



University of Fort Hare
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**EVALUATION OF SOME WASTEWATER TREATMENT FACILITIES IN CHRIS
HANI AND AMATHOLE DISTRICT MUNICIPALITIES AS POTENTIAL SOURCES
OF *ESCHERICHIA COLI* IN THE ENVIRONMENT**

SINAZO NOMATHAMSANQA MAZWI

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Department of Biochemistry and Microbiology
Faculty of Science and Agriculture
University of Fort Hare, Alice, 5700, South Africa

SUPERVISOR: PROF.A.I OKOH

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DECLARATION

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I would like to thank God Almighty for giving me strength throughout this journey, without Him I would not have made it this far.” Through him all things are possible”

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DEDICATION

This Dissertation is dedicated to:

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You guys are my pillar of strength, I love you so much

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LIST OF ABBREVIATIONS

ADS	Application Data Sheet
AEMREG	Applied & Environmental Microbiology Research Group
BOD	Biological Oxygen Demand
CCME	Canadian Council of Minister of the Environmental
CDC	Centre of Diseases Control and Prevention
CFU	Colony Forming Unit
CLIS	Clinical and Laboratory Standards Institute
COD	Chemical Oxygen Demand
CSIR	Council of Scientific and Industrial Research
DEC	Diarrhoeagenic <i>Escherichia coli</i>
DAEC	Diffusely Adherent <i>E. coli</i>
DNA	Deoxyribose Nucleic Acid
DWAF	Department of Water Affair and Forestry
EAEC	Enterotoaggregative <i>E. coli</i>
EHEC	Enterohemorrhagic <i>E. coli</i>

EIEC	Enteroinvasive <i>E. coli</i>
ELISA	Enzyme-linked Immunosorbent Assay
EPEC	Enteropathogenic <i>E. coli</i>
ETEC	Enterotoxigenic <i>E. coli</i>
ExPEC	Extraintestinal Pathogenic <i>E. coli</i>
FAO	Food and Agricultural Organisation
HIV	Human Immunodeficiency Virus
HUS	Haemolytic Uremic Syndrome
JMP	Joint Monitoring Programme
LT	Heat-Labile Toxin
MARI	Multiple Antibiotic Resistance Index
MARP	Multiple Antibiotic Resistance Phenotype
MF	Membrane Filtration
NMEC	Neonatal Meningitis <i>E. coli</i>
PCR	Polymerase Chain Reaction
ST	Heat-Stable Toxin
TOC	Total Organic Carbon

UN	United Nations
UNICEF	United Nation Children’s Fund
USEPA	United States Environmental Protection Agency
UTI	Urinary Tract Infection
VTEC	Verotoxin-producing <i>E. coli</i>
WHO	World Health Organisation
WWTP	Wastewater Treatment Plant

ABSTRACT

Access to clean and safe water is essential for the survival of human beings. Pollution of freshwater sources constitutes a major problem hindering access to safe water for drinking and other domestic uses. Wastewater effluent discharges often impact the microbiological qualities of surface waters with its attendant health and environmental problems. This study evaluated the microbiological qualities of the discharged effluents of four selected wastewater treatment plants in Amathole and Chris Hani District Municipalities of the Eastern Cape Province over a twelve-month sampling period. Microbiological analysis (faecal coliform, *Escherichia coli* and *Escherichia coli* O157:H7) was done using standard methods and polymerase chain reaction method was used to confirm identities of bacterial isolates. Presumptive bacteria counts ranged as follows: faecal coliforms 0 to 1.6×10^3 CFU/100 ml, *E. coli* 0 to 1.4×10^3 CFU/100 ml and *E. coli* O157:H7 0 to 9.6×10^2 CFU/100 ml. Forty eight percent (305/626) of the presumptive *E. coli* isolates were confirmed using species-specific *uidA* gene which code for β -glucuronidase enzyme in *E. coli*. Antibiotic susceptibility profile of the isolate using a panel of 10 antibiotics shows 100% (150/150) resistance to antibiotics rifampicin and penicillin G while 49.3% (74/150) of the isolates and 46.7% (70/150) were susceptible to streptomycin and cefotaxime respectively. Multiple antibiotic resistance phenotypes (MARP) of the isolates showed resistance to two or more test antibiotics while the calculated multiple antibiotic resistance index (MARI) for the tested isolated is 0.49. The detection of potentially pathogenic *E. coli* in the final effluents suggests potential danger to the receiving water bodies where the effluents are discharge. The high MARI valued obtained in this study indicates that the isolates are form environment where

the tested antibiotics are being used and may further lead to the spread of multiple antibiotics resistance among other pathogens that may be present in the same environment.

CHAPTER ONE

INTRODUCTION

1.1 General Introduction

Water is essential for the survival of all life forms and nothing can exist on earth without it (Agrawal *et al.*, 2010). Water also forms the backbone of the world's economy and is essential for living systems, industrial processes, agricultural production, and domestic uses (World Water Day, 2007). Fresh-water make up only about 0.01% of the global water and approximately 0.8 % of the earth's surface. This tiny portion of global water supports at least 100 000 species out of approximately 1.8 million, which is almost 6% of all described species (Dudgeon *et al.*, 2006). Inland waters and freshwater biodiversity constitute an inestimable natural resource, in economic, cultural, aesthetic, scientific and educational terms, and their conservation and management are critical to the interests of all humans, nations and governments (Strayer and Dudgeon, 2010). Yet, this precious heritage is in crisis (Dudgeon *et al.*, 2006) due to uncontrolled exploitation and dumping of hazardous wastes like acid mine drainage and partially treated or raw wastewater effluents leading to pollution. Water pollution may be described as any form of impairment in water's natural characteristics through the addition of anthropogenic pollutants to such levels that it either cannot serve humans usage and/or affect aquatic biotic communities, such as fish and other aquatic life (Agrawal *et al.*, 2010). Water pollution is a major cause of global concern as it leads to the outbreak of numerous fatal waterborne diseases (Daniel, 2006).

In most countries of the world the major public health risks associated with consumption of polluted water are microbiological in nature. However, the importance of chemical pollutants

cannot be underestimated. An estimated 80% of all diseases and over one-third of deaths in developed countries results from the consumption of contaminated water and on average as much as one-tenth of each person's productive time is lost to water-related diseases (WHO, 1997).

South Africa is among the driest countries on earth and it has been predicted that, due to massive worldwide increases in the human population, water will become one of the scarcest resources in the 21st century (Muller *et al.*, 2009). As human numbers increase, greater strains will be placed on available resources and pose even greater threat to environmental sources.

Pollution of freshwater can occur from either point or nonpoint sources (La Bella, 2009). Point source pollution refers to the contamination that occurs in a waterway from a single, identifiable source, such as a pipe, ditch or pollution from discharged effluents while non-point source pollution refers to diffuse contamination that does not come from a single discrete source e.g. runoff from rain and melting ice (La Bella, 2009; Mane *et al.*, 2013).

Municipal wastewater remains a concern because of its constituents and the amount of it that is discharged (Mara and Horan, 2003). Municipal wastewater is a mixture of human excreta (sewage), grit, suspended solids, debris, pathogens such as bacteria and viruses; decaying organic waste which reduces the amount of oxygen available in a water body; nutrients such as nitrogen and phosphorus and a variety of chemicals that originate from residential, commercial and industrial activities (Tchobanoglous *et al.*, 2003; Argaw, 2004; CCME, 2006). It therefore remains that municipal wastewater must undergo treatment before its effluents are disposed of into the environment. One of the priorities in the treatment of wastewater is the removal of pathogenic microorganisms in order to comply with the required discharge standards for the

treated effluent and thus protects the receiving water-bodies from such pathogens (Osode and Okoh, 2009). In general, the proper implementation of this management strategy results in the protection of the quality of water sources, reduction of the cost of drinking water treatment, and the control or prevention of waterborne disease (Bekal *et al.*, 2003).

In spite of improved access to better sanitation systems, most of the wastewaters collected through the sewer systems do not undergo proper treatment processes, and significant amounts of faecal pollution indicators and pathogenic microorganisms are released into receiving watershed, causing negative alteration in the quality of various water resources (Bahlaoui *et al.*, 1997; Momba and Mfenyana, 2005). Discharged wastewater effluents remain a major source of pathogenic bacteria and viruses in the natural environments as they carry lots of faecal matter especially that of human and animal origin. Assessment of water and wastewater is therefore very crucial to safeguard public health and the environment.

Some studies have reported the poor operational state and inadequate maintenance of most municipalities' sewage treatment works in South Africa leading to the pollution of various water bodies thereby posing very serious health and socio-economic threats to the dependants on such water bodies (Momba *et al.*, 2006; Okoh *et al.*, 2005; 2007). When wastewater treatment systems are not working efficiently, sewage discharges contribute to oxygen demand and nutrient loading of the water bodies, promoting toxic algal blooms (eutrophication) and leading to a destabilised aquatic ecosystem (Ogunfowokan *et al.*, 2005).

Bacteria are the most common of microbial pathogens found in wastewater. A wide range of bacterial pathogens and opportunistic pathogens associated with wastewater are enteric in origin and have been reported in literature (Simpson and Charles, 2000). Gastrointestinal

infections are amongst the most common diseases caused by bacterial pathogens in wastewater (LeChevallier and Au, 2004). Waste-water associated infections generally include diarrhoea, dysentery, dysentery-like infections, *Leptospira interrogans* infections, typhoid, human enteritis, legionellosis, melioidosis, stomach ulcer and cancer (Liang *et al.*, 2006).

Escherichia coli was first described by Theodor Escheria a German paediatrician in 1885 as a *Bacterium coli commune*, which he isolated from the faeces of human and neonates (Todar, 2008). *Escherichia coli* are member of the Enterobacteriaceae and belong to the order Eubacteria, (Berg, 1978; Leclerc *et al.*, 2001). These bacteria are Gram negative, rod shaped facultative anaerobes that can grow under both aerobic and anaerobic respiration (Chapelle, 2001). In cases where molecular oxygen is present, the bacteria rely on respiratory metabolism to survive, while in the absence of molecular oxygen; the organisms use fermentation as an alternate means of survival (Berg, 2000; Chapelle, 2001).

The bacterium has been recognised as an important cause of food and water-related diseases since its discovery. Strains of *E. coli* were shown to be the causative agent in outbreaks of diarrhoea in infants, although for many years it was considered simple commensal bacteria of the large intestines (Todar, 2007). *Escherichia coli* is now used as an indicator of faecal pollution that originates from human and warm-blooded animals; this was based on the ground that *E. coli* is found in large quantities in human and animal faeces at a concentration of roughly 10^9 colony forming units per gram of faeces (Omar and Barnard, 2010). The bacterium belongs to the coliform groups, which are a common part of the normal facultative anaerobic microflora of the intestinal tracts of most mammals, including humans (Todar, 2008). Coliforms include all the aerobic and facultative anaerobic, Gram-negative, non-spore forming, rod-shaped bacteria that ferment lactose with gas formation within 48 hours at 35°C (Edberg *et al.*, 2000).

