphenicol, cephalothin, streptomycin, trimethoprim and penicillin. Previous studies have shown that streptomycin, rifampicin, kanamycin, tetracycline, polymyxin B were active against *Vibrio* species.<sup>37</sup> But this was contrary in our findings where we observed resistances to streptomycin, tetracycline and polymyxin B in the *Vibrio* strains studied. However, Ottaviani et al.<sup>38</sup> showed that *V. parahaemolyticus* were resistant to penicillin, carbenicillin, ampicillin, cephalothin, kanamycin and rifampicin. Besides, their results also showed that increase in salt concentration cause changes of sensitivity toward antibiotics from the resistant to susceptible phenotypes.

In this study, the observed 100% resistance to ampicillin of analyzed strains differ from other studies that have been reported, which ranged from 44.4% to 98%.<sup>39, 40</sup> but corroborates the findings of French and coworker<sup>41</sup> who reported similar antibiotics susceptibility profile for *V. parahaemolyticus*.

There are few factors that may contribute to the *Vibrio* species antibiotic resistance. Firstly, a mutation in cellular DNA could modify the antibiotics target site or transport mechanism, resulting in decreased action of the antibiotic on the cell.<sup>40</sup> Another factor is an extra gene product target site or transport mechanism. About 10-20% of the studied strains showed a 5-10 MAR pattern (i.e., resistance to 5-10 of 21 antibiotics tested). The multiple antibiotics resistance (MAR) index of *Vibrio* strains are shown in Table 7.3. In this study, all the antibiotics resistant isolates have MAR index of 0.30 or more which can be grouped under origination from high risk sources of contamination like

humans and commercial industry where antibiotics are often used. Donlan and Costerton<sup>42</sup> also reported the acquisition of inherent resistance to antimicrobial agents by attached bacterial species, which suggests that attachment to plankton at one point or the other may have enhanced the multiple resistances of our isolates to several of the antibiotics tested against. The physicochemical character of the wastewater effluent may have influenced the level of resistance displayed by the *Vibrio* species isolated in this study. It has been widely reported that conventional wastewater treatment plants lack the capacity to effectively remove antibiotics and a number of other chemicals from wastewater, thereby increasing the chances of bacterial pathogens resident in such wastewater effluents to develop resistance to common antibiotics due to selective pressure.<sup>11, 43, 44</sup>

Resistance to antimicrobial agents in bacteria can be mediated by several mechanisms.<sup>45</sup> On the basis of the prevailing assumption that gene carriage equals resistance, genetic tests aimed at detecting resistance genes in bacterial isolates by using DNA probes or PCR methods have been developed.<sup>46-49</sup>

In this present study, all strains exhibited multiple resistances to five antibiotics. The strains of *Vibrio* species are known to carry plasmids, which encode for drug resistance.<sup>50, 22, 51</sup> A study carried out in India between 1997 and 1998 on a total number of 94 isolates of *V. cholerae* observed that 43 strains belonging to non-O1 and non-O139 serogroups contained plasmids that contributed to the multiple antibiotic resistance and exhibited resistance to ampicillin, neomycin, tetracycline, gentamicin, streptomycin, sulfonamide, furazolidone, and chloramphenicol.<sup>50</sup> The drug resistant conjugative plasmid pMRV150 has been reported in *V. cholerae* O139 from China which mediated multiple-drug resistance (MDR) to at least six antibiotics, including ampicillin, streptomycin, gentamicin, tetracycline, chloramphenicol and trimethoprim-sulfamethoxazole.<sup>51</sup> Among the *Vibrio* species, resistance to streptomycin and furazolidone were previously observed for the first time in *V. cholerae* O139 isolated after 1992.<sup>25</sup>, and then these phenotypes associated with resistance to chloramphenicol were detected in many Asian clinical *V. cholerae* O1 El Tor and O139 isolates,<sup>29</sup>

and in some clinical isolates of non-O1, and non-O139.<sup>50</sup> The spread of these resistances in *V. cholerae* is mainly due to a new type of conjugative transposon called the SXT element or constin.<sup>25</sup> There were differences in the antibiotics resistance gene clusters in the SXT element in the *Vibrio* strains in our study, corroborating the results of earlier workers<sup>29</sup> who reported differences in antibiotics resistance and suggested that these genes were not intrinsic features of this family integrase, but rather appeared to have been inserted into these elements, becoming transmissible bacterial population.

Strains AL024, AL038, AL054 AL056 and AL009 lack SXT integrase, therefore the entire element. TMP, STR and COT resistance can then be associated to any other mobile element. This could easily be class 1 integrons, already described in Africa, both in *V. cholerae* and *V. parahaemolyticus*. SXT-like element devoid of the resistance cluster could be represented by strain AL016, positive for the integrase but not for the gene cassettes.

The *dfr18* and *dfrA1* genes cassettes coding for trimethoprim resistance, found among several *Vibrio* strains in this study, have also been detected among the strains isolated in Thailand<sup>15</sup>, and India.<sup>50</sup> Similarly, the *strB* gene for aminoglycoside resistance (streptomycin) found in our collection has been detected by Falbo et al.<sup>28</sup> among the outbreak strains in Albania and Italy in 1994, and Calcutta, India during the period 1997 to 1998 by Thungapathra et al.<sup>50</sup> The presence of multiple determinants for trimethoprim resistance is alarming, because this drug is used for the treatment of cholera in children and pregnant women.<sup>50</sup> The presence of a *strB* gene cassette is also of concern, because streptomycin is also used in the treatment of cholera in children.<sup>50</sup> Although the antibiotics resistance patterns of these strains were not identical, it appeared from observations described (Table 3) that these isolates could have a common origin. Our findings thus showed that SXT element bearing drug resistance markers were distributed fairly widely in the *Vibrio* species strains isolated from our study sites. It also revealed the presence of the gene cassettes, *floR*, *tetA*, *dfr18*, *strB*, *dfrA1*, and *sul2*. Since there are progressively reports of cholera-like diarrhoea being caused by non-O1

strain<sup>52</sup>, and since these strain appear to survive better than O1 strain in a wide range of foods<sup>53</sup>, it is important to monitor the distribution of SXTs in emerging *Vibrio* stains.

## **7.5 Conclusion**

To the best of our knowledge, this is the first study that describes detection of antibiotics resistance genes known to confer resistance to common classes of antibiotics in a rural community of South Africa. The mobile pool of resistance genes shared by bacteria of the wastewater effluents analysed even includes resistance genes that have only recently been described for clinical isolates, indicating genetic exchange between clinical and environmental bacteria. Further, detection of these newer resistance genes isolated from bacterial inhabitants of wastewater final effluents confirms that these determinants are released into the environment, which subsequently facilitates further dissemination among environmental bacteria. Moreover, it appeared that the wastewater purification processes operating in the wastewater treatment facility under study are not appropriate to significantly reduce the spectrum of resistance genes that are detectable in the final effluents. PCR can be used effectively to detect antibiotics resistance genes and could be used in surveillance of the spread of antibiotics resistance in epidemiological and environmental studies.

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## 7.7 Transparency declarations

None to declare.

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## CHAPTER 8

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**Detection of antibiotic resistant *Vibrio* strains and their  
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treatment plant of the Eastern Cape Province, South Africa**

*This chapter has been submitted in this format for publication in Applied and environmental*

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## CHAPTER 8

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## CHAPTER 8

### **Detection of antibiotic resistant *Vibrio* strains and their resistance genes in final effluents from sub-urban wastewater treatment plant of the Eastern Cape Province, South Africa**

#### **Abstract**

Final effluents of a wastewater treatment plant (WWTP) in a sub-urban community of South Africa have become a major reservoir for antibiotic-resistant *Vibrio* strains carrying a wide pool of antibiotic resistant genes in the effluent system. *V. fluvialis* (24), *V. vulnificus* (20), and *V. parahaemolyticus* (14) strains were isolated from this final effluents of the WWTP. The disk diffusion method was used for the characterization of the antibiograms of the isolates. Polymerase chain reaction (PCR) was utilized to assess the presence of established antibiotic resistance genes using specific primers. The *Vibrio* strains showed the typical multi-antibiotic-resistance of an SXT element. They were resistant to sulfamethoxazole (Sul), trimethoprim (Tmp), cotrimoxazole (Cot), chloramphenicol (Chl) and streptomycin (Str), as well as other antibiotics such as ampicillin (Amp), penicillin (Pen), erythromycin (Ery), tetracycline (Tet), nalidixic acid (Nal), and gentamicin (Gen). The antibiotic resistance genes detected includes *dfr18* and *dfrA1* for trimethoprim; *tetA*, *strB*, *floR*, *sul2* *blaP1*, for tetracycline, streptomycin, chloramphenicol, sulfamethoxazole and  $\beta$ -lactams respectively. A number of these genes were only recently described from clinical isolates, demonstrating genetic exchange between clinical and environmental *Vibrio* species. These results demonstrate that the final effluents are reservoirs for various antibiotic resistance genes which could be disseminated to habitats downstream of the sewage plant and pose health risk to the communities who are dependent on the watershed for domestic and recreational purposes.

**Key words:** Antibiotic resistance, *Vibrio* species, genes, wastewater effluents.

## 8.1 Introduction

Resistance to antibiotics has been described as a major threat to public health (39). Antibiotics contribute to the development of resistance through ‘selective grooming’ (28), from three sources: medical prescription to humans, veterinary prescription and nonprescription use, and waste antibiotics reaching the environment (27).

Antibiotics are used extensively to prevent or to treat microbial infections in human and veterinary medicine. Apart from their use in aquaculture, they are also employed to promote more rapid growth of livestock (27). Most of the compounds used in medicine are only partially metabolized by patients and are then discharged into the hospital sewage system or directly into municipal wastewater if used at home. Along with excreta, they flow with municipal wastewater to the sewage treatment plant. They may pass through the sewage system and end up in the environment, mainly in the water compartment (27). An important step in coping with this threat is to elucidate and to understand pathways for resistance gene spread. Many resistance genes are located on mobile genetic elements such as plasmids, transposons and integrons, which function as vectors for these determinants and promote their dissemination (4, 15, 16, 22, 30, 31, 34). Moreover, inappropriate use of antimicrobial drugs favours spread of resistance genes by selection for resistant microorganisms (8, 9, 38).

Multiple drug resistance in *Vibrio cholerae* has been reported frequently, usually after acquisition by strains of conjugative plasmids (17, 18, 35). Other genetic elements, such as the class 1 integron and SXT element, have also been reported to carry genetic determinants for antimicrobial resistance (13, 14, 23, 24, 26, 36). Integrative and conjugative elements (ICEs) form a large class of mobile genetic elements able to encode many properties, such as drug resistance (7). The SXT element is an ICE that contributes to horizontal transmission and rearrangement of resistance gene in *V. cholerae*. It was originally found in the chromosome of *V. cholerae* O139 MO10 from India

(SXT<sup>MO10</sup>) in 1992 (2, 37). This element is able to mobilize plasmids and resistance to chloramphenicol (coded by *floR*), streptomycin (*strA* and *strB*), sulfamethoxazole (*sul2*), and trimethoprim (*dfrA18*). Increased incidence of multiple antibiotic resistant *Vibrio* strains isolated from treated final effluents environment sources have been reported in South Africa and they may pose a serious problem in chemotherapy (unpublished report). However, resistance to cotrimoxazole and tetracycline has been reported (14).

The ability of bacteria to develop multiple-drug resistance is due in part to their ability to acquire new antibiotic resistance genes. Mobile elements called integrons determine a site-specific recombination system that is responsible for the acquisition of many antibiotic resistance determinants (21, 22). A large number of antibiotic resistance genes (conferring resistance to aminoglycosides,  $\beta$ -lactams, chloramphenicol, and trimethoprim), as well as several unidentified open reading frames, have been found as inserts in integrons (20).

To the best of our knowledge, there is paucity of information on antibiotic resistance profiles of *Vibrio* species isolated from wastewater final effluents in South Africa. In this paper, we report on the prevalence of antibiotic resistant *Vibrio* strains in the final effluents of wastewater treatment facility in a typical sub-urban community of the Eastern Cape Province of South Africa.

## **8.2 Material and Methods**

### *8.2.1 Study site*

The wastewater treatment plant is located in sub-urban community of the Eastern Cape Province, South Africa with the geographical coordinates of 32°51'28"S, 27°35'29"E. The plant is designed to treat an average of dry weather flow of 7 000 m<sup>3</sup>/day and an average wet weather flow of 21 000 m<sup>3</sup>/day, and receives domestic sewage, industrial wastewater and run-off water. It discharges its final effluents into a stream that empties into a nearby river.

