Assessment of the prevalence of virulent *Escherichia coli* strains in the final effluents of wastewater treatment plants in the Eastern Cape Province of South Africa

by

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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 Science, 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, either in part or in its entirety, for the award of any degree.

Name: _____ **Augustina Nwabuje Osode**_____

Signature: _____  _____

Date: _____10 April 2010_____

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DEDICATION

This Thesis is

Dedicated to my parents: Mr. and Mrs. C. N. Osode

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Mary Chukwudumebi Udeh and

Brothers: Mr. Peter Ikechukwu Osode, Mr. Victor Chukwudi Osode,

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LIST OF ABBREVIATIONS

AE	–	Adhesion-effacing
ATCC	–	American Type Culture Collection,
Bp	–	Base pair
cAMP	–	Cyclic adenosine monophosphate
CFU	–	Colony forming units
DAEC	–	Diffusively adherent *E. Coli*
dATP	–	deoxyadenosine triphosphates
dCTP	–	deoxycytidine triphosphates
dGTP	–	deoxyguanosine triphosphates
dTTP	–	deoxythymidine triphosphates
DNA	–	Deoxyribonucleic acid
DWAF	–	Department of Water and Forestry
e.g.	–	*exempli gratia*, for example
eae	–	Attaching and effacing gene
EAEC	–	Enteroaggregative *Escherichia coli*
EC	–	Eastern Cape
EDTA	–	Ethylenediaminetetraacetic acid

EHEC	–	Enterohaemorrhagic *Escherichia coli*
EIEC	–	Enteroinvasive *Escherichia coli*
EMBA	–	Eosin Methylene Blue Agar
EPA	–	Environmental Protection Agency
EPEC	–	Enteropathogenic *E. coli*
et al	–	(*et alii*) and others
EtBr	–	Ethidium bromide
ETEC	–	Enterotoxigenic *E. coli*
fliC	–	Flagellin gene
g	–	Gram
h	–	Hour
HUS	–	Hemolytic-uremic syndrome
LT	–	Heat-labile enterotoxin
$MgCl_2$	–	Magnesium Chloride
mg	–	Milligram
MHA	–	Mueller-Hinton Agar
min	–	Minute
ml	–	Millilitre
mM	–	Millimole
NB	–	Nutrient Broth
NA	–	Nutrient Agar
NaCl	–	Sodium Chloride
NCCLS	–	National Committee for Clinical Laboratory Standards

№	–	Number
O	–	Somatic antigen
H	–	Flagella antigen
K	–	Capsular antigen
^{o}C	–	Degrees Celsius
PCR	–	Polymerase Chain Reaction
rfb	–	Gene cluster encoding biosynthesis of the O-antigen (pO157)
SA	–	South Africa
SAS	–	Statistical Analysis System
SLT	–	Shiga-like toxin
ST	–	Heat-stable enterotoxin
STEC	–	Shiga Toxigenic *Escherichia coli*
Stx	–	Shiga toxin
TAE	–	Tris-acetate
Taq	–	*Thermus aquaticus*
Tris-HCl	–	Trishydroxymethylaminomethane-Hydrochloric acid
UK	–	United Kingdom
UNDP	–	United Nations Development Programme
US	–	United States
USA	–	United States of America
USEPA	–	United States Environmental Protection Agency
UV	–	Ultra Violet

V	–	Voltage
WHO	–	World Health Organization
µg	–	Microgram
µm	–	Micrometre
µℓ	–	Microlitre

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GENERAL ABSTRACT

General Abstract

Escherichia coli (*E. coli*) is a common inhabitant of surface waters in the developed and developing worlds. The majority of *E. coli* cells present in water are not particularly pathogenic to humans; however, there are some present in small proportion that possess virulence genes that allow them to colonize the digestive tract. Pathogenic *E. coli* causes acute and chronic diarrheal diseases, especially among children in developing countries and in travelers in these locales.

The present study, conducted between August 2007 and July 2008, investigated the prevalence and distribution of virulent *E. coli* strains as either free or attached cells in the final effluents of three wastewater treatment plants located in the Eastern Cape Province of South Africa and its impact on the physico-chemical quality of the receiving water body. The wastewater treatment plants are located in urban (East Bank Reclamation Works, East London), peri-urban (Dimbaza Sewage Treatment Works) and in rural area (Alice Sewage Treatment Works).

The effluent quality of the treatment plants were acceptable with respect to pH (6.9-7.8), temperature (13.8-22.0 °C), dissolved oxygen (DO) (4.9-7.8 mg/L), salinity (0.12-0.17 psu), total dissolved solids (TDS) (119-162 mg/ L) and nitrite concentration (0.1-0.4 mg/l). The other

physicochemical parameters that did not comply with regulated standards include the following: phosphate (0.1-4.0 mg/L); chemical oxygen demand (COD) (5-211 mg/L); electrical conductivity (EC) (237-325 µS/cm) and Turbidity (7.7-62.7 NTU). Results suggest that eutrophication is intensified in the vicinity of the effluent discharge points, where phosphate and nitrate were found in high concentrations.

Presumptive *E. coli* was isolated from the effluent samples by culture-based methods and confirmed using Polymerase Chain Reaction (PCR) techniques. Antibiogram assay was also carried out using standard *in vitro* methods on Mueller Hinton agar. The viable counts of presumptive *E. coli* for the effluent samples associated with 180 µm plankton size ranged between 0 – 4.30 × 10^1 cfu/ml in Dimbaza, 0 – 3.88 × 10^1 cfu/ml in Alice and 0 – 8.00 × 10^1 cfu/ml in East London. In the 60 µm plankton size category *E. coli* densities ranged between 0 and 4.2 × 10^1 cfu/ml in Dimbaza, 0 and 2.13 × 10^1 cfu/ml in Alice and 0 and 8.75 × 10^1 cfu/ml in East London. Whereas in the 20 µm plankton size category presumptive *E. coli* density varied from 0 to 5.0 × 10^1 cfu/ml in Dimbaza, 0 to 3.75 × 10^1 cfu/ml in Alice and 0 to 9.0 × 10^1 cfu/ml in East London. The free-living presumptive *E. coli* density ranged between 0 and 3.13 × 10^1 cfu/ml in Dimbaza, between 0 and 8.0 × 10^1 cfu/ml in Alice and between 0 and 9.5 × 10^1 cfu/ml in East London.

Molecular analysis successfully amplified target genes (*fliC*$_{H7}$, *rfbE*$_{O157}$, *ial* and *aap*) which are characteristic of pathogenic *E. coli* strains. The PCR assays using *uidA*-specific primer confirmed that a genetic region homologous in size to the *E. coli uidA* structural gene, including the regulatory region, was present in 3 of the *E. coli* isolates from Alice, 10 from Dimbaza and 8 from East London. Of the 3 *E. coli* isolates from Alice, 1 (33.3%) was positive for the *fliC*$_{H7}$ genes and 3 was positive for *rfbE*$_{O157}$ genes. Out of the 10 isolates from Dimbaza, 4 were

positive for *fliC*$_{H7}$ genes, 6 were positive for the *rfbE*$_{O157}$ genes and 1 was positive for the *aap* genes; and of the 8 isolates from East London, 1 was positive for *fliC*$_{H7}$ genes, 2 were for the *rfbE*$_{O157}$ genes, 6 were positive for the *ial* genes.

Antimicrobial susceptibility profile revealed that all of the *E. coli* strains isolated from the effluent water samples were resistant (R) to linezolid, polymyxin B, penicillin G and sulfamethoxazole. The *E. coli* isolates from Dimbaza (9/10) and East London (8/8) respectively were resistant to erythromycin. All the isolates were found to be susceptible (S) to amikacin, ceftazidime, ciprofloxacin, colistin sulphate, ceftriaxone, cefotaxime, cefuroxime, ertapenem, gatifloxacin, gentamycin, imidazole, kanamycin, meropenem, moxifloxacin, neomycin, netilmicin, norfloxacin and tobramycin.

The findings of this study revealed that the Alice wastewater treatment plant was the most efficient as it produced the final effluent with the least pathogenic *E. coli* followed by the Dimbaza wastewater treatment plant. In addition, the findings showed that the wastewater treatment plant effluents are a veritable source of pathogenic *E. coli* in the Eastern Cape Province watershed. We suggest that to maximize public health protection, treated wastewater effluent quality should be diligently monitored pursuant to ensuring high quality of final effluents.

CHAPTER 1

INTRODUCTION

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GENERAL INTRODUCTION

Throughout history, consumption of drinking water supplies containing enteric pathogenic bacteria has been linked to illnesses in human populations. These illnesses commonly present as gastrointestinal-related symptoms, such as diarrhea and nausea. Diarrheal diseases are major causes of morbidity and mortality in the developing world, more especially in young children (Kosek *et al*., 2003). In South Africa it has been estimated that diarrheal diseases are the primary causes of death in infants that are younger than 5 years, leading to about 160-200 deaths per day (Nemarude *et al*., 2008). Diarrhea is a condition that results when there are increased amounts of water in stools. This occurs when the stomach or the small intestine secrete too much fluid, such that the distal small intestine and colon do not absorb enough water, or the undigested, liquid food passes too quickly through the small intestine and colon for them to remove enough water.

In almost all South African metropolitan areas, the consumer is provided with high-quality drinking water. However, in many rural communities, the situation is very different. In 1994, an estimated 14 million people had no access to clean or safe water. Although initiatives were undertaken and improvement measures implemented, 7 million of the 14 million people in rural areas still lack safe and clean water (Duse *et al*., 2003). The population of the Eastern Cape Province is largely non-urban, poor and with an inadequate water supply infrastructure. Rural communities of this Province comprise both scattered villages and subsistence farmers, and formalised towns serving subsistence farmers. The poverty rate in 1998 was 78% and only 25% of the households had a pipe-borne water supply inside their dwellings (Mey, 1998). This implies that many people depend on surface and/or groundwater sources for their daily water needs. Water from these sources is used directly by communities and in many cases the water sources

are faecally contaminated and devoid of treatment (Momba and Notshe, 2003). Many of these water bodies are often impacted by inadequately treated effluents from municipal wastewater plants as receiving water bodies (Fatoki *et al*., 2003).

Strains of *E. coli*, which are capable of causing diarrhea, under certain conditions, for example, when the immune system is compromised, or due to environmental exposure, is referred to as diarrheagenic *E. coli*. Six groups of *E. coli* that could potentially cause diarrheal diseases are now recognized and they are enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). Each group has its unique virulence factors and are classified as shown in Figure 1. Other diarrheagenic *E. coli* pathotypes have been proposed, such as cell detaching *E. coli* (CDEC); however their significance remains uncertain (Clarke, 2001; Abduch-Fabrega *et al*., 2002).

Diarrheagenic *E. coli* strains possess specific fimbrial antigens that enhance their intestine-colonizing ability and allow adherence to the small mucosa bowel. Once having colonized, the strains use very different pathogenic strategies to cause changes in the arrangement of the bowel's mucosa (Donnenberg, 1999); this is depicted in Figure 2 below.

The variety of pathogenic strategies exhibited by this *E. coli* strains is attributable to differences in genetic background with each strain carrying unique plasmids or pathogenicity islands (Keskimäki *et al*., 2001). Standard methods used in the detection of diarrheagenic *E. coli* are based on unique sets of virulence factors, such as toxins [heat-labile (LT) and heat-stable (ST) enterotoxins (ETEC)] (Kuhnert *et al*., 2000) and Shiga-like toxins SLT1 and SLT2 (STEC/EHEC) (Nataro and Kaper, 1998), intimin (Cravioto *et al*., 1996) and EPEC adherence

factor (EAF) (Donnenberg *et al*., 1997) (EPEC)] and virulent factors (EIEC) (Robins-Browne, 1987), and their cell-adherence characteristics (Clarke, 2001; Nataro and Kaper, 1998). Detection techniques include bioassays (e.g. cell culture), immunologic assays (e.g. immunoblotting or EIA), and DNA assays (e.g. PCR, probing) (Thompson *et al*., 2003).

Marine research has provided news about the health aspects of pathogens living in association with plankton. Dixon (2004) found evidence that the colonization of zooplankton by organisms capable of causing human disease is a widespread phenomenon. The survey assessed the occurrence of species of *Campylobacter*, *Vibrio* and other genera in Italy's coastal waters, together with comparisons of free-living bacteria and those associated with zooplankton and of plankton-bound organisms with selected pathogens. The findings revealed that not only *Vibrio* and *Aeromonas* spp. but also *E. coli*, enterococci; *Campylobacter* and *Arcobacter* spp. (agents of human diarrhea) were linked with zooplankton. An abundance of both free-living and plankton-associated *E. coli* and enterococci confirmed that the Straits of Messina were indeed seriously polluted (Dixon, 2004). The results indicated that potentially pathogenic organisms living in close association with zooplankton have considerable epidemiological (and ecological) implications.

While it appears that a lot of emphasis have been placed on issues of compliance in wastewater research with regards to monitoring such classical pollution indicator organisms like culturable total and faecal coliforms, not much is being done regarding the survival and molecular epidemiology of pathogenic strains of *E. coli* in wastewater effluents, either as free or an attached (planktonic) cells. This even more so as the habits of antibiotic usage have been known to influence the spectrum and susceptibility pattern of virulent pathogens (Lõivukene *et*

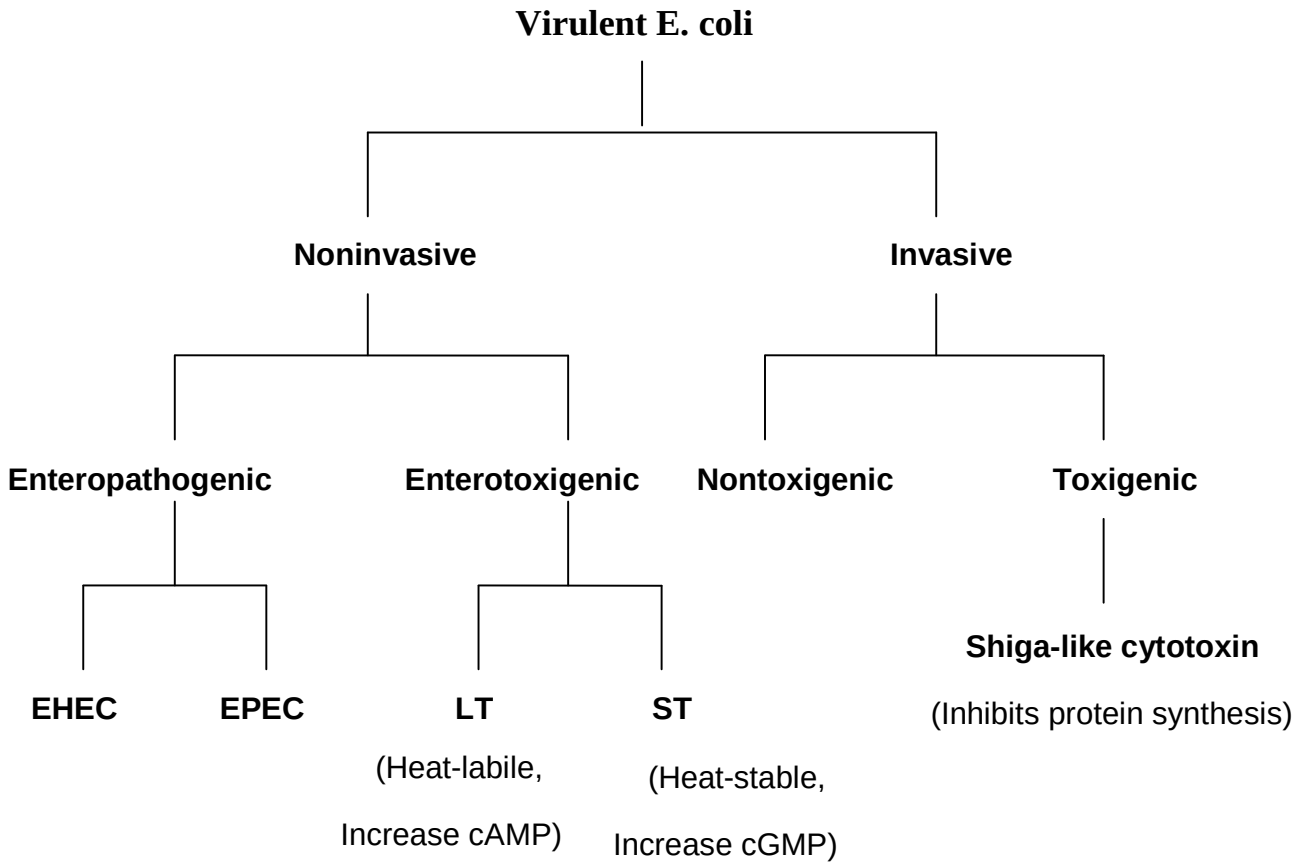

Figure 1: Different types of virulent *E. coli* strains (Source: Evans and Evans, 1990).

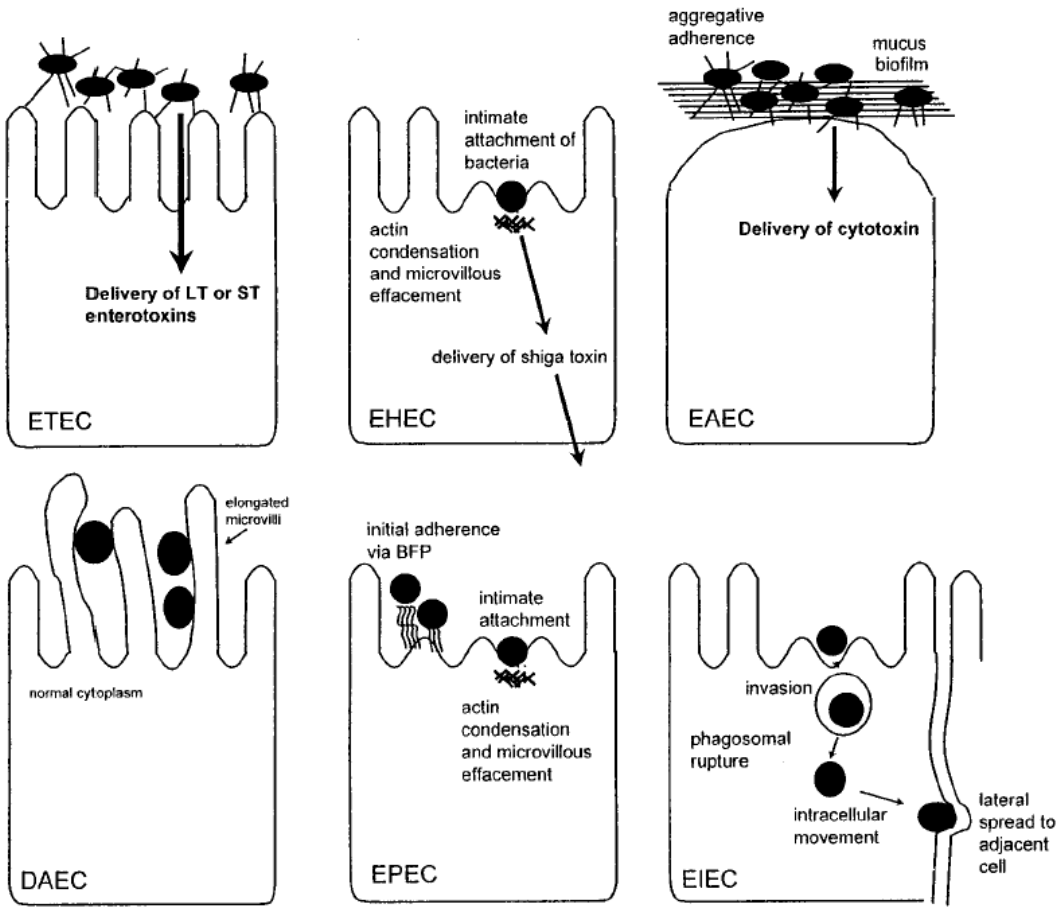

Figure 2: Pathogenic schemes of the diarrheagenic *E. coli*, each with a unique feature in its interaction with eukaryotic cells (Source: Nataro and Kaper, 1998).

al., 2006). Besides, some pathogens under environmental conditions have been shown to be capable of entering a viable but nonculturable state thus significantly underestimating their population (Xu *et al*., 1983). Hence the broad aim of this study was to evaluate the hypothesis that virulent pathogenic *E. coli* strains very easily survive the treatment processes of the activated sludge system of wastewater treatment facilities in the Eastern Cape Province either as free cells or as plankton associated entities and secondly that these treatment facilities are veritable sources of pathogenic *E. coli* and abiotic pollutants in the receiving watershed. The specific objectives of the study include:

- To investigate the prevalence and distribution of the virulent *E. coli* strains as free and plankton associated cells in the final effluents of the wastewater treatment plants in the Eastern Cape Province.

- To ascertain the prevalence and distribution of virulent *E. coli* strains in the final effluents of the wastewater treatment plants and the receiving water bodies in the Eastern Cape Province.

- To assess the survivability of the different *E. coli* strains in the various stages of the wastewater treatment processes.

- To elucidate the antibiotic susceptibility profiles in the isolated *E. coli* strains.

- To compare data obtained from typical urban, semi-urban and rural settings, and in relation to the microbiological qualities of the effluents.