Most *E. coli* are non-pathogenic in humans and other warm-blooded animals. Nevertheless, certain pathovars can cause health problems when they come in contact with humans. Pathogenic *E. coli* are aetiological agents of three types of infections in human's urinary tract infections (UTI), neonatal meningitis, and gastroenteritis (Bekal *et al.*, 2003; Elizaquivel *et al.*, 2011). Some virulence factors involved in pathogenic mechanisms of *E. coli* include adhesins, host cell surface-modifying factors, invasins, toxins, and secretion systems (Bekal *et al.*, 2003).

Escherichia coli is the most common bacterial pathogen associated with endemic forms of childhood diarrhoea in developing countries (Okeke *et al.*, 2000) including South Africa, especially in regions with poor sanitation. Based on the occurrence of different chromosomal or plasmid - encoded virulence genes, their pattern of interaction with epithelial cells and tissue culture monolayers, Strains of *E. coli* can be classified as (i) commensal, (ii) intestinal pathogenic (enteric/diarrheagenic), or (iii) extraintestinal pathogenic *E. coli* (ExPEC) (Russo and Johnson, 2000). These commensal *E. coli* strains rarely cause disease except in immunocompromised hosts or where the normal gastrointestinal barriers are breached (Kaper *et al.*, 2004). Diarrheagenic *E. coli* is further categorized into the following six pathotypes; enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (Okeke *et al.*, 2000; Nataro and Kaper, 1998).

1.2 Justification of this Research

Waterborne pathogenic microorganisms infect an average number of 250 million people yearly resulting in about 10 to 20 million cases of mortality world-wide (Anon, 1996). This

highlights the potential of infection due to waterborne pathogens. More than 7 million people (approximately 17% of the population) do not have access to potable water supply and nearly 21 million (about 54% of the population) lack basic sanitation in South Africa (DWAF, 1996; Zamxaka *et al.*, 2004). Although there have been improvements in these figures according to a more recent study carried out by the World Health Organisation and United Nations Children's Fund Joint Monitoring Programme (JMP) for Water Supply and Sanitation (2010), lesser improvements have been achieved on sanitation (WHO/UNICEF, 2010).

In South Africa nearly 80% of the population rely on surface water as the main source of water (Venter, 2001; Zamxaka *et al.*, 2004). Many of these water bodies are often impacted by inadequately treated effluents from municipal wastewater treatment plants (Fatoki *et al.*, 2003). In September 2007, there was a reported case of cholera outbreak in Ilinge location; a semi-urban area located about 20 km from Queenstown in the Eastern Cape province of South Africa. Five of the reported cases presented mild diarrheal symptoms (Chris Hani District – Department of Health, 2007). According to this report the number of diarrheal cases increased from 1 to 23 for the period between the 10th and the 15th of September 2007, although, there was notable decrease in the number of cases between the 16th and 17th of the same month. A report by Department of Water Affairs (DWA, 2009) points out that the Queenstown and Whittlesea wastewater treatment plants fall short in meeting the required standards for *E. coli* (a subgroup of the faecal coliforms) and Streptococcus, and *E. coli* and ammonia (NH₄), respectively.

Although some work have previously be done on some wastewater treatment plants in the Eastern Cape, those work have only reported the failure of a few wastewater treatment facilities in producing discharged effluents of acceptable standards in terms of physicochemical and other microbiological indicators (Igbinsa and Okoh, 2009; Odjadjare and Okoh 2009). There is a

dearth of information on the occurrence of *E. coli* in the final effluents of wastewater treatment plants in Amathole and Chris Hani District Municipalities of the Eastern Cape Province. Thus, we aim at using molecular based technique (PCR) to detect *E. coli* concentrated from wastewater samples using membrane filtration and determine antibiotic susceptibility profile of the *E. coli* isolates.

1.3 Hypothesis

The working hypothesis of this research is that final effluents of four wastewater treatment plants in Amathole and Chris Hani District municipalities of the Eastern Cape Province are potential sources of pathogenic *E. coli* strains in the environment.

1.4 Aim and Objectives

The main aim of the study was to evaluate the incidences of pathogenic *E. coli* in the final effluents of four selected wastewater treatment plants in Amathole and Chris Hani District Municipalities in the Eastern Cape Province, the specific objectives which were to:

1. investigate the incidence of faecal coliforms, presumptive *E. coli* and presumptive *E. coli* O157:H7 in the final effluents of the wastewater treatment plants;
2. isolate, purify and identify the presumptive *E. coli* in the wastewater effluents using molecular based technique and
3. to determine the antibiogram and multiple antibiotic resistance phenotypes (MARP) and multiple antibiotic resistance index (MARI) of the identified *E. coli* isolates.

CHAPTER TWO

LITERATURE REVIEW

2.1 Wastewater

One key problem of our current age is the shortage of drinking water. Most freshwater sources are becoming polluted thus, decreasing access to clean and safe water for various human use (Dixit *et al.*, 2005). Water pollution occurs when contaminants are discharged into water bodies. One main source of pollutants in freshwater bodies is the release of pollutants from untreated or inadequately treated wastewater treatment facilities (Owili, 2003). Wastewater is a water-based waste that is removed from domestic, commercial and industrial establishments (Sonune and Ghate, 2004). Wastewater is any water that the quality has been altered by the addition of anthropogenic materials (Norzatulakma, 2010). Municipal wastewater is usually made up of a combination of sewage, suspended solids, debris and a variety of chemicals that come from residential, commercial and industrial processes (Argaw, 2004; Tchobanoglous *et al.*, 2003). It typically consists of about 99.93% water and 0.07% total dissolved and suspended solids. Out of the 0.07% total solids, only half are organic in nature, the other half is inorganic (Ellis, 2004). Pathogenic microorganisms in wastewater are excreted in faecal matter and urine of disease infected humans and animals, and they may be bacteria, enteric viruses, protozoans and helminths (Okoh *et al.*, 2007). Wastewater treatment history dated back to the late 1800s and 1900s and treatment methods vary from one country to another. Nonetheless, wastewater treatment objective remains the same, which is to remove contaminants from wastewaters before they are discharged back into the natural environment or used for other purposes such as

irrigation or aquaculture (Chow *et al.*, 1972, Okoh *et al.*, 2007). One of the prime focuses of treatment of wastewater is the removal of pathogens in order to safe guard the health of the public by ensuring discharged effluents comply with the required standards. Generally, the proper implementation of this management process results: in the protection of source water quality; the reduction in drinking water treatment cost; and the control or prevention of waterborne disease (Bekal *et al.*, 2003).

A few countries of the world including South Africa have incorporated access to clean and safe water in their constitutions as a basic right for everyone (Dubreuil, 2006; Heleba, 2011). However, some problems persist in terms of the ability to keep up with provision of water-related services such as lack of attention to maintenance of wastewater treatment infrastructure. Although, South Africa has a strong water industry with a good track record of innovations, worries related to the discharge of effluent of poor quality from various municipal wastewater treatment plants around the country remains a major challenge bothering on the problem of sanitation (WHO/UNICEF, 2008). Many local communities in most developing countries including South Africa still rely on untreated surface and ground water sources for their domestic and other water needs. Water from these sources is often contaminated by faecal pollution from wastewater effluents (Toze, 2004). Economy development and population growth has overwhelmed many wastewater treatment plants in many countries causing them to operate under stress, a situation which in turn places much pressure on water usage and hygiene a situation which presents regulatory authorities with challenges in sustaining the quality of water resources (Mema, 2009).

One obvious instance of the detrimental effect of increasing anthropogenic activities on an essential natural water sources is the case of Lake Victoria, which is the second largest lake in

the world and has a surface area of 68 800 km² distributed between Tanzania, Uganda, and Kenya (Amelia, 2001). The type of stress facing the lake and its wetlands from direct and indirect anthropogenic activities was described by Rutashobya (1996) and Kassenga (1997). The Lake was extremely polluted by indiscriminate wastewater effluent discharges from the shoreline communities, agriculture and industry. Obvious changes in the Lake ecosystem include: a twofold increase in algal growth causing turbidity in the Lake; domination of zooplankton by cyanobacteria; excessive phosphates concentration beyond algal requirements and about half of the lake bottom is anoxic. As a result of eutrophication, the lake was infested with a free-floating macrophyte, the water hyacinth (*Eichhornia crassipes*) which is almost covering over 600 ha of the shoreline on the side of Tanzania (Amelia, 2001). Eutrophication of natural waters is one of the most significant causes of a decline in water quality in recent years. It usually results in large quantity of plant material in the water. It is fair to state that the control of nutrients (nitrates and phosphates) discharge is important in controlling aquatic plant growth (Dixit *et al.*, 2005).

More than 95 percent of South Africa's available freshwater resources had already been allocated by the year 2005 (CSIR, 2010). The quality of these resources has also deteriorated due to increased pollution caused by industry, urbanisation, afforestation, mining, agriculture and power generation (Ashton *et al.*, 2008). Some factors contributing to the situation in the country include outdated and inadequate water and wastewater treatment infrastructure and unskilled man labour (CSIR, 2010). Effluent discharges from municipalities and industrial areas, as well as leakage and discharges from mines and intensive agriculture placed several noticeable changes in water quality. In effect, these changes in water quality have critical implications for all of society and the natural ecosystems that depend on the water resources. A large fraction of the sewage emanating from South African urban areas is not treated adequately before discharge, because the

sewer systems are incomplete or broken, or sewage treatment plants are overloaded and mismanaged. Without a fundamental intervention in water quality management approaches and treatment technologies, progressive deterioration of water quality will continue to reduce the benefits and increase the costs associated with use of the country's water resources (CSIR, 2010). Poor quality of water not only reduces its usefulness; it equally places additional economic burden on society through both the primary treatment costs and the secondary impacts on the economy, the more polluted the water resource, the higher the treatment costs. Contamination of groundwater by viruses and bacteria has caused a number of disease outbreaks in South Africa, for example at Delmas in 2005 and 2006 (Griesel *et al.*, 2006).

The prime focus of treating wastewater is by and large to allow human and industrial effluents to be disposed of without hazard to human health or deplorable damage to the natural environment (FAO, 1992). In order to achieve this; a basic wastewater treatment plant consists of the mechanical and biological processes that result in the removal of solids, organic matter and nutrients from the wastewater (Sonune and Ghate, 2004). These processes are grouped into the preliminary, primary, secondary and tertiary stages (Doorn *et al.*, 2006). Sewage may be treated in *on-site* septic systems involving wastewater from one or several households consisting of an anaerobic underground tank and a drainage field for the treatment of effluent from the tank (UNEP, 2002). However, there are communities without wastewater treatment facilities, and in some cases existing infrastructure is faltering; and even in areas with a high level of treatment, pathogens and some chemicals, many with unknown ecological consequences, may still be released into the environment (LeChevallier and Au, 2004; Paillard *et al.*, 2005). The understanding of the negative effects of inadequately treated sewage has led to moves by

environmental groups and governments in many countries to undertake initiatives aimed at reducing sewage pollution.

2.1.1 Stages in wastewater treatment

Wastewater treatment processes often consist of a series of physical, chemical and biological processes. Degrees of wastewater treatment are usually described preliminary, primary, secondary and tertiary or advanced wastewater treatment. Disinfection process which inactivates pathogenic microorganisms sometimes follows the last treatment step (FAO, 1992). A brief description of some of the stages involved in wastewater treatment processes are given below.