### 8.2.2 Isolation and identification of *Vibrio* species

Sample collection and treatments of samples has been described in our previous work (25). Aliquots of the plankton free and plankton associated samples were inoculated into alkaline peptone water (APW Pronadisa) and incubated aerobically at 37 °C for 18-24 h. Turbid cultures were streaked onto thiosulphate citrate bile salts sucrose (TCBS Pronadisa) agar incubated at 37 °C for 24 h. Five to ten isolated colonies per plate were randomly picked from each sample and subsequently subcultured on fresh TCBS agar plates. The pure isolates were subjected to Gram staining and oxidase test. Only Gram-negative, oxidase-positive isolates were selected for biochemical identification using API 20 NE kit. The strips were then read and the final identification was made using API lab plus software (bioMerieux, Marcy l'Etoile, France). Only excellent identification reports were accepted. Polymerase chain reaction (PCR) was used to confirm the identities of the *Vibrio* species using the species-specific primers described in our previous study (25).

### 8.2.3 Bacterial strains

A total of 58 confirmed *Vibrio* isolates were included in this study. Of these, 14 were *V. parahaemolyticus*, 20 were *V. vulnificus* and 24 were *V. fluvialis*. *V. parahaemolyticus* strain SABS PM ATCC Vbr 1, *V. vulnificus* DSM 10143; *V. fluvialis* DSM 19283 were used as the PCR positive control for the *blaPI*  $\beta$ -lactamase cassette and SXT, *sul2*, *dfrA1*, *strB*, *floR*, *dfr18*, and *tetA* (tetracycline) as described and listed in Table 8.1. All strains were maintained in tryptic soy broth supplemented with 30% glycerol and stored at -80°C.

#### 8.2.4 Antibiotic susceptibility testing

Bacterial susceptibility to antimicrobial agent was performed by disk diffusion method using guidelines established by Bauer et al. (3) as recommended by the Clinical and Laboratory Standards Institute (11) using commercial antimicrobial discs. A total of 21 antibiotic discs (Mast Diagnostics, Merseyside, United Kingdom) which includes ampicillin (25 µg), cotrimoxazole (25 µg), amikacin (30 µg), imipenem (10 µg), erythromycin (15 µg), meropenem (10 µg), streptomycin (25 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), cephalothin (30 µg), nalidixic acid (30 µg), tetracycline (30 µg), trimethoprim (30 µg), norfloxacin (10 µg), sulfamethoxazole (25 µg), gentamicin (10 µg), neomycin (30 µg), penicillin G (10 unit), nitrofurantoin (200 µg), polymyxin B (300 units) and cefuroxime (30 µg) were employed. Characterization of the resistance or susceptibility profile of the isolates was determined by measuring zones of inhibition and comparing with the interpretative chart to determine the sensitivity of the isolates to the antibiotics.

#### 8.2.5 Isolation of genomic DNA

Genomic DNA was extracted following a modified scheme of Maugeri et al. (29). Single colonies of *Vibrio* species strains grown overnight at 37 °C on TCBS agar plates were picked, suspended in 200 µl of sterile Milli-Q PCR grade water (Merck, SA) and the cells were lysed using Dri-block DB.2A (Techne, SA) for 15 min at 100 °C. The cell debris was removed by centrifugation at 11,000 × g for 2 min using a MiniSpin micro centrifuge (Merck, SA). The cell lysates (10 µl) were used as template in the PCR assays immediately after extraction placed on ice for 5 min or following storage at -80 °C. Sterile Milli-Q PCR grade water (Merck, SA) was included in each PCR assay as a negative control.

### 8.2.6 PCR amplification assay

Polymerase chain reaction (PCR) was used to detect antibiotic resistant genes in the *Vibrio* species using the specific primers pairs and PCR conditions for detection of the SXT, *floR*, *strB*, *sul2*, *dfrA18*, *dfrA1*, and the *blaP1*  $\beta$ -lactamase cassette and *tetA* are listed in Table 8.1. All PCRs were done in 50  $\mu$ l volume of reaction buffer containing 0.05 unit/ $\mu$ l *Taq* polymerase as directed by the manufacturer (Fermentas Life Sciences). Cycling conditions (Bio-Rad My Cycler<sup>TM</sup> Thermal Cycler) were the following: initial denaturation at 94 °C for 2 min was followed by 35 cycles of 94 °C for 1 min, 60.5 °C for 1 min and 72 °C for 1 min with a final extension at 72 °C for 10 min and cooling to 4 °C. Electrophoresis of amplicons was performed with 1% agarose gel (Hispanagar, Spain) containing 0.5 mg/L Ethidium Bromide (EtBr) (Merck, SA) for 1 h at 100 V in 0.5 $\times$  TAE buffer (40 mM Tris-HCl, 20 mM Na-acetate, 1 mM EDTA, pH 8.5) and visualized under an UV transilluminator (BioDoc-It System, UVP Upland, CA 91786, USA).

## 8.3 Results

### 8.3.1 Antibiotic susceptibility profile

All tested *Vibrio* species were 100% susceptible to imipenem, and meropenem and uniformly resistant to chloramphenicol and erythromycin. All isolates, except 50% of *V. vulnificus* strains, were also susceptible to tetracycline (Table 8.2). All isolates of *V. fluvialis*, *V. vulnificus* and *V. parahaemolyticus* were 70-80% susceptible to neomycin, gentamicin, and amikacin. More than 90% of isolates showed a resistance to cefuroxime, nitrofurantoin, norfloxacin, cephalothin and more than 80% nalidixic acid and ciprofloxacin without appreciable variation between different species. All *V. fluvialis* strains were 80-90% resistant to the  $\beta$ -lactams antibiotics penicillin G and ampicillin. More than 90% of the isolates showed resistance to streptomycin, trimethoprim, sulfamethoxazole and cotrimoxazole. *V. vulnificus* and *V. parahaemolyticus* strains (> 60%) were susceptible to

Table 8.1: List of primers used for detection of antibiotic resistance gene and the SXT element.

Primer	Sequence (5' to 3')	Target gene	Amplicon size (bp)	Reference
SXT-F	ATGGCGTTATCAGTTAGCTGGC	SXT	1035	(5)
SXT-R	GCGAAGATCATGCATAGACC			
SUL2-F	AGGGGGCAGATGTGATCGC	<i>sul2</i>	625	(24)
SUL2-B	TGTGCGGATGAAGTCAGCTCC			
FLOR-F	TTATCTCCCTGTCGTTCCAGCG	<i>floR</i>	526	(26)
FLOR-2	CCTATGAGCACACGGGGAGC			
TMP-F	TGGGTAAGACACTCGTCATGGG	<i>dfr18</i>	389	(24)
TMP-B	ACTGCCGTTTTTCGATAATGTGG			
TetA-F	GTA ATT CTG AGC ACT GTC GC	<i>tetA</i>	950	(33)
TetA-R	CTG CCT GGA CAA CAT TGC TT			
strB-F	GGCACCCATAAGCGTACGCC	<i>strB</i>	470	(12)
strB-R	TGCCGAGCACGGCGACTACC			
dfr1-F	CGAAGAATGGAGTTATCGGG	<i>dfrA1</i>	372	(26)
dfr1-B	TGCTGGGGATTTTCAGGAAAG			
In-F	GGCATCCAAGCAGCAAG	<i>blaP1</i>	874	(10)
blaP1-B	CTGGTTCATTTTCAGATAGCG			

Table 8.2: Antibiotic susceptibility profile of some *Vibrio* species isolated from final effluent of the wastewater treatment plant.

Antibiotics	Percentage (%) Response to antibiogram profile								
	<i>V. parahaemolyticus</i> (n=14)			<i>V. fluvialis</i> (n=24)			<i>V. vulnificus</i> (n=20)		
	R	I	S	R	I	S	R	I	S
Ampicillin (25 µg)	55	45	0	90	10	0	60	40	0
Cotrimoxazole (25 µg)	30	5	65	90	10	0	30	10	60
Amikacin (30 µg)	20	10	70	25	5	70	20	0	80
Imipenem (10 µg)	0	0	100	0	0	100	0	0	100
Erythromycin (15 µg)	100	0	0	100	0	0	100	0	0
Meropenem (10 µg)	0	0	100	0	0	100	0	0	100
Streptomycin (25 µg)	15	20	65	90	10	0	30	10	60
Chloramphenicol (30 µg)	100	0	0	100	0	0	100	0	0
Ciprofloxacin (5 µg)	80	20	0	85	15	0	75	25	0

Cephalothin (30 µg)	90	10	0	95	5	0	90	10	0
Nalidixic acid (30 µg)	80	20	0	85	15	0	75	25	0
Tetracycline (30 µg)	0	0	100	0	0	100	40	5	55
Trimethoprim (30 µg)	65	30	5	95	5	0	60	40	0
Norfloxacin (10 µg)	90	0	10	95	0	5	90	10	0
Sulfamethoxazole (25 µg)	60	40	0	90	10	0	55	45	0
Gentamicin (10 µg)	30	0	70	20	10	70	0	20	80
Neomycin (30 µg)	10	20	70	15	5	80	0	30	70
Penicillin G (10 unit)	55	45	0	90	10	0	60	40	0
Nitrofurantoin (200 µg)	90	10	0	95	5	0	90	10	0
Polymyxin B (300 units)	60	40	0	70	30	0	50	45	5
Cefuroxime (30 µg)	90	10	0	95	5	0	90	10	0

---

Legend: R- Resistant; I- Intermediate; S-Susceptible

streptomycin, trimethoprim, sulfamethoxazole and cotrimoxazole. Many strains (> 50%) of *V. vulnificus* and *V. parahaemolyticus* were resistant to penicillin G, and ampicillin. All strains showed resistance to at least one antibiotic. The *Vibrio* strains displayed a wide range of multiple antibiotics resistances ranging from 7 to 10 antibiotics (Table 8.3) suggesting that the strains have originated from the environment where antibiotics were often used.

### 8.3.2 The antibiotic resistance gene cluster and SXT element of the *Vibrio* species

The SXT element was found to contain genes encoding resistance to sulfamethoxazole, trimethoprim, cotrimoxazole and streptomycin (37). As most isolates in our study were resistant to each of these antibiotics (Table 8.2), we decided to investigate whether our isolates contained the SXT element by using PCR specific primers set that produce a 1035 bp fragment of the integrase of the SXT element. PCR for the SXT element showed that 24 *V. fluvialis* strains tested were positive for the SXT element (Table 8.4). Of the 14 *V. parahaemolyticus* isolates tested for the SXT element, 11 were positive (Table 8.4). For *V. vulnificus* PCR revealed 7 positive strains that harbor the SXT element (Table 8.4).

Some tetracycline-resistant *V. vulnificus* strains screened by PCR for the *tetA* gene yielded the expected amplicon size of the gene (950 bp). *V. parahaemolyticus* and *V. fluvialis* strains did not harbor *tetA* resistant gene (Table 8.4), which was also corroborated by the phenotypic characterization (Table 8.2). The  $\beta$ -lactamase resistant gene (*blaP1*) was present in most of the *V. fluvialis*, *V. parahaemolyticus* and *V. vulnificus* strains yielding the expected amplicon size of 874 bp (Table 8.4).

Table 8.3: Multiple antibiotics resistances of *Vibrio* species isolated from the wastewater final effluents.

Antibiotics	n = 58	Percentage (%)
AMP, PEN, STR, SUL, TMP, COT, CHL , ERY, CIP, PB	10 <sup>a</sup>	17.24
STR, SUL, TMP, COT, CHL , ERY, AK, CIP, CEP	6 <sup>a</sup>	10.34
STR, SUL, TMP, COT, CHL , ERY, NAL, NOR, PB	8 <sup>a</sup>	13.79
AMP, PEN, STR, SUL, TMP, COT, CHL , ERY, GEN	6 <sup>b</sup>	10.34
AMP, PEN, STR, TMP, CHL, ERY, AK, CEP, CXM	3 <sup>b</sup>	5.17
STR, SUL, TMP, COT, CHL , ERY, NOR, GEN, CEP	4 <sup>b</sup>	6.90
AMP, PEN, TMP, SUL, COT, CHL , ERY,	1 <sup>b</sup>	1.72
AMP, PEN, STR, SUL, TMP, CHL, TET, CHL , ERY	1 <sup>c</sup>	1.72
STR, SUL, TMP, CHL, TET, CHL , ERY, NOR	3 <sup>c</sup>	5.17
STR, SUL, TMP, CHL, CHL, ERY, CIP, NIT	2 <sup>c</sup>	3.45
AMP, PEN, CHL , ERY, TET, AK, NIT, PB, CEP, CXM	5 <sup>c</sup>	8.62
AMP, PEN, STR, SUL, TMP, CHL , ERY, NEO	1 <sup>c</sup>	1.72
AMP, PEN, CHL , ERY, TET, TMP, NIT, PB	4 <sup>c</sup>	6.90
AMP, PEN, CHL , ERY, NOR, NIT, PB, CXM	4 <sup>c</sup>	6.90
Total	58	100.00

Legend: AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; NAL, nalidixic acid; NEO, neomycin; NOR, norfloxacin; STR, streptomycin; SUL, sulfamethoxazole; COT, cotrimoxazole; TET, tetracycline; TMP, trimethoprim; ERY, erythromycin; CEP, cephalothin; CXM, cefuroxime; AK, amikacin; PB, polymyxin B; PEN, penicillin G; NIT, nitrofurantoin. <sup>a</sup>*V. fluvialis*; <sup>b</sup>*V. parahaemolyticus*; <sup>c</sup>*V. vulnificus*.