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CHAPTER 2

ENTEROTOXIGENIC *ESCHERICHIA COLI* (ETEC): A RECURRING DECIMAL IN INFANTS' AND TRAVELERS' DIARRHEA

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhea in infants and in travelers from developed to underdeveloped countries, especially in regions of poor sanitation. The ETEC are acquired by the ingestion of contaminated food and water, and adults living in endemic areas develop immunity. The disease condition manifests as a minor discomfort to a severe cholera-like syndrome and requires colonization by the microorganism and the elaboration of one or more enterotoxins. The ETEC attach to the epithelial cells of the gastrointestinal tract and release substances that affect the normal functioning of the tract, thereby resulting in diarrhea, and subsequently millions of deaths everyday, particularly in children. The prevention of the spread of this strain of diarrheagenic *E. coli* depends on ensuring appropriate sanitary measures; hand-washing and proper preparation of food; chlorination of water supplies; and appropriate sewage treatment and disposal. Parenteral or oral fluid and electrolyte replacement is used to prevent dehydration, and broad-spectrum antibiotics are used in chronic or life-threatening cases, but in most cases, should be avoided because of severe side effects.

Keywords: *E*nterotoxins, Colonization factors, Epidemiology, Pathogenesis, Diagnosis, Treatment

2.1 INTRODUCTION

The microorganism *Bacterium coli commune* was discovered in 1885 by Dr. Theodor Escherich during his work on bacteria in stools of infants with enteritis /1/. The bacterium has been recognized as an important cause of food and water-related diseases since its discovery and is now known as *Escherichia coli*. *Escherichia coli* belong to the coliform group of microorganisms, which are a common part of the normal facultative anaerobic microflora of the intestinal tracts of most mammals, including humans. This flagellated gut flora is mainly found in the colon /2/. Coliforms include all the aerobic and facultatively anaerobic, Gram-negative, non-spore forming, rod-shaped bacteria that ferment lactose with gas formation within 48 hours at 35°C /3/. *Escherichia coli* belongs to the genus *Escherichia* which in turn is part of the tribe *Escherichiae* belonging to the family *Enterobacteriaceae.* The genus *Escherichia* contains four other species besides *E. coli* and includes *E. hermanii, E. fergusonii, E. vulneris,* and *E. blattae*. *Escherichia blattae* were isolated from cockroaches, whereas *E. hermanii, E. fergu-sonii,* and *E. vulneris* were isolated from both intestinal and extra-intestinal human sources /2/. Most *E. coli* serotypes are non-pathogenic in humans and other warm-blooded animals. Nevertheless, certain serotypes, if present in the body, can cause health problems. Pathogenic *E. coli* are responsible for three types of infections in humans: urinary tract infections (UTI), neonatal meningitis, and intestinal diseases (gastroenteritis) /4/. It is therefore of clinical importance to be able to differentiate between various serotypes of *E. coli.*

Bacterial serotypes are defined by antibodies in the serum of the patients or animals that identify the specific type of antigen presented by the bacteria. Three major surface antigens enable the serotyping of *E. coli*. The *types* of antigens are designated by letters. Numbers refer to the known *subtypes* of antigens that can be differentiated by the use of specific antibodies and

thus are used to identify bacterial serotypes. The “O” antigens are somatic cell-wall phospholipids-polysaccharide complexes, whereas the “H” antigens are components of the flagella /2/. The H antigens are heat-labile protein antigens found in flagellin, the protein that constitutes the flagella of motile *E. coli* /2/. The “K” antigens are surface or capsular antigens that are acidic polysaccharides /5/, which were originally further divided into three classes: A, B, and L. Only the A-type K antigens are now considered important for typing antigens because they are mainly associated with the pathogenic strains of *E. coli* that cause extra-intestinal infections and not those associated with diarrheal disease /2/.

Currently, determining only O and H antigens is considered necessary to serotype strains of *E. coli* associated with diarrheal disease. Specific virulence factors like enterotoxins and colonization factors differentiate ETEC from other categories of diarrheagenic *E. coli*. Enterotoxigenic *E. coli* belongs to a heterogeneous family of lactose-fermenting *E. coli* belonging to a wide variety of O antigenic types that produce enterotoxins, which may be heat labile and/or heat stable, and colonization factors that allow the organisms to readily colonize the small intestine and thus cause diarrhea /6/.

Diarrheal diseases are major causes of morbidity and mortality in the developing world, especially in young children. In South Africa, estimates are that diarrheal diseases are the primary causes of death in infants that are younger than 5 years of age, leading to about 160-200 deaths per day /7/. Diarrhea is a condition that results when increased amounts of water are present in stools. This increase occurs when the stomach or the small intestine secrete too much fluid, such that the distal small intestine and colon do not absorb enough water, or the undigested, liquid food passes too quickly through the small intestine and colon for them to remove enough water.

Diarrheagenic *E. coli* are a leading cause of children's diarrhea in developing countries /8/, and some strains are increasingly being recognized as important enteropathogens in developed countries /9/. Diarrheagenic *E. coli* is categorized into the following six pathotypes:

1. Enteropathogenic *E. coli* (EPEC),
2. Enterohaemorrhagic *E. coli* (EHEC),
3. Enteroinvasive *E. coli* (EIEC),
4. Enteroaggregative *E. coli* (eaggec),
5. Enterotoxigenic *E. coli* (ETEC), and
6. Diffusively adherent *E. coli* (DAEC) /8/.

Other diarrheagenic *E. coli* pathotypes have been proposed, such as the cell-detaching *E. coli* (CDEC); yet, their significance remains uncertain /10-11/. Each pathotype has distinguishing characteristics related to its epidemiology, pathogenesis, clinical manifestations, and treatment. Among these pathotypes, ETEC is the most common, particularly in the developing world /12/, and is increasingly recognized as an emerging enteric pathogen. Enterotoxigenic *E. coli* is the second most common cause of traveler's diarrhea and a common cause of acute diarrheal illness in children and adults (4.5%) presenting to emergency departments and inpatient units in the United States (USA) /9,13/. Because ETEC is a major cause of traveler's diarrhea in persons who journey abroad, the organism is regularly imported into the developed world /14-18/. ETEC diarrhea occurs in all age groups, but mortality is most common in infants, particularly in the most undernourished or mal-nourished infants in developing nations /19/. The disease is characterized by watery stool, abdominal cramps, fever, malaise, and vomiting /20/. In this

paper, we present a comprehensive overview of ETEC-mediated diarrheal disease with regard to its epidemiology, diagnosis, treatment, and prevention through the use of vaccines.

2.2 EPIDEMIOLOGY

In developing countries, enterotoxigenic *E. coli* (ETEC) is the most recognized cause of infectious diarrhea. Worldwide, the incidence of ETEC infections is estimated to result in 650 million cases of diarrhea and 380,000 deaths in children under the age of 5 years. The pathogen is also an important cause of travelers' diarrhea in persons traveling to endemic regions of the world /21/. The ease with which people move around the world has dramatically increased the frequency of traveler's diarrhea, which now affects up to one-third of individuals who visit developing areas, such as Africa, South Asia, Latin America, and the Middle East. Because ETEC has endemic and epidemic potentials, the pathogen is a major cause of relatively serious disease during natural disasters.

Traveler's diarrhea has been defined as the passage of at least three unformed stools in a 24-hour period during travel from an industrialized nation to a less developed country, or during the first 7 to 10 days after returning home /22/. Associated symptoms can include nausea, vomiting, abdominal pain, fecal urgency, tenesmus, and bloody or mucoid stools. Individuals at highest risk include young children; adults aged 15-29 years, and those with high gastric pH (achlorhydria, post-gastrectomy, and proton-pump inhibitor use) /23/. The spectrum of infectious agents varies from country to country, but overall, the most common pathogens in order of decreasing frequency include ETEC, EAEC, Shigella species, *Campylobacter jejuni*, rotavirus, *Aeromonas* species, *Plesiomonas shigelloides, Salmonella species, non-cholera vibrios, and the norovirus* /24/.

2.2.1 Sources of infection

All infectious agents causing traveler's diarrhea are efficiently spread by the fecal-oral route. According to epidemiologic investigations, fecally contaminated food and water are the most common vehicles for ETEC infection /25/. Although most travelers fear contaminated water as the source of disease, contaminated food could be a much more common vehicle of transmission for both bacteria and viruses /26/. In rural and peri-urban areas of most developing countries, the use of sewage and wastewater for irrigation is a common practice. As wastewater is often the only source of water for irrigation in these areas, eating fruits and vegetables that have been irrigated with contaminated water and eaten raw is one way that *E. coli* can be ingested. *E. coli* can also be found in raw milk from cows or other milk-producing animals that carry the bacteria on unclean udders. Finally, *E. coli* can be found in fresh meat /27/.

2.2.2 Geographic distribution

Enterotoxigenic infections are common in areas that have high levels of fecal contamination of water and food supplies. Enterotoxigenic *E. coli* strains are associated with two major clinical syndromes—weaning diarrhea among children and traveler's diarrhea in the developing world. Immunity develops in exposed individuals, which explains why natives of endemic areas can drink the water, yet visitors are prone to infection /25/. Enterotoxigenic *E. coli* traveler's diarrhea occurs most commonly during the warm and wet months and among first-time travelers to the developing world /28/.

Up to 50% of those traveling from developed to developing countries are expected to have at least one episode of acute diarrhea during a 2-week stay. The risk of travelers' diarrhea is

not uniform throughout the developing world. For instance, Latin America, Africa, Asia, and parts of the Middle East have reported attack rates for traveler's diarrhea ranging between 20% and 75% /29/. Attack rates of between 8% to 20% have been recorded among travelers to China, southern Europe, Israel, South Africa, Russia, and several Caribbean islands (especially Haiti and the Dominican Republic), whereas 5% have been recorded in Canada, the USA, Australia, New Zealand, Japan, northern European countries, and a few Caribbean Islands /15/.

In the developed world, ETEC infections are not an important cause of diarrhea in either children or adults /30/. Nevertheless, several infantile diarrhea outbreaks did occur in England, Scotland, and the USA, incriminating the serogroups O6, O78, and O159 as the causative agent /31/. The sources and routes of transmission were not clarified, although cross-infection was very important. Some outbreaks in Japan were associated with contaminated well water (cited in /31/). Food outbreaks associated with contaminated turkey, imported French cheese, and salad vegetables occurred in England, Japan, and the USA /32/. Infected food handlers have also been implicated as vehicles for transmission for certain outbreaks /2, 33/.

In regions of poor hygiene, especially in the tropics /34/, ETEC strains are reportedly significant causes of infantile diarrhea and death /35/. Children up to 2 years of age are particularly infected, and a decline in diarrhea in older children and adults ensues due to a progressive development of immunity. The infective dose of ETEC can be significantly lowered by the development of clinical malnutrition brought about by diarrhea of other etiology /2/. Contaminated weaning foods, latrine-contaminated unprotected water supplies, or sewage contaminated rivers are some of the vehicles of transmission /35-36/.

Enterotoxigenic *E. coli* are zoonotic because pathogenic strains shed from healthy livestock, including pigs and cattle, can contaminate the environment. Asymptomatic human

carriers form the principal reservoir of ETEC strains /35/. Enterotoxigenic *E. coli* have also been shown to cause travelers' diarrhea or gastroenteritis among travelers coming from temperate regions with good sanitation and hygiene to visit tropical countries /5/.

2.3 ETEC INFECTION

2.3.1 Clinical manifestations

Symptoms of ETEC infection include abdominal cramping, fever, nausea, with or without vomiting, chills, loss of appetite, headache. Muscle aches and bloating can also occur but are less common. The illness develops 1-3 days after exposure, usually lasting 3-4 days.

2.3.2 Pathogenesis

The pathogenesis of ETEC diarrhea involves two steps: intestinal colonization, followed by elaboration of diarrheagenic enterotoxin(s).

Colonization.

Enterotoxigenic *E. coli* strains are characterized by their specialized pili, antigenically unrelated to common pili, which act as ligands to bind the bacterial cells to specific complex carbohydrate receptors on epithelial cell surfaces of the small intestine. As this interaction results in colonization of the intestine by ETEC, with subsequent multiplication on the gut surface, these pili are termed *colonization-factor antigens* (CFAs) /19/. Enterotoxigenic *E. coli* possess organelles called *fimbriae* that are species-specific. Different types of ETEC fimbrial adhesions are used by the bacteria to colonize the gastrointestinal tract. These strains are non-invasive, but produce enterotoxins /8/. The CFAs can be subdivided based on their morphological

characteristics. Three major morphologic varieties exist: rigid rods, bundle-forming flexible rods, and thin flexible wiry structures. The prototype rigid rod-shaped fimbriae, CFA/I, are composed of a single protein assembled in a tight helical configuration; CFA/III is a bundle-forming pilus; and the CFA/II and CFA/IV are composed of multiple distinct fimbrial structures. The CFA-type pili play a major role in host specificity /19/.

Diarrheagenic enterotoxin(s).

Enterotoxigenic *E. coli* carry the gene for enterotoxin production, which causes diarrhea in humans and animals. ETEC strains cause diarrhea through the action of two types of enterotoxins—a heat-labile toxin (LT) and a heat-stable toxin (ST). These strains can express an LT only, an ST only, or both an LT and an ST. The genes coding for the production of CFAs reside on the ETEC virulence plasmids, usually on the same plasmids that carry the genes for one or both of the two types of *E. coli* enterotoxin, LT and ST. In most cases of ETEC infections, the diarrhea is caused by CFA and both LT and ST; fewer are caused by those possessing a CFA and only one toxin (usually LT); and the fewest are caused by *E. coli* lacking a CFA and possessing only ST /2/. Infection requires colonization and the release of one or more enterotoxins.

The heat-stable ST toxin is a non-immunogenic protein comprising 18-20 amino acids functionally and structurally related to the mammalian protein guanylin /37/, and thus binds to the guanylin receptor. The binding results in an elevation of cyclic adenosine monophosphate (cAMP), ultimately leading to the secretion of chloride, which results in diarrhea /28/. The STs are small, monomeric toxins containing multiple cysteine residues, whose disulfide bonds account for the heat stability of these toxins. Two unrelated classes of STs (STa and STb) differ

in structure and in mechanism of action. The genes for both classes are found predominantly on plasmids, and some ST-encoding genes have been found on transposons.

The STa class binds to a membrane-spanning enzyme receptor called *guanylate cyclase* C (GC-C), an enzyme that converts guanosine 5'-triphosphate (GTP) to cyclic guanosine 5'-monophosphate (cGMP) /38/. Guanylate cyclase C (GC-C) is located in the apical membrane of intestinal epithelial cells, and the binding of ligands to the extracellular domain stimulates the intracellular enzymatic activity. This receptor is normally used by guanylin, which is presumed to play a role in normal gut homeostasis, and GC-C is apparently used opportunistically by STa to cause diarrhea. The binding of STa to GC-C stimulates GC activity, leading to increased intracellular cGMP levels /39/. This activity leads to the stimulation of chloride secretion and/or the inhibition of sodium chloride (NaCl) absorption, resulting in net intestinal fluid secretion. The latter is due to the activation of the chloride channel, leading to secretion of Cl_2 ions into the intestinal lumen. In contrast to the 15- to 60-min lag time required for LT to translocate and activate the basolateral adenylate cyclase complex, STa acts much faster because of the apical location of its cyclase receptor. The toxins termed STb do not seem to cause diarrhea by the same mechanism as STa /19/.

The STb protein is associated primarily with ETEC strains isolated from pigs, although certain human ETEC isolates expressing STb have been reported. The STb protein sequence has no homology to that of STa, although it does contain four cysteine residues that form disulfide bonds. Unlike STa, STb induces damage in the intestinal epithelium by causing the loss of villus epithelial cells and partial villus atrophy. Although previous studies suggest that the toxin may bind non-specifically to the plasma membrane before endocytosis /40-41/. Unlike the chloride ion secretion elicited by STa, STb stimulates the secretion of bicarbonate from intestinal cells.

The STb toxin does not stimulate increases in intra-cellular cAMP or cGMP concentrations, although it does stimulate increases in intracellular calcium levels from extracellular sources /2/. The heat-labile toxin (LT) causes diarrhea by activating the chloride channel. The chloride channel can also be activated by simulating prostaglandin synthesis and by the enteric nervous system, both of which can stimulate secretion and inhibit the absorption of water. The LT is an immunogenic protein structurally, functionally, and antigenically related to the cholera toxin /42/. The *E. coli* LT proteins are oligomeric toxins that are closely related in structure and function to the cholera enterotoxin (CT) expressed by *Vibrio cholerae* and have a similar mechanism of action. The LT and CT toxins share common antigenic determinants, and their primary amino acid sequences are similar. The two major serogroups of LT, termed LT-I and LT-II, do not cross-react immunologically. LT-I is expressed by *E. coli* strains that are pathogenic for both humans and animals. LT-II is found primarily in animal *E. coli* isolates and rarely in human isolates, but in neither animals nor humans has it been associated with disease /8/.

An LT protein is composed of two types of subunits. One type of subunit (the B subunit) binds the toxin to the target cells via a specific receptor that has been identified as Gm1 ganglioside. The other type of subunit (A subunit) is then activated by its own peptide bond cleavage and internalized. Once inside the epithelial cells, the A subunit catalyzes the ADP-ribosylation (transfer of ADP-ribose from nicotinamide adenine dinucleotide [NAD]) of a regulatory subunit of membrane-bound adenylate cyclase, the enzyme that converts ATP to cAMP. The ADP-ribosylation activates adenylate cyclase, which produces excess intra-cellular cAMP, thereby leading to a hypersecretion of water and electrolytes into the bowel lumen, resulting in diarrhea /19/.

2.4 DIAGNOSIS AND DETECTION OF ETEC

To detect outbreaks effectively, public health surveillance and diagnostic procedures for ETEC require both sensitivity and specificity. During diarrheal outbreaks, subculturing techniques of stool samples serve as the first step in the identification of ETEC strains, followed by genetic-based detection methods /43/. Whereas *V. cholerae*, *Shigella* spp., and the rotavirus can be readily detected by standard assays, ETEC is more difficult to recognize and therefore is often not appreciated as a major cause of either infantile diarrhea or of cholera-like disease in all age groups /28/. Hence, definitive diagnosis remains largely confined to research laboratories and requires the identification of a specific toxin by EIA (enzyme immunoassay) or by a DNA probe of the toxin gene.

2.4.1 Culture-based detection methods.

This approach involves stool-sample collections from individuals with diarrhea, and the swabs containing the sample are transferred onto nitro-cellulose paper /19/. The sample is inoculated into MacConkey or Eosin Methylene Blue (EMB) agar by overlaying the paper onto the agar plates, followed by incubation overnight. Colonies yielding typical results for *E. coli* will have a pink to red color.

To confirm for the presence of *E. coli*, the IMViC (Indole, Methyl red, Voges-Proskauer, and Citrate) test should be conducted. The IMViC test examines the ability of an organism to (1) produce indole; (2) produce sufficient acid to change the color of a methyl red indicator; (3) produce acetoin, (a positive result of the Voges-Proskauer test), and (4) grow on citrate as the sole source of carbon. *E. coli* is positive in the first two tests and negative in the second two; non-fecal coliforms give the opposite result /4/. *E. coli* colonies are inoculated into tryptic soy

broth, casamino acid yeast extract salts (CA-YE) broth or Luria broth. After incubation, the supernatant is collected for further identification procedures /44/.

No reliable biomarkers, such as serotype or biotype, exist for enterotoxigenity. Serotyping was found to be of limited use in Bangladesh /45/ because a very large number of *E. coli* serotypes could be enterotoxigenic. Nevertheless, a demonstration of the toxin is necessary to identify ETEC strains. The assays used earlier for the direct identification of ETEC enterotoxins include physiological assays like the rabbit ileal loop model for LT /46/ and the infant mouse assay /47/ for ST. Commonly used biological assays are the Y-1 adrenal assay, suckling-mouse assay, and the Chinese hamster ovary (CHO) cell assay. The suckling-mouse assay used to detect the ST enterotoxin entails the measurement of intestinal fluid in CD4 infant mice after injecting culture supernatants. The supernatant from the cultured cells is administered to infant 6-days-old mice. The presence of the enterotoxin is assessed based on a scoring system incorporating the ratio of intestinal weight, the remaining body weight; and the production of diarrhea /44/. Either the Y1 adrenal cell assay or the CHO cell assay detects the LT enterotoxin. In the Y1 assay, ETEC culture supernatants are added to Y1 cells and the cells are examined for rounding. In the CHO cell assay, the presence of LT is indicated by cell elongation /8/.

2.4.2 Diagnostic assays

Simpler diagnostic assays developed over the years include an enzyme-linked immunosorbent assay (ELISA) technology /48/, immunoprecipitation in agar and the Biken test /49/, passive latex agglutination /50/, and staphylococcal coagglutination /51/.

Enzyme-linked immunosorbent assays.