2.1.1.1 Preliminary treatment

The wastewater that goes into the treatment plant first undergoes the preliminary treatment phase (Okoh *et al.*, 2007). At this stage, the preliminary treatment process typically includes coarse screening and grit removal. Most small wastewater treatment plants often exclude the grit removal step (FAO, 1992). This treatment removes any solids coarse, large, entrained, and suspended or floating (Sonune and Ghate, 2004). Some treatment plants may employ devices that also include a grinder along with the screen, known as comminators. Such devices retain the solids and then grind into smaller materials, which are returned back into the wastewater flow and later removed in the primary treatment stage (Okoh *et al.*, 2007).

2.1.1.2 Primary treatment

Primary wastewater treatment typically involves screening, grit chamber and sedimentation tank. Screening removes large material such as stones, sticks, and plastics etc. that could block lines or tank inlets (ADS, 2004). In the primary treatment stage the organic and inorganic solids of about 50-70%; along with 25-50% of biochemical oxygen demand and 65% of oil or/and grease are removed. All these removals are achieved using the physical methods of sedimentation and flocculation (FAO, 1992; Sonune and Ghate, 2004). Grit chamber slows down the flow to allow grit to fall out. Sedimentation tank allows settleable solids to settle out and are pumped away, while oils floats to the top and are skimmed off (ADS, 2004). Primary settling tanks are usually furnished with mechanically driven scrapers that continually drive the collected sludge towards a hopper in the base of the tank where it is pumped to sludge treatment facilities (US EPA, 2004). In more advance primary treatments, chemicals are added or filtration is performed, furthermore, in order to enhance sedimentation and the removal of lighter suspended solids and some dissolved solids (Metcalf and Eddy, 2003).

2.1.1.3 Secondary treatment

This involves biological treatment process that removes dissolved organic matter from the primary effluent. Ninety percent of this removal is achieved through microorganisms absorbing the organic matter in the wastewater as their food source, mostly using aerobic biological treatment processes (FAO, 1992; Sonune and Ghate, 2004; Doorn *et al.*, 2006; Okoh *et al.*, 2007). Microorganisms (mostly bacteria) are often engaged at this stage to convert the colloidal and dissolved carbonaceous organic matter into various gases and into cell tissue which is then removed in sedimentation tanks. Biological processes are usually used together with physicochemical processes, with the sole aim of reducing the organic content (measured as biological oxygen demand, total organic carbon or chemical oxygen demand) and nutrient

content (nitrogen and phosphorus) of wastewater (UN, 2003(b)). Secondary treatment takes place in fixed or suspended growth reactors using any or variants of activated sludge, biofiltration, rotating biological contactors, and constructed wetlands processes (US EPA, 1997). Under ideal conditions the microorganisms produced by this process will aggregate to form a settleable stable floc structure.

2.1.1.4 Tertiary treatment

Tertiary treatment stage may include processes to further remove nutrients such as nitrogen and phosphorus, and carbon adsorption to remove chemicals (ADS, 2004). These treatments usually involve improved removal of dissolved and suspended solids as well as the removal of nutrients such as nitrogen, phosphorus, heavy metals etc. (FAO, 1992). The treatment can be achieved using one or a combination of the following processes: polishing ponds, biological processes, advanced filtration, carbon adsorption, ion exchange and/or disinfection (Sonune and Ghate, 2004; Doorn *et al.*, 2006).

2.1.1.5 Disinfection

Disinfection is the commonest tertiary treatment stage employed in wastewater treatment plants (Osode and Okoh, 2010). Disinfection is an important method for the inactivation of pathogenic microorganisms that may persist in the treated effluent in order to prevent the spread of waterborne disease to downstream user of receiving water bodies as well as protect the environment (US EPA, 1999b). Some commonly used methods of disinfection include physical agents such as heat and light; mechanical processes such as screening, sedimentation, filtration, radiation, mainly gamma rays; chemical agents including chlorine and its compounds, bromine, iodine, ozone, phenol and phenolic compounds, alcohols, heavy metals, dyes, soaps and

synthetic detergents, quaternary 13 ammonium compounds, hydrogen peroxide, and various alkalis and acids. Chlorine is the most commonly used oxidizing chemical disinfectants (UN, 2003b)

The importance of sewage or wastewater treatment for the protection of the health of the public as well as the conservation of the ecosystem cannot be overemphasised (WHO, 2006). Considering the health and economic implications of the consumption of polluted water and the imbalances caused in aquatic ecosystem by the emission of pollutants from inadequately treated wastewater; it is imperative that efforts are made to ensure that all forms of biological and physicochemical pollutants in wastewater are properly removed before they are released back into the natural environment (UNEP, 2010).

2.2 Microbial indicators in wastewater

Indicator organisms have often been used as basic tools to suggest the presence and estimate microbial contaminants of faecal origin in wastewater (Bitton, 2005; Odorkor and Ampofo, 2013). Although these bacterial indicators are not pathogenic themselves, their presence in water is often correlated to the possible presence of pathogenic microorganisms including enteric viruses and bacteria (Myers and Sylvester, 1997). Bacteria indicators are important in water quality assessment because it is usually hectic to attempt detecting and enumerating all diseases-causing microorganisms that may be present in water. Indicator organisms are therefore used to save time and resources, based on their supposed correlation to the presence of pathogens. According to USEPA(2006) an indicator must have the following qualities to be considered as a useful water quality indicator for faecal contamination:(1) The organism should be found in the faeces of humans and other warm-blooded animals in large

numbers, (2) it must be quick and readily detectable by simple methods, (3) it must not grow in natural waters, the general environment or water distribution systems (4) its persistence in water and the extent of its removal by water treatment must be similar to those of waterborne pathogens.

Bacteria species selected as indicators are native to the digestive tracts of warm-blooded animals and indicate the potential presence of pathogens that can cause diseases (EPA, 2012). Bacterial Indicator including total coliforms (TC), faecal coliform (FC), enterococci, faecal streptococci (FS) and *E. coli* have all been used conventionally for microbial water quality monitoring (Myers and Sylvester, 1997; Ashbolt *et al*, 2001).

Table 2.1: Description of microbial indicator classes.

Group	Description
Process indicator	A group of microorganisms that indicate the efficiency of a process, such as total heterotrophic bacteria or total coliforms for chlorine disinfection.
Faecal indicator	A group of bacteria that shows the presence of faecal contamination, such as the thermotolerant coliforms or <i>E. coli</i> . Their presence may only infer the possible presence of pathogens.
Index and model organisms	A group or species indicative of pathogen presence and behavior respectively, such as <i>E. coli</i> as an index for <i>Salmonella</i> and F-RNA coliphages as model of human enteric viruses.

Source: Odonkor and Amplolo, 2013.

2.2.1 Total coliforms

The total coliform bacteria include *Escherichia coli*, *Enterobacter*, *Klebsiella*, *Citrobacter*, *Budvicia*, *Erwinia*, *Leclercia* and *Serratia* all belonging to the family

enterobacteriaceae. They are mostly present in large numbers as part of the intestinal flora of human and other warm-blooded animals, hence their presence in faecal polluted wastewater (Bitton, 2005). According to the 20th edition of Standard Methods for Examination of Water and Wastewater, total coliforms have been defined as either:

1. Facultative anaerobes that are Gram-negative, non-spore forming, rod-shaped bacteria and can ferment lactose that results in the production of gas and acid at 35°C for 48 hours;
2. Facultative anaerobes that are Gram-negative, non-spore forming, rod-shaped bacteria and can develop red colonies with a metallic sheen on an Endo-type medium containing lactose at 35°C for 48 hours; or
3. Simply bacteria that possess the β -galactosidase enzyme that can cleave a chromogenic substrate (e.g. ortho-nitrophenyl- β -D-galactopyranoside) resulting in the release of a chromogen (ortho-nitrophenol), also known as β -galactosidase-positive enterobacteriaceae (Tallon *et al.*, 2005).

The presence of total coliform in source water can indicate the general quality of that water and likely that the water is faecally contaminated (USEPA, 2009). Hence, they are used as indicators of the potential presence of pathogens in water and have been used since the end of the 19th century (Rompre *et al.*, 2002; Bitton, 2005).

2.2.2 Faecal coliforms

Faecal coliform group, which is distinguished from the total coliform group by its ability to grow at elevated temperatures of 44.5 °C, has been mostly used as indicator in microbial

waterquality investigation (Harwood *et al.*, 1999).These coliforms are mainly comprised of *Escherichia coli* and *Klebsiella pneumoniae*, with *E. coli* being the most dominant (Bitton, 2005).These indicators are released by humans and animals, and they are members of the normal microbial flora hence they are always present in sewage polluted water with numbers relatively close related to the levels of faecal pollution (DWAF, 1996).

2.3 Microbial pathogens in wastewater

Microbial pathogens may be possibly present in wastewater can be divided into three major groups. These groups are the viruses, bacteria and the pathogenic protozoan/helminths (LeChevallier and Au, 2004). Most of these microorganisms are of enteric origin, that is, they are mostly excreted in faeces, contaminate the environment including soil and water, and then infect new hosts by ingestion (Toze, 1997).Bacteria are the commonest of these microorganisms and they mostly cause gastrointestinal infections in their host. Infections caused by pathogenic bacteria include various forms of diarrhoea, cholera (caused by *Vibrio cholerae*), salmonellosis (caused by *Salmonella* species), dysentery (caused by *Shigella* species and some *Salmonella* species), and typhoid (caused by *Salmonella typhi*) (Grant *et al*, 1996; Toze, 1997).

Detection of viruses in water started over five decades ago, with scientists trying to detect poliovirus in water samples. Ever since then, other important intestinal viruses capable of causing gastroenteritis and hepatitis, among a great variety of virus strains, have taken the place of enteroviruses as the main target for detection in the water (Bosch, 1998).Enteric viruses are one group of pathogens usually found in very high titre in wastewater (Gerba *et al.*, 1975).

Human and animal faecal materials in wastewater contain various groups of enteric viruses that cause gastrointestinal infections, hepatitis or neurological diseases. These viruses include adenoviruses, astroviruses, enteroviruses, noroviruses, rotaviruses and hepatitis A and E viruses, and are collectively known as enteric viruses. They are transmitted mainly via the faecal-oral route and present a significant public health hazard in water environments (Metcalf *et al.*, 1995).

Enteric viruses are usually released in high concentrations in faeces of infected human and are discharged into sewage which may contaminate surface water sources for drinking water, recreational activities, aquaculture and irrigation (Wyn-Jones and Sellwood 2001). An increased understanding of the diversity and sources of pathogenic enteric viruses and their persistence in water environments have raised critical questions about the suitability of the current bacterial indicator approaches for assessing the human waste contamination of waters (Savichtcheva and Okabe 2006).

2.3.1 *Escherichia coli* pathotypes

Escherichia coli belongs to the faecal coliform group and is a more specific bacterial indicator of faecal pollution than other faecal coliforms (Anderson *et al.*, 2005; Odorkor and Ampofo, 2013). *Escherichia coli* is often used as a more dependable indicator of faecal pollution in place of thermotolerant coliforms. Currently, *E. coli* has been adopted as the best bacterial indication of faecal contamination in drinking water (Odorkor and Ampofo, 2013).