Table 8.4: Antibiotics resistance gene markers profiles in *Vibrio* strains isolated from the wastewater final effluents.

Antibiotics resistance genes	Proportion of <i>Vibrio</i> species carrying the resistance genes		
	<i>Vibrio fluvialis</i>	<i>Vibrio parahaemolyticus</i>	<i>Vibrio vulnificus</i>
<i>floR</i>	24 (100)	14 (100)	20 (100)
<i>dfr18</i>	24 (100)	11 (78.57)	5 (25)
<i>blaP1</i>	10 (41.66)	10 (71.43)	15 (75)
<i>tetA</i>	0 (0)	0 (0)	13 (65)
<i>strB</i>	24 (100)	13 (92.86)	7 (35)
<i>dfrA1</i>	24 (100)	3 (21.42)	6 (30)
<i>sul2</i>	24 (100)	11 (78.57)	7 (35)
SXT	24 (100)	11 (78.57)	7 (35)

Values in parentheses represents percentages.

#### 8.4 Discussion

The alarming world-wide increase of bacterial resistance to antibiotics threatens their chemotherapeutic application. Since some of the resistant factors are also transferable to sensitive bacteria, frequent assessment of antimicrobial activity of commonly used antibiotics is desirable. The focus of this work was the analysis of a final effluent wastewater treatment plant as a reservoir for antibiotics resistance genes. The results of Polymerase Chain Reaction (PCR) profiles of the *Vibrio* species isolates from the final effluents (Table 8.4) strongly suggest that the vibrios pathogens possess a high degree of genetic diversity within the same species in the study community.

Also, the study assessed the antibiotic resistant vibrio pathogens for the presence of SXT element, tetracycline-resistance (*tetA*) and  $\beta$ -lactams resistance (*blaPI*) genes. Most of the *Vibrio* species under study exhibited resistance to these antibiotics. The fact that these organisms could create a widespread antibiotics-resistance gene pool and the ability of microorganism especially the pathogenic organism to acquire new resistance genes through horizontal transfer (6), can subsequently have detriment consequences for the health system and the economics of the Province. It is therefore important to track antibiotics-resistant genes in a variety of commensal pathogen and environmental bacteria in order to measure the environmental gene pool for resistance and to understand the molecular ecology of antibiotics resistance (1, 19).

The SXT element is a 62 kb, conjugative, self-transmissible integrating element encoding resistance to sulfamethoxazole, trimethoprim and/or streptomycin (37). Furthermore, SXT encodes an integrase at its 5' end required for SXT transfer. The SXT<sup>MO10</sup> element was first detected in the newly emerged O139 serogroup of *V. cholerae* in 1992. Since 1994 *V. cholerae* O1 strains isolated from India, Bangladesh, Mozambique and Laos have been found to contain the SXT element (2, 14, 24, 26).

In this study we could clearly show that the gene cassette arrays isolated from *Vibrio* strains residing in final effluent systems are flanked by SXT element specific sequences, since part of 5'- and 3'- conserved segment of SXT element are present on the amplification products generated by SXT element specific PCR, suggesting a role in the acquisition of antibiotic resistance genes encoding sulfamethoxazole, trimethoprim and/or streptomycin resistance. There were also differences in the antibiotics resistance gene clusters in the SXT element in the *Vibrio* strains investigated, corroborating the findings of earlier workers (24) who reported differences in antibiotics resistance and suggested that these genes were not intrinsic features of this family

of integrase, but rather appeared to have been inserted into the elements becoming transmissible bacterial populations.

Overall, from the result obtained from literature and data presented in this work, it becomes clear that SXT element specific gene cassettes are widespread in natural and man-managed environments and those bacteria from different habitats serve as reservoirs for SXT element and SXT specific gene cassettes. It is very likely that the mobile gene cassette pool contributes significantly in environments where antimicrobial drugs are present. Considering the abundance of different resistance gene cassettes, it appeared that cassettes encoding different aminoglycosides-modifying enzymes and dihydrofolate reductases for trimethoprim resistance were found frequently by previous authors (32, 33). This is in agreement with our analyses where we found *dfr18* and *dfrA1* for trimethoprim resistance, *sul2*, *strB* and *floR* encoding for sulfamethoxazole, streptomycin, and chloramphenicol respectively (Table 8.4). In addition, cassette mediating  $\beta$ -lactams resistance and tetracycline resistance were identified. The result of this study show that a pool of antibiotic-resistant genes exists within this final effluent system of the wastewater treatment facility.

In conclusion, *Vibrio* strains containing the SXT element and some specific antibiotics resistance gene including those for some  $\beta$ -lactam antibiotics are released from the final effluents of the study wastewater treatment plant into the receiving watershed. This allow us to speculate with respect to SXT element mediated resistance gene capture, dissemination and recombination of resistance gene cassettes in the final effluent system and in habitats downstream of the plant. The calls for studies to determine the extent to which transmission of antibiotics-resistant *Vibrio* occurs and the extent to which such transfer impacts on the efficacy of anti-vibrios use in human medicine is imperative. Studies on the virulence genes and sequencing of these *Vibrio* strains are subjects of ongoing project in our research group. We propose to identify the specific genes

and/or polymorphisms that are correlated with expression of virulence and disease through comparative genomic analysis. This work will increase our knowledge of the virulence of this understudied biothreat pathogen and provide a foundation for the development of diagnostic and therapeutic counter measures.

## 8.5 Acknowledgement

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## CHAPTER 9

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***Vibrio fluvialis*: an unusual enteric pathogen of increasing  
public health concern**

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## CHAPTER 9

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## CHAPTER 9

### ***Vibrio fluvialis*: an unusual enteric pathogen of increasing public health concern**

#### **Summary**

*Vibrio fluvialis* is a halophilic *Vibrio* species associated with acute diarrhoeal illness in humans. It has the potential to cause outbreaks and has an association with paediatric diarrhoea. *Vibrio fluvialis* is an important cause of cholera-like bloody diarrhoea and rarely causes wound infection with primary septicaemia in immunocomprised individuals from developed to underdeveloped countries, especially in regions with poor sanitation. A number predispose factors have been associated with *Vibrio fluvialis* pathogen such as liver disease (especially alcoholic cirrhosis), immunocomprised states such as HIV/AIDS, iron overload (hemochromatosis), and diabetes mellitus. The prevention of the spread of *Vibrio fluvialis* infections depends on ensuring appropriate sanitary measures and efficient disposal of sewage effluents. Parenteral or oral fluid electrolyte replacement is used to prevent dehydration, and broad-spectrum antibiotics are used in chronic or life threatening cases. This paper addresses the epidemiology of this pathogen, pathogenesis of its disease and its clinical manifestations in humans.

## 9.1 Introduction

*Vibrio fluvialis* is a Gram-negative bacterium, straight to slightly curved rod that is motile by means of polar flagella. It is a sodium chloride-requiring, oxidase-positive, nitrate-positive organism that ferments D-glucose and other carbohydrates with the production of acid and gas; has 50 mol% guanine plus cytosine in its DNA (Lee *et al.*, 1978b); and is isolated from water, animal feces, human feces, sewage, and seafood product.

In 1977, Furniss *et al.* (1977) reported the isolation of a new group of bacteria, designated, group F, in Bahrain from humans suffering from severe diarrhoea. The newly described bacteria possessed phenotypic properties intermediate between those of *Vibrio* spp. and *Aeromonas* spp. and were associated with gastroenteritis. Subsequently, results of a numerical taxonomy study of 154 strains of *Vibrio* spp., *Aeromonas* spp., and group F organisms were reported by Lee *et al.* (1978b). Group F strains were found to cluster within a single group, with two sub-clusters separable on the basis of gas production from glucose. All strains isolated from diarrhoeal patients were found to be anaerogenic, whereas those from the environment included both aerogenic and anaerogenic strains. The authors concluded that group F organisms are more closely related to *Vibrio* spp. than to *Aeromonas* spp. Group F organisms have been reported to be widely distributed in the marine and estuarine environment around Britain (Lee *et al.*, 1978b).

The distribution of *V. fluvialis* is global phenomenon (McNicol *et al.*, 1980) and this organism is not only isolated from human diarrhoeal cases which has been reported by previous authors (Huq *et al.*, 1980; Hodge *et al.*, 1995) but also from aquatic environments (Seidler *et al.*, 1980; Lee *et al.*, 1981; Morris and Black, 1985). We recently reported *V. fluvialis* from a treated effluent system in South Africa (Igbinosa *et al.*, 2009). There are reports of food poisoning caused by this organism (Kobayashi and Ohnaka, 1989; Thekdi *et al.*, 1990), especially due to consumption of raw shellfish (Levine and Griffin, 1993). *V. fluvialis* is also associated with

extra-intestinal infections (Yoshii *et al.*, 1987; Albert *et al.*, 1991). The clinical indication of the disease include mild to moderate dehydration, vomiting, fever, abdominal pain and diarrhoea (Seidler *et al.*, 1980).

Given its public health implication, *V. fluvialis* has been the subject of intensive study for the last two decades, since it was isolated in 1975 and described in 1977 by Furniss and his group, yet many questions remain unanswered about its microbiological characteristics, virulence-engendering factors, and possible methods of attenuation. Since *V. fluvialis* enteritis is reported infrequently, the epidemiology of this infection is not adequately understood. There is very little information available on the virulence factors associated with infection and much less information on the mechanism of pathogenicity of this organism. In our laboratory, microbial water/wastewater quality studies are in progress and directed at assessing the hazards implications of this organism in the aquatic milieu. This ‘super bug’ has been isolated from treated final effluent from wastewater treatment plants in our recently conducted study both at the rural, sub-urban and urban communities of the Eastern Cape Province of South Africa (Igbiosa *et al.*, 2009; Igbiosa and Okoh, unpublished report). After referring to recent scientific research on *Vibrio fluvialis*, the review stresses the need for better understanding of the risk factors of emerging *Vibrio* infection as a result of *Vibrio fluvialis* in order to establish adequate prevention measures for control of human diseases. The prevention of this infection is of immense public health significance.

## **9.2 Classification of *V. fluvialis* strains**

As earlier highlighted *Vibrio fluvialis* were first described by Furniss *et al.* (1977). The organisms, designated group F, were isolated in 1975 from a patient with diarrhoea in Bahrain, from patients with diarrhoea in Bangladesh, and from shellfish and estuarine waters in England.

Group F required salt and had a number of properties compatible with or midway between those of vibrios and aeromonads. In a numerical taxonomic study, Lee and co-workers showed that six group F strains were a distinct phenon that probably represented a new species (Lee *et al.*, 1978a) and that the group contained two subgroups on the basis of gas production during fermentation of glucose (Lee *et al.*, 1978b). Huq *et al.* (1980) studied a large number of strains associated with an outbreak of diarrhoea in Bangladesh as well as strains isolated from patients with diarrhoea in Indonesia, strains from sewage in Brazil, and U.S. strains that had been called group EF-6 in the Special Bacterial Reference Activity at the Centers for Disease Control. By both phenotypic tests and DNA relatedness, they found that the organism was closer to the genus *Vibrio* than to the genus *Aeromonas*. All of their strains produced no gas from the fermentation of glucose (were anaerogenic) and formed a single DNA relatedness group (Brenner *et al.*, 1983). Thus, the EF-6 group appeared to be identical to group F. Group F strains isolated from several parts of the world were compared phenotypically and genetically by Seidler *et al.* (1980). They confirmed and extended the observation that group F was more closely related to *Vibrio* than to *Aeromonas*. They further showed that the aerogenic group F strains were in a different DNA relatedness group from the anaerogenic strains, and they recommended that the two biogroups be considered as two separate species within the genus *Vibrio*. Lee *et al.* (1981) proposed the name *V. fluvialis*, which included both aerogenic and anaerogenic strains of group F and the synonymous group EF-6. An anaerogenic strains was chosen as the type strain of *V. fluvialis*. These authors noted that both aerogenic and anaerogenic strains of *V. fluvialis* were found in the environment but that only anaerogenic strains had been isolated from humans with diarrhoea (Lee *et al.*, 1981).