A capture ELISA has been developed that can be used to detect the heat-labile LT-I toxin produced by enterotoxigenic *E. coli* strains. This solid-phase assay is performed using the immunoglobulin G (IgG) enriched fraction of anti-LT-I antiserum and IgG2b as a 'capture' antibody to bind as much of the toxin as possible, and an anti-LT-I monoclonal antibody (MAb) obtained from mice or rabbits serve as the recognition agent for the bound toxin. As each Mab detects only a single epitope in the polyclonal anti-LT-I IgG fraction, this method provides an inherent monospecificity that allows the fine detection and quantitation of small differences in antigen. Microtiter plates are coated with the anti-rabbit LT IgG enriched fraction in carbonate-bicarbonate-buffer, and the supernatant of bacterial cultures is inoculated. Unbound toxins are removed by washing three times with phosphate buffered saline. Toxins bound to the solid-phase anti-rabbit LT IgG-enriched fraction are then detected with an IgG2b Mab, followed by peroxidase-labeled anti-mouse IgG peroxidase. The substrate hydrogen peroxide is added and converted by the enzyme to a detectable form. The estimated accuracy of the assay is 78% for sensitivity, 94% for specificity, and 92% for efficiency. The capture assay is considered an excellent tool for detecting LT-producing strains and could be employed in the diagnosis of diarrhea caused by LT-producing ETEC strains /52/.

ST gangliosides GERM CELLS1-ELISA.

Monoclonal Abs prepared against the heat-stable ST obtained from human *E. coli* isolates can also be used in another immunodetection assay denoted the ST gangliosides GERM CELLS1-ELISA. This assay is based on the ability of the Sta present in culture filtrates from ST-

producing *E. coli* to inhibit specific anti-ST antibodies from binding to a solid-phase-bound ST gangliosides (GERM CELLS1-bound ST-cholera B subunit). One example of a MAb is immunoglobulin G1 (IgG1); all IgG1 MAbs can be completely inhibited by the addition of free ST /51/. When the IgG1 MAbs were tested in the ST GERM CELLS1-ELISA, ST could be detected in culture filtrates from human stock *E. coli* isolates with 100% sensitivity and specificity. The presence of ST in filtrates from fresh stool cultures was demonstrated with higher sensitivity using the MAbs ST GERM CELLS1-ELISA than with the conventional infant mouse test /53/.

Reverse passive latex agglutination (RPLA).

The RPLA assay is used to detect the presence of soluble LT/ST enterotoxins in culture filtrates. In RPLA tests, the antibody is attached to latex particles and reacts with the soluble LT/ST antigen, unlike in the conventional latex agglutination method, whereby the soluble antibody is reacted with the bacterial LT/ST toxin. In this assay, the samples (*E. coli* bacterial cultures) are inoculated into 96-well microtitre plates and sensitized latex particles are added. The plates are covered and shaken for 24 h. If the LT/ST toxin is present, a visible molecular lattice and a diffuse layer at the base of the well will form due to agglutination /54/.

2.4.3 Molecular methods

Assays employing DNA probes and DNA amplification have proven useful for identifying ETEC. Oligonucleotide gene probes for LT and ST-1 with non-radioactive enzyme markers are available and provide a sensitive and specific detection method /55/. Direct ETEC diagnosis of

fecal material as well as of isolated colonies has been made possible with the polymerase chain reaction (PCR) /56/.

ETEC colonization factors.

A number of different methods have been used during the years. Initially, the capacity of *E. coli* CFs to agglutinate certain species of erythrocytes in a mannose-resistant manner was used to demonstrate CFA/I and CS1, CS2, and CS3 /57/. This non-precise method was soon replaced by the more-specific slide agglutination and immunodiffusion tests, initially using polyclonal sera and subsequently MAbs against different CFs /58/. Traditional methods, including non-specific salting-out tests /59/ and binding to tissue culture cell lines /60/, have now been replaced by molecular methods, for example, DNA probes and the PCR to detect most of the known CFs, or dot blot assays using several different anti-CF MAbs /36,61/.

Genetic-based methods.

This approach to ETEC detection relies on the presence of the genes encoding LT and/or ST enterotoxins. DNA probes and PCR assays are very sensitive and useful in the detection of LT- and ST-encoding genes in stool samples. The LT polynucleotide probe provides good sensitivity and specificity when labeled with radioisotopes or with enzymatic, non-isotopic detection systems /62/. Lately, the use of a highly reliable alkaline phosphatase-based detection system in polynucleotide probe colony-blot hybridization is more popular /8/. The ST polynucleotide probes have had problems of poor sensitivity and specificity, presumably because of the small size of the gene. Hence, oligonucleotide probes that are generally more sensitive and specific for ST detection have been developed /63/.

Multiplex polymerase chain reaction (PCR).

A useful adaptation of the PCR is the *multiplex* PCR assay, so that the simultaneous diagnosis of LT- and ST-producing organisms as well as other diarrheagenic *E. coli* can be accomplished /56/. Several PCR primers are combined with the aim of detecting one or more of several different diarrheagenic *E. coli* pathotypes in a single reaction. After multiplex PCR, various reaction products can usually be differentiated by product size, but a second detection step (for example, nonisotopic probe hybridization) is generally performed to identify definitely the respective PCR products /64/.

2.5 TREATMENT

At present, the recommendations for treating ETEC can only be stated for surety in the treatment of traveler's diarrhea for which ETEC are known to be the most frequent cause /65/. An effective treatment of diarrheal disease has the potential to substantially lower morbidity and mortality. The reduction of mortality from diarrheal disease is primarily related to the effective management of dehydration /66/. In general, oral rehydration plus bismuth subsalicylate or loperamide is adequate therapy for mild to moderate diarrhea (less than four stools per day). Several prophylactic and treatment drug regimens have been described for ETEC diarrhea1 disease /67-68/, with quinolones being the current drugs of choice for both prophylaxis and treatment. Yet, the use of quinolones in the pediatric population remains controversial. Antibiotics should generally be reserved only for persons with traveler's diarrhea who have moderate to severe symptoms. Double-blind randomized studies have demonstrated the efficacy of several antibiotic regimens in treating acute traveler's diarrhea: single doses of either levofloxacin 500 mg or azithromycin 1000 mg, or twice-daily dosing of rifaximin 200 mg or

ciprofloxacin 500 mg, for three days, appear to be roughly equivalent /69-70/. In countries where the bacteria are likely to be resistant to the fluoroquinolones, azithromycin or rifaximin have been recommended for use in empiric treatment.

More than half the enteric bacterial isolates from patients with traveler's diarrhea are resistant to trimethoprim-sulfamethoxazole, this has limited utility for treating traveler's diarrhea. Studies have demonstrated that ETEC strains from Egypt are routinely resistant to ampillicin, streptomycin, and chloramphenicol (David *et al*., unpublished data). Some investigators have reported an association between multiresistance and enterotoxin phenotype. Multiresistance occurred more often in ST-producing strains /71/, whereas such resistance was observed to be more common in LT-producing strains /72/. One study in Bangladeshi adults in which tetracycline was used to treat ETEC diarrhea (determined retrospectively) showed only a minimal effect on the severity or duration of diarrhea /73/. When ETEC were first recognized, the bacteria were usually highly sensitive to all antimicrobials, including the tetracyclines and trimethoprim-sulfamethoxazole /74/. With time, however, antibiotic resistance emerged, necessitating the use of newer antimicrobials for treating traveler's diarrhea. Antimicrobials that have been used in effective treatment include doxycycline, trimethoprim-sulfamethoxazole, erythromycin, norfloxacin, ciprofloxacin, ofloxacin, azithromycin, and rifamycin /18/.

Due to the increasing microbial resistance of ETEC, newer drugs have been used. Fluoroquinolones such as ciprofloxacin, levofloxacin, or ofloxacin are currently the drugs of choice because no significant resistance to these drugs has yet developed /18, 75/. A newer non-absorbed drug, rifaxamin, has also been shown to be as effective as the fluoroquinolones and has only recently been approved for use in the United States /69/.

2.6 PREVENTION

2.6.1 Public health interventions

The prevention of the spread of this strain of diarrheagenic *E. coli* depends on ensuring appropriate sanitary measures like hand-washing, proper food preparation, chlorination of water supplies, and efficient sewage treatment and disposal. A recent systematic review and analysis revealed that interventions to improve water quality are generally effective for preventing diarrhea in all age groups, including those less than 5 years of age /76/. Therefore, proper surveillance of water, food, and sanitation facilities, using public health diagnostic and detection procedures as mentioned before is necessary to protect infants and travelers from infection.

Environmental health protection measures that can be applied in the agricultural use of wastewater for irrigation include wastewater treatment, crop restriction, control of wastewater application and human exposure, and promotion of hygiene. Because consumers of irrigated crops that are likely to be eaten uncooked are at high risk for direct contact with pathogens leading to infection, the irrigation of fruit trees should cease two weeks before the fruit is picked. No fruit should be picked off the ground, and sprinkler irrigation should not be used /77/. 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 aquaculture (farming of fish, shellfish and aquatic plants in fresh or salt water), the quality of the water is of paramount importance to prevent the contamination of fish or plants grown in wastewater ponds. Reliance has been placed primarily on minimizing the risk of pathogen transmission by thorough cooking of the products, but this approach has not always

been satisfactory and, where the pond products are eaten uncooked, no health protection is provided.

Overall, most interventions have been found to reduce the levels of diarrheal illness significantly, with the greatest impact being seen for hygiene and household treatment interventions /78/. Personal preventive measures include the following:

- not drinking tap water;
- not using ice in beverages (including alcoholic drinks);
- not eating salads or other forms of raw vegetables;
- not eating fruits that cannot be peeled on the spot; and
- not eating mayonnaise, unpasteurized dairy products, uncooked fish, or undercooked shellfish.

2.6.2 Vaccines

Limited and often outdated information compounded by increasing drug resistance has made empirical treatment difficult. Therefore, the development of vaccines has been aggressively pursued for the control of ETEC infections (for comprehensive review, see reference /28/). The use of short-term chemoprophylaxis and self-treatment for diarrhea are effective for travelers' who are unwilling to accept even a short period of illness because of the serious impact it may have on their overall mission /79/. The routine use of pharmaceutical anti-microbial prophylaxis for the general traveler is not recommended, however, because of the potential for associated adverse drug reactions and the potential to worsen the problem of antibiotic resistance of enteric bacteria /24,29,80/. All these factors make the development of vaccines against ETEC a priority. To develop a vaccine offering the broadest protective potential and to assess the extent of

antibiotic resistance, the characterization of representative ETEC strains from different geographic regions is a necessity /81/. Nevertheless, the development of a vaccine will not eliminate the need for effective antibiotics to treat diarrhea caused by ETEC.

2.7 CONCLUSION

Enterotoxigenic *E. coli* remains a threat to both humans and animals because children, particularly those under 5 years of age, adults, and animals die every day from infections caused by this strain. The biggest challenge in preventing the spread of these pathogens is poverty, which leads to lack of sanitization. Hence, developing countries are affected at higher rates than developed nations. The development of vaccines is being aggressively pursued to stop the spread of this pathogenic strain. Most important, the proper surveillance of water, food, and sanitation facilities must be implemented. For this purpose, rapid, efficient, and specific detection techniques are required, and this is a subject of intensive investigation in our laboratory.

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CHAPTER 3

IMPACT OF DISCHARGED WASTEWATER FINAL EFFLUENT ON THE PHYSICO-CHEMICAL QUALITIES OF A RECEIVING WATERSHED IN A SUB-URBAN COMMUNITY OF THE EASTERN CAPE PROVINCE

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ABSTRACT

Failures of sewage treatment systems both within and outside South Africa are most commonly ascribed to inadequate facilities and other factors resulting in the production of poor quality effluents with attendant negative consequences on the receiving watershed. The impact of the final effluent of a wastewater treatment facility in a sub-urban community of the Eastern Cape Province of South Africa on the physico-chemical qualities of the receiving watershed was assessed between August 2007 and July 2008. Water quality parameters were analyzed according to South African Department of Water Affairs and Forestry standards. The effluent quality was acceptable with respect to the pH (6.9-7.8), temperature (13.8-22.0 °C), dissolved oxygen (DO) (4.9-7.8 mg/L), salinity (0.12-0.17 psu), total dissolved solids (TDS) (119-162 mg/ L) and nitrite concentration (0.1-0.4 mg/l). The other physicochemical parameters that did not comply with regulated standards include the following: phosphate (0.1-4.0 mg/L); chemical oxygen demand (COD) (5-211 mg/L); electrical conductivity (EC) (237-325 μS/cm) and Turbidity (7.7-62.7 NTU). Results suggest that eutrophication is intensified in the vicinity of the effluent discharge points, where phosphate and nitrate were found in high concentrations. The discharged final effluents had detrimental effects on the receiving water body, thus suggesting the need for regular and consistent intervention by appropriate monitoring and compliance agencies to ensure adherence to acceptable standards for discharged effluents.

Keywords: effluent, physico-chemical qualities, sub-urban community, wastewater treatment.

3.1 Introduction

Water is perhaps South Africa's most critical resource. Located largely in a semi-arid part of the world, the country's water resources are, in global terms, scarce and extremely limited [1] and a key environmental problem facing the country is water pollution. This pollution arises from many sources, including industrial, mining and municipal effluents, and runoff of biocides, nutrients and pathogens from agricultural lands, urban areas and informal settlements with their characteristic poor sanitation [2, 3]. Biological and chemical contaminants [4, 5, 6] represent a health risk if suitable health protection measures are not taken. Typical examples of those of greatest concern are shown in Table 1.

An epidemiologic study in South Africa [7] found an increase in methemoglobin levels in infants that are fed water with nitrate > 20 mg/L nitrate-N. However, clinical methemoglobinemia was rarely found. Severe methemoglobinemia has been documented in India [8] and was found to be related to elevated nitrate in drinking water. More recently, a retrospective, nested case-control study in Romania found an association between nitrate exposure from drinking water and clinical methemoglobinemia, as well as some evidence of an association with diarrheal and respiratory disease [9, 10, 11].

Since 2004 a spate of surveys and technical papers have noted that up to 70 percent of municipal waste treatment facilities in South Africa face collapse for lack of proper maintenance and extension, while about a third require "immediate intervention" and another third intervention "within the short to medium term" [12, 13]. Experts point out that the country’s local authorities are increasingly unable to cope with the constant demand for effective sewage treatment [14, 15]. Recent studies on wastewater treatment plants have shown that small

Table 1. Selected Contaminants of Health Concern Identified in Untreated Municipal Wastewater.

Contaminant	Health Effect	Maximum Contamination Level (mg/L)
Heavy metals		
Arsenic	Gastrointestinal, skin, and nerve damage, cancer	0.01
Cadmium	Gastrointestinal, kidney and lung damage	0.005
Chromium	Lung and skin damage, cancer	0.1
Mercury	Brain and kidney damage, embryo/fetotoxic	0.002
Nickel	Lung, brain, kidney, liver, spleen and skin damage, cancer	0.1
Inorganic chemicals		
Cyanide	Brain and heart damage, shortness of breath, death	0.2
Fluoride	Dental and skeletal fluorosis	4
Nitrate	Methemoglobinemia	10
Organic chemicals		
Benzene	Anaemia, dizziness, leukaemia	0.005
Toluene	Brain and kidney damage	1
Xylenes	Confusion, dizziness, memory loss, embryo/fetotoxic	10
Nutrients		
Nitrite (as Nitrogen)	Cause eutrophication which facilitates the growth of toxin-producing cyanobacteria and other harmful algae	1

Source: U. S. Environmental Protection Agency (USEPA), Drinking Water Standards and Health Advisories (2006).

wastewater treatment plants are often situated in far-flung peri-urban or rural areas where technical and management capacity is hard to come by [16, 17, 18]. With sewage pollution, rivers and dams become eutrophic and algal blooms could develop, rendering the water difficult to treat with normal water treatment methods, and consequently impacting the water with unpleasant taste and odors. In this paper, we report on the physicochemical qualities of discharged final effluents of a sub-urban wastewater treatment facility in the Eastern Cape Province of South Africa and its impact on the receiving watershed.

3.2 Materials and Methods

3.2.1 Plant description: The wastewater treatment plant located in a sub-urban settlement in the Eastern Cape Province within the geographical coordinates 32°51'274"S and 27°14'167"E, accepts municipal domestic sewage and wastewater containing a heavy industrial contribution. The wastewater treatment system is of a basic design; the inlet works comprises of two screens, three grit channels and a flow recorder. The plant has two aeration tanks, each equipped with three vertically mounted mechanical aerators, two anaerobic tanks and two sedimentation tanks. There is a return activated sludge (RAS) pump station which lifts the recycle sludge from the sedimentation tanks to the aeration tanks. A splitter box controls the flow of the raw sewage and RAS to the aeration tank. The plant has a waste mixed liquor pump station which pumps the waste mixed liquor from the aeration tank to two sludge lagoons. Chlorine contact is carried out by means of a water pressure operated, wall mounted, gas chlorinator in a baffled reinforced concrete contact tank. Thereafter the final effluent is pumped to a pair of final effluent reservoirs and into Tembisa sewerage dams. The plant is designed to treat an average dry weather flow of 7

000 m^3/day and an average wet weather flow of 21 000 m^3/day. The plant accounts for large daily inflow due to the high number of industries located in the area and high population of residents. According to the Geospatial Analysis Platform (GAP) – The Presidency, dti and CSIR, July 2007, estimates that the population per mesozone in 2004 of Dimbaza was between 30,000 to 80, 000.

3.2.2 Sampling: Water samples were collected once monthly from August 2007 to July 2008 from the final effluent, discharge point, 500 m upstream and 500 m downstream of the discharge points. The samples for chemical analyses were collected in clean Nalgene bottles according to standard procedures [19, 20]. Before sampling, the sample bottles were cleaned by soaking in detergent for 24 h, followed by rinsing several times with tap water until free of detergent, rinsed with 5% nitric acid and then thoroughly with distilled-deionised water. All samples collected were transferred in ice to the laboratory and analysed within 2 to 4 h of collection.

3.2.3 Physico-chemical analysis: Eleven physicochemical parameters considered as priority pollutants by the Department of Water Affairs and Forestry of South Africa were selected as target indices in this study. pH, temperature, electrical conductivity (EC), total dissolved solids (TDS), salinity and dissolved oxygen (DO) of the samples were determined *on site* using a multiparameter ion specific meter (Hanna instruments, version HI9828) equipped with three different probes for pH; electrical conductivity, salinity and total dissolved solids; and dissolved oxygen and temperature. A one point calibration with the customized buffers was used for each sampling day as recommended by the manufacturer. Turbidity was determined using the

microprocessor turbidity meter (Hach Instruments). The Chemical Oxygen Demand (COD), concentrations of orthophosphate as phosphate, nitrate and nitrite were determined by the standard photometric method [21] using the Spectroquant NOVA 60 photometer (Merck). Samples for COD analyses were digested with a Thermo reactor Model TR 300 (Merck) and then analysed by the Spectroquant NOVA 60 photometer (Merck).

3.2.4 Data analysis: The data were analyzed using analysis of variance (ANOVA) and Duncan Multiple Range Tests (DMRT) to test differences among all possible pairs of treatment means. Statistical test of the effect of season on the physico-chemical parameters was conducted. Correlation was performed using Proc Corr procedure of SAS (SAS version 8, SAS Institute, Cary, NC).

3.3 Results and Discussion

3.3.1 Results

The profile of the physico-chemical parameters measured for the water samples are given in Table 2. In addition, nitrate concentration and chemical oxygen demand (COD) values of the samples are presented in Figs. 1. and 2.

3.3.1.1 pH

The pH of the samples varied between 6.9-7.6 in autumn; 7.1-7.6 in spring; 7.1-7.8 in summer; and 7.5-7.8 during winter (see Table 2). These variations are nevertheless not significant.

Table 2. Seasonal changes in physico-chemical properties of Dimbaza wastewater treatment facility over a period of twelve months.