The pathogenicity of *E. coli* was first demonstrated in 1935 when some strains of the bacteria were shown to be the causative agent in an outbreak of diarrhea in infants (Koba, 2013). Pathogenic *E. coli* have been grouped into numerous categories based on their possession of

virulence factors, clinical symptoms and sites of pathogenesis to the host (Gyles and Fairbrother, 2004; Milonet *et al.*, 1999; Nataro and Kaper, 1998).

The pathogenic strains of *E. coli* that can cause enteric infections are called diarrheagenic *E. coli*(DEC), a group which includes emerging pathogens of public health importance worldwide (Nataro and Kaper, 1998; Vidal *et al.*, 2005). Pathogenic strains of *E. coli* are largely classified either as diarrheagenic *E. coli* or extraintestine pathogenic *E. coli* (ExPEC) (Kaper *et al.*, 2004) Within each of these groups are sets of strains known as pathotypes or pathovars (pathogenic variants) that share common virulence factors and elicit similar pathogenic outcomes (Mars *et al.*, 2005).

Diarrheagenic *E.coli*is an important agent of infant diarrhea which represents a major public health problem in developing countries (Nataro and Kaper, 1998; Soltan-Dallal, 2001; Mitchell *et al.*, 2005; Akinjogunla *et al.*, 2009). The diversity among diarrheagenic *E.coli* pathotypes and antigens means that children may be subjected to repeated infection by different subtypes without immune protection (Okeke, 2009).

Enteropathogenic *E. coli*have been classified based on different virulence factors such as entotoxins, their pattern of interaction with epithelial cells and tissue culture monolayers (Vidal,*et al.*, 2005). About six pathotypes of DEC have been described. These includes enteropathogenic *E. coli* (EPEC); enterotoxigenic *E. coli* (ETEC); enteroinvasive*E.coli* ; (EIEC), enterohemorrhagic *E.coli*(EHEC); enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (Okeke *et al.*, 2000; Nataro and Kaper, 1998; Vidal *et al.*, 2005). Uropathogenic *E. coli* (UPEC) and Neonatal Meningitis *E. coli* (NMEC) are twoExPEC strains that have been characterised (Wiles *et al.*, 2008; Dubois *et al.*, 2009). These are able to cause

infections in the urinary tract, bloodstream and in the central nervous system which leads to sepsis and meningitis (Nataro and Kaper, 1998).

2.3.1.1 Enteropathogenic *E. coli* (EPEC):

Enteropathogenic *E. coli* (EPEC) strains are the oldest known group of diarrhoeagenic *E. coli* (Zuber, 1999), and is a major cause of potentially fatal diarrhoea in infants in developing countries (Kaper *et al.*, 2004 ;Trabulsi *et al.*, 2002; Tennant *et al.*, 2009). Enteropathogenic *E. coli* pathotype belongs to a family of pathogens that form attaching and effacing (A/E) lesions on intestinal epithelial cells that enables localized adherence of bacteria to intestinal cells and a non fimbrial adhesin designated intimin, which is an outer membrane protein that mediates the final stages of adherence. (Tennant *et al.*, 2009). Enteropathogenic *E. coli* induces watery diarrhoea that may contain mucus but it does not typically contain blood in it. Symptoms associated with EPEC includes fever, vomiting, malaise and dehydration and may last for a brief period of several days, although instances of long, chronic EPEC disease have been noted. (Koba, 2013). The 12 serogroups originally recognised as EPEC included; O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158 and these were classified and defined on the basis of O and H serotypes (Hernandez *et al.*, 2009). Outbreaks of water-borne diseases have been linked to the consumption of contaminated drinking water as well as some meat products. A frequent infant diarrhoea outbreak was once caused by EPEC in the United Kingdom (UK), US and there have been reported outbreaks in the last two decades and were reported to occur frequently (Bower *et al.*, 1989; Robins-Browne 1987; Paulozzi *et al.* 1986 ; Rothbaum *et al.*, 1982).

2.3.1.2 Enterotoxigenic *E. coli* (ETEC):

Enterotoxigenic *E. coli* is the commonest pathovar, largely in developing countries and is recognised as an emerging intestinal pathogen (Croxen and Finlay, 2010; Osode and Okoh, 2008). Enterotoxigenic *E. coli* strains produce heat-labile toxin (LT), heat-stable toxin or both toxins (Benenson, 1995) and colonization factors allowing the organism to colonize the small intestine with subsequent development of diarrhoea especially among children in developing countries and of traveller's diarrhoea (Kaper *et al.*, 2004; Levine *et al.*, 1977; Osode and Okoh, 2008; Wolf, 1997). Characteristic symptoms of disease include watery stool, abdominal cramps, fever, malaise, and vomiting. One out of every six traveller's to endemic areas has been observed to be infected with ETEC (Steffen *et al.*, 2005). The major virulence factors of ETEC are intestinal colonization factors, for example, fimbriae, and the enterotoxins (Todar, 2008). The host specificity of individual strains is determined by the type of colonization factors and fimbriae produced (Kaper and Nataro, 1998). An outbreak was largely attributed to ST-producing ETEC in the US nursery from 1974 – 1975 (Okeke, 2009). Another documented information of 16 outbreaks of ETEC infections in the United States and on cruise ships were confirmed with the noted reported symptoms by all passengers, diarrhoeal being the highest (99%) followed by abdominal cramps (78%) and nausea (63%) (Beatty *et al.*, 2004).

2.3.1.3 Enteroinvasive *E. coli* (EIEC):

Enteroinvasive *E. coli* (EIEC) was firstly well-known to be one of the etiological agents of diarrheal diseases in 1971 (DuPont *et al.*, 1971). Enteroinvasive *E. coli* causes shigellosis-like symptoms in both adults and children (Vieira *et al.*, 2007). *Shigella* are highly infectious bacteria that cause bacillary dysentery and bloody diarrhoea (Kaper *et al.*, 2004). Clinic symptoms include watery diarrhoea prior to onset of dysentery with a low volume of stools containing blood and mucus. Other symptoms may include headache, fever, and cramping. Enteroinvasive *E. coli* are

vastly invasive and employ adhesin proteins to attach to and enter intestinal cells (Maurelli *et al.*, 1998). Virulence is largely due to a 220 kb plasmid that encodes a T3SS on the Mxi–Spa locus that is required for invasion, cell survival and apoptosis of macrophages (Vieira *et al.*, 2007). The incidence of the disease caused by EIEC is generally low in developed countries (Maurelli *et al.*, 1998). The epidemiology of EIEC is not well studied in Africa (Okeke, 2009).

2.3.1.4 Enteroaggregative *E. coli* (EAEC):

Enteroaggregative *E. coli* (EAEC) was discovered in 1985 and is recognized by its distinctive adherence to HEp-2 cells in an aggregative, stacked brick-like pattern (Harrington *et al.*, 2006). Aggregative adherence is the defining characteristic of EAEC (Nataro *et al.*, 1987). Enteroaggregative *E. coli* strains have been implicated in acute as well as persistent diarrhoea among adults and children (Huang and Dupont, 2004; Okeke and Nataro, 2001). Enteroaggregative *E. coli* has also been identified as a principal cause of diarrheal disease in Brazil, Germany, the United Kingdom and the United States (Kaur *et al.*, 2010; Araujo *et al.*, 2007; Cohen *et al.*, 2005).

Enteroaggregative *E. coli* pathogenesis typically involves three steps : (1) adherence to the intestinal mucosa by aggregative adherence fimbriae (AAF) and adherence factors, (2) increased production of mucus that encrusts EAEC on the surface of enterocytes; and (3) release of toxins and elicitation of an inflammatory response, mucosal toxicity, and intestinal secretion (Kaur *et al.*, 2010; Nataro, 2005). Symptoms of EAEC infections include watery diarrhea with or without blood and mucus, abdominal pain, nausea, vomiting, and low-grade fever and these clinical symptoms vary between individuals (Kaur *et al.*, 2010; Jiang *et al.*, 2003; Adachi *et al.*, 2002).

2.3.1.5 Diffusely adherent *E. coli* (DAEC):

Diffusely adherent *Escherichia coli* strains are those that diffuse adherence (DA), on the Hep-2 cell surface (Scaletsky *et al.*, 2002). Diarrheagenic *E. coli* has also been observed to be the sixth class of diarrheagenic *E. coli* (Lopes *et al.*, 2005). Diffusely adherent *Escherichia coli* is most commonly associated with age-dependent diarrhoea and in children less than 12 months of age (Scaletsky *et al.*, 2002). However, most case-control studies have demonstrated that the association of DAEC as a diarrhoea causing agent remains controversial. Diffusely adherent *Escherichia coli* is believed to comprise a heterogeneous group of organisms of variable enteropathogenicity (Arikawa *et al.*, 2005). There is limited information on epidemiology and pathogenesis of the diffusely adherent *E. coli*. Diffusely adherent *Escherichia coli* strains as agents of diarrhea and clinical symptoms include watery diarrhoea in some instances with mucus and blood with vomiting being more prominent as compared to diarrhoea among children (Meraz *et al.*, 2008; Levine *et al.*, 1977).

2.3.1.6 Enterohemorrhagic *E. coli* (EHEC):

Enterohemorrhagic *E. coli* were first discovered in 1977 by the production of cytotoxin, verotoxin (VT), lethal to vero cells, which led to these pathogens being called verocytotoxigenic *E. coli* (VTEC) (Konowalchuk *et al.*, 1977). It is also known as *E. coli* 0157:H7 surfaced in the last decade as an important food-borne pathogen with 73000 cases of annual infection in the United States (Rangel *et al.*, 2005; Zhao *et al.*, 2006). These cytotoxins are mediated by genes carried by lysogenic bacteriophages and act in a similar manner by interfering with protein synthesis in eukaryotic cells (Todar, 2008). The toxins belong to two major antigenically distinct groups, Stx1 and Stx2, with various subgroups. Enterohemorrhagic *E. coli* strains are

characterized by verotoxin production which have been linked to life-threatening diseases such as severe haemolytic-uremic syndrome (HUS) complication; an important cause of acute renal failure in children and morbidity and mortality in adult humans and thrombo-cytopenic purpura (Karmali, 1985). Toxins formed by EHEC are described as Shiga-like toxins which are toxins produced by *Shigella dysenteriae*. These strains are also referred to as Shiga-toxin producing *E. coli*, or STEC. Other strains are described as non-O157 EHEC (Nataro and Kaper, 1998; Tozzi *et al.*, 2003).

Enterohemorrhagic *E. coli* O157:H7 designated by its somatic, O, and flagellar, H, antigens is the most important EHEC serotype in relation to public health. Enterohemorrhagic *E. coli* O157:H7 was first discovered in 1982 as a highly virulent human pathogen following two outbreaks of hemorrhagic colitis (Sanjar *et al.*, 2014) while other members of some non-O157 serotypes have frequently been involved in sporadic cases and outbreaks, and are increasingly recognized as causes of hemorrhagic colitis and HUS. Some of these non O-157 serotypes may be as significant in human disease as EHEC O157:H7. For instance, EHEC O153 has been linked to a disease that resembles HUS in rabbits (Paton and Paton, 2002; Mallick, 2012).