### **9.3 Sources and routes of *Vibrio fluvialis* infection transmission to human**

There is a complex interaction of environmental and behavioural factors of both animals and humans, which facilitate the spread of *Vibrio fluvialis* infections. Poor sanitation and hygiene conditions as well as lack of or little environmental awareness among people is considered as the major cause of source water contamination. An example of such ways is agricultural practices that involve usage of sewage water and/or cattle manure on farms. Another practice is uncontrolled waste disposal, bathing and swimming in water sources such as rivers and dams, which serve as sources to municipal water supplies, answering calls of nature in velds and grazing of cattle next to catchment areas (WHO, 2006). Enteric infections cause considerable morbidity and mortality worldwide, especially among children in developing countries. *Vibrio fluvialis* infections are common in areas that have high levels of fecal contaminated water, food supplies and consumption of raw seafood or contaminated seafood products (WHO, 2006). The causative microbes are environmentally determined, with transmission occurring through fecal contamination of food or water or by person-to-person contact (Igbinosa and Okoh, 2008). Infection rates are highest where general standards of living, water supply, and sanitary conditions are low or inadequate. Microbial contamination of water is the largest and most immediate health hazard. Surface water quality is subjected to frequent dramatic changes in microbial quality as a result of the variety of activities on the watershed (Okoh *et al.*, 2005; Igbinosa and Okoh, 2009). These changes could be caused by discharges of municipal raw waters or treated effluent at a specific point-source into the receiving waters (Okoh *et al.*, 2007).

Okoh *et al.* (1996) reported on the qualities of wastewater effluents of some crude oil flow stations in the Niger delta which are channeled into the saver pits prior to discharge into the environment including water bodies such as streams or rivers and suggested that such saver pits could be potential sources of pathogens in the watershed. Also, in rural and sub-urban

communities of most developing nations, the reuse of sewage and wastewater is often the only source of water for irrigation in these areas, and eating fruit and vegetables that have been irrigated with inadequately treated wastewater is a most likely route of contracting *V. fluvialis* infection (Okoh *et al.*, 2007; Igbinosa and Okoh, 2008; 2009; Igbinosa *et al.*, 2009). The practice of direct discharge of effluents into receiving water bodies is of major concern as it could result amongst other things in the substantial increase in organic load and consequently depletion of the dissolved oxygen content of the receiving water body (Okoh *et al.*, 1996, 2007; Igbinosa and Okoh 2009).

There are ongoing debates about the origins of this emerging *Vibrio fluvialis* strains, but most attribute at least some causation to global changes impacting on the social and natural environments. There is a speculation that *Vibrio fluvialis* infection strains evolved from highly polluted waters with intense population pressure in large urban concentration, intensification of farming to feed growing populations, and widespread poverty and inequalities in the region. Tons of pesticides, millions of gallons of industrial waste and raw sewage, and millions of tons of chemical fertilizers are dumped daily into local rivers flowing into the bay. It was previously thought that pollution dumped at sea would quickly degrade as it sunk downwards, but there is growing evidence that microbes and other biological material can survive in suspended animation in the ocean depths, capable of resurfacing via the food chain or ocean currents (McKie, 1999). This human-induced degradation of the local environment is also leading, it is believed, to widespread changes in the coastal ecology (Lee, 2001).

An alternative environmentally-focused theory argues that patterns of cholera-like diarrhoea epidemics in South Asia can be linked to patterns of global climates changes. Human-induced climates changes may be creating favourable conditions such as water, temperature,

nutrient concentration and plankton production, for the growth and reproduction of the bacterium (Lee, 2001).

#### **9.4 Epidemiology**

*Vibrio fluvialis* is considered to be one of the foodborne pathogenic bacteria and has been implicated in outbreaks and sporadic cases of diarrhoea (Oliver, and Kaper, 2001). Existing naturally in warm, salty, and brackish water, *V. fluvialis* survive in temperature between 9 °C and 31°C, but thrives when water temperature rises above 18 °C. *V. fluvialis* infections demonstrate a seasonal pattern with the majority of clinical illnesses when temperature and salinity factors are most favourable to the bacteria's proliferation (Igbinosa *et al.*, 2009). In Bahrain, *V. fluvialis* was identified for the first time as group F *Vibrio* in 1975 in a patient with diarrhoea (Furniss *et al.*, 1977), and it was later named *V. fluvialis* (Lee *et al.*, 1980). Between October 1976 and November 1977, the largest outbreak of *V. fluvialis* infection was reported in Bangladesh (Huq *et al.*, 1980). The outbreak involved more than 500 patients, with 50% of them being young children.

In the United States, *V. fluvialis* has been associated with enterocolitis in infants (Bellet *et al.*, 1989), and has also accounted for 10% of clinical cases in a survey of vibrio infections along the Gulf Coast (Levine and Griffin 1983). It has also been detected in the stools of persons with diarrhoea in Jordan, Yugoslavia (Lee *et al.*, 1981), and Bahrain (Furniss *et al.*, 1977). In the United States, the organism has been isolated from a wound of a patient in Hawaii; from water and sediment in the New York Bay (Seidler *et al.*, 1980); from shellfish in Louisiana; and from water and shellfish in Pacific Northwest estuaries (Tison, *et al.*, 1982). Recently it was reported to be associated with acute diarrhoea in Indonesia (Lesmanaa *et al.*, 2002). Currently, *V. fluvialis* has an infectious importance because its clinical symptoms of gastroenteritis are very similar to

that of *V. cholerae*. The matter became more serious after the recent characterization of an enterotoxigenic El Tor-like hemolysin in *V. fluvialis*, which represents one of the virulence factors of *V. cholerae* (Kothary *et al.*, 2003). *V. fluvialis* have been reported as causing necrotising fasciitis and septicaemia in the Gulf of Mexico and Southeast Asia, associated with minor trauma and exposure to fish, raw oyster, shellfish, crabs or seawater, especially in the summer months (Chakraborty *et al.*, 1997; Morris 2003; Tsai *et al.*, 2004).

Infections due to *V. fluvialis* most commonly present as gastroenteritis and diarrhoea (Klontz *et al.*, 1994; Kothary *et al.*, 2003; Lesmana *et al.*, 2002). *V. fluvialis* produces various extracellular toxic factors, such as lipase, protease, and haemolysin (Kothary *et al.*, 2003; Rahim and Aziz, 1996; Wong *et al.*, 1992). Baffone *et al.* (2001) reported that *V. fluvialis* has weak adhesiveness and no bacterial cytotoxicity, but Wong *et al.* (1992) found it had strong haemolytic and proteolytic activity. Two cases of fatal infection due to *V. fluvialis* have been reported (Klontz *et al.*, 1994). However, *V. fluvialis* rarely causes wound infection with primary septicaemia (Morris, 2003). It accounted for 10% of *Vibrio* gastroenteritis cases in a US survey (Altekruse *et al.*, 2000). Unlike other *Vibrio* spp., which have commonly been reported to cause extraintestinal infections, *V. fluvialis* is uniquely associated with gastroenteritis, with only rare reports of extraintestinal infections such as hemorrhagic cellulitis with cerebritis, bacteremia, and peritonitis (Huang and Hsu, 2005; Lai *et al.*, 2006; Ratnaraja *et al.*, 2005; Tacket *et al.*, 1982; Lee *et al.*, 2008).

### **9.5 Survival of *Vibrio fluvialis* and other vibrios in environments**

*Vibrio* spp. are, in most cases, found as free planktonic bacteria in the environment but in complex multispecies biofilm structures attached to various biotic and abiotic surfaces (Donlan, 2002; Schembri, *et al.*, 2002; Igbinosa *et al.*, 2009). Many *Vibrio* spp. are often found attached to

chitinaeous exoskeleton of zooplankton (Donlan, 2002; Schembri, *et al.*, 2002). Biofilms contribute to the survival of bacterial communities by promoting interspecies metabolic and genetic cooperation as well as protection against diverse environmental stresses, such as starvation and predation (Costerton *et al.*, 1995; Wai *et al.*, 1999; Watnick and Kolter, 2000).

Usually, lack of nutrient is the most common environmental stress which microorganisms routinely encounter in natural ecosystems. However, it was found that *Vibrio* spp. can survive for a long time during starvation by sequential changes in cell physiology and gradual changes in morphology (Albertson *et al.*, 1990; Morita, 1993; Östling *et al.*, 1993). Moreover, it was reported that some species develop the so-called viable but non culturable (VBNC) state in response to certain stress conditions (Oliver and Wanucha, 1989; Biosca *et al.*, 1996; Jiang and Chai, 1996). It has been proposed that the VBNC state is an adaptative strategy of microorganisms against stress from which cells may be able to recover once optimal conditions are restored (Huq and Colwell, 1995; Nybroe, 1995; Oliver, 1995). VBNC state was described for many *Vibrio* species [*V. anguillarum*, *V. campbellii*, *V. cholerae*, *V. fischeri*, *V. harveyi*, *V. mimicus*, *V. natriegens*, *V. parahaemolyticus*, *V. proteolyticus*, *V. vulnificus*] (McDougald *et al.*, 1998). Amel *et al.* (2008) studied survival of *V. fluvialis* in seawater under starved condition and found that *V. fluvialis* have developed strategies that allow it to survive in seawater in the absence of nutrients and outside its natural host during a long period of time. Under these conditions, this bacterium maintains its virulence factors expressed.

A long-term starvation survival has been described for both *V. anguillarum* and *V. salmonicida* that are able to survive for more than 60 weeks in seawater at a temperature of 6-8 °C (Hoff, 1989). Bacterial survival is further enhanced when surfaces are available for their attachment or when they are located in sediment (Dawson *et al.*, 1981). *V. salmonicida* was isolated in the sediment of a fish farm more than 18 months after an outbreak of vibriosis (Enger *et al.*, 1989).

Many enzymes, which can metabolize aquatic substrates and contribute to bacterial survival in the environment, have been identified in several *Vibrio* spp. Agarases are enzymes that degrade agar, a compound found in the cell walls of algae, releasing a metabolisable product that is used as an energy source (Sugano *et al.*, 1993; Sugano *et al.*, 1994). Chitinases degrade chitin, a homopolymer of N-acetylglucosamine, which is the major component of the cell walls of many organisms such as fungi, crustaceans and insects. Chitin is the largest pool of amino sugars in the oceans and the ability to degrade it confers an important survival advantage (Riemann and Azam, 2002). Chitinase activity has been detected notably in *V. anguillarum*, *V. furnissi* and *V. cholerae* and more than 10 enzymes with chitinase activity are produced by *V. harveyi* (Bassler *et al.*, 1991; Conchas *et al.*, 1991; Hirono *et al.*, 1998). *In vitro*, *V. cholerae* uses chitin as a sole carbon source for growth (Garay *et al.*, 1985), providing to the bacterium the potential to use a readily available nutrient source in aquatic environments and to colonise ubiquitous marine environments. These long-term starvation strategies in seawater or attachment to surfaces in the aquatic environment indicate that most of the pathogenic vibrios are endemic species in the marine environment. They can survive during a relative long period in various milieus and re-infect their respective marine host when the conditions become favourable (Huq and Colwell, 1995).

## **9.6 Clinical manifestations**

*Vibrio fluvialis*-related illness is characterized by gastroenteritis, nausea, loss of appetite, vomiting, watery bloody diarrhoea with abdominal cramps or significant fever. Moderate to severe dehydration, hypokalemia, metabolic acidosis, and occasionally, hypovolemic shock can occur in 4 to 12 hours if fluid losses are not replaced. Stools are colourless, with small flecks of mucus and contain high concentrations of sodium, potassium, chloride, and bicarbonate.

*V. fluvialis* rarely causes wound infection with primary septicaemia. The wound infection (cellulitis) that is caused by direct inoculation of bacteria into the skin or exposure of a wound to contaminated water (Huang and Hsu, 2005). The bacterium (and its associated toxins) rapidly cause local tissue necrosis associated with hemorrhagic bullae and erosions (Huang and Hsu, 2005). Cellulitis may occur when an abrade area of skin is inoculated through bathing in marine waters where *Vibrio fluvialis* thrives, or through exposure to liquid from harvested raw seafood (Morris, 2003; Huang and Hsu, 2005). This type of exposure typically occurs while sucking or handling raw oysters. Since the organism causes obliterating vasculitis and vascular necrosis, therapeutic levels of antibiotics may not reach the organism and rapid amputation may be necessary to prevent progression.

The primary septicaemia syndrome consists of high fever and chills, often with vomiting, diarrhoea, abdominal pain and extremities pain (Morris, 2003) with no apparent focus of infection. Major diagnostic clues for *V. fluvialis* sepsis syndrome are hemorrhagic bullae which can be seen both in sepsis and cellulitis (Morris, 2003). It is believed that the bacteria most likely enter the circulation through the intestine (Morris, 2003).