Parameter	Location				
	Season[a]	Final Effluent	Discharge Pt	Upstream (500 m)	Downstream (500 m)
pH	Autumn	6.9 ± 0.2	7.4 ± 0.1	7.6 ± 0.4	7.1 ± 0.1
	Spring	7.1 ± 0.3	7.4 ± 0.2	7.6 ± 0.1	7.4 ± 0.1
	Summer	7.1 ± 0.2	7.5 ± 0.2	7.8 ± 0.1	7.5 ± 0.1
	Winter	7.1 ± 0.5	7.5 ± 0.2	7.8 ± 0.4	7.3 ± 0.6
Temperature	Autumn	18.8 ± 0.3	18.8 ± 0.7	16.7 ± 0.8	18.0 ± 0.2
	Spring	19.9 ± 0.1	20.0 ± 0.1	20.3 ±0.2	20.9 ± 0.5
	Summer	21.7 ± 1.3	21.6 ± 1.8	21.7 ± 1.9	22.0 ± 1.6
	Winter	16.9 ± 1.9	15.4 ± 1.3	13.8 ± 2.3	15.6 ± 1.9
Phosphate	Autumn	4.0 ± 0.4	3.9 ± 0.5	1.6 ± 1.5	3.4 ± 0.6
	Spring	0.8 ± 0.2	0.9 ± 0.2	0.2 ± 0.1	0.7 ± 0.2
	Summer	2.6 ± 1.0	2.7 ± 1.0	0.5 ± 0.2	2.0 ± 0.8
	Winter	0.4 ± 0.5	0.4 ± 0.5	0.1 ± 0.1	0.3 ± 0.4
Dissolved oxygen (DO)	Autumn	5.3 ± 0.8	6.1 ± 0.7	6.5 ± 0.4	5.7 ± 0.4
	Spring	5.1 ± 0.2	6.3 ± 0.1	5.9 ± 0.3	5.5 ± 0.2
	Summer	4.9 ± 0.3	5.6 ± 0.5	5.5 ± 0.5	5.0 ± 0.4
	Winter	4.9 ± 0.3	7.3 ± 0.6	7.8 ± 0.9	6.7 ± 1.0
Electrical Conductivity (EC)	Autumn	237 ± 10	243 ± 2	309 ± 17	264 ± 11
	Spring	295 ± 20	298 ± 1	325 ± 7	311 ± 10
	Summer	245 ± 27	263 ± 7	322 ± 14	281 ± 7
	Winter	252 ± 36	264 ± 13	315 ± 12	284 ± 35
Nitrite	Autumn	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
	Spring	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.1
	Summer	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1
	Winter	0.3 ± 0.3	0.3 ± 0.3	0.1 ± 0.0	0.4 ± 0.2
Salinity	Autumn	0.12 ± 0.01	0.16 ± 0.07	0.16 ± 0.01	0.13 ± 0.01
	Spring	0.15 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	0.16 ± 0.01
	Summer	0.12 ± 0.02	0.14 ± 0.02	0.16 ± 0.01	0.14 ± 0.01
	Winter	0.13 ± 0.02	0.14 ± 0.02	0.17 ± 0.01	0.15 ± 0.02
Total Dissolved Solids (TDS)	Autumn	119 ± 5	122 ± 4	155 ± 8	132 ± 5
	Spring	148 ± 10	149 ± 9	162 ± 4	155 ± 5
	Summer	123 ± 13	133 ± 18	161 ± 7	140 ± 4
	Winter	126 ± 18	133 ± 17	154 ± 10	144 ±21
Turbidity	Autumn	10.4 ± 2.0	16.3 ± 2.0	45.6 ± 10.3	25.4 ± 5.7
	Spring	8.5 ± 0.7	8.0 ± 0.7	31.4 ± 0.5	17.1 ± 2.1
	Summer	7.7 ± 1.7	15.1 ± 7.0	62.7 ± 21.1	36.7 ± 11.6
	Winter	19.4 ± 12.4	21.2 ± 12.9	24.7 ± 11.4	21.7 ± 9.3

*Values are means of triplicate determination ± standard deviations (SD). [a] Summer (November to March); autumn (April to May); winter (June to August); spring (September to October).

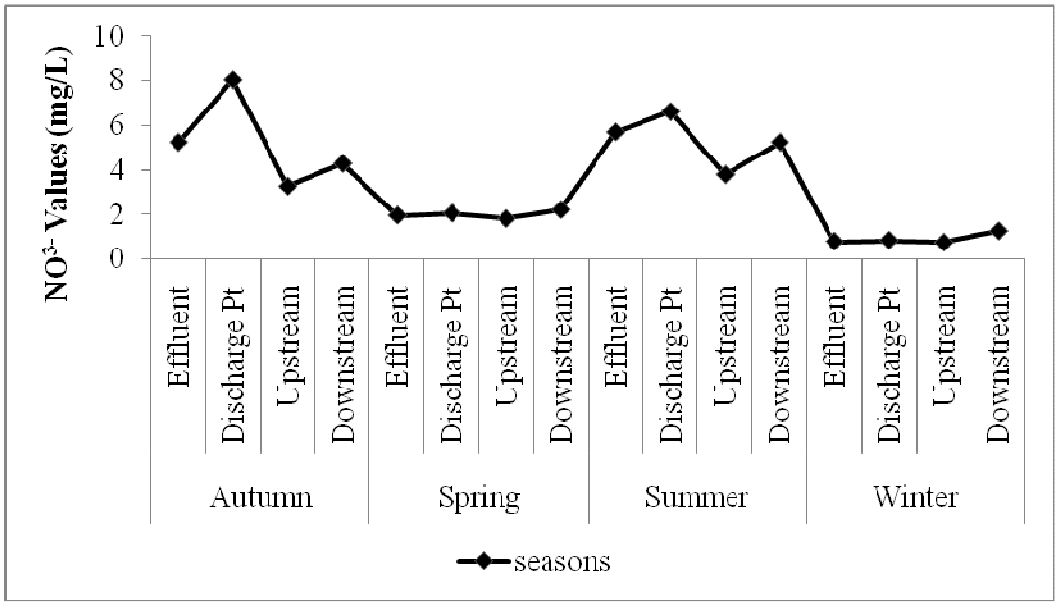

Figure 1**:** Seasonal nitrate profile across the four sampled points.

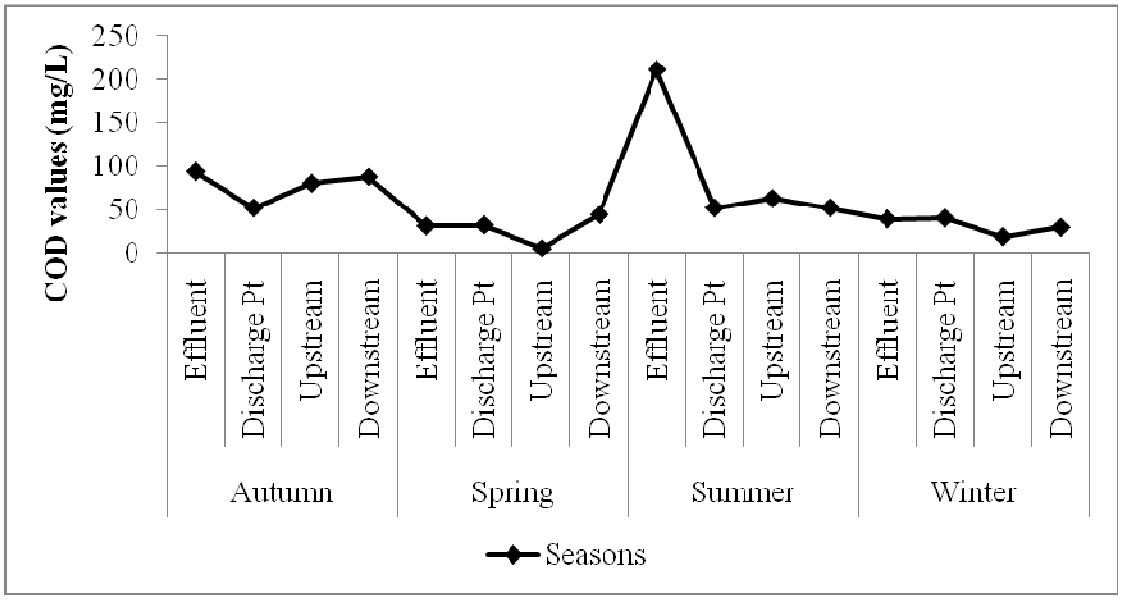

Figure 2**:** Seasonal chemical oxygen demand profile across the four sampled points.

The highest pH values were obtained at the upstream location throughout the seasons. Generally the pH ranges obtained fall within the water quality range of 6.5 to 8.5 for any purpose [22].

3.3.1.2 Temperature

As seen in Tab. 2, the temperature regimes of the water samples ranged between 16.7-18.8 °C in autumn, 20.1-21.0 °C in spring, 21.6-22.0 °C in summer and 13.8-16.9 °C in winter. The lowest temperature values were recorded during winter and autumn while the highest was during summer and spring period. Higher water temperatures can reduces the dissolved oxygen concentrations in water and hence its availability to aquatic organisms [23]. The temperature profiles observed in this study are comparable to those reported by Feng [24] and are in compliance with the recommended limit for no risk of below 25 °C according to the *South African Water Quality Guidelines for Domestic Use* [25].

3.3.1.3 Phosphate

The orthophosphate levels ranged from 0.1-4.0 mg/L in all the sample locations (see Table 2). The major peaks in orthophosphate concentrations were found during autumn, followed by a decline in concentration in winter. Orthophosphates are generally limiting factors in aquatic environments. At concentrations above 0.1 mg/L in water bodies, orthophosphates usually leads to increased eutrophication [26]. The South African guidelines do not specify the target water quality ranges for phosphate in water for domestic use and recreational purposes [27, 28]. However the level of phosphate in water systems that will reduce the likelihood of algal and other plant growth is 0.005 mg/L [23]. Hence, the orthophosphate limits were exceeded in the final effluent of the plant, thus suggesting inadequate removal of phosphate by the wastewater

treatment facility. The final effluent discharge is therefore considered a main source of phosphate to the receiving watershed. Locations where high concentrations of orthophosphate were detected could be due to point source pollution, and where low orthophosphate concentrations were detected could be due to non-point sources of pollution.

3.3.1.4 Dissolved Oxygen (DO)

In the case of dissolved oxygen (DO), the concentrations varied between 4.9-5.3 mg/L (final effluent); 5.6-7.3 mg/L (discharge point); 5.5-7.8 mg/L (upstream); and 5.0-6.7 mg/L (downstream). For all the locations, the maximum DO values were obtained in summer and the minimum in winter with the exception of autumn season final effluents. Dissolved oxygen concentrations in unpolluted water normally ranged between 8 and 10 mg/L [29, 30]. Dissolved oxygen standard for drinking purposes is 6 mg/L whereas for sustaining fish and aquatic life a concentration of 4 - 5 mg/L [23] is stipulated. For water quality variable such as dissolved oxygen, water quality criteria are set at the minimum acceptable concentration to ensure the maintenance of biological functions. Dissolved oxygen is an important parameter 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 to maintaining biological life within a system [26]. Our result suggests that the mean DO concentrations of the final effluents were acceptable.

3.3.1.5 Electrical conductivity (EC)

The electrical conductivity (EC) values for all sample points appear to be similar and ranged between 237 and 325 μS/cm (see Table 2). This observation corroborates reports of another

study on the nearby Keiskamma River [31]. Electrical conductivity of water which is a useful indicator of its salinity or total salt content is high in the upstream and downstream points compared with the final effluent, thus suggesting a contribution to EC levels from source(s) outside the treatment plant. The South African guideline for conductivity in effluent that could be discharged into the receiving water bodies is 250 μS/cm [32] and based on this guideline; the effluent quality does not appear to be compliant with the regulation for electrical conductivity. Also, the South African acceptable limit for conductivity in domestic water supply is 70 μS/cm [27]. This limit was exceeded in the receiving water body thus posing a risk to direct domestic usage of the water from these sources.

3.3.1.6 Nitrite

The United States Environmental Protection Agency (USEPA) safe limit for nitrite is 1.0 mg/L as N [33]. The mean nitrite concentrations obtained during the study was 0.1-0.3 mg/L in both the final effluent and discharge point; 0.1-0.2 mg/L in the upstream; and 0.2-0.4 mg/L in the downstream. In all locations, minimum values were obtained during spring while elevated concentrations were obtained in the effluent and upstream points in summer and the discharge point and downstream locations in winter. The wastewater treatment facility did not exceed the regulatory limit and thus nitrite is not considered to pose a risk to the communities when the effluents are used for domestic and recreational purposes. Nutrient enrichment of river water can contribute to algal blooms and die-offs and to decomposition and depletion of dissolved oxygen, reducing water quality and creating unfavorable conditions for other aquatic life [34].

3.3.1.7 Salinity

Salinity levels ranged from 0.12-0.15 psu in the final effluents; 0.14-0.16 psu in the discharge point; 0.16-0.17 psu in the upstream; and 0.13-0.16 psu in the downstream samples as shown in Tab. 2. Freshwater has a salinity of ~0 psu whereas seawater is approximately 35 psu [35] and the samples were in compliance based on the limit of ~0 psu. The highest values were obtained in spring while the lowest was in autumn with the exception of the discharge point being low in summer and winter.

3.3.1.8 Total dissolved solid (TDS)

Total dissolved solids (TDS) levels varied between 119 and 148 mg/L in the final effluent and between 122 and 149 mg/L in the discharge point. Upstream and downstream TDS levels ranged between, 154-162 mg/L and 132-155 mg/L respectively (see Tab. 2). The maximum values were obtained in spring while the minimum was in autumn with exception for the upstream location in winter. The TDS values are in compliance with the permissible limits of 0 to 450 mg/L [36].

3.3.1.9 Turbidity

The turbidity values obtained in this study ranged from 7.7-19.4 NTU in the final effluent; 8.0-21.2 NTU in the discharge point; 24.7-62.7 NTU in the upstream and 17.1-36.7 NTU in the downstream. There is no South African guideline for turbidity in effluent discharge [32]. The South African Target Water Quality Range for turbidity in water for domestic water supply is 0 to 1 NTU [27] while World Health Organization standard is 5 NTU [22]. These values are grossly exceeded in the water samples in all the seasons and it disqualifies the effluent for direct domestic use. Turbidity may be comprised of organic and/or inorganic constituents. Organic

particulates may harbor microorganisms in the effluent. Thus, high turbid conditions may increase the possibility for waterborne diseases since particulate matter may harbor microorganisms and may stimulate growth of bacteria [37] thereby posing some health risk to the effluent users. Also, the excessive turbidity in water can cause problems with water purification processes such as flocculation and filtration, which may increase treatment cost [19]. Elevated turbid waters are often associated with the possibility of microbiological contamination, as high turbidity makes it difficult to disinfect water properly [19]. When highly turbid waters are chlorinated there is a tendency for an increase in trihalomethane (THM) precursor formation [38]. The high turbidity also makes the sight of the receiving water bodies where the effluent is being discharged unpleasant for full-contact recreation [28].

3.3.1.10 Nitrate

The concentrations of nitrate vary appreciably (0.7 and 8.0 mg/L) with season (Fig. 1). The mean nitrate concentrations obtained during the study period exceeded the regulatory limit of 0 to 0.5 mg/L as N [27, 25] and thus nitrate is considered to be a potential nuisance to the communities when the effluents are used for domestic and recreational purposes. It is important to note that the nitrate levels in the final effluents could be a source of eutrophication for the receiving water bodies as the value obtained in the effluent exceeded the recommended maximum.

3.3.1.11 Chemical Oxygen Demand (COD)

The COD concentrations obtained in this study ranged from 31 to 211 mg/L in the final effluent and from 32 to 52 mg/L at the discharge point. Upstream of the discharge point the COD vary

from 5 to 80 mg/L while downstream concentrations range between 29 and 87 mg/L (see Fig. 2). The new South African water quality guidelines do not specify the COD concentrations for domestic, recreational, aquatic ecosystems and agricultural purposes. A standard for drinking water purposes is 4 mg/L [39]. The COD guidelines available are for industrial purposes and ranges between 0 and 10 mg/l [40]. The mean COD values in all effluents were above the acceptable limit of no risks [40], thus also suggesting the inefficiency of the wastewater treatment facility in removing the chemical oxygen-demanding substances.

3.3.1.12 Correlation Matrix of Physicochemical Parameters

The correlations among the physicochemical properties were assessed and results presented in Table 3. There were no significant correlations observed between pH and temperature as well as COD and nitrate concentrations. However, there were significant ($P<0.01$) positive correlations between pH, electrical conductivity, turbidity, salinity, TDS and DO, while pH correlated negatively with nitrite and phosphate ($r = -0.44$, -0.37 at $P<0.01$, respectively). Increased temperature exhibited a significant positive correlation with the turbidity at $P<0.05$ and with concentrations of nitrate and phosphate at $P<0.01$. Temperature with DO indicated a negative correlation ($r = -0.69$ at $P<0.01$). Non-point contamination events of the watershed in the seasons could be contributing to these results. Conductivity exhibited negative significant correlation with nitrate, nitrite and phosphate ($r = -0.35$,-0.51 and -0.48 at $P < 0.01$) and positive significant correlation with salinity, TDS and DO ($r = 0.98$, 0.57 and 0.30 at $P < 0.01$, respectively). This will help to understand the nature of these physicochemical variables and their speciation in the effluent and receiving watershed.

Table 3. Correlation coefficient of physico-chemical parameters**.**

Variables	pH	Temperature	Electrical Conductivity	Turbidity	Salinity	TDS	DO	COD	Nitrate	Nitrite	Phosphate
pH	1	0.08 ns	0.74 **	0.67 **	0.72 **	0.69 **	0.32 **	-0.13 ns	-0.16 Ns	-0.44 **	-0.37 **
Temperature		1	-0.01 ns	0.21 *	-0.07 ns	-0.02 ns	-0.69 **	0.08 ns	0.43 **	0.02 ns	0.29 **
Electrical Conductivity			1	0.57 **	0.98 **	0.98 **	0.30 **	-0.07 ns	-0.35 **	-0.51 **	-0.48 **
Turbidity				1	0.53 **	0.56 **	0.06 ns	-0.14 ns	0.04 Ns	-0.21 ns	-0.26 *
Salinity					1	0.97 **	0.34 **	-0.09 ns	-0.36 **	-0.53 **	-0.49 **
TDS						1	0.29 **	-0.08 ns	-0.35 **	-0.49 **	-0.49 **
DO							1	-0.11 ns	-0.39 **	-0.27 *	-0.41 **
COD								1	0.11 Ns	0.22 ns	0.11 Ns
Nitrate									1	0.36 **	0.55 **
Nitrite										1	0.24 *
Phosphate											1

= P < 0.05; ** = P < 0.01; ns = not significant.

Turbidity with salinity, TDS and DO exhibited significant positive correlation and negative correlation with concentration of phosphate ($r = -0.26$ at $P<0.05$). During high watershed flow, a dilution effect of the concentrations of some of the measured pollutants was observed downstream of the discharge point e.g. dissolved oxygen and TDS. There was an observable depletion of dissolved oxygen in the downstream point of the discharge point, which was possibly brought up by decomposition of settling organic matter which may include algal bloom biomass.

3.4 General Discussion

The challenges of effective environmental protection from the impacts of domestic sewage disposal in recently developed and rural areas are a matter of international concern. Surface waters that receive wastewater treatment plant effluents are abstracted for domestic and other purposes in many local communities of South Africa. As far as irrigation is concerned, the two major factors to be considered when determining water's suitability for that use are salinity (measured by electrical conductivity (EC) or the concentration of total dissolved solids (TDS). With regards to TDS the effluents appear to be suitable for irrigation without any form of further treatment [35]. The pH, temperature and dissolved oxygen levels observed in this study were still within recommended limits. Across the seasons, final effluent discharge into the receiving water posed critical risk to the inherent ecology with regards to electrical conductivity (EC) and nitrate concentrations. A similar trend was also observed in terms of phosphate concentration except in the autumn season. The overall picture suggest that eutrophication is intensified in the vicinity of the effluent discharge points, phosphate and nitrate were observed in high concentrations.The results of this comprehensive one-year river physicochemical quality monitoring effort have

demonstrated the necessity for continuous monitoring programme of surface waters and confirm what previous isolated studies have suggested, that the source of nutrient loading was contributed significantly by the wastewater treatment facility. The values of chemical oxygen demand (COD) in the current study were above the recommended limit during most of the seasons, not minding the turbulence of the water upstream and downstream of the discharge point.

The increase in turbidity as the water flows downstream suggests that the final effluent of the treatment plant could be an important source of turbidity. Good quality effluent is a reasonable expectation provided adequate provision is made for the necessary expenditure on maintenance, skilled operation and meaningful quality monitoring. Both within South Africa and abroad, failures of sewage treatment systems are most commonly ascribed to poor design, construction and operation, insufficient or no maintenance and mechanical breakdowns. Most municipal sewerage systems in South Africa are 30 to 50 years old, and the ageing process is taking its toll. In addition to these problems, treatment plant manufacturers face the challenge of high variability of sewage influent amongst others [17].

3.5 Concluding Remarks

In this study, the quality of the final effluent was acceptable with respect to the pH, temperature, dissolved oxygen, salinity, total dissolved solids and nitrite concentration. On the other hand, the phosphate and nitrate concentrations of the final effluents being outside the acceptable limits could contribute to eutrophication of the receiving watershed and if not controlled could result in other serious pollution problems. The effects of polluted water on human health, the aquatic ecosystem and on various sectors of the economy, including agriculture, industry and recreation,

can be disastrous. Deteriorating water quality leads to increased treatment costs of potable and industrial process water, and decreased agricultural yields. Also, the turbidity, electrical conductivity and chemical oxygen demand did not comply with regulated standards.

Continuous pollution of source waters is a global problem that is particularly debilitating to rural communities that are directly dependent on untreated source water for all their domestic and other needs. To reduce pollution and conserve what is left of this precious resource, there is need to ensure that wastewater is properly treated before discharge into the environment. The communities must be informed of the impact of effluent outfalls that result in deterioration in the water quality. Since several areas of the receiving water body in this study are used for recreational and agricultural purposes, the public health implications need to be addressed. Approaches and methods on how to eliminate or mitigate the problems associated with municipal sewerage systems includes the proper planning, design, construction, operation and rehabilitation of municipal sewerage systems. Adequate measures to remove the nutrients as well as the oxygen demanding components from the wastewater should be adopted, and proper maintenance of the treatment facility are suggested to avoid further deterioration of the receiving watershed's quality.