An outbreak in 1998 of O111 EHEC occurred in Nigeria (Okeke *et al.*, 2003). Some of the symptoms of infections caused by *E. coli* O157:H7 include severe or acute hemorrhagic diarrhea which may in some cases advance to bloody diarrhea (haemorrhagic colitis), and abdominal cramp. Fever and vomiting may also occur and, nonhemorrhagic diarrhea have been reported in some cases also.

Clinical cases can be diagnosed by finding these organisms in fecal samples because humans do not normally carry EHEC. Selective and differential media have been developed for

detection based on its lack of β -glucuronidase activity and the inability of most strains to rapidly ferment sorbitol (Nataro and Kaper, 2004).

2.3.1.7 Uropathogenic *E. coli* (UPEC):

Uropathogenic *Escherichia coli* (UPEC) strains are known cause of about 80% of uncomplicated community-acquired uropathogenic infection. Uropathogenic *Escherichia coli* has the challenge of moving from the intestinal tract to establish an infection in the urinary tract, where it uses peptides and amino acids as the primary carbon source for fitness (Altalhi and Hassan, 2009). Uropathogenic *Escherichia coli* strains of *E. coli* has a compound life cycle, replicating and persisting in intracellular and extracellular niches (Lane *et al.*, 2006; Gawel and Seed, 2011). The major reservoir is the intestinal tract for various groups of bacteria, including UPEC. UPEC strains from the anus gain access to the periurethral area and establish infection in an ascending manner (Lane *et al.*, 2006). Uropathogenic *Escherichia coli* strains harbour the gene that encodes filamentous adhesive organelles called type 1 pili. These structures facilitate both bacterial attachment to and colonisation of bladder epithelial cells. Nevertheless, the mechanism by which type 1 pilus-mediated bacterial invasion plays in UPEC pathogenesis of a urinary tract infection is unknown. UPEC can also form both extra- and intracellular biofilm-like communities within the bladder (Blango and Mulvey, 2010). The fimbriae bind not only to red cells but to a specific galactose disaccharide that is found on the surfaces of uroepithelial cells in approximately 99% of the population (Todar, 2008).

2.3.1.8 Neonatal Meningitis *E. coli* (NMEC):

Neonatal meningitis due to *Escherichia coli* remains important challenge to the clinicians and scientists. In spite of advances in antimicrobial chemotherapy, the combined mortality and morbidity of neonatal meningitis *E. coli* (NMEC) remains high (Robbinset *al.*, 1974). Neonatal meningitis is a common inhabitant of the gastrointestinal tract and it is the most frequent cause of

Gram-negative-associated meningitis in newborns. Fatality rates can approach 40% (Kaper *et al.*, 2004). Majority of NMEC strains carry virulence factors such as antigen K1 and the fimbria adhesion (Wang *et al.*, 2011). The S. fimbria enhance efficient adherence of the bacterium to epithelial cells lining the choroid plexus and brain ventricles, and also vascular endothelium in the brain (Prasadarao *et al.*, 1994). Further research has revealed an 8.2-kDa protein (Ibe 10) which is associated with *E. coli* K1 invasion of brain microvascular endothelial cells and K-1 may not be the only determinant of virulence, however, as siderophore production and endotoxin are also likely to be involved (Huang *et al.*, 1995; Todar, 2008).

CHAPTER THREE

METHODOLOGY

3.1 Study area and plant description

Four wastewater treatment plants (WWTPs), one (WWTP-W) located in Chris Hani District Municipality and three (WWTP-Z, WWTP-S, and WWTP-R) located in Amatole District Municipality in the Eastern Cape Province were selected for this study. Table 3.1 below gives the description of some of the characteristics of the WWTPs.

3.2 Sampling

Wastewater samples were collected once monthly for a period of twelve months (September 2012 to August 2013) from the final effluents as well as the discharge points of the wastewater treatment plants using grab sampling method. Sterile plastic bottles of about 1.7 L capacity to which 1.7 ml of 0.1 M sodium thiosulphate has been added were used for the collection of the samples. After collection, the sample bottles were covered tightly with screw caps and transported in cooler boxes containing ice to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, Alice for analyses within 6 h of collection.

Table 3.1 Description of the four wastewater treatment plants selected for the study (DWA Green Drop Report 2012).

Wastewater treatment works	Technology	Designed capacity (MI/d)	Operational capacity (%)	River where effluents is discharged
WWTP-S	Activated sludge, biofilters, anaerobic digestion and sludge drying beds	4.8	66.2	Buffalo River
WWTP-Z	Biofilters, anaerobic digestion and sludge drying beds	9.3	84.9	Buffalo River
WWTP-R	Activated sludge and sludge lagoons	2.5	44	Buffalo River
WWTP-W	Biofilters	4.99	54	Kliplaat River

3.3 Bacteriological analysis

Bacteriological analysis of the samples was done using standard methods as follows:

3.3.1 Detection and enumeration of faecal coliforms, *Escherichia coli* and *Escherichia coli* O157:H7

3.3.1.1 Membrane filtration (MF) method:

Wastewater samples collected were serially diluted to 10^{-1} , 10^{-2} and 10^{-3} ; using sterile distilled water after which 100 ml aliquots of each dilution were filtered through membrane filters (MF) of 0.45 μ m pores, using a vacuum pump. The membrane filters were aseptically transferred onto prepared agar plates containing the appropriate medium for the target indicator bacteria. The medium used for faecal coliforms detection and enumeration was m-FC agar (biolab, Merck) while presumptive *E. coli* detection and enumeration was done on *E. coli*-Coliforms Chromogenic Medium (Conda) and the isolation of *E. coli* O157:H7 was done with enterohemorrhagic *E. coli* O157:H7 chromogenic agar base (Conda; Cat. No. 1588.00) with CefiximeTellurite supplement (Mast; Ref. SV48) added. The plates were then incubated in inverted positions at the appropriate temperatures and time period; the agar plates for *E. coli* and *E. coli* O157:H7 were then incubated at 37°C for 24 h while the m-FC plates (for faecal coliforms) were incubated at 44.5°C for 24 h.

3.3.1.2 Identification and counting of presumptive faecal coliforms, *E. coli* and *E. coli* O157:H7 colonies

After the 24 h of incubation, the target bacteria colonies on the agar plate were identified based on the manufacturer's instructions, Distinctive blue colonies on m-FC agar were counted as faecal coliforms; blue colonies on *E. coli*-Coliforms Chromogenic Medium were also counted as *E. coli* while pale pink colonies on enterohemorrhagic *E. coli* O157:H7 chromogenic agar base

were counted as *E.coli* 0157:H7. The counts were recorded as presumptive colony forming units per 100 ml (cfu/100ml) of effluent samples analysed.

3.4 DNA extraction and PCR

Deoxyribonucleic acid extraction was done by boiling method following the description of Torres *et al.* (2003), Maugeri *et al.* (2004), with little modifications. Single colony of freshly grown cultures was picked using sterile inoculating loop to avoid agar contamination, an important cause of erratic amplification and suspended in 200µl of sterile nuclease free water. The suspension was vortexed and the cells were lysed by heating for 10 min at 100°C using a MS2 a Dri-Block DB.2A (Techne, SA). The suspension was then centrifuged at 13 000 rpm for 10 min to pellet the cell debris. Thereafter, the lysate supernatant was incubated on ice for 5min and 5.0 µl of it was used as template with 12.5 µl PCR master mix, 0.5 µl of each of forward and reverse primers and 6.5 µl of nucleases free water to make a 25.0 µl total reaction volume for the PCR assays immediately after the extraction.

3.4.1 Molecular confirmation of presumptive *E. coli* isolate

Polymerase chain reactions (PCR) protocols were run to confirm the identities of the presumptive *E. coli* isolates using specific oligonucleotide primer which targeted the *uidA* gene (the gene which encodes the beta glucuronidase enzyme in all *E. coli* pathotypes). The protocol for the PCR included a 35 cycle of denaturation at 94°C for 90 sec, an annealing at 60°C for 90 sec and extension at 72°C for 90 sec and a final extension step at 72°C for 10 min following the description of Moyo *et al.* (2007) and Guionet *et al.* (2008). The primer sequences and the respective amplification size are shown on the Table 2 below.

3.4.2 Gel electrophoresis

The amplified PCR products (5 µl aliquots) were resolved in 1.8 % agarose gel (Merck, SA) containing 0.5 µg/Ethidium bromide (EtBr) (Merck, SA) in 0.5X TBE buffer (44.5 mM Tris base, Boric acid, 44.5 mM, 1mM EDTA, pH 8.0) (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 DNAladder (Promega, White Head Scientific) was included on each gel as a molecular size marker. The electrophoresis was carried out at 100 V for 1 h.

3.5 Antibiotic susceptibility profiling of *E. coli* isolates

The antibiotic susceptibility profile of the *E. coli* was determined by the standard disc diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLIS, 2006) using Muller-Hinton agar (MH). The test antibiotics included Streptomycin (10 µg), Cefotaxime (30 µg), Penicillin G (10 µg), Rifampicin (5 µg), Erythromycin (15 µg), Chloramphenicol (30 µg), Cefuroxime (30 µg), Neomycin (10 µg), Cefepime (30 µg) and Norfloxacin (5 µg). Zones of inhibition around the antibiotic disc were measured and classified using CLSI interpretative tables as susceptible, intermediate or resistant. Multiple antibiotic resistance phenotypes (MARP) were generated for isolates that showed resistance to three or more antibiotics while multiple antibiotic resistance index (MARI) of the confirmed isolates was calculated by the formula given below as previously described by (Krumperman, 1983).

MARI = $a / (b \times c)$ where;

a = the aggregate antibiotic resistance score of all isolates;

b = number of antibiotics;

c = number of isolates

Table 3.2: Primer sequence and expected size of PCR-amplified target gene of the *E. coli*.

Target strains	Target genes	Primer sequence (5'→3')	Amplicon size (bp)	References
<i>E. coli</i>	<i>uidA</i>	AAA ACG GCA AGA AAA AGC AG ACGCGT GGT TAACAGTCTTGC G	147	Tsai <i>et al.</i> , 1993

Source: Vidal *et al.*, 2005; Osode *et al.*, 2010

CHAPTER FOUR

RESULTS

4.1 Incidence and distribution of faecal coliforms across the sampling points

The incidence and monthly distributions of the faecal coliforms at the various sampling points are shown in Figure 4.1. At all sampling points, faecal coliforms count ranged between 0 and 1.6×10^3 CFU/100 ml. Zero faecal coliforms counts were observed in the samples analysed in some months (mostly in spring) while the highest coliforms count was observed in the samples taken from WWTP-S in November 2012 also in spring.

4.2 Incidence and distribution of presumptive *E. coli* across the sampling points

Figure 4.2 show the incidence of presumptive *E. coli* counts in the samples at all sampling points and their monthly distribution. The counts generally ranged between 0 and 1.4×10^3 CFU/100 ml. Similar to faecal coliforms, zero counts were observed in the samples in September 2012 (spring) at both WWTP-W and WWTP-R while the highest presumptive *E. coli* counts was observed in March 2013 (autumn) at WWTP-W.