A number of host factors predispose patients to severe infection with *V. fluvialis*. Known adverse host factors include liver disease (especially alcoholic cirrhosis), immunocompromised states such as HIV/AIDS, iron overload (e.g., hemochromatosis), and diabetes mellitus (Morris, 2003).

## **9.7 Pathogenesis of disease**

### *9.7.1 Virulence factors*

In spite of a significant volume of published research effort to elucidate virulence factors of *V. fluvialis* that are responsible for the notable disease process, very little definitive information has

been achieved. Several virulence factors have been identified in *V. fluvialis*, but the majority of them are only partially characterized and their precise role in virulence remains to be known. From the enzyme-linked immunosorbent assay, Chikahira and Hamada (1988) have reported that several *V. fluvialis* strains isolated from environmental and human sources produced an enterotoxin which is immunologically indistinguishable from cholera toxin (CT).

*V. fluvialis* produces several toxins that may be important in pathogenesis including an enterotoxin-like substance, protease, cytotoxin, and hemolysin (Wall *et al.*, 1984). Endotoxin activity of *V. fluvialis* has been demonstrated *in vitro* using Chinese hamster ovary (CHO) cells. Lockwood *et al.* (1982) reported that at least four biologically active substances could be found in culture supernatants of *V. fluvialis* strain 5489. CHO cell elongation factor, CHO cell killing factor (CKF), and cytotoxin active against rabbit erythrocytes were identified when the bacterium was grown without lincomycin. Finally, CHO cell rounding toxin, which is known to be a protease, was found. CKF was internalized and cell death was induced by disruption of cellular function (Wall *et al.*, 1984). These four active substances were heat-labile and each crude concentrate caused fluid accumulation in the small intestines of infant mice. Of many virulence factors produced from *V. fluvialis*, hemolysin was thought to be most important.

Hemolysin has been known to be an important virulence factor in the pathogenic processes of many clinical microorganisms, causing hemorrhagic septicemia and diarrhoea (Honda *et al.*, 1976; Kreger and Lockwood, 1981). It can lyse erythrocytes and a variety of other cells including mast cells, neutrophils, and polymorphonuclear cells as well as enhance virulence by causing tissue damage or by dissolving material that would prevent spreading of the pathogen throughout the tissue. Several extracellular hemolysins have been characterized from *Vibrio* spp. The thermostable hemolysin from *Vibrio parahaemolyticus* exhibited enterotoxic effects on human and rat cell monolayers (Honda and Iida, 1993; Raimondi *et al.*, 2000). To

date, *Vibrio* hemolysin genes have been isolated from *V. cholerae* (Alm *et al.*, 1988), *V. parahaemolyticus* (Nishibuchi and Kaper, 1985), *V. anguillarum* (Hirono *et al.*, 1996) and *V. mimicus* (Kim *et al.*, 1997). However, the role and biological properties of hemolysin from *V. fluvialis* have been studied by Han *et al.* (2002). They found that hemolysin of *Vibrio fluvialis* (VFH) forms pores in erythrocyte membrane and by using osmotic protectants. The authors estimate the diameter of the pores to be 2.8–3.7 nm. This size seems larger than those formed by other *Vibrio* hemolysins such as *V. cholerae* (Zitzer *et al.*, 1995; Krasilnikov *et al.*, 1992), *V. parahaemolyticus* (Honda *et al.*, 1992), *V. metschnikovii* (Miyake *et al.*, 1989), *V. mimicus* (Shinoda *et al.*, 1993), and *V. vulnificus* (Yamanaka *et al.*, 1987). They suggest that VFH, a major hemolysin of *V. fluvialis*, is a pore forming toxin and induces osmotic lysis in erythrocytes. Chakraborty *et al.* (2005) report that *V. fluvialis* showing cytotoxic and vacuolating activity on HeLa cells. These strains are also capable of causing haemolysis of sheep red blood cells. The utilization of heme compounds by *V. fluvialis*, although an iron acquisition system mediated by the catecholate siderophore fluvibactin has been reported (Yamamoto *et al.*, 1993). Expression of iron-regulated proteins in *Vibrio* spp. has been related to increased virulence in animal models, but the role of heme utilization proteins in bacterial survival under oxidative stress and their effect on the production of pathogenic factors such as hemolysin is unknown. Ahn *et al.* (2005) report the identification of the heme utilization protein HupO, which mediates the acquisition of iron from hemin in *V. fluvialis* and has amino acid sequence homology to bacterial outer membrane heme receptors.

## **9.8 Antimicrobial resistance**

An increase in the emergence of multi-antibiotics resistant bacteria in recent years is worrisome and the presence of antibiotics resistance genes on bacterial plasmids has further helped in the transmission and spread of drug resistance among pathogenic bacteria (Zulkifli *et al.*, 2009).

Antimicrobial resistance has become a major medical and public health problem as it has direct link with disease management (Ramamurthy, 2008). Antibiotics such as tetracycline, doxycycline, norfloxacin, ciprofloxacin, streptomycin and fluoroquinolones may be used as an adjunct to rehydration therapy and are critical in the treatment of septicemia patient (Lima, 2001; Bhattacharya, 2003; Chiang and Chuang, 2003).

Multiple antibiotics resistance gene cluster with the same genetic locus (Resistance Island) can be transferred to other organisms. Spread of antibiotic resistance in microbes has been attributed to the mobilization of drug resistance marker by a variety of agent like plasmid, transposons, integrons and SXT element (Waldor *et al.*, 1996; Hochhut *et al.*, 2001). Ahmed *et al.* (2004) reported that *Vibrio fluvialis* have showed typical multidrug resistance phenotypes of SXT. In their findings, they observed that *V. fluvialis* was resistant to chloramphenicol, streptomycin, cotrimoxazole (trimethoprim and sulfamethoxazole), ampicillin, furazolidone, nalidixic acid, and gentamicin, and concur with our recently conducted research (Okoh and Igbinosa, unpublished report). There are few factors that may contribute to the *Vibrio* species antibiotic resistant. Firstly, a mutation in cellular DNA could modify the antibiotics target site or transport mechanism, causing a decreased action of the antibiotic on the cell (Saitanu *et al.*, 1994; Zulkifli *et al.*, 2009). Other factors were an extra gene product target site or transport mechanism (Son *et al.*, 1997).

## **9.9 Treatment**

An effective treatment of diarrhoeal disease has the potential to substantially lower morbidity and mortality. The reduction of mortality from diarrhoeal is primarily related to the effective management of dehydration. In general, oral rehydration plus bismuth subsalicylate or loperamide is adequate therapy for mild to moderate diarrhoea (less than four stools per day)

(Ericsson and DuPont, 1993). The gastroenteritis syndrome is usually self-limited and does not require parenteral therapy (Bellet *et al.*, 1989; Altekruise *et al.*, 2000), the sepsis syndrome and the cellulitis syndrome are potentially life and limb threatening and require aggressive antibiotics therapy. Any delay in treatment in the later two syndromes increase the likelihood of poor outcomes for the patient especially if hypotension ensues. There is debate over which antibiotic regime is most effective given to multiple resistance pattern of *V. fluvialis* pathogen. Haq and Dayal (2005) recommend 100 mg doxycycline intravenously every twelve hours, combined with two grams ceftazidime intravenously every eight hours. A group in Taiwan performed *in vitro* antibacterial testing and found several cephalosporin antibiotics effective in killing *Vibrio* infections including ceftazidime, ceftriaxone and cefotaxime (Chiang and Chuang, 2003). They also found imipenem and a variety of quinolones to be equally efficacious. Several prophylactic and treatment drug regimes have been described for *Vibrio* infections with quinolones being the current drugs of choice for both prophylaxis and treatment (Chiang and Chuang, 2003). Yet, the use of quinolones in the pediatric population remains controversial. The combined therapy with doxycycline and ceftazidime is the recommended therapy according to CDC website. For treatment of children in whom doxycycline is contraindicated, the CDC website recommends a combination of trimethoprim-sulfamethoxazole and aminoglycoside (McCann, 2006).

The initial antibiotic choice for bacterial peritonitis often is empiric, based on the most likely pathogens, and 3<sup>rd</sup> generation cephalosporin or ampicillin plus aminoglycoside are commonly used. Third-generation cephalosporins, doxycycline, amoxicillin / clavulanate, and fluoroquinolones were commonly used for extra-intestinal *Vibrio* infections, mainly with *V. vulnificus* (Huang and Hsu, 2005). The treatment guideline for extra-intestinal *V. fluvialis* infection are not established, and the antibiotics were different in each case: cefuroxime and trimethoprim/sulfamethoxazole, gentamicin and ciprofloxacin, ceftazidime and oxytetracycline

(Huang and Hsu, 2005; *Lai et al.*, 2006; Ratnaraja *et al.*, 2005). Further survey is needed to decide which is the treatment of choice for extra-intestinal infection by *V. fluvialis*.

## **9.10 Prevention**

### *9.10.1 Public health implication*

The prevention of the spread of *V. fluvialis* strain cholera-like diarrhoea depends on ensuring appropriate sanitary measure like hand-washing, proper food preparation, efficient sewage treatment system disposal. Proper surveillance of water, food, and sanitation facilities, using public health diagnostic and detection procedures is necessary to protect individuals including infants from this cholera-like diarrhoea.

Environmental health protection measures that can be applied in agricultural use of wastewater for irrigation including wastewater treatment, crop restriction, control of wastewater application and human exposure, and promotion of hygiene (Okoh and Osode, 2008), since consumers of irrigated crops that are likely to be eaten uncooked are at high risk for direct contact with pathogens leading to this disease. As with drinking water quality surveillance, finding affordable ways of monitoring the presence of harmful contaminants in wastewater that can accrue in soil and crops is essential.

In evaluating the degree of the *Vibrio fluvialis* risk attention should be directed to control of fish and shellfish at the point of capture/harvest, a good sanitary management of farms and new regulatory initiative should be adopted both to limit losses caused by emerging pathogens and avoid the transmission of foodborne diseases to consumers, and also consumers should be informed of the risk they incur in eating raw or undercooked fish and seafood because, even when all preventive measure have been applied, pathogenic bacteria may have accumulated in the live animals.

The essential step to protect against emerging diseases caused by *Vibrio fluvialis* is an effective global surveillance system to give early warning of infections. Medical practitioners must be aware of *Vibrio fluvialis* infections and contribute to the identification of the risk factor associated with these pathogens in order to suggest specific preventive measures.

### **9.11 Concluding remarks**

A number of circumstances could influence the emergence and re-emergence of different *Vibrio* species as significant pathogens in both developing and developed countries. *Vibrio fluvialis* ranks very high as a human public health hazard amongst bacterial pathogens as well as a contaminant in marine foods and food products, and causing havoc in both freshwater and marine environments. The prime challenge in preventing the spread of these pathogens is poverty, which goes with poor sanitization, which has always been a bane of developing nations. There is need for proper surveillance of water, food and sanitation facilities to eradicate *Vibrio fluvialis* malaise.

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## **GENERAL DISCUSSION**

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## CHAPTER 10

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## GENERAL DISCUSSION

The increasing scarcity of water in the world along with rapid population increase in urban areas gives reason for concern and the need for appropriate water management practices. Very little investment has been made in the past on sewage treatment facilities; water supply and treatment often received more priority than wastewater collection and treatment. However, due to the rising rural-urban development, wastewater treatment deserves greater emphasis. Currently there is a growing awareness of the impact of sewage contamination on aquatic milieu; wastewater treatment is now receiving greater attention from the World Bank and government regulatory bodies (Looker, 1998) as it has become crucial that sanitation systems have high levels of hygienic standards to prevent the spread of diseases.

Assessment of water and wastewater is very crucial to safeguard public health and the environment (Okoh *et al.*, 2005; 2007). However, water quality data on fresh and marine waters in South Africa are still sparse and poorly coordinated. Therefore, monitoring these parameters is important for safety assessment of the environment and human public health in particular (Okoh *et al.*, 2007). Physicochemical parameters such as temperature, pH, DO, salinity, and nutrient loads have been reported to influence biochemical reactions within water systems such that changes in the concentration of these parameters are indicative of changes in the condition of the water system (Hacioglu and Dulger, 2009). Wastewater discharges may contain health compromising pathogens, carcinogenic substances (e.g., heavy metals, trihalomethanes, etc), and /or chemical substances which may cause adverse environmental impact such as changes in aquatic habitats and species composition, decrease in biodiversity, impaired use of recreational waters and shellfish harvesting areas, and contaminated drinking water (Environment Canada 2001; CCME, 2006). All of these impact leads to a less valuable environment, poor health, a less prosperous economy, and ultimately, a diminished quality of life (Environment Canada 2001).

Recent studies on wastewater treatment plants have shown that small wastewater treatment plants are often situated in remote sub-urban or rural areas where technical and management capacity is hard to come by (Vuuren *et al.*, 2009).