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CHAPTER 4

SURVIVAL OF FREE-LIVING AND PLANKTON-ASSOCIATED *ESCHERICHIA COLI* IN THE FINAL EFFLUENTS OF A WASTEWATER TREATMENT FACILITY IN A PERI-URBAN COMMUNITY OF THE EASTERN CAPE PROVINCE OF SOUTH AFRICA

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ABSTRACT

Escherichia coli remains a major threat in many places around the globe as a major causative agent of diarrhea, and its reservoir in the estuarine environment may play an important role in the survival and transport of pathogenic strains. The final effluents of a peri-urban wastewater treatment facility were assessed for surviving *E. coli* community as free-living or plankton-associated cells in relation to some physicochemical parameter for a year period. Standard culture and molecular based techniques were employed. The free-living *E. coli* population densities varied from 0 to 3.13×10^1 cfu/ml, while the plankton-associated *E. coli* densities vary with plankton sizes as follows: 180 µm (0 - 4.30×10^1 cfu/ml); 60 µm (0 - 4.20×10^1 cfu/ml); 20 µm (0 - 5.00×10^1 cfu/ml). The seasonal variations in the *E. coli* densities among the plankton size categories were significant ($P < 0.05$). Correlation analysis suggested that the counts of *E. coli* correlated negatively with salinity ($P< 0.001$) and positively with temperature, pH, turbidity and dissolved oxygen ($P < 0.001$) in the final effluent. The study suggested that wastewater treatment final effluents could be a significant source of pathogenic *E. coli* in the receiving watershed.

Keywords: *Escherichia coli*, free-living, plankton-associated, wastewater final effluent.

4.1 Introduction

Escherichia coli is a member of the *Enterobactericeae* (the intestinal bacteria) and belong to the order *Eubacteria* [1−3]. These bacteria are facultatively anaerobic, Gram negative rods that can grow under both aerobic and anaerobic conditions [3]. If molecular oxygen is available, the bacteria rely on respiratory metabolism to survive. In the absence of molecular oxygen, the organisms use fermentation as an alternate means of survival [2−3]. Bacteria belonging to the genus Escherichia are motile by means of peritrichous/multiple flagellum and it is unable to form spores to survive unfavourable environmental conditions [4].

Escherichia coli is an important cause of disease in animals and humans worldwide. Strains of *E. coli* can be classified as (i) commensal, (ii) intestinal pathogenic (enteric/diarrheagenic), or (iii) extraintestinal pathogenic *E. coli* (ExPEC) [5]. An increasing number of categories of pathogenic *E. coli* isolates have been identified over the past few decades, which has led to the current situation in which there are now at least 11 recognized pathotypes of *E. coli* in humans [6]. These pathotypes are defined by the presence of combinations of virulence and virulence-related genes; conversely, the pathotype of an uncharacterized strain can be inferred from its virulence gene profile [6]. These pathotypes are defined by the presence of combinations of virulence and virulence-related genes; conversely, the pathotype of an uncharacterized strain can be inferred from its virulence gene profile [6]. Pathogenic *E. coli* strains are also divided into pathotypes on the basis of their distinct clinical symptoms of the host [7]. Three main types of clinical syndrome can result from infection with one of these pathotypes: enteric and diarrheal diseases, urinary tract infections, and sepsis/meningitis.

The *E. coli* pathotypes responsible for intestinal infections include enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli*, necrotoxic *E. coli*, and cell-detaching *E. coli*. Three additional *E. coli* pathotypes, collectively called extraintestinal pathogenic *E. coli* [5], are responsible for extraintestinal infections. Extraintestinal pathogenic *E. coli* is composed of uropathogenic *E. coli* (UPEC) isolates that cause urinary tract infections, neonatal meningitis-associated *E. coli* (MNEC), and *E. coli* strains that cause septicemia [5, 8]. Diarrheal diseases continue to be a health problem worldwide [9−10]**,** especially in developing countries, where they are estimated to be responsible for 2.5 million infant deaths per year, with an annual mortality rate of 4.9 per 1,000 children and an incidence of 3.2 episodes per child per year among children under 5 years of age [10]. In South Africa it has been estimated that diarrheal diseases are the primary cause of death in infants that are younger than 5 years, leading to about 160-200 deaths per day [11].

Pathogenic *E. coli* bacteria are known to be associated with food-borne diseases; contamination of drinking or recreational waters with some pathotypes has resulted in waterborne disease outbreaks and associated mortality. Outbreaks of pathogenic *E. coli* in Canada [12] have been associated with contaminated drinking-water obtained from rural catchments. In 2000, in the town of Walkerton, Ontario, Canada, an estimated 2300 people became ill and 7 died from exposure to EHEC contaminated drinking-water [13] and a recreational water outbreak in 2001 at a beach in Montreal, Quebec, resulted in the hospitalization of 4 children [14]. During October 1992, a large outbreak of bloody diarrhea affecting thousands of individuals, some of whom died, occurred in South Africa and Swaziland [15]. *Escherichia coli* O157 was isolated from 22.5% of 89 stool samples, and epidemiological

investigations implicated waterborne spread. In some areas, cases were mainly men, who drank surface water from fields. *Escherichia coli* O157 was also isolated from 14.3% of 42 samples of cattle dung confirming that agricultural animals can serve as a vector for *E. coli* 0157:H7 [16−17].

Uropathogenic *E. coli* strains are frequently isolated from biofilms formed in the lumen of catheters, where they resist antibiotic treatment and shear forces [18]. Pathogenic *E. coli* is found in aquatic ecosystems physically isolated from any source of faecal contamination [19]. Studies have been performed to determine their distribution in environmental surface waters including ponds [20], recreational waters [21], lagoons [22], rivers [23], streams [24] and lakes [25]. Isolation of EHEC from municipal sewage has been reported [26−27]. Prevention of the spread of strains of diarrheagenic *E. coli* depends on ensuring appropriate sewage treatment and disposal [28]. Marine research has also provided news about the health aspects of pathogens living in association with plankton. Previous studies have demonstrated strong relationships between abundance of both free-living and plankton-associated *E. coli* [29] and indicating that potentially pathogenic organisms living in close association with zooplankton have considerable epidemiological and ecological implications. In this paper, we report the prevalence of potentially pathogenic *Escherichia coli* as free living and plankton associated entities in the final effluents of a wastewater treatment facility in a typical peri-urban community of the Eastern Cape Province of South Africa, as well as their relationship with some physiochemical variables.

4.2 Results and Discussion

4.2.1 Physicochemical analysis

In our previous study [30] we reported the temperature of the final effluents to range from (13.8 - 22.0 °C), while the pH, turbidity, salinity and dissolved oxygen varied from 6.9 - 7.8; 7.7 - 9.4 NTU; 0.12 - 0.15 psu and 4.90 - 5.33 mg/l respectively (Table 1). Also water temperatures of the effluent samples were high in February and the lowest was in July which was characterized with low *E. coli* density. A positive correlation between *E. coli* densities and water temperature was observed (r = 0554; $p < 0.001$). The highest pH was observed in the month of August which had high density of *E. coli*. There was an association between $\log_{10}$ *E. coil* density and pH. The lowest pH was observed in July. The highest turbidity value was observed in the month of August and the lowest in the month of January and there was a similar trend with pH during the same period. Also, increasing turbidity was associated with increasing *E. coli* abundance, suggesting a positive correlation.

The highest salinity value was observed in the month of September and the lowest in the months of January to April and June to July. Salinity negatively correlated with *E. coli* density (r = - 0.982; $p < 0.001$) over the entire sampling period. Also, dissolved oxygen levels were highest in summer, and lowest in winter with the exception of the autumn season final effluents. Dissolved oxygen negatively correlated with *E. coli* densities in the final effluent, and water temperature correlated the most with *E. coli* abundance.

Table 1. Profile of some physicochemical parameters of the final effluent of the wastewater treatment facility over a period of twelve months (extracted from Osode and Okoh, 2009 [30]).

Variables	Season [a]	Final Effluent	*F*-value	Pr > F
pH	Autumn	6.88 ± 0.19	75.98	0.0001
	Spring	7.05 ± 0.29	1154.20	<0.0001
	Summer	7.11 ± 0.18	3516.90	<0.0001
	Winter	7.05 ± 0.47	957.59	<0.0001
Temperature (°C)	Autumn	18.82 ± 0.25	5876.36	<0.0001
	Spring	19.91 ± 0.14	804.79	<0.0001
	Summer	21.65 ± 1.28	4558.87	<0.0001
	Winter	16.91 ± 1.91	113.66	<0.0001
Salinity (psu)	Autumn	0.12 ± 0.01	598.85	<0.0001
	Spring	0.15 ± 0.01	2839.89	<0.0001
	Summer	0.12 ± 0.02	389.55	<0.0001
	Winter	0.13 ± 0.02	768.93	<0.0001
Turbidity (NTU)	Autumn	10.42 ± 1.91	16584.6	<0.0001
	Spring	8.51 ± 0.75	857.21	<0.0001
	Summer	7.68 ± 1.65	998.80	0.001
	Winter	19.45 ± 12.37	14965.85	<0.0001
Dissolved oxygen (mg/l)	Autumn	5.33±0.79	674.58	0.0001
	Spring	5.11±0.20	1434.70	0.0001
	Summer	4.90±0.32	529.20	0.0001
	Winter	4.91±0.27	1349.85	0.0001

*Values are means of triplicate determination ± standard deviations (SD). [a] Summer (November to March); autumn (April to May); winter (June to August); spring (September to October).

4.2.2 Free chlorine residual

The profile of the chlorine residual in the final effluents is as shown in Figure 1. Chlorine residual varied significantly ($P < 0.05$) from 0.10 in the month of September to the highest level of 3.85 mg/L in the month of October. In this study, free chlorine residual range for domestic water (0.3 to 0.6 mg/l) [31] was considered as standard, since the South African guidelines do not specify any standard for final effluents in sewage treatment plants.

4.2.3 Abundance of Escherichia coli

The abundance of presumptive *E. coli* in the effluents varied appreciably between sampling period and plankton sizes and is presented in Figure 2. Presumptive *E. coli* associated with 180 µm plankton size ranged from 0 to 4.30×10^1 cfu/ml. The highest density was observed in September while low densities were observed from October to February, April, May and July (Figure 2). In the 60 µm plankton size category *E. coli* densities ranged between 0 and 4.2×10^1 cfu/ml being highest in September and low in the months of October to May and July. Also in the 20 µm plankton size category presumptive *E. coli* density varied from 0 to 5.0×10^1 cfu/ml with the highest counts in September and low counts in the months of October to December and July. The free-living presumptive *E. coli* density ranged between 0 and 3.13×10^1 cfu/ml with the highest in January and lower counts in the months of October to December and July. With regards to season, low densities of presumptive *E. coli* was observed in summer (October to February) as well as the later end of spring (July) and were found to be more associated with 20 µm plankton size. There was no significant correlation between presumptive *E. coli* abundance and seasons either as free-living or plankton-associated cells. The densities of free-living *E. coli* in summer varied significantly with those of spring ($P < 0.05$), but not with other seasons. In

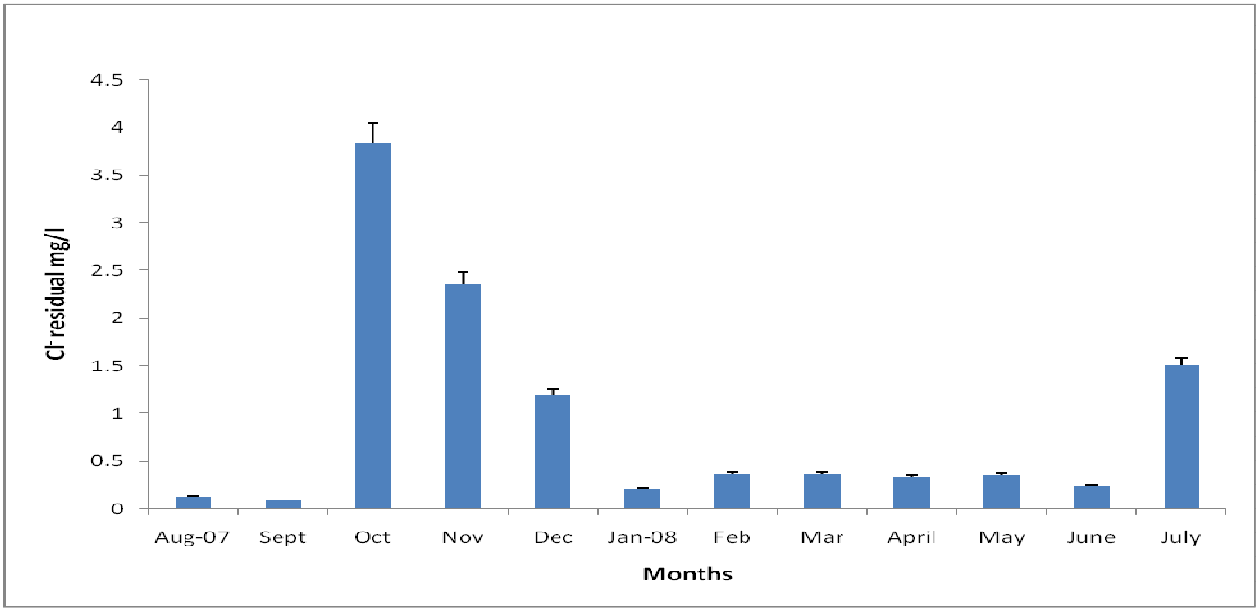

Figure 1. Residual chlorine profile of the final effluents of the wastewater treatment plant.

general, the presumptive *E. coli* was most predominant as 20 µm plankton associated entities (40 %) followed by the 180 µm (25 %), 60 µm (20 %) plankton associated entities respectively, while the free-living *E. coli* constituted only 15 %.

Escherichia coli found in domestic sewage come predominantly from human fecal material [32]. Thus, sewage isolates may serve as representatives of the strains of *E. coli* present within the human population in a given locale. A major goal of wastewater treatment facilities is to reduce pathogen loads in order to decrease public health risks associated with exposure. The effectiveness of pathogen control is indirectly assessed through routine monitoring of the final effluent by using grab samples to detect standard indicator bacteria such as total or faecal coliforms. In this study we used *E. coli* as an indicator of the presence of pathogens from faecal contamination.

The final effluent outfalls in October, November and December 2007 and July 2008 only complied with the South African General and Special Standards which stipulate that treated sewage effluents must have a standard of 0 faecal coliforms/100 ml (Act 96 of 18 May 1984 No. 9225, Regulation 991), and according to [33] the maximum limit for no risk of faecal coliforms is 0 cfu/100 ml. The standard of The World Health Organization for drinking water is no *E. coli* in a 100 ml sample [34]. High *E. coli* counts suggest inefficiency of the wastewater treatment plant in removing the bacteria. Crops such as vegetables can become contaminated with human and animal pathogens when irrigated with water containing this organism [35].

Disinfection is a common final step in wastewater treatments. In recent years, disinfection by Ultra violet radiation or micro-filtration has been proposed because of the sanitary and ecological risks associated with using chemical compounds.

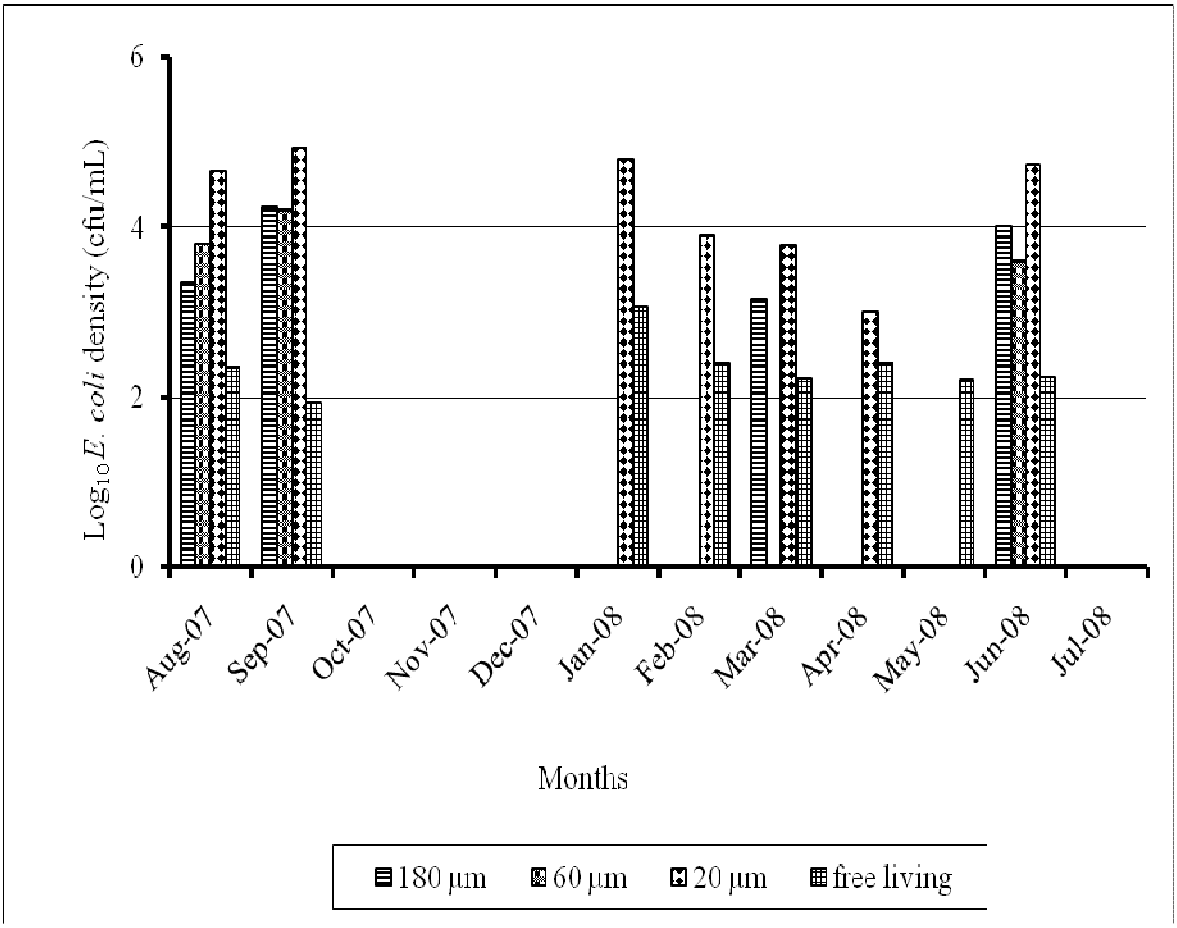

Figure 2. Abundance of *Escherichia coli* in the final effluents of the wastewater treatment plant.

However, in South Africa, disinfection by chemical agents, mainly chlorine, is still the most widespread process [36]. In this study we considered as standard, the free chlorine residual range of 0.3 to 0.6 mg/l for domestic water and 0.6 to 0.8 mg/l as good free chlorine residual concentration with insignificant risk of health effects [31] since the South African guidelines do not specify any standard for final effluents in sewage treatment plants. Based on this concentration, the free chlorine residual in the effluents complied with the regulatory standard in about 33.3% of the sampling periods. The higher densities of *E. coli* in August and September 2007 as well as in January and June 2008 were due to chlorination activity which was under dosed at the wastewater treatment plant. Chlorine overdosing was observed in the October to December 2007 and July 2008 sampling periods, consequently resulting in lower densities of presumptive *E. coli*. It is evident from the result that there is a relationship between chlorine residual and densities of *E. coli*. The wastewater treatment plant contained high densities of *E. coli* in the final effluent which was supposedly disinfected. However, densities of *E. coli* remained high after treatment due to the inefficiency of the wastewater treatment plants in removing the bacteria. For sewage treatment plants to meet national and international standards, there is a need to improve treatment processes and to adopt stringent policies in terms of monitoring and control of the quality of the final effluent. This includes the use of effective methods for the detoxification and disinfection of the sewage effluent.

The abundance of *E. coli* in the final effluent was been found to be associated with temperature. Water temperature fluctuated through the seasons thereby affecting *E. coli* abundance and corroborating previous studies by [37−38] which reported correlations between *E. coli* abundance and water temperature.

However, the fact that plankton-associated *E. coli* were more abundant in the final effluent compared to free-living *E. coli,* suggests that bacterial attachment may play a role in the indecisive effect of the chlorine residual on *E. coli* populations in the final effluent. Some other factors that may affect the efficiency of disinfectants such as chlorine include contact time, temperature and pH [39]. There is little or no report in literature with regards to the occurrence and distribution of *E. coli* as free-living and /or plankton-associated cells in the wastewater final effluents and its receiving watershed. The dynamics observed in the sizes of the most numerous plankton-associated cells describes a system that alternates between populations dominated by large cells (20 µm, possibly cryptophytes or diatoms). Bacteria associated with particles have been shown to survive in aquatic environments for longer times than suspended forms [40−41]. This includes bacterial species of concern to public health such as *Vibrio* and *Enterococcus* [42−44]. Particle attachment of pathogens can play an important role in the ultimate fate of these microbes and the dynamics of the fraction attached may be as important as the total population numbers. For example, aggregation and particle settling presents an efficient mode of bacterial removal from surface waters [45], potentially altering the exposure of local populations using the affected water body [46].

E. coli abundance correlated significantly ($P < 0.01$) with seasons in this study either as free-living or plankton-associated cells. Similar observations have been reported by other authors [46−48]. The counts of free-living *E. coli* during spring varied significantly with those of summer ($P < 0.01$), but not with other seasons or plankton sizes. The findings of this study suggests that bacterial discharge into aquatic systems through inadequately treated wastewater posses a potential health hazard to communities depending on such receiving watershed for domestic and other uses. Moreover, even when most of the *E. coli* cells are destroyed or at least

injured by chlorination to make them non-culturable, evidence have shown that after chlorination, non-culturable cells of enteropathogenic strains of *E. coli* retain their enterotoxigenic activity and can recover and express pathogenicity *in vivo* [49].