4.3 Incidence and distribution of presumptive *E. coli* O157:H7 across the sampling points

The incidence and monthly distribution of presumptive *E. coli* O157:H7 counts are as shown in Figure 4.3. Counts generally ranged between 0 and 9.6×10^2 CFU/100 ml over the four seasons of sampling. The least counts were observed mainly in the months of spring at WWTP-S, WWTP-Z and WWTP-W while the highest count was observed at WWTP-S in November 2012 (spring).

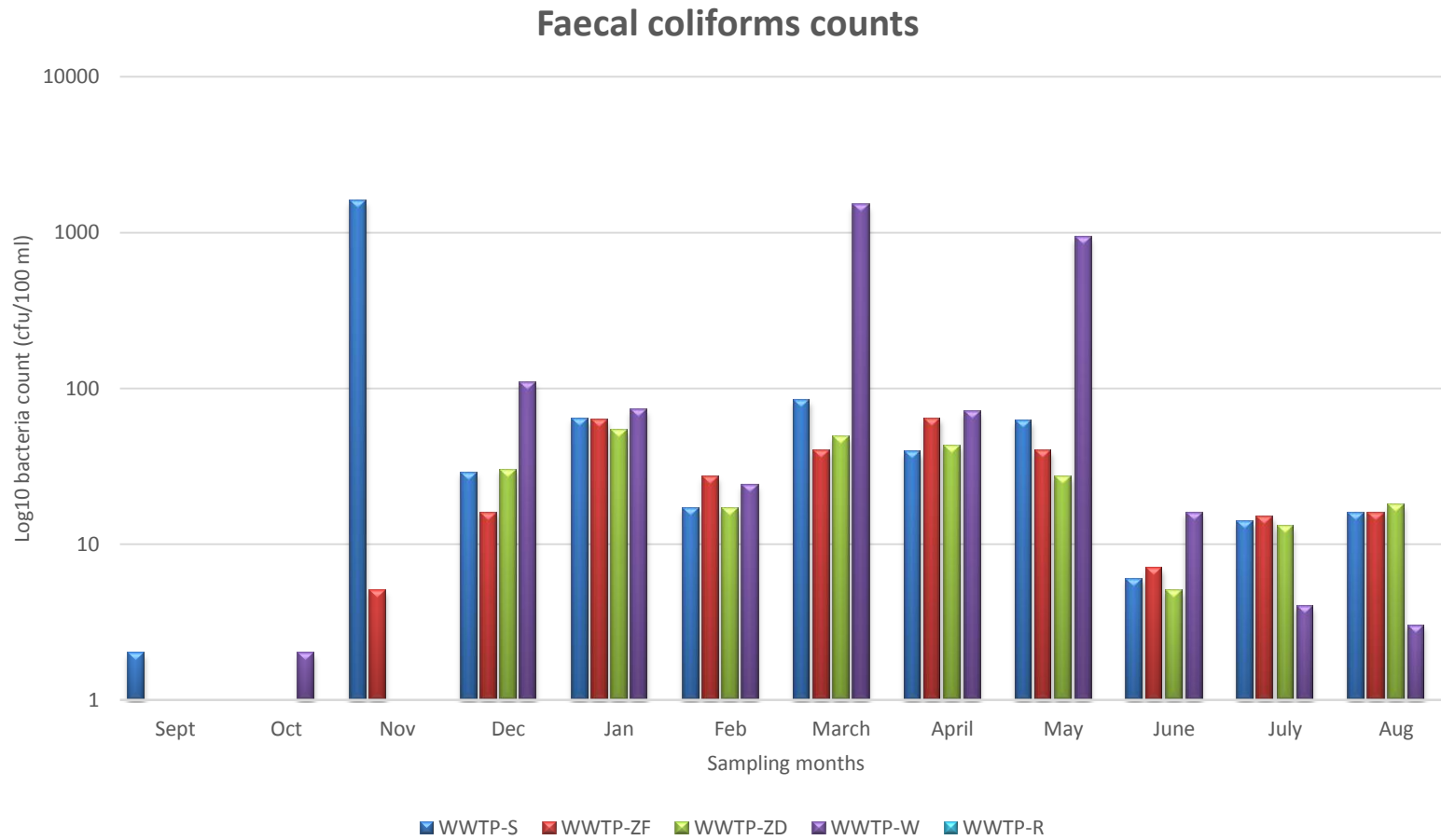


Figure 4.1: Incidence of faecal coliforms across the sampling points

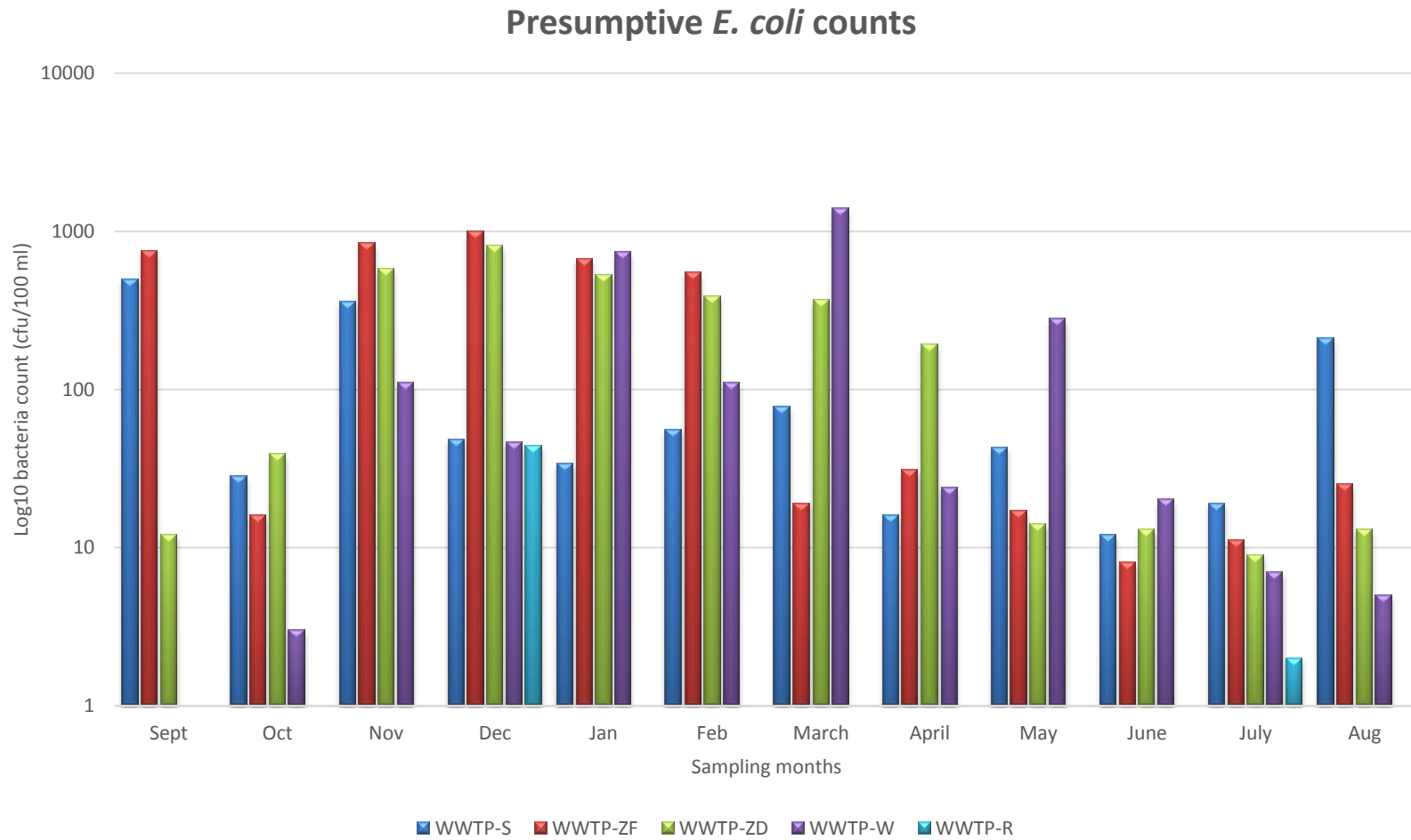


Figure 4.2: Incidence of presumptive *E. coli* counts across the sampling points

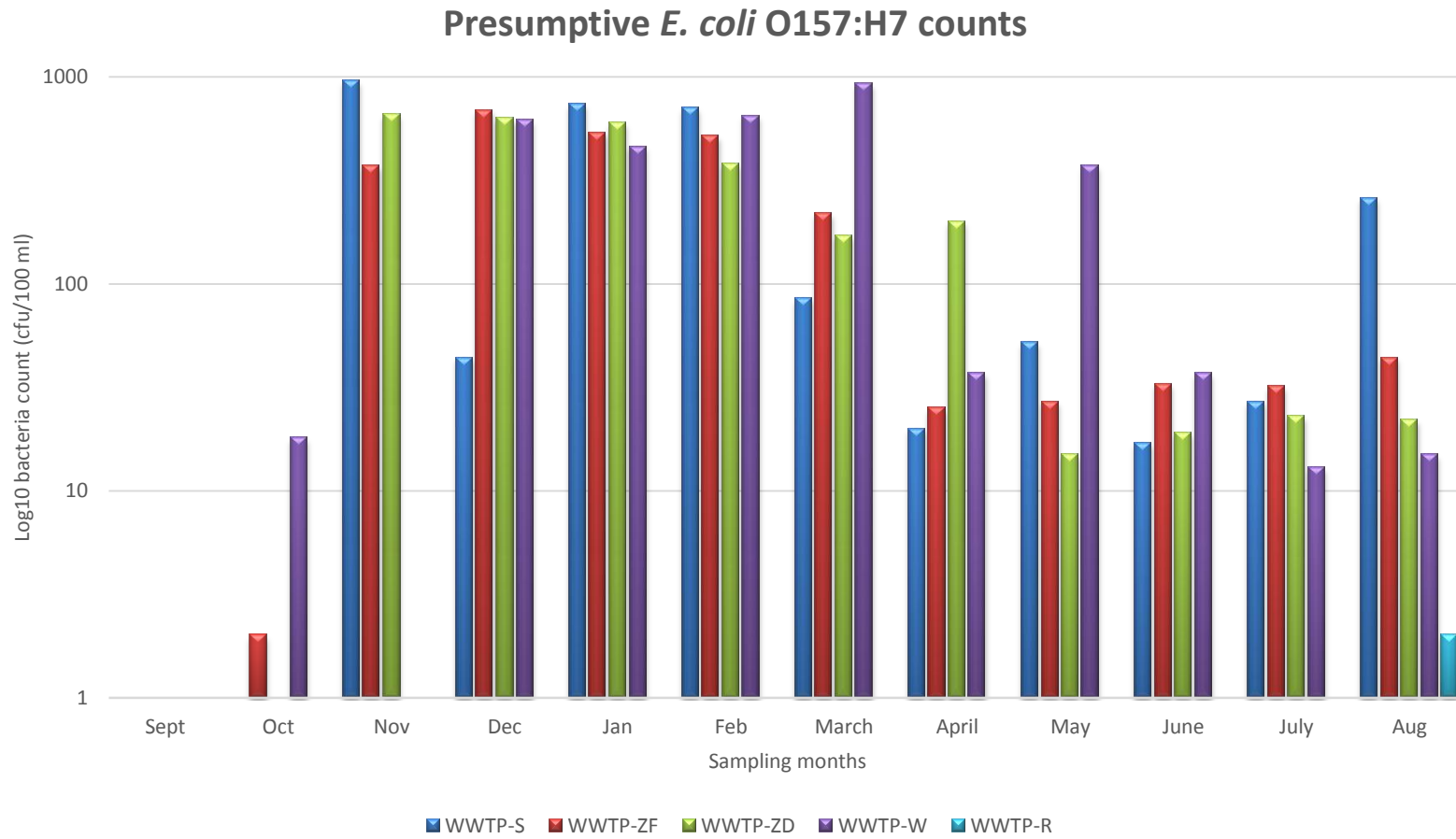


Figure 4.3: Incidence of *E. coli* O157:H7 counts across the sampling points

4.4 Molecular confirmation of presumptive *E. coli* isolates with PCR

Three hundred and five (305) out of the total number of 626 presumptive *E. coli* isolates (48.7%) tested positive for the presence of *uidA* gene and therefore confirmed as *E. coli*. The gel picture in Figure 4.4 shows some of the confirmed isolates with the amplicon size of 147 bp.