Hydrogen ion concentration (pH) is an important parameter in assessing water quality. pH has significance in controlling corrosion and scaling tendency of water, and its measurement is useful in monitoring for compliance with set standards and to ensure that industrial effluents meet certain specific criteria. pH measures hydrogen ion activity in water, a characteristic that is related to the water's alkalinity or acidity. The pH values from this study varied significantly ( $P < 0.05$ ) in rural sampling stations throughout the study periods and ranged from 6.10 to 7.92 irrespective of seasons. The pH in the sub-urban community study location also varied significantly ( $P < 0.05$ ) and range from 6.88 - 7.61 across sampling stations and seasons, while in the urban study location the pH values varied significantly ( $P < 0.01$ ) between 6.8 and 8.3 across the sampling stations. Similar pH ranges have been reported in the literature for final effluents and their receiving waters (Manios *et al.*, 2006). On the other hand, Ogunfowokan *et al.* (2005) reported lower pH ranges (5.23 - 6.32) and Akan *et al.* (2008) reported higher pH values (8.94 - 10.34) for wastewater effluents and their receiving watersheds in Nigeria. The composition of wastewater effluent varies from facility to facility according to the level of treatment, type of households, businesses, industries, and public facilities discharging into the aquatic system (Environment Canada, 2001) and this could be an important contributory factor to the observed differences in pH regimes.

Generally the pH values obtained fell within the World Health Organization standard of 7.0 to 8.5 and the water quality ranges of 6.5 to 8.5 for drinking water and water meant for full contact recreation, respectively (WHO, 2004; DWAF, 1996b). The European Union also sets pH protection limits of 6.0 to 9.0 for fisheries and aquatic life (Chapman, 1996). The neutral to alkaline pH values obtained in most sampling sites and points are similar to those reported

elsewhere (Morrison *et al.*, 2001). The pH values observed in this study across all sampled stations and locations fell within the recommended standards irrespective of seasons. This suggests that the effluents may not negatively impact significantly on the receiving watersheds for domestic, fishery and recreational purposes with reference to pH regulations.

Temperature is an important water quality parameter due to its influence on other parameters. Temperature affects the solubility and consequently the availability of oxygen in water (Akan *et al.*, 2008); it also affects the toxicity of some chemicals in water systems as well as the sensitivity of living organisms to toxic substances (Dojlido and Best, 1993; Mayer and Ellersieck, 1988). The water temperature profile from the study range between 13.79 and 24.73 °C and were below 25 °C acceptable limit of no risk for domestic water uses in South Africa (DWAF, 1995). Based on these guidelines, the water temperature of the effluent does not appear to pose any threat to the homeostatic balance of the receiving water bodies, in conformity with the report of Jaji *et al.* (2007). Higher water temperatures can decrease the dissolved oxygen concentrations in water and consequently its availability to aquatic organisms (DWAF, 1996c).

Electrical conductivity (EC) is a measure of dissolved ions in water systems; it has also been reported to be a useful and easy indicator of salinity or total salt content of water systems (Morrison *et al.*, 2001). The South African guideline for conductivity in effluent that could be discharged into the receiving waterbodies is 250  $\mu\text{Scm}^{-1}$  (Government Gazette, 1984), and based on this guideline the effluent quality does not appear to be compliant with the regulation for electrical conductivity. The South African acceptable limit for conductivity in domestic water supply is 70  $\mu\text{Scm}^{-1}$  (DWAF, 1996a). This limit was exceeded in the receiving water bodies thus posing a risk to direct domestic usage of the water from these sources. The electrical conductivity values obtained in this study is similar to the findings of previous study on the nearby Keiskamma River (Fatoki *et al.*, 2003).

Turbidity is a measure of suspended particles in water systems. These suspensions could be inorganic and / or organic matters. Microorganisms are usually associated with high concentrations of organic matter; hence high turbidity will more often than not support the growth of pathogens and increase the chances of infection (Obi *et al.*, 2007). The presence of suspended particles in a water bodies could also render it unfit for full-contact recreational uses (DWAF 1996a). Turbidity of the final effluents across the sampling sites irrespective of seasons varies between 3.68 and 9.64 NTU, but there is no standard for turbidity for effluent discharge in South Africa (Government Gazette, 1984). The mean turbidity values for final effluents and the receiving waterbodies (2.7 - 35 NTU) across all sampled sites and locations in this study fell short of the target water quality limit (0 - 1 NTU) of no risk for domestic water uses in South Africa (DWAF, 1996b); implying that the water system under study is not suitable for domestic uses with reference to turbidity. These values are grossly exceeded in the water samples and it disqualifies the receiving watersheds for direct domestic use. Also, the excessive turbidity in water can cause problem with water purification processes such as flocculation and filtration, which may increase treatment cost (DWAF, 1998). There may be a tendency for an increase in trihalomethane (THM) precursors, where highly turbid waters are chlorinated (Fatoki *et al.*, 2003). Trihalomethane is a carcinogenic compound formed as a by-product of chlorine and organic matter reaction in water systems and has serious health implications for aquatic life and humans if exposed to it (Environment Canada, 2001). High turbid waters are often associated with the possibility of microbiological contamination, as high turbidity makes it difficult to disinfect water properly (DWAF, 1998; Obi *et al.*, 2007). However the turbidity values were generally lower during the winter and spring seasons compared to summer and autumn. The increase in values during the summer and autumn season could be attributed to surface runoff and erosion carrying soil/silt (Morokov, 1987). The turbidity values obtained in this study were

higher than those reported for Umtata River and Keiskamma River in South Africa (Fatoki *et al.*, 2001; 2003).

High salt content of effluents discharged into a receiving watershed could cause serious ecological disturbance that may result in adverse effects on the aquatic biota (Morrison *et al.*, 2001). The salinity of a water system as in total dissolved solids (TDS) may affect the amount of oxygen that can dissolve in a water system; under normal circumstances, it relates inversely with dissolved oxygen and may lead to oxygen depletion in water systems (Akan *et al.*, 2007). Although there are no set standard for salinity level for effluent discharge into the aquatic ecosystems in South Africa, the water quality criteria for South African coastal zones (SACOR, 1984) put the acceptable range of salinity in marine ecosystem for all biological activity at 33-36 psu, while Whitfield and Bate (2007) gave a multipurpose limit of ~0 psu for freshwater and ~35 psu for marine waters. Some of the impacts of excess salinisation on water resources include reduced crop yield, increased formation of scale, and added corrosion in domestic and increased requirements for pretreatment of water for selected industrial use such as boiler feed water (DEAT, 2000).

Total Dissolved Solids (TDS) is an expression for the combined content of all inorganic and organic substances contained in a liquid which are present in a molecular, ionized or micro-granular suspended form. Total dissolved solids like electrical conductivity is a measure of salinity in water systems (FAO, 1992). Plant growth, crop yield, and quality of produce are affected by the TDS in the irrigation water (FAO, 1992). This is remarkable as the effluent from the wastewater facility located in the urban community is used as water resource for a fish pond as well as to irrigate a nearby golf course. Elevated TDS can be toxic to freshwater animals by causing osmotic stress and affecting the osmoregulatory capability of the organisms. The TDS values in this study fell within acceptable limits ( $\leq 2000$  mg/l) for effluents discharged into

surface waters by WHO standards (Akan *et al.*, 2008). It also fell within acceptable limits (0 - 450 mg/l) for South African water systems applied in domestic uses (DWAF, 1996b).

Dissolved oxygen (DO) is essential in maintaining the oxygen balance in an aquatic biota. Low dissolved oxygen levels in a water system have adverse effects on the aquatic life. It affects the survival of fish by increasing their susceptibility to disease, hampering swimming ability, altering feeding, migration, and reproductive behavior. Further, extreme oxygen depletion levels results in rapid death of aquatic life (Environment Canada, 2001). The observed faster depletion of DO content in final effluents across the sampling sites than DO from the receiving waterbodies could be attributed to the presence of degradable organic mater which resulted in a tendency to be more oxygen demanding. The DO values obtained from this study are similar to those reported elsewhere (Fatoki *et al.*, 2003; Obire *et al.*, 2003; Jaji *et al.*, 2007; Akan *et al.*, 2007). Dissolved oxygen is an important factor used for water quality control. The effect of waste discharge on a surface water source is largely determined by the oxygen balance of the system and its presence is essential in maintaining biological life within a system (DFID, 1999). Dissolved oxygen concentrations in unpolluted water normally range between 8 and 10 mg/l and concentrations below 5 mg/l adversely affect aquatic life (DFID, 1999; Rao, 2005). DO standard for drinking purpose is 6 mg/l whereas for sustaining fish and aquatic life is 4 -5 mg/l (Rao, 2005). The DO value from this study fell short of the recommended standard. For water quality variable such as dissolved oxygen, water quality criteria are set at the minimum acceptable concentration to ensure the maintenance of biological function.

Chemical oxygen demand (COD) is a measure of the capacity of water to consume oxygen during the decomposition of organic matter and the oxidation of inorganic chemicals such as ammonia and nitrite. COD is a vital test for assessing the quality of effluents and wastewaters prior to discharge. The Chemical oxygen demand test predicts the oxygen requirement of the effluent and is used for monitoring and control of discharges, and for

assessing treatment plant performance. The impact of an effluent on the receiving water is predicted by its oxygen demand. This is because the removal of oxygen from the natural water reduces its ability to sustain aquatic life. Elevated levels of COD in water systems lead to drastic oxygen depletion which adversely affects the aquatic biota (Fatoki *et al.*, 2003). The values obtained in all seasons were higher than the South African guideline for COD in effluents to be discharged into the receiving waterbodies which is 30 mg/l (Government Gazette, 1984) and suggests that the effluents may negatively impact on the receiving environment. Higher levels of COD were observed upstream and downstream of the discharge points in summer. The increased COD concentrations during summer season could be attributed to run-off washed into waterbodies. This is undesirable since continuous discharge of effluents has impacted the receiving watersheds to some extent, and this may have negative effects on the quality of the aquatic environments and subsequently cause harm to the aquatic life especially fish, downstream (Morrison *et al.*, 2001). When this present result was compared with results of COD of the treated final effluent and receiving watersheds from developed countries, it was observed that the concentrations of COD differ as reported by UNEP (1993). According to Ogunfowokan *et al.* (2005) this increase in COD could be attributed to an increase in the addition of both organic and inorganic substance from the environment as well as organic contaminant entering the systems from the municipal sewage treatment plants. In the same light, our observation agrees with the previous works of Fatoki *et al.* (2003) and Morrison *et al.* (2001) who reported that the contribution of COD to the effluent and receiving waterbodies in South Africa appears to be significant.

Nitrate, the most highly oxidized form of nitrogen compounds is commonly present in surface and groundwater because it is the end product of aerobic decomposition of organic nitrogenous matter. Unpolluted natural waters usually contain only minute amounts of nitrate (Jaji *et al.*, 2007). The South African guideline for nitrate in sewage effluent is 1.5 mg NO<sub>3</sub><sup>-</sup> as

N/l (Government Gazette, 1984). The effluents did not meet this standard. Nitrates are inorganic sources of nitrogen that support the growth and development of living organisms at appropriate concentrations. It is important to note that nitrate level in the final effluent could be a source of eutrophication for receiving waters as the values obtained exceeded the recommended limit. The effluent from the treatment works could be said to be a source of nitrate into the receiving waterbodies. The high nutrient levels in the upstream discharge point of the receiving water may be as a result of diffuse sources from settlement and agricultural runoff. It has also been reported that nitrate concentration above 45 mg/l may result in anaemia in infants and pregnant women and formation of carcinogenic nitrosamines (Akan *et al.*, 2007).

Nitrite which is alike to nitrate is a source of nutrient that could have adverse effects on aquatic ecosystems at elevated concentrations. Their effects on water systems are generally alike to those described for nitrate. The South African limit (0 - 6  $\text{NO}_2^-$  as N/l) of no adverse effect for nitrite in domestic water supply is the same as for nitrate (DWAF, 1996b) and suggests that the entire water system under study was fit and safe for domestic uses based on their nitrite concentrations (0.06 - 2.4  $\text{NO}_2^-$  as N/l). The nitrite levels recorded in the entire water system across the sampling sites in spring and in the final effluent in winter however, exceeded the regulatory limits of the South African Standard (< 0.5  $\text{NO}_2^-$  as N/l) for the conservation of the aquatic milieu (DWAF, 1996c) and therefore put the aquatic environment at risk of eutrophication and also poses a problem to communities that depend on the receiving waterbody for domestic purposes from the perspective of the potential for the development of methaemoglobinemia (Fatoki *et al.*, 2003).