4.3 Materials and methods

4.3.1 The study area

The wastewater treatment plant located in a sub-urban settlement in the Eastern Cape Province within the geographical coordinates 32°51'274"S and 27°14'167"E, accepts municipal domestic sewage and wastewater containing a heavy industrial contribution. The plant is designed to treat an average dry weather flow of 7 000 m^3/day and an average wet weather flow of 21 000 m^3/day. The plant accounts for large daily inflow due to the high number of industries located in the area and high population of residents. The wastewater treatment system is of a basic design; the inlet works comprises of two screens, three grit channels and a flow recorder. The plant has two aeration tanks, each equipped with three vertically mounted mechanical aerators, two anaerobic tanks and two sedimentation tanks. There is a return activated sludge (RAS) pump station which lifts the recycle sludge from the sedimentation tanks to the aeration tanks. A splitter box controls the flow of the raw sewage and RAS to the aeration tank. The plant has a waste mixed liquor pump station which pumps the waste mixed liquor from the aeration tank to two sludge lagoons. Chlorine contact is carried out by means of a water pressure operated, wall mounted, gas chlorinator in a baffled reinforced concrete contact tank. Thereafter the final effluent is pumped to a pair of final effluent reservoirs and into Tembisa sewerage dams.

4.3.2 Treatments of samples

All samples were collected aseptically using sterile 1L 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. Samples were stored at 4 °C until analyses were complete. All samples were processed after 24 h of collection.

The procedure was as described by Alam *et al.* [19], 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 × g to dislodge the attached bacteria, and the homogenates used for direct plating.

4.3.3 Physicochemical analysis

The measurement of sample pH, temperature, turbidity, salinity and dissolve oxygen has been described elsewhere [30]. 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.

4.3.4 Estimation of Escherichia coli densities

For direct plate count analyses of plankton-free samples, the samples were serially diluted and appropriate aliquots used to inoculate Eosine Methylene Blue agar (Merck, South

Africa) (EMBA) agar and incubated at 37 ^{o}C for 24 h. For the plankton-associated samples, the *E. coli* densities were obtained using the same agar and in accordance with the description of Alam *et al.* [19]. Colonies showing greenish metallic sheen in transmitted light were considered total presumptive *E. coli* and counted as described elsewhere [50].

4.3.5 Statistical analysis

In assessing the relationship between *E. coli* abundance in the final effluent and physicochemical variables of the environment, linear regression was performed on the collected data. *E. coli* 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 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).

4.4 Conclusion

The finding of this study further reaffirms the existence of association between planktons and potentially pathogenic *E. coli*. Association of general, pollution-indicator and pathogenic bacteria with zooplankton is a common feature [29, 44, 51−53]. Also, the survival of *E. coli* at densities outside acceptable range suggest that the final effluents as veritable sources of pathogens in the watershed. This observation and ensuing inferences of this study are useful for managing effluent outfall into coastal ecosystems and demonstrated the necessity for regular monitoring of effluent quality prior to discharge into the environment. We conclude that when

evaluating disinfection efficiency, the effect of disinfected wastewater effluents on the self depuration process and bacterial survival in receiving waters should be considered, and this is a subject of ongoing investigation by our group.

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CHAPTER 5

PREVALENCE OF POTENTIALLY PATHOGENIC *ESCHERICHIA COLI* IN THE FINAL EFFLUENTS OF A WASTEWATER TREATMENT FACILITY IN THE EASTERN CAPE PROVINCE OF SOUTH AFRICA

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ABSTRACT

Escherichia coli remains a major threat in many places around the globe as the causative agent of diarrhea, and its reservoir in the estuarine environment may play an important role in the survival and transport of pathogenic strains. The final effluent samples of three wastewater treatment facilities were screened for the presence of pathogenic *E. coli* strains as free-living or planktonic cells. The screening was carried out monthly for one year period, the samples were collected according to standard guidelines and extraction was grouped by size into three fractions (180 µm, 60 µm and 20 µm) based on culture method and mdecular technique. In Dimbaza location, the free-living presumptive *E. coli* densities varied from 0 to 3.13×10^1 cfu/mL, while plankton-associated presumptive *E. coli* densities varied from 0 to 4.30×10^1 cfu/mL (180-µm); 0 to 4.20×10^1 cfu/mL (60-µm); 0 to 5.00×10^1 cfu/mL (20-µm). In Alice, the free-living presumptive *E. coli* counts ranged between 0 to 6.20×10^1cfu/mL (180 µm), 0 to 3.88×10^1 cfu/mL (60 µm), 0 to 3.75×10^1 cfu/mL (20 µm) and 0 to 8.0×10^1 cfu/mL (free-living sample). Lastly the presumptive *E. coli* densities in East London location were between 0 to 8.00×10^1 cfu/mL (180 µm), 0 to 8.75×10^1 cfu/ml (60 µm) cfu/mL, 0 to 9.0×10^1 cfu/ml (20 µm) and 0 to 9.5×10^1 cfu/ml (free-living sample). The antibiogram revealed that 100% of *E. coli* isolates from Dimbaza and East London were resistant to linezolid, polymyxin B, penicillin G and sulfamethoxazole. Nine of the ten *E. coli* isolates from Dimbaza and all 8 isolates from East London were resistant to erythromycin. Target genes (*fliC*$_{H7}$, *rfbE*$_{O157}$, *ial* and *aap*) that encode pathogenicity for *E. coli* were successfully amplified by PCR confirming that the isolates were pathogenic strains.

Keywords: Antibiotic, *Escherichia coli*, free-living, plankton-associated, virulence markers, wastewater effluent discharge.

5.1 Introduction

There are two fundamental reasons for treating wastewater: to prevent pollution, thereby protecting the environment; and perhaps more importantly, protecting public health by safeguarding water supplies and preventing the spread of waterborne diseases (Gray, 2004). Proper wastewater treatment is particularly important in South Africa where majority of the populace rely on surface water sources such as rivers for their daily water needs (Venter, 2001). In developing countries, a large population depends on untreated water from rivers, lakes, wells, and other surface water resources for drinking, bathing, laundry, recreation, and other domestic purposes (Qadri *et al*., 2005; Ashbolt, 2004). Surface water resources have emerged as reservoirs of fecal coliforms including diarrheagenic forms of *E. coli* exhibiting virulence genes and resistance to multiple antimicrobial agents due to addition of municipal sewage and wastes from animal production industries and hospitals (Ram *et al*., 2007, Hamelin *et al*., 2006, Edge and Hill, 2005).

Wastewater treatment plant effluents can be an important source of pathogenic bacteria in surface waters (Okoh *et al*., 2007; Auerbach *et al*., 2007). Previous reports on the microbial quality of effluent outfalls in the Eastern Cape Province of South Africa showed that they were unsafe for human consumption or recreational purpose and *Escherichia coli* was one of the predominant pathogens isolated (Osode, 2006). Infections due to pathogenic *E. coli* may be limited to the mucosal surfaces or can disseminate throughout the body. Three general clinical syndromes result from infection with inherently pathogenic *E. coli* strains: (i) urinary tract infection, (ii) sepsis/meningitis, and (iii) enteric/diarrheal disease (Nataro and Kaper, 1998). Basically, the causes of diarrhea are well known and can be summarized as poor access to a good water source and poor sanitation. In South Africa, the provision of water and sanitation to the

previously unserved is a priority development goal and one of the important Millennium Development Goals as set by the United Nations Millennium Summit in 2000. An estimated 3.7 million people have no access to any form of water supply infrastructure and an additional 5.4 million people who have some access have to be brought up to a basic level of service (Info, 2006). With regard to sanitation services, 16 million people (3.9 million households) are without adequate sanitation services (Info, 2006). In terms of targets the UN aims to halve, by 2015, the proportion of people without sustainable access to safe drinking water and basic sanitation. In South Africa the government is committed for reducing the backlog in services by 2008 in the case of water and 2010 in the case of sanitation (DWAF, 2003).

Each year diarrheal disease affects children in developing countries some 5 billion times, claiming the lives of nearly 1.8 million (UNDP, 2006). In South Africa, the prevalence and incidence statistics in general for diarrhea is estimated to affect 44,448,470 (US Census Bureau, International Data Base, 2004). In addition, estimates are that diarrheal diseases are the primary cause of death in infants that are younger than 5 years of age, leading to about 160-200 deaths per day (Nemarude *et al*., 2007). Although the provision of clean water supplies will reduce the levels of infection in the short term, in the long term it is vital that the environment is protected from faecal pollution. 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 conducted 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% were meeting all the set discharge limits. Of particular concern are the high levels of

indicator organisms in some of the effluent. Some of the treated effluents in this area showed faecal coliform counts that were 100 times the discharge limit (Antrobus, 2003).

Antibiotic resistance is a major public health threat, and the presence of resistant organisms in environmental waters is an emerging concern around the world. Antibiotics are released daily into the natural environment with treated wastewater effluent and through use in animal husbandry, leading to increasing concerns with regard to their contribution to the abundance and persistence of antibiotic resistance in populations of pathogenic, commensal, and nonpathogenic microorganisms (Auerbach *et al*., 2007, Yang and Carlson, 2003). The overuse of antibiotics, chemicals such as disinfectants, antiseptics, pesticides together and the practice of sewage discharge that is improperly treated into receiving waters, has resulted in a significant increase of antibiotic resistant bacteria in aquatic environments. These antimicrobial agents are washed off into streams and rivers during rainfall events resulting in development and spread of antibiotic resistant bacteria (Schwartz *et al.,* 2003). Bacteria may be defined as resistant when they are not susceptible to a concentration of antimicrobial agent such as antibiotics and this is indicative of the selection pressure exerted on bacteria (Cloete, 2003). The occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater have been reported in other parts of the world (Brown *et al.,* 2006; Miao *et al.,* 2004; McArdell *et al.,* 2003; Alder *et al.,* 2001). Reinthaler and co-workers (2003) reported antibiotic resistance of *E. coli* in sewage and sludge. There are no reports (to my knowledge) available on antibiotics susceptibility patterns in pathogenic *Escherichia coli* with reference to treated final effluent ecological niches in the Eastern Cape Province of South Africa. This study therefore hypothesize that the final effluents of wastewater treatment facilities in this Province are potential reservoirs of multi resistant antibiotics and pathogenic *Escherichia coli* strains. This study also reports on the

prevalence of pathogenic *Escherichia coli* strains in the final effluents of some wastewater treatment facilities in the Eastern Cape Province, South Africa as either free or attached cells.

5.2 Materials and methods

5.2.1 Plant description

The wastewater treatment plants that serve the Buffalo City (Dimbaza and East London) and Nkonkobe (Alice) Municipal areas in the Eastern Cape Province of South Africa were investigated in the present study. The wastewater treatment plants are located in urban (East Bank Reclamation Works, East London), peri-urban (Dimbaza Sewage Treatment Works) and in rural area (Alice Sewage Treatment Works).

5.2.2 Sample collection and treatment

Water samples were collected once in every month from the final effluent over a period of one year from August 2007 to July 2008. The 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. The samples were collected aseptically using sterile 1L Nalgene bottles and transported on ice from the sampling site to the laboratory and analyzed within 24 h of collection.

As described by Alam *et al.* (2006) one liter of wastewater was filtered successively through 180 µm, 60 µm and 20 µm nylon nets (Millipœre 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 × g to dislodge the attached bacteria, and the homogenates used for direct plating of *Escherichia coli.* The filtrate water from the 20-µm nylon net was collected as representative of water, to be analyzed for planktonic (unattached, free-living) *E. coli.*

5.2.3 Microbiological analysis of the effluent samples

For direct plate count analysis of plankton free samples, the samples were serially diluted in sterile distilled water and used to inoculate Eosine Methylene Blue Agar (Merck, South Africa) according to standard pour plate culture technique and incubated at 37 ^{o}C for 24 h. For the plankton associated samples, the *Escherichia coli* densities were obtained using the same culture medium and in accordance with the description of Alam *et al.* (2006). The samples were enriched in Nutrient Broth (NB) 35 ± 2°C for 18-24 hours before plating as described previously by (Obi *et al*., 2004; Maier *et al*., 2000). Briefly, 1 mL of homogenate were inoculated unto Nutrient Broth (NB) (Merck, South Africa), and incubated at 35 ± 2°C for 18-24 hours before streaking onto eosine methylene blue agar (Merck, South Africa), and incubated at 35 ± 2°C for 18-24 hours. For the direct plating, 0.1 ml of the samples was used to inoculate the plates in triplicates. Colonies showing greenish metallic sheen in transmitted light were considered presumptive *E. coli* and counted as described elsewhere (Obi *et al.,* 2004).

5.2.4 Identification of *Escherichia coli*

Aliquots of the plankton-free and plankton associated samples were inoculated into Nutrient Broth (NB) and incubated aerobically at 37 ^{o}C for 18-24 h. Turbid cultures were streaked onto EMBA and incubated at 37 ^{o}C for 24 h. Five to ten isolated colonies per plate were randomly picked from each sample and subsequently subcultured on fresh EMBA 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 20E kits. The strips were then read and the final identification was secured using API LAB PLUS computer software (BioMerieux, Marcy l'Etoile, France). Only excellent identification reports were accepted.

5.2.5 Antimicrobial Susceptibility Tests

The identified *E. coli* isolates were subjected to antibiotic sensitivity testing by the disc diffusion method (Bauer *et al.*, 1966). The inocula of the *E. coli* isolates were prepared using the colony suspension method (EUCAST, 2003). Colonies picked from 24 h old cultures grown on Nutrient Agar (NA) were used to make suspension of the test organisms in saline solution to give an optical density of approximately 0.1 at 600nm. The suspension was then diluted 1:100 by transfer of 0.1 ml of the bacterial suspension to 9.9 ml of sterile Nutrient Broth (NB) before use. Isolates were subcultured onto Mueller-Hinton agar (MHA) (BD Bioscience, Sparks, MD) and screened for susceptibility to locally produced commercial antimicrobial discs (Davies Diagnostics Pty Ltd) by the disk diffusion method (NCCLS 2000). *E. coli* strain 25922 (American Type Culture Collection) was used as a reference control strain. Isolates were also tested for susceptibility to 37 antibiotics as shown in Table 5.3. Plates were incubated at 35^{o}C

and zones of inhibition were interpreted as resistant or sensitive using the interpretative chart of the zone sizes of the Kirby – Bauer sensitivity test method (Cheesbrough, 2000).

5.2.6 Molecular characterization of *E. coli* using the polymerase chain reaction (PCR)

5.2.6.1 DNA extraction

DNA was extracted from identified *E. coli* and from a positive control strains for *E. coli* (ATCC 8739) (SABS No ESC 20) purchased from the South African Bureau of Standards (SABS), Pretoria, South Africa. The extraction was done following the method of Maugeri *et al.* (2004) and Torres *et al.* (2003) with little modification. Single colonies of presumptive *E. coli* grown overnight at 37 ^{o}C on EMBA plates were picked, suspended in 200 µl of sterile nuclease free water (Fermentas Life Sciences, SA), vortexed using a MS2 Minishaker (IKA Works Incorporation) and the cells were lysed using a Dri-Block DB.2A (Techne, SA) for 15 min at 100 ^{o}C. The cell debris was removed by centrifugation at 10 000 rpm for 5 minutes using a MiniSpin microcentrifuge (Eppendorf) to remove any particulate material that might still be present after processing. The lysate supernatant was placed on ice for 5 min. Nuclease free water (Fermentas Life Sciences, SA) was included in each PCR assay as a negative control. The cell lysates (10 µl) were used as template in the PCR assays immediately after extraction.

5.2.6.2 Amplification of *uidA*, *fliC*$_{H7}$, *rfbE*$_{O157}$, *aap*, and *ial* genes

Oligonucleotide primers targeting the *uidA* structural gene, *fliC*$_{\mathrm{H7}}$ gene encoding for Enterohemorrhagic *E. coli* structural flagella antigen H7, *rfbE*$_{\mathrm{O157}}$ gene encoding Enterohemorrhagic *E. coli* somatic antigen, *aap* gene encoding for antiaggregation protein (dispersin) of Enteroaggregative *E. coli* and *ial* gene encoding for invasion-associated locus of

Enteroinvasive *E. coli* was used in the polymerase chain reaction (PCR). The primers (Southern Cross Biotechnology, SA) sequences that were used to identify the target genes and the expected amplifications sizes are listed on Table 5.1. The PCR assays were carried out in a 25 µl reaction volume. The PCR Master Mix (2X) which was composed of 0.05 units/µl Taq DNA polymerase in reaction buffer, 4mM $MgCl_2$, 0.4mM dATP, 0.4 mM dCTP, 0.4mM dGTP and 0.4mM dTTP (Fermentas Life Sciences, SA). The PCR reaction was carried out in the Eppendorf model AG 22331 Thermocycler (Merck, SA).

The PCR was used to confirm the identities of the *Escherichia coli* strains using the specific primers targeting the *uidA* structural gene as described by Tsai *et al*. (1993). The following PCR conditions for *uidA* genes optimized in our laboratory were similar to those previously used by Tsai *et al*. (1993). The thermal cycling profile was as follows: a 2 minutes denaturation at 94 ^{o}C followed by 25 cycles at 94 ^{o}C for 1 minute, 58 ^{o}C for 1 minute and 72 ^{o}C for 1 minute and final extension at 72 ^{o}C for 2 minutes. The amplified products were held at 4 ^{o}C after completion of the cycles.

To confirm the virulence of the *Escherichia coli* strains, the specific primers described in Table 5.1 were used. The following PCR conditions for $fliC_{H7}$, $rfbE_{O157}$ genes optimized in our laboratory were similar to those previously used by Wang *et al*. (2002). The following PCR conditions for *aap* genes optimized in our laboratory were similar to those previously used by Samie *et al*. (2007) and the following PCR conditions for *ial* genes optimized in our laboratory were similar to those previously used by Presterl *et al*. (2003).

Table 5.1: Primer sequences and expected size of PCR-amplified gene targets of the pathogenic strains of *Escherichia coli*.

Target strain	**Gene target**	**Primer sequence (5' → 3')**	**Amplicon size (bp)**
E. coli	*uidA*	AAA ACG GCA AGA AAA AGC AG	147
		ACG CGT GGT TAA CAG TCT TGC G	
EAEC strain	*aap*	CTT GGG TAT CAG CCT GAA TG	232
		AAC CCA TTC GGT TAG AGC AC	
EHEC strain	$rfbE_{O157}$	CTA CAG GTG AAG GTG GAA TGG	328
		AATT CCT CTC TTT CCT CTG CGG	
EHEC strain	$fliC_{H7}$	TAC CAT CGC AAA AGC AAC TCC	247
		GTC GGC AAC GTT AGT GAT ACC	
EIEC strain	*ial*	CTG GAT GGT ATG GTG AGG	320
		GGA GGC CAA TTA TTT CC	

For enteroaggregative *E. coli* the PCR condition consisted of 1 cycle for 2 minutes at 50 ºC; 1 cycle for 5 minutes at 95ºC; 40 cycles for 45 seconds at 95ºC, 45 seconds at 55ºC, and 45 seconds at 72ºC; and a final extension step for 10 minutes at 72ºC. For enterohaemorrhagic and enteropathogenic *E. coli* the PCR condition consisted of initial denaturation at 95ºC for 8 minutes followed by 30 cycles of heat denaturation at 95ºC for 30 seconds, primer annealing at 58ºC for 30 seconds and DNA extension at 72ºC for 30 seconds. After the last cycle, the samples were kept at 72ºC for 7 minutes to complete the synthesis of all strands. For enteroinvasive *E. coli* the PCR condition consisted of 1 cycle for 2 minutes at 50 ºC; 1 cycle for 5 minutes at 95ºC; 40 cycles for 45 seconds at 95ºC, 45 seconds at 55ºC, and 45 seconds at 72ºC; and a final extension step for 10 minutes at 72ºC to complete the synthesis of all strands. Amplifications were carried out using a Bio-Rad MyCycler thermal cycler with the specified conditions.

5.2.6.3 DNA electrophoresis

The PCR products (10 µl aliquots) were resolved in 1.8 % agarose gel (Merck, SA) containing 0.5 µg/Ethidium bromide (EtBr) (Merck, SA) in 1X TAE buffer (40 mM Tris–HCl, 20 mM Na-acetate, 1 mM EDTA, pH 8.5) (Cagney *et al*., 2004; Wang *et al*., 2002) before being visualized and photographed under the BioDoc-It System (UVP Upland, CA 91786, USA). A 100-bp DNA ladder (Promega, White Head Scientific) was included on each gel as a molecular size standard. The electrophoresis was carried out at 76 V for 1 h.

5.3 Results

5.3.1 Abundance of *Escherichia coli*

The abundance of presumptive *E. coli* in the effluents varied appreciably between sampling period and plankton sizes and is as presented in Table 5.2. Presumptive *E. coli* associated with 180 µm plankton size ranged from 0 to 4.30×10^1 cfu/ml in Dimbaza, 0 to 3.88×10^1 cfu/ml in Alice and 0 to 8.00×10^1 cfu/ml in East London. The highest density in Dimbaza was observed in September 20007 while low densities were observed from October 2007 to February, April, May and July 2008. The highest density in Alice was observed in November while low densities were observed from January to February and April to July (Table 5.2). The highest density in East London was observed in April while low densities were observed only in November 2007 (Table 5.2).