4.5 Antibiotic susceptibility profile of confirmed *E. coli* isolates

Out of the 305 confirmed *E. coli* isolates, 150 were randomly selected and tested for antibiotic susceptibility against a panel of 10 antibiotics. The results of the antibiotic susceptibilities are as shown in Table 4.1. The results revealed that all of the isolates were resistant to rifampicin and penicillin G while 90% were resistant to erythromycin and norfloxacin. The majority (52.7%) of the isolates showed an intermediate susceptibility to cefuroxime, while 25% showed intermediate susceptibility to streptomycin, and 18% to neomycin. Exactly 92.7% of the isolates were found to be susceptible to cefepime while 78.7% were susceptible to chloramphenicol, and 49.3% susceptible to streptomycin and 46.7% to cefotaxime. Figure 4.5 shows the pattern of resistance to tested antibiotics.

4.6 Multiple antibiotic resistance phenotypes and index of confirmed *E. coli* isolates

The results of the antibiotic susceptibility profiling of 150 randomly selected confirmed *E. coli* isolates showed resistance to two or more tested antibiotics. Table 4.2 shows the multiple antibiotic resistance phenotypes (MARPs) of the isolates to the tested antibiotics. Multiple antibiotic resistance phenotypes are shown for isolates that were resistant to any three or more antibiotics.

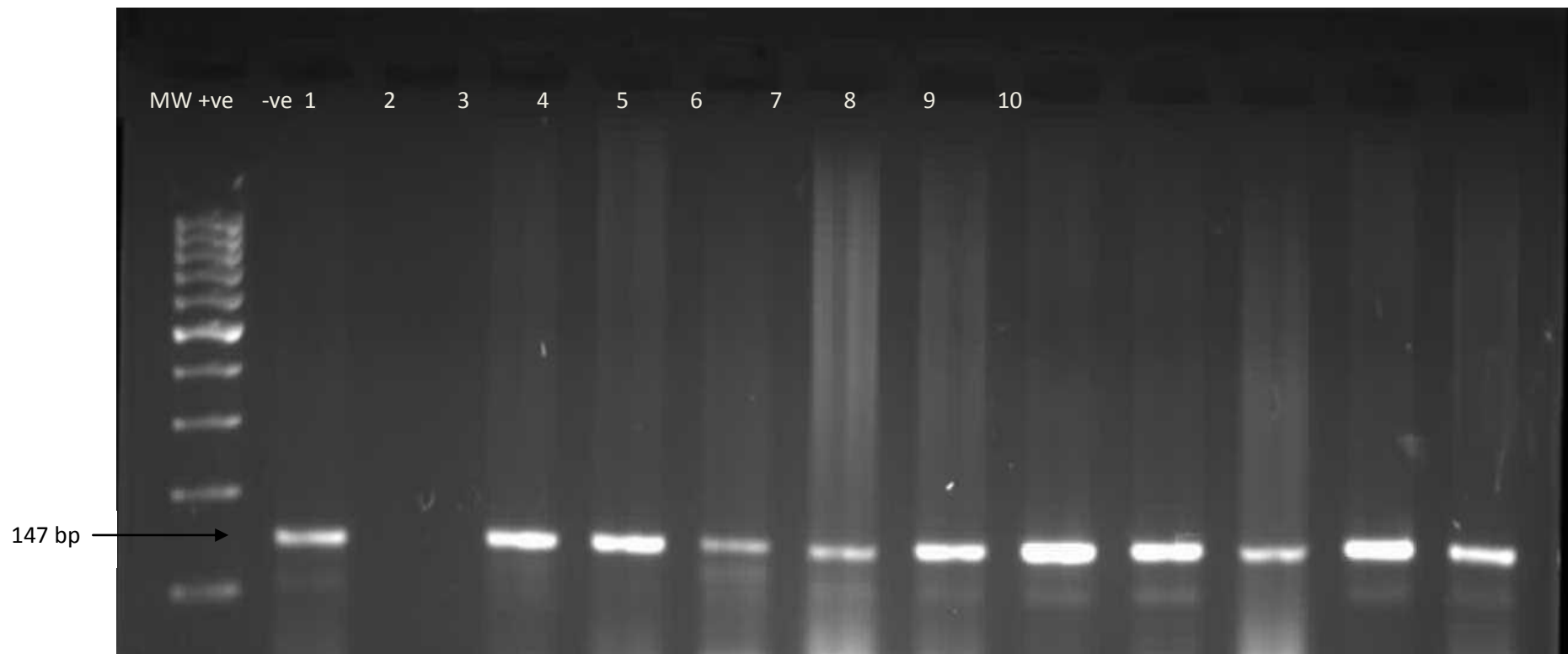


Figure 4.4: Gel picture of confirmed *E. coli* isolates with *uidA* gene. Legend: Lane 1: 100 molecular weight marker; lane 2: positive control (*E. coli* ATCC 25922 strain); lane 3: negative control; lane 4 to 13 *E. coli* isolates

Table 4.1: Antibiotic susceptibility pattern of *E. coli* isolates recovered from the final effluents

Antibiotic	<i>E. coli</i> (n=150)		
	S (%)	I (%)	R (%)
Chloramphenicol(30 µg)	78.7	19.3	2
Streptomycin(5 µg)	49.3	24.7	26
Rifampicin (5 µg),	0	0	100
Cefotaximine (30 µg)	46.7	34	19.3
Cefuroxime (30 µg)	36.7	52.7	10.7
Neomycin (10 µg)	36	18	46
Cefepime (30 µg)	92.7	2	5.3
Penicillin G (10 µg)	0	0	100
Norfloxacin (10 µg)	6	0.7	93.3
Erythromycin (15 µg)	0.7	8.7	90.7

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

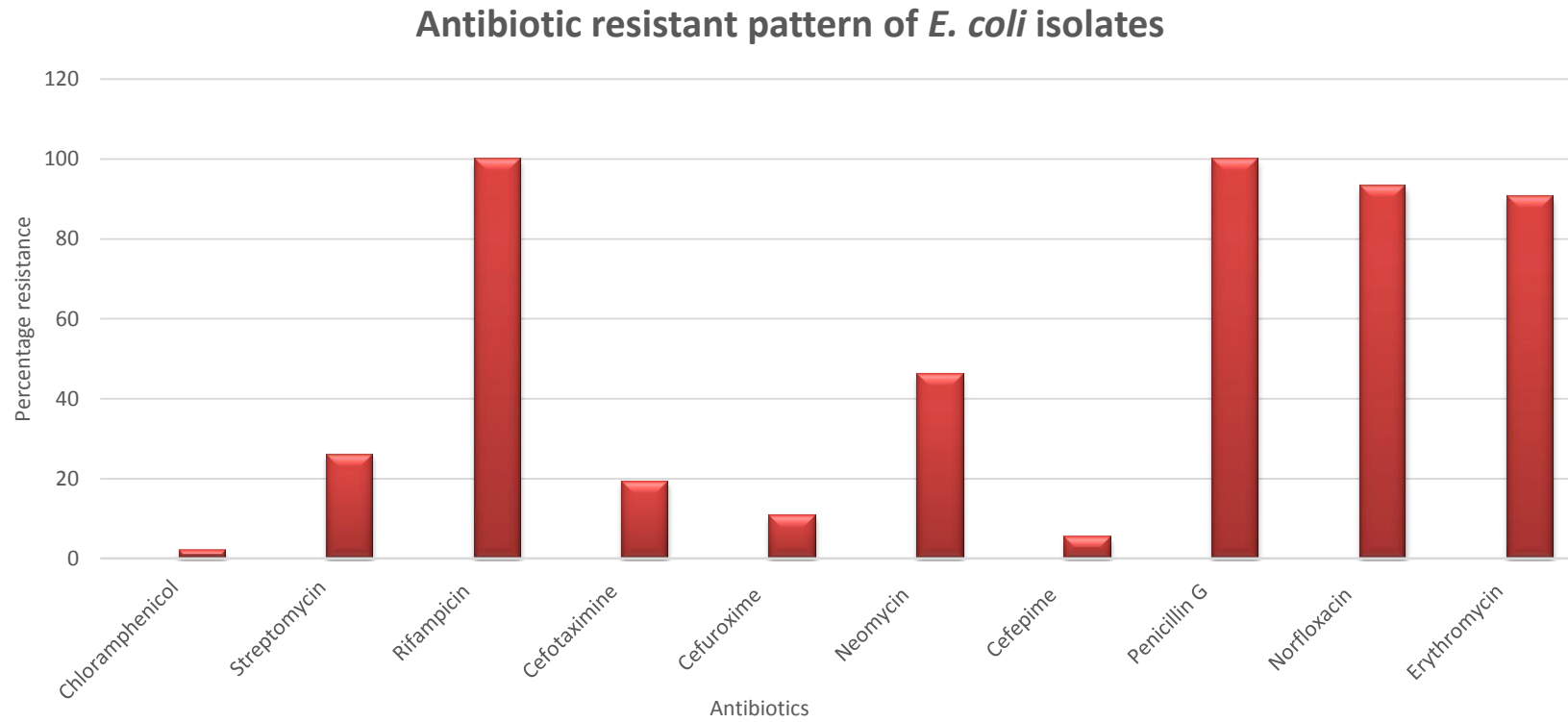


Figure 4.5: Antibiotic resistant pattern of confirmed *E. coli* isolates

Table 4.2: Multiple antibiotic resistant phenotypes and multiple antibiotic resistance index of *E. coli* isolates