The orthophosphate - P contents in this study varied from 0.07 mg  $\text{PO}_4^{3-}$  as P/l to 4.81 mg  $\text{PO}_4^{3-}$  as P/l across seasons and sampling sites. The phosphate level in this study exceeded the South African target limit of 5  $\mu\text{g/l}$  (0.005 mg  $\text{PO}_4^{3-}$  as P/l) for phosphate level in water systems that will reduce the growth of algae and other plants; and suggests that the water is

polluted and pose serious threat to the aquatic biota in particular and the ecosystem in general. Other investigators have pointed out that eutrophication-related problems in temperate zones of aquatic systems begin to increase at ambient total P concentrations exceeding 0.035 mg P<sup>-1</sup>. In warm-water systems, the values range between 0.34 and 0.70 mg P<sup>-1</sup> (Rast and Thornton, 1996). These represent nutrient threshold levels beyond which there will be a corresponding increase in the risk and intensity of plant-related water quality problems (OECD, 1982). Generally, the phosphate-P values were higher during summer seasons compared to other seasons, across the sampling sites for the downstream and upstream of the discharge points. This could be attributed to phosphorus in runoffs from domestic, municipal and agricultural waste (non-point sources) flowing into rivers as well as washing along the riverside with detergent (Correll, 1998).

Vibrios are halophilic bacteria mostly found in effluents environments associated with domestic sewage. Several studies have reported their presence in final effluents and its receiving watershed (Gugliandolo *et al.*, 2005; Maugeri, *et al.*, 2000, 2004). This study showed that the investigated areas are subject to a wide spatial fluctuation of *Vibrio* pathogens contamination probably as a consequence of inefficiently treated effluent discharged into the receiving watershed. Abundances of free-living vibrios, small and large plankton-associated *Vibrio* species show higher seasonal fluctuation.

The wide spatial distribution of *Vibrio* species found in this study is comparable to the report by Gugliandolo *et al.* (2005). Free-living vibrios abundance directly correlated with temperature, while vibrios associated with 20 µm plankton showed a negative relationship with temperature, indicating a different seasonal behavior. Also, the statistically significant differences between the densities of free-living and plankton-associated vibrios suggest that *Vibrio* species in the final effluents and the receiving watersheds habitats are of independent existence.

This study revealed that free-living *Vibrio* species were the less prevalent (45%) in both treated effluents and receiving surface water samples. This was followed by *Vibrio* cells associated with planktons sizes 20  $\mu\text{m}$  (68%), 180  $\mu\text{m}$  (65%), and 60  $\mu\text{m}$  (55%), respectively. This observation is consistent with that of Maugeri *et al.* (2004), who reported high prevalence for free-living and plankton-associated bacterial species including *Vibrio* spp., *E. coli*, *Aeromonas* spp., *Enterococcus* spp., *Campylobacter* spp. and *Arcobacter* spp.; and concluded that although prevalence varied from one bacterial species to another, plankton-associated vibrios were generally more prevalent compared to the free-living vibrios. Also consistently with our observation, these workers (Maugeri *et al.*, 2004) reported higher prevalence in bacterial cells associated with larger plankton (> 200  $\mu\text{m}$ ) than those associated with smaller planktons (> 64  $\mu\text{m}$ ). However, contrary to the observation of Maugeri and co-workers our study showed higher prevalence for *Vibrio* species associated with a relatively larger (180  $\mu\text{m}$ ) plankton size compared to those attached to smaller (20  $\mu\text{m}$ ) plankton size, thus suggesting that the *Vibrio* species have more affinity for small or relatively large size plankton compared to planktons of medium sizes. Maugeri *et al.* (2004) reported higher abundance for plankton-associated bacteria compared to their free-living counterparts in coastal waters of Italy.

Free-living vibrios abundance was found directly correlated with temperature, while vibrios associated with 20  $\mu\text{m}$  plankton size showed a negative relationship with temperature, indicating a different seasonal activity, There was a positive relationship between densities of 180 and 60  $\mu\text{m}$  plankton sizes, thus strongly suggesting that *Vibrio* species in these habitats survive the wastewater treatment process as plankton-associated biofilms. This generally indicates that the *Vibrio* species associated with 20, 60 and 180  $\mu\text{m}$  plankton sizes, occupy the same niches in the ecosystem separate from those occupied by free-living *Vibrio* species. Consistent with this observation, Maugeri *et al.* (2004) reported no significant correlation between free-living bacteria and plankton-associated bacterial density in a marine coastal zone in

Italy. Contrary to the observation of this study, Hsieh *et al.* (2007) reported a negative correlation between planktonic *Vibrio* cells and attached populations. The authors explained that this trend could possibly mean that attachment provided shelter for cells under harsh conditions, thereby increasing the population of attached cells during such conditions while the abundance of planktonic cells decrease; on the other hand detachment of cells from planktons during favourable conditions would likely increase the planktonic population while reducing the abundance of attached cells.

The occurrence of *Vibrio* species as plankton-associated entities confirms the role of planktons as a potential reservoir of *Vibrio* pathogens (Gugliandolo *et al.*, 2005; Tamplin, *et al.*, 1990) and this is corroborated by previous findings on the association of pathogenic bacteria with planktonic organisms in the Mediterranean environment (Carbone *et al.*, 2005; Fera *et al.*, 2004; Maugeri *et al.*, 2004). The major free-living *Vibrio* species identified in the final effluents during this study was *V. vulnificus* which is considered a pathogenic *Vibrio* species, particularly of wound infections, gastroenteritis, or a syndrome known as primary septicemia in individuals exposed to marine environment contaminated with aqueous effluents (Gugliandolo *et al.*, 2005). *V. fluvialis* was the predominant *Vibrio* isolated across all locations and it has been shown to produce an enterotoxin known to cause serious infections with clinical symptoms of gastroenteritis very similar to those caused by *V. cholerae* O1 and non-O1 strains.

The results of this study demonstrate seasonal patterns for the *Vibrio* densities for the period August 2007 to July 2008. Strong correlation was demonstrated between *Vibrio* densities and water temperatures. The seasonal patterns of vibrios growth prevailed during the current study and are strongly supported by other studies (Lobitz *et al.*, 2000; Nishiguchi, 2000). *Vibrio* species were isolated more frequently and huge densities occurred during the summer months, which are consistent with the trends for clinical cases of *Vibrio* infections (Parveen *et al.*, 2008), but did not correspond with the low survival rates reported for summer temperatures *in vitro*

(Zimmerman *et al.*, 2007). Some previous studies reported higher levels of *V. parahaemolyticus* in oysters (plankton) during the warmer months and detectable or lower levels of this bacterium during winter months (Cook *et al.*, 2002; DePola *et al.*, 2003; Duan and Su, 2005). The mean water temperature (13 - 27 °C) of the final effluents and its receiving waterbodies during the summer period is ideal for the prolonged survival of *Vibrio* pathogens.

The ecological studies on the distribution of *Vibrio* species narrate a clear temperature dependent seasonal distribution in the final effluents and the receiving watersheds. These organisms were reported in high number during summer months and declined gradually towards winter in the majority of the field studies carried out in many parts of the world. The results of this study showed the same pattern reported elsewhere (DePaola *et al.*, 1990, 2003; Cook *et al.*, 2002; Duan and Su, 2005; Igbiosa *et al.*, 2009). The temperature dependent seasonal distribution was discernible in all sampled sites analysed. The statistical analyses also revealed a significant positive relationship between *Vibrio* densities and temperature.

In Southern African region, the maximum number of clinical cases of *Vibrio* related infections also occurs in the summer months (National Department of Health, 2003), when precipitation tends to be high. These results differ from the results of other environmental studies, wherein the detection of vibrios peaked in late autumn and winter months (Duan and Su, 2005). However, not all strains of *V. parahaemolyticus* are pathogenic. It has been demonstrated that the Kanagawa phenomenon, a beta-hemolysis in high-salt blood agar (Wagatsuma agar), is associated with most clinical strains but with very few environmental strains (Tada *et al.*, 1992). In this study it was observed that *V. fluvialis* was the most predominant species from the rural, sub-urban and urban sampling locations and this is alarming given that *V. fluvialis* is an emerging strain which has been shown to produce enterotoxin and clinical symptoms of gastroenteritis similar to those of *Vibrio cholerae* O1 and non-O1 strains, more so with the recent

characterization of an enterotoxigenic El Tor-like haemolysin in *V. fluvialis*, which represents one of the virulence factors of *V. cholerae*. (Kothary *et al.*, 2003).

The role of the environment in the emergence and spread of antibiotics-resistant vibrios, their possible pathway, and the way in which environmental vibrios contribute to the spread of resistance genes are not yet clear. In this study, both conventional cultivation methods for the detection and identification of resistant bacteria, and genetic test to detect *Vibrio* resistance genes in the final effluents were used to study various locations and sampling sites in the Eastern Cape Province.

This study describes the detection of antibiotics resistance genes known to confer resistance to common classes of antibiotics in a rural community of South Africa. The results reveal the high individual and multiple antibiotics resistant *Vibrio* species strains from the treated final effluents. Data on antibiotic resistant zones indicates that *Vibrio* species were 100% resistant to ampicillin and sulfamethoxazole and more than 50% resistance to cotrimoxazole, erythromycin, chloramphenicol, cephalothin, streptomycin, trimethoprim and penicillin. Previous studies have shown that streptomycin, rifampicin, kanamycin, tetracycline, polymyxin B were active against *Vibrio* species (Li *et al.*, 2003). But this was contrary to our findings since we observed resistances to streptomycin, tetracycline and polymyxin B in the *Vibrio* strains studied. However, Ottaviani *et al.* (2001) showed that *V. parahaemolyticus* were resistant to penicillin, carbenicillin, ampicillin, cephalothin, kanamycin and rifampicin. Besides, their results also showed that increase in salt concentration cause the changes of sensitivity toward antibiotics from the resistant to susceptible phenotypes.

In this study, resistance to ampicillin was observed to be 100% of analyzed *Vibrio* strains and this differs from other studies that have been reported, which ranged from 44.4% to 98% (Son *et al.*, 1998; Lesmana *et al.*, 2001). These results were also similar to those of French *et al.* (1989) who reported similar antibiotics susceptibility profile for *V. parahaemolyticus*. In their

study, most isolates were resistant to ampicillin but were susceptible to chloramphenicol and tetracycline. Low percentage of resistance has also been reported against cephalothin (Pedersen *et al.*, 1996; Lesmans *et al.*, 2001; Roque *et al.*, 2001). There are few factors that may contribute to the *Vibrio* species antibiotic resistance. Firstly, a mutation in cellular DNA could modify the antibiotics target site or transport mechanism, resulting in decreased action of the antibiotic on the cell. Another factor is an extra gene product target site or transport mechanism. About 10-20% of the studied strains showed a 5-10 MAR pattern (i.e., resistance to 5-10 of 21 antibiotics tested). In this study, all the antibiotics resistant isolates have MAR index of 0.30 or more which can be grouped under origination from high risk sources of contamination like humans and commercial industry where antibiotics are often used.

Donlan and Costerton (2002) also reported the acquisition of inherent resistance to antimicrobial agents by attached bacterial species, which suggests that attachment to plankton at one point or the other may have enhanced the multiple resistances of our isolates to several of the antibiotics tested against. The physicochemical character of the wastewater effluents may have influenced the level of resistance displayed by the *Vibrio* species isolated in this study. It has been widely reported in the literature that conventional wastewater treatment plants lack the capacity to effectively remove antibiotics and a number of other chemicals from wastewater, thereby increasing the chances of bacterial pathogens resident in such wastewater effluents to develop resistance to common antibiotics due to selective pressure (Golet *et al.*, 2003; Giger *et al.*, 2003; Volkmann *et al.*, 2004).

In this present study, all stains exhibited multiple resistance to more than five antibiotics. The strains of *Vibrio* species are known to carry plasmids, which encode for drug resistance (Thungapathr *et al.*, 2002; Ashraf *et al.*, 2004; Pan *et al.*, 2008). A study carry out in India between 1997 and 1998 on a total number of 94 isolates of *V. cholerae* observe that 43 strains belonging to non-O1 and non-O139 serogroups contained plasmids that contributed to the

multiple antibiotic resistance and exhibited resistance to ampicillin, neomycin, tetracycline, gentamicin, streptomycin, sulfonamide, furazolidone, chloramphenicol (Thungapathr *et al.*, 2002). The drug resistant conjugative plasmid pMRV150 has been reported from China in *V. cholerae* O139 which mediated multiple-drug resistance (MDR) to at least six antibiotics, including ampicillin, streptomycin, gentamicin, tetracycline, chloramphenicol and trimethoprim-sulfamethoxazole (Pan *et al.*, 2008). Among the *Vibrio* species, resistance to streptomycin and furazolidone were previously observed for the first time in *V. cholerae* O139 isolated after 1992 (White and McDermott, 2001), and then these phenotypes associated with resistance to chloramphenicol were detected in many Asian clinical *V. cholerae* O1 El Tor and O139 isolates (Falbo *et al.*, 1999), and in some clinical isolates of non-O1, non-O139 (Thungapathr *et al.*, 2002). The spread of these resistances in *V. cholerae* is mainly due to a new type of conjugative transposon called the SXT element or constin (White and McDermott, 2001). Our findings are in good agreement with these previous studies.