In the 60 µm plankton size category *E. coli* densities ranged between 0 and 4.2×10^1 cfu/ml in Dimbaza, 0 and 2.13×10^1 cfu/ml in Alice and 0 and 8.75×10^1 cfu/ml in East London. The highest density observed being in September and low in the months of October to May and July in Dimbaza. The highest density observed being in August 2007 and low in the months of January to July in Alice. The highest density observed being in August 2007 and low in the months of November 2007 and January to March in East London.

Also in the 20 µm plankton size category presumptive *E. coli* density varied from 0 to 5.0×10^1 cfu/ml in Dimbaza, 0 to 3.75×10^1 cfu/ml in Alice and 0 to 9.0×10^1 cfu/ml in East London. The highest densities from Dimbaza were observed in September and low counts in the months of October to December and July. The highest counts from Alice were observed in August 2007 and low counts in the months of April to July. The highest density from East

TABLE 5.2: Population densities of *Escherichia coli* in the final effluents for one year period.

Months	Locations	180 µm	60 µm	20 µm	Free-living
2007 Aug	Dimbaza	3.29×10^1	3.80×10^1	4.65×10^1	2.34×10^1
	Alice	1.63×10^1	2.13×10^1	3.75×10^1	5.80×10^1
	East London	1.00×10^1	8.75×10^1	6.25×10^1	7.50×10^1
Sept	Dimbaza	4.30×10^1	4.20×10^1	5.00×10^1	1.92×10^1
	Alice	0	0	0	0
	East London	7.50×10^1	2.50×10^1	0	9.50×10^1
Oct	Dimbaza	0	0	0	0
	Alice	ND	ND	ND	ND
	East London	ND	ND	ND	ND
Nov	Dimbaza	0	0	0	0
	Alice	3.88×10^1	1.08×10^1	1.98×10^1	1.20×10^1
	East London	0	0	1.18×10^1	5.50×10^1
Dec	Dimbaza	0	0	0	0
	Alice	1.25×10^1	1.25×10^1	0	8.00×10^1
	East London	6.13×10^1	2.13×10^1	9.00×10^1	2.20×10^1
2008 Jan	Dimbaza	0	0	4.79×10^1	3.13×10^1
	Alice	0	0	0	0
	East London	1.25×10^1	0	1.25×10^1	2.60×10^1
Feb	Dimbaza	0	0	3.87×10^1	2.38×10^1
	Alice	0	0	0	0
	East London	1.25×10^1	0	5.00×10^1	4.00×10^1
Mar	Dimbaza	3.14×10^1	0	3.10×10^1	2.16×10^1
	Alice	2.50×10^1	0	1.25×10^1	0
	East London	2.05×10^1	6.13×10^1	5.00×10^1	1.20×10^1
Apr	Dimbaza	0	0	2.99×10^1	2.40×10^1
	Alice	0	0	0	0
	East London	8.00×10^1	3.50×10^1	1.60×10^1	7.20×10^1
May	Dimbaza	0	0	0	2.14×10^1
	Alice	0	0	0	0
	East London	4.75×10^1	1.00×10^1	0	5.00×10^1
Jun	Dimbaza	3.95×10^1	3.59×10^1	4.73×10^1	2.21×10^1
	Alice	0	0	0	0
	East London	2.38×10^1	1.35×10^1	4.75×10^1	8.00×10^1
Jul	Dimbaza	0	0	0	0
	Alice	0	0	0	0
	East London	1.50×10^1	2.63×10^1	0	0

London were observed in December 2007 and low densities in the months of September, 2007 and May and July 2008.

The free-living presumptive *E. coli* density ranged between 0 and 3.13×10^1 cfu/ml in Dimbaza, between 0 and 8.0×10^1 cfu/ml in Alice and between 0 and 9.5×10^1 cfu/ml in East London. The highest density in Dimbaza was observed in January and lower densities in the months of October to December and July. The highest density in Alice was observed in December, 2007 and lower densities in the months of January to July. The highest density in East London was observed in September, 2007 and lower density in the month of July. High densities of presumptive *E. coli* was found to be more associated with 20 µm plankton size. There was no significant correlation between presumptive *E. coli* abundance and seasons either as free-living or plankton-associated cells.

5.3.2 Antimicrobial Susceptibility Profiles of the *E. coli* isolates

The antibiotic susceptibility tests (Table 5.3) revealed that 100% of *E. coli* isolates from Dimbaza (10/10) and East London (8/8) respectively were resistant to linezolid, polymyxin B, penicillin G and sulfamethoxazole. The *E. coli* isolates from Dimbaza (9/10) and East London (8/8) respectively were resistant to erythromycin. The isolates from Dimbaza showed an intermediate susceptibility to tetracycline (60%), oxytetracycline (60%) and carbenicillin (80%). The isolates from East London showed an intermediate susceptibility to cephalexin (12.5%), doxycycline (62.5%) and nalidixic acid (12.5%). All the isolates were found to be susceptible to amikacin, ceftazidime, ciprofloxacin, colistin sulphate, ceftriaxone, cefotaxime, cefuroxime, ertapenem, gatifloxacin, gentamycin, imidazole, kanamycin, meropenem, moxifloxacin, neomycin, netilmicin, norfloxacin and tobramycin.

Table 5.3: Antibiograms of *E. coli* isolates from final effluent samples.

Antibiotics	**Antibiotic disc content (µg)**	**Antibiotic resistant no. (%)**	
		Dimbaza (n = 10)	**East London (n = 8)**
Amikacin	30	0 (0)	0 (0)
Amoxicillin	25	2 (20)	6 (75)
Ampicillin	25	2 (20)	6 (75)
Aztreonam	30	0 (0)	1 (12.5)
Carbenicillin	100	2 (20)	5 (62.5)
Ceftazidime	30	0 (0)	0 (0)
Ceftriaxone	30	0 (0)	0 (0)
Cefotaxime	30	0 (0)	0 (0)
Cefuroxime	30	0 (0)	0 (0)
Cephalexin	30	0 (0)	2 (25)
Cephalothin	30	0 (0)	2 (25)
Chloramphenicol	30	2 (20)	4 (50)
Ciprofloxacin	5	0 (0)	0 (0)
Colistin sulphate	25	0 (0)	0 (0)
Doxycycline	30	7 (70)	2 (25)
Ertapenem	10	0 (0)	0 (0)
Erythromycin	15	9 (90)	8 (100)
Gatifloxacin	5	0 (0)	0 (0)

Gentamycin	10	0 (0)	0 (0)
Imipenem	10	0 (0)	0 (0)
Kanamycin	30	0 (0)	0 (0)
Linezolid	30	10 (100)	8 (100)
Meropenem	10	0 (0)	0 (0)
Moxifloxacin	5	0 (0)	0 (0)
Nalidixic acid	30	0 (0)	0 (0)
Neomycin	10	0 (0)	0 (0)
Netilmicin	30	0 (0)	0 (0)
Nitrofurantoin	200	0 (0)	1 (12.5)
Norfloxacin	10	0 (0)	0 (0)
Oxyteyracycline	30	3 (30)	2 (25)
Polymyxin B	300	10 (100)	8 (100)
Penicillin G	10	10 (100)	8 (100)
Streptomycin	25	2 (20)	6 (75)
Sulfamethoxazole	25	10 (100)	8 (100)
Tetracycline	30	3 (30)	2 (25)
Trimethoprim	5	0 (0)	5 (62.5)
Tobramycin	10	0 (0)	0 (0)

5.3.3 Molecular Characterization of *E. coli*

The *E. coli* isolates identified by biochemical profiles that were positive by PCR for $fliC_{H7}$, $rfbE_{O157}$, *aap*, and *ial* genes are summarized in Table 5.4. The molecular characterization confirmed that 3 of the presumptive *E. coli* isolates from Alice were positive for the *E. coli uidA* structural gene. The molecular analysis using *uidA*-specific primer confirmed that a genetic region homologous in size to the *E. coli uidA* structural gene, including the regulatory region, was present in 3 of the *E. coli* isolates from Alice, 10 from Dimbaza and 8 from East London. Of the 3 *E. coli* isolates, 1 (33.3%) was positive for the $fliC_{H7}$ genes and 3 was positive for $rfbE_{O157}$ genes. Out of the 10 isolates from Dimbaza, 4 were positive for $fliC_{H7}$ genes, 6 were for the $rfbE_{O157}$ genes and 1 was positive for the *aap* genes; and of the 8 isolates from East London, 1 was positive for $fliC_{H7}$ genes, 2 were for the $rfbE_{O157}$ genes, 6 were positive for the *ial* genes. *Escherichia coli* isolates from Dimbaza (10) were confirmed using the specific primers targeting the *uidA* structural gene. The entire target genes of *E. coli* O157 ($fliC_{H7}$, $rfbE_{O157}$) were noticed in the isolates obtained from all the final effluent samples. Representative gel electrophoresis profiles of amplified products of target genes for pathogenic *E. coli* strains are illustrated in Figures 5.1 to 5.5.

Table 5.4: Occurrence of pathogenic *E. coli* isolates from the wastewater treatment plants as indicated by presence of the target gene marker.

Location	**Amplified genes**				
	***fliC*$_{H7}$**	***rfbE*$_{O157}$**	***aap***	***ial***	***uidA***
Alice	+ (1)	+ (3)	-	-	+ (3)
Dimbaza	+ (4)	+ (6)	+ (1)	-	+ (10)
East London	+ (1)	+ (2)	-	+ (6)	+ (8)

"+" target gene present, "-" target gene absent. n = 28 Representative number of presumptive *E. coli* characterized by PCR. In parenthesis are numbers of isolates carrying the gene markers.

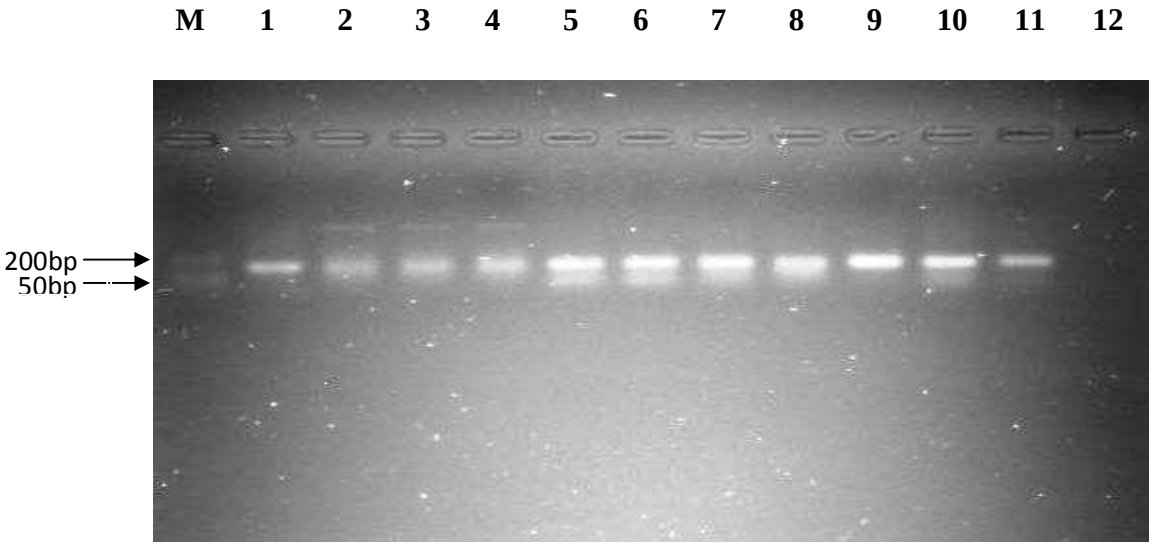

Figure 5.1: The amplification product for the *uidA* gene. Lane M: Ultra Low Range DNA Ladder (Fermentas Life Sciences, SA); Lane 1 PCR positive control (*Escherichia coli* ATCC 8739 - SABS No ESC 20); Lane 2 to Lane 4 (Samples from Alice), Lane 5 to Lane 8 (Samples from Dimbaza), Lane 9 to Lane 11 (Samples from East London), Lane 12 negative control. The expected molecular size of *uidA* fragments was 147 bp.

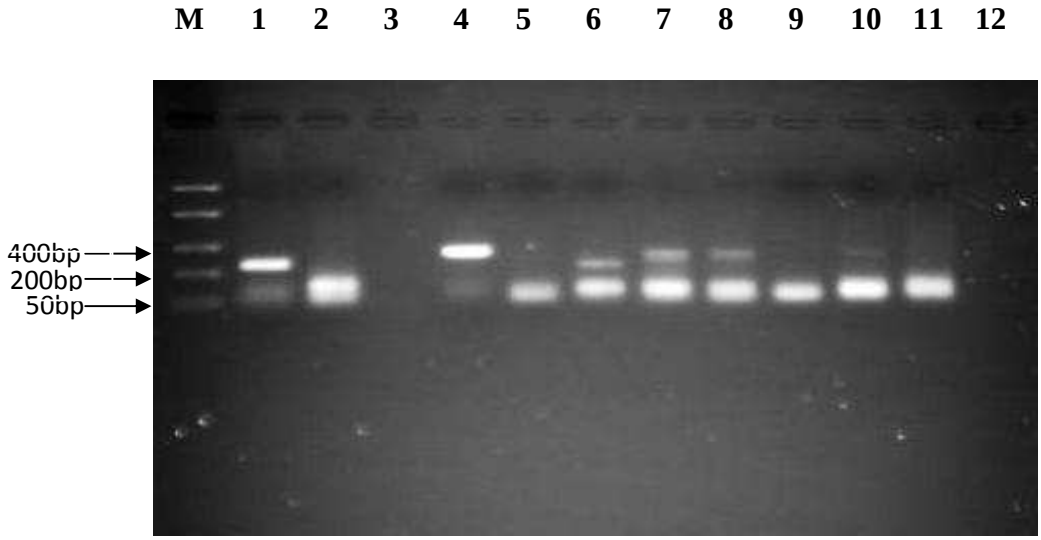

Figure 5.2: The amplification product from the oligonucleotide primer pair for the *fliC*$_{H7}$ gene. Lane M: Low Range DNA Ladder (Fermentas Life Sciences, SA); Lane 1 PCR positive control (Enterohaemorrhagic *Escherichia coli* NSCC ESCCO 06), Lane 2 to Lane 6 (samples from Dimbaza) Lane 7 to Lane 11 (samples from East London), Lane 12 negative control. The expected molecular size of *fliC*$_{H7}$ fragments was 247 bp.

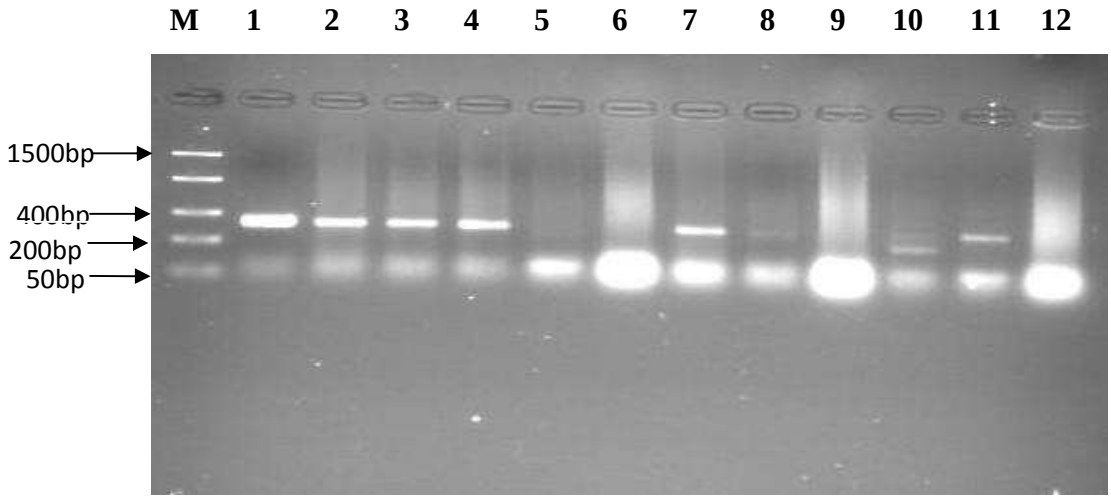

Figure 5.3: The amplification product from the oligonucleotide primer pair for the *rfbE*$_{O157}$ gene. Lane M: Low Range DNA Ladder (Fermentas Life Sciences, SA); Lane 1 PCR positive control (Enterohaemorrhagic *Escherichia coli* NSCC ESCCO 06), Lane 2 to Lane 4 (samples from Alice), Lane 5 to Lane 11 (samples from Dimbaza), Lane 12 negative control. The expected molecular size of *rfbE*$_{O157}$ fragments was 328 bp.

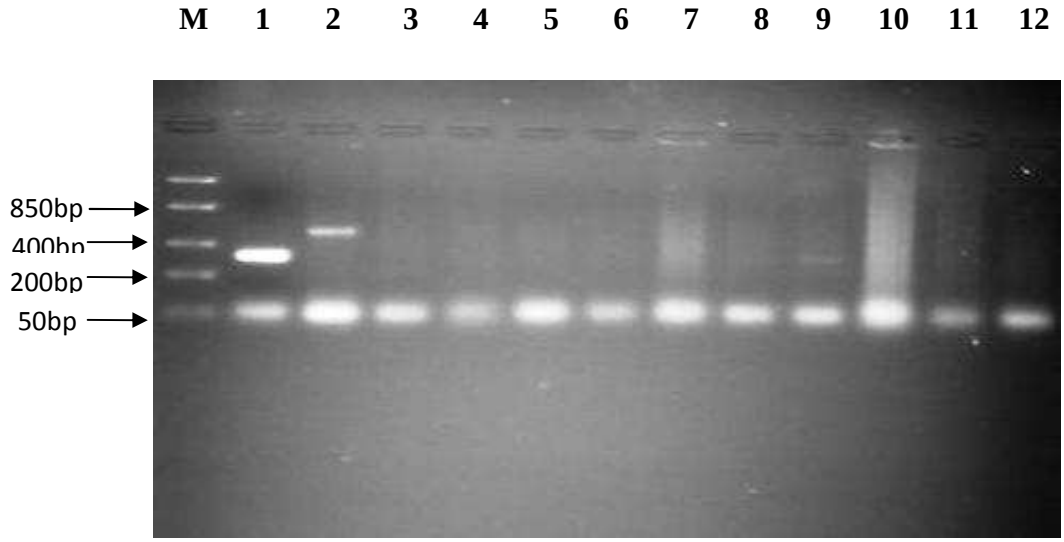

Figure 5.4: The amplification product from the oligonucleotide primer pair for the *aap* gene. Lane M: Low Range DNA Ladder (Fermentas Life Sciences, SA); Lane 1 PCR positive control (Enteroaggregative *Escherichia coli* NSCC ESCCO 14), Lane 2 to Lane 10 (samples from Dimbaza), Lane 11 (sample from East London), Lane 12 negative control. The expected molecular size of *aap* fragments was 232 bp.

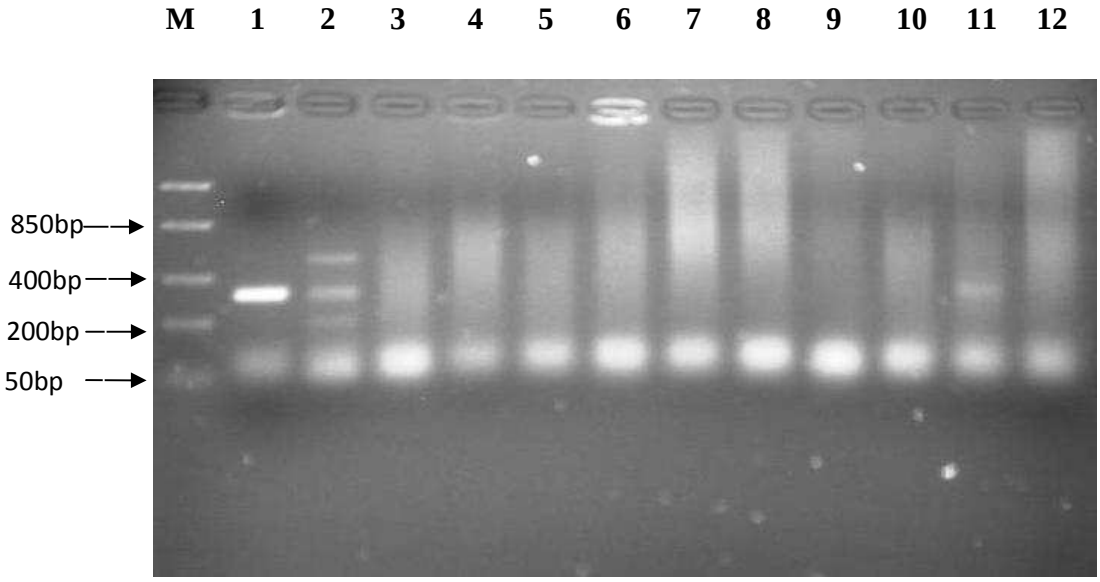

Figure 5.5: The amplification product from the oligonucleotide primer pair for the *ial* gene. Lane M: Low Range DNA Ladder (Fermentas Life Sciences, SA); Lane 1 PCR positive control (Enteroinvasive *Escherichia coli* NSCC ESCCO 03), Lane 2 to Lane 11 (samples from East London), Lane 12 negative control. The expected molecular size of *ial* fragments was 320 bp.