Randomly selected confirmed <i>E. coli</i> isolates (N = 150)			
MAR phenotypes	Number observed	Percentage	MAR index
Rp-P-Nor	2	1.3	0.49
Rp- P-E	2	1.3	0.49
Rp-P-Nor-E	33	22	0.49
S-P-Nor-E	1	0.7	0.49
Rp-Cpm-P-Nor	1	0.7	0.49
Rp-N-P-Nor	1	0.7	0.49
Ro-Ctx-P-E	1	0.7	0.49
RP-N-P-E	1	0.7	0.49
S-Rp-P-Nor-E	15	10	0.49
Rp-Ctx-P-Nor-E	9	6	0.49
Rp-N-P-Nor-E	32	21.3	0.49
S-N-P-Nor-E	2	1.3	0.49
Rp-Cxm-P-Nor-E	5	3.3	0.49
Rp-Cpm-P-Nor-E	1	0.7	0.49
C-Rp-P-Nor-E	1	0.7	0.49
Rp-Ctx-N-P-Nor	1	0.7	0.49
S-Rp-N-P-Nor-E	13	8.7	0.49
Rp-Ctx-Cpm-P-Nor-E	2	1.3	0.49
S-Rp-Ctx-P-Nor-E	2	1.3	0.49
Rp-Cxm-N-P-Nor-E	5	3.3	0.49
Rp-N-Cpm-P-Nor-E	3	2	0.49
Ro-Ctx-N-P-Nor-E	5	3.3	0.49
Rp-Ctx-P-Nor-Cxm-E	1	0.7	0.49
Rp-S-Ctx-N-P-Nor	1	0.7	0.49
Rp-S-N-P-Nor-E	1	0.7	0.49
Rp-Ctx-N-Cpm-P-E	2	1.3	0.49
Rp-Ctx-Cxm-Cpm-P-Nor-E	1	0.7	0.49
C-Rp-Cxm-N-P-Nor-E	1	0.7	0.49
S-Rp-Ctx-N-P-Nor-E	3	2	0.49
S-Rp-Ctx-Cxm-P-Nor-E	1	0.7	0.49

Legend: Streptomycin (S), Cefotaxime (Ctx), PenicillinG (P), Rifampicin (Rp), Erythromycin (E), Chloramphenicol (C), Cefuroxime (Cxm), Neomycin (N), Cefepime (Cpm) and Norfloxacin (Nor).

Multiple antibiotic resistance index (MARI) for the selected isolates was calculated by:

MARI = $a / (b \times c)$ where;

a = the aggregate antibiotic resistance score of all 150 isolates = $(3 + 39 + 150 + 29 + 16 + 69 + 8 + 150 + 140 + 136) = 740$;

b = number of antibiotics = 10;

c = number of isolates = 150

MARI = $740 / (10 \times 150) = 0.49$

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 Discussion

The primary purpose of wastewater treatment is to permit domestic and industrial effluents to be disposed of without hazard to public health or unacceptable damage to the natural environment (FAO, 2013). Wastewater treatment and/or reclamation holds promise as an important water resource in arid and semi-arid regions as increasing human population and economic expansion continues to place increasing demands on finite water resources (Salgot *et al.*, 2006). Even though attention has been given to the detrimental effects of poorly treated sewage effluent discharges, attempt to obtain pathogen-free effluent discharge is still a daunting task.

Faecal coliform bacteria was used as indicator in this study to suggest the presence of pathogenic microorganism of faecal origin in discharged effluents of selected wastewater treatment plants in Amathole and Chris Hani District Municipality in Eastern Cape Province. The general range of faecal coliform counts obtained in this study varied between 0 and 1.6×10^3 CFU/100 ml. Although, the coliforms counts were in line with DWAF's limit of 1000 CFU/100 ml for most parts of the sampling seasons, exceptions from this compliance were observed at WWTP-S in November 2012 and also at WWTP-W in March 2013 where faecal coliform counts of 1.6×10^3 and 1.5×10^3 were obtained respectively. An important observation was made at WWTP-R where zero coliform count was recorded for the whole sampling period. The reason for this could be due to high chlorine dosages consistently used at this treatment site. While the high chlorine dosages may have completely inactivated the coliforms and possibly other

pathogens of faecal and non-faecal origins, the detrimental impacts of high chlorine concentrations from discharged effluents on aquatic lives in receiving surface water environments cannot be overemphasized. High chlorine concentration from discharged effluents is toxic to fish and other aquatic animals and can cause fish-kill (Jensen, 1992).

Escherichia coli is often used more specifically as indicator of faecal contamination because it is exclusively found in the gut of human and other warm-blooded animals where it exists as part of the normal intestinal flora. However some strains have acquired virulence genes which make them to become pathogenic. The presumptive *E. coli* counts obtained in this study generally ranged between 0 and 1.4×10^3 CFU/100 ml throughout the sampling period. Zero counts of *E. coli* were obtained at WWTP-R for all the sampling months exception in December 2012 and July 2013 where counts of 4.4×10^1 CFU/100 ml and 2 CFU/100 ml were observed respectively. There is no specific seasonal trend in terms of the distribution of *E. coli* during the sampling months. However, the highest presumptive *E. coli* count was observed in March 2013 (autumn) at WWTP-W.

Enterohaemorrhagic *E. coli* is one of the virulent human pathogen that has emerged over the past years. *Escherichia coli* O157:H7 serotype has been implicated in outbreaks of bloody diarrhoea and haemolytic uremic syndrome. Presumptive *E. coli* O157:H7 were recovered from the effluent samples analysed in this study and the counts generally ranged between 0 and 9.6×10^2 CFU/100 ml with the highest count observed at WWTP-S in November 2012.

Six hundred and twenty six presumptive *E. coli* isolates identified by cultural characteristics were subjected to molecular analysis (PCR) to confirm their identities using the *uidA* gene, the gene that codes for beta-glucuronidase in all *E. coli* species. Out of the 626

presumptive *E. coli* isolates subjected to PCR, 305 (48.7%) were positive for the presence of the *uidA* gene confirming their identities as *E. coli*. The PCR analysis shows more specificity in identifying pathogen than the cultural based techniques. The result of the molecular confirmation of some of the *E. coli* isolated is shown in Figure 4.4 under the results section. The molecular confirmation of the isolates suggests the potential danger that the discharged effluent pose to persons that may come in contact directly or indirectly with the effluent and the receiving surface water bodies. This can also suggest the possible presence of other potential pathogens of enteric sources that might equally have escape the various treatment stages including the disinfection process at the treatment plants.

One hundred and fifty of the confirmed *E. coli* isolates were randomly selected for antibiotic susceptibility profiling using a panel of 10 antibiotics that are commonly used in the treatment of diarrhoea and other enteric diseases caused by pathogenic *E. coli*. The susceptibility testing was done following the CLSI Kirby-Bauer (disk diffusion) method. The result of the analysis is shown in Table 4.1 under the result section. From the table, 78.7 % (118/150) of the test isolates were susceptible to chloramphenicol (30 µg) and only 2% of the isolates (3/150) were resistant to the antibiotic. For Streptomycin (5 µg) 74 of the 150 isolates (49.3%) were susceptible while 26% (39/150) of the test isolate were resistant. All the isolate 100% (150/150) showed complete resistance to both Rifampicin (5 µg) and penicillin G (10 µg). This reflects the importance of the use of these two antibiotics in the treatment of infections that may arise from these potential pathogens. The 100% resistance to penicillin was similar to the report of Eapen *et al.* (2005) who reported that Gram-positive bacteria were more susceptible to penicillin rather than Gram-negative bacteria. The resistance percentages observed for the remaining antibiotic used in the study (cefotaxime (30 µg), cefuroxime (30 µg), neomycin (10 µg). cefepime (30 µg),

norfloxacin (10 µg) and erythromycin (15 µg)) were 19.3%, 10.7%, 46%, 5.3%, 93.3% and 90.7% respectively.

All tested isolates showed multiple antibiotic resistances (MAR) to two or more antibiotics. The frequencies of the multiple antibiotic resistance phenotypes (MARP) and the combinations of the antibiotics to which they were resistant are given in Table 4.6. The table shows Multiple antibiotic resistance phenotypes of minimum of 3 and maximum of 7. For isolates with MARP 3, Rp-P-Nor and Rp- P-E showed the same frequency of 1.3 %. Rp-P-Nor-E was the most common MARP4 with percentage frequency of 22%. Isolates with MARP 5, Rp-N-P-Nor-E shows the highest frequency of 21.3% followed by S-Rp-P-Nor-E with frequency of 10% and Rp-Ctx-P-Nor-E with the frequency of 6%. For Isolates with MARP 6, S-Rp- N-P-Nor-E has the highest frequency of 8.7 % followed by Rp-Cxm-N-P-Nor-Cxm- E and Rp – Ctx – P-Nor- Cxm- E with frequencies of 3.3 %. Isolates with MARP 7, S-Rp-Ctx-N-P-Nor-E had the highest frequency of 2%.

Literature reveals that the sub-therapeutic use of antibiotics in the mass production of farm animals and related products has led to the emergence of MAR *E. coli* in the faecal environment of these animals (Novick, 1981; Mutsami, 2012). The indiscriminate use of antibiotics in human therapy have also produced MAR *E. coli* in the faeces of humans (Feary *et al.*, 1972; Krumperman, 1983). An important implication of MAR is the provision of microbial source tracking tool in the identification of *E. coli* contaminations of food and water originating from these high-risk environments by MAR indexing of *E. coli* isolates obtained from food and water (Krumperman 1983). Antibiotic resistance in bacteria often comes by the acquisition of genetic elements such as plasmid or transposon (e.g., extended spectrum β-lactamases (ESBL)) that carries resistance genes (Gold and Moellering, 1996; Christopher *et al.*, 2013). An

alternative mechanism for the generation of multiple antibiotic resistant (*mar*) mutants in *Escherichia coli* involves activation of a regulatory *mar* locus. The *mar* locus confers drug resistance by interfering with the expression of multiple genes located on the bacterial genome. Since the early description of the *mar* locus, other intrinsic regulatory mechanisms that bacteria use to resist the lethal effects of a wide range of toxic agents have been described (Aleksun and Levy, 1999).

The value of the multiple antibiotic index (MARI) obtained for the tested isolates is 0.49. The MARI value is a tool used in the analysis of health risk and associated with the spread of bacterial resistance in a given population where there is resistance to more than three antibiotics. (Sambrook *et al.*, 1982; Christopher *et al.*, 2013). A MARI value of 0.2 is used to differentiate between low and high risk. MAR index greater than 0.2 indicates that the isolates are from an environment where several antibiotics are used.

5.2 Conclusion

South Africa has been described as a water scarce country. The country's average rainfall of about 450 mm per annum falls short of the global average rainfall of 860 mm per annum. South Africa has a limited amount of water resource and several factors, such as climate change, water pollution particularly from inadequately treated effluents and economic expansion adds to water scarcity (CSIR, 2010). Assessment of wastewater effluents is an important method used to supervise water quality for the management of point-source contaminations (WRC, 1997).

Although, faecal coliform counts obtained in this study was generally in compliance with set guideline for discharged effluents, the discovery of potentially pathogenic *E. coli* which were confirmed by molecular methods shows the potential hazards that contact with these effluents

pose. This may also be an indication of the possible presence of other pathogens that were not assessed in this study. High chlorine concentrations used for disinfection at WWTP-R throughout the sampling period may have detrimental effect on the aquatic biomes of the receiving water body. There is need for proper monitoring of chlorine dosing at this site to forestall the effect of excessive chlorine concentration on fish and other aquatic lives of the receiving water body. The high frequencies of MAR *E. coli* isolate observed in the study is a reflection of indiscriminate use of antibiotics leading to increasing drug resistance shown by the isolates. Constant antibiotic sensitivity monitoring is necessary to control the selection for antibiotic resistance bacteria.

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