The SXT element is a 62 kb, conjugative, self-transmissible integrating element encoding resistance to sulfamethoxazole, trimethoprim and /or streptomycin (Waldor *et al.*, 1996). Furthermore, SXT encodes an integrase at its 5' end required for SXT transfer. The SXT<sup>MO10</sup> element was first detected in the newly emerged O139 serogroup of *V. cholerae* in 1992. Since 1994 *V. cholerae* O1 strains isolated from India, Bangladesh, Mozambique and Laos have been found to contain the SXT element (Amita *et al.*, 2003; Dalsgaard *et al.*, 2001; Hochhut *et al.*, 2001; Iwanaga *et al.*, 2004).

In this study we could clearly show that the gene cassette arrays isolated from *Vibrio* strains residing in the wastewater final effluent systems are flanked by SXT element specific sequences, since part of 5'- and 3'- conserved segment of SXT element are present on the amplification products generated by SXT element specific PCR, suggesting a role in the acquisition of antibiotic resistance genes encoding sulfamethoxazole, trimethoprim and/or

streptomycin resistance. There were also differences in the antibiotics resistance gene clusters in the SXT element in the *Vibrio* strains investigated, corroborating the results of earlier workers (Hochhut *et al.*, 2001) who reported differences in antibiotics resistance and suggested that these genes were not intrinsic features of this family of integrase, but rather appeared to have been inserted into the elements becoming transmissible bacterial populations.

Overall from the result obtained from literature and data presented in this work, it becomes clear that SXT element specific gene cassettes are widespread in natural and man-managed environments and that bacteria of different habitats serve as reservoir for SXT element and SXT specific gene cassettes. It is very likely that the mobile gene cassette pool contributes significantly in environments where antimicrobial drugs are present. Considering the abundance of different resistance gene cassettes, it appeared that cassettes encoding different aminoglycosides-modifying enzymes and dihydrofolate reductases for trimethoprim resistance were found frequently by previous authors (Schmidt *et al.*, 2001a; 2001b). This is in agreement with our analyses where we found *dfr18* and *dfrA1* for trimethoprim resistance, *sul2*, *strB* and *floR* encoding for sulfamethoxazole, streptomycin, chloramphenicol respectively. In addition, cassette mediating  $\beta$ -lactams resistance and tetracycline resistance were identified. The result of this study show that a pool of antibiotic-resistant genes exists within this final effluent system of the Eastern Cape Province wastewater treatment facilities.

The observation that *Vibrio* strains containing the SXT element specific antibiotics resistance gene, tetracycline and the  $\beta$ -lactams resistance genes are released from the final effluent wastewater treatment plant under study. This allow us to speculate with respect to SXT element mediated resistance gene capture, dissemination and recombination of resistance gene cassettes in the final effluent system and in habitats downstream of the plant. Addressing antibiotic resistance from an environmental standpoint can promote a better understanding of the

ecology and evolution of antibiotic resistance, and may provide an early detection system for the development of antibiotics resistance mechanisms in clinically relevant bacteria.

The abundance of *Vibrio* species appear to be generally affected by environmental variables, although on some occasion vibrios abundance appeared to fluctuate independently of temperature. The correlation between temperature and total vibrios density in this study corroborates reports of other similar finding from Atlantic, Pacific and Gulf Coasts, as well as from Japan and Germany (Cook *et al.*, 2002; DePola *et al.*, 2003; Duan and Su, 2005; Lhafi and Kuhne, 2007). The abundance of vibrios were higher during summer than during winter. In this study, *Vibrio* species appear to be more abundant at a temperature range between 17.07 and 27.15 °C, indicating a strong dependence of the culturable forms of the pathogens on temperature. Similarly, the salinity profile observed in this study was consistently lower than the maximum requirement for optimal growth of vibrios (Kaspar and Tamplin, 1993). Depending on the range of salinities encountered, some previous studies (Cook *et al.*, 2002; DePola *et al.*, 2003) have found a significant relationship between salinity and the abundance of total *Vibrio* species. One study (Zimmerman *et al.*, 2007) has also reported a positive correlation between *Vibrio* species and turbidity, which is also consistent with the observation reported by Watkins and Cabelli (1985) that found *V. parahaemolyticus* levels in water to be strongly correlated with turbidity during summer, when water temperatures are relatively constant. It has been hypothesized that higher nutrient level associated with highly turbid and polluted waters may stimulate growth of vibrios (Zimmerman *et al.*, 2007). Our results also appear consistent with those of Watkins and Cabelli (1985) who found poor correlation between *V. parahaemolyticus* levels and pH of the water.

Turbidity was relatively higher at rural and sub-urban than at the urban location, and analyses indicated a positive association between turbidity and vibrios densities at the rural and sub-urban sites. Lower levels and less variability in turbidity may have obscured the effects of

turbidity at the urban site. The association between turbidity and total vibrios densities at the rural and sub-urban study locations was generally consistent and statistically significant ( $P < 0.05$ ;  $P < 0.0001$  respectively). Increase in turbidity was associated with increasing *Vibrio* species abundance in the study. Recent studies (Zimmerman *et al.*, 2007; Igbiosa *et al.*, 2009) have also reported a positive correlation between *Vibrio* species and turbidity. This is also consistent with the observations obtained by Watkins and Cabelli (1985) that found *V. parahaemolyticus* levels in water to be strongly correlated with turbidity during the summer, when water temperatures are relatively constant. These authors hypothesized that higher nutrient levels associated with more turbid and polluted waters may have stimulated *V. parahaemolyticus* growth.

Watkins and Cabelli (1985) reported *V. parahaemolyticus* levels in water to be strongly correlated with dissolved oxygen, which is in agreement, with our present study. However, the Watkins and Cabelli (1985) study was restricted to summer season, when water temperatures were relatively constant and during which the importance of other environmental parameters could likely increase.

An interesting phenomenon noticed in this study was negative statistical relationship between salinity and vibrios at the rural and sub-urban sites. This relationship was significant at the urban site. This may be attributed to the fact that the rural and sub-urban sites are freshwater habitat carried high nutrients which in turn enhanced the proliferation of *Vibrio* species in coastal area. Such relationship was reported by Watkin and Cabelli (1985). Differences in salinity levels between these sampling sites during the study period may account for some of the discrepancies in *Vibrio* species densities. A significant correlation between salinity and total vibrios densities in effluent was identified at the urban site, and the opposite trend observed at the rural and sub-urban sites, is likely a consequence of the lower and narrow range of salinity observed therein. Previous studies indicated that *V. parahaemolyticus* densities decrease as salinity increases

(Anonymous, 2005, DePaola *et al.*, 1990, 2003). In the present study, the densities of total *Vibrio* species were generally positively associated with increasing salinity. Depending on the range of salinities encountered, some previous studies (Cook *et al.*, 2002; DePaola *et al.*, 2003) have found a significant relationship between salinity and the abundance of total *V. parahaemolyticus* which other studies (DePaola *et al.*, 1990; Ristori *et al.*, 2007) have not. These differences between studies are likely a consequence of the difference in the range of variation of salinities and the sample sizes of the studies. In general, a wide range (5.6-34 ppt) of salinities was observed in the Cook *et al.* (2002) study compared to other studies where no significant salinity effect was identified.

According to White (1992) the most prevalent practice of disinfection is the use of free chlorine which dissociate into molecular hypochlorite and the hypochlorite ion. Most South African wastewater treatment plants disinfect wastewater by chlorination prior to discharge into receiving waterbodies. The aim is to eliminate pathogens from wastewater. To attain this aim, residual chlorine is maintained at sufficient levels and in contact with the microbial community in the chlorination tank. There is no recommended standard for residual chlorine concentration for wastewater effluents in South Africa at the moment. This study considered those for domestic water supplies which recommended a range of 0.3 to 0.6 mg/l as ideal free residual chlorine concentration and 0.6 - 0.8 mg/l as good free residual chlorine concentration with insignificant risk of health effects (Mooijiman *et al.*, 2001). Based on this concentration, the free residual chlorine in the effluents complied with the regulatory standard, but failed to eliminate the *Vibrio* pathogens. Even when there was over dose in October and November 2007 at the sub-urban location plant, high densities of *Vibrio* pathogens were observed. Similar ranges have been reported for residual chlorine concentration in South African water works (Obi *et al.*, 2007, 2008; Igbiosa *et al.*, 2009), suggesting that some South African water works do not comply with stipulated standards with reference to free residual chlorine concentration. Residual chlorine

concentration did not significantly affect the abundance of *Vibrio* species in this study ( $P > 0.05$ ). Previous studies have reported the potentials of bacterial growth in the treated water with chlorine residual concentrations up to 1 mg/l (Obi *et al.*, 2007, 2008). Hence, there is need for a more efficient disinfection procedure to eliminate the *Vibrio* pathogens from the final effluents.

## **Conclusion**

The working hypothesis of this research was that wastewater treatment facilities in the Eastern Cape Province of South Africa are a significant source of *Vibrio* pathogens in the aquatic environment. To this end, our findings revealed that the treatment plants exhibited effluent qualities that meet acceptable standards in some parameters, like pH, temperature, and total dissolved solid (TDS) but fell short of standard requirements that are critical to the provision of clean and safe water such as organic waste (measured as turbidity DO, COD, orthophosphate, nitrate and nitrite). These findings therefore suggest that the effluents negatively impacted on the receiving watershed and pose a significant health and environmental risk on the biota including the communities that rely on the receiving watershed for domestic purposes.

Also, this study revealed that *Vibrio* pathogens survive the wastewater treatment processes either as free-living organisms or as plankton-associated entities. Moreover, the *Vibrio* pathogens appear to be resistant to chlorine disinfection at normal recommended concentrations, thus posing a potential health risk to the communities which depend on the watershed for domestic and recreational purposes. Also, the *Vibrio* pathogens survived the treatment process mostly as planktonic-associated entities than the free-living vibrios cells, thus reaffirming the role of plankton as a potential reservoir of *Vibrio* pathogens in aquatic environment. Notwithstanding, the prevalence of SXT element specific antibiotics resistance gene, tetracycline and the  $\beta$ -lactams resistance genes in the *Vibrio* pathogens isolated from the final effluents allow us to speculate on the potentials of dissemination and recombination of resistance

gene cassettes in the final effluents system and in habitats downstream of the plant. There is need for regular monitoring of the efficiency of wastewater treatment facilities in South Africa to ensure compliance to regulatory standards.

### **Potential for future development of the study**

Concerns about the potential health risk from *Vibrio* species especially with regards to their association with wastewater will continue into the foreseeable future. There is limited information in the literature on the microbial risk assessment of this pathogen in the inhabitants in respect of wastewater contributes to the epidemiology of the pathogen. Thus the call for future studies to focus on this area is imperative. Whereas this study show that *Vibrio* pathogens existed as free-living and plankton-associated entities, the identities of the specific planktons and their interactions involved in this association were not investigated in the course of this study. Information from such research may add on our present knowledge of the pathogen-host-environment relationship and will help to improve our understanding and approach to disease prevention, control and surveillance of *Vibrio* infections. In addition it may be necessary to investigate and identify the specific genes and/or polymorphisms that are correlated with expression of virulence and disease via comparative genomic analysis. This work will increase our knowledge of the virulence of this biothreat pathogen and provide a foundation for the development of diagnostic and therapeutic countermeasures. Finally, a number of *Vibrio* species has been isolated from final effluents that appear to be environmental isolates. Identifying the genomic content of this broad representation of *Vibrio* species will contribute towards a broad understanding of the genus and potentially reveal common genes that are perhaps shared and transferred among diverse strains.

## Recommendations

Based on the results of the study, the following recommendations could be suggested:

- a) The study showed a need for a continuous pollution monitoring programme of the surface waters in rural, sub-urban and urban communities in the Eastern Cape Province of South Africa. In addition the provincial government and all agencies concerned with environmental matter in South Africa should evolve measures to ascertain and ensure that discharge effluents comply with laid down rules and regulations.
- b) There is need for the training and re-training of operators of wastewater treatment facilities pursuant to ensuring efficiency of the wastewater treatment processes towards meeting recommended standards.
- c) The environment and health departments should be informed of the emergence of pathogenic non *Vibrio cholera* bacteria in the aquatic milieu of the Eastern Cape Province and be proactive to prevent future outbreaks of diseases from these pathogens.

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