5.4 Discussion and conclusion

This study revealed that the final effluent outfalls in October, November and December 2007 and July 2008 only complied with the South African General and Special Standards which stipulate that treated sewage effluents must have a standard of nil faecal coliforms/100 ml (Act 96 of 18 May 1984 No. 9225, Regulation 991), and according to (DWAF, 1998) the maximum limit for no risk of faecal coliforms is 0 cfu/100 ml. The standard of The World Health Organization for drinking water is no *E. coli* in a 100 ml sample (WHO, 2003). However during the study period, the *E. coli* counts were above the recommended limit indicating poor microbiological quality of the effluent and its unfitness for human consumption without prior treatment. High *E. coli* counts suggest inefficiency of the wastewater treatment plants in removing the bacteria. Crops such as vegetables can become contaminated with human and animal pathogens when irrigated with water containing this organism (DWAF, 1996). This study revealed important findings on the prevalence of pathogenic *E. coli* strains in the final effluent discharge to the communities of Alice, Dimbaza and East London. The presence of virulent *Escherichia coli* strains in the effluents is a cause for concern as most communities in the region rely on surface waters for drinking, recreational, domestic and irrigation purposes.

The results of this study confirm the poor microbiological quality of the effluents that is produced by many of the wastewater treatment plants in the Eastern Cape Province. The PCR assays successfully amplified the target genes (*fliC*$_{H7}$, *rfbE*$_{O157}$) which are characteristic of the Enterohaemorrhagic *Escherichia coli* O157:H7; (*ial*) characteristic of the Enteroinvasive *Escherichia coli*; and (*aap*) characteristic of the Enteroaggregative *Escherichia coli*. *Escherichia coli* O157:H7 has been associated with water related outbreaks. It has been isolated from surface and ground waters (Hamner *et al*., 2007; Gagliardi and Karns, 2000), and it is capable of survival

in water for days or weeks (Nwachukwu and Gerba, 2008). Enteroinvasive *Escherichia coli* (EIEC) cause dysentery; however, it is less widely reported than other etiological agents in studies of diarrhea worldwide. EIEC has principally been associated with contaminated food and water (Gordillo *et al*., 1992; Marier, *et al*., 1973) although cases of person-to-person transmission of EIEC have been noted (Harris *et al*., 1985). Enteroaggregative *Escherichia coli* is an emerging diarrheagenic pathogen associated with diarrheal illnesses among patients in developed and developing countries. Enteroaggregative *Escherichia coli* has been increasingly isolated and characterized around the world from human clinical, animal, and environmental samples (Kahali *et al*., 2004; Falcao *et al*., 2004; Yamamoto and Nakazawa, 1997). However, frequencies of Enteroaggregative *Escherichia coli* among patients with diarrhea in the Eastern Cape Province, South Africa, are not known. The outcome of the findings of Samie and co-workers (2007) highlights the need for the design of surveillance strategies to control Enteroaggregative *Escherichia coli* diarrhea and to prevent the transmission of Enteroaggregative *Escherichia coli* in the region through water or food contamination. Considering the microbiological quality of the effluent outfalls in the Eastern Cape Province in general and the Buffalo City and Nkonkobe Districts in particular and the profile of the molecular results of the present study, monitoring and strict compliance with effluent standard should be re-enforced.

The antibiotic resistance patterns of the isolates in this study corroborate results from previous investigations (Olaniran *et al*., 2009; Obi *et al*., 2004). Most of the strains were resistant to linezolid, polymyxin B, penicillin G and sulfamethoxazole. Tetracycline has sequentially been replaced by trimethoprim-sulfamethoxazole and, more recently, quinolones, because of the emergence and spread of resistant strains. *Escherichia coli* is the most frequently isolated etiological agent of urinary tract infections (UTIs), and trimethoprim-sulfamethoxazole (TMP-

SMZ) is one of the primary antibiotics empirically prescribed for the treatment of community-acquired UTIs (Karlowsky *et al*., 2002). In the United States, there has been a notable increase in the isolation of uropathogenic *E. coli* strains resistant to TMP-SMZ (Karlowsky *et al*., 2002).

Travelers to developing areas are often concerned with the development of traveler's diarrhea and may seek a means of preventing it. Doxycycline and trimethoprim-sulfamethoxazole have been shown to be effective in this regard, although increasing resistance would suggest that fluoroquinolones administered once daily would be more effective (Heck *et al*., 1994). However, the growing problem of antibiotic resistance and the possibility of adverse effects from antimicrobial agents weigh strongly against recommending antimicrobial prophylaxis routinely. Rather, experts have recommended (i) avoiding potentially contaminated food and drink while traveling, (ii) bismuth subsalicylate given four times daily, and (iii) the use of antibiotics empirically if significant diarrhea develops (DuPont and Ericsson, 1993).

The adequate long-term protection of South Africa's water sources is of vital importance for sustained economic growth and development. It is thus critical that no more watersheds be destroyed and that the existing ones are protected particularly in the Buffalo City and Nkonkobe communities and in the Eastern Cape in general. If wastewater effluent quality is not maintained, it is not only the environment that will suffer the commercial and recreational values of surface water resources will also diminish. The personnel's in all the wastewater treatment plants should be armed with skills that will aid improved running of the facilities, maintaining and thereby uplifting the standard of the effluent discharge into the Eastern Cape community as well as protecting it against waterborne diseases.

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CHAPTER 6

GENERAL DISCUSSION

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GENERAL DISCUSSIONS

The activated sludge process is the most widely used biological wastewater treatment for both domestic and industrial plants in the world, and it has been in use in many sewage treatment plants around the world (Chen *et al.,* 2004) and one of the most used in South Africa (Samie *et al.,* 2008). In the activated sludge process, a bacterial biomass suspension (the activated sludge) is responsible for the removal of pollutants. Depending on the design and the specific application, an activated sludge wastewater treatment plant can achieve biological nitrogen (N) and phosphorus (P) removal, besides removal of organic carbon substances (Gernaey *et al.,* 2004). The process considerably reduces the pathogen concentration of the effluent. Fecal coliform bacterial (FEB) populations along with other pathogens are destroyed in significant numbers in the course of wastewater treatment. Such removal of FEB has been distinguished from combined physical, chemical and biological processes and from purposeful disinfection process such as chlorination and UV irradiation in a wastewater treatment plant. Some studies show that overall removal efficiency for FEB in the municipal wastewater treatment plants may vary in the range of 90%–99% (Koivunen *et al.,,* 2003; Rose *et al.,,* 1996; James, 1985; Miescer and Cabelli, 1982). Even though high removal efficiency may be achieved for FEB in a wastewater treatment plant, high concentrations of FEB are still discharged in treated effluents to receiving water bodies due to abundance in raw influents.

The quality of wastewater discharges has traditionally been controlled by the use of physical and chemical parameters (Harty, 2001). It is important that wastewater discharge is characterized so that its impact on the receiving environment is assessed and to assist in the identification of priority actions to eliminate or reduce hazardous substances. When wastewater

has been characterized license conditions can be drafted to protect the aquatic environment which receives treated wastewater (Environmental Protection Agency, 1999).

In this study, the quality of the final effluents of some wastewater treatment facilities in the Eastern Cape Province, South Africa were noted to be acceptable with respect to the pH, temperature, dissolved oxygen, salinity, total dissolved solids and nitrite concentration. pH is an indicator of the acidity or basicity of water but is seldom a problem by itself. The normal pH range for irrigation water is from 6.5-8.4; pH values outside this range are a good warning that the water is abnormal in quality. The pH of most mineral waters is 6-9. The pH remains reasonably constant unless the water quality changes due to natural or anthropogenic influences, adding acidity or basicity. As most ecological life forms are sensitive to pH it is important that the anthropogenic impact of the effluent discharged be minimized (Kiely, 1997).

Water temperature is a fundamental and important water quality variable; for example, most chemical and biological activities are a function of temperature, and fish habitat in particular is sensitive to water temperature. Wastewater from municipal treatment plants is probably the largest anthropogenic heat source for surface water bodies. Its effect on stream temperature depends on the temperature and volume of wastewater added to the stream (Kinouchi *et al.,* 2007). In this study, the quality of the final effluent was acceptable with respect to temperature. The results of the dissolved oxygen levels from this study indicate the final effluent could support the oxygen requirement of the aquatic organisms (Belmont *et al*., 2004).

Solids concentration is another important characteristic of wastewater (Lee and Lin, 1999). The solids may in fact consist of algal growths and hence be indicative of severely eutrophic conditions; they will reduce light penetration in surface waters and interfere with aquatic plant life; they will seriously damage fishery waters and may affect fish life; they may

form deposits on the bed of rivers and lakes which in turn give rise to septic and offensive conditions; and they may indicate the presence of unsatisfactory sewage effluent discharges. In this study, the quality of the final effluent was acceptable with respect to the total dissolved solids concentration.

Increased nutrient loading can lead to eutrophication (Gücker *et al.,* 2006) and temporary oxygen deficits (Rueda *et al.,* 2002). Increased organic matter can alter energy relationships in the stream, disrupting biotic community structure and function (Spänhoff *et al.,* 2007; Gücker *et al.,* 2006; Dyer *et al.,* 2003). In this study, nutrient concentration particularly phosphate and nitrate concentrations of the final effluents were outside the acceptable limits which could contribute to eutrophication of the receiving watershed and if not controlled result in other serious pollution problems. The effects of polluted water on human health, the aquatic ecosystem and on various sectors of the economy, including agriculture, industry and recreation, can be disastrous. Deteriorating water quality leads to increased treatment costs of potable and industrial process water, and decreased agricultural yields. This is worthy to note as wastewater accounts for 2–10% of the total water production in water treatment plants (Le Gouellec *et al.,* 2004; Nasser *et al.,* 2002; Cornwell and MacPhee, 2001; Dotremont *et al.,* 1999; Vigneswaran *et al.,* 1996). The turbidity, electrical conductivity and chemical oxygen demand did not comply with regulated standards.

Contamination of natural aquatic ecosystems by wastewater is a major human health and environmental issue. Contaminated water from wastewater treatment plants and or animal operations has been implicated in disease outbreaks (e.g., DPLG 2001; MacKenzie *et al.,* 1994, Drenchen and Bert, 1994). In South Africa, studies have shown the outbreak of diarrhea originated in the town's water supply, suspected to have been contaminated with human faeces

(Osode 2006; DPLG 2001). Certain types of *Escherichia coli* are among the pathogens commonly found in wastewater effluents which can cause gastrointestinal illness. For example, enterotoxigenic *E. coli* causes diarrhea in children in developing countries and in travelers to those countries, whereas enterohemorrhagic *E. coli* (EHEC) may cause bloody diarrhea and hemorrhagic colitis as well as hemolytic uremic syndrome (HUS) because of the production of Shiga toxins (STx). Enteropathogenic *E. coli* (EPEC) shares several key virulence determinants with the most common varieties of EHEC but does not produce Shiga toxins (STx) nor cause hemorrhagic colitis or hemolytic uremic syndrome. Instead, it causes nonspecific gastroenteritis, especially in children in developing countries (Trabulsi *et al.,* 2002; Robins-Browne, 1987). In several reports, from countries as diverse as Iran, Norway, Peru, Poland, South Africa, the United States, and the United Kingdom (Afset *et al.,* 2003; Paciorek, 2002; Galane and Le Roux, 2001; Knutton *et al.,* 2001; Bouzari *et al.,* 2000; Bokete *et al.,* 1997; Nataro *et al.,* 1985), as well as Australia (Robins-Browne *et al.,* 2004; Kukuruzovic *et al.,* 2002), atypical EPEC strains have been identified in children with acute diarrheal disease.

The current study investigated the prevalence of pathogenic *E. coli* strains in the final effluents. The high levels of presumptive *E. coli* corroborate the findings of other studies (Samie *et al.,* 2007). This study revealed that the final effluents discharged from all the wastewater treatment plants were above the recommended limit of the South African General and Special Standards which stipulate that treated sewage effluents must have a standard of 0 faecal coliforms/100 ml (Act 96 of 18 May 1984 No. 9225, Regulation 991), and according to (DWAF, 1998) the maximum limit for no risk of faecal coliforms is 0 cfu/100 ml. This indicates poor microbiological quality of the effluent and its unfitness for human consumption without prior treatment

The findings of this study revealed that the Alice wastewater treatment plant located in the rural area was found the most efficient as it produced the final effluent with the least pathogenic *E. coli* followed by Dimbaza wastewater treatment plant which is located in the peri-urban area of the Eastern Cape Province of South Africa. This led to the conclusion that although urban wastewater treatment plants may be better equipped than those in rural areas, rural wastewater treatment plants can achieve relatively good microbiological qualities of effluents by taking good care of the resources available.

To date, chlorination is the most widely used means to inactivate pathogenic micro-organisms in water and wastewater and is the principal method for preventing waterborne infectious diseases throughout the world. However, several studies have reported that the effectiveness of the process is reduced by turbidity, suspended solids and the presence of nitrogen compounds such as ammonia and nitrite (Lazarova *et al.,* 1999). Furthermore, the use of chlorine in wastewater gives rise to undesirable by-products suspected to pose a hazard to humans and the environment (Minear and Amy, 1996). In view of these problems, alternative technologies have been developed in recent years such as ozonization, Ultra Violet (UV) disinfection, and paracetic acid (Liberti *et al.,* 2000). Membrane technologies offer an alternative to the disinfection process. Disinfection by reagent or by UV irradiation requires a pre-treatment stage to remove suspended solids or reduce turbidity (Lazarova *et al.,* 1999). Similarly, membrane technologies require suitable pretreatment in order to maintain membrane efficiency by preventing fouling and module damage (Marcucci *et al.,* 2002). Traditionally, the most frequently applied pre-treatment for tertiary treatment has consisted of macrofiltration processes (Mujeriego and Asano, 1999), which have proven effective in reducing certain pathogen groups

(Gómez *et al.,* 2003). The study revealed that the free chlorine residual concentration in the effluents complied with the regulatory standard in about 33.3% of the study period.

The emergence and spread of antibiotic resistance in bacteria is a major public health issue (Levy, 2002). Previous studies have correlated an increased incidence of antibiotic resistance among culturable bacteria in surface water (Reinthaler *et al.,* 2003; Harwood *et al.,* 2000) and groundwater (McKeon *et al.,* 1995), with wastewater treatment plant effluent discharge. While much effort has been directed toward management and monitoring of antibiotic use and the prevalence of bacterial resistance within communities, bacterial resistance to antibiotics in the aquatic environment has received comparatively little attention. Bacterial contamination of surface waters, particularly contamination with fecally derived bacteria, has long been a water quality issue due to the potential for disease transmission. Because of this and the potential for antibiotic resistance, there is a new level of risk associated with these bacteria. Recent studies have also identified antibiotics themselves in surface waters (Batt *et al.,* 2006; Costanzo *et al.,* 2005; Calamari *et al.,* 2003; Kolpin *et al.,* 2002), and the role of these antibiotics in the development, transfer, and maintenance of resistance is largely unknown. In a limited number of studies workers have identified antibiotic-resistant bacteria in the aquatic environment. In a study of 16 United States Rivers, antibiotic-resistant bacteria were found to be widespread, and the resistance included resistance to chemically modified and synthesized antibiotics (Ash *et al.,* 2002). Parveen and coworkers (1997) showed that the frequency of antibiotic-resistant and multiple-antibiotic-resistant *Escherichia coli* isolates was higher close to point source discharges.

Resistance is common where antibiotics are heavily used, and additionally antibiotic resistant bacteria are present in wastewater, surface water, ground water, sediments and soils, and increasingly in aquatic environments (Zhang *et al.,* 2009; Baquero *et al.,* 2008; Martinez, 2008; Kummerer, 2004; Klare *et al.,* 1995). Antibiotic use selects for existing resistance mechanisms and for novel resistance mutations (Bywater, 2004, 2005; Finch, 2004; Wassenaar, 2005). Resistance can also be acquired through horizontal gene transfer via uptake of resistance determinants via conjugation, transduction and transformation (Davison, 1999; Lorenz and Wackernagel, 1994; Thomas and Nielsen, 2005). Wastewater treatment plants are important reservoirs of commensal human and animal bacteria in which antibiotic resistant organisms, and/or, determinants persist in the final effluent and are released to the environment (Davison, 1999; Lorenz and Wackernagel, 1994; Thomas and Nielsen, 2005). By linking different environmental components including municipal sewage and surface water, wastewater treatment plants may facilitate spread of antibiotics, antibiotic resistance genes, and antibiotic resistant bacteria between these compartments (Schluter *et al.,* 2007). Furthermore, the high microbial density and diversity of biofilms and activated sludge may facilitate genetic exchange in wastewater treatment plants (Schluter *et al.,* 2007) and antimicrobial agents were found present in wastewater (Kummerer, 2003). These conditions may lead to selection of antibiotic resistant bacterial populations in wastewater treatment plants.

The antibiotic resistance patterns of the isolates in this study corroborate results from previous investigations (Obi *et al.,* 2004; Olaniran *et al.,* 2009). Most of the strains were resistant to linezolid, polymxin B, penicillin G and sulfamethoxazole. Tetracycline has sequentially been replaced by trimethoprim-sulfamethoxazole and, more recently, quinolones, because of the emergence and spread of resistant strains. *Escherichia coli* is the most frequently isolated

etiological agent of urinary tract infections (UTIs), and trimethoprim-sulfamethoxazole (TMP-SMZ) is one of the primary antibiotics empirically prescribed for the treatment of community-acquired UTIs (Karlowsky *et al.,* 2002). In the United States, there has been a notable increase in the isolation of uropathogenic *E. coli* strains resistant to TMP-SMZ (Karlowsky *et al.,* 2002).

PCR amplification using *uidA* specific primers confirmed that a genetic region homologous in size to *uidA* structural genes of *Escherichia coli* was present in the *E. coli* isolated from the effluents. An explanation for the extra bands in three lanes is that the primers are hybridized to secondary sites of template and as a result of miss priming (Titus, 1991). The PCR amplification using $fliC_{H7}$ and $rfbE_{O157}$ confirmed that a genetic region homologous in size to $fliC_{H7}$ and $rfbE_{O157}$ structural genes of Enterohemorrhagic *E. coli* somatic antigen was present in the *E. coli* isolated from the effluents. This was equally observed for the PCR amplification using *aap* and confirmed that a genetic region homologous in size to *aap* structural genes of Enteroaggregative *E. coli* somatic antigen was present in the *E. coli* isolated from the effluents. Similar observation was noted for the PCR amplification using *ial* and confirmed that a genetic region homologous in size to *ial* structural genes of Enteroinvasive *E. coli* somatic antigen was present in the *E. coli* isolated from the effluents.

The broad aim of this study was to evaluate the hypothesis that virulent pathogenic *Escherichia coli* strains very easily survive the treatment processes of the activated sludge system of wastewater treatment facilities in the Eastern Cape Province either as free cells or as plankton associated entities and secondly that these wastewater treatment facilities are veritable sources of pathogenic *E. coil* and abiotic pollutants in the receiving watershed.

In summary this chapter presents a series of concluding remarks regarding the set objectives. The prevalence and distribution of virulent *E. coli* strains was ascertained. The occurrence of the virulent *E. coli* strains as free and plankton associated cells in the final effluents was investigated. The antibiotic susceptibility profiles of the isolated *E. coli* strains were elucidated. Lastly a comparison of the data from urban, semi-urban and rural settings in relation to the microbiological quality of the effluent was accomplished.

6.2 CONCLUSION

Assessing the effects of effluent discharge on the health of receiving waterways is of considerable environmental consequence, especially in catchments with variable stream flows and where population pressure through urbanization and peri-urbanization is placing increasing pressure upon wastewater treatment plant infrastructure and the health of freshwater ecosystems (Daniel *et al.,* 2002; Singer and Battin 2007; Taylor *et al.,* 2002). South Africa and the Eastern Cape Province in particular need to address the impact of wastewater effluent on its water resource to enhance the wellbeing of the populace and especially those in the rural communities of Buffalo City and Nkonkobe.

Recommendations:

- We suggest that to maximize public health protection, wastewater treatment system effluent quality should be monitored diligently pursuant to ensuring high quality of final effluents.

- Wastewater treatment plant problems should not be allowed to continue after they have been detected. A deterioration of treated water quality at the treatment plant should be cause for immediate, careful, and informed remedial measures.
- Design engineers can enhance the ability of plant operators to cope with difficult treatment situations by providing for operational flexibility and comprehensive plant performance monitoring capability at wastewater treatment plants.
- Due to the heightened risk involved in times of changing source water or changing treated water quality, wastewater treatment plant operators should be particularly diligent to detect deviations from normal operating conditions. A deterioration of source water quality should signal the need for special care in treatment plant operations.
- To ensure compliance with future discharge requirements, upgrading of the existing wastewater treatment facilities and implementation of new technologies is envisaged as the next step in improvement of wastewater treatment.
- The wastewater treatment plant staff needs to be aware of sources of contamination in the watershed. Examples include wastewater treatment plant outfalls, combined sewer overflow points, sanitary sewer overflows, and farm animal waste sources.
- Proper technical training for wastewater treatment operators will be needed to enhance competence in proper water sampling and wastewater treatment facility operations.